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CELL BIOPHYSICS

The Kinetics of Thermal Denaturation of Acetylcholinesterase of the Rat Red Blood Cell Membrane during Moderate Hypothermia

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Abstract—The thermostability of acetylcholinesterase of rat erythrocyte membranes in the norm and moderate hypothermia was studied. It is shown that the kinetics of the thermal denaturation of acetylcholinesterase is nonlinear and corresponds to a model that involves two-step denaturation, fast and slow, of the enzyme's native form. The rate constants of the fast phase, k_1, are much higher than those of the slow phase, k_2, while the energy of the fast phase activation is lower by only 19.4% compared to that of the slow one. Short-term moderate hypothermia is shown to increase k_1 and decrease the index of relative activity of the intermediate form of acetylcholinesterase (parameter β), leading to significant lowering of the activation energies of both stages; parameter β becomes more temperature dependent. The prolongation of hypothermia up to 3 h mainly contributes to a decrease in k_1 and k_2 relative to short-term hypothermia and the activation energy of denaturation increases. These data support the hypothesis according to which the structure of acetylcholinesterase is labilized at the initial stages of the development of the hypothermic state and stabilized during prolonged hypothermia.

Keywords: acetylcholinesterase, kinetics of thermal denaturation, erythrocyte membrane, hypothermia, rats
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INTRODUCTION

Temperature is one of the most important environmental factors that determine the rate of chemical, physical, and biological processes [1]. The stability of biological structures and, first of all, the stability of molecular structures depends on temperature. A change in temperature may lead to a disturbance of the balance between the two important qualities of macromolecules: stability and lability, which may lead to impairment of their functions. For this reason, it is necessary to develop appropriate adaptive mechanisms that preserve their functional properties when the temperature of the environment and body is changed [2].

In warm-blooded animals, homeostasis of body temperature occurs in a certain temperature range. At low temperatures of the environment, when intense heat transfer occurs, the thermoregulatory mechanisms may not cope with this task and the temperature of the body can be significantly reduced. These conditions, called hypothermic, are very dangerous for homoiothermal organisms and demand an immediate reaction for survival [3]. In homoiothermal organisms, acute reaction to cold stress involves the initial contractile thermogenesis controlled by the nervous system, followed by non-contractile thermogenesis controlled by hormones. The systematic effect of these reactions is an increase in the consumption of oxygen, oxidative metabolism, and heat production. The resulting vasoconstriction, on the one hand, with an increase in the viscosity of the blood and an increase in the levels of fibrogen and the hematocrit on the other hand, impairs microcirculation, which leads to a decrease in the rate of blood circulation and the availability of oxygen for tissues, leading to disorder of their functions [4, 5]. The activation of oxidative metabolism and the resulting hypoxia and acidosis lead to an increased production of reactive oxygen species (ROS) and the development of oxidative stress [6–8]. The investigations we conducted allowed us to establish that during the short-term moderate hypothermia (the temperature of the body 30°C) and its prolongation during 1.5 h the processes of oxidative modification of both lipids and membrane proteins of rat erythrocytes are significantly activated [9]. The oxidative destruction of lipids and membrane proteins affect the structure and the properties of associated membranes enzymes, receptors, and transport proteins. One of the enzymes anchored on the outer membrane of erythrocytes is acetylcholinesterase (AChE), whose "non-neuronal" functions have received great attention in recent years. It was found that AChE can bind to the subunits of the G-protein ($G\alpha_{1/2}$ and $G\beta$) of erythrocytes and thereby influence the degree of phospholipidation of the C-terminus of the band 3 protein, which

may lead to modulation of the oxygenation processes of the erythrocyte and the metabolism of the nitrogen oxide in it [10]. Such an important role of AChE in erythrocytes suggests the need to study the mechanisms of its functioning under hypothermal conditions.

Previously, we have shown that the activity and the kinetic characteristics of AChE in the membrane of erythrocytes are significantly altered during short-term moderate hypothermia; prolongation of hypothermia up to 3 hours leads to their normalization [11]. However, the mechanisms of these changes are not completely clear. One of the approaches to studying the mechanisms of changes in the activity of enzymes at different physiological states of an organism is the investigation of their thermal stability. Thermal stability is an important characteristic of an enzyme, which is quite sensitive to the structural changes in the molecule itself and its environment [12].

The purpose of this work was to study the kinetic parameters of the thermodenaturation of AChE of erythrocyte membranes during hypothermia and its prolongation.

MATERIALS AND METHODS

Animals. The experiments were performed on male rats of the Wistar line (at the age of 3.5 months and a mass of 200–220 g). The animals were kept in the standard conditions of a vivarium with free access to water and food. During this study, all animal-testing regulations were observed (Directive 2010/63/EEC on the protection of animals used for experimental and other scientific purposes).

Hypothermia modeling . Animals were divided into three groups with eight animals in each: group 1, control (body temperature 37°C) and groups 2 and 3, animals exposed to hypothermia. Hypothermia was caused by external cooling of animals in plexiglass chambers with a sleeve with circulating cold (5°C) water. The body temperature of the rats was evenly decreased at a rate of 0.23°C/min to 30°C (short-time moderate hypothermia) and maintained at this level of hypothermia for 3 h (prolonged moderate hypothermia). The temperature was measured in the rectum at a depth of 4–5 cm with a rectal digital thermometer MS6501.

