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EXPERIMENTAL ARTICLES

The Effect of Hypothermia on Some Structural and Functional Characteristics of Lactate Dehydrogenase of the Rat Brain

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Abstract—We studied the activity and some physicochemical parameters of LDH in the rat brain under normal and hypothermic conditions. It was found that during hypothermia the activity of LDH in the rat brain increases, while this increase is less pronounced in an enzyme preparation purified from ballast proteins. A study of LDH self-fluorescence showed a decrease in the intensity of total fluorescence of LDH, in which the main contribution is made by tryptophan residues. Analysis of the second derivatives of the fluorescence spectra showed that hypothermia affects tryptophanyls, which are located on the periphery of the LDH molecule. Study of the binding kinetics of the fluorescent probe ANS with LDH showed the presence of at least two types of binding sites of the probe, which differ in polarity. Hypothermia leads to a decrease in the fluorescence intensity of the ANS, a decrease in the number of binding sites, an increase in the probe dissociation constants, and a shift in the inflection position on the graph of the temperature dependence of the ANS fluorescence to higher temperatures. The study of the content of sulfhydryl and carbonyl groups in the LDH molecule suggests that one of the causes of the changes in the activity and spectral characteristics of LDH in the brain of hypothermic rats is the modification of the enzyme by reactive oxygen species.

Keywords: rats, hypothermia, brain, lactate dehydrogenase, intrinsic fluorescence, ANS binding kinetics, carbonyl groups, sulfhydryl groups

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INTRODUCTION

Lactate dehydrogenase (LDH, EC 1.1.1.27) is a key enzyme in the glycolytic pathway of carbohydrate oxidation, which occurs at the junction of aerobic and anaerobic metabolism [1]. LDH is a tetrameric enzyme that performs the interconversion of pyruvate into lactate and reduced nicotinamide adenine dinucleotide (NADH) to oxidized (NAD^+) [2]. LDH has the ability to regenerate NAD^+, which makes it an especially important enzyme under conditions of oxygen deficiency, since it allows acceleration of the flow of carbon through the glycolytic oxidation pathway [3].

Many researchers believe that the main function of LDH is not the reduction of pyruvate or lactate oxidation but regulation of the NAD^+/NAD·H ratio, since it affects the rate of many catalytic reactions, as well as the transcription of genes associated with metabolism and circadian rhythms [4–6]. However, LDH can perform many other non-classical functions [2]. It has been proposed that LDH participates in the cell cycle [7] and the regulation of the activity of ATP-dependent K+ channel [8].

Special attention is drawn to the question on the functional importance of various LDH isoforms in the brain. According to the theory of the astrocyte-neuronal lactate shuttle, in the brain, glucose is metabolized to lactate by astrocytes using the enzyme LDH-5. Lactate is then secreted into the extracellular space, transported to neurons, where it is metabolized by LDH-1 to pyruvate, and acts as the main source of energy [9]. It was found that neurons in the period of their high frequency activity predominantly use lactate rather than glucose as an energy substrate [10]. More detailed studies have shown that lactate in the brain can function as a signaling molecule through the 1-hydroxycarboxylic acid receptor (HCAR1) [11] and, since it is able to quickly diffuse over long distances, act as a "volume" glio- and neurotransmitter [12]. In addition, lactate can modulate neuronal activity by acting on NMDA receptors and G-protein-coupled receptors [13].

This variety of various physiological and biochemical processes directly or indirectly associated with LDH activity suggests the need to study the molecular mechanisms of the functioning of this enzyme in various physiological states of the body, in particular, in hypothermia.

We have previously shown that after moderate (30°C) short-term (30 min) hypothermia LDH activ-

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ity in the brain significantly increased. However, the study of the kinetic characteristics suggested that the observed changes in the efficiency of enzyme catalysis are due to its structural modifications [14, 15]. However, the molecular mechanisms of these modifications are still unknown. One of the most likely causes of changes in the enzyme structure after a short period of hypothermic state (30 minutes) is modification by reactive oxygen species (ROS). It is known that moderate hypothermia, as an extreme condition for a homoiothermal organism, is accompanied by a stress response that provokes oxidative stress, a process associated with the activation of redox reactions and, accordingly, intensification of free radical processes [16, 17].

Structural modifications of LDH by oxygen radicals may be detected by changes in some of its physicochemical parameters. Both indices of the self-fluorescence of the enzyme and probe fluorescence and markers of its oxidative modification may serve as these parameters. The purpose of our study was to analyze the effect of moderate short-term hypothermia on the self-fluorescence of rat brain LDH, the kinetics of enzyme binding by the fluorescent probe 8-anilino-1-naphthalenesulfonic acid (ANS), and the content of sulfhydryl and carbonyl groups.

MATERIALS AND METHODS

Object of study. These studies were performed with white outbred rats (of both sexes) weighing 150–200 g. Animals were kept in vivarium conditions on a normal diet. The time of the experiments (from 9:00 to 12:00 a.m.) was strictly followed, in order to avoid the influence of circadian fluctuations on the result of the experiment. The animals were divided into two groups (eight animals each): (1) intact control and (2) short-term moderate hypothermia (30°C).

Hypothermic conditions. The hypothermal state was caused by external cooling of animals in plexiglass chambers with a jacket with cold (5°C) water circulation. The body temperature of the rats was reduced evenly at a rate of 0.25°C/min. Under these conditions, the body temperature of the animal reached 30°C in 25–30 min, after which decapitation was performed (short-term moderate hypothermia). The body temperature of the rats was measured in the rectum at a depth of 4–5 cm using an MS6501 rectal digital thermometer.

