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# Thermostability of Lactate Dehydrogenase in Rat Brain under Conditions of Short-Term Moderate Hypothermia

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Thermostability of rat brain lactate dehydrogenase (LDH) was studied in intact animals and animals subjected to moderate short-term hypothermia. Two exponential stages, rapid and slow, were distinguished in the thermodenaturation kinetics. The contribution of the rapid phase to the lactate dehydrogenase denaturation kinetics was more significant: the energy of activation for this phase was 2.33 times lower than that for the slow phase. Moderate short-term hypothermia led to a significant decrease of lactate dehydrogenase thermostability: thermodenaturation rate constants for the rapid ($k_1$) and slow ($k_2$) phases increased. Significant changes in parameters *a* and *b* reflecting the initial proportion of the two native forms of the enzyme developed only at 40°C. As hypothermia caused no appreciable changes in the energy of activation of lactate dehydrogenase denaturation, a significant contribution of the entropic factor to the decrease of free energy of enzyme denaturation was hypothesized. The data indicated significant labilization of lactate dehydrogenase structure under conditions of moderate hypothermia.

**Key Words:** *rats; brain; hypothermia; lactate dehydrogenase; thermostability*

Hypothermia is a state of homoeothermic organism with reduced body temperature. These states naturally result from excessive cooling under the effect of an extreme thermal factor [10]. Hypothermia is more and more often used for the protection of the brain from traumatic, ischemic, and reperfusion injuries [1,7,12]. In clinical practice, moderate hypothermia (30-34°C) is primarily used, as deep hypothermia promotes the development of various side effects. The main effect of hypothermia consists in reduction of metabolism intensity in various organs or the whole organism in order to prevent threatening hypoxia or control it when it has emerged.

On the other hand, moderate hypothermia is an extreme condition for a homoeothermic organism, involving for this reason the development of many pathological processes, associated with vasoconstriction and microcirculatory disorders in tissues, shift of the oxyhemoglobin dissociation curve to the left, and activation of free radical processes [2,6]. Despite wide use of hypothermia in medicine, the molecular mechanisms of its protective effects remain not quite clear. One of the most interesting problems is the relationship between low body temperature and the work of enzymes essential for survival of animals during exposure to an extreme factor. One of these factors is lactate dehydrogenase (LDH).

LDH is the key enzyme of anaerobic glycolysis realizing mutual conversions of pyruvate and lactate and promoting recirculation of glycolysis. In addition, LDH can be indirectly involved in regulation of other catalytical reactions through modulation of the $NADH/NAD^+$ proportion in the cell [11]. We have found previously that activity of LDH in the brain increases significantly under conditions of moderate hypothermia [4,5]. The pattern of changes in the kinetic parameters of the enzyme attests to possible modification of its structure. One of the methods for studies of the enzyme molecule restructuring is evaluation of their thermostability, a protein characteristic highly sensitive to even minor changes in its structure [3].

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We evaluate the possible conformations in the brain LDH of rats subjected to short-term moderate hypothermia by analyzing the thermostability of the enzyme.

## MATERIALS AND METHODS

The study was carried out on outbred albino male rats (150-200 g). Hypothermia was induced by external cooling of animals in Plexiglas boxes with a shell through which cold (5°C) water was circulating. The rat body temperature was reduced to 30°C at a rate of 0.25°C/min (short-term (30 min) moderate hypothermia).

Mitochondrion-free cytosol from the brain of decapitated rats was prepared by differential centrifugation. The enzyme was purified from cytosol proteins by fractionation with the use of ammonium sulfate. The purified enzyme preparation was dialyzed and chromatography on Sephadex was performed.