Obtaining erythrocyte membranes. After the decapitation of the animal, the blood was collected in a test tube with heparin. The blood was centrifuged at 3000 rpm for 10 min in the cold to precipitate the erythrocytes. The erythrocyte was washed three times with cold physiological solution. The membranes of the washed erythrocytes were prepared according to the Dodge method [13] after osmotic hemolysis. The white ghosts of the erythrocytes were frozen at –20°C and used the next day. The protien concentration in membrane preparation was measured by the method of Lowry et al. [14].

Determination of acetylcholinesterase activity. The activity of AChE was determined by Ellman's method [15], using acetylthiocholine iodide as a substrate (Sigma, United States). The 37°C thermostatic cuvette of a DU700 spectrophotometer (Beckman Coulter, United States) was filled with 1.45 mL of 0.1 M phosphate buffer at pH 8.0 and 0.05 mL of the suspension of erythrocyte membranes (40–50 μg protein). Next, 0.05 mL of a 0.01 M solution of 5,5'-dithiobis (2-nitrobenzoic acid) (Sigma, United States) was added. The reaction was started via the addition of 0.02 mL of an acetylthiocholine iodide solution. The change in the optical density of the solution in the course of the hydrolysis of acetylthiocholine iodide catalyzed by AChE was registered at 412 nm for 2 min at 10 s intervals relative to the control probe that contained all the components of the reaction medium except the membrane suspension. At the same time, the change in the optical control density was recorded, in which the corresponding volume of 0.1 M phosphate buffer was introduced instead of the membrane suspension. Calculation of the enzyme activity (in μmol/mg protein/min) was performed using the molar absorption coefficient of the 5-thio-2-nitropenzoate anion, which is $1.36 \cdot 10^4$ M^{-1} cm^{-1}.

The study of the kinetics of thermal denaturation of acetylcholinesterase. To determine the thermal stability of AChE, the suspension of erythrocyte membranes was incubated in a 0.1 M phosphate buffer, pH 8.0 at 40, 43, 46, and 49°C. The temperature was maintained with an accuracy of ±0.1°C

After the specified time intervals an aliquot was selected from the suspension and put on ice to stop the reaction. The residual activity of AChE with acetylthiocholine iodide was then determined as a substrate (concentration 0.5 mM) at incubation temperature of 37°C in 0.1 M phosphate buffer, pH 8.0. According to the data, the kinetic curves of the thermal denaturation of AChE were plotted in coordinates (A_t/A_0, t), where A_0 is the initial value of the enzyme activity before denaturation, A_t is the enzyme activity at the time t, and t is the incubation time at the denaturation temperature. Kinetic curves were used to calculate the rate constants of enzyme denaturation at different incubation temperatures. According to the data we obtained, the temperature dependence of the denaturation rate constants as linear anamorphoses in Arrhenius plots ($\ln K$, $1000/T$) were built and the effective activation energies were calculated based on their slopes.

Statistical processing of the results. The data was processed using single-factor analysis (ANOVA) and the Statistica software package (StatSoft, United States). The validity of the difference was determined using Fisher's criterion at the level of significance of $P = 0.05$. Each curve on the graphs of concentration

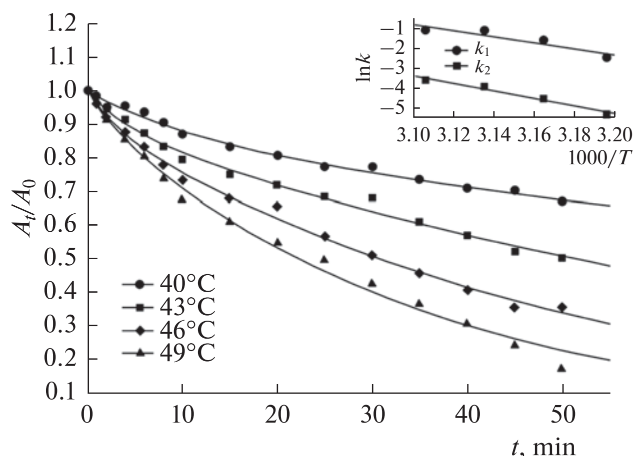
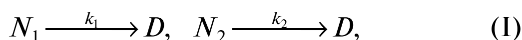


Fig. 1. Graphs of the dependence of the residual activity (A_t/A_0) of the erythrocyte membrane AChE of control rats on the incubation time at various denaturation temperatures. The dots on the graphs correspond to the experimental data. The lines are constructed by the least-squares method in accordance with the regression equation (IIa). Inset: the temperature dependence of the denaturation rate of fast (k_1) and slow (k_2) phases in the Arrhenius coordinates.

dependence is the average of eight independent experiments. The data in the table are given in the form of the mean ± error of the mean.

RESULTS AND DISCUSSION

Earlier, in the study of AChE of rat brain synaptic membranes it was shown that the kinetics of the thermal denaturation of the enzyme has nonlinear characteristics [16]. We suggested that such kinetics can correspond to a parallel scheme with two native states that denature with different rate constants:



$$N_1 \xrightarrow{k_1} D, \quad N_2 \xrightarrow{k_2} D, \tag{I}$$

where N_1, N_2 is the native form, D are the denatured forms of the enzyme, and k_1 and k_2 are the denaturation rate constants.

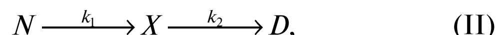
This scheme corresponds to the kinetics of denaturation, as described by the following equation [17]:

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

where k_1 and k_2 are the denaturation rate constants of two native (N_1, N_2) forms, a and b are the initial fractions of two native forms ($a + b = 1$).

