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Comparative Study on the Conformational Mobility of Muscle Lactate Dehydrogenase from Loach Fish Adapted to Different Temperatures Using Differential Scanning Microcalorimetry

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Abstract—Differential scanning microcalorimetry was used to assess the conformational stability of muscle lactate dehydrogenase (M_4-LDH) from skeletal muscle of *Misgurnus fossilis* (loach) fishes adapted to low (the "cold" enzyme) and high (the "warm" enzyme) environmental temperatures. It was found that the denaturation temperature T_d was the same for both forms of lactate dehydrogenase, whereas the denaturation enthalpy ΔH^{cal} of the "warm" enzyme was higher at all pH values studied. For the operating pH values, three stages of lactate dehydrogenase denaturation were found. These results agree with the X-ray data obtained for the dogfish muscle. The main difference between the "warm" and the "cold" enzyme forms is exhibited mostly at the first stage.

Key words: lactate dehydrogenase, temperature adaptation, differential scanning microcalorimetry, protein thermal stability, conformational transitions

INTRODUCTION

Structural and functional transformations of proteins upon changes in the environmental temperature is one of the basic mechanisms of metabolic adaptation of poikilothermic animals. The evolutionary aspects of the adaptation of poikilothermic animal proteins to different temperature conditions have been studied in detail [1–4]. The phylogenetic changes in protein thermal stability are determined, on the one hand, by amino acid substitutions, and on the other hand, by protein interactions with stabilizing factors such as cations, coenzymes, membranes, and peptides [5–7].

Conversely, ontogenetic thermal adaptation developing over the course of several weeks has been studied inadequately. It has been found that adaptation of poikilothermic animal enzymes to different temperatures is attended by changes in their kinetic features [3, 4], thermal stability, and resistance to denaturing agents [8–10].

Among other examples, we have found differences in the thermal inactivation of lactate dehydrogenase (LDH) from skeletal muscle of fishes adapted for several weeks to low and relatively high environmental temperatures [8–10]. These data, however, did not allow us to decide whether the changes taking place in LDH are microscopic (local) or macroscopic (integral). This problem was solved using the data obtained by differential scanning microcalorimetry (DSC). We have demonstrated that there is a difference in the thermodynamic parameters of denaturation of these two forms of enzyme. Specifically, the heat capacity of the native LDH form C_p(N) measured at 25°C was higher for the enzymes extracted from fishes adapted to the low temperature ("cold" enzyme) than for the enzymes extracted from fishes adapted to the high temperature ("warm" enzyme). The denaturation temperature T_d was the same for both LDH forms, whereas the specific denaturation enthalpy Δh_d was higher for the "warm" enzyme form than for the

Abbreviations: LDH, lactate dehydrogenase; DSC, differential scanning microcalorimetry.

"cold" one [11]. These data indicate that the protein undergoes a macroscopic change. It should be noted that we have not found any difference in the electrophoretic mobility between the "cold" and the "warm" LDH forms, as well as in their amino acid composition [10, 11].

It is known that the thermodynamic parameters of protein denaturation are determined both by its structure and by the environmental conditions [12]. In particular, the pH of the solution is one of the most important factors influencing the state of a protein molecule. Even a small-scale variation of the pH can result in substantial changes in the values of parameters characterizing the protein state.

In this study we used DSC for a comparative study of the influence of pH on the thermodynamic properties of LDH from skeletal muscle of loaches adapted to low and relatively high environmental temperatures.

EXPERIMENTAL

LDH extraction. *Misgurnus fossilis* loaches were kept at low (5°C) and comparatively high (18°C) temperatures for 25 days to study their thermal adaptation. Skeletal muscle tissue was minced, homogenized in the cold in 0.1 M Tris-HCl (pH 6.8), and centrifuged 10 min at 15,000 *g*. LDH was purified in two stages: fractionation with ammonium sulfate (0.72, 0.55, 0.52, 0.50) saturation [13] and column chromatography on FPLC (Pharmacia); column monoS; the initial buffer was 0.01M Na cacodylate; the NaCl gradient was from 10 mM to 250 mM; the flow rate was 1ml/min. Protein concentration determined by UV absorption at 280 nm, using a molecular extinction coefficient $\varepsilon = 1.38$ mg^{-1} ml sm^{-1}, amounted to 0.9–2.4 mg/ml.

