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Conformational features of lactate dehydrogenase: temperature effect in presence of small molecules, mathematical model

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ABSTRACT

The aim. To study the conformational changes of lactate dehydrogenase under the influence of different concentrations of intermediates (pyruvate, oxaloacetate) in the temperature gradient with the subsequent building of a mathematical model.

Materials and methods. Thermolability of lactate dehydrogenase was studied using the method of differential scanning fluorimetry to determine the change in endogenous fluorescence of tryptophan and tyrosine under the conditions of stable concentration of lactate dehydrogenase and changing concentrations of pyruvate and oxaloacetate. Further, a mathematical model was developed for a more in-depth consideration of the behavior of the catalytic protein.

Results. We found that pyruvate and oxaloacetate in low concentrations have a thermostabilizing effect on lactate dehydrogenase conformation; the effect of pyruvate is statistically more significant in comparison with oxaloacetate ($p < 0.05$). The studied ligands in high concentrations reduce the thermal stability of lactate dehydrogenase.

Conclusion. Understanding the role of small molecules in the regulation of biological and catalytic processes has long remained in the background of scientific interest, but today the work in this direction is reaching a new level. The data obtained indicate the possibility of small molecules acting as ligands when interacting with enzymes.

Key words: lactate dehydrogenase, conformation, differential scanning fluorimetry, oxaloacetate, pyruvate.

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Конформационные особенности лактатдегидрогеназы: влияние температурного фактора в присутствии малых молекул, математическая модель

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РЕЗЮМЕ

Цель. Исследовать конформационные изменения лактатдегидрогеназы под действием различных концентраций интермедиатов (пируват, оксалоацетат) в температурном градиенте с последующим построением математической модели.

Материалы и методы. Изучение термолабильности лактатдегидрогеназы проводили с использованием метода дифференциальной сканирующей флуориметрии по изменению эндогенной флуоресценции триптофана и тирозина в условиях стабильной концентрации лактатдегидрогеназы и изменяющихся концентраций пирувата и оксалоацетата. Далее была разработана математическая модель для более углубленного рассмотрения поведения каталитического белка.

Результаты. Было выявлено, что пируват и оксалоацетат в низких концентрациях оказывают термостабилизирующее воздействие на конформацию лактатдегидрогеназы, влияние пирувата статистически более значимо в сравнении с оксалоацетатом ($p < 0,05$). Изучаемые лиганды в высоких концентрациях снижают термостабильность лактатдегидрогеназы.

Заключение. Понимание роли малых молекул в регуляции биологических и каталитических процессов долгое время оставалось в тени научного интереса, но сегодня работа в данном направлении выходит на качественно новый уровень. Полученные данные свидетельствуют о возможности малых молекул выступать в качестве лигандов при взаимодействии с каталитическими белками.

Ключевые слова: лактатдегидрогеназа, конформация, дифференциальная сканирующая флуориметрия, оксалоацетат, пируват.

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Источник финансирования. Авторы заявляют об отсутствии финансирования.

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INTRODUCTION

Recently, more attention has been paid to small molecules, unique chemical "fingerprints" that can change gene expression and flows in metabolic pathways, leading to changes in specific processes in intra- and intercellular spaces [1]. Signal proteins, such as catalytic ones, are therefore an interesting area for the study of protein-ligand interactions, as they are highly sensitive to external stimuli such as endogenous metabolites or small molecules [2].

Molecules with a small molecular weight, located at the intersection of metabolic pathways for the exchange of proteins, fats, carbohydrates and serving as the "metabolic currency" of a living cell are such key bioenergetic intermediates as pyruvic and oxaloacetic acids.

Oxaloacetate (OA), a four-carbon keto acid with tautomerism phenomenon, is a valuable and quite rare molecule; its concentration in mitochondria does not exceed 10^{-6} M, and it is involved in many metabolic pathways, including gluconeogenesis, citric acid cycle, glyoxylate cycle, urea cycle and amino acid metabolism. Oxaloacetate is a critical component in ATP production and should be continuously regenerated to maintain the required level of oxidation processes in the citric acid cycle and the electron transfer chain [3].

