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Oxaloacetate: Transmitter Function, Contribution to the Neurophysiological Processes of the Body, Prospects for Therapeutic Application. Experimental data

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Abstract

The focus of our attention is directed towards the small molecule of oxaloacetate. This work is dedicated to the investigation of the role played by natural intermediates as metabolism switches in intermolecular interactions. The full range of biological activity for oxaloacetate has been unveiled through the utilization of computer modeling methods. Furthermore, the interaction partner proteins have been characterized. It has been demonstrated that small molecules act as metabolic intermediates, serving as points of intersection for numerous metabolic pathways encompassing protein, carbohydrate, and lipid metabolism. Concurrently, a coordinating role is assumed by these molecules in the execution and modulation of mediator, hormonal, receptor responses, immunological, inflammatory, antibacterial, and antiviral reactions, thus manifesting anticarcinogenic properties. Through the application of differential scanning fluorimetry and microcapillary thermophoresis, the interaction between lactate dehydrogenase and ligands of endogenous origin has been established. The calculated Kd value obtained for the interaction between oxaloacetate and lactate dehydrogenase was determined to be 0.5±0.01 μM. The thermalstability of LDH is enhanced by final concentrations of oxaloacetate ranging from 0.5 to 1 μM, whereas a concentration of 16 μM of the metabolite diminishes its thermostable characteristics. In intermolecular processes in in vitro cell culture, the stimulating effect of oxaloacetate 33,8 % ($p=0.028$) on the primary culture of human dermal fibroblasts was shown.

Keywords: protein-metabolite interactions, oxaloacetate, computer modelling, differential scanning fluorimetry, microcapillary thermophoresis, human dermal fibroblasts

1. Introduction

Currently, it is important to study the role of metabolites in the systems of intercellular interaction. In particular, protein - metabolite interactions can regulate and control a variety of cellular processes: transport of substances, signaling, playing a role in maintaining cellular homeostasis (Zhao *et al.* 2021). The small molecule of oxaloacetate is the focus of our attention. It is a linking compound of carbohydrate and protein exchanges. Oxaloacetate quantitatively determines the intensity of the tricarboxylic acid cycle. It is necessary to constantly regenerate oxaloacetate to ensure that the citric acid cycle and the electron transfer process in the mitochondrial respiratory chain are not interrupted (Campos *et al.* 2012; Pesi *et al.*2018). Scientific research has shown that oxaloacetate affects lifespan. It activates FOXO/DAF-16 transcription factors and protein kinase activity. These processes are required in adenosine monophosphate (Williams *et al.* 2009; Al-Homsi *et al.* 2012; Edwards *et al.* 2013). In addition, oxaloacetate stimulates the growth of neurons of the hippocampal gyrus, which leads to a decrease in the intensity of cell division. Active cell division in this area has a beneficial effect on cognitive processes and mnemonic functions (Wilkins *et al.*2014). Intermediate effects play a crucial role in several vital functions - it has a general promitochondrial effect. Which is expressed by in increasing the content of mitochondrial markers COX4|1 and PGC1α. Along with preventing neuroinflammation and neurodegeneration (Lu *et al.* 2018; New M. 2019; Zerr *et al*. 2019). Oxaloacetate can penetrate through the blood-brain barrier into the central nervous system. This finding became a prerequisite for preclinical trials of drugs against Alzheimer's disease and therapy in ischemic stroke neurodegeneration (Vidoni *et al.* 2021; Shahouzehi *et al.* 2023).

Experimentally assessing the intermolecular interaction of millions of chemical compounds with thousands of ligands is difficult both from an economic and practical point of view; therefore, it is advisable to conduct a preliminary assessment of the biological activity of specific chemicals *in silico* (Chakraborty *et al.* 2019). The use of computer simulation can reduce the amount of necessary experiments by a factor of ten compared to blind search (Filimonov *et al.* 2018). The study of structure-functional features of small molecules includes the

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following stages of research: determination of predicted spectrum of biological activity using computer technologies; identification of potential protein partners to confirm the scientific hypothesis; setting up *in vitro* and *in vivo* model experiments revealing these or those properties of the studied molecule.

