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TROUT ERYTHROCYTE MEMBRANES
Thermoresistivity and thermally induced structural transitions

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Abstract

Differential scanning microcalorimetry and equilibrium thermohemolysis procedure were used to study the effect of acclimation temperature on thermally induced transitions and thermoresistivity of fish (trout) erythrocyte membranes. Strong correlation has been found between the rates and activation energies of erythrocyte thermohemolysis and acclimation temperature. Transition temperatures of five thermodynamically irreversible and one partially reversible transitions at about 87°C as well as the overall shape of microcalorimetric curves of the erythrocyte ghosts do not vary with acclimation temperature. The results suggest an essential conservation of phospholipid microenvironment of membrane skeleton proteins despite the compensatory response in lipid composition of erythrocyte membrane bilayer.

Keywords: microcalorimetry, thermohemolysis, trout erythrocyte membranes

Introduction

According to the concept of 'homeoviscous adaptation' [1–3] the poikilotherms, unlike homeotherms, are able to adjust their cell membrane bilayer order to altered temperature conditions. This is aimed at the independence from variations of temperature of the membrane functions. Despite its importance for the understanding of the molecular basis of membrane function underlying cellular sensitivity to heat, not much has been reported on the details of the maintenance of the thermal stability characteristics of those membranes which experience disturbances imposed by variations in temperature. So, the effect of regulation of bilayer order is not clearly revealed. A major factor of the regulation is most likely the adjustment of the membrane lipids as it was widely accepted that poikilotherms change their cell lipid composition in response to temperature alterations.

The substantial body of knowledge obtained mostly on model systems indicate that the denaturational parameters of proteins incorporated into various phospho-

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lipids is determined by the properties of the lipid environment [4, 5]. The most feasible approach to consider the relationship between the adaptive regulations of membrane lipid composition and denaturational and structural properties of membrane is to study the cells of poikilotherms acclimated to different ambient temperatures. The studies of human erythrocytes using DSC and thermoresistivity measurements in parallel have been aimed so far at elucidation of thermohemolysis mechanisms and have brought a considerable evidence for the critical event in thermosensitivity associated with the erythrocyte membrane [6, 7]. Differential scanning microcalorimetry (DSC) also allowed to investigate protein involvement in structural transitions of various mammalian erythrocyte membranes [8–12]. In the present study monitoring the alterations of the erythrocytes capacity to resist hemolysis which is referred to as resistivity, and characterization of protein involvement in thermotropic transitions of erythrocyte membranes by means of DSC were employed to observe the response of the native membrane structures of fish to varying temperature conditions.

Experimental procedures

Materials

Reagents for preparation of buffers, erythrocyte suspensions and erythrocyte ghost samples: NaCl, KCl, $CaCl_2$, NH_4Cl, $MgSO_4 \cdot 7H_2O$, $NaH_2PO_4 \cdot H_2O$, $NaHCO_3$ were of the reagent grade, Hepes was purchased from Serva (Heidelberg, Germany).

Erythrocytes and their ghost preparation

The study was carried out using trout (*Salmo gairdneri*) red blood cells taken at seasonal acclimatizations to altered ambient temperatures of water over the range 0 to 21°C. Trout blood has been obtained from a local fish-factory at Suna river, Karelia, Russia monthly during a two-year period. Acclimatization of fish and specifically trout to altered temperatures have been widely reported to lead to a correction of the cell plasma membrane bilayer order probably due to the rising of the level of unsaturated lipids [3, 13]. Blood was taken from the caudal stem. Heparine solution (5000 units ml^{-1}) was added to the blood to prevent coagulation (100 µl/10 ml blood). Blood was immediately centrifuged (2000 rpm, 10 min) and after removing plasma and buffy coats, the erythrocytes were washed three times using Cortland solution with glucose missing (124 mM NaCl, 5 mM KCl, 1.6 mM $CaCl_2$, 1 mM $MgSO_4$, 3 mM NaH_2PO_4, 12 mM $NaHCO_3$, pH 8.0) [14]. Intact erythrocyte and nucleated and enucleated erythrocyte ghost samples were prepared according to the procedures for nucleated red blood cells [15, 16] using isotonic buffer (145 mM NH_4Cl, 4 mM KCl, 10 mM $NaHCO_3$, 5 mM $CaCl_2$, 1 mM $MgSO_4$, 15 mM Hepes, 1 mM PMSF, pH 7.5).

Membrane content was controlled by drying the suspension at 100°C.

Calorimetric measurements

A differential adiabatic scanning calorimeter DASM-4 (Bureau of Biological Instrumentation, Russian Academy of Sciences, Pushchino) [17] was employed to obtain the temperature curves of excess specific heat absorption by erythrocyte membranes (erythrocyte ghosts) in suspension.

