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Study of dephosphorylated 2'-5'-linked oligoadenylates impact on *apo*-S100A1 protein conformation by heteronuclear NMR and circular dichroism

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Low molecular weight natural mediators, 2'-5'-linked oligoadenylates, play an important role in interferon-based antiviral mechanism; participate in growth, apoptosis and other important cellular processes. Taking into account their concentration within living cells, the 2'-5'A_3 oligoadenylates may act as additional biologically active substrates, capable of regulating the S100A1 protein functioning in vivo. ***Aim****. Find the evidence for the interaction of human apo-S100A1 with 2'-5'-linked oligoadenylates.* ***Methods****. Using the circular dichroism (CD) and heteronuclear NMR spectroscopy.* ***Results****. The obtained results demonstrated the occurrence of the secondary structure changes in human S100A1 protein upon the interaction with 2'-5'-linked oligoadenylates as well as indicated specific residues involved in this process.* ***Conclusions****. Our study points to the 2'-5'-linked oligoadenylates as possible additional elements of the complex system of fine regulation of the Ca^{2+}-signal transduction pathway in human cells.*

Keywords: 2'-5'-linked oligoadenylates, S100A1.

Introduction. Triphosphorylated 2'-5'-linked oligoadenylates, which can be defined by the general formula ppp(2'- 5')A_n, are being synthesized in the cell through interferon-induced enzyme – 2'-5'-oligoadenylate synthase (OAS). These compounds play a key role in the antiviral innate immunity mechanism, participate in the cell differentiation and apoptosis processes, at the same time being involved in diabetes and atherosclerosis pathogenesis. It was demonstrated, that they can be used as effective therapeutic compounds for oncology and hematology treatment [1, 2]. Their biological activity is mainly related to the ribonuclease L (RNase L) activation: only phosphorylated 2'-5'-linked oligoadenylates are capable of activating the enzyme due to the presence of phosphate groups though. dephosphorylated, or so-called «core» 2'-5'-linked oligoadenylates, do not bind to RNase L thus leaving the enzyme inactive [1–3].

The long-term research of 2'-5'-linked oligoadenylates is based on the «interferon hypothesis» of biological activity [1–4]. It assumes that the antiviral activity of these compounds is based on the formation of phosphorylated 2'-5'-linked oligoadenylates and further activation of RNase L, which specifically cleaves viral mRNAs, therefore rendering their antiviral properties. Nevertheless, phosphorylated oligoadenylates possess a low stability within the cell: they are cleaved by phosphorylases, thus forming «core» oligoadenylates, which display a set of other activities that have nothing to do with the interferon–RNase L system. In particular, 2'-5'-linked triadenylates (2'-5'A_3) and their analogues possess cardio protector potential and may act as graft versus host disease (GVHD) inhibitors [5]. It is worth

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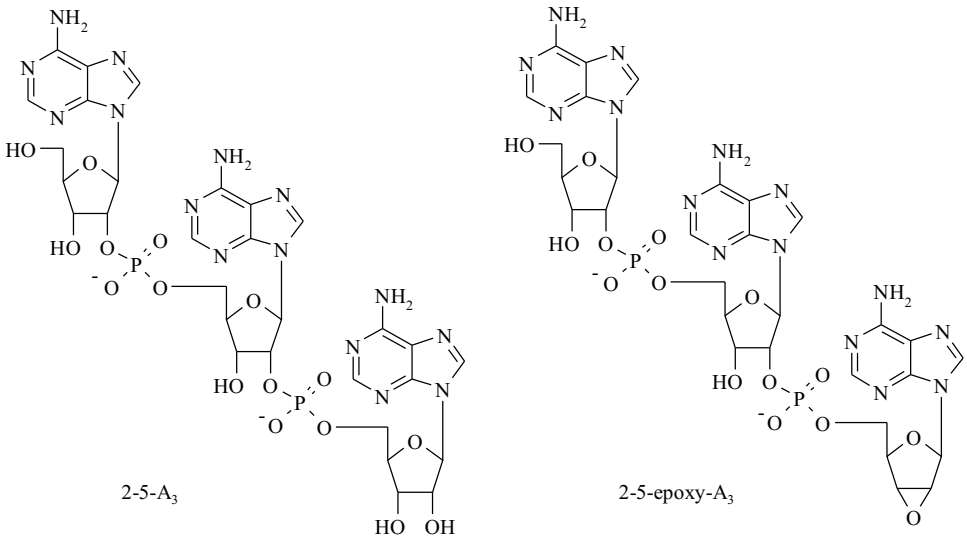

