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Regulation of liver lactate dehydrogenase by reversible phosphorylation in response to anoxia in a freshwater turtle

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ABSTRACT

Lactate dehydrogenase (LDH) is the terminal enzyme of anaerobic glycolysis and key to hypoxia/anoxia survival by most animals. In this study, the effects of anoxic submergence (20 h at 7 °C in nitrogen-bubbled water) were assessed on LDH from liver of an anoxia-tolerant freshwater turtle, the red-eared slider (*Trachemys scripta elegans*). Liver LDH from aerobic and anoxic turtles was purified to homogeneity in two steps. The kinetic properties and thermal stability of purified LDH were analyzed, revealing significant differences between the two enzyme forms in V_{max}, K_m pyruvate, and I_{50} pyruvate as well as melting temperature determined by differential scanning fluorimetry. The phosphorylation state of aerobic and anoxic forms of LDH was visualized by ProQ Diamond phosphoprotein staining, the results indicating that the anoxic form had a higher phosphorylation state. Incubation studies that promoted protein kinase versus protein phosphatase actions showed that changes in the phosphorylation state of aerobic and anoxic forms mimicked the anoxia-responsive changes in K_m pyruvate and I_{50} pyruvate. The high phosphate form of liver LDH that occurs in anoxic turtles appears to be a less active form. Turtle liver LDH was also subject to another form of posttranslational modification, protein acetylation, with a 70% higher content of acetylated lysine residues on anoxic versus aerobic LDH. This is the first study to show that LDH function in an anoxia-tolerant animal can be differentially modified between aerobic and anoxic states via the mechanism of posttranslational modification.

1. Introduction

Lactate dehydrogenase (LDH; EC 1.1.1.27) catalyzes the NAD(H)-dependent interconversion of pyruvate and lactate. The enzyme is encoded on three genes in mammals and birds: LDH-A, B and C encoding the subunits that typify muscle (M), heart (H) and testes, respectively (Li, 1990). However, only LDH-A and B genes have been reported in reptiles (Mannen et al., 1997; Liao et al., 2001). The gene products assemble to form a tetrameric enzyme; in non-testicular tissues, five subunit compositions have been identified in varying proportions in different tissues (M_4, M_3H_1 M_2H_2 M_1H_3 and H_4), including in turtle species (Javed, 1990; Mannen et al., 1997; Liao et al., 2001). The M_4 LDH isoform is prominent in skeletal muscle and liver where it is poised to work in the pyruvate reductase direction. By converting pyruvate to lactate, the reaction regenerates the NAD^+ that is needed to sustain glycolytic flux (Hochachka and Somero, 2002). The glycolytic pathway is almost universally employed for ATP production by animals under low oxygen (hypoxia, anoxia) conditions but has two limitations. The first is that use of the pathway typically results in significant cellular acidification which is a challenge to homeostasis during long-term anoxia (Storey and Storey, 2004). The second is the low energy yield (2 ATP per glucose) of glycolysis compared with aerobic oxidative phosphorylation; indeed, to partially counteract this, various alternative anaerobic pathways that have higher ATP yields have been developed by facultative anaerobes (Storey and Storey, 2004). With such importance and also these limitations, the glycolytic pathway for conversion of glucose to lactate must be carefully regulated in anoxia-tolerant animals. Four enzymes of glycolysis/glycogenolysis (glycogen phosphorylase, hexokinase, phosphofructokinase, pyruvate kinase) are well-known to be regulated in most tissues but LDH, the final enzyme in the pathway, is typically considered to be non-regulatory. However, some studies have shown that the LDH function can be modified by shifting between dimer and tetramer forms (Yamamoto and Storey, 1988), by allosteric effectors (Brown and Christian, 1974), or by peptides that affect the interaction between LDH subunits and thereby influence enzyme kinetic properties (Döbeli and Schoenenberger, 1983). We reasoned that functional control of LDH might be most prominent in a good facultative anaerobe that both survives long term anoxia exposure and accumulates extremely high concentrations of lactate.