Isolation and purification of LDH. Mitochondria-free cytosol was obtained from the brain of decapitated rats using differential centrifugation. Purification of the enzyme from cytosol proteins was performed by the method of fractionation using ammonium sulfate. Desalination of the purified enzyme preparation was performed using gel chromatography on a Sephadex G-50. Further purification of the preparation was performed by chromatography on a carboxymethylcellulose (KM-cellulose) and subsequent affinity elution. To this end, the protein solution was applied to a CM-cellulose column, which was equilibrated with a phosphate buffer at pH 7.2. The column was then washed with 100 mL of the same buffer and then with a buffer at a pH of 8.0. During this process, different protein impurities were eluted and LDH remained bound with the carrier. Five–six volumes of buffer were passed through the column. Each time, the optical density of the eluate was measured at 280 nm. When the optical density of the eluate had already stopped decreasing, we applied buffers for elution of only LDH to the column. In each obtained fraction, we determined the LDH activity and the protein content. The study included fractions with the highest activity of LDH [18].

Determination of the activity of purified LDH. The LDH activity was determined by a decrease in the NADH content in the reaction mixture as a result of the enzymatic reduction of pyruvate to lactate, which was recorded spectrophotometrically (at 340 nm for 2 minutes). The reaction mixture contained 2.4 mL 0.1 M phosphate buffer (pH 7.4), 0.3 mL sodium pyruvate solution (Sigma, United States), 0.3 mL 1 mM $NADH_2$ solution (Sigma, United States), and 0.05 mL enzyme preparation containing 25 μg protein. LDH activity was expressed in nanomols of NADH oxidized in 1 min per mg of protein (nmol/min mg of protein).

Measurement of protein content. The protein content was determined by the method of Lowry [19].

Measurement of carbonyl groups. The content of carbonyl groups in the enzyme preparation was determined by their reaction with 2.4-dinitrophenylhydrazine (Sigma, United States) [20]. The calculation was made using a molar extinction coefficient of 22000 $(M/L)^{-1}$ cm^{-1}. The level of carbonyl groups was expressed in nmol per mg protein.

The measurement of the content of SH-groups. The content of SH-groups in the enzyme preparation was measured by a colorimetric method by their reaction with 5.5'-dithio-bis-2-nitrobenzoic acid (Sigma, United States) [21]. Concentrations were expressed in nmol per mg protein using a molar absorption coefficient of 13600 $(M/L)^{-1}$ cm^{-1}.

Measurement of LDH self-fluorescence. The self-fluorescence of lactate dehydrogenase was measured on a Hitachi F-7000 spectrofluorometer (Japan) with automatic correction of spectra. The fluorescence spectrum was recorded in the range of 290 nm $\leq \lambda \leq$ 400 nm with an excitation at 280 nm (total fluorescence) and 295 nm (tryptophan fluorescence). The spectra were processed using the Origin 8.6 program.

Analysis of probe fluorescence of lactate dehydrogenase. 1-aniline naphthalen-8-sulphate (ANS) probe (Sigma) was used as a fluorescent probe. We used enzyme preparations with the protein content of 0.05 mg/mL. One mL of the suspension containing the enzyme was incubated with a probe for 15 seconds on a magnetic stirrer. The analysis was performed on

Table 1. The LDH activity in the mitochondria-free cytosol and purified enzyme preparation from rat brain in the norm and after hypothermia ($M \pm m$, $n = 8$)

Enzyme source	LDH activity, nmole/min mg protein	
	norm	after hypothermia
Mitochondria-free cytosol	208.0 ± 16.21	342.4 ± 12.76*
Purified preparation	5986.13 ± 162.3	8986.2 ± 224.3*

* $p < 0.05$ compared to the control.

a Hitachi F7000 spectrofluorometer, λ_{exc} = 360 nm and $\lambda_{emission}$ in the range of 400–550 nm.

The dependence of the fluorescence intensity on the ANS concentration, which was added to the LDH preparation, was studied in the range of probe concentrations of 2.5–25 μM at a temperature of 25°C.

The enzyme preparation was incubated with ANS at a concentration of 12.5 μM at temperatures of 5–50°C to study the temperature dependence.

The optimum fluorescence intensity of the probe was determined from the obtained fluorescence spectra of ANS. We then plotted the dependence between the optimum fluorescence intensity and the concentration of the probe in the sample. We used nonlinear multidimensional regression analysis to calculate the probe dissociation constant using the nonlinear estimation option $y = y^*_{max}[ANS]/(K_d + [ANS])$, where y is the fluorescence intensity and y_{max} is the maximum fluorescence intensity [22].

Statistical processing of results. Data processing was performed using the one-way analysis of variance (ANOVA) using Statistica software. The significance of the differences was evaluated using the Fisher test at a significance level of $P = 0.05$. The average fluorescence spectra were obtained by averaging the spectral lines obtained in repeated experiments ($n = 8$) using Fourier filtering (5 points). Each curve in the graphs of concentration dependence and temperature dependence of the ANS fluorescence intensity is the average of eight independent experiments. The data in Table 1 are given as the mean ± the standard error of the mean.