Fig. 1. Chemical formula of native 2'-5'A_3 (left) and its epoxy derivative (right). The oxygen atom, involved in epoxy-group formation, is high-lighted in blue

mentioning, that these compounds are also capable of executing the inhibiting effect on smooth muscle contraction [6].

The biological action of «core» 2'-5'A_3 is not fully understood yet. Up to now, all target proteins are unknown. Our previous experiments identified a set of protein targets, with which the «core» 2'-5'A_3 and its analogues interact: strong binding to albumin and interferon and weak binding to immunoglobulin were demonstrated [7]. In order to investigate the ability of «core» 2'-5'A_3 to interact with α-interferon – the key protein of 2'-5'OAS/RNAase L system, responsible for antiviral cell defense – MALDI-TOF mass spectrometry was applied. It was shown, that naturally occurring 2'-5'A_3 and its epoxy-modified analogue (2'-5'A_3-epo) bind to a-interferon, so 3'-5'-linked triadenylate does. Simultaneous binding of up to five ligand molecules was demonstrated [8].

It is a matter of common knowledge that Ca^{2+} signal transduction is an essential mechanism, ensuring various cell processes regulation. Multifunctional Ca^{2+}-transducing protein Calmodulin (CaM) is one of the key proteins involved in it. As it has recently been shown in our group, native 2'-5'A_3 binding to human CaM caused an alteration of its functional profile [9]. The experimental data revealed considerable (2–3 times) increase of its Ca^{2+} affinity upon interaction with «core« 2'-5'A_3, its epoxy and 3'-cordycepin modified analogues.

Using these data as a background, we decided to perform a study, focused on another Ca^{2+}-binding protein, human S100A1. It is the protein with known activity during neurological disorders and several types of cancer; its expression level serves as one of the key indicators for heart failure [10, 11]. As shown recently, the S100A1 interacts with ryanodine receptor (RyR) in heart (RyR1) and skeletal (RyR2) muscles [12]. The S100A1 shares with CaM exactly the same binding site to interact with RyR receptors [13]. The S100A1, an important regulator of calcium cycling, revealed the modulated activity of RyR in calcium-dependent way. The model activation of calcium release by RyR receptor in both hearts and skeletal muscles included knock-out RyR inhibitor (CaM).

Earlier, we have shown that dephosphorylated 2'-5'A_3 and its chemically modified analogues possess similar activity towards stimulating the Ca^{2+}-currents inactivation kine tics in HVA cell cultures [14]. Other experimental results, obtained by us, demonstrate that 2'-5'A_3 stimulates the RyR mediated Ca^{2+} release from the sarcoplasmatic reticulum in the smooth muscle cells [6].

Current report demonstrates the recently obtained experimental data, proving the possibility of interaction between the «core» 2'-5'A_3 and human S100A1 protein in *apo*-(Ca^{2+} free) form.

Materials and methods. *Synthesis of 2'-5'-linked oligoadenylates*. Natural 2'-5'A_3 and its epoxy modified analogue (2'-5'A_3-epoxy) were synthesized from a solution modified by phosphotriether method [15] (Fig. 1).