The study aims to predict *in silico* biological properties, determine potential protein partners of the interaction of oxaloacetate, and study its effect on the conformation and thermolability of lactate dehydrogenase (LDH) and on the viability of human dermal fibroblasts. An important step in studying the effect of low molecular weight ligands on protein structures is to conduct experiments involving individual proteins. The monocatalytic protein lactate dehydrogenase (lactate dehydrogenase catalytic system) was chosen as the object of our further study, and oxaloacetate was chosen as a low molecular weight ligand. In its structure, this metabolite is similar to one of the substrates of the lactate dehydrogenase reaction, pyruvate, differing in the presence of an additional carboxyl group. Fibroblast culture is an adequate experimental model for assessing the metabolic profile, exo- and endometabolome variability associated with changes in the incubation environment during the study (Stunova, Vistejnova, 2018).

2. Materials and Methods

2.1. Computer modeling of the biological activity of oxaloacetate using PASS

The software PASS version 1.917 (Prediction of Activity Spectra for Substances) is intended for prediction of the biological activity spectrum of a compound by its structural formula based on the analysis of structure-activity relationships. The spectrum of biological activity predicted by PASS includes molecular mechanisms of action, pharmacological effects, specific toxicity, side effects, effect of molecules on metabolism, molecular transport, gene expression, and identification of undesirable targets. The prediction result is presented as Pa "to be active" and Pi "to be inactive" probabilities with values from 0 to 1. We took Pa over 0.5 as the optimal probability value for the presence of activity. The prediction of the biological activity spectrum was obtained as an ordered list of Pa and Pi probability estimates.

2.2. Evaluation of probable partner proteins for oxaloacetate in STITCH

Potential protein interaction partners for small molecules were identified using Search Tool for Interactions Chemicals (STITCH) version 5.0. http://stitch.embl.de. The standard SMILES entry was used to search for identifiers and common names of chemicals that are stored in the small molecule information database. The program calculates the parameter p - the probability of small molecule-protein interaction. In STITCH, the interaction network can be displayed and adjusted using different settings: by degree of evidence, confidence, molecular action or bond affinity. In our work, we used the bond affinity score. The program predicts intermolecular interactions in a confidence threshold from 0 to 1 (low, medium, high, highest); we used a medium confidence threshold of $p>0.4$

2.3. Method for evaluation by microcapillary thermophoresis of LDH interaction with oxaloacetate

Experiments on the influence of oxaloacetate (Sigma, product number O4126) on the conformation and thermostability of lactate dehydrogenase (Sigma, product number L1254) were carried out in the laboratory of molecular and radiation biophysics at the Kurchatov Institute (Russia). We used reagents Sigma-Aldrich, USA: lactate dehydrogenase, oxaloacetate, Tris-HCl buffer 50mM, pH 7.5. We measured the pH value before and after adding the oxaloacetate solution to the samples using a Mettler Toledo (USA) pH-meter. The pH variation of the medium had fluctuations within the range of 0.01-0.02.

Experiments using the method of microcapillary thermophoresis to establish the fact of ligand-protein interaction, as well as to calculate the dissociation constant. The procedures were performed using Monolith NT.115 equipment (NanoTemper Technologies GmbH). Lactate dehydrogenase labeling was performed using a standard L001 protein labeling kit "Monolith NT Protein Labeling Kit RED-NHS". The unreacted "free" dye was removed by gel filtration. To calculate the dissociation constant (Kd) by microthermophoresis, a series of dilutions were prepared, where the final concentration of LDH was chosen empirically to be 1.65 μM, and the amount of added lactate varied from 40 μM to 0.0012 μM.

2.4. Study of the influence of oxaloacetate on the conformational structure of LDH using differential scanning fluorimetry

Determination of changes in the conformational structure of lactate dehydrogenase under the influence of oxaloacetate was performed using differential scanning fluorimetry based on changes in intrinsic fluorescence of protein tryptophan and tyrosine at 330 and 350 nm. Prometheus NT.48 device (NanoTemper Technologies, Germany) was used. Protein conformational stability was described by its average denaturation temperature Tm (°C), which is the point where half of the protein unfolds. Six dilutions were prepared. In which the final concentration of LDH was constant - 1 μM. The final concentration of oxaloacetate varied, 0.5 μM, 1 μM, 2 μM, 4 μM, 8 μM, 16 μM. Capillaries were scanned at 30% laser intensity, heating range from 20°C to 95°C, 1°C/min step. The effect of oxaloacetate concentration range on the lactate dehydrogenase fluorescence ratio (350/330 nm) in the physiological temperature range (36.5-37.5°C) was analyzed. The pH value was monitored before and after adding the solutions of the studied small molecules to the samples using a Mettler Toledo pH-meter (USA). The pH variation ranged from 0.01-0.02.