The thermally induced structural transitions in trout erythrocytes and erythrocyte ghosts were studied according the procedures described by Zhadan *et al.* [11] and Akoev *et al.* [18] with microcalorimeter platinum cell volumes of 0.464, loaded by suspensions of intact erythrocytes or their ghosts. Scanning rate was always 1 K min^{-1}. The noise level and the reproducibility of the baseline were within 0.5 and 3 μW, respectively. An overpressure of 2 atm was always imposed over the content of the cells during the measurements to prevent possible degassing of the samples on heating. A background scan collected with a buffer in both cells was subtracted from each scan. A repeated heating of the samples after cooling was used to examine the reversibility of the thermal transitions and this secondary calorimetric trace was subtracted from the first one to obtain the temperature dependence of the excess heat capacity of erythrocytes and their ghosts for further analysis and plotting. The temperature of thermally induced structural transitions of erythrocyte ghosts was determined as the temperature at which the heat capacity peaks.

Thermohemolysis measurements

Thermoresistivity of intact erythrocytes was studied using equilibrium thermohemolysis procedure [19]. Erythrocyte suspension was placed in a water bath at the desired temperature (36–46°C). After incubation for time τ, samples were immediately cooled down for 3–4 h at 4°C and centrifuged (3000 rpm, 10 min). The extent of hemoglobin egress was then registered spectrophotometrically from the absorbance at 540 nm wavelength using Specoll 11 (Carl Zeiss, Jena). The extent of hemolysis was calculated as a ratio of the amount of hemoglobin released into solution to the total hemoglobin. Kinetic curves of erythrocyte thermohemolysis were of sigmoidal shape characteristic of the normal distribution and indicative of cooperativity of the process. The rate of 50% lysis of the suspension cells $k_{50}=1/\tau_{50}$ (min^{-1}), where τ_{50} is the time of 50% lysis of suspension red cells, was determined from a non-linear regression analysis using sigmoidal models of the lysis curves and used as an index of thermoresistivity. The activation energy taken from Arrhenius plots of the rate were also calculated to characterize thermosensitivity of erythrocyte membranes. Data analysis was performed using MicroCal Origin 5 Software.

Results and discussion

Microcalorimetry

Figures 1 and 2 present records of excess specific heat absorption by trout intact erythrocytes and erythrocyte ghosts. Each DSC curve reveals a series of structural

transitions. In the case of intact erythrocytes (Fig. 1) the large double peak of the hemoglobin denaturation transition dominated the scan and covered the most of both the temperature range and structural transitions of erythrocyte membrane.

An original DSC scan obtained from isolated trout erythrocyte membranes (Fig. 1) of nucleated ghosts containing no hemoglobin transition, shows a number of

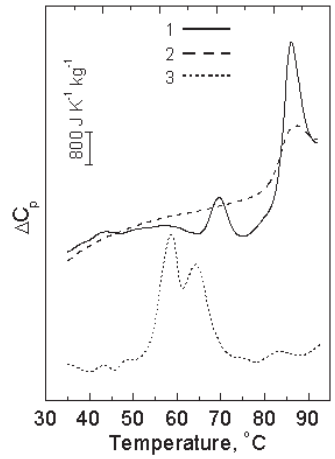

Fig. 1 Microcalorimetric profile of suspensions of trout erythrocytes membranes (ghosts) (1, 2 – second scan) and intact erythrocytes (3). Peaks indicate thermal transitions of different membrane domains including proteins and corresponding membrane region as well as the cell content. The assignment of peaks is shown in Table 1. Ambient water temperature 15°C

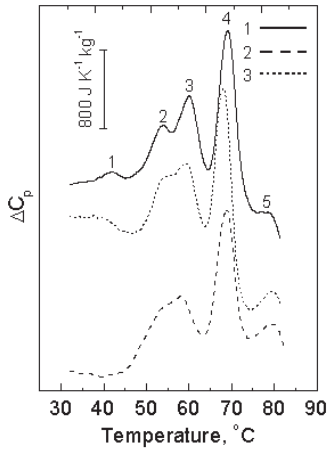

Fig. 2 Temperature dependences of excess specific heat absorption of trout erythrocyte ghost membrane suspension at different acclimation water temperatures: 1 – 14°C, 2 – 21°C, 3 – 1°C

peaks between 40 and 90°C. A major peak of this scan at about 87°C has not been ever registered for human or other enucleated erythrocytes [8–12, 18]. The most high-temperature transition in erythrocyte ghost curves is registered at about 80°C and has not been yet conclusively attributed to any known membrane domain. The second scan of the trout erythrocyte ghosts obtained in the heating of the sample immediately after cooling from the first scan (Fig. 1), shows that unlike the other transitions, the last one at 87°C is essentially reversible. This high-temperature peak is probably due to structural transitions of trout erythrocyte nucleus. It has been established much earlier that in the process of tissue heating in the temperature range of 75–90°C, the melting of chromatin complex is found with clear maximum at 82–86°C [20].

