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## **KINETIC DIFFERENCES IN FISH MUSCLE LACTIC DEHYDROGENASE ON TEMPERATURE ADAPTATION\***

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On adaptation of the loach for 25 days to low (5°C) and relatively high (18°C) temperatures of the environment, the kinetics of thermo-inactivation of lactic dehydrogenase of the skeletal muscles significantly differs. In experiments on thermal inactivation, the enzyme of the skeletal muscles of fish acclimatized to low temperatures of the environment is more stable. Also characteristic of this enzyme is higher specific activity (E/mg protein). The LDH of the muscles of the loach adapted to low and high temperatures has an identical temperature optimum and similar activational energy ( $E_a$ ).

Adaptation of poikilothermal animals to different ambient temperatures is accompanied by a change in the functional and structural properties of the enzymes [1, 2]. While the mechanisms of evolutionary adaptations of the enzymes to temperature conditions have been explored in detail [1, 3, 4] the ontogenetic temperature adaptations (acclimatization) formed over several weeks have been insufficiently studied. It has been established that acclimatization of poikilothermal animals to different temperatures changes the kinetic properties of the enzymes, in particular, the Michaelis constant ( $K_{M}$ ). The magnitude  $K_{M}$  depending on the environmental temperature has a minimum in the temperature region of acclimatization [5], and the  $K_{M}$  minimum, as is known, corresponds to maximum enzyme-substrate affinity. The temperature dependence of  $K_{\rm M}$  for some enzymes (acetylcholinesterase, isocitric dehydrogenase, pyruvate kinase, lactic dehydrogenase) of different organs of invertebrates and fish acclimatized to low environmental temperatures has a minimum  $K_{M}$  in the low temperature region of measurement, while on adaptation to high temperatures this comes in the high temperature region [6-8]. In some cases these features of enzymatic kinetics are associated with the appearance of new isoenzymes with change in the temperature of the surroundings [7, 9]. However, for other enzymes, in particular, LDH, the different character of the temperature dependence of  $K_{\rm M}$  at different temperatures of the environment is unrelated to synthesis of new isoforms [10-12]. It should be noted that the kinetic differences of the two LDH variants compared disappeared after they were treated with urea followed by reactivation [11, 12]. This muscle enzyme of fish acclimatized to low temperatures possesses greater stability on thermal inactivation and on

# exposure to urea. However, the mechanisms leading to change in the kinetic properties of LDH on

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temperature acclimatization have not been established. The present work studies the kinetic properties of LDH of the skeletal muscles of the loach acclimatized to low and relatively high ambient temperatures.

## MATERIALS AND METHODS

The work was undertaken on the skeletal muscle of the loach, *Misgurnis fossilis*. To study the temperature influences the fish were adapted for 25 days to low (5°C) and relatively high (18°C) ambient temperatures. The comminuted muscle tissue was homogenized in the cold in 0.1 M *Tris*-HCl buffer, pH 6.8 and centrifuged at 15,000 g for 10 min. The LDH was purified in two stages. Ammonium sulphate with different degrees of saturation (0.72, 0.55, 0.52, 0.50) was used in the first stage for fractional salting out of the fish skeletal muscles [13]. The second stage of purification included chromatography on a FPLC column of the mono S system (LKB). The protein was eluted in the NaCl gradient (from 10 to 250 mM). The purity of the LDH preparation obtained was determined by electrophoresis [14, 15]; its activity was measured by spectrophotometry [16]. The protein concentration was determined by the Lowry method [17]. The value  $K_{\rm M}$  was calculated by the Lineweaver-Burke method using the reciprocals of the reaction rates against the substrate concentration. The experiments with thermal inactivation of LDH were conducted in a medium with 0.1 M phosphate buffer, pH 7.0.

## **RESULTS AND DISCUSSION**

For the analysis of the process of thermal inactivation of LDH of fish muscle, high temperatures were used followed by cooling of the samples of the enzyme and determination of its activity at 20°C. In addition, the LDH preparations were incubated at high temperatures for 30 min and their activity determined at these temperatures.

Study of the kinetics of thermal inactivation of LDH of the skeletal muscle of the loach acclimatized to 5 and 18°C revealed definite differences. For all inactivation temperatures studied (66, 68, 70 and 72°C) these two enzyme preparations differed in the degree of resistance to heat. The LDH isolated from the skeletal muscle of fish adapted for 25 days to 5°C ("cold" enzyme) possessed higher thermal stability than that of fish acclimatized to 18°C ("warm" enzyme) (Fig. 1). Next, it should be noted that the process of thermal inactivation of LDH of fish skeletal muscle in most cases is of a biphasic nature (Fig. 1) peculiar to enzymes with a quaternary structure [18]: a rapidly developing presteady phase and a comparatively slow steady one. The biphasic nature of thermal inactivation of the enzyme becomes manifest when the data are presented in semilogarithmic coordinates of the reaction rate ln ( $v/v_0$ ) against time where  $v_0$  is the reaction rate at the initial moment of time, and v is the reaction rate at the moment of time t. Thermal inactivation of the enzyme is characterized by the inactivation constant  $K_{in}$  estimated from the dependences of  $\ln(v/v_0)$  on time. As may be seen from Table 1 the  $K_{in}$  value for the warm enzyme is higher than that for the cold enzyme at all the temperatures studied. It should also be noted that at 66°C for the cold enzyme in contrast to the warm enzyme,  $K_{in}$  is equal to 0. At 68°C the kinetic curve of thermal inactivation of the cold enzyme, unlike the warm enzyme, consists of three portions: an initial rapidly developing stage followed by a slower one for which  $K_{in}$  was also calculated and a third stage when the enzyme activity did not change with the incubation time as already noted at 66°C in the case of thermal inactivation of the cold enzyme. Thus, the kinetics of thermal inactivation of the cold enzyme at 68°C is transitional in nature between 66 and 70°C. As Fig. 1 shows, the biphasic character of thermal inactivation at 70°C is distinctly marked for both the cold and warm forms of

