Comparative Biochemistry and Physiology, Part B 202 (2016) 67–74

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part B

journal homepage: www.elsevier.com/locate/cbpb

Regulation of crayfish, *Orconectes virilis*, tail muscle lactate dehydrogenase (LDH) in response to anoxic conditions is associated with alterations in phosphorylation patterns

Stuart R. Green, Kenneth B. Storey *

Institute of Biochemistry & Department of Biology, Carleton University, 1125 Colonel By Drive, Ottawa, Ontario K1S 5B6, Canada

ARTICLE INFO

Article history:
Received 6 June 2016
Received in revised form 10 August 2016
Accepted 15 August 2016
Available online 18 August 2016

Keywords:
Anoxia-tolerance
Muscle glycolysis
Orconectes virilis
Posttranslational modification

ABSTRACT

Lactate dehydrogenase (LDH), the terminal enzyme of anaerobic glycolysis, has a crucial role in sustaining ATP production by glycolysis during periods of anoxia via regenerating NAD^+ through the production of lactate. The present study examined the effects of prolonged (20 h) anoxic submergence on LDH from the tail muscle of an anoxia-tolerant crayfish (*Orconectes virilis*). LDH was purified to homogeneity from tail muscle of both aerobic control and anoxic crayfish in a three step process. Analysis of the kinetic parameters and the stability of LDH showed that the V_{max} in the pyruvate-reducing direction was significantly higher for the enzyme from anoxic crayfish whereas in the lactate-oxidizing direction the V_{max} was significantly higher for the control enzyme. Differential scanning fluorimetry was used to assess thermal unfolding of crayfish LDH. The results showed that the enzyme from control muscle had a significantly higher melting temperature (greater thermal stability) than the anoxic enzyme form, suggesting that there was a structural difference between the two enzyme forms. Immunoblotting of purified LDH implicated post-translational modification as the reason for this difference; purified LDH from aerobic control crayfish showed significantly higher amounts of serine/threonine phosphorylation than did the anoxic enzyme form. This study provides evidence for anoxia-induced modifications of crayfish muscle LDH that may contribute significantly to modulating enzyme function under anoxic conditions.

1. Introduction

The northern crayfish (*Orconectes virilis*) is a common species in streams, lakes and wetlands across southern Canada and the northern USA. In their native habitat the animals may be subject to constraints in oxygen availability at different times of the year. For instance during the summer crayfish may face hypoxic conditions due to high water temperatures and low water levels that impede the ability of the animals to aerate their gills whereas during the winter they may face oxygen limitation in ice-locked bodies of water when oxygen is depleted by the biomass of respiring organisms in the water (Bonvillain et al., 2012; Dawson and Storey, 2011). These pressures ensure that this crayfish is capable of living without oxygen for extended periods of time. These animals can use anaerobic glycolysis as the main source of ATP through consumption of stored glycogen during oxygen deprivation thereby resulting in a large increase in the concentration of intracellular lactate in the muscle (Gäde, 1984). In response to the rising levels of lactate and acidosis associated with anaerobic glycolysis, crayfish buffer acidification by releasing HCO_3^- ions from the carapace as well as exporting and sequestering some of the excessive lactate via complexation to Ca^{2+} into the carapace (Galler and Moser, 1986; Jackson et al., 2001). Furthermore, studies have also shown that freshwater crayfish are able to suppress the activity of ATP-consuming pathways when oxygen is limited in order to conserve energy reserves (Cowan and Storey, 2001).

Due to the importance of anaerobic glycolysis as the main supplier of ATP under anoxic conditions, lactate dehydrogenase (LDH), the terminal enzyme of glycolysis, is an interesting target for study in anoxia-tolerant species. LDH (EC 1.1.1.27) catalyzes the following reversible reaction:

$$\text{pyruvate} + \text{NADH} + H^+ \leftrightarrow \text{lactate} + NAD^+.$$

Efficient operation of LDH is needed for aiding glycolysis under anoxic or hypoxic conditions since the NADH produced by the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) step of glycolysis cannot be regenerated to form NAD^+ by the mitochondrial electron transport system in the absence of oxygen. Lactate formation via LDH is

Abbreviations: DEAE, Diethylaminoethyl; DSF, Differential scanning fluorimetry; EDTA, Ethylenediaminetetraacetic acid; EGTA, Ethylene glycol tetraacetic acid; HEPES, 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid; K_m, Michaelis constant; LDH, Lactate dehydrogenase; PTM, Posttranslational modification; PVA, Polyvinyl alcohol; SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; TBS(T), Tris base salt (Tween) buffer; T_M, Protein unfolding temperature; V_{max}, Maximal enzymatic activity.

