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Thermodynamic Properties of Muscle Lactate Dehydrogenase from *Misgurnus fossilis* Fish, Adapted to Different Temperatures

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Abstract—Adaptation of loach *Misgurnus fossilis* for 25 days to low (5ºC) and relatively high (18ºC) temperatures changed the thermodynamic properties of lactate dehydrogenase (LDH) from skeletal muscle, as revealed by differential scanning microcalorimetry. The enzyme purified from fish adapted to low temperature had greater heat capacity. The denaturation temperature was the same for both LDH forms. The denaturation enthalpy for the enzyme from fish adapted to high temperature was greater than for the enzyme from fish adapted to low temperature. The number of thermodynamic cooperative units (the ratio $\Delta H_d^{cal}/\Delta H_d^{eff}$) was about two for both LDH forms.

Key words: lactate dehydrogenase, thermostability

INTRODUCTION

Protein thermal stability is often correlated with the environment temperature [1–5]. However, its functional activity may be modified by brief temperature exposures [1, 6–10]. Thus, a study of the influence of temperature on the structural and functional properties of proteins is important for understanding the mechanisms of thermal adaptation and stability of these molecules.

The change in protein thermal stability may result from the amino acid substitutions and from interaction of the protein with stabilizing factors (cations, coenzymes, membranes, peptides) [3]. Different mechanisms seem to determine the thermal stability of homologous proteins from the species living in different thermal environments, and the change in thermal stability upon short-term changes in temperature.

Thermal adaptation of the enzymes from poikilothermic species is accompanied by changes in their functional (kinetic) and physicochemical properties (thermal stability, tolerance to denaturing agents) [6–10]. Particularly, we have studied lactate dehydrogenase (LDH) from fish skeletal muscle. The fish had been adapted for several weeks to high and low environment temperature. We have found differences in the position of the Michaelis constant (K_m) minima. Activity of LDH sample affected by temperature decreased in varying degree, indicating differences in thermal stability [8, 10]. However, the physicochemical, particularly thermodynamic causes of such differences are still to be found.

Here we present a comparative study of LDH thermodynamic properties for the samples from skeletal muscle of loaches adapted to low and relatively high environment temperature, using the differential scanning microcalorimetry approach.

EXPERIMENTAL

Migurnus fossilis loaches were kept at low (5ºC) and comparatively high (18ºC) 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 for 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) [11]

and column chromatography on CM-Toyopearl. Protein concentration was determined according to Lowry [12].

We used DASM-4 differential scanning microcalorimeter (Institute for Biological Device Design, RAS) for microcalorimetric studies of LDH solutions. The temperature range was 10–100ºC at the protein concentration of 0.7–7.5 mg/ml; the scanning speeds were 0.5, 1.0, 2.0, 4.0ºC/min at excess pressure of 4 atm. The heat capacity scale was independently calibrated for each experiment using Joule's law. Methanol–water solutions were used as the calorimetric standard to check additionally the reliability of the electric calibration. Thermodynamic parameters for thermal denaturation of the protein: T_d (denaturation temperature), ΔH_d^{cal} (calorimetric denaturation enthalpy), ΔH_d^{eff} (effective (van't Hoff) denaturation enthalpy) were calculated from experimental calorimetric curves according to the Privalov's technique [13, 14]. The effective denaturation enthalpy was calculated as

$$\Delta H_d^{eff} = 2RT_d[C_p^* - 0.5\Delta C_{p,d}(T_d)]^{1/2}, \qquad (1)$$

where C_p^* is the maximum thermogram ordinate, $\Delta C_{p,d}$ is the difference between protein heat capacities in native and denatured states. The number of cooperative units R (energy domains) was determined as the ratio $\Delta H_d^{cal}/\Delta H_d^{eff}$.