Activity of LDH was evaluated by the decrease in NADH content in the reaction mixture as a result of enzymatic reduction of pyruvate into lactate, which was recorded by spectrophotometry (λ=340 nm, 2 min). The reaction mixture contained 2.4 ml 0.1 M PBS (pH 7.4), 0.3 ml sodium pyruvate solution (Sigma), 0.3 ml 1 mM $NADH_2$ solution (Sigma), and 0.5 ml enzymatic preparation containing 12.5 μg protein. Thermostability of LDH was evaluated by kinetics of its thermal denaturation, for which the enzyme preparation was incubated in 0.1 M PBS (pH 7.4) at 40, 45, 50, and 55°C. Aliquots were collected from the suspension at preset intervals and the residual LDH activities were measured in them. The data were used to plot kinetic curves of LDH thermal denaturation in $A_t/A_0$-t coordinates (where $A_0$ was the initial activity of the enzyme before denaturation, $A_t$ enzyme activity during t moment, and t was duration of incubation at denaturation temperature), and the kinetic parameters were calculated.

The results were statistically processed by ANOVA unifactorial analysis of dispersions with the use of Statistica software. The significance of differences was evaluated by Fisher's test at $p$=0.05. Each thermodenaturation kinetic curve is the mean of 8 independent experiments.

## RESULTS

Thermostability of the cerebral LDH in intact rats and rats subjected to short-term hypothermia was evaluated by the kinetics of thermal denaturation of the enzyme over a period of 50 min at various temperatures of incubation. The LDH thermodenaturation curve was nonlinear for intact and hypothermic rats: the decrease of the initial activity of the enzyme was rapid during the first 10-20 min, which was followed by its slow decrease (Figs. 1, 2). Hence, the entire process of the enzyme denaturation could be subdivided into two stages, differing by duration: rapid and slow.

The nonlinear time dependence of the denaturation rate could be attributed to various causes. For example, transition of one native form of the enzyme first into an intermediate (partially denatured) state, in which a certain activity was still retained, and only then transition to completely denatured state. According to this viewpoint, the slow stage of denaturation most likely reflected transition of subunits in the tetramer into a state of melted globule with complete loss of enzyme activity.

On the other hand, it was shown that all the known five LDH isoforms were present in the brain. Of these, LDH1 (in the neurons) and LDH5 (in the glia) were characterized by the greatest functional significance and highest levels [9]. Hence, presumably, these two