RESULTS

Previously, all our studies of the activity and kinetic characteristics of LDH were performed using the mitochondria-free cytosol as the source of the enzyme [14, 15]. For a more detailed study of the structural and functional parameters of the enzyme it became necessary to purify the enzyme from ballast proteins. The study showed that the activity of purified rat brain LDH, which was measured at a pyruvate concentration of 3.2 mM (optimal concentration), after moderate hypothermia increases by 50.1%. At the same time, the increase in the activity of untreated LDH in the mitochondria-free cytosol with hypothermia is 64.6% of the control level (Table 1).

Fluorescence spectroscopy was used to determine the possible mechanisms of change in the activity of purified LDH. The results of the analysis of the intensity of the total (λ_{exc} = 280 nm) and tryptophan (λ_{exc} = 295 nm) LDH fluorescence in the emission range from 290 to 450 nm are shown in Figs. 1 and 2.

Figure 1 shows that the maximum intensity of the total LDH fluorescence occurs at λ = 333 nm, which may indicate the main contribution of tryptophan residues to the fluorescence spectrum of the protein [23]. This is possible in the case where the majority of the tryptophan residues of LDH are located approximately in the same hydrophobic or in a relatively rigid environment. It was shown that the core of the LDH molecule is characterized by a rather high degree of hydrophobicity [6]. In the spectra we obtained, the fluorescence of tyrosine residues was not detected, since a significant fraction of the excitation energy from tyrosine residues migrates to tryptophanyl and fluoresces as the tryptophan component [24]. After moderate hypothermia, a slight decrease occurred in the intensity of the total fluorescence of the enzyme, while there are no changes in the character of the spectra (the half-width of the spectrum and the asymmetry of the spectrum).

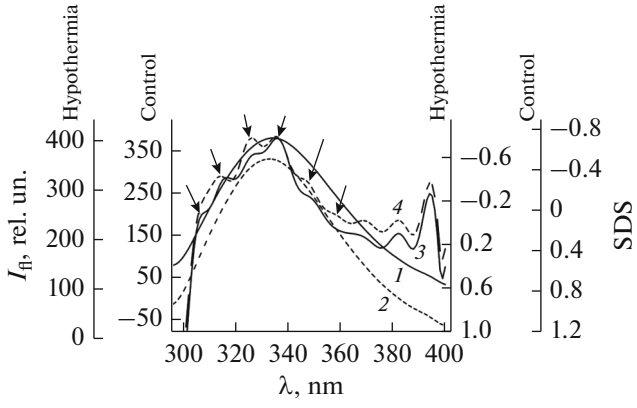

Fig. 1. The total fluorescence spectra and second derivatives of spectra (SDS) of total LDH fluorescence of rat brain in the norm and after hypothermia (spectra: (*1*) control; (*2*) hypothermia; second derivatives of spectra: (*3*) control; (*4*) hypothermia).

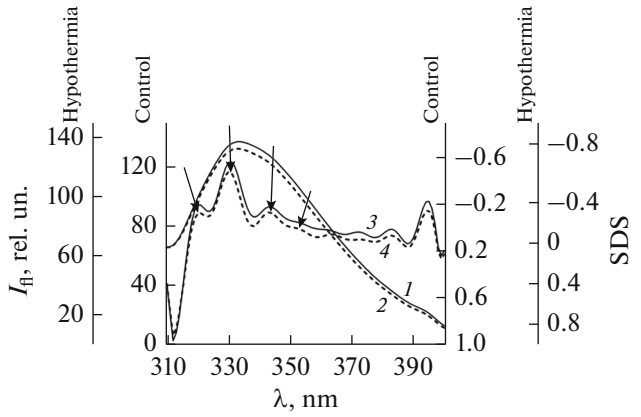

Fig. 2. Spectra of tryptophan fluorescence and second derivatives of the spectra of tryptophan fluorescence LDH of rat brain in the norm and after hypothermia (spectra: (*1*) control; (*2*) hypothermia; second derivatives of spectra: (*3*) control; (*4*) hypothermia).

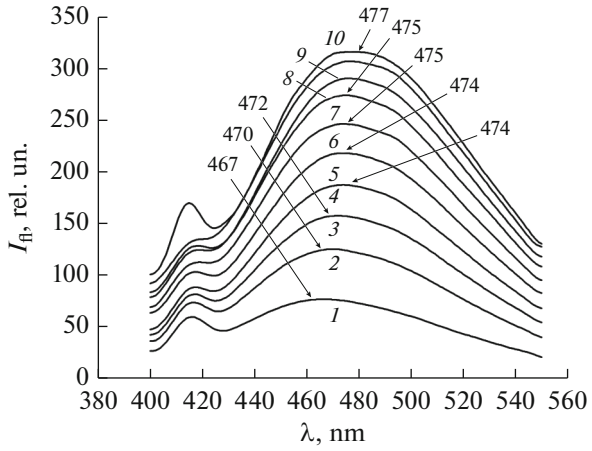

Fig. 3. The fluorescence spectra of ANS incubated with rat brain LDH at different probe concentrations: (*1*) 2.5, (*2*) 5.0, (*3*) 7.5, (*4*) 10.0, (*5*) 12.5, (*6*) 15.0, (*7*) 17.5, (*8*) 20.0, (*9*) 22.5, (*10*) 25.0 μm. The arrows indicate the wavelengths at which the ANS fluorescence intensity is at a maximum.

To analyze the fluorescence spectra, the initial spectra were analyzed, as well as their second derivatives. The second derivatives of the fluorescence spectra, compared to the initial spectra, give more detailed information on the state of the tryptophan microenvironment in proteins and help to separate the contribution of tyrosine and tryptophan fluorescence in the total spectrum [23].