Whatever the acclimation temperature, the temperatures of denaturation transitions for hemoglobin were 57.1±0.2 for the first peak and 62.3±0.1°C for the second peak. Position of the maximum of the most high temperature trout erythrocyte ghost transition was 87±0.5°C and did not depend on acclimation temperature as well.

Figure 2 shows also a typical excess heat capacity curve of suspension of trout erythrocyte ghosts. It reveals five thermally induced structural transitions in ghost membranes and does not show the transition of the nuclei due to high reversibility of the latter. This overall five-peak shape of erythrocyte DSC curve appears to be much the same in such diverse species as mammalians (human, rat, dog, pig) [8–12, 18] and fish (trout). There are quantitative differences: the first three transitions occur at slightly different temperatures and has different relative intensities. The identification of the specific erythrocyte ghost components involved in thermally induced transitions can not be carried out exactly by means of DSC itself, but the data obtained can be correlated to others by virtue of transition temperature and overall shape similarities. The analogy in the transition sequence, their number and relative positions allows to associate them with the corresponding groups of membrane proteins as is done for human erythrocytes (Table 1).

Table 1 Transition temperatures (°C) for different protein groups of trout intact erythrocytes and their ghosts

Peak No.	Erythrocytes		Ghosts		
	trout	human**	trout	human**	protein group
1	43.5–44.0	50.0	40.0–42.0	49.5	α, β-spectrin – actin
2	47.5–48.0	56.8	53.0–55.0	56.0	ankyrin, b.4.1, 4.2
3	57.0–58.0*	63.8	59.0–60.0	62.0	cytopl. domain b.3
4	62.0–64.0*	72.4*	68.0–70.0	68.0	membr. domain b.3
5	76.0	–	78.0–79.0	78.0	unknown proteins

*Denaturation transition of hemoglobin
**Data for human erythrocytes are taken from [8, 9, 18]

Another considerable difference is that the low-temperature denaturation peaks (40–42°C for ghosts and 43.5–44°C for intact cells) probably associated with

spectrin-actin, appear only slightly. This is consistent with the considerable difference observed in the electrophoretic patterns of human and trout erythrocyte ghosts [15, 21, 22]. It was shown that the spectrin band was substantially less intensive, while band 5 was more intensive in the case of trout. In other cases the electrophoretic patterns of fish erythrocytes did not show the bands corresponding to high molecular mass components 215–240 kD at all that was explained by the destruction of spectrin in the course of membrane degradation [23].

On the other hand, comparing these peaks for intact erythrocytes and their ghosts can demonstrate that isotonic lysis used for preparing the ghost samples (with the complete egress of hemoglobin from the cell) does not lead to complete irreversible degradation of this group of proteins, but changes enthalpy and denaturation temperature of the transition. This indicates a structural alteration of the main proteins of cytoskeleton in the process of ghost formation. Hence, the differences in low-temperature part of ghost curves can be attributed to distinctive features of fish erythrocytes.

Results obtained indicate that no significant changes in transition temperatures of different membrane domains can be correlated with the acclimation temperature variations for the trout erythrocytes. The overall shape of the thermal curves also does not show distinct dependence on the environmental temperature conditions both in the case of intact erythrocytes and in the case of their ghosts. Variations in relative intensities of the heat adsorption peaks (Fig. 2) that are due most likely to changes in relative amounts of the specific proteins in membrane does not follow the temperature variations as well.

Thermohemolysis

Figure 3 shows pronounced seasonal variations of the erythrocytes thermohemolysis rate k_{50}. These data reveal the correlation of thermoresistivity of the cells with accli-

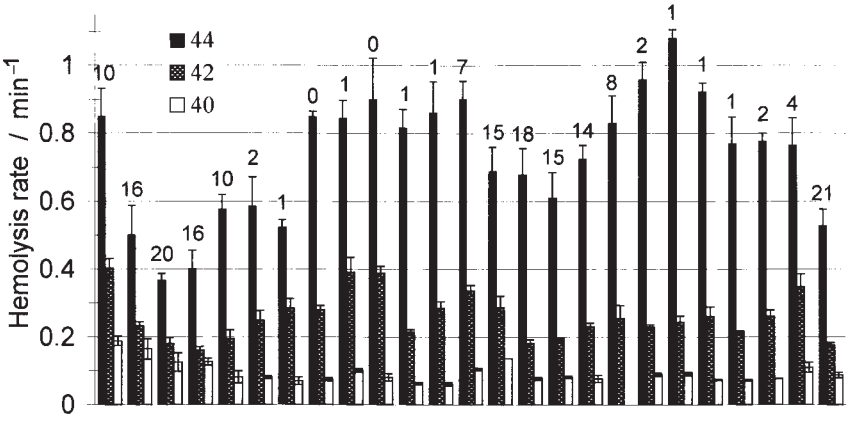

Fig. 3 Effect of acclimation temperature on hemolysis rate (k_{50}) of trout erythrocytes at different temperatures of hemolysis: 40, 42 and 44°C. Acclimation temperature is shown at the top of the columns (°C)

mation water temperature: maximum structural stability to thermal lysis is observed at higher acclimation temperatures (summer period), and it was minimum at about 0°C (fall-spring period). Such correlation was most distinct at the highest temperature of hemolysis.