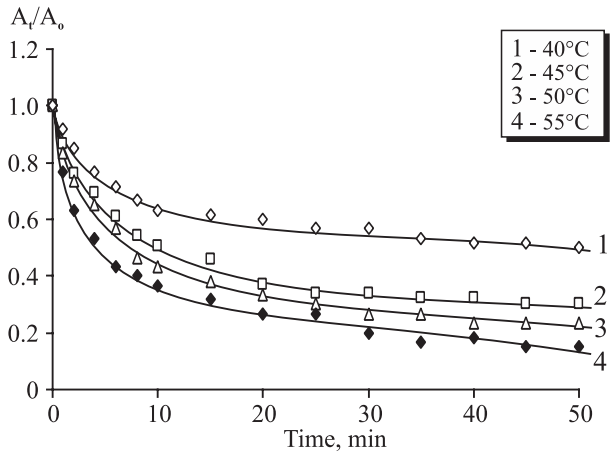


**Fig. 1.** Kinetic curves of thermodenaturion of rat brain LDH under normal conditions.

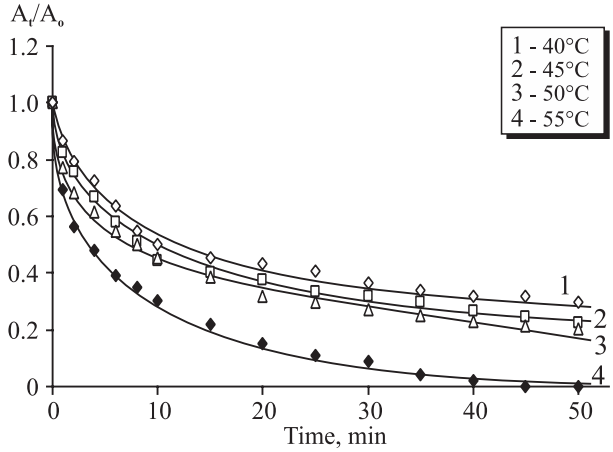


**Fig. 2.** Kinetic curves of thermodenaturion of rat brain LDH under conditions of hypothermia.

molecular forms of LDH, denaturation at different velocities, made the main contribution to the overall kinetics of LDH thermodenaturation. In addition, nonlinear kinetics of denaturation could be attributed to the presence of two types of protomers A and B, forming (in certain proportions) various isoforms of the enzyme. These hypotheses suggest the following elementary scheme of LDH denaturation:

$$N_1 \xrightarrow{k_1} D,$$
$$N_2 \xrightarrow{k_2} D$$

where $N_1$ and $N_2$ were native, $D$ denatured forms of the enzyme, $k_1$ and $k_2$ were denaturation rate constants. The specific activities of both forms were assumed to be the same in this scheme, while the denaturation rate constants differed.

This scheme conforms to denaturation kinetics, described by equation:

$$\frac{A_t}{A_0} = [a \times \exp(-k_1 t) + b \times \exp(-k_2 t)],$$

where $a$ and $b$ were initial shares of two native forms of the enzyme ($a+b=1$).

The kinetic curves (Figs. 1, 2) were plotted with the use of this equation as a regression model. Negligible deviations of experimental points from the curves indicated good agreement of the results with this theoretical model. The patterns of kinetic curves depended on the incubation temperature and the animal status.

The kinetic parameters of the brain LDH denaturation in health were calculated by nonlinear multidimensional regression analysis with the use of the above equation for the nonlinear evaluation option. The $k_1$ values were by an order of magnitude higher than $k_2$ ones at all incubation temperatures (Table 1). Hence, the rapid phase made the most significant contribution to the kinetics of LDH denaturation. Thermodenaturation rate constants increased for the rapid and slow phases with elevation of temperature: $k_1$ increased by 28.3% at 45°C, by 59.2% at 50°C, and 3.1 times at 55°C in comparison with that at 40°C; $k_2$ increased 1.8, 2.85, and 4.72 times, respectively.

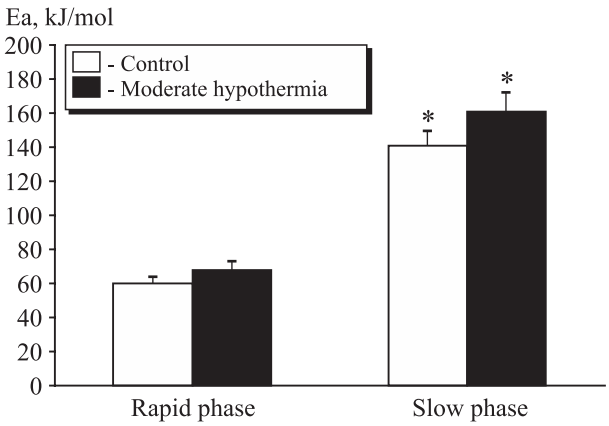


**Fig. 3.** Energies of activation of rapid and slow phases of LDH denaturation in intact rats and rats with moderate hypothermia. *$p<0.05$ in comparison with rapid phase.

Temperature dependences of $a$ and $b$ parameters shifted as follows: at 45°C the contribution of the native form $a$ to denaturation kinetics decreased by 30.5%, while the contribution of $b$ form increased by 55.1%. Further increase of incubation temperature was inessential for these parameters.