In this scheme, in the implicit form it is assumed that the specific activities of both forms are identical, but their denaturation rate constants differ, which indicates the presence of two different AChE isoforms. However, the published data indicate that erythrocytes express only one molecular form of AChE on the external surface of membranes, that is, as a dimer anchored in the outer sheet of the membrane by phosphatidylinositol [18, 19]. Therefore, such a scheme and model cannot adequately describe the dynamics of denaturation of the AChE of the erythrocytes.

Thus, we analyzed the experimental data in accordance with the sequential pattern, which suggests the denaturation of one native form of the enzyme in two stages: first, the native form transits to the intermediate state; this state then transits to the denatured state [20]:



$$N \xrightarrow{k_1} X \xrightarrow{k_2} D, \tag{II}$$

where N, X and D are the native form, the intermediate state, and the denatured form of the enzyme, respectively.

The mathematical model that corresponds to this scheme has the following form:

$$\frac{A_t}{A_0} = \exp(-k_1 t) + \frac{\beta k_1(\exp(-k_2 t) - \exp(-k_1 t))}{k_1 - k_2}, \tag{IIa}$$

where $0 \le \beta \le 1$ is the relative activity of the intermediate form in comparison with the native form, which is assumed to be equal to 1.

In Fig. 1 the kinetic curves of denaturation of membrane AChE of rat erythrocytes, constructed in coordinates (A_t/A_0, t) using equation (IIa) are presented. It is seen that as the temperature of incubation increases denaturation is faster. At the same time, the kinetic curves have non-linear characteristics, which indicates complex dynamics of the denaturation process. From Fig. 1 it can be seen that the kinetics of denaturation consists of two parts, that is, an initial rapid and a second slow one.

Thus, regression analysis showed the good agreement between the experimental data of the theoretical model (Fig. 1). The parameters of the regression equation are given in Table 1.

From Table 1, it can be seen that as the incubation temperature increases, the denaturation rate constants increase, especially for the slow stage. An increase in the rate of denaturation with an increase in the temperature of incubation indicates that the rate of transition from the native state to the intermediate state and from the intermediate to the denatured state increases. In this case, the k_1 values for all incubation temperatures are higher than those of k_2. Thus, the fastest phase significantly contributes to the kinetics of AChE denaturation. The parameter β is slightly dependent on the temperature.

In the Arrhenius coordinates, the temperature dependences of the denaturation rate constants are approximated by straight lines (Fig. 1, inset), from the slope of which the corresponding activation energies (E_a) were calculated. Table 2 indicates that E_a of the fast phase is 19.4% lower than that for the slow phase. The proximity of the two AChE denaturation stages indicates that the structures of the native and interme-

Table 1. The kinetic characteristics of the thermal denaturation of AChE of rat erythrocyte membranes in the norm and under hypothermia ($M \pm m$, $n = 8$)

The parameters of the regression equation	The temperature of denaturation, °C			
	40	43	46	49
Control				
k_1, min^{-1}	0.0892 ± 0.0036	0.2259 ± 0.0102	0.3597 ± 0.0203	0.3571 ± 0.0427
k_2, min^{-1}	0.0050 ± 0.0003	0.0115 ± 0.0008	0.0198 ± 0.0008	0.0282 ± 0.0014
β	0.8217 ± 0.0592	0.8587 ± 0.0398	0.8712 ± 0.0443	0.8657 ± 0.0312
Short-term hypothermia				
k_1, min^{-1}	0.3181 ± 0.0250*	0.3031 ± 0.0134*	0.4739 ± 0.0383*	0.4180 ± 0.0242
k_2, min^{-1}	0.0068 ± 0.0008	0.0077 ± 0.0008*	0.0118 ± 0.0007*	0.0197 ± 0.0009*
β	0.7211 ± 0.0145	0.6438 ± 0.0136*	0.6443 ± 0.0110*	0.5646 ± 0.0119*
Prolonged hypothermia				
k_1, min^{-1}	0.2692 ± 0.0159*	0.2837 ± 0.0227*	0.2924 ± 0.0288^{+}	0.5414 ± 0.0591*$^{+}$
k_2, min^{-1}	0.0054 ± 0.0003	0.0070 ± 0.0005*	0.0105 ± 0.0010*	0.0281 ± 0.0014^{+}
β	0.7443 ± 0.0065	0.6605 ± 0.0115*	0.5829 ± 0.0176*	0.5725 ± 0.0180*

*, Reliable differences with respect to the control; +, reliable differences with respect to moderate short-term hypothermia.

Table 2. Energies of activation of fast and slow stages of kinetics of denaturation of AChE of rat brain in norm and under hypothermia ($M \pm m$, $n = 8$)

Condition of animals	E_a, kJ/mol	
	Fast stage	Slow stage
Control	128.60 ± 7.11	159.40 ± 9.22
Short-term hypothermia	35.28 ± 1.05*	90.23 ± 6.21*
Prolonged hypothermia	59.09 ± 1.59*$^{+}$	148.94 ± 6.98^{+}

*, Reliable differences with respect to the control; +, reliable differences with respect to moderate short-term hypothermia.

diate states are similar to each other. However, they differ from each other in their catalytic activities.

In Fig. 2, the kinetic curves of AChE denaturation in rat erythrocytes are presented for short-term moderate hypothermia. They demonstrate the fact that hypothermia promotes significant changes in the dynamics of AChE denaturation.