Pyruvic acid (pyruvate) is one of the intermediate components of metabolism, the precursor of the most important intermediates of anabolic and catabolic pathways, including gluconeogenesis, lipogenesis *de novo*, cholesterol synthesis, as well as maintenance of the citric acid cycle. Pyruvate is formed as a result of anaerobic glycolysis in pyruvate kinase reaction, and can also be synthesized from various precursors: lactate in lactate dehydrogenase reaction, malate in cytosol malate dehydrogenase reaction, from alanine [4]. Serine, threonine, glycine, cysteine and tryptophan can also be converted to pyruvate [5].

Lactate dehydrogenase (EC 1.1.1.27), a tetrameter protein with catalytic activity, belongs to the oxidoreductase class. It catalyzes reversible transformation of pyruvate into lactate with NADH oxidation. The following genes are known to encode this enzyme: LDHA, LDHB, LDHC and LDHD [6]. Gene transcription of this enzyme is regulated by more than 20 different factors, including HIF1 protein. Recently, it was shown that LDH, along with enzymatic activity, also regulates the cell cycle; suppression of LDH accelerates cell transition into the G2 stage, while an increase in the LDH activity delays the cell in S-period [7]. LDH molecules are found in the cytoplasm and the nucleus, where they play the role of a transcription factor and influence DNA synthesis [8]. There are indications that lactate dehydrogenase has a direct effect on potassium ion channels of myocardium and liver cells in hypoxia [9].

The aim of our work is to reveal conformational changes of lactate dehydrogenase under the influence of different intermediates (pyruvate, oxaloacetate) concentrations in a temperature gradient with the subsequent building of a mathematical model.

MATERIALS AND METHODS

The experiments were performed at the Department of Molecular and Radiation Biophysics of National Research Center "Kurchatov Institute".

The following reagents were supplied from Sigma-Aldrich, USA: lactate dehydrogenase (EC 1.1.1.27, LDH, L-Lactic Dehydrogenase) from rabbit muscle, type XI, lyophilizate, 848 U/mg protein; pyruvate, oxaloacetate, Tris-HCl buffer 50 mM, pH 7.5. The enzyme and small molecules were diluted in tris-HCl buffer.

Differential Scanning Fluorimetry (DSF) was performed on Prometheus NT.48 (NanoTemper Technologies, Germany). This device allows the quick and accurate evaluation of protein folding, as well as its chemical and thermal stability. The method is based on changes of tryptophan and tyrosine endogenous fluorescence at wavelengths of 330 and 350 nm. The result is recorded in degrees Celsius, corresponding to the temperature of protein melting (*Tm*) [10]. This parameter is dependent on the forces of non-covalent intermolecular interactions: electrostatic, hydrophobic, Van der Waals forces, as well as the presence and number of hydrogen bonds.

Six dilutions were prepared, in which the final LDH concentration remained unchanged: 1 μM, and the final concentration of pyruvate and oxaloacetate varied: 16 μM, 8 μM, 4 μM, 2 μM, 1 μM and 0.5 μM respectively. We placed 10 μM of the solution in Prometheus NT.48 capillaries (nanoDSF grade). Scanning fluorimetry was performed at laser

intensity of 30%, heating diapason from 20 °C to 95 °C, step 1 °C/min.

Two approaches were used to study the dependence of the relative fluorescence on the protein melting temperature and the ligand concentration: the pre-installed software (Promethus NT.48 software), which allowed the determination of the protein melting point and the maximum melting rate (the first derivative), and the nonlinear regression model in the SPSS 21 statistical package (IBM SPSS Statistics, USA, license No 20130626-3). We used a four-parameter S-shaped curve:

$$f(t) = d + \frac{c - d}{1 + e^{-a(t-b)}},$$

where: *f(t)* is a dependent variable, fluorescence ratio at wavelengths 330 and 350 nm; *t* – independent variable, temperature, °C; *a*, *b*, *c*, *d* – equation parameters, or regression coefficients with the following substantial interpretation.