The temperature dependences of denaturation rate constants in Arrhenius coordinates were approximated by straight lines, the respective activation energy were calculated from the slope of these lines. The activation

**TABLE 1.** Kinetic Parameters of Brain LDH Thermodenaturation in Intact and Hypothermic Rats ($M \pm m$, $n=8$)

| Parameter | Denaturation temperature | | | |
|---|---|---|---|---|
| | 40°C | 45°C | 50°C | 55°C |
| Control | | | | |
| $k_1$, min$^{-1}$ | 0.162±0.013 | 0.208±0.027 | 0.258±0.030$^{+}$ | 0.504±0.035$^{+}$ |
| a | 0.644±0.058 | 0.448±0.036$^{+}$ | 0.432±0.022$^{+}$ | 0.454±0.018$^{+}$ |
| b | 0.356±0.046 | 0.552±0.032$^{+}$ | 0.568±0.031$^{+}$ | 0.546±0.026$^{+}$ |
| $k_2$, min$^{-1}$ | 0.0055±0.00044* | 0.0099±0.00025*$^{+}$ | 0.0157±0.0014*$^{+}$ | 0.026±0.0018*$^{+}$ |
| Moderate hypothermia | | | | |
| $k_1$, min$^{-1}$ | 0.228±0.0268$^{\circ}$ | 0.294±0.015$^{\circ+}$ | 0.609±0.059$^{\circ+}$ | 0.849±0.069$^{\circ+}$ |
| a | 0.497±0.031$^{\circ}$ | 0.506±0.045 | 0.429±0.039 | 0.417±0.029 |
| b | 0.503±0.028$^{\circ}$ | 0.494±0.027 | 0.571±0.024 | 0.583±0.017$^{+}$ |
| $k_2$, min$^{-1}$ | 0.0119±0.0015$^{\circ}$* | 0.0169±0.0017$^{\circ*+}$ | 0.0251±0.0019$^{\circ*+}$ | 0.0719±0.0027$^{\circ*+}$ |

**Note.** $p<0.05$ in comparison with $k_1$, $^{+}$40°C, $^{\circ}$control.

energy of rapid phase of denaturation was 2.33 times lower than that of the slow phase (Fig. 3).

Moderate short-term hypothermia promoted significant changes in the patterns of kinetic curves of denaturation (Fig. 2) and in the kinetic parameters of the enzyme denaturation (Table 1). The $k_1$ value increased by 40.7% at 40°C, by 41.4% at 45°C, 2.4 and 1.68 times at 50°C and 55°C, respectively. The $k_2$ values increased as well: 2.2 times at 40°C, 1.7 times at 45°C, 1.59 times at 50°C, and 2.76 times at 55°C. Parameters *a* and *b* changed significantly only at 40°C: *a* decreased by 22.8% and *b* increased by 41.3%.

Despite significant shifts in the constants of denaturation velocities in hypothermia, activation energies of the slow and rapid stages did not change much: activation energy of the rapid stage increased by just 12.7% and that of slow stage increased by 14.5% (Fig. 3).

Hence, our study showed that short-term moderate hypothermia promoted a significant increase in the rate of LDH denaturation. This fact indicated a decrease in the thermal stability of the enzyme, that is, labilization of its structure. The structural stability of enzymes and their catalytical activity formed a negative correlation [8], this implying that labilization of the brain LDH structure at low body temperature in a homoeothermic animal could promote an increase of enzyme activity. This hypothesis was in good agreement with our previous data on an increase in the LDH catalytical activity at low body temperature [4,5]. Interestingly that despite significant changes in the denaturation rate constants, the activation energy and hence, activation enthalpy of the respective stages changed little. Hence, the increase in the brain LDH denaturation rate constants in hypothermic animals was due to a considerable contribution of the entropic factor to the decrease of the free energy of denaturation. Presumably, the structure of LDH became more "loose" under conditions of hypothermia, this allowing its greater conformational mobility and hence, activation of the enzyme. Hence, the changes in LDH stability under conditions of hypothermia seemed to be adaptive and aimed at compensation for the effects of low temperature on the organism of a homoeothermic animal. As these changes developed within a rather short period of body temperature decrease (30 min), they were most likely caused by posttranslation modification of the enzyme.

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