From Table 1 it is seen that the degree of change in the parameters of the regression equation in hypothermia depends on the temperature. Thus, at 40°C, k_1 increases by 3.6 times, at 43°C by 34%, at 46°C by 31.5%, and at 49°C by 17.1%. In this case k_2, at 40°C it increases by 36%, and at 43, 46, and 49°C, in contrast, it decreases by 33.1, 40.5, and 30.2%, respectively. The β parameter experiences insignificant changes. It decreases by 12.3% at 40°C, by 25.2% at 43°C, by 26.1% at 46°C, and by 34.8% at 49°C. At the same time this parameter is more temperature dependent in comparison with the control; it increases when the temperature is decreased.

From Fig. 2 (inset), it is seen that in hypothermia the character of the temperature dependences of denaturation rate constants changes. This affects the E_a values of the corresponding stages. The E_a of the rapid stage of the hypothermia is reduced by 72.7%, while the E_a of the slow stage of the hypothermia is reduced by 43.4% (Table 2).

The kinetic curves of the AChE denaturation in rat erythrocytes under prolonged hypothermia are presented in Fig. 3. It can be seen that the prolongation of hypothermia changes the character of the dependence of the denaturation rate on time, both with respect to the control and with respect to short-term hypothermia.

From Table 1 it can be seen that when hypothermia is prolonged, the k_1 at 40°C is decreased by 15.4%, at 43°C by 6.7%, at 46°C by 38.4%, and at 49°C by

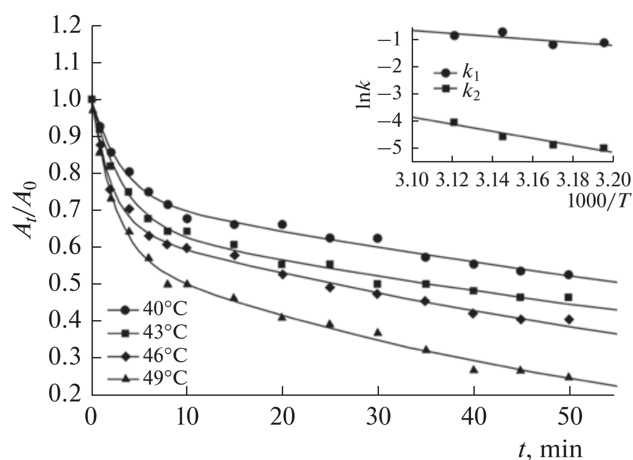


Fig. 2. Graphs of the dependence of the residual activity (A_t/A_0) of erythrocyte membrane AChE of control rats exposed to short-term hypothermia, on the incubation time at various temperatures of denaturation. The dots on the graphs correspond to the experimental data. The lines are constructed by the least-squares method in accordance with the regression equation (IIa). Inset: the temperature dependence of the denaturation rate of fast (k_1) and slow (k_2) phases in the Arrhenius coordinates.

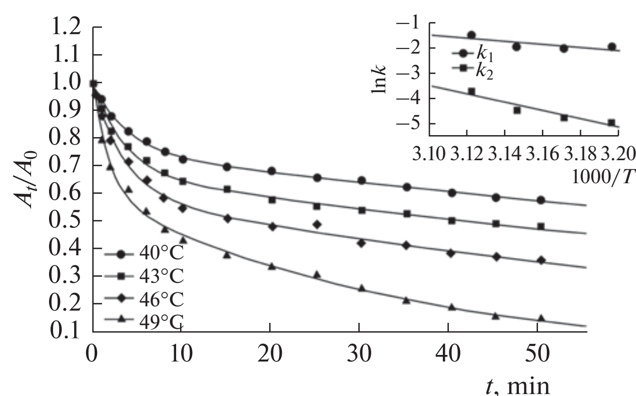


Fig. 3. Graphs of the dependence of the residual activity (A_t/A_0) of erythrocyte membrane AChE of control rats exposed to prolonged moderate hypothermia on incubation time at various temperatures of denaturation. The dots on the graphs correspond to the experimental data. Lines are constructed by the least-squares method in accordance with the regression equation (IIa). Inset: the temperature dependence of the denaturation rate of fast (k_1) and slow (k_2) phases in the Arrhenius coordinates.

29.5% with respect to short-term hypothermia. In this case, at 40, 43, and 46°C, k_2 is decreased insignificantly as well, and at 49°C, in contrast, it increases by 42.9%, achieving the level of the control. Prolongation of the hypothermic state does not have a significant effect on the β parameter with respect to short-term hypothermia (Table 1); therefore, just as in the case of short-term hypothermia, this indicator decreases with an increase in incubation temperature.

Figure 3 (inset) shows the temperature dependence of the denaturation rate constants in the Arrhenius coordinates. The character of the temperature curves differs significantly from that of short-term hypothermia.

Calculation of the thermodynamic characteristics of AChE showed that when the hypothermia is prolonged the E_a of the fast phase increases by 67.4%, while the slow phase increases by 53.7% relative to short-term hypothermia (Table 2). The obtained E_a values of the slow phase reach the control level, while the fast phase is less than the control by 54.4%.

