THERMAL DENATURATION STUDY OF STRESS-INDUCED SODIUM PUMP DAMAGE IN THE RAT HEART AND THE ROLE OF LIPID PEROXIDATION

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Lipid peroxidation (LPO) is regularly activated in emotional-painful stress (DPS) [3] and injuries to cardiomyocyte membranes develop at the same time [1, 6], and are accompanied by lowering of ATPase activity [2, 4, 5]. This last fact is very important because Na,K-ATPase plays a key role in maintenance of the transmembrane potential and electrical stability of the heart. However, in most investigations of the sodium pump in biomembranes, changes only in the hydrolytic activity of the enzyme in various pathological states have been studied, and on the basis of these alone it is impossible to evaluate fine conformational changes in the molecule which could influence the resistance of Na,K-ATPase in vivo to external factors, and its transport, cooperative, regulatory, and other properties.

A simple way of detecting these changes is to analyze the kinetics of thermal denaturation of the enzyme. Thermal denaturation is an accepted method of estimating conformational stability of DNA, RNA, and soluble and membrane-bound proteins.

The aim of this investigation was to study the effect of stress on the thermostability of Na,K-ATPase and to assess the role of LPO in these changes.

EXPERIMENTAL METHOD

Experiments were carried out on male Wistar rats weighing 180-200 g, in which EPS was induced in the form of an anxiety neurosis by the method in [8] in the course of 6 h. The animals were decapitated 2 h after the end of stress.

The sarcolemmal fraction was isolated by the writer's original method. The hearts were removed, homogenized twice in a homogenizer of the Polytron type, for 7 sec each time in 10 volumes of medium A (20 mM imidazole, 20 mM sodium pyrophosphate, 1 mM EDTA, pH 7.8, at 4°C. The homogenate was filtered through two layers of gauze and centrifuged for 15 min at 1300g. The residue was washed in 10 volumes of medium A and centrifuged under the same conditions. This operation was repeated twice. The final residue was suspended in 2 volumes of medium A + 1 M KCl, pH 7.2, incubated at 4°C with mixing for 2 h, and centrifuged for 15 min at 2300g. The residue was washed in 20 mM imidazole, pH 7.2, incubated, reprecipitated, and suspended in storage medium (20 mM imidazole, 1 mM EDTA, 25% glycerol, pH 6.8). The sarcolemmal fraction thus obtained possessed Na,K-ATPase activity up to 15 μmoles inorganic phosphate (P_i) per milligram protein per hour and the ratio between Mg- and Na,K-ATPase activities was not more than 1:0.8. ATPase activity was determined by measuring accumulation of P_i [12] in medium with and without 0.5 mM ouabain.

LPO was induced in a suspension of sarcolemmal vesicles with protein in a concentration of 1 mg/ml, in medium containing 20 mM Tris-HCl, pH 7.0, at 25°C and with an Fe^{++} + ascorbate system in concentrations of 0.1 and 0.2 mM, respectively. The reaction was stopped by addition of the antioxidant ionol (10 nmoles/mg protein) and rapid cooling. The degree of oxidation of the membranes was verified by measuring accumulation of malonic dialdehyde (MDA) [11].

Thermal denaturation was carried out within the temperature range 50-60°C, the temperature being kept constant with an activity of ±0.1°C. The kinetics of thermal denaturation was studied and the thermodynamic parameters calculated as described in [9].

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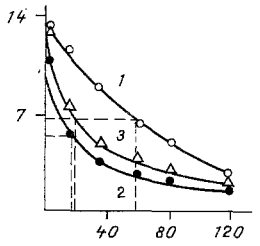

Fig. 1

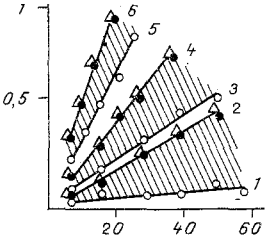

Fig. 2

Fig. 1. Thermal inactivation of Na,K-ATPase of myocardial sarcolemma at 52°C in control (1), after EPS (2), and in membranes oxidized in vitro (3). Abscissa, duration of thermal denaturation (in min); ordinate, Na,K-ATPase activity (in μmoles P_i/mg protein/h). Broken lines show time of inhibition of enzyme to half the maximal level.

Fig. 2. Change in velocity constant of thermal denaturation of myocardial Na,K-ATPase depending on temperature (K_{td} = 2.303 × tan α). Abscissa, duration of thermal denaturation (in min); ordinate, log A_o/A_t, where A_o denotes enzyme activity at initial moment of time, A_t at moment of time t. Empty circles — control, filled circles — EPS, triangles — oxidation in vitro. Temperature of thermal denaturation: 1, 2) 52°C; 3, 4) 55°C; 5, 6) 58°C.