Partial heat capacities of protein solutions at 25ºC temperature were calculated by the equation [13, 14]:

$$C_{p,prot} = \left[\frac{C_{p,H_2O}}{V_{H_2O}} - \frac{\Delta C_{p,sol/H_2O}}{v}\right]V_{prot} - \frac{\Delta C_{p,prot/sol}}{m}, \qquad (2)$$

where V_{H_2O} and C_{p,H_2O} are respectively partial specific volume and heat capacity of water; $\Delta C_{p,sol/H_2O}$ and $\Delta C_{p,prot/sol}$ are respectively differences in heat capacity between solvent and water and between protein solution and solvent; V_{prot} is the protein's partial volume; v is the operation volume of the calorimeter unit, and m is the mass of the protein in the calorimeter unit.

The partial specific volume for LDH was calculated by adding group impacts of amino acid residues [15].

The data on the amino acid composition of skeletal muscle LDH from loach adapted for 25 days to 5ºC and to 18ºC were obtained in cooperation with I.A. Kashparov, Protein Research Institute, RAS.

RESULTS AND DISCUSSION

The heat capacity of native LDH in solution at 25ºC, its denaturation parameters, calorimetric and effective (van't Hoff) denaturation enthalpies have been determined to compare the thermodynamic properties of skeletal muscle LDH from fishes adapted to low ("cold" enzyme) and high ("warm" enzyme) temperatures. We have also calculated the number of cooperative units or energy domains (R) for the two forms of the enzyme.

First of all, we determined C_p for the cold and warm LDH forms. To do this, we have substituted ΔC_p experimental values into equation (2). Note that ΔC_p and hence C_p do not depend on the enzyme concentration (Fig. 1). Values have been compared for two forms of LDH. For the "cold" enzyme form C_p is 1.39 ± 0.03 J g^{-1} K^{-1} and for the "warm" form it is 1.14 ± 0.05 J g^{-1} K^{-1}. The difference in the C_p for two enzyme forms indicates the "cold" enzyme to contain more total heat at 25ºC relative to the "warm" form, and seems to relate to the variations in the enzyme surface properties. The "cold" enzyme appears to have higher conformational mobility than the "warm" form, which allows the "cold" form to perform the reaction at lower temperature.

To study the thermal stability for the skeletal muscle LDH upon temperature adaptation of the fishes, we measured the dependence of the excess heat capacity on temperature for both "cold" and "warm" enzyme forms (Fig. 2). Each curve has one rather narrow heat absorption peak in the range of 70–80ºC. It corresponds to molecule transition from native to denatured state. The denaturation temperature (T_d) is the same for the two forms regardless of heating rate (table).

Our previous enzyme activity measurements indicated similar thermal inactivation of the "warm" and "cold" forms of LDH from loach skeletal muscle. Both enzyme forms were found to have the same temperature optimum [10]. As evident from the table, the denaturation temperature of these two LDH form is also the same. The apparent denaturation temperature is almost constant at low heating rates (0.5 and 1.0ºC/min), but increases slightly as the heating rate is raised.

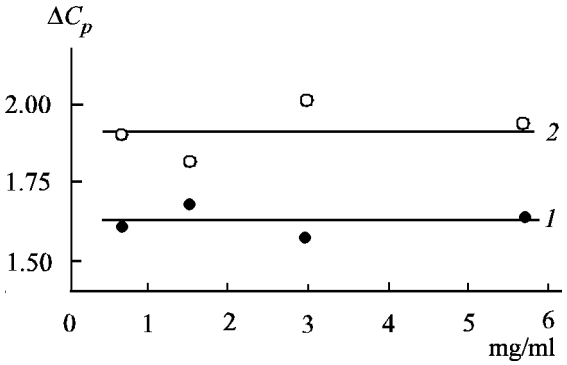

Fig. 1. Excess heat capacity for (*1*) "cold" and (*2*) "warm" LDH forms at different protein concentration.