Differential scanning microcalorimetry. We used a DASM-4 differential adiabatic scanning microcalorimeter (Institute for Biological Instrument Design, RAS) with the cell volume of 0.468 ml for microcalorimetric studies of LDH solutions. The temperature range was 10–110°C at the scanning speed of 2.0°C/min and excess pressure of 4 atm. The curves obtained were analyzed as proposed by Privalov [14, 15]. The calorimetric denaturation enthalpy ΔH^{cal} was calculated via numerical integration of the area bounded from above by the curve of the excess heat capacity and from below by a sigmoid baseline defined as the difference in the heat capacities of the native and the denatured states. The effective denaturation enthalpy was calculated using the formula:

$$\Delta H^{\text{eff}} = 4RT_{\text{d}}^2 \frac{C_p^* - 0.5\Delta C_p}{\Delta H^{\text{cal}}}, \qquad (1)$$

where T_{d} is the denaturation temperature; R is the gas constant; C_p^* is the maximal ordinate of the thermogram; ΔC_p is the difference in protein heat capacities in its native and denatured states. The number of cooperative units r (energy domains) was determined as the ratio

$$r = \Delta H^{\text{cal}} / \Delta H^{\text{eff}} . \qquad (2)$$

Deconvolution. We applied the CpCalc program (Applied Thermodynamics) to perform deconvolution of the temperature dependences of the excess heat capacity. The overall excess heat capacity was represented as a sum over n independent thermal transitions, each of which corresponded to a two-state model. The heat capacity of every such transition was determined as a derivative of the enthalpy change over the temperature:

$$C_p^i(T) = \frac{\partial H^i(T)}{\partial T} . \qquad (3)$$

For the ith transition, the change in the enthalpy is determined as the total enthalpy of the ith transition multiplied by the share of molecules having transited to the new phase:

$$H^i(T) = \alpha_i(T)\Delta H^i . \qquad (4)$$

The share of molecules in the new phase (the fraction occupancy) is determined by the equilibrium constant:

$$\alpha_i(T) = \frac{k_i(T)}{1 + k_i(T)} , \qquad (5)$$

$$k_i(T) = \exp\left[-\frac{\Delta H^i}{R}\left(\frac{1}{T} - \frac{1}{T_{\text{d}}^i} \right) \right] . \qquad (6)$$

Two parameters are subject to variation during analysis: the total enthalpy ΔH^{f} and the temperature of the ith transition T_{d}^i.

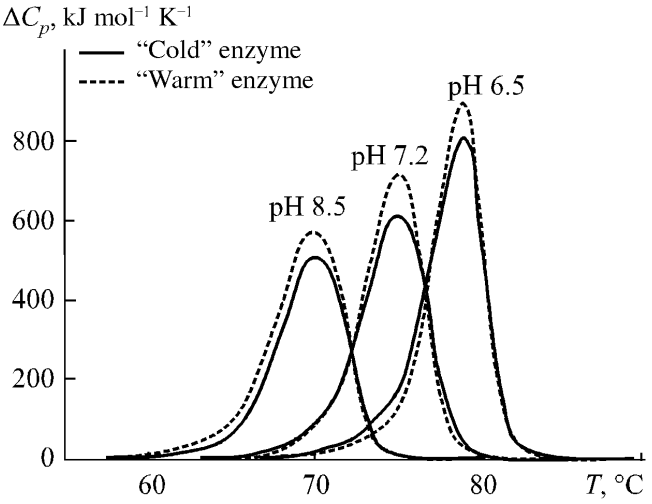

Fig. 1. Temperature dependences of the excess heat capacity of LDH. The position of the curves on the ordinate axis is arbitrary.

RESULTS AND DISCUSSION

The characteristic calorimetric melting curves of the "warm" and "cold" lactate dehydrogenase forms are displayed in the Fig. 1. They contain only one rather narrow peak of heat absorption, which corresponds to the phase transition of the molecule from its native to its denatured form. As the pH increases from 6.5 to 8.5, this peak is displaced to a lower temperature region. The results obtained are summarized in the table.

Thermodynamic parameters of denaturation of LDH from skeletal muscle of loaches adapted to 5 and 18°C at different pH values

pH	LDH form	T_d, °C	ΔH^{cal}, kJ/mol	ΔH^{eff}, kJ/mol	r
4.0	Cold	69.2	1842	728	2.5
	Warm	69.2	1988	795	2.5
4.5	Cold	76.8	2829	906	3.1
	Warm	76.8	2867	960	3.0
5.0	Cold	78.7	3212	1013	3.2
	Warm	78.7	3311	1163	2.9
5.6	Cold	79.1	3388	1153	2.9
	Warm	79.1	3612	1336	2.7
6.5	Cold	78.8	3473	934	3.7
	Warm	78.8	3656	1061	3.5
7.0	Cold	77.3	3360	854	3.9
	Warm	77.3	3570	952	3.8
7.2	Cold	75.3	3210	773	4.1
	Warm	75.3	3408	844	4.0
8.0	Cold	71.4	2982	777	3.8
	Warm	71.4	3192	781	4.1
8.5	Cold	70.0	2917	754	3.9
	Warm	70.0	3124	758	4.1