The study showed that the kinetics of AChE denaturation of rat erythrocytes are nonlinear. These results are consistent to a certain extent with the literature. However, it should be noted that the kinetics of the thermal denaturation of AChE obtained from different sources (both in the composition of membranes and in the purified form) demonstrates significant differences. It was shown in [21] that the thermal denaturation of solubilized phosphatidylinositol-specific phospholipase C and the purified dimeric form of AChE obtained from the electric organs of *Torpedo California* has one-exponential kinetics with a high (145 kcal/mol) activation energy. Studying the same form of AChE from *Torpedo Californica*, the authors of [22] discovered two-stage kinetics of thermodenaturation. In [23], the thermodenaturation of monomeric solubilized form of AChE from lancelet muscles was studied. In this case, the kinetics of thermodenaturation of AChE also had a two-exponential character. The addition of AChE inhibitors led to a change in the kinetics of the thermal denaturation of AChE: the fast stage disappeared and the rate of the second stage constant decreased, i.e., the kinetics became monophasic. In studying the tetrameric AChE of synaptic membranes of the rat brain we also discovered the two-exponential kinetics of denaturation of the enzyme [16].

Analyzing the data, the authors of [22] concluded that the two-stage kinetics of AChE denaturation are caused by the fact that between the native (folded) state of the enzyme and the full unfolded state there is a state of partial unfolding, called a melted globule. In this state, the three-dimensional structure is broken, while the secondary structure still exists. This model of denaturation of AChE is described well by an equation of the (IIa) type. The catalytic activity of AChE in the state of the melted globule is zero. Therefore, the X state in scheme (II) is an intermediate state between the native state and the melted globule. Thus, between the native state and the total denatured state of the tetramer there are two stable states: the X state ("pseudonative" and catalytically active) and the melted globule (catalytically inactive). If we proceed from this assumption, the scheme of AChE denaturation can be more complicated.

The intermediate state is catalytically active; however, its specific activity is lower than in the native state. At the same time, this state is separated from the native state and denatured state by rather high energy barriers, since in contrast, it could not be manifested kinetically, i.e., it could not have been sufficiently populated. Thus, there are three potential wells, corresponding to three stable configurational states of AChE. The energy path profile from the native state to the denatured one in this case can be represented in the form of the following scheme (Fig. 4).

One of the important issues that arises in the analysis of thermodenaturation kinetics of erythrocyte membrane AChE is the nature of the intermediate state. Since it is catalytically active, the transition from the native state to the intermediate state may be one of the mechanisms for regulation of the enzyme activity in vivo. The dimeric structure of the erythrocyte membrane enzyme suggests that the intermediate state represents one of the dimer conformations. If the same monomeric forms of AChE can have two-exponential kinetics of thermal denaturation, then it is necessary to determine how the energy landscape of the enzyme tertiary structure depends on temperature. Due to different dependences of the electrostatic and hydrophobic interactions on the temperature, the energy landscape for the enzyme molecule can be significantly changed by changing temperature.

It is necessary to understand the nature of these states. It was shown that the tetrameric form of G4 AChE from the electric organ of *Electrophorus electricus*, depending on pH and temperature, can be crystallized in vitro in two structures: compact and more free [24]. In the compact form, two of the four active centers are shielded by neighboring subunits, and therefore are inaccessible to the substrate. The authors of [25], using methods of molecular dynamics, showed that dimers of the tetramer must have considerable mobility. They estimated that in the solution the average shielded time of two active centers in the tetramer is 20%, while the open state corresponds to 80%. The average time for maintaining an open state by a tetramer is 300 ns, while for a closed state it is 50 ns. Thus, in the solution, the compact and free forms of the tetramer are mutually transformed. We do not have any information on the existence of an opened and closed form of dimer AChE of erythrocytes. However, in this case, it can be suggested that the fast thermodenaturation stage of AChE can be connected with the transition of a "looser" form of dimer to a compact one with a partial loss of enzyme activity. The slow stage of thermal denaturation may reflect the transition of dimer subunits to the state of the melted globule with a total loss of activity.

The authors of [23], in analyzing the set of data obtained with respect to denaturation of monomeric solubilized AChE obtained from the muscles of the lancelet, assumed that the monomeric form of AChE

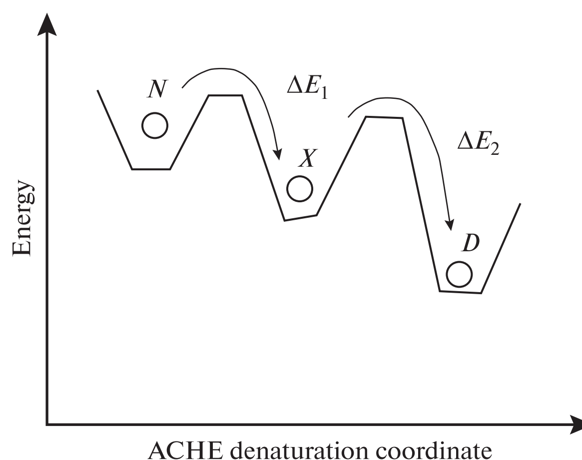


Fig. 4. The scheme of the AChE denaturation process.

can be folded into two different structures: stable and unstable. Thus, there are two stages of the thermodenaturation of the enzyme preparation. The addition of inhibitors, according to the authors, transfers the unstable conformation into the stable one. Consequently, kinetics becomes monophase. This hypothesis, if it is correct, explains our results. Based on the fact that there are no literature data on the existence of two genetic variants of AChE in the erythrocyte membranes of mammals, we attempted to explain the two-phase kinetics of the AChE thermodenaturation at the level of dimers. Since the AChE dimer is composed of two monomers, it can be assumed that one monomer is in stable conformation, and the other one is in an unstable conformation. Such thermodenaturation kinetics of the erythrocyte AChE should be described in our case by a parallel scheme and, respectively, by equation (Ia).