Among vertebrate animals, anoxia tolerance is most highly developed among selected species of freshwater turtles, the red-eared slider (*Trachemys scripta elegans*) being one of them (Ultsch, 1985). High anaerobic capacity is not only related to the diving lifestyle of this

Abbreviations: LDH, lactate dehydrogenase; PKA, cyclic AMP dependent protein kinase; PKC, calcium and diacylglycerol dependent protein kinase C; PKG, cyclic GMP dependent protein kinase; DSF, differential scanning fluorimetry.

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species but also supports extended weeks of underwater hibernation without breathing during the winter, often in ice-locked ponds that prevent the animals from surfacing. Some turtle species have effective mechanisms of extra-pulmonary oxygen uptake, but this capacity is low in *T. s. elegans* (Ultsch, 1985) and the primary mechanism for survival during long-term breath-hold submergence is their well-developed anoxia tolerance. The main components of turtle anaerobiosis include (a) large stores of glycogen fuel, especially in liver that synthesizes and releases glucose for use by other organs, (b) high buffering capacity to deal with acidosis that is achieved by release of calcium and magnesium carbonates from the shell as well as sequestering lactate into the shell (Jackson, 1999; Jackson et al., 2000), and (c) strong metabolic rate depression to just 10–20% of the aerobic rate at the same temperature that greatly reduces the ATP demands of metabolism (Herbert and Jackson, 1985; Storey, 2007). The unique methods for buffering and storing lactate are particularly critical to turtle survival and allow plasma lactate concentrations to rise to 150 mM or more during prolonged submergence in cold water but still maintain a blood pH above 7.0 (Jackson et al., 2000).

Reversible protein phosphorylation is a prominent mechanism for the posttranslational modification and regulatory control of enzyme/proteins and is involved in virtually every aspect of cell life. For example, phosphorylation or dephosphorylation of an enzyme can influence its activity, kinetic parameters, thermal stability, and protein–protein or subunit–subunit interactions (Cohen, 2002), often modifying enzyme function in response to different environmental or physiological stresses. Multiple studies by our lab and others have shown that reversible enzyme phosphorylation is an important regulatory mechanism in animal response to environmental stress both in general (Storey and Storey, 2004; Storey and Storey, 2007) and specifically during turtle anaerobiosis (Brooks and Storey, 1993; Storey, 1996, 2007). The present study investigates the properties of turtle liver LDH and documents a role for reversible phosphorylation in regulating enzyme response to anoxia exposure.

2. Material and methods

2.1. Chemicals and animals

Chemicals were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA) or Boehringer Mannheim Corporation (Montreal, QC, Canada). Adult female red-eared sliders (*T. s. elegans*), 700–1500 g, were acquired from local suppliers and held at 5±1 °C in large plastic tubs (2 turtles per tub) filled with dechlorinated tap water for at least a week before use. Control turtles were sampled from this condition. For anoxia exposure, turtles were transferred to large buckets at 5±1 °C that had been previously bubbled with N_2 gas for 1 h; 2–3 turtles were added per bucket in 30 min intervals. Bubbling was continued for 1 h after the last turtle was added and was reinitiated again during sampling of the animals. A wire mesh was fitted into the tank about 5 cm below the water surface so that turtles remained submerged throughout the 20 h experimental anoxic submergence. All animals were killed by decapitation and then tissues were rapidly dissected out, frozen in liquid nitrogen and stored at −80 °C until use. All animals were cared for in accordance with the guidelines of the Canadian Council on Animal Care and all experimental procedures had the prior approval of the Carleton University Animal Care Committee.