Analysis of the second derivatives of total LDH fluorescence revealed a main negative peak at 338 nm and shoulders at 327 and 352 nm, which correspond to tryptophan fluorescence, and shoulders at 307 and 312 nm, which correspond to tyrosine fluorescence. Hypothermia contributes to the formation of a pronounced peak of tyrosine fluorescence in the region of 312 nm and the appearance of a clear peak at 327 nm.

Figure 2 shows the spectra of tryptophan fluorescence of the rat brain LDH in the control and after hypothermia. It may be seen from the figure that during hypothermia the tryptophan fluorescence of membrane proteins decreases slightly, while the character of the spectra does not undergo any significant change.

The second derivatives of the tryptophan fluorescence spectrum of LDH from the control animals have the main negative peak at 330 nm and additional peaks at 320 (corresponding to tryptophanyl in a hydrophobic environment) and 345 nm (corresponding to tryptophanyl accessible for polar solvent. Short-term hypothermia led to the formation of a shoulder at 355 nm. The data we obtained indicate that hypothermia affects tryptophanyls on the periphery of the LDH molecule, whereas tryptophan residues in the center of the globule do not change their position. Thus, the data of self-fluorescence of LDH indicate certain changes, both in the structure of the chromophores of the enzyme, and in its spatial configuration.

One of the most informative methods to study the conformational state of biomolecules is the method of fluorescent probing. The ANS fluorescent probe is widely applied for the characterization of the structural-dynamic properties of protein molecules [25]. ANS has a very low quantum yield in water, whereas in combination with proteins and lipids it increases considerably [26]. Thus, the fluorescence quantum yield of ANS depends on the polarity of its environment and increases in hydrophobic environments. This allows the use of ANS as a sensitive indicator of protein folding/unfolding, conformational changes, and the state of the molten protein globule. All these states of the macromolecule modify the binding parameters of ANS, which is reflected in its spectral characteristics [27].

To calculate the kinetic parameters of ANS binding with brain LDH of control and hypothermic rats, we studied the concentration dependence of the ANS fluorescence intensity in the concentration range of 2.5–25 μM during incubation of the probe with the enzymatic preparation. From Fig. 3, where the corresponding fluorescence spectra of the ANS are given, it may be seen that the type of the spectra and the position of the fluorescence maximum depend on the probe concentration.

Special attention is attracted by the fact that an increase in probe concentration shifts the maximum fluorescence intensity to the long-wavelength region. Thus, the maximum fluorescence intensity at an ANS concentration of 2.5 μM corresponds to an emission wavelength of 467 nm, while at an ANS concentration of 25 μM it is 477 nm; thus, the shift to the long-wavelength region is 10 nm. This may be due to differences in the polarity of the environment of the probe bound with the protein, which reflects the heterogeneity of

the ANS binding sites on the protein molecule. This study suggests the presence of at least two types of probe binding sites that have different affinities for ANS, some of which provide a more polar environment for the probe, while others are less polar. This is confirmed by the presence of two separate negative peaks in the second derivatives of the fluorescence spectra of the ANS (figure not shown).

Figure 4 shows the dependence of the maximum fluorescence intensity of ANS on its concentration. It is non-linear and is represented by two straight lines that intersect in the vicinity of one point. It may be seen from the figure that an increase in probe concentration leads to a linear increase in its fluorescence intensity; however, in the 10–12.5 μM concentration range, the character of the concentration dependence changes and it becomes less pronounced. The study showed that during hypothermia the fluorescence intensity of ANS incubated with LDH decreased throughout the entire range of studied probe concentrations. The presence of two linear sites on the concentration curve also supports the idea that there are at least two different binding sites.

For each linear section of the presented dependency graph we used the method of regression multidimensional nonlinear analysis with the equation $y = y^*_{max}[ANS]/(K_d + [ANS])$ (where y is the fluorescence intensity, y_{max} is the maximum fluorescence intensity, and K_d is the dissociation constant) [22] to calculate the kinetic parameters of probe binding y_{max} and K_d. y_{max} is a value that depends on the number of probe binding sites (N) and, thus, may indirectly reflect this number.

Table 2 shows that the dissociation constants of two heterogeneous ANS binding sites of rat brain LDH normally differ significantly: K_{d2} is 2.29 times higher than K_{d1}. The apparent number of ANS binding sites (N_1 and N_2) is also different. Thus, the number of the binding sites of the first type is smaller than the number of the second type by 31.9%.

Hypothermia reduces the apparent number of ANS binding sites and increases the dissociation constants. Table 2 shows that the decrease of N_1 is 21.8% and that of N_2 is 17.8%. The increase of K_{d1} is 31.2% and that of K_{d2} is 27.8%.

We evaluated the temperature dependence of the ANS binding to LDH on the basis of the probe fluorescence intensity in the temperature range from 5 to 50°C. We found that in Arrhenius coordinates it may be approximated by two lines that intersect in a neighborhood of one point. Figure 5 shows that an increase in the incubation temperature of the probe with LDH leads to a decrease in the fluorescence intensity until a certain critical temperature (in the control 33.34 ± 1.12). After this point, the fluorescence intensity begins to weakly depend on temperature and even tends to increase with a further increase in temperature.