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Fig. 1. Kinetics of the thermal inactivation of LDH of the skeletal muscle of the loach adapted to 5°C (1) and 18°C (2). Inactivation at (°C) 66 (a),

## 68 (b), 70 (c) and 72 (d).

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## Table 1. Inactivation constants $(K_{in})$ for the cold and warm forms of LDH of loach skeletal muscle.

LDH form	66°C	68°C	70°C	72°C
Cold enzyme	0	0,0101 ±0,0007	0,0110±0,0004	0,0719±0,0040
Warm enzyme	0,0030±0,0003	0,0142±0,0003	0,0272±0,0011	0,0910±0,0060

LDH. At 72°C the first and second thermal inactivation phases do not differ in practice for either the cold or warm enzymes by virtue of the high rates of their temperature degradation although cold LDH, as at other temperatures, is more resistant to heat than warm LDH.

To analyse the mechanism of the differences in the forms of LDH isolated from the muscles of fish adapted to different temperatures, we also determined the specific activity of the enzyme (*E*/mg protein). The specific activity of the cold enzyme measured at 20°C was higher than that of the warm enzyme. While the activity of the cold enzyme was  $176 \pm 24$ , for the warm enzyme it was  $141 \pm 14$ .

Thus, LDH of the muscles of fish adapted to low temperatures possesses not only greater stability on thermal inactivation but also higher specific activity. In general, the stability of proteins to denaturing is higher the more saturated the protein molecule by intramolecular, primarily hydrogen bonds, and hydrophobic interactions [19]. Besides these factors, the reason for the differences between the cold and warm forms of LDH of piscine muscle may be the binding of ligands and also the post-synthesis covalent modification of protein.

The reason for the higher thermal stability of the cold form of LDH than of the warm form has not been cleared up. It was established earlier that the cold enzyme of loach muscle is more resistant to the action of temperature and urea [11, 12]. These studies also showed that 3 M urea removes the differences in the kinetic properties of the two forms of LDH. From this result it was concluded that the differences noted are due to those interactions in proteins influenced by urea.

These are, as is known, hydrogen bonds [20], hydrophobic interactions [21] and the hydrate sheath [22] of protein molecules. However, the specific mechanism of the differences of LDH of loach muscle detected has not been established.

To analyse the thermal stability of the enzyme, we also studied its inactivation by high temperatures, followed by measurement of activity at these temperatures. The thermal stability of LDH of the muscle of fish adapted to 5 and 18°C was determined within the temperature range  $20-55^{\circ}$ C and it was found that the character of the dependence of activity on temperature for the cold and warm enzymes does not differ (Fig. 2). The activity of both forms of the enzyme rises in the temperature interval from 20 to 40°C, reaches a maximum at  $40-42^{\circ}$ C and drops sharply at higher temperatures, a not unexpected result, since differences in the thermal stability of homologous proteins are usually detected in organisms dwelling in different temperature conditions in nature. These differences are usually related to amino acid substitutions in homologous proteins as has been shown, in particular, for glyceraldehyde-3-phosphate dehydrogenase, LDH and ferredoxin [4]. Apparently the changes occurring in the LDH molecule of loach skeletal muscle during temperature acclimatization for 25 days are not so significant as to affect the level

# of thermal stability of this enzyme. We also determined the dependence of the maximum reaction rate (V<sub>max</sub>) on temperature in Arrhenius coordinates and calculated the activational energy (E<sub>a</sub>) for the cold and warm enzymes. In the temperature interval used (from 3 to 28°C) the Arrhenius curves were linear, indicating an absence of conformational transitions in the enzyme molecule. In this temperature interval the

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Fig. 2. Temperature optimum of the activity of LDH of the muscle of fish adapted to 5°C (1) and 18°C (2).



Fig. 3. Dependence of  $V_{max}$  on temperature in Arrhenius coordinates. Activity of LDH of the muscles of fish adapted

to 5°C (1) and 18°C (2).

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activational energy is close in value for both LDH forms (Fig. 3). For the enzyme cleared of fish muscle after adaptation to low temperature,  $E_a$  is 11.33 kcal/mole, and on adaptation to relatively high temperatures, 11.78 kcal/mole. The absence of differences in the activational energies on temperature acclimatization had earlier been shown for acetylcholinesterase of the carp brain [7] and also for isocitric dehydrogenase of trout muscle [8].

Thus, the differences in the thermal inactivation of the two forms of LDH of loach skeletal muscle do not affect the energy parameters of the reaction catalysed by this enzyme.

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