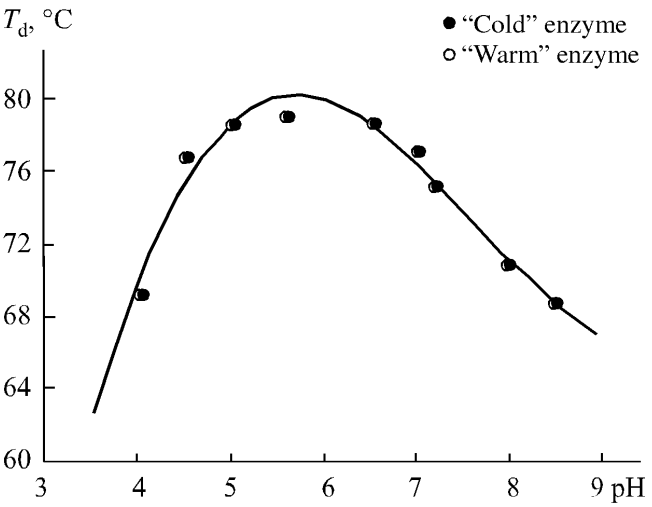

Fig. 2. The temperature of LDH denaturation transition for the "warm" and "cold" LDH forms as dependent on the pH of the solution.

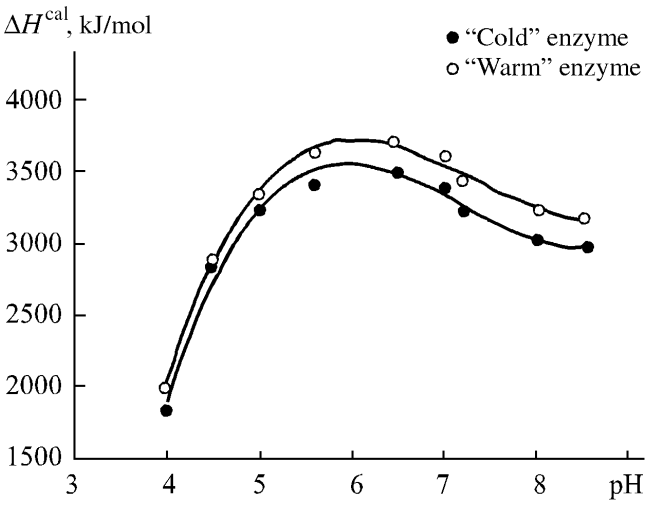

Fig. 3. The dependence of the enthalpy of denaturation transition on the solution pH for "cold" and "warm" LDH.