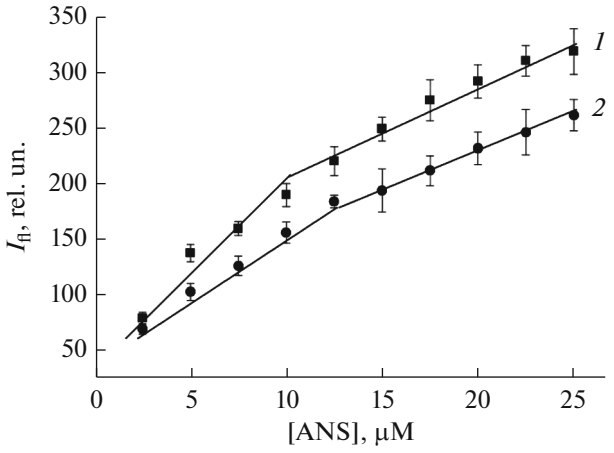

Fig. 4. The concentration dependence of ANS fluorescence during incubation with LDH of the rat brain in norm and after hypothermia: (*1*) (■) control; (*2*) (●) hypothermia.

The curve of the temperature dependence of ANS fluorescence during hypothermia is significantly lower than normal, that is, the ANS fluorescence intensity when it is incubated with LDH from the brain of hypothermic rats is lower than in the control. The graphs also demonstrate the fact that the dependence of the probe fluorescence intensity on temperature during hypothermia of rats becomes more pronounced, with a shift in the break point to higher temperatures (38.52 ± 1.52; $P < 0.05$).

The decrease in the fluorescence intensity of ANS bound to LDH after hypothermia, the increase in the dissociation constants, the decrease in the number of probe binding sites, and the change in the character of the temperature dependence may reflect structural changes in the enzyme molecule. Hypothetically,

Table 2. The kinetic parameters of ANS binding with LDH from the rat brain in the norm and after hypothermia ($M \pm m$, $n = 8$)

Animal state	N_1, arb. un.	N_2, arb. un.	K_{d1}, μM	K_{d2}, μM
Control	352.4 ± 20.4	584.8 ± 36.8	7.45 ± 0.22	17.10 ± 0.92
Hypothermia	257.5 ± 10.7*	480.5 ± 18.7*	9.78 ± 0.34*	21.84 ± 1.47*

* $p < 0.05$ compared to the control.

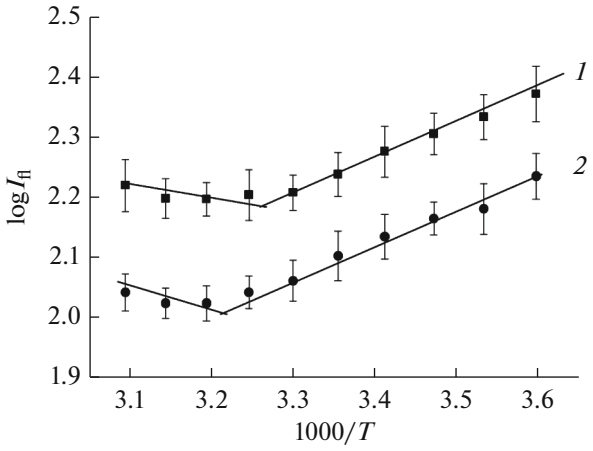

Fig. 5. The temperature dependence of the ANS fluorescence during incubation of rat brain with LDH in normal conditions and after hypothermia: (*1*) (■) control; (*2*) (●) hypothermia.

ROS, whose generation increases during hypothermia, may play a definite role in this modification.

Oxidation of the side radicals of proteins leads to the formation of products such as carbonyl groups and disulfides, which are used as markers of oxidative damage of proteins [27]. The content of disulfides increases due to the oxidation of sulfhydryl groups of cysteine residues. Thus, using the content of sulfhydryl and carbonyl groups in the enzyme molecule, it is possible to judge the degree of its oxidative modification under pathological conditions of the body.

This study showed that the content of sulfhydryl groups in rat brain purified LDH slightly decreased (by 21.5%) (Table 3). The concentration of carbonyl groups increased by 49.8%.

DISCUSSION

This study showed that with moderate short-term hypothermia, LDH activity in mitochondria-free rat brain cytosol increases, which corresponds to the previously obtained experimental data [14, 15]. Interestingly, the purification of LDH leads to a decrease in the strength of the effect of hypothermia on the enzyme activity recorded in vitro. This may support the assumption that certain soluble factors influence the activity of the enzyme in hypothermic animals and their release during the purification process led to a decrease in the activity of the enzyme. Hypothetically, these factors may include chaperones. It was found that during the acclimation of fish, chaperones contribute to the formation of various LDH conformers [29]. Hypothermia is known to increase the level of the Hsp70 chaperone [30].

β-Alanine may be proposed as another potential candidate that reversibly binds to LDH and is separated during its purification. It is known that the level of β-alanine increases under stress in various tissues of the body [31]. It possesses a chaperone-like activity and can suppress the thermoactivation of LDH in vitro [32].

In addition, it should be taken into account that LDH functions in vivo as part of a glycolytic metabolon. [33]. LDH, glyceraldehyde 3-phosphate dehydrogenase, and aldolase have been shown to form associates with F-actin [34]. Let us suppose that during hypothermia metabolon proteins associated with LDH undergo some chemical modifications, which lead to a change in the conformation of the enzyme and an increase in the rate of catalysis. In this case, LDH fractionation separates it from other cytoplasmic proteins, which may also be one of the reasons for the differences that we observed in the effects of hypothermia on the rate of catalysis of purified and unpurified LDH. It should be noted that these differences are not highly significant, that is, only 14.5%. This means that the contribution of soluble factors that are easily separated during the enzyme purification to the total change in its activity during hypothermia is negligible.