Synthesis and purification of the recombinant human apo-S100A1. The synthetic gene encoding human S100A1 was cloned into pET-30a + plasmid and ex-

pressed in *Escherichia coli* utilizing the T7 expression system. Bacterial cells were grown at 37 °C in LB medium. Expression was induced by addition of 0.4 mM IPTG at OD_{600} = 0.8. Bacterial culture was grown for 2 h afterward. Human S100A1 was isolated using the classical method of ammonium sulfate precipitation [16, 17] followed by the purification procedure using reversed-phase HPLC on a semi preparative Vydac C18 column [18]. The final product was identified by the electrospray ionization mass spectrometry using a Macromass Q-Tof spectrometer. The concentration in solution was estimated from its absorbance at 280 nm. For the synthesis of the recombinant ^{15}N-labeled human S100A1 protein, the *E. coli* cells were grown in M9 media containing $(^{15}NH_4)_2SO_4$ as the sole nitrogen source and unlabeled glucose as the sole carbon source.

CD Spectroscopy. The far-UV CD spectra were recorded over 260–200 nm range on Jasco J-815 CD spectropolarimeter at 298 K in a 2 mm light path length cuvette. For the measurements, 8 μM human Ca^{2+}-free form of S100A1 protein (*apo*-S100A1) was dissolved in a buffer that contained 5 mM Tris-HCl and 100 mM NaCl (pH 7.5). The concentration of human S100A1 in solution was controlled spectrophotometrically by measuring its UV absorbance at 280 nm using extinction coefficient 10200 ($mol^{-1}cm^{-1}$) on Cary Eclipse spectrophotometer. The titration experiments were performed by adding the small amounts of natural 2'-5'A_3 or 2'-5'A_3-epoxy concentrated stock solution, prepared in the same buffer. The final oligoadenylate concentration was 56 μM.

The oligoadenylate-*apo*-S100A1 solution ellipticity values [θ] were corrected by subtracting the corresponding values for natural 2'-5'A_3 or 2'-5'A_3-epoxy only and converted into the molar ellipticity [$\theta_{molar,\lambda}$], using the following equation:

$$[\theta]_{molar,\lambda} = \frac{100\theta_\lambda}{md},$$

where θ_λ is the observed ellipticity (degrees) at wavelength λ, *m* is the molar concentration of protein solution and *d* is the path length in centimeters. The secondary structure content of human S100A1 was extracted using CDNN deconvolution software [19].

NMR Spectroscopy and S100A1 titration with 2'-5'A_3. All NMR data sets were acquired on Varian Unity + 500 NMR spectrometer (^{1}H resonance frequency 500,606 MHz) equipped with three channels, *z*-gradients unit and ^{1}H/^{13}C/^{15}N triple resonance probe head with inverse detection. NMR sample was prepared by dissolving 0.3 mM ^{15}N-labeled human *apo*-S100A1 protein in 90 %/10 % H_2O/D_2O, 20 mM TRIS-d11 and 150 mM NaCl buffer solution. Spectra were recorded at 293 K using sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as external indirect reference with coefficients Ξ = = 0.251449530 and Ξ = 0.101329118 for ^{13}C and ^{15}N resonance frequencies respectively [20]. In order to obtain the detailed information about the interaction between apo-S100A1 and 2'-5'A_3, the concentrated stock solution containing 130 mM of 2'-5'A_3 was prepared in the same buffer. The NMR titration experiment was done as four consecutive injections (10 μL each) of stock solution to previously prepared NMR sample. Every addition was followed by acquiring 2D ^{1}H-^{15}N HSQC spectra. All recorded data were processed with the NMR Pipe [21] and analyzed with the Sparky [22] software.