This form of analytic dependence refers to sigmoidal curves, recommended for the approximation of the growth phenomena with saturation [11], including for various medical and biological needs [12–14]. The choice of the given mathematical model for the present research was determined by a convenient substantial interpretation of its parameters: *a* – reflects the melting rate; *b* – corresponds to the theoretical inflection point and the temperature when the melting rate is maximum; *c* – asymptotically minimal absorption ratio of wavelengths; *d* – asymptotically maximum absorption ratio of wavelengths.

Adequacy assessment of the built models was carried out by a graph-analytical method on the analysis of the obtained and estimated values by the regression equation; regression residues were analyzed and their distribution was normal. The quality of the approximation was estimated by the statistical significance of the model, determination coefficients and standard regression errors. After building regression models (one mathematical model for each intermediate in different concentrations), analysis of their parameters was performed. According to the estimated statistical package 95% confidence intervals of the regression coefficients were assessed to be statistically significant between the LDH melting curves with different ligand concentrations and two ligands

RESULTS

The enzyme in solution is simultaneously present both in a folded and partially unfolded state ("molten globule"). At high temperature, proteins, including enzymes with ordered structure, unfold [15, 16], which leads to changes in the orientation of aromatic residues. These changes can be detected by means of differential scanning fluorimetry.

Thermostability of lactate dehydrogenase when adding oxaloacetate and pyruvate had the following features (Fig. 1, Fig. 2).

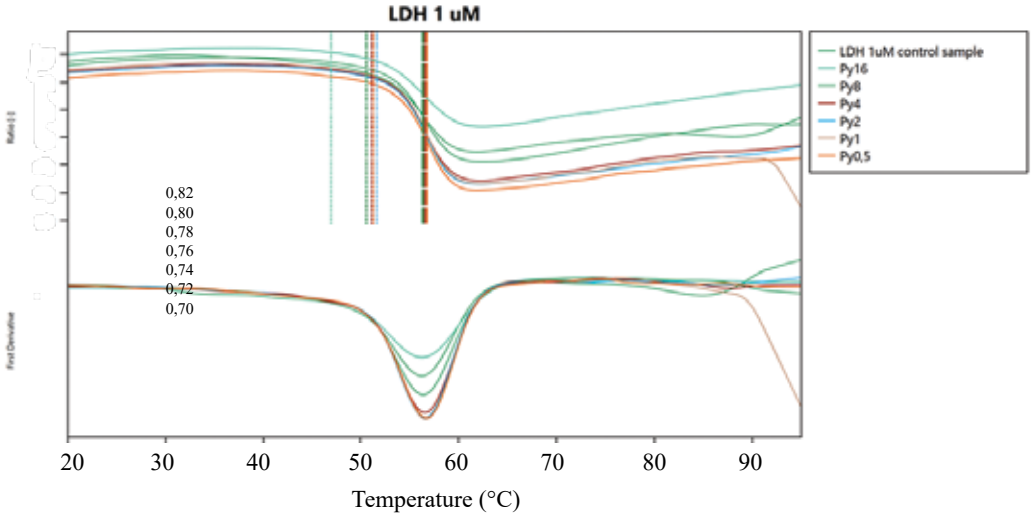

Fig. 1. Melting curves and the first derivate of lactate dehydrogenase (1 μM) in combination with oxaloacetate in different concentrations (upper chart). The concentration of oxaloacetate in the mixture was 16; 8; 4; 2; 1 and 0.5 μM, respectively. The fluorescence value (upper panel) at 350/330 nm (Y axis) is presented depending on temperature (X axis). The colored lines at the transition points correspond to the maximum peak *Tm* of the first derivate (bottom panel)

