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Increased transcript levels and kinetic function of pyruvate kinase during severe dehydration in aestivating African clawed frogs, *Xenopus laevis*

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ABSTRACT

The African clawed frog, *Xenopus laevis*, can withstand extremely arid conditions through aestivation, resulting in dehydration and urea accumulation. Aestivating *X. laevis* reduce their metabolic rate, and rely on anaerobic glycolysis to meet reduced ATP demands. The present study investigated how severe dehydration affected the transcript levels, kinetic profile, and phosphorylation state of the key glycolytic enzyme pyruvate kinase (PK) in the liver and skeletal muscle of *X. laevis*. Compared to control frogs, severely dehydrated frogs showed an increase in the transcript abundance of both liver and muscle isoforms of PK. While the kinetics of muscle PK did not differ between dehydrated and control frogs, PK from the liver of dehydrated frogs had a lower K_m for phosphoenolpyruvate (PEP) (38%), a lower K_a for fructose-1,6-bisphosphate ($F1,6P_2$) (32%), and a greater activation of PK via $F1,6P_2$ (1.56-fold). PK from dehydrated frogs also had a lower phosphorylation-state (25%) in comparison to the enzyme from control frogs in the liver. Experimental manipulation of the phosphorylation-state of liver PK taken from control frogs by endogenous protein phosphatases resulted in decreased phosphorylation, and a similar kinetic profile as seen in dehydrated frogs. The physiological consequence of dehydration-induced PK modification appears to adjust PK function to remain active during a metabolically depressed state. This study provides evidence for the maintenance of PK activity through elevated mRNA levels and a dephosphorylation event which activates frog liver PK in the dehydrated state in order to facilitate the production of ATP via anaerobic glycolysis.

1. Introduction

The African clawed frog, *Xenopus laevis*, survives severe loss of aquatic habitat during arid periods. The seasonal drying and loss of water sources forces *X. laevis* to either migrate to find other aquatic habitats or burrow into the ground, entering a state termed aestivation. Aestivation is characterized by whole body water loss, urea accumulation, and entrance into a metabolically depressed state that can last for months (Hillman, 2009; Storey and Storey, 2004a, 2004b; Tinsley et al., 1996). During aestivation, *X. laevis* endures severe dehydration losing up to 35% of total body water (excluding bladder water) (Romspert, 1976). Some anurans (*Rana pipiens, Heleioporus eyrei*) can withstand daily fluctuations of > 22% of total body water (Dole, 1967; Lee, 1968). This remarkable tolerance to water loss is crucial for the survival of most anurans because the skin typically offers little resistance to evaporative water loss. In fact, the extent of dehydration endurance correlates strongly with the terrestrial nature of anuran habitats (Hillman, 1980; Joergensen, 1997; Thorson and Svihla, 1943; Thorson, 1955). Regulation of water balance to facilitate the endurance of dehydration involves a suite of behavioral, physiological, and biochemical responses (Churchill and Storey, 1993; Malik and Storey, 2009; Pinder et al., 1992). Dehydration-induced variability in body water content also leads to wide variations in body fluid osmolality and ionic strength and, among vertebrates, anurans show some of the greatest tolerance to variation in these parameters (Hillman, 1978, 1988, 2009). Another consequence of severe dehydration is reduced blood volume and increased blood viscosity which leads to impairment of aerobic cardiovascular capacity and performance, including a decline in arterial pressure, pulse rate, and oxygen consumption (Gatten Jr, 1987; Hillman, 1987). Due to decreased blood flow, tissues and organs can become hypoxic during severe dehydration, which induces an increased use of anaerobic energy production via glycolysis (Churchill and Storey, 1994a; Churchill and Storey, 1995; Hillman, 1978, 1987).

When aerobic energy production via oxidative phosphorylation is limited, such as in aestivating frogs, metabolic ATP production shifts from primarily oxidative phosphorylation to anaerobic production of ATP. Pyruvate, generated during glycolysis from the intermediate phosphoenolpyruvate, is converted to lactate under anaerobic

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conditions to regenerate the NAD^+ needed to sustain glycolysis. Pyruvate kinase (PK; E.C. 2.7.1.40) is responsible for catalyzing the following reaction:

phopshoenolpyruvate + ADP → pyruvate + ATP

This reaction is an important regulatory step in glycolysis, leading to production of pyruvate that can either be reduced (forming lactate) or oxidized (forming acetyl CoA), depending on conditions. In most cases, this reaction is essentially irreversible, representing one of the major points of control for glycolysis. Hence, mediation of PK activity is critical for regulating levels of ATP and glycolytic intermediates, while also serving as a metabolic control point, regulating the use of PEP by gluconeogenic or glycolytic pathways in tissues such as liver (Jurica et al., 1998; Mattevi et al., 1996; Valentini et al., 1995). Regulation of PK activity occurs via multiple mechanisms, including tight allosteric regulation and regulation via insulin-mediated cell signaling pathways (Jurica et al., 1998; Mattevi et al., 1996; Valentini et al., 1995). In this way, PK activity is responsive to the metabolic requirements of an organism, which may change depending on the energy state, tissue, or cell type.

