A SPECTROSCOPIC INVESTIGATION OF LACTATE DEHYDROGENASE THERMAL STABILITY AND ITS INTERACTION WITH NADH

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Fluorescence spectroscopy was used to study some aspects of thermal denaturation of lactate dehydrogenase (LDH) from rabbit muscle, in the absence and presence of urea and NaCl. Thermal denaturation of LDH seems to be one step and a slightly reversible process. The solution pH has an important influence on the LDH thermal denaturation. The interaction of LDH enzyme with NADH ligand was also investigated. The LDH-NADH complex is characterized by a moderate affinity constant and its formation is entropically driven.

Key words: protein-ligand interaction, denaturation, dynamic quenching, affinity constant, thermodynamic parameters.

1. INTRODUCTION

Lactate dehydrogenase (LDH) is a widely spread enzyme in all living organisms (prokaryotes and eukaryotes). LDH is found in several types of cells in the form of monomer, dimer, and tetramer, mainly in the heart, kidneys, liver, brain, and muscles [1]. LDH catalyzes the conversion of pyruvate into lactate, in the last step of the anaerobic glycolysis, having the key role in cell respiration [2]. LDH is also a non-specific marker for the detection of affected tissues and joint diseases [3]. Determination of blood LDH level is used in the diagnosis of myocardial infarction [4] or in retrospective detection of lung problems [5]. The concentration of LDL in the body increases by physical exercises, and also during intense labour [6]. To identify the increased LDH concentration, electrophoretic methods are performed by dosing the five different fractions of the enzyme, resulted from the combination, in different proportions, of the two types of LDH monomers, H and L [7].

The conversion of pyruvate to lactate, which is enzymatically catalyzed by LDH, is presented in Figure 1. Nicotinamide adenine dinucleotide (NAD/NADH) is a coenzyme involved in the metabolism of living cells. The reduced coenzyme (NADH) binds to proteins associated with both aerobic and anaerobic metabolism, while the oxidized coenzyme (NAD^+) is a cofactor, involved in redox reactions, carrying electrons from one molecule to another, but also in some post-translational modifications in proteins [8].

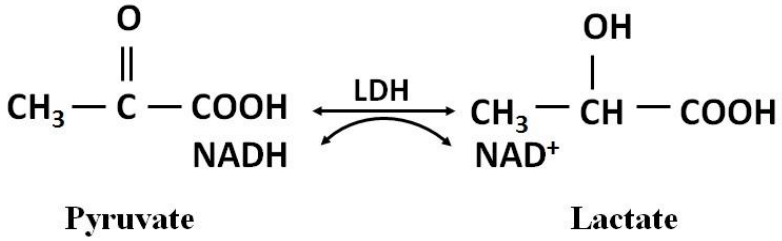

Fig. 1 – The reduction reaction of pyruvate to lactate, catalyzed by the LDH enzyme.

In this experimental study, the behaviour of the LDH enzyme was followed by changing the pH and temperature of the environment. The binding mechanism of the reduced cofactor NADH to the enzyme site was also characterized.

2. MATERIALS AND METHODS

Protein. The LDH enzyme, from rabbit muscle, purchased from Sigma, has a purity of 98 % and a molecular weight of 36,565 g/mol. In the primary structure, LDH protein contains two fluorescent aromatic amino acids (6 Trp and 7 Tyr) so that it can be studied by fluorescence spectroscopic methods.

Ligand. NADH (the reduced form of nicotinamide adenine dinucleotide) a substance with a molecular weight of 741.62 g/mol and a purity of 98 %, was purchased from Sigma.

Buffer solution. All samples were dissolved in HEPES buffer (molecular weight 238.3 g/mol), in a concentration of 100 mM, at different pH values.

Steady state fluorescence spectroscopy. Fluorescence emission spectra were recorded with a Perkin Elmer LS55 spectrophotometer at λ_{ex} = 290 nm, using five spectra accumulation, with a scan speed of 500 nm/min, and a system of slits of 5.0 nm for the excitation monochromator and 2.5 nm for emission monochromator.

pH-metry. The pH of the buffer and all investigated samples was determined using a pH-meter, 720 InoLab.

3. RESULTS AND DISCUSSIONS

3.1. STUDY OF THE THERMAL DENATURATION OF THE LDH

If a protein dissolved in a buffer solution is heated, a process called thermal denaturation it changes some properties, like the absorption in UV radiation range, the viscosity and the optical rotation, even at very small change in temperature. By denaturation, the native structure of the protein is disorganized in a cooperative way, this meaning that one disordered state in a certain region of the polypeptide chain stimulates the destabilization of the whole protein structure which collapses in a disordered structure, which corresponds to a denatured state [9].