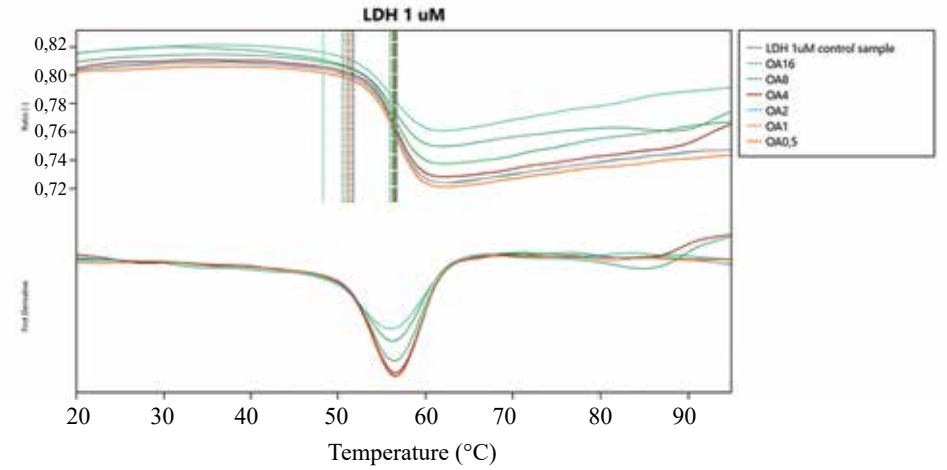

Fig. 2. Melting curves and the first derivate of lactate dehydrogenase (1 μM) in combination with pyruvate in different concentrations (upper chart). The concentration of oxaloacetate in the mixture was 16; 8; 4; 2; 1 and 0.5 μM, respectively. The fluorescence value (upper panel) at 350/330 nm (Y axis) is presented depending on temperature (X axis). The colored lines at the transition points correspond to the maximum peak *Tm* of the first derivate (bottom panel)

Increased concentrations of oxaloacetate and pyruvate (16 μM) promotes unfolding of the protein molecule, expressed in a decrease in the thermal stability: lower melting point (oxaloacetate – 48.3 °C, pyruvate – 47 °C compared to 50.5 °C for the reference sample), faster temperature inflection point (oxaloacetate – 56.0 °C, pyruvate – 56.2 °C compared to 56.3 °C for the reference sample). In contrast, the addition of oxaloacetate and pyruvate at the minimum concentration (0.5 μM) changes protein conformation to a more folded one, leading to an increase in the thermodynamic stability of lactate dehydrogenase. The starting melting point increased in comparison with the control sample and was 51.1 for oxaloacetate and 51.3 °C for pyruvate; there was a shift in the melting point of 56.6 °C for oxaloacetate, 56.7 °C for pyruvate. The total amplitude of the fluorescence signal during the unfolding process also changed significantly, indicating different conformational states of the complexes. We built a mathematical model for a more detailed characterization of catalytic protein condition in a temperature gradient when adding small molecules.

Before building a mathematical model that approximates the experimentally obtained points of the fluorescence ratio during protein heating, a visual analysis of initial scatterograms was carried out (Fig. 3). In contrast to the curves smoothed by the Prometheus NT.48 software shown earlier for oxaloacetate and pyruvate in Figures 1 and 2, it should be noted that fluorescence ratio spreads along the ordinate axis were higher before melting and after reaching the inflection point and decreased immediately when protein melting temperature was reached.

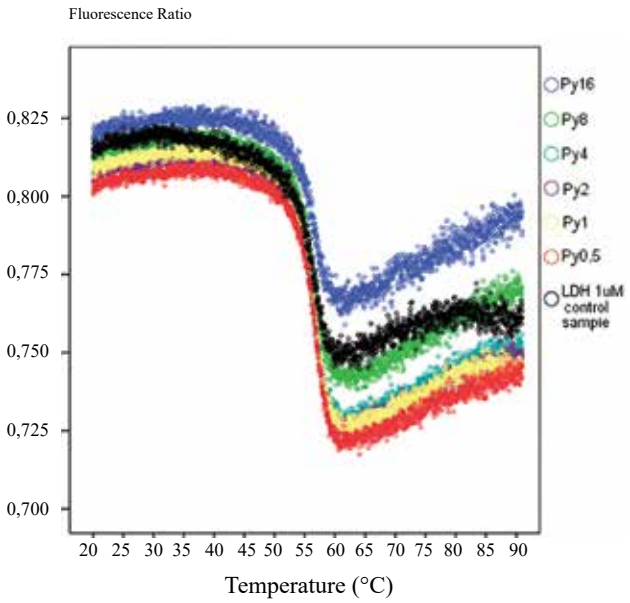

Fig. 3. Melting curves of lactate dehydrogenase (1 μM) in combination with pyruvate in different concentrations: non-smoothed fluorescence ratios

Temperature range from 48 to 63 °C was selected. Determination coefficients of the obtained models were in the range from 98.5% to 99.7%, and standard regression errors, respectively, from 0.0026 to 0.0018 (Table 1). The regression coefficients for different ligand concentrations are given in Table 2.