2.5. Determination of viability of human dermal fibroblasts

The effect of oxaloacetate on viability of human dermal fibroblasts was assessed at the Institute of Experimental Medicine and Biotechnology of Samara State Medical University, Ministry of Health of Russia, using reagents for cultivation of human cells produced by Biolot LLC (Russia) with MTT-test using a Tecan Infinite M200 PRO multirider (Tecan Austria GmbH, Austria) at 570 nm. Statistical analysis of the obtained parameters was performed using SPSS 25.

3. Results and discussion

3.1. Biological activity of oxaloacetate determined by the PASS

We have revealed the influence of oxaloacetate on the regulation of metabolism using the PASS computer environment (Table1).

Table 1. The interaction of oxaloacetate with target enzymes

Target enzymes	Enzyme code number	Pa oxaloacetate
Alanine transaminase inhibitor	EC 2.6.1.2	0.916
Oxaloacetate tautomerase inhibitor	EC 5.3.2.2	0.895
Oxaloacetate tautomerase inhibitor	EC 5.3.2.2	0.895
Glutamine-phenylpyruvate transaminase inhibitor	EC 2.6.1.64	0.856
Malate dehydrogenase acceptor inhibitor	EC 2.7.3.9	0.851
Pyruvate decarboxylase inhibitor	EC 4.1.1.1	0.827
Aspartate-phenylpyruvate transaminase inhibitor	EC 2.6.1.70	0.818
Oxaloacetate decarboxylase inhibitor	EC 4.1.1.3.	0.815
Phenylpyruvate decarboxylase inhibitor	EC 4.1.1.43	0.807
Pyruvate dehydrogenase inhibitor	EC 1.2.4.1.	0.802
L-lactate dehydrogenase (cytochrome) inhibitor	EC 1.1.2.3	0.794
L-lactate dehydrogenase (cytochrome) inhibitor	EC 1.1.2.3	0.794
Phosphoenolpyruvate carboxykinase inhibitor	EC 4.1.1.38	0.786
D-lactate dehydrogenase (cytochrome) inhibitor	EC 1.1.2.4	0.783
D-lactate dehydrogenase (cytochrome) inhibitor	EC 1.1.2.4	0.783
Succinate dehydrogenase inhibitor	EC 1.3.5.1	0.755
Serine-3-dehydrogenase inhibitor	EC1.1.1.276	0.733
Malate dehydrogenase inhibitor	EC 1.1.1.37	0.708
Malate dehydrogenase inhibitor	EC 1.1.1.37	0.708
Glycerol-3-phosphate dehydrogenase (NAD+) inhibitor	EC 1.1.1.8	0.675
Tryptophan transaminase inhibitor	EC 2.6.1.27	0.647
Glycerol-3-phosphate oxidase inhibitor	EC 1.1.3.21	0.628

Pa - probability of presence

Oxaloacetate inhibits enzymes of protein metabolism: alanine transaminase, serine-3-dehydrogenase, tryptophantransaminase. It is known that the realization of oxaloacetate action on carbohydrate metabolism is carried out by inhibiting effect on a number of key enzymes: lactate dehydrogenase, malate dehydrogenase, malatoxidase, pyruvate dehydrogenase. It was revealed that oxaloacetate is involved in the regulation of lipid metabolism. It reduces the activity of enzymes: trans-2-enoyl-CoA reductase, acylcarnitine hydrolase, glycerol-3-phosphate dehydrogenase, cytochrome-b5 reductase.

It should be emphasized that oxaloacetate has an inhibitory effect on succinate dehydrogenase, which is not only a part of the Krebs cycle but also an important element in the electron transfer chain. At the same time, it has been determined that impaired succinate dehydrogenase function accompanies a number of pathological conditions, such as Lee syndrome (Finisterer, 2008), familial paraganglioma syndrome (Her, 2015), and neuroendocrine tumors (Armstrong, 2009).