An increase in the efficiency of LDH catalysis in hypothermia may occur due to either the redistribution of enzyme isoforms in the brain or structural modifications in the enzyme molecules themselves. All five known isozyme forms of LDH were found in the brain, among which LDH1 and LDH5 have the highest contents and functional significance; their distribution in different cells and subcellular structures of the brain varies. According to the theory of the astrocyte-neuronal lactate shuttle, glucose is metabolized to lactate in the brain by astrocytes using LDH5. Lactate is then transported to neurons, where it is metabolized by LDH1 to pyruvate and acts as the main source of energy [9]. However, the study by O'Brien et al. [35] experimentally demonstrated that the LDH1 content in brain synaptosomes is 5 times higher

Table 3. The contents of sulfhydryl and carbonyl groups in the LDH preparation from rat brain after hypothermia ($M \pm m$, $n = 8$)

Animal state	SH-group content, nmole/mg protein	Carbonyl group content, nmole/mg protein
Control	760.66 ± 45.98	85.24 ± 4.97
moderate hypothermia	597.86 ± 14.97*	127.93 ± 8.01*

* $p < 0.05$ compared to the control.

than in the cytosol of cortical neurons or astrocytes. In contrast, the content of LDH5 in the synaptosol is insignificant, while in the cytosol of neurons it is 37.5%. The authors showed that in the cytosol of neurons, the spectrum of LDH isoforms has similarity with the spectrum of LDH isoforms in astrocytes. Thus, there is a differential subcellular localization of LDH isoforms in the bodies of neurons and subcellular terminals. In the terminals, due to its extremely high functional activity, LDH1 predominates to rapidly convert lactate, which comes from neighboring glial cells or from the neuronal soma, to pyruvate. A part of LDH1 is found in mitochondria and it participates there in the oxidation of lactate into pyruvate at a high rate. Mitochondrial LDH is located on the outer side of the inner mitochondrial membrane and is associated with an integral protein of the inner membrane, monocarboxylate transporter 1, which is associated with the glycoprotein basigin (CD147) and cytochrome oxidase [36]. Which LDH isoform does our enzyme preparation mainly contain? Since the mitochondria and synaptosomes (which contain the main portion of LDH1) were precipitated at a certain stage of differential centrifugation, we assume that the product we studied contains mainly LDH5.

Hypothetically, after hypothermia, the redistribution of isozyme forms of LDH in the brain may occur as a result of a change in the level of their expression or, on the contrary, via proteosomal degradation. However, in the time frame of hypothermia (the state of moderate hypothermia was reached in 30 minutes), this possibility is limited to some extent. In addition, lowering the temperature of mammals significantly slows the generation of ATP, which affects the rates of all synthetic reactions [37]. It has been experimentally shown that during hypothermia, expression of many proteins is suppressed [38, 39]. During hypothermia, not only the rate of synthesis of proteins, but also the rate of their proteasomal degradation, can decrease. This may be due to the fact that the main protein degradation element in the cell, the proteasome, is also ATP-dependent [40].

Since the probability of quantitative regulation of LDH activity in the rat brain during hypothermia is very small it may be assumed that the detected changes in LDH activity are most likely due to modification of existing LDH molecules. The mechanisms that regulate the activity of mammalian LDH still remain unclear. Rapid regulation, whose signs remain in vitro, is most likely performed by chemical modification of the enzyme, for example, by phosphorylation/dephosphorylation of an enzyme protein. It was found that oncogenic signals increase the LDH activity in mammalian tumor cells by acting through the receptor tyrosine kinase FGFR1, which directly phosphorylates LDH at tyrosine residues Y10 and Y83 [41]. A study by Zhao et al. uncovered the key role of acetylation in the post-translational modification of LDH in pancreatic tumor cells [42]. It was previously shown that LDH phosphorylation may occur not only in transformed cells but also in normal cells. In this case, a special role is assigned not to tyrosine but to Ca/calmodulin-dependent protein kinases [43].

One of the most likely causes for modification of the LDH structure during hypothermia is the oxidation of individual amino-acid residues by ROS [17]. A decrease in the body temperature of homeothermic organisms causes a stress response, which initially involves contractile thermogenesis, replaced by hormone-driven non-contractile thermogenesis. The general effect of these reactions is the intensification of oxygen consumption and an increase in the activity of oxidative metabolism. The resulting vasoconstriction, an increase in blood viscosity, and a shift of the oxyhemoglobin dissociation curve to the left leads to a decrease in the availability of oxygen for tissues, that is, hypoxia [44, 45]. Activation of oxidative metabolism during hypoxia and acidosis contributes to the development of oxidative stress and increased ROS production [16, 17, 46]. The literature data indicate the intensification of ROS formation and an impaired prooxidant–antioxidant balance in tissues during hypothermia, which stimulates the oxidative modification of cellular lipids and proteins [46]. Due to the peculiarities of their structure, proteins are among the main targets of ROS [47].

The literature data on the effect of ROS on LDH activity are contradictory, since the media that generate ROS used in experiments had different compositions. It was shown that in Fenton medium that contains Cu^{2+} ions (100 μM) and hydrogen peroxide (0.525%), the enzyme activity is significantly reduced [48]. However, in vivo ROS concentrations are different from those used in model systems; thus, their effects on the activity of the enzyme may also differ. A positive correlation was found between the LDH activity and the content of free radicals in the lizard *Uromastyx aegyptius* during acclimation to cold. On this basis it was suggested that free radicals contribute to LDH activation [49]. The results of our study on some structural and functional characteristics of LDH also suggest that changes in LDH detected during hypothermia are a result of its oxidative modification.