The LDH 3D structure is influenced by the temperature, but the completely loss of the spatial structure is observed only at greater temperature values, its structure being irreversible lost in some organisms at ~ 90 ^{o}C [10]. Since the primary structure of LDH contains aromatic amino acids, biophysical and biochemical properties of this protein can be investigated by steady-state fluorescence spectroscopy.

Figure 2 presents the fluorescence emission spectrum of the LDH (3.2 μM), recorded at room temperature by excitation at 290 nm (in order to avoid as much as possible the excitation of Tyr residues). The emission spectrum presents a maximum at 349 nm, characteristic for Trp residues.

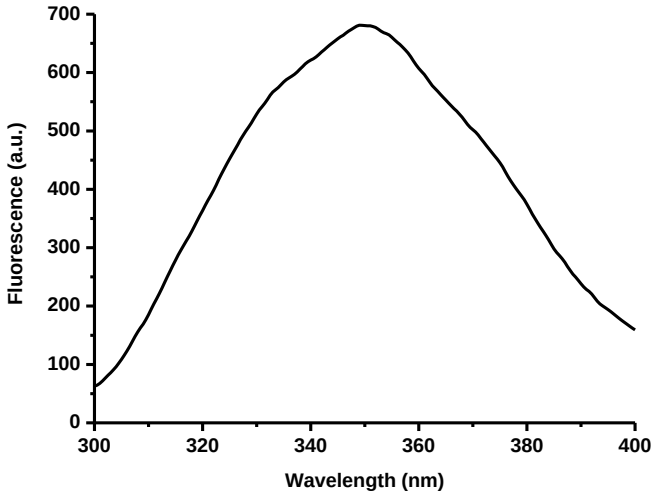

Fig. 2 – The fluorescence emission spectrum of LDH (3.2 μM), in 100 mM HEPES buffer, at λ_{ex} = 290 nm, pH = 7.4.

Thermal denaturation of LDH was investigated by recording emission fluorescence spectra for LDH sample at λ_{ex} = 290 nm, in the 25–85 ^{o}C range,

waiting for 3 minutes in order to be reached the desired temperature by the sample. LDH fluorescence emission maxima are represented in Figure 3.

Many globular proteins recover partially their spatial structure after the action of the denaturant factors. Figure 3 shows both thermal denaturation and renaturation of the LDH structure, after gradually lowering the temperature from 85 ^{o}C to 25 ^{o}C. It is noted that in denaturation process, an inflection point (at 50 ^{o}C) is put in evidence, not visible on the thermal renaturation curve.

LDH secondary and tertiary structures are lost almost completely beyond 80 ^{o}C, but these structures are partly recovered by the thermal renaturation. The percentage of protein structure renaturation was calculated, at each temperature, by ratio of the fluorescence emission intensity, in the renaturation process, to the fluorescence emission intensity, in the denaturation process.

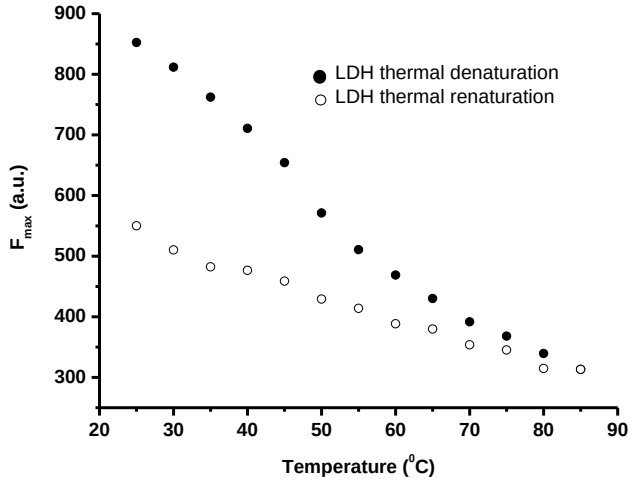

Fig. 3 – Thermal denaturation (●) and renaturation (○) of LDH (3.2 μM), monitored by the fluorescence emission of Trp. Protein was dissolved in 100 mM HEPES buffer, at pH = 7.4.

From Fig. 3, one can see that, at 50 ^{o}C, thermal renaturation percentage is about 75 %. The effect of the temperature on the folded structure of LDH is slightly reversible, because by removal of the denaturant factor, the hydrogen bonds are partially reconstituted.