The effects of oxaloacetate on gene expression are noteworthy (Table 2): the increase in expression of the HMOX1 gene encoding the hemoxygenase-1 protein and the JAK2 gene, which regulate the viability, proliferation, and differentiation of many cell types. There is an interesting fact that oxaloacetate enhances the expression of the TP53 gene. The p53 protein acts as a suppressor of malignant tumor formation, so the TP53 gene is an anti-oncogene (Chavez-Perez et al., 2011). The inhibitory effect of oxaloacetate on the expression of the MMP9 gene, a protein of matrix metalloproteinase family, was predicted. In addition, oxaloacetate was shown to reduce the expression of the gene for tumor necrosis factor (TNF), which encodes a multifunctional proinflammatory cytokine that is mainly secreted by macrophages and is involved in the regulation of a wide range of biological processes. In addition, oxaloacetate was modeled to inhibit HIF1A factor, which functions as the main regulator of transcription of the adaptive response to hypoxia.

Table 2. Effect of oxaloacetate on gene expression

Effect on gene expression	Pa	Pi
BRAF expression inhibitor	0.683	0.004
JAK2 expression inhibitor	0.661	0.022
MMP9 expression inhibitor	0.601	0.015
TP53 expression enhancer	0.599	0.05
HMOX1 expression enhancer	0.574	0.023
EIF4E expression inhibitor	0.518	0.008
TNF expression inhibitor	0.514	0.026
HIF1A expression inhibitor	0.506	0.055
APOA1 expression enhancer	0.483	0.032

Pa - probability of presence; Pi - probability of absence

3.2. Predicted proteins of interaction with oxaloacetate

We used the STITCH v.5.0 to study intermolecular interactions between proteins and oxaloacetate. It is a well-known fact that oxaloacetate interacts as substrates with malate dehydrogenase and its various isoforms (Halestrap and Wilson, 2012). Oxaloacetate with a high degree of probability interacts with proteins of the Solute Carrier Family (SLC25). The data that coincide with those from the PASS system on the relationship of oxaloacetate with the succinate dehydrogenase complex are interesting; oxaloacetate has been shown to mediate the ADP-dependent inhibition of mitochondrial Complex II-induced respiration (Fink *et al*., 2018) (Table 3).

Table 3. Predicted proteins - partners of interaction with oxaloacetate

Proteins as partners		p
ACLY	ATP citrate lyase	0.992
CS	Citrate synthase	0.985
PCK1	Phosphoenolpyruvate carboxykinase 1	0.985
PC	Pyruvate carboxylase	0.984
MDH2	Malate dehydrogenase 2, mitochondrial	0.979
MDH1	Malate dehydrogenase 1	0.976
FH	Fumarate hydratase	0.937
ACACA	Acetyl-CoA carboxylase alpha	0.923
SLC25A10	Solute carrier family 25 (dicarboxylate transporter), member 10	0.916
SLC25A11	Solute carrier family 25 (oxoglutarate transporter), member 11	0.916
SLC25A1	Solute carrier family 25 (citrate transporter), member 1	0.911
ME3	Malic enzyme 3, NADP(+)-dependent	0.864
ME1	Malic enzyme 1, NADP(+)-dependent, cytosolic	0.859
ME2	Malic enzyme 2, NAD(+)-dependent	0.848
SDHB	Succinate dehydrogenase complex, subunit B	0.834
SDHA	Succinate dehydrogenase complex, subunit A	0.818
SDHC	Succinate dehydrogenase complex, subunit C	0.811
SDHD	Succinate dehydrogenase complex, subunit D	0.765
OGDH	Oxoglutarate dehydrogenase (lipoamide)	0.611

p - the probability of interaction between a small molecule and a protein

It was noted that oxaloacetate can be a partner in the interaction of aspartate transaminases of types 1 and 2, providing a neuroprotective effect (Martin *et al*., 2014). Oxaloacetate can bind to interleukin 4; alpha-subunit of hypoxia-inducible factor (HIF1). This allows it to indirectly influence gene expression. Which are responsible for cell adaptation to hypoxic conditions (Semenza, 2004), which is consistent with the PASS data. Determination of the interaction between oxaloacetate and lactate dehydrogenase by microcapillary thermophoresis

The next step was a series of *in vitro* experiments designed to determine the interaction of oxaloacetate with lactate dehydrogenase by microcapillary thermophoresis. We prepared a series of experiments of 16 dilutions in which the final concentration of dye-labeled LDH remained unchanged at 1.65 μM and the final concentration of oxaloacetate varied from 0.0012 to 40μM. The software automatically plots the fluorescence as a function of time (Fig. 1). Then, the software calculates the degree of lactate dehydrogenase binding to oxaloacetate by plotting the ratio between fluorescence before and after switching on the laser as a function of oxaloacetate concentration.