Thus, the experimental data we obtained are equally well described by both equation (Ia) and by equation (IIa). Therefore, based only on the results that we have it is difficult to make a choice between these options.

It is interesting to note that the rate constants of thermal denaturation of monomeric AChE from the lancelet muscle [23] and the rat erythrocyte membranes presented in our study are significantly different. The thermal denaturation in [23] was carried out at 37°C, and in the control the thermal denaturation rate constant was 0.41 min^{-1} for k_1 and 0.06 min^{-1} for k_2. In our experiments, the minimum incubation temperature when studying thermal denaturation was 40°C, while k_1 = 0.0892 min^{-1}, and k_2 = 0.0050 min^{-1}. Consequently the stability of AChE in the dimer is much higher. In a study of the kinetics of AChE thermal denaturation from rat and mice diaphragm muscles it was discovered that the monomeric forms of the enzyme are more thermolabile than tetrameric forms [26]. The authors linked these differ-

ences to the presence of disulfide bonds, which connects the subunits. What is the biological meaning of the AChE dimerization? Oligomerization usually leads to an increase in protein stability [27]. In addition, the complication of the quaternary structure gives additional possibilities for regulation of the enzyme activity.

The revealed differences in the rates of denaturation of monomeric AChE from lancelet muscle and dimer rat erythrocyte AChE may be related not only to oligomerization of the enzyme increasing its stability, but also to the effects of the lipid matrix bound to enzyme. It is known that different AChE isoforms in mammals are generated by the alternative allelic splicing of a single gene. However, the catalytic domain in all forms is the same, and the differences concern the short C-terminal domain. This domain is involved in the formation of various polymeric forms of the enzyme [28]. It was shown that the dimeric AChE forms of erythrocytes and the electric organ of the *Torpedo Californica* were inserted into the membrane by a glycophosphoinositol anchor. The glycophosphoinositol anchor of AChE is insets into membrane lipid rafts, that is, microdomains rich in glycosphingolipids and cholesterol [29].

Milkani et al. [30], studied AChE isolated with the glycophosphoinositol anchor from human erythrocytes. They discovered that the AChE activity in the buffer without a detergent is two times lower than in its presence. The activity of the enzyme was also higher in the case of its incorporation into liposomes, lipid mono- and bilayers. The authors concluded that AChE is more active in the presence of detergent due to the amphiphilic glycophosphoinositol anchor embedded in detergent micelles, thus leading to the stabilization of the enzyme.

Actually, in spite of the fact that the catalytic AChE subunits are found in the extracellular space, the enzyme “feels” the lipid microenvironment of the membrane [31]. Consequently it can be suggested that the kinetics of denaturation of membrane AChE we showed is connected not only with changes in the structure of the enzyme, but also with its lipid substrate. The effect of the lipid composition of the membrane on the temperature dependence of the AChE activity [32] corresponds to such predisposition. Changes in the character of the dependence of the denaturation rate on time can be caused by phase transitions in the rafts in a certain narrow time range, or by their lateral movements, aggregation or, in contrast, decomposition. The membrane can then fix two possible dimer configurations.

The change in the AChE thermal stability that we discovered under hypothermia probably reflects local changes in the physico-chemical state of the erythrocyte membrane in the area of enzyme attachment during transition from normothermia to hypothermia. Earlier, using a fluorescent pyrene probe, we estimated the physico-chemical state of the rat erythrocyte membrane in hypothermia. These data demonstrate that the structural-dynamic characteristics of the erythrocyte membranes depend on the duration of the hypothermia. After 1.5 h hypothermia the fluidity of the lipid bilayer of the erythrocyte membranes increases compared to the norm [11]. It is interesting that the changes in the kinetic characteristics of the AChE correlated with the indications of probe fluorescence.

The detected changes in the structural–dynamic characteristic of the erythrocyte membranes in moderate hypothermia (30° C) could be associated with a change in the lipid composition of the membranes. Analysis of the composition of erythrocyte membrane phospholipids in hypothermia showed that the amount of choline-containing phospholipids decreases, while that of phosphatidylethanolamine and its plasmogenic form increases [33]. The amount of lysophospholipids increases in membrane lipids as the temperature of the body decreases, which can have a significant effect on the viscosity of the lipid membranes. However, the question of how the lipid microenvironment influences the structural parameters of AChE, which is bound to it only by the glycophosphoinositol anchor, remains open. The data we obtained allow us to propose a hypothesis about the important role of the physico-chemical state of a lipid substrate in the maintenance of the structural stability of AChE.

It should be noted that in acute hypothermia, not only can the phospholipid and fatty-acid composition of the erythrocyte membranes change, their oxidative modification can also occur. It has been shown that the content of peroxide oxidation products of lipids increases in short-term hypothermia in erythrocytes and blood plasma [34]. Accumulation of hydrophilic products of peroxide oxidation of lipids in membranes can result in destabilization of the AChE bound with the membrane. AChE is adjacent to the phospholipid head of the membrane; electro-static interactions can occur between the amino-acid residues of the enzyme and the phospholipid heads. Oxidation of phospholipids in hypothermia may lead to a disturbance of electrostatic interactions and a decrease in the energy of the activation of the protein transition to a quasinative state.