3.2. pH INFLUENCE ON THE LDH THERMAL DENATURATION

Some proteins undergo large rearrangements of their space structure when pH is changed, leading to the alteration of the 3D shape of the native protein. This process appears because the ionization states of the side chains of amino acids are modified by the pH changes. As a result, the charge distribution in the polypeptide

chain changes and the formation of ionic bonds is induced. We monitored the effect of pH on the process of thermal denaturation of LDH, by excitation of LDH (2 μM) at 290 nm and recording the emission at 354.9 nm, at different values of pH (4.2, 6.5, 7.4, and 8.0).

Figure 4 shows the effect of acidic (4.2 and 6.5), physiological (7.4) and basic (8.0) pH values on the denaturation of LDH 3D structure. One can see a very important and different behaviour of the LDH structure with the temperature at the physiological pH in comparison to those at the acidic and basic values.

The denaturation of LDH at pH = 7.4 is one slow step process. For pH values much lower than the corresponding isoelectric point (8.4–8.6 for rabbit muscle LDH), the protein loses its negative charges and presents only positive ones [11]. The charges of the same kind prevent LDH molecules to form aggregates. In LDH domains with a high density of charge, the intramolecular repulsion can be sufficiently strong to provoke the unfolding of the protein, a similar effect to that of the gentle heat denaturation. The optimal pH for rabbit muscle LDH function is 7.5 [11].

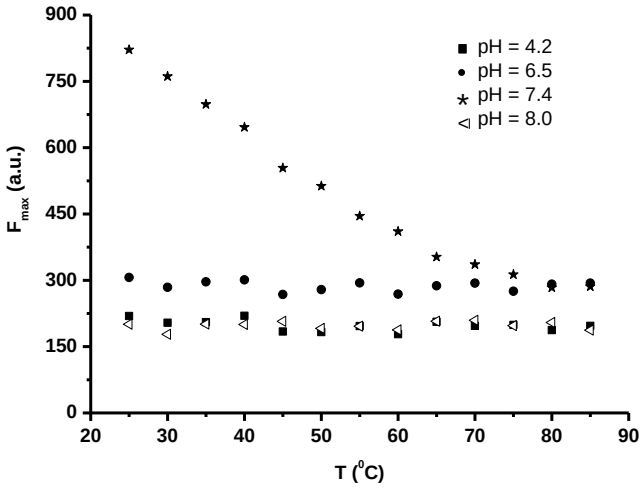

Fig. 4 – The influence of pH on thermal denaturation of LDH (2 μM) in 100 mM HEPES buffer at room temperature.

3.3. THE INFLUENCE OF OTHER FACTORS ON THE LDH SPATIAL-CONFORMATION

Proteins can be denatured not only by temperature or pH, but also by certain solutes as urea. In the literature, two models of interaction between urea and globular proteins are proposed [12]. In the first model, the direct interaction between urea and protein is considered the main cause of protein denaturation. The second model is based on indirect interaction that alters the structure of water

around the protein, leading to an unpacked protein. Although recent studies support the model of direct interaction, it is still unclear whether the non covalent bonds of the polypeptide chain are broken by interaction with urea. Urea in high concentrations denatures the proteins by the chaotropic effect. At low urea concentrations, the structure of the target protein tends to be stabilized, by an unclear mechanism, leading to inhibition of protein aggregation during refolding [13, 14].

The presence of salts in the buffer solutions in which a protein is dissolved affects its thermal transition and also its kinetic properties. Neutral salts induce association or dissociation of proteins, in function of their *salting out* or *salting in* properties [15, 16]. The literature presents indications that NaCl, a neutral salt, could be a destabilizing factor of the folded structure of proteins, its chaotropic ions pertaining to the Hofmeister series [17].

In this study, the LDH thermal denaturation, in the presence of urea and NaCl, was monitored at pH = 7.4 (Fig. 5 A and B). It was found that by adding urea (5 M) and NaCl (1 mM), the 3D structure of LDH was modified. This can be seen in fluorescent intensities at room temperature, which are much lower as compared to fluorescent intensity of native LDH.

In the presence of urea, the thermal denaturation is very aggressive as one can see (Fig. 5A), the process being characterized by a curve having an inflection point at ~ 43 ^{o}C. In the presence of NaCl (Fig. 5B), the inflection point of the thermal denaturation curve occurs at ~ 63 °C. This is an indication that urea is a very aggressive denaturation agent, in comparison with NaCl. Therefore, LDH is easier to be thermally denatured in the presence of urea than in the presence of NaCl.