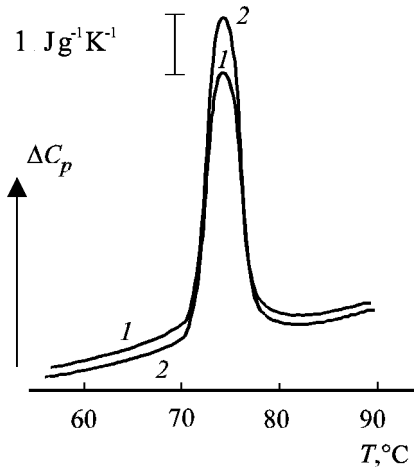

Fig. 2. Temperature dependence of the excess heat capacity for (*1*) "cold" and (*2*) "warm" LDH forms.

To characterize the denaturation process, we have determined the specific denaturation enthalpy (Δh_d) for both "cold" and "warm" enzyme forms at different heating rates. Note that Δh_d for the "warm" form is higher than for the "cold" form at all heating rates. As evident from the table, Δh_d grows with the increase of the heating rate, but the difference between the two enzyme forms remains virtually constant.

We have also calculated the number of the cooperative units (energy domains) for the "cold" and "warm" enzyme forms. In both cases R is 1.8–1.9, which corresponds to two energy domains in the LDH molecule. This calculation agrees with the two-domain organization of the fish LDH molecule found by X-ray analysis [16], and with the study of pig LDH [17]. For rabbit muscle LDH we have obtained an R value of unity [18]. However, in that study a high protein concentration was used for calorimetric measurements (19–94 mg/ml); this often results in a high impact of aggregation on the denaturation process, affecting the experimental value of R.

The comparative study of "cold" and "warm" enzyme forms, reveals differences in their C_p and Δh_d. The question of what modification in the enzyme structure during temperature adaptation causes such differences remains open.

The LDH molecule does not contain metal ions [19, 20], hence it is unlikely that they act as ligands changing the enzyme properties during fish temperature adaptation. However, LDH has been reported to bind zinc ion [21, 22]. By means of X-ray fluorescence technique, we have determined the content of calcium in the "cold" and "warm" forms of loach skeletal muscle LDH. This study has been performed in cooperation with I.A. Yamskov, Organoelement Com-

Denaturation parameters for LDH from skeletal muscle of loaches adapted to 5°C and 18°C

LDH form	Heating rate, °C/min	T_d, °C	Δh_d, J/g	ΔH^{cal}, kJ/mol	ΔH^{cal}, kJ/mol	R
Adapted to 5°C	0.5	73.7	20.1	2814	1505	1.87
	1	73.8	21.4	2996	1666	1.80
	2	75.2	20.4	2856	1626	1.76
	4	76.2	23.8	3320	1731	1.92
Adapted to 18°C	0.5	73.7	21.6	3024	1634	1.85
	1	73.8	22.8	3192	1760	1.81
	2	75.2	23.4	3276	1764	1.86
	4	76.2	25.5	3570	1848	1.93

pounds Institute, RAS. Calcium is the frequent protein ligand. The content of this element does not differ in the two enzyme forms. Neither enzyme form contained phosphorus, hence the enzyme was not phosphorylated.

The results obtained indicate a stable difference between the thermodynamic parameters of two LDH forms extracted from skeletal muscle of fishes adapted to low and relatively high environment temperatures. The denaturation transition temperatures do not vary, but the enthalpy and heat capacity values are lower for the "cold" enzyme at all heating rates studied. This may relate to minor conformational changes in the thermodynamically important enzyme regions. The other possible reason is the modification of the enzyme quaternary structure. It consists of four subunits arranged in space according to symmetry of 222 in substrate-bound apo and holo forms and according to symmetry of 2 in the complex with cofactor (NAD) [16].

It is interesting that the number of cooperatively melting regions equals not the number of protein subunits, but the number of structural domains.

Thus, the difference in thermodynamic parameters for enzymes obtained from skeletal muscle of fish adapted to high and low environment temperature may indicate structural differences of the enzyme forms, which we believe to be localized at the surface regions of the LDH molecule.

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