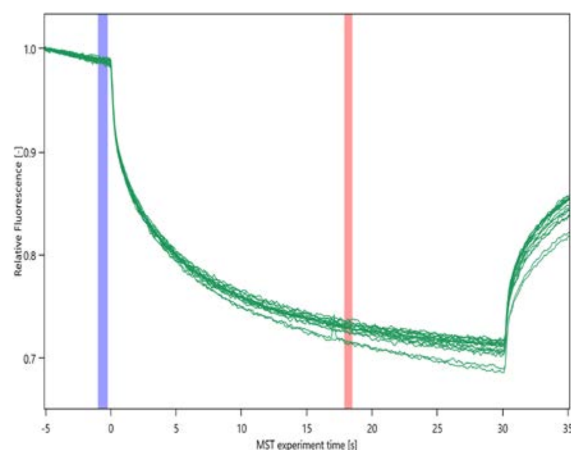

Figure 1. Analysis of the interaction between LDH (at a constant concentration (1.65 μM) and oxaloacetate at varying concentrations.

To estimate the degree of affinity, we used a stoichiometric parameter, the dissociation constant (Kd), numerically equal to the ligand concentration at which the free and bound partner molecules are equal. The calculated Kd value obtained for oxaloacetate with lactate dehydrogenase was 0.5±0.01 μM. It should be noted that in most cases the equilibrium dissociation constant numerically corresponds to the Michaelis-Menten constant, which is used to describe the affinity of the protein-ligand bond and is applicable only at the steady state (Lasseter, 2019).

3.3. Assessment of the influence of oxaloacetate on the thermostability of lactate dehydrogenase by differential scanning fluorimetry

Differential scanning fluorometry (DSF) is one of the methods for assessing the thermostability of proteins under various conditions, including after binding to small ligand molecules. It is used to determine the melting point (Tm) (transition temperature) of a protein. This is when half of the protein is in a folded conformation and the other half has undergone thermal denaturation.

We performed an experiment to evaluate the formation of the spatial structure of lactate dehydrogenase under the influence of oxaloacetate using differential scanning fluorimetry (Table 4).

Table 4. Melting initiation temperature and temperature inflection points of LDH upon addition of oxaloacetate.

Ligand concentration, μM	Melting point,°C	Inflection point, °C
16	48.3	56.0
8	51.1	56.4
4	51.7	56.5
2	51.5	56.6
1	50.8	56.6
0.5	51.1	56.6

LDH control: Melting point = 50.5°C, Inflection point = 56.3°C

It is remarkable that with a small difference in the melting temperature of LDH a different state of LDH-oxaloacetate complexes is observed, which is manifested by a different depth of the peak. The melting temperature of LDH with different concentrations of oxaloacetate

corresponds to the deepest point of the formed peak on the first derivative curve. The observed change in the depth of the formed peaks can be explained by the different influence of oxaloacetate concentrations on the thermal stability of the molecule.

3.4. Mathematical Modelling

After a visual analysis of the original scattergrams, mathematical modeling of the fluorescence values obtained during the experiment on heating the protein molecule was performed.

We performed a more detailed simulation of the lactate dehydrogenase melting curve functioning under the influence of different concentrations of oxaloacetate in the physiological temperature range of 36.5-37.5°C (Fig.3, Table 5).

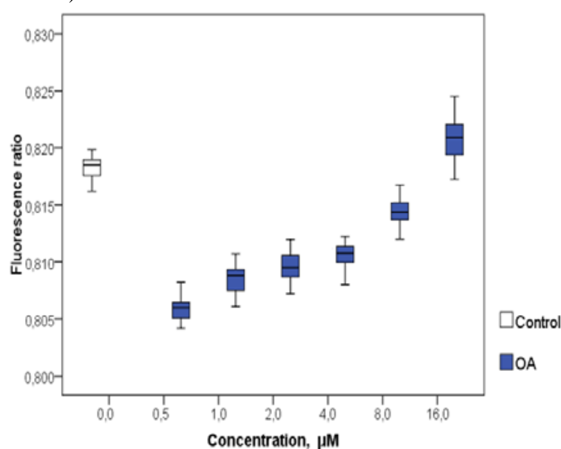

Figure 2 .Comparison of the effect of different oxaloacetate concentrations on LDH thermostability at 36.5-37.5°C.