* Corresponding author.
E-mail address: kenneth_storey@carleton.ca (K.B. Storey).

http://dx.doi.org/10.1016/j.cbpb.2016.08.004
1096-4959/

not an exclusive or universal strategy for anaerobic survival, e.g. the glycolytic end product of yeast is ethanol generated by alcohol dehydrogenase (Wills, 1990) and many marine invertebrates substitute one of the opine dehydrogenases as the terminal enzyme of anaerobic glycolysis (Harcet et al., 2013). However, LDH is undoubtedly the most common terminal enzyme of anaerobic glycolysis across species, being almost exclusive among vertebrate and crustacean species.

Anaerobic glycolysis typically leads to acidification of the cell due to an imbalance between proton production during ATP hydrolysis and proton consumption when glycolysis is the sole producer of ATP; by contrast, there is no net accumulation of protons when ATP hydrolysis is balanced by ATP synthesis from oxidative phosphorylation (Hochachka and Mommsen, 1983). Though the crayfish is capable of buffering against these changes to a degree via carbonate release from the carapace to prevent fatal acidosis, pronounced decreases in hemolymph pH are known to occur in the early stages of oxygen limiting conditions in crayfish as well as other aquatic crustaceans while the animals acclimate to the new conditions (Taylor and Wheatly, 1981; Truchot, 1975). Therefore the impact of pH change is of interest with regards to the activity and functional parameters of crayfish LDH during anoxia.

In this study, kinetic parameters of the LDH pyruvate reducing and lactate oxidizing reactions were assessed for LDH purified from tail muscle of aerobic control and anoxia-exposed (20 h in nitrogen-bubbled water) crayfish. The potential involvement of posttranslational modifications in altering enzyme stability and kinetic properties were also evaluated. Reversible protein phosphorylation has long been a subject of interest in the regulation of enzymes in response to anoxia (Storey and Storey, 2007), most recently including studies of LDH (Storey, 2015). For example, skeletal muscle LDH from an anoxia-tolerant freshwater turtle, *Trachemys scripta elegans*, was shown to be phosphorylated and acetylated in response to anoxia which resulted in a decrease in activity in the pyruvate reducing direction of the reaction (Xiong and Storey, 2012). The role of protein phosphorylation in regulating the activity of enzymes has been long understood to depend upon the enzyme in question with some enzymes becoming active with the addition of a phosphate group to hydroxyl bearing residues, such as glycogen phosphorylase, while others may become inactivated when phosphorylation occurs, such as the pyruvate dehydrogenase complex (Krebs and Beavo, 1979). When understanding the regulation of any enzyme, it is important to examine the influences of relevant natural conditions, as this may play an important role in modulating enzymatic activity. For example, one recent study demonstrated that LDH substrate affinity is sensitive to the presence of urea in the frog, *Xenopus laevis*; indeed, the K_M values of LDH from dehydrated frogs for pyruvate, lactate, and NAD^+ was seen to be significantly higher than the control frog LDH under standard assay conditions, but these trends were abolished with the addition of 150 mM urea (a metabolite accumulated during dehydration in the frog) resulting in the dehydrated K_M values of LDH becoming nearly identical to those seen in the control in the absence of urea (Katzenback et al., 2014). Relevant physiological fluctuations in the crayfish include a decrease in cellular pH under hypoxic/anoxic conditions and changes in temperature due to large seasonal variation in water temperature between summer and winter months. The present study purified LDH from crayfish tail muscle and evaluated changes in enzyme properties and thermal stability between aerobic and anoxic states as well as the roles of protein phosphorylation in enzyme control.