PK has tissue specific isozymes, each of which exhibit different kinetic properties to meet the particular metabolic requirements of the expressing tissue. Although multiple PK isoforms can be detected in some tissues, cells generally express only one isoform at appreciable levels (Cardenas and Dyson, 1978; Imamura and Tanaka, 1972). The biochemical properties of the different PK isozymes are best characterized in mammals, which typically have four isozymes: PKL, which is the major isozyme in the liver and minor isozyme in the kidney; PKM1, which is associated with metabolically-demanding tissues like muscle, heart and brain; PKR, which is found exclusively in erythrocytes; and the embryonic PKM2, which is also found in proliferating cells (Cardenas and Dyson, 1978; Imamura and Tanaka, 1972; Imamura and Tanaka, 1982; Noguchi et al., 1986; Noguchi et al., 1987). The *pklr* gene encodes the liver and erythrocyte isozymes of PK (PKL, PKR), while the *pkm* gene encodes the two muscle isozymes (PKM1, PKM2) (Noguchi et al., 1986; Noguchi et al., 1987). In *X. laevis*, both *pklr* and *pkm* have been identified, but not all transcripts and isoforms have been characterized (Klein et al., 2002). Work so far suggests that the regulation of liver and muscle isozymes differs considerably. The muscle isozyme PKM1 is typically regarded as non-allosterically regulated, as it shows no responses to the binding of its inhibitors and/or activators under most conditions (Jurica et al., 1998); whereas, PKM2 is allosterically regulated (Israelsen and Vander Heiden, 2015). In contrast PKL, the liver isozyme is tightly regulated by the product of the phosphofructokinase 1 (PFK) reaction, fructose-1,6-bisphosphate ($F1,6P_2$) (Jurica et al., 1998). PKL is also allosterically regulated via feed-back inhibition by ATP, the product of the PK reaction, and by phosphorylation by cyclic AMP-dependent protein kinase (PKA) (Birnbaum and Fain, 1977; El-Maghrabi et al., 1982).

Aside for the action of inhibitors, activators and feedback loops, post-translational modifications, such as reversible protein phosphorylation, also play an important role in regulating key proteins in metabolic pathways in organisms that experience periods of dormancy (Dawson et al., 2013; Dawson et al., 2015; Dawson and Storey, 2011, 2012a, 2012b, 2016; Malik and Storey, 2009; Storey and Storey, 2004a). Post-translational modification of PK specifically has been reported in previous studies examining dormancy and metabolic rate depression in other animals such as the aestivating land snail (*Otala lactea*) and the freeze-tolerant goldenrod gall fly (*Eurosta solidaginis*) (Abboud, 2015; Whitwam and Storey, 1990). However, few studies have examined multiple isoforms, and no work to date has characterized the role of regulation of PK activity in aestivating frogs.

The purpose of this study is to understand the transcriptional, allosteric and post-translational regulation of pyruvate kinase under dehydration stress in *X. laevis*, which may facilitate increased glycolytic production of ATP in a metabolically depressed state. Here we report on the characterization of PK from hind limb muscle and liver of control and dehydrated *X. laevis* by investigating the differences in mRNA levels, enzyme kinetics, and post-translational modifications. In addition, we examine the effect of phosphorylation on the function of PK from control and dehydrated animals.

2. Materials and methods

2.1. Animals

African clawed frogs, *Xenopus laevis*, were donated from the Department of Zoology, University of Toronto. All animals were initially held in tanks of dechlorinated water, acclimated to 22 °C without food for at least 1 week. Animals were exposed to various levels of dehydration and were housed as described previously (Churchill and Storey, 1993). The frogs were divided into two groups; a control group (n = 4), and a severe dehydration group (> 25% body water lost) (n = 4). Control frogs were left in the tanks as previously described (Churchill and Storey, 1993). Dehydration groups were placed in closed containers and allowed to dry until ~25% of their body water was lost. To facilitate drying, a layer of silica gel desiccant was present in the container of the dehydration group; the silica gel desiccant was kept physically separate from the frogs using a perforated divider. Water loss was monitored for both groups over a period of 6–7 days by removing the animals at set intervals and weighing each animal. No significant changes in body mass were observed for the control groups. The change in body mass was used as a measurement of water loss using the following equation; % water lost = [(Wi − Wd) / (Wi × BWCi)] × 100, where Wi is the initial mass of the frog, Wd is the measured mass at each interval, and BWCi is the initial body water content of frogs prior to dehydration. For sampling, animals were killed by pithing and liver and hind limb skeletal muscle tissues were quickly harvested, immediately frozen in liquid nitrogen, and stored at −70 °C.

All experiments were conducted in accordance with the Canadian Council on Animal Care guidelines and The Carleton University Animal Care Committee.

2.2. Chemicals

Chemicals, biochemicals, chromatography media and coupling enzymes were from Sigma Chemical Co. (St. Louis, MO) whereas ProQ Diamond Phosphoprotein stain was from Invitrogen (Eugene, OR).

2.3. Preparation of tissue lysates for protein purification

Frozen liver and skeletal muscle samples were homogenized using a Polytron PT1000 homogenizer (1:5 w:v) on ice in buffer A (50 mM Tris, 25 mM NaF, 2.5 mM ethylenebis(oxyethylenenitrilo)tetraacetic (EGTA), 2.5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), pH 8.0). PMSF crystals were added before homogenization process. Liver and skeletal muscle homogenates were centrifuged at 13500 × *g* at 4 °C for 20 min. The resulting supernatant was decanted and held on ice until use.

2.4. Sephadex G-50 and $DEAE^+$ chromatography of PK

A 5 cm column of Sephadex G-50 in a syringe barrel was equilibrated in buffer A and centrifuged at 500 × *g* in a bench-top centrifuge for 2 min to remove excess buffer. 500 µl aliquots of either liver or skeletal muscle extracts (1:5 w:v in buffer A) were centrifuge through the columns at 500 × *g* for 2 min. The resulting eluant was collected and 1 ml was applied to a $DEAE^+$ Sephadex G50 column (1.5 cm × 20 cm) equilibrated in buffer A, washed with 50 ml of buffer to remove unbound protein, and then eluted with a linear KCl gradient (0–1 M in 50 ml) in the same buffer. Fractions of ~0.6 ml were collected and 5 µl from each fraction was assayed to detect PK activity. The top 3 fractions