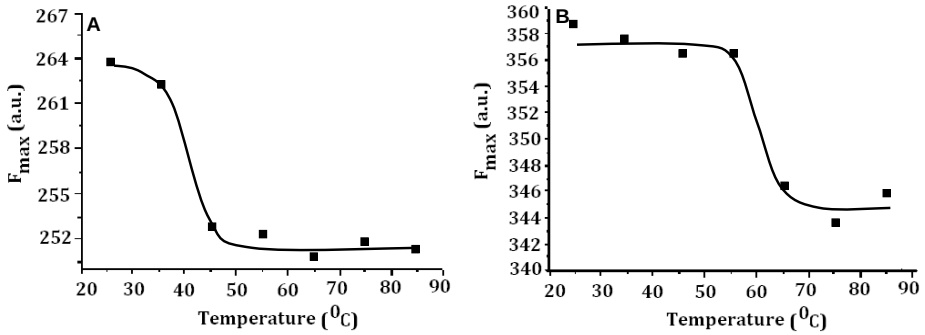

Fig. 5 – Fluorescence emission maxima of LDH (3.2 μM) in the presence of urea (A) and NaCl (B) under the influence of temperature. Protein was dissolved in 100 mM HEPES buffer, at pH = 7.4.

In the presence of NaCl, the protein structure is more difficult to be degraded by increasing the temperature, as compared to the presence of urea.

3.4. CHARACTERIZATION OF THE LDH-NADH INTERACTION

In order to quantitatively analyze the binding mechanism of NADH to LDH, the Scatchard equation was used:

$$F_0/F = 1 + K_A[Q]^n, \tag{1}$$

where K_A is the affinity constant (the binding constant), n is the stoichiometry index, which represents the number of the binding sites, and [Q] is the quencher concentration.

The slopes of the Scatchard plots for the interaction between the LDH (3.2 μM) and NADH (0–5.5 μM) at 25 °C and 35 °C can be seen in Figure 6. The linearity of the plots allows the calculation of the number of binding sites, n = 0.98, and the affinity constant, $K_A = 8.59 \times 10^3$ M^{-1} at 25 °C and $K_A = 9.17 \times 10^3$ M^{-1} at 35 °C. K_A values give indications on the strength of the binding. The affinity constant values show that the ligand binding to the site of LDH is moderate. One can see that there is a slight increase of the affinity constant with the temperature increase.

It is known that the variations of the thermodynamic parameters: Gibbs free energy (ΔG) enthalpy (ΔH), entropy (ΔS), and heat capacity (ΔC_p) show the binding mode of NADH to LDH site.

The variation of Gibbs free energy and the affinity constant are determined by the well-known equation:

$$\Delta G = -RT\ln K_A, \tag{2}$$

where R is the universal gas constant.

The values obtained for the change in Gibbs free energy are $\Delta G_1 = -21.75$ kJ mol^{-1} at 25 °C and $\Delta G_2 = -22.97$ kJ mol^{-1} at 35 °C.

The variation of enthalpy, ΔH, can be obtained from the equation:

$$\ln \frac{K_{A1}}{K_{A2}} = \left(\frac{1}{T_1} - \frac{1}{T_2}\right)\frac{\Delta H}{R}, \tag{3}$$

where K_{A1} and K_{A2} are the binding constants at T_1, respectively T_2 temperatures.

The changes of the entropic factor, $T\Delta S$, for the interaction of NADH with LDH, were calculated according to the well-known equation:

$$\Delta G = \Delta H - T\Delta S. \tag{4}$$

The interaction of proteins with ligands depends on the buffer solutions, through the enthalpy of the buffer ionization. For LDH-NADH complex, in HEPES buffer, the variation of the enthalpy is small but negative ($\Delta H = -4.75$ kJ mol^{-1}).

The entropic factors are positive ($T\Delta S$ = 17.00 kJ mol^{-1} at 25 °C and $T\Delta S$ = 18.22 kJ mol^{-1} at 35 °C). The positive changes of the entropy are an

indication that water molecules have been released from the LDH-NADH complex surface [18].