Table 1

Quality assessment of regression models approximating LDH melting curves in the presence of pyruvate and oxaloacetate in different concentrations, μM

Concentrations	Pyruvate		OA	
	Determination coefficient, R^2	Standard regression error	Determination coefficient, R^2	Standard regression error
16	0.985	0.0026	0.988	0.0025
8	0.995	0.0019	0.995	0.0021
4	0.996	0.0020	0.996	0.0021
2	0.997	0.0018	0.997	0.0018
1	0.997	0.0019	0.996	0.0020
0,5	0.996	0.0020	0.996	0.0019
0	0.992	0.0022	0.992	0.0022

Note: all built models are statistically significant at $p < 0.001$.

Table 2

Parameters of regression models approximating LDH melting curves in the presence of pyruvate and oxaloacetate in different concentrations, μM

Concentrations	Pyruvate				OA			
	a	*b*	*c*	*d*	*a*	*b*	*c*	*d*
16	0.724 (0.685–0.762)	56.2 (56.1–56.3)*	0.765 (0.765–0.766)*	0.819 (0.818–0.820)*	0.732 (0.697–0.767)	55.9 (55.8–56.0)	0.759 (0.758–0.759)	0.814 (0.814–0.815)
8	0.760 (0.737–0.783)	56.2 (56.2–56.3)	0.741 (0.740–0.741)*	0.811 (0.811–0.812)*	0.780 (0.756–0.804)	56.3 (56.2–56.3)	0.736 (0.735–0.736)	0.807 (0.807–0.808)
4	0.776 (0.755–0.797)	56.4 (56.4–56.4)	0.727 (0.726–0.727)	0.806 0.805–0.806)*	0.788 (0.768–0.809)	56.4 (56.3–56.4)	0.726 (0.726–0.727)	0.804 (0.803–0.804)
2	0.787 (0.768–0.806)	56.4 (56.4–56.4)	0.724 (0.724–0.725)*	0.805 (0.805–0.806)*	0.778 (0.759–0.797)	56.4 (56.4–56.4)	0.722 (0.722–0.723)	0.802 (0.802–0.803)
1	0.776 (0.757–0.796)	56.5 (56.4–56.5)*	0.724 (0.724–0.725)*	0.807 (0.806–0.807)*	0.767 (0.746–0.788)	56.3 (56.3–56.4)	0.722 (0.722–0.723)	0.802 (0.801–0.802)
0.5	0.761 (0.741–0.782)	56.5 (56.4–56.5)*	0.719 (0.719–0.720)	0.801 (0.801–0.801)	0.755 (0.735–0.775)	56.3 (56.3–56.4)	0.719 (0.719–0.720)	0.800 (0.799–0.800)
0	0.728 (0.698–0.757)	56.1 (56.0–56.1)	0.748 (0.747–0.748)	0.809 (0.808–0.809)	0.728 (0.698–0.757)	56.1 (56.0–56.1)	0.748 (0.747–0.748)	0.809 (0.808–0.809)

Note. The table shows regression coefficients and their 95% confidence intervals. Asterisks mark the statistically significantly different parameters between pyruvate and OA.