The study of the spectral characteristics of LDH showed that during hypothermia the intensity of its own total fluorescence decreases; tryptophan fluorescence makes the main contribution to this decrease. The decrease in tryptophan fluorescence may be explained by a change in the conformation of proteins, which leads to globule unfolding and higher accessibility of the chromophore groups of tryptophan residues to water molecules with quenchers dissolved in it [50]. On the other hand, a decrease in tryptophan fluorescence may be due to its direct oxidation by ROS. It is known that tryptophan residues are the most sensitive to modification under oxidative stress conditions [28].

It was found that W248 is located in the active center of the enzyme and is involved in the binding of the substrate [51]. Oxidation would most likely lead to a decrease in the enzyme activity. Consequently, hypothermia affects peripheral tryptophan residues and not deeply buried ones. In fact, the second derivatives of the fluorescence spectra indicate that changes in hypothermia occur only in the longer wavelength region of the spectrum that is related to the fluorescence of the tryptophan residues that are closer to the surface of the protein molecule.

Interestingly, after hypothermia, the second derivatives of the total fluorescence spectra have a distinct peak of tyrosine fluorescence. Taking the well-known fact into account that the fluorescence spectra of tyrosine chromophores practically do not differ in position and shape after significant changes in the properties of the environment [24], the observed changes may be due to the fact that the distance between tyrosine and tryptophan residues increased; this may indicate conformational changes in protein structure. Changes in the LDH conformation are also reflected by changes in the nature and location of peaks in the graphs of the second derivatives of tryptophan fluorescence, which points to an increase in the availability of this chromophore to the solvent.

The parameters of probe fluorescence may also reflect the structural modification of LDH. The study of the binding kinetics of ANS with LDH suggested the existence of at least two heterogeneous sites. An increase in the fluorescence intensity of ANS during its incubation with protein is primarily due to the hydrophobicity of the binding sites and the limited mobility of the probe in the hydrophobic "pockets" of the protein [52]. However, in addition to hydrophobic interactions with aromatic amino acids in the pockets of the protein, the probe may be linked by electrostatic interactions with peripheral amino acids of the enzyme molecule. These interactions are formed between the negatively charged sulfonic ANS group with positively charged amino acids, for example, histidine, lysine, or arginine [53]. It has been shown that additional forces are needed to stabilize ion pairs, such as van der Waals interactions. At the same time, when measuring stationary fluorescence, it was found that the contribution to ANS fluorescence from external binding sites is much smaller than from deeply buried hydrophobic sites [25].

Kinetic analysis of ANS fluorescence also demonstrates the presence of at least two binding sites that differ in polarity. This is most likely a consequence of the ANS binding to both the hydrophobic pockets of the protein (with a higher affinity for the probe) and the residues of positively charged amino acids (with a lower affinity for the probe). Hydrophobic binding sites create a less polar environment for the probe and may have a higher affinity for ANS. Therefore, they saturate more quickly at lower substrate concentrations and are characterized by relatively low values of dissociation constants. In contrast, external positively charged amino-acid sites provide a more polar environment for the probe and have lower affinity. Therefore, they are saturated at higher probe concentrations and are characterized by relatively high dissociation constants. This study showed that after hypothermia the ANS fluorescence intensity decreases. This decrease is due to both an increase in the dissociation constant of the ANS and a decrease in the number of sites of its binding.

The study of the dependence of the ANS fluorescence intensity on the temperature of incubation with LDH was undertaken based on the following assumption: in the temperature range 5–50°C, significant conformational changes may occur in the enzyme molecule, which may affect the character of its binding with the ANS probe, which is often used to study conformational changes in proteins. If the hypothesis about the chemical modification of LDH during hypothermia is correct, then the enzymes from the brain of hypothermic and intact rats should have different sensitivities to temperature. Thus, structural modifications of LDH in hypothermia may affect the conformational changes in LDH over a wide temperature range, which may be detected using the change in the ANS fluorescence intensity.

Our study showed that in different temperature ranges, the character of the dependence of the ANS fluorescence intensity on its incubation temperature is significantly different. Thus, in the low temperature range, as the incubation temperature rises, the ANS fluorescence intensity decreases, while at the high temperatures it changes only slightly.

This unusual character of the temperature dependence of the ANS fluorescence is most likely due to changes in the strength and character of weak interactions, which may also reflect the heterogeneity of ANS binding sites. Each type of site (polar and non-polar) has a different character of weak interactions with the probe and the relationship between the probe fluorescence and its incubation temperature depends on the total contribution of every interaction. A study by Begatolli et al. [54] showed the presence of two distinct binding sites for ANS in serum albumin, that is, a high affinity site located in a more hydrophobic environment and a low affinity site, where the binding of the ANS anion probe to the protein is regulated by electrostatic attraction.

It is known that hydrophobic interactions increase with temperature; therefore, one would expect an increase in the probe fluorescence intensity (binding to the hydrophobic pockets of the protein) with an increase in its incubation temperature. Since, on the contrary, the results of our study demonstrate a decrease in the ANS fluorescence intensity in the lower temperature range (in the control, 5.0–33.34°C), this may indicate a more significant contri-

bution to the binding of the probe to electrostatic interactions, rather than hydrophobic ones. This assumption is based on the work of Latipov et al. [55] who investigated the effect of temperature on the interaction between ANS and interleukin-1 and showed that at 25°C the values of K_d for ANS are lower than at 37°C. This, according to the authors, reflects the weakening of ligand–protein interactions due to increased thermal fluctuations and indicates that the ANS–interleukin-1 association is not due to hydrophobic interactions, which, as expected, should increase with temperature but due to electrostatic interactions that decrease with increasing temperature.