It is interesting that the differences in the values of the denaturation rate constants of rapid and slow stages that we found are significant, while those in the energies of activation of the corresponding stages are not strongly pronounced; the rate constant of the chemical reaction depends on E_a in accordance with the Arrhenius equation:

$$k = k_0 e^{\frac{-\Delta E_a}{RT}}.$$

This fact can be explained by elementary representations of chemical kinetics if we turn to the Eyring equation. According to this equation, the rate of the

chemical process is determined by the variance in its free energy [1]:

$$k = \frac{k_B T}{\hbar} \exp(-\Delta G^* / RT),$$

where k is the rate constants, k_B is the Boltzmann constant, $\hbar$ is the Planck constant, and ΔG^* is the free energy of activation.

At the same time, variance in the free energy of any process can be calculated according to the following formula:

$$\Delta G = \Delta H - T\Delta S.$$

It is seen from the formula that the enthalpy of activation ΔH and the activation entropy ΔS can change the free energy. The change in enthalpy can be calculated from the formula:

$$\Delta H = E_a - RT.$$

This means that it is mainly connected with the change of E_a. In our case, the E_a of the rate constants of the fast and slow phases is not significantly different, compared with the rates by themselves. This indicates that these two stages of thermal denaturation differ not only in energies (activation enthalpies), but also by activation entropies. In other words, the second stage of thermal denaturation not only has a high E_a, but also has a high activation entropy. This means that the transition from the intermediate to the denatured state (or into the state of the melted globule) is accompanied by more significant changes in the structure of the enzyme molecule than the transition from the native to the intermediate state. This is consistent with the fact that the structure of the intermediate state is closer to the native state than to the denatured one, since the intermediate state is catalytically active.

This study showed that moderate hypothermia results in a decrease in the stability of the erythrocyte AChE. This is shown by the significant increase in the rate of AChE denaturation at all incubation temperatures (Fig. 2). At the same time, the temperature dependence of the denaturation rate constants decreases significantly; consequently, the calculated effective activation energies decrease as well. It is interesting that the changes in the denaturation rate constant of the slow phase are not as expressed and unambiguous. Nevertheless, the dependence of this parameter on the temperature also decreases, as in case with the rate constant of the fast phase. Since the main contribution to the kinetics of denaturation is made by the fast stage it can be concluded that the stability of the AChE under hypothermia is significantly reduced. At the same time, the proportion of the enzyme present in the intermediate state decreases, which may be related with the decrease in the lifetime of the intermediate state.

It is important to note that hypothermia promotes the decrease in the specific activity of the intermediate form. This can be explained by the fact that in the erythrocyte AChE of rats exposed to hypothermia, the native condition is more rapidly transformed into a less stable intermediate state. At the same time, the parameter β becomes a temperature-dependent value: it decreases with an increase in incubation temperature. This can be explained by the fact that at different incubation temperatures the structure of the intermediate state is different, i.e., the trajectory from the native state to the denatured one depends on the temperature. It is natural to expect that at higher temperatures the less compact conformation of the intermediate state, which has a lower specific activity, is stabilized.

The activation energy of the first stage under hypothermia is reduced by 93.3 kJ/mol. This difference roughly corresponds to two additional weak bonds, which stabilize the original state in the control. The E_a of the second stage decreases by 69.1 kJ/mol. This value is also found in the energy dissipation regions of weak interactions. Thus, the decrease in the stability of the AChE under hypothermia is likely related to the disintegration of several weak bonds, due to this, less energy is required for the denaturation of the enzyme.

Short-term 30°C hypothermia leads to an increase in the rate constants of both stages of denaturation compared with the control, where the incubation temperature is 40°C. However, at higher incubation temperatures, the rate constants in the control and in hypothermal states are closer to each other. Why does the difference between the control and hypothermia decrease at high temperatures? It can be suggested that high temperatures destabilize the AChE structure; thus, the effects of hypothermia on the kinetics of denaturation are not as pronounced as at low denaturation temperatures.

It is well known that structural stability and catalytic activity have a negative correlation [2], i.e., the more rigid the structure of the protein is, the slower it works. Therefore, the stabilization of the structure can be accompanied by a decrease in catalytic activity and vice versa. It should be noted that in the case of moderate short-term hypothermia the catalytic efficiency of AChE of erythrocyte membranes is significantly increased [11]. At the same time, the main contribution to the change in the efficiency of catalysis was caused by a decrease in the Michaelis constant, which supported the hypothesis of the structural changes in the enzyme molecule.

What are the molecular mechanisms of the changes in the thermal stability of erythrocyte AChE that are associated with changes in its activity for such a short

period (0.5–3 h) as the body temperature decreases? The thermostability of the enzyme is always sensitive to changes in the structure of the enzyme [12]. Since there is no protein-synthesizing apparatus in erythrocytes and genetically determined structural variants of the enzyme are not known, the changes in the thermal stability of the enzyme are probably connected with its chemical modification. Phosphorylation–dephosphorylation of the enzyme protein may be one such mechanism. As shown in [35], phosphorylation leads to a several-fold increase in AChE activity but does not change the K_M value of the enzyme. Although there are ecto-protein kinases and phosphatases in the blood [36], whether phosphorylation of AChE subunits exposed to the plasma of erythrocytes can occur remains unknown.