EXPERIMENTAL RESULTS

Previous investigations [4, 5] showed that the Na,K-ATPase activity of the heart is depressed by 20% as a result of stress compared with the control. However, the effect of stress is evidently not limited to a very small decrease in hydrolytic activity of the sodium pump, but it is accompanied by a change in its interaction with the lipid microenvironment in the membrane. This, in turn, is the cause of the decrease in conformational stability of the enzyme, which can be revealed by analysis of the thermal denaturation process.

The kinetics of inhibition of Na,K-ATPase as a result of thermal denaturation of the control and experimental samples of sarcolemma is illustrated in Fig. 1. Inhibition of the enzyme in both cases obeyed a first order reaction kinetics, but it will be evident that after EPS the rate of inhibition depending on temperature increased.

Values of the velocity constants of thermal inactivation of Na,K-ATPase were found from the semilogarithmic dependences of the degree of inhibition of the enzyme on the time of thermal denaturation (Fig. 2). With a rise of temperature the velocity of thermal denaturation increased in both the control and experimental samples, but the velocity of inactivation of Na,K-ATPase, at each concrete temperature after stress, was always higher than in the control.

By subjecting Na,K-ATPase to thermal denaturation at different temperatures it is possible to determine the thermodynamic parameters of this process. Values of the activation energy (E_a) were found from dependence of the logarithm of the velocity constant of the reaction on the reciprocal of temperature, after which changes in enthalpy (ΔH*), entropy (ΔS*), and free energy (ΔF*) of the process were calculated by the usual method (Table 1).

As a result of EPS the values of E_a, ΔH*, and ΔS* of the reaction fell significantly, but ΔF* remained virtually constant. These results are evidence that in stress the conformation of the protein is disturbed so that it resembles the partially denatured state. In fact, the decrease in ΔS* observed in EPS can be explained on the grounds that the thermal denaturation process, in both control and experimental membranes, leads to the same denaturation state of the enzyme, characterized by the identical entropy value, for ΔS* will depend only on the initial state of the protein molecule. Thus entropy increases after EPS, i.e., the protein loses its high degree of structural organization and denaturation is accompanied by a smaller change in entropy on conversion of the polypeptide chain into a chaotic state.

The stability of membrane-bound proteins is largely determined by the character of hydrophobic protein-lipid interactions. As was shown previously [3], as a result of EPS LPO in the myocardium is activated, and this must lead to the appearance of polar LPO products in the sarcolemma and in the microenvironment of Na,K-ATPase. These polar products have a detergent

TABLE 1. Thermodynamic Parameters of Thermal Denaturation of Myocardial Sarcolemmal Na,K-ATPase in Control, after stress, and after Induction of LPO (M ± m)

Parameters studied	Control	Stress	LPO
E_a, kcal/mole	75,6±2,3	60,7±2,0	59,2±1,9
ΔH^*, kcal/mole	75,0±2,3	60,1±2,0	58,6±1,9
ΔS^*, cal/(mole · deg	156,8±0,6	111,3±3,6	104,6±1,2
ΔF^*, kcal/mole	24,4±1,5	23,7±1,9	23,4±2,1

action [7, 10], and they increase the orderliness of the lipid bilayer, disturb protein-lipid interactions, and reduce the conformational stability of the lipoprotein complexes in the membrane. These findings agree with the production of energy expenditure which we found during conversion of the protein into a disordered denatured state.

Considering the facts described above, it was decided to construct an in vitro model of the process injuring Na,K-ATPase in vivo during stress. Native membranes were oxidized by an Fe^{++} + ascorbate system until 20% of their activity was lost, which corresponds to inhibition of Na,K-ATPase during EPS. MDA accumulation under these circumstances was 3.5 nmoles/mg protein. Vesicles of sarcolemma treated in this way were subjected, along with native vesicles, to thermal denaturation (Figs. 1 and 2), after which thermodynamic calculations were carried out (Table 1). The data given are evidence of identity of changes in the parameters of thermal denaturation of the enzyme after exposure to stress and induction of LPO in the membrane.

The study of the kinetics of thermal denaturation of Na,K-ATPase thus revealed significant changes in the intramolecular properties of the enzyme, even associated with very small decreases in its hydrolytic activity. In this way profound changes were found in the Na,K-ATPase molecule of the myocardial sarcolemma during stress. The most probable cause of these injuries is activation of LPO, observed in the heart in EPS.

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