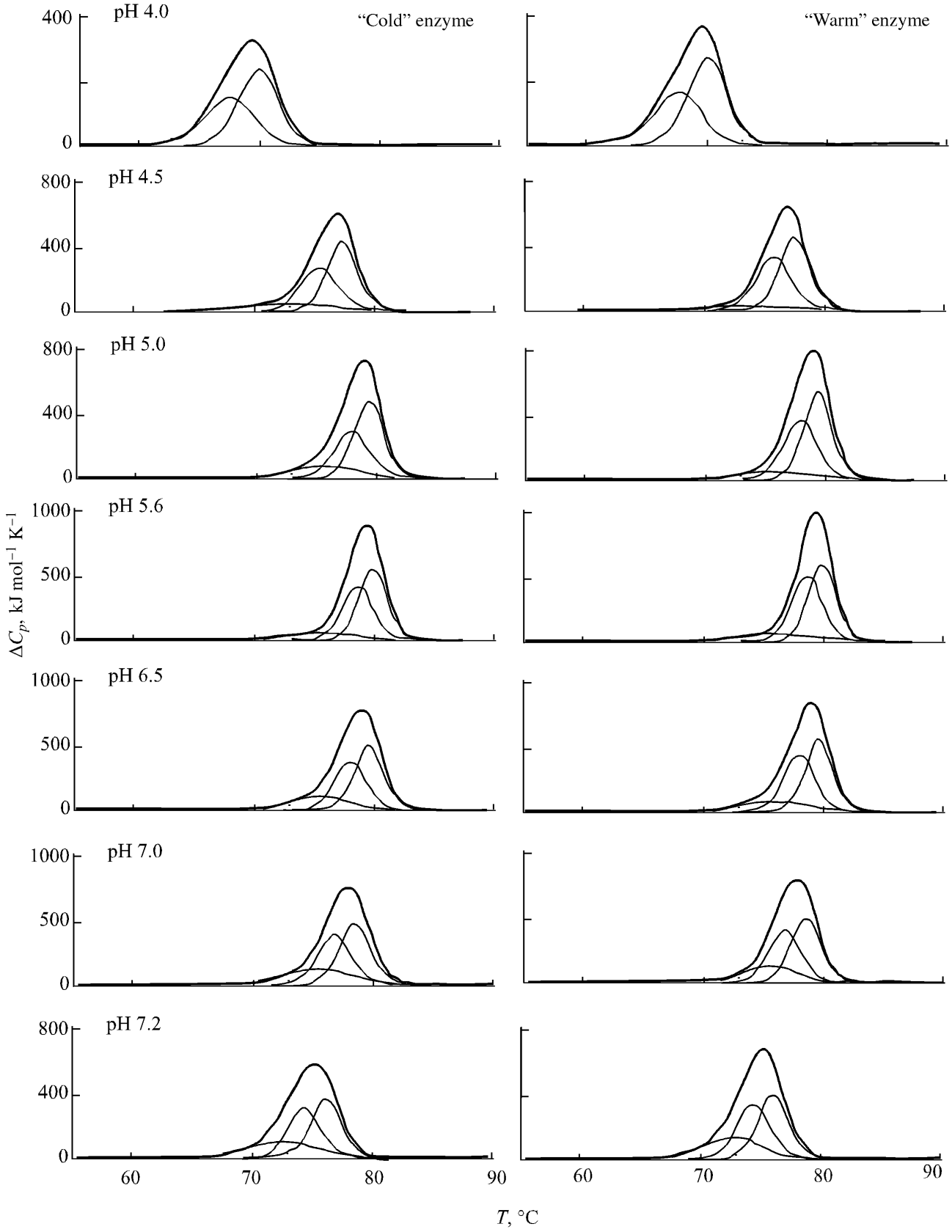

Fig. 4. The results of melting curve deconvolution for "warm" and "cold" LDH at different pH values.

It was found that the temperature of protein transition from its native to its denatured state practically does not differ for the "cold" and "warm" enzymes over the entire pH range, and attains its maximum at pH 5.0–6.5 (Fig. 2). The denaturation enthalpy is higher for the "warm" enzyme at all pH values studied, and has its maximum at pH 6.0 (Fig. 3). At pH 4.0 the specific denaturation enthalpy amounts to half of its maximal value, and the temperature of the denaturation transition is decreased by 10 K. We did not observe a phase transition at the pH 3.0, which indicates that the molecule loses its native conformation even at room temperature.

The number of cooperative units r is decreased with the pH, which indicates enhanced cooperativity of the LDH denaturation process. It should be noted that in all cases the r was somewhat higher for the "cold" enzyme form. A question arises of what intermediate states the LDH molecules passes through during the process of its denaturation. Kube *et al.* [17] assumed that the transition of LDH into its denatured form with an increase of temperature takes place in two stages: first the tetramer dissociates into dimers, which, in their turn, dissociate into monomers. For a more detailed analysis, we performed deconvolution of the calorimetric curves obtained in the pH range 4.0–8.5.

The results of deconvolution are displayed in Fig. 4. One can see that practically over the entire pH range the denaturation transition for both LDH forms consists of three independent transitions, each of which is of the "all-or-none" type, and thus corresponds to the two-state model. The data obtained agree with the X-ray studies of LDH from *Squalus acanthias* (dogfish) muscle [18], which allowed one to suggest that the molecule of this enzyme contained three energy domains. The transition temperatures T_d^i corresponding to the midpoints of the corresponding transitions are practically equal for the "cold" and the "warm" enzymes over the whole pH range.

The change in the enthalpy ΔH^i is greater for the "warm" enzyme in all cases. Therewith, the enthalpies of the second and the third transitions (as well as the overall denaturation enthalpy ΔH^{cal}) attain their maxima at a pH of about 6.0,. The dynamics of the first transition reveals a different pattern. The enthalpy of the first transition increases almost monotonously with the pH. At pH 4.0 there is practically no contribution of the first transition into ΔH^{cal}, whereas at pH 7.2 the enthalpy of the first transition amounts to 22% of the overall enthalpy (690 and 761 kJ/mol for the "cold" and the "warm" enzyme forms respectively).

Thus, the temperature denaturation of muscle LDH includes three stages, each of which takes place according to the "all-or-none" principle. Short-term adaptation of fishes to low and high environmental temperatures results in significant changes in the protein energy parameters, probably caused by alterations in the hydrate shell of this macromolecule.

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