2.2. Preparation of tissue extracts and purification

Frozen liver samples were quickly weighed and mixed 1:4 w/v in ice-cold buffer A containing protein kinase and protein phosphatase inhibitors (30 mM HEPES, pH 7.0, 10% v/v glycerol, 5 mM 2-mercaptoethanol, 1.5 mM EDTA, 1.5 mM EGTA, 15 mM β-glycerophosphate); a few crystals of phenylmethylsulfonyl fluoride were added and the sample was immediately homogenized with a Diamed Pro 200 homogenizer. Homogenates were centrifuged at 10,000 *g* at 4 °C for 30 min and then supernatant was isolated and stored on ice. Low molecular weight metabolites/ions were removed from the supernatant by a 2 min low speed centrifugation (2000 rpm) through small columns of Sephadex G50 equilibrated in buffer A. The eluate was then loaded onto a 5×1 cm column of Cibacron blue equilibrated in buffer A and eluted with 40 mL of 0–2 M KCl gradient in buffer A. The fractions with highest activity were pooled and concentrated in a Centricon to 1 mL. The concentrated sample was then applied to an N(6-aminohexyl) oxamate column followed by washing with 10 mL of 10 mM NADH in 50 mM phosphate buffer and then elution of LDH with 10 mL of 50 mM phosphate buffer. Purified LDH was collected for enzyme assay and a portion was further concentrated for SDS-polyacrylamide gel electrophoresis (PAGE).

2.3. Enzyme assays

LDH activity was measured by monitoring the consumption or production of NADH at 340 nm using a Multiskan Spectrum microplate reader. Standard assay conditions for the forward reaction were 50 mM HEPES pH 7.0, 0.15 mM NADH, and 0.5 mM pyruvate in a 200 μL total volume; assays were started by the addition of 10 μL of enzyme sample. Standard assay conditions for the reverse reaction were 50 mM HEPES pH 8.0, 3 mM NAD^+, and 22.5 mM L-lactate. Routine assays were run at 22 °C. K_m and I_{50} values for pyruvate or lactate were determined at constant co-substrate, 0.15 mM NADH or 3 mM NAD^+. For temperature studies (from 5 to 37 °C), HEPES buffer was used and the microplate reader was placed in a Thermal Scientific Revco adjustable temperature incubator. Filled microplates were held in the incubator for ~10 min to equilibrate to temperature before enzyme was added; assay temperature was confirmed both before and after assay by inserting a thermistor into a well containing buffer.

For analysis of the pH dependence of LDH activity, purified enzyme was assayed under optimal substrate concentrations in 100 mM phosphate buffer adjusted to different pH values at room temperature.

Soluble-protein concentrations were determined using the Coomassie blue G-250 method with the BioRad Laboratories prepared reagent and bovine serum as the standard. Spectrophotometric quantification was performed at 595 nm.

2.4. Incubations to stimulate endogenous protein kinase and phosphatase activities

To assess the potential effects of reversible phosphorylation on LDH, aliquots of enzyme were incubated under conditions that facilitated the activities of selected endogenous protein kinases or protein phosphatases. Homogenization (1:5 w:v in buffer A) and centrifugation were as described above. The supernatant was isolated and low molecular weight metabolites/ions were removed by a 2 min low speed centrifugation (600 *g*) through small columns of Sephadex G50 equilibrated in buffer A. Then aliquots were mixed with 1 volume of an appropriate incubation buffer. Each buffer contained 60 mM HEPES, pH 7.0, 10 mM 2-mercaptoethanol with the additions as follows:

(A) "stop" conditions: 40 mM β-glycerophosphate, 4 mM EDTA and 4 mM EGTA to inhibit both protein kinase and protein phosphatase activities.
(B) promotion of endogenous protein kinases: 40 mM β-glycerophosphate, 2 mM cAMP, 10 mM ATP, 2 mM cGMP, 2.6 mM $CaCl_2$, 14 μg/mL phorbol myristate acetate, 20 mM $MgCl_2$ and 10 mM Na_3VO_4 to stimulate PKA, PKG and PKC.
(C) promotion of endogenous protein phosphatases: 10 mM $MgCl_2$ and 10 mM $CaCl_2$ to promote the activities of protein phosphatases.