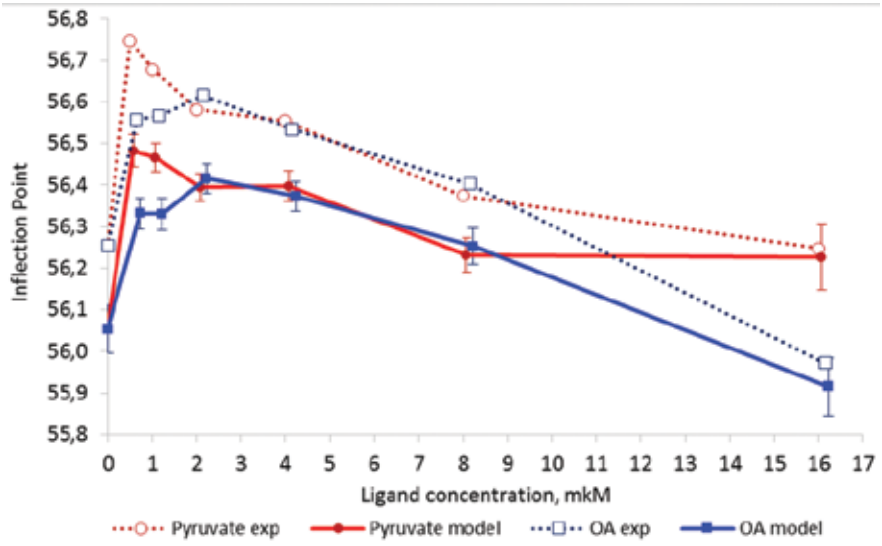

Fig. 4. Position of inflection points in the melting curves of lactate dehydrogenase in combination with pyruvate and oxaloacetate in different concentrations

For the convenience of comparison of the parameters between different ligand concentrations, we have drawn dependency plots.

The parameter *b*, numerically equal to the inflection point, is considered in Figure 4. The data obtained for the mathematical model, as well as data from Prometheus NT.48 Software, are presented. The nature and magnitude of the inflection point shift, estimated by different methods, are the same. Concentrations of ligands from 0.5 to 8 μM cause an increase in the melting point of the LDH, and a concentration of 16 μM leads to a decrease in the thermal stability of the protein. The maximum change of the melting temperature when adding pyruvate was noted at its concentration of 0.5 μM and in the case of oxaloacetate it was at a concentration of 2 μM. Shift estimates obtained by modeling the nonlinear regression and the Prometheus NT.48 software differ slightly: the discrepancy with the data obtained by the software is 0.2 °C.

When comparing the influence of two ligands on the melting point shift, it was found that the influence of oxaloacetate and pyruvate differs statistically significantly in the area of low concentrations (0.5 and 1 μM) and high concentrations (16 μM). Pyruvate at low concentrations causes a statistically significant ($p < 0.05$) increase in the melting point than oxaloacetate, while at high concentrations it causes a greater reduction in the inflection temperature. In concentrations of 2–8 μM the ligands' effect on the melting point shift according to the regression model built was indistinguishable.

To analyze the maximum melting rate of LDH in the presence of ligands (Fig. 5), the first derivatives of the inflection points were obtained from the instrument software report.

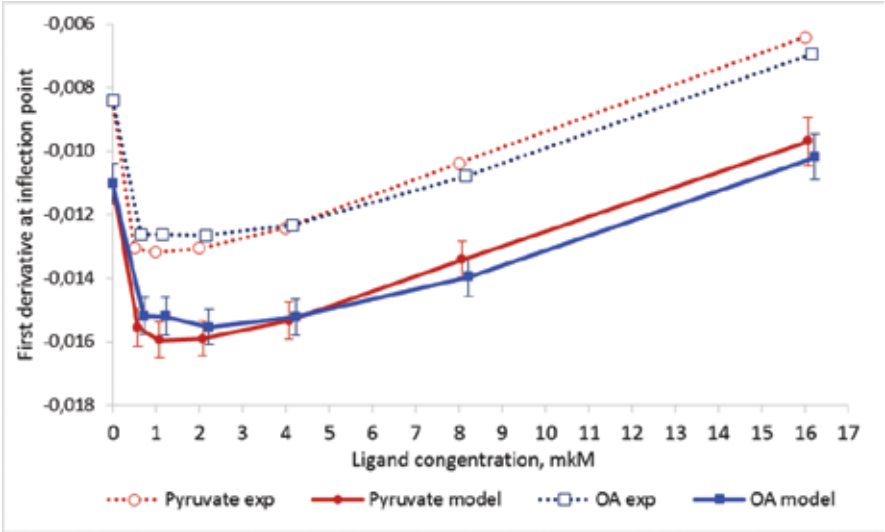

Fig. 5. Maximum melting rates of lactate dehydrogenase in combination with pyruvate and oxaloacetate in different concentrations

First derivatives of the temperature of reaction mixture $t = b$ were calculated from the parameters of the built mathematical models by the formula $f'(t) = -0.25a(d–c)$. The errors of the received results are estimated by the rules of errors calculation in arithmetic operations. Negative values of the derivatives reflect a decrease in the fluorescence ratio during protein unfolding. The higher the absolute value of the first derivative, the higher the rate of protein melting.