Hypothermia contributes to a decrease in the ANS fluorescence intensity at the high and low temperatures. This suggests that both hydrophobic binding sites and polar binding sites undergo changes in hypothermia. The increase in both probe dissociation constants during hypothermia that we found confirms this assumption.

The unusual character of the temperature dependence of the ANS fluorescence incubated with LDH may have another explanation. With an increase in the incubation temperature, the LDH molecule becomes looser and the number of hydrophobic pockets in it decreases. According to Matulis et al., the ANS fluorescence is mainly associated with the ANS localization in the region of these pockets in the protein molecule. The authors indicate that ANS that binds to the surface charged groups of the protein does not fluoresce. Denaturation of the protein is accompanied by a decrease in the number of hydrophobic pockets, which contributes to a decrease in the number of ANS binding sites and, accordingly, to a decrease in the intensity of its fluorescence [56]. Based on these considerations, it may be assumed that with an increase in incubation temperature, the structure of the LDH molecule becomes less tightly packed, the number of hydrophobic pockets for ANS binding decreases, and thus the fluorescence intensity decreases to a certain critical temperature, above which the number of hydrophobic pockets does not change. The LDH molecules of control and hypothermic animals hypothetically may differ in structure; the dynamics of their loosening as the incubation temperature rises may also differ, which affects the character of the temperature dependence of ANS fluorescence intensity.

The decrease in fluorescence of ANS incubated with LDH from hypothermic animals may be due to oxidation of aromatic amino acids that form the hydrophobic pockets of the enzyme or a change in LDH conformation, for example, by compaction, which changes the availability to hydrophobic pockets to ANS. The data on LDH self-fluorescence indicate a decrease in the intensity of its tryptophan fluorescence after hypothermia. Moreover, this decrease may be due to both the oxidation of tryptophan and its exposure to the protein surface due to a conformational transition, as well as an increase in the distance between tyrosine and tryptophan residues, which significantly reduces the likelihood of inductive-resonant energy transfer to tryptophanyls. The most likely explanation that satisfies both the data on self and probe-based fluorescence is that tryptophan residues are oxidized by ROS, which are formed during hypothermia.

Since ANS may interact not only with hydrophobic protein sites but also with positively charged amino acids, for example, histidine, lysine, or arginine [53], modification of these amino acids during hypothermia may have a significant effect on the spectral characteristics of the probe. It is known that as a result of metal-catalyzed oxidation of the proline, arginine, lysine, and histidine amino-acid residues, their carbonyl derivatives are formed [28, 57]. Compared with other forms of oxidative modification of proteins, the mechanism of their carbonylation is much more complicated and this reaction is irreversible [58]. Therefore, carbonyl groups of proteins are reliable markers of oxidative modification.

The results of our study indicate an increase in the content of carbonyl groups in proteins, which may be one of the reasons for the decrease in the fluorescence intensity of ANS in the LDH solution of the brains of hypothermic rats. It is known that residues R171 and H195, which are located in the LDH active center far from the surface of the enzyme, play an important role in catalysis [6]. It was found that their chemical modifications may lead to a significant decrease in enzyme activity [59]. Since in our case the activity of LDH increases, this suggests that only positively charged amino-acid residues on the surface undergo oxidation by ROS.

In addition to carbonyl groups, another important marker of the oxidative modification of membrane proteins is the level of sulfhydryl (thiol) groups of cysteine residues. Thiol groups play a key role in the structure and catalysis of enzymes; however, due to their reactive nature, they are often targets of free radicals [60, 61]. Under oxidative stress, SH-groups of proteins are deprotonated to form disulfides. Moreover, this oxidation may be reversible [28]. Although cysteine residues are not directly involved in catalysis, they may play an important role in the formation of the functionally significant conformation of LDH [59]. It was shown that the modification of five key cysteine residues in the LDH molecule by free radicals contributes to a change in its spatial configuration [62].

Our study has demonstrated a decrease in the level of thiol groups in LDH molecules during hypothermia; however, the activity of the enzyme increases. It is necessary to pay special attention to the fact that the difference in the content of sulfhydryl groups in LDH of control and hypothermic rats is insignificant and the level of carbonyl groups is relatively high. How-

ever, the thiol groups of proteins are more reactive; therefore, in aggregate, they form one of the links of the non-enzymatic antioxidant system of the body. The reason for the observed imbalance of markers of oxidative modification may be the reversible nature of the oxidation of cysteine residues, which is performed by antioxidants, in particular glutathione. It was previously found that during short-term moderate hypothermia the content of glutathione in the brain decreases [16, 46]. It may be assumed that a certain proportion of glutathione is spent on the regeneration of functionally important thiol groups of LDH.

Thus, our experimental data on LDH intrinsic fluorescence, kinetics, and thermodynamics of ANS binding, sulfhydryl groups, and carbonyl derivatives allow us to conclude that its modification by free radicals may be one of the causes of changes in LDH activity.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest. The authors declare that they have no conflict of interest.

Ethical approval. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution at which the studies were conducted. This article does not contain any studies with human participants performed by any of the authors.

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