AChE is quite sensitive to ROS. Their effects on AChE depend on the type of ROS, their concentration, and the time of exposure. Thus, it was shown that the incubation of brain homogenates in a medium with $FeSO_4$ (84 μM) and ascorbic acid (400 μM) causes enzyme activation during the first 15 min and inhibition after 1.5 h [37]. It was found that hydrogen peroxide has a dose-dependent effect on the activity and kinetic characteristics of AChE. The incubation of the soluble and membrane-bound erythrocyte AChE [38], as well as the recombinant human AChE [39], in the medium with low concentrations of H_2O_2 (10^{-6} M), led to an increase in the enzyme activity, while high concentrations of H_2O_2 (10^{-3} M) had a significant inhibitory effect. The authors assume that the observed effects of H_2O_2 are linked with the modification of the lipid matrix of the erythrocyte membranes [38], or with a change in the degree of oxidation of the methionine, cysteine, and tryptophan residues in the enzyme [39]. It was shown that the activity of AChE from human neuroblastoma cells greatly increased after 24-h incubation with H_2O_2 (in the range of concentrations 1–1000 μM). The results of a kinetic study showed that H_2O_2 affects V_{max}, rather than K_M, and changes the sigmoid kinetics observed in the control samples to hyperbolic kinetics [40]. In addition, H_2O_2 (in the range of 100–1000 μM) reduced the total content of AChE and modified its isoenzyme profile.

Thus, a set of experimental data indicates that H_2O_2 can play a regulatory role in the functioning of AChE.

In the light of these data, it should also be noted that in the case of short-term hypothermia in rats, we found a positive correlation between the increase in the AChE activity [11] and the degree of oxidative modification of lipids and membrane proteins of erythrocytes [9]. We suggested that AChE activation under hypothermia may be the result of a "soft" oxidative modification of the enzyme under the action of ROS at the initial stages of the hypothermia. In this case, the "soft" oxidative modification of the enzyme can lead it to a quasi-native state, as in the modification of AChE thiol groups of the electric organ of *Torpedo California* by mercury chloride [21]. Under an increase in temperature this condition can transit to the melted globule state. In the quasi-native state, due to the influence of active oxygen forms, AChE undergoes faster denaturation than in the native state, as shown by the values of the denaturation rate constants and the activation energy of the AChE denaturation of erythrocytes of animals exposed to hypothermia. Weiner's work, in which it was shown that during the incubation of purified AChE from the electric organ of *Torpedo Californica* for 2 to 5 h in a medium that generates oxygen radicals (ascorbate–H_2O_2–Fe^{3+}) the transition of the enzyme to a partially unfolded state similar to the melted globule with increased sensitivity to proteolysis occurs, may support of such a prediction [41]. It can be suggested that, in this situation, the enzyme will be very sensitive to these effects and other physico-chemical factors, including the temperature.

We note that the decrease in the thermal stability of the AChE that we discovered during short-term hypothermia turned out to be reversible in the case of 3-h prolonged hypothermia. It was shown that prolongation of moderate hypothermia during 3 h reduces the intensity of free-radical processes in cells, including erythrocytes, due to an increase in the content of antioxidant protection components (glutathione and sulfhydryl groups of membrane proteins) and an increase in the activity of superoxide dismutase [42]. This is consistent with the data in [43], in which the direct correlation between the overall antioxidative activity of the blood plasma and the activity of the AChE of the human erythrocyte membrane was discovered: the higher the antioxidative activity is, the higher the activity of the enzyme is.

Since the AChE catalytic subunits in erythrocytes are oriented into the extracellular space it is quite logical that the activity of the enzyme is regulated by the antioxidant status of the plasma. The prolongation of hypothermia by increasing the active antioxidant protection of plasma and erythrocytes probably leads to recovery of oxidized amino-acid residues and to an increase in the effective activation energy of the AChE thermodenaturation to the level of control. It has been revealed that the thermal stability of drosophila AChE can be changed by the reduction and oxidation of the most important amino-acid residues [44]. Thus, the changes in the activity of AChE and its kinetic characteristics, as well as the AChE thermostability that we revealed earlier correlate well with the data on the change in the intensity of free-radical processes in the erythrocytes in moderate hypothermia of different durations.

On the other hand, red blood cells that are old or were damaged in short-term hypothermia can be attacked by phagocytes [45] and removed from the bloodstream and undergo intravascular hemolysis, while new erythrocytes can enter the blood from the bone marrow [34]. The structural–functional characteristics of AChE, including the thermostability of the younger population of red blood cells, may differ from the long-circulating population, since it has been shown that the activity of AChE depends on the age of the erythrocyte [43].

Thus, the results of this study, which indicates the decrease in the stability of the AChE structure, and consequently an increase in its lability, are consistent with the earlier data on the enhancement of the catalytic efficiency of the enzyme at low body temperatures. Thus, it can be suggested that the change in the thermolability of the AChE under hypothermia is adaptive and allows compensation of the effect of low-temperature by labilization of the enzyme structure. This form of adaptation is widely known in poikiloterms and is well described in the classical works of Hochachka and Somero [1].

It is of particular interest that the prolongation of hypothermia to 3 h leads to an increase in the thermal stability of AChE, as shown by an increase in E_a denaturation, thus achieving a control value. In a study of the activity and kinetic characteristics of AChE we showed that the prolongation of hypothermia results in their normalization [11]. Thus, the data of this study are in good agreement with the results of the previous studies and indicate that changes in the thermal stability and activity of AChE depend on the duration of hypothermia and appear to be reversible.

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