Both studied ligands caused a significant increase in the melting rate of LDH in concentrations of 0.5–4 μM ($p < 0.05$ compared to no ligands) and its subsequent decrease in concentrations of 8–16 μM. The effect of different concentrations of pyruvate and oxaloacetate on the maximum melting rate was the same: no statistically significant differences between the parameters *a*, or between the first derivatives at the inflection point for pyruvate and oxaloacetate were found ($p > 0.05$).

DISCUSSION

When comparing the maximum melting velocities obtained by different methods (according to software and mathematical modeling), a systematic difference of 0.003 on average was revealed. We

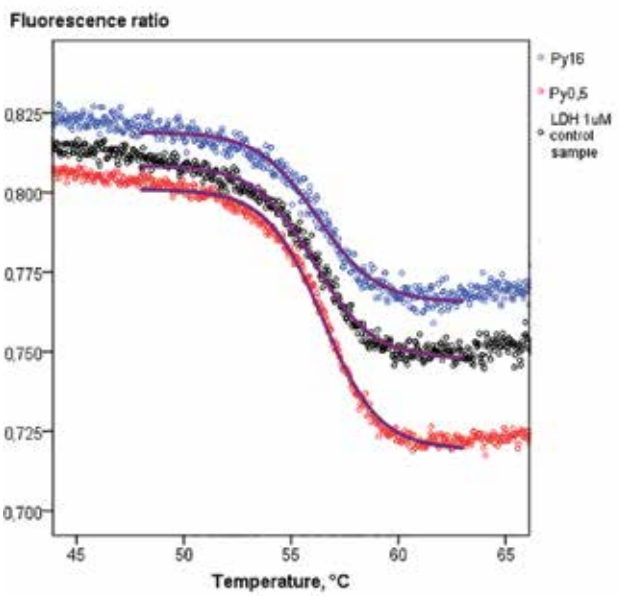

Fig. 6. Empirical and theoretical melting curves of lactate dehydrogenase without pyruvate in combination with pyruvate in concentrations of 16 and 0.5 μM.

assume that this is due to the fact that the proposed form of the analytical dependence is well consistent with the empirical data only in the area with the maximum melting rate, but not before and after it. It is because of this fact that when constructing the regression model, a rather narrow temperature range was chosen at which melting occurs. Figure 6 shows the fluorescence ratios observed in the experiment and the theoretically calculated points for pyruvate concentrations of 0.5 and 16 μM according to the models constructed. The figure shows that the beginning of the theoretical curves has more divergence with the experimentally observed points than in the LDH melting section. Therefore, insufficient estimations of the parameter *d* of the developed regressions lead to an insignificant shift in the estimation of the maximum melting rate. However, from the point of view of this study these corrections have no statistically significant effect.

CONCLUSION

When studying the conformational state of lactate dehydrogenase by differential scanning fluorimetry with the subsequent building of a mathematical model, it was found that pyruvate and oxaloacetate in low concentrations (0.5–2 μM) have a thermostabilizing effect on the LDH structure, and at high concentrations (16 μM), on the contrary, they reduce the thermal stability of LDH. Despite the co-directed effects of the studied metabolites, the effect of pyruvate was more pronounced in comparison with oxaloacetate ($p < 0.05$).

The ability of intermediates to take part in parametabolic interactions and, in particular, to bind and influence the conformation of catalytic proteins, determines a wide range of their biological action, the mechanisms of which have yet to be studied in more detail. Of undoubted interest is the further study of pyruvate and oxaloacetate as molecules of protectors and stabilizers, an increase of the studied concentrations and protein partners, which is especially promising in view of bioenergetical and "mitochondrial" medicine concepts.

These are the unknown pages of a known enzyme.

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