The activation energy (E_{act}) of trout erythrocytes thermohemolysis also shows reliable correlation with acclimation temperature (Fig. 4). In consistence with the numerous early results [24, 25], the values of E_{act} are well in the range of activation energies of protein denaturation. However, when E_{act} and hemolysis rate (Fig. 2) are considered as thermoresistivity indices, these two parameters come into conflict: the minimum values of hemolysis rate at highest acclimatization temperatures correspond not to maximum, but to minimum activation energies. This could be an indication of protein involvement in thermohemolysis, if there were any evidence from

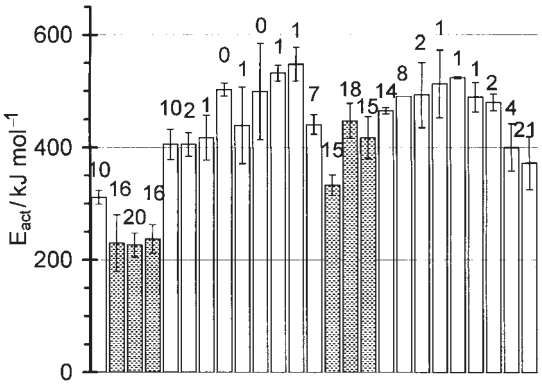

Fig. 4 Effect of acclimation temperature on activation energy (E_{act}) of trout erythrocytes thermohemolysis. The values of E_{act} are obtained by the data of Fig. 3. Acclimation temperature is shown at the top of the columns (°C)

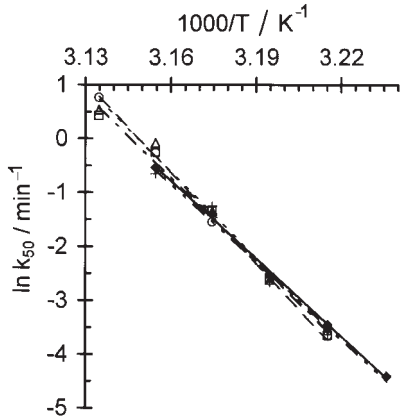

Fig. 5 Arrhenius plots of thermohemolysis rate (k_{50}) of trout erythrocytes over the temperature range 36 to 44°C. Different symbols correspond to different blood samples taken at temperatures 0–2°C

DSC measurements of an effect of acclimation temperature on the denaturational parameters of membrane components.

Arrhenius plot of erythrocytes thermohemolysis rate constant (Fig. 5) also does not show a break in the region of spectrin denaturation. Unlike human and mammalian erythrocytes, temperature range of intensive thermohemolysis of trout erythrocytes is much lower than the spectrin transition temperature. Complete hemolysis occur at 36–38°C for about 90 min that is well below the transition temperatures of membrane proteins and hemoglobin. These results support the notion that membrane protein denaturation can be considered neither as a process that initiate erythrocytes hemolysis nor as that markedly affecting its course [6, 7].

The data of thermohemolysis show no interplay of thermoresistivity of the cells with thermally induced transitions of erythrocytes. This indicates that lipid bilayer is crucial for structural stability of erythrocyte to thermal lysis. It is generally accepted that cold adaptation is accompanied by greater incorporation of unsaturated fatty acids in membrane lipids. Taking into account also a relationship between the plasma membrane bilayer order and the habitat temperature [1–3], the correlation of erythrocytes resistivity and acclimation temperature is obviously determined by the adjustment of the phospholipid composition of membrane bilayer by poikilotherms to accommodate to the new temperature. This suggests a compensatory adaptation of erythrocytes: acclimation of fish to altered temperature leads to a tuning of resistivity.

On the other hand, despite the fact that temperature denaturation of membrane-bound proteins is strongly affected by the properties and composition of the lipid environment [4, 5], the shape of calorimetric profiles and positions of thermally induced transitions of erythrocyte membranes do not show significant alterations. That is the lipid microenvironment of membrane proteins is likely to be materially the same.

Thus, temperature adaptation of trout erythrocyte membranes can result in a compensatory response of their phospholipid bilayer structural stability to thermal lysis as well as to conservation of the thermostability of the membrane proteins and intracell content.

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