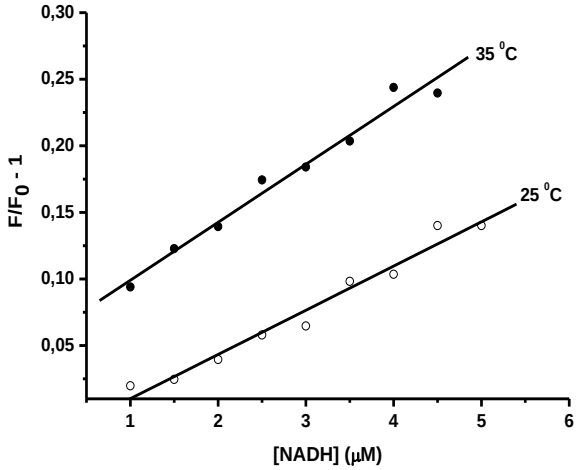

Fig. 6 – Scatchard plots of the LDH and NADH binding, at 25 °C (●) and 35 °C (■).

The values of the thermodynamic parameters demonstrate that the interaction between the LDH and NADH is a spontaneous process ($\Delta G < 0$) and also a slight exothermic one ($\Delta H < 0$). The entropic term, at each temperature value, is higher than the enthalpic one. Therefore, the LDH-NADH interaction is driven by the entropy, both at 25 °C and 35 °C, as one can see in Figure 7.

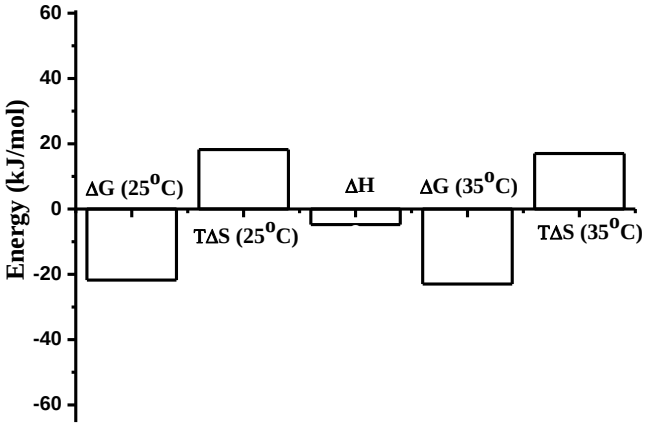

Fig. 7 – Thermodynamic parameters: ΔG, ΔH, and $T\Delta S$ for the LDH-NADH complex at 25 °C and 35 °C, in HEPES buffer, pH = 7.4.

The variation of the heat capacity, ΔC_p, is strongly related to the change in exposed solvated surface area during a biochemical reaction. The change in the heat capacity, ΔC_p, of the protein molecules that are linked to change of temperature variation by one degree, was calculated according to equation:

$$\Delta C_p = \frac{\mathrm{d}(\Delta H)}{\mathrm{d}T} \cong \frac{\Delta(\Delta H)}{\Delta T}, \tag{5}$$

where $\Delta T = T_2 - T_1$.

The negative value for the heat capacity change, ΔC_p = – 0.47 kJ mol^{-1} K^{-1}, indicates that the complex has a lower heat capacity than the free partners. Taking into consideration the positive entropic term, this is associated with hydrophobic interactions and conformational changes upon NADH binding to the LDH site.

4. CONCLUSIONS

This study aimed to characterize some properties of lactate dehydrogenase (LDH) under the influence of environmental factors, such as temperature, pH, urea, NaCl by the interaction with the ligand (NADH). The analysis and interpretation of fluorescence emission spectra of LDH and LDH-NADH complex, led to the following conclusions:

1. When one we use heat to disrupt hydrogen bonds and non-polar hydrophobic interactions, LDH secondary and tertiary structures are almost completely lost at great temperatures (*e.g.*, beyond 80 ^{o}C), but are recovered by thermal renaturation, in a relatively high percentage. Therefore, the influence of the temperature on the spatial structure of LDH is almost reversible.

2. For pH below the physiological value, the intermolecular repulsion can be sufficiently strong to achieve unfolding of the LDH, as in the case of a gentle thermal denaturation.

3. In the presence of urea, thermal denaturation of LDH is an enhanced process. Over 43 ^{o}C, the protein structure is completely denatured. The synergic effects of urea and temperature lead to an inflection point, at 63 ^{o}C, of LDH denaturation curve. In the presence of NaCl, LDH thermal denaturation starts beyond 50 ^{o}C, the whole LDH structure being denatured over 63 ^{o}C.

4. The strength of NADH-LDH binding is moderate and the complex formation is driven by entropy, as it was indicated by the affinity constant and the thermodynamic parameters.

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