2. Material and methods

2.1. Chemicals and animals

Biochemicals were purchased from Sigma Chemical Company (St. Louis, MO) or BioShop (Burlington, ON). Northern or virile crayfish, *O. virilis*, were purchased from bait shops in Ottawa, Ontario and treated as described in Lant and Storey (2011). The crayfish were placed in large tubs containing 10 L of water that was continuously aerated and kept at 15 °C for 7 days in incubators. Following this, groups of 10 crayfish were transferred into smaller tubs; controls were placed in containers at 15 °C with continuous air-bubbling whereas for anoxia exposure, crayfish were transferred into containers that had been previously bubbled with nitrogen gas for 45 min. These containers had closed lids that were fitted with two ports, one to accommodate a nitrogen gas bubbler and the other to vent gas. Nitrogen bubbling was continued over the course of a 20 h anoxia exposure at 15 °C. Following anoxia exposure, oxygen content was measured at <1 Torr in the water. Both control and anoxic crayfish were killed by decapitation and then samples of tail muscle were quickly dissected, frozen in liquid nitrogen, and transferred to a −80 °C freezer for storage.

2.2. Preparation of tissue extracts and purification of enzyme

Each enzyme preparation was prepared from the muscle of at least two crayfish. Frozen samples of crayfish tail muscle were quickly weighed and then homogenized 1:4 w/v in ice-cold buffer A, containing protein phosphatase and protein kinase inhibitors (25 mM Tris-HCl, pH 8.0, 2.5 mM EDTA, 2.5 mM EGTA, 15 mM 2-mercaptoethnaol, 25 mM β-glycerophosphate, and 10% v:v glycerol) using a Janke & Kunkel IKA-Werk Ultra Turrax homogenizer. A few crystals of phenylmethylsulfonyl fluoride (a protease inhibitor) were also added immediately prior to homogenization. Homogenates were then centrifuged in an Eppendorf 5810R at 10,000 rpm at 4 °C for 30 min. The supernatant was collected and pellet was discarded.

LDH was purified in a 3-step process. A column (3 cm high × 1 cm wide) of diethylaminoethanol-Sepharose (DEAE-Sepharose) was prepared and equilibrated by running 15 mL of buffer A through the column. Supernatant was then loaded onto the column followed by eluting with 30 mL buffer A. Fractions of approximately 2 mL each were collected with a Gilson Micro-Fractionator fraction collector. Very little LDH bound to this column but many other proteins did. The eluted fractions with the highest LDH activity were then pooled and loaded onto a Cibacron blue column prepared as above. Following a 30 mL wash with buffer A, LDH was eluted using 30 mL of a solution containing 1 mM NADH and 1 mM pyruvate in buffer A. The fractions with the highest activity were again pooled, then diluted 1:4 in buffer A and loaded onto a second Cibacron blue column prepared identically to the first one. After a wash with 30 mL of buffer A, LDH was eluted with 30 mL of a 0–2 M KCl gradient in buffer A. Fractions with the highest activities were pooled and then analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) to demonstrate purity of the LDH sample. A total of 6 purifications were performed for the control and anoxic LDH in order to perform subsequent kinetic and physical studies.

To prepare the LDH for SDS-PAGE, aliquots of purified enzyme 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) with 10% v:v β-mercaptoethanol added fresh. Samples were boiled for 5 min, then cooled on ice and stored in a freezer at −20 °C. Aliquots of samples were loaded into gel wells and were run together with a GeneDirex 10.5–175 kDa protein ladder on a 10% polyacrylamide gel for 45 min at 180 V in running buffer (25 mM Tris-base, 250 mM glycine, 0.1% w:v SDS). Silver staining was used according to the procedure described by Gromova and Celis (2006).

2.3. Enzyme assays

LDH activity was measured with a Multiskan Spectrum microplate reader to monitor the amount of NADH consumed or produced via absorbance at 340 nm. All assays were performed in a total volume of 200 μL. Standard assay conditions for the forward direction (the pyruvate-reducing direction) were 20 mM HEPES buffer, pH of 7.2, 0.2 mM NADH and 1 mM pyruvate. Standard assay conditions for the