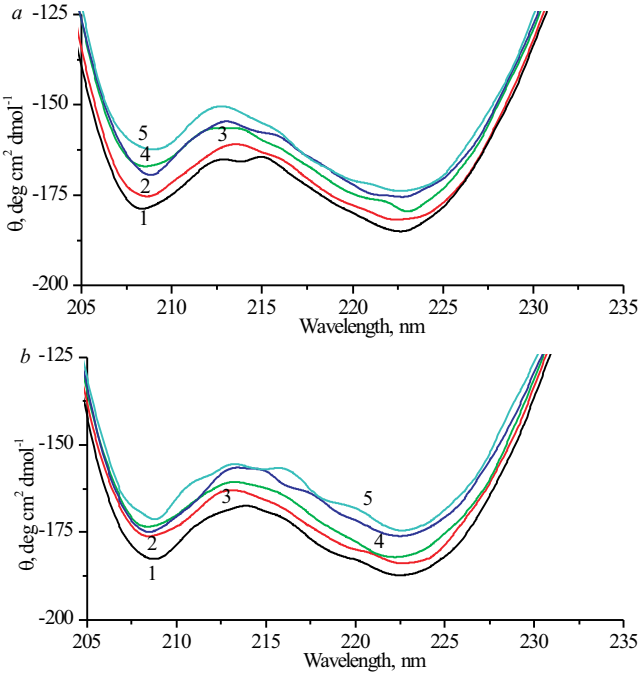

Fig. 2. CD spectra of *Apo*-S100A1 (black/1) in the presence of 8 μM (red/2), 24 μM (green/3), 40 μM (blue/4) and 56 μM (cyan/5) of 2'-5'A3-nat (*a*) or 2'-5'A3-*epo* (*b*).

Results and discussion. *CD spectroscopy*. Far-UV circular dichroism (CD) in the 260–200 nm range is widely used for the protein structure analysis [23]. In case of human S100A1, the recorded CD spectrum demonstrates two strong bands at around 222 and 208 nm (Fig. 2,

Changes in human apo-S100A1 protein's secondary structure content upon interaction with natural 2'-5' A3 or 2'-5'A_3-epoxy

Concentration of oligoadenylates, μ M	2'-5'A_3, %[a]			2'-5'A_3-epoxy, %[a]		
	α-Helix	β-Turn	Random coil	α-Helix	β-Turn	Random coil
0	54.5	13.7	21.1	54.5	13.7	21.1
8	53.2	13.9	21.7	52.9	13.9	21.8
24	50.2	14.2	23.1	51.9	14.0	22.4
40	49.8	14.3	23.4	50.6	14.2	23.0
56	48.8	14.4	23.9	49.6	14.3	23.5

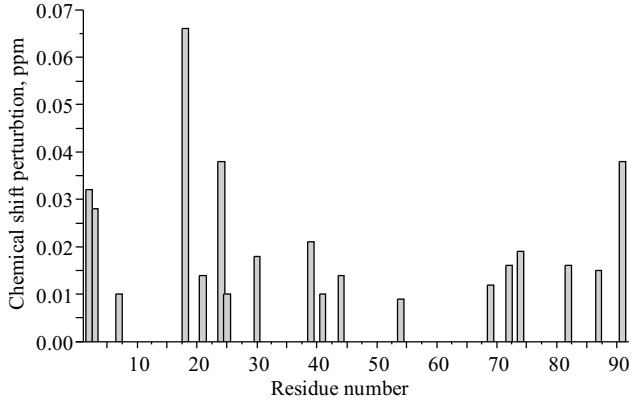

Fig. 3. *A* – chemical shift perturbation (csp) plot on 2D ^{1}H-^{15}N HSQC spectra upon titration with 2'-5'A_3 (only residues with notable csp values are presented)

a, *b*), which is an essential feature for the proteins with high content of α-helical structure [24]. According to the literature sources, the 3D structure of *apo*-S100A1 homodimer is dominated by four long α-helices with two short antiparallel β-sheets in C-terminal parts of both Ca^{2+}-binding loops [25].

The secondary structure analysis revealed that more than 50 % of the protein's secondary structure content is presented by α-helical elements. The contents of β-turns and randomly coiled elements are very low (Table). The presented CD spectra are very similar to those previously obtained in our group or published for structurally similar S100B protein [26].

