Purification and Kinetic Properties of Skeletal Muscle Lactate Dehydrogenase from the Lizard *Agama stellio stellio*

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Abstract—Lactate dehydrogenase isoenzyme LDH-5 (M4) was purified to homogeneity from the skeletal muscle of lizard *Agama stellio stellio* as a poikilothermic animal, using colchicine-Sepharose chromatography and heat inactivation. The purified enzyme showed a single band after SDS-PAGE, corresponding to a molecular weight of 36 kD. The $K_m$ values for pyruvate, NADH, lactate, and NAD$^+$ were 0.020, 0.040, 8.1, and 0.02 mM, respectively. Pyruvate showed maximum activity at about 180 µM, with a decline at higher concentrations. The enzyme was stable at 70°C for 30 min, but was rapidly inactivated at 90°C. The optimum pH for the forward reaction (pyruvate to lactate) was 7.5, and for the reverse reaction (lactate to pyruvate) was 9.2. Oxalate, glutamate, Cu$^{2+}$, Co$^{2+}$, Mn$^{2+}$, and Mg$^{2+}$ were inhibitory in both forward and reverse reactions.

Key words: lactate dehydrogenase, skeletal muscle, lizard, *Agama stellio stellio*

Most poikilotherms exhibit biochemical and physiological adaptation which tend to maintain vital function at a relatively constant level against external temperature. These adaptation can be categorized with regard to the time span over which they function as instantaneous, acclimation, or evolutionary and may involve biogeographic, behavioral, physiological metabolic, or genetic characteristics of these species [1-4].

Acclimation changes in cellular metabolic rates may be in part explained by changes in the activities of the enzymes involved in the major pathway of energy metabolism, glycolysis and the Krebs cycle. Moreover, isoenzyme patterns of some enzymes such as lactate dehydrogenase were reported to be affected by various acclimations of poikilotherms [5-8].

Lactate dehydrogenase (L-lactate:NAD-oxidoreductase, EC 1.1.1.27; LDH) is a glycolytic enzyme that catalyses the conversion of pyruvate and lactate.

The structure of LDH was investigated by Cahn et al. [9], who showed the enzyme to be tetramer composed of two subunits, types M (or muscle) and H (or heart) which combined to form five isoenzymes, LDH-1 (H$_4$), LDH-2 (H$_3$M$_1$), LDH-3 (H$_2$M$_2$), LDH-4 (H$_1$M$_3$), and LDH-5 (M$_4$).

LDH isoenzymes are not formed by random subunit combination, and differences in the proportions of isoenzymes in different organs suggest a physiological basis for their existence [10-12]. Thus LDH-4 and LDH-5, which contain mainly M subunits, permit rapid accumulation of lactate and are found in tissues such as skeletal muscle where anaerobic glycolysis predominates, whereas LDH-1 and LDH-2, containing mainly H subunits, are found in tissues such as heart, where pyruvate is oxidized via the tricarboxylic acid cycle [5, 13]. Very little information is available on purified LDH from reptiles, especially lizards, as poikilothermic animals which exhibit biochemical and physiological adaptations and tend to maintain vital functions at a relatively constant level against the environment conditions. In this study we describe for the first time the basic properties of this enzyme from a skeletal muscle of the lizard *Agama stellio stellio* and show that they differ from the properties of this enzyme in other species.

**MATERIALS AND METHODS**

The lizard *A. stellio stellio* of either sex having weight ranging from 38-71 g were obtained from the north area of Jordan. All chemicals used were of analytical grade and purchased from Sigma (USA) or Fluka (Germany).

Skeletal muscle was dissected from freshly killed specimens. All extraction and purification procedures of LDH
were performed at 0-4°C unless indicated otherwise. Muscles were homogenized (1 : 5 w/v) in cold 0.05 M potassium phosphate buffer containing 1 mM EDTA (KPE buffer, pH 7.2). After centrifugation for 1 h at 20,000g, the supernatant was fractionated with ammonium sulfate (40-80% saturation) and the protein precipitates were dissolved in a minimum volume of the KPE buffer. The extract was dialyzed overnight at 4°C against the same buffer, centrifuged for 30 min at 10,000g, then applied to a colchicine-Sepharose column (2 × 1.5 cm) pre-equilibrated with 50 mM KPE containing 2.5 M NaCl. In order to remove glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.1), the column was washed with KBE buffer till $A_{280}$ became zero. LDH was then eluted from the column using 1 mM NADH in KPE, and the fractions containing maximum LDH activity were pooled and dialyzed against KPE buffer to remove NADH. The purified LDH was heated in a water bath at 80°C for 30 min and then centrifuged for 10 min at 10,000g. The supernatant was used for LDH activity measurements.

To resolve the type of LDH isoenzyme in the muscle extract and fractions, SDS-PAGE (7.5%) was used [14]. LDH isoenzymes were visualized with a staining solution containing 10 mM Tris-HCl buffer (pH 7.5), 0.75 mM NAD+, 90 mM lithium lactate, 0.37 mM nitroblue tetrazolium, and 8 µM phenazine methosulfate. The stained gels were fixed in 5% acetic acid.

Enzyme activity was measured in the forward reaction (pyruvate reduction in the presence of NADH with NAD+ formation) at 25°C in 1 ml reaction mixture containing 50 mM potassium phosphate buffer (pH 7.5), 0.18 mM NADH, 0.6 mM sodium pyruvate, and a suitable amount of enzyme to obtain a measurable decrease in absorbance. The production of NAD+ was followed by a decrease in absorbance at 340 nm. One unit (U) of enzyme is defined as the amount of enzyme that produced 1 µmole of NAD+ per 1 min under the assay conditions. The molar absorption coefficient for NADH of 6.2·10³ M⁻¹·cm⁻¹ was used [7]. The specific activity of LDH was calculated by the method of Narang and Narang [15]. For the reverse reaction (lactate oxidation in the presence of NAD+ with NADH formation), the enzyme activity was determined by measuring the increase in absorbance at 340 nm. The final concentration of the reactants in 1 ml reaction mixture was 50 mM Tris-HCl buffer (pH 9.5), 50 mM lithium lactate, 0.1 mM NAD+ and suitable amount of enzyme. In the determination of $K_m$ for the forward reaction, NADH concentration was varied in the range of 3-96 µM at a constant concentration of pyruvate (400 µM), or the pyruvate concentration was varied in the range of 3-96 µM at a constant concentration of NADH. For the reverse reaction, NAD+ concentrations of 0.1-1.0 mM were used at a constant concentration of lactate (20 mM), or the lactate concentrations 1-10 mM were used at NAD+ concentration of 2 mM. Protein concentration was determined by Bradford’s method [16] with bovine serum albumin as a standard protein.

RESULTS AND DISCUSSION

When dialyzed extract of the skeletal muscle of A. stellio stellio was applied on a colchicine-Sepharose column, the entire LDH activity was retained by the column. Addition of 1 mM NADH in KBE buffer resulted in the elution of a sharp peak of active LDH.

The LDH could not be separated into its isoenzymes by the methods normally used for mammalian