We analyzed 29 measurements of fluorescence ratios at different temperature points of the stated temperature corridor. The effect of oxaloacetate concentration on LDH conformation was found to be statistically significant ($p < 0.005$), as manifested by changes in fluorescence intensity (table 5). No statistically significant dependence was found for oxaloacetate concentrations of 2 and 4 µM. Oxaloacetate in concentration range from 0.5-2 µM causes decrease of fluorescence ratio in comparison with control ($p < 0.001$). In this case, the most pronounced effect is exerted by the lowest ligand concentration studied - 0.5 µM. At a concentration of 8 µM, the fluorescence ratios are at the control level, while the oxaloacetate concentration of 16 µM leads to an increase in the fluorescence ratio significantly higher than the control ($p < 0.001$).

Table 5. Comparison of the effect of oxaloacetate on LDH thermostability at 36.5-37.5°C

Ligand concentration, µM	OA M±SD
Control (LDH)	0.818±0.0011
0.5	0.806±0.0009
1	0.809±0.0013
2	0.810±0.0011
4	0.811±0.0012
8	0.814±0.0012
16	0.821±0.0018
Comparison of different ligand concentrations, p_{ANOVA}	
$p_{K-0,5}$	<0.001
p_{K-1}	<0.001
p_{K-2}	<0.001
p_{K-4}	<0.001
p_{K-8}	<0.001
p_{K-16}	<0.001

High content 16 µM of oxaloacetate is reflected in decrease of thermostability of protein: decrease of the melting onset temperature and faster onset of temperature inflection point. The content of minimum concentrations of oxaloacetate is 0.5-2 µM. As a result, the melting point and the inflection point of the protein molecule are increased. It is interesting to note the ability of low concentrations of oxaloacetate to have a thermostabilizing effect, which is manifested not only by a general shift of the melting temperature in the direction of increasing its value, but also by the manifestation of the protective effect on the section of the curve corresponding to the normal temperature of the human body.

3.5. The influence of oxaloacetate on the viability of human dermal fibroblast

There is a variety of possible interactions of natural metabolites with intra- and extracellular structures *in vivo* poorly studied. The next stage of our work is to evaluate the effect of oxaloacetate on the viability of human dermal fibroblast cell culture. It was found that the differences in LDH-test values between the control and experimental samples are not significant, which suggests the absence of cytotoxicity or protective effect of oxaloacetate in this test. The value of the MTT test in culture after the addition of oxaloacetate was 0.74±0.09 compared to control cells 0.55±0.03. Oxaloacetate was shown to have a 33,8 % ($p=0.028$) stimulating effect on the primary culture of human dermal fibroblasts, which was expressed in an increase in cell viability parameters in the MTT-test. Being a natural cell component, oxaloacetate has a low cytotoxicity profile, which indicates the ability to play a regulatory role and effectively modulate cell energy flows, maintaining internal homeostasis and even optimizing metabolism under the influence of external factors.

4. Conclusion

We revealed the full range of biological activity of oxaloacetate *in silico*. We characterized the interaction partner proteins. It has been shown that it serves as points of intersection of many metabolic pathways of protein, carbohydrate, and lipid metabolism. At the same time, it is playing a coordinating role in functioning and modulating mediator, hormonal, receptor responses, immunological, inflammatory, antibacterial and antiviral reactions, having anticarcinogenic action. We established the interaction of lactate dehydrogenase with oxaloacetate using microcapillary thermophoresis and calculated dissociation constant for interacting substances. The change in protein melting point temperature at different concentrations of oxaloacetate reflects the presence of changes in the conformational stability of the enzyme during protein-metabolite interactions. The degree of change is proportional to the affinity of the bond between the protein and the ligand. A change in the conformational structure of lactate dehydrogenase under the influence of oxaloacetate in the temperature range was revealed. Final concentrations of oxaloacetate 0.5-1 μM increase the thermal stability of the protein, while the concentration of 16 μM of the metabolite reduces the thermostable properties. The effect of oxaloacetate on the proliferative properties of human dermal fibroblasts was shown. Oxaloacetate increased the viability of dermal fibroblast cell culture.

It is interesting to use the knowledge about the activating effect of low doses of oxaloacetate in the development of "mitochondrial" and "bioenergetic" medicine approaches. It is possible to influence the metabolic processes of the cell by changing the direction of energy flows by controlling the content of intracellular and, in particular, mitochondrial metabolites. In conclusion, the fundamental research carried out has provided evidence for the participation of oxaloacetate in intra- and intercellular interactions.

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