An addition of either natural 2'-5'A_3 or its epoxy-modified derivative to the protein solution did not provide any dramatic changes in initial protein CD spectra shape. Nevertheless, it is demonstrated that 2'-5'A_3 oligoadenylates caused tiny alterations in 3D structure reflected in a small decrease of molar ellipticity within both helical bands (Fig. 2, *A*, *B*). The analysis of *apo*-S100A1 secondary structure demonstrated that addition of the oligoadenylate at maximum concentration, either natural or epoxy-modified, led to a decrease in the α-helical conformation by 6 % (Table). The value of error was determined by calculating the differences in evaluation of the secondary structure content within different regions of the CD spectra by the software.The value turned out to be around 2 % at the average in all cases.

NMR spectroscopy of human apo-S100A1 protein and its titration with natural 2'-5'A_3 oligoadenylates. The high-resolution 3D structure of *apo*-S100A1 is presented as X-type homodimeric biomolecule. Each subunit has two helix-loop-helix Ca^{2+}-binding EF-hand motifs of 93 amino acid residues. The spatial structure S100A1 in solution was solved independently by several groups under slightly different conditions (buffer, pH, ionic strength) and temperatures which could lead to small differences in Ca^{2+} affinity [25, 27, 28]. In the present study, the NMR data sets were acquired on NMR sample containing 300 μM of ^{15}N-labeled human *apo*-S100A1 at pH 7.2 under the same conditions as we have used recently [28]. The sequence-specific assignments of ^{1}H and ^{15}N resonances observed on 2D ^{1}H-^{15}N HSQC spectrum were transferred from previously deposited chemical shifts (bmrb code 18089).

A titration curve was obtained after four successive additions (10 μL each) of 130 mM natural 2'-5'A_3 stock solution prepared in the same buffer. The chemical shift perturbations (csp) values were calculated from ^{1}H and ^{15}N chemical shifts according to the following equation [29]:

$$\Delta\sigma = \sqrt{0.2(\Delta\sigma_N)^2 + (\Delta\sigma_H)^2}.$$

An inspection of the ^{1}H and ^{15}N HSQC NMR data sets exhibited several amino acid residues, which de-

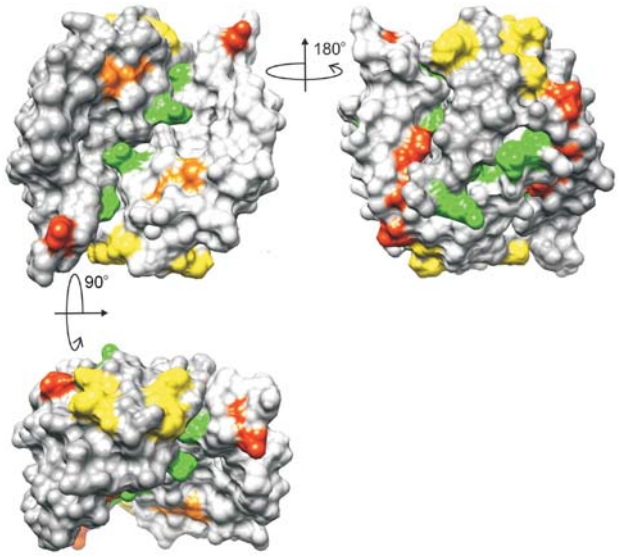

Fig. 4. Surface representation of 3D structure of human *apo*-S100A1 protein (pdb 2LLU) solved at the same experimental conditions [28]. Residues exhibited notable csp are colored as follows: located within N-terminal (His18, Lys21, Asp24, Lys25) and C-terminal (Val69, Gln72) Ca^{2+}- binding loops are highlighted in yellow and orange, respectively; residues within linker region and C-terminal part of helix IV (Glu39, Leu41, Phe44, and Glu91) are colored in red; at the intersubunit interface (Ser2, Glu 3, Ala7, Val54, Tyr74, Thr82, and Asn87) are highlighted in green

monstrated notable csp values for amide groups (Fig. 3, *A*). The recorded experimental data could be used to obtain information about binding constants of native 2'-5'A_3 oligoadenylates to *apo*-S100A1 [30]. The dissociating constants extracted from the chemical shifts curves (Fig. 3, *B*) fall in the moderate 10^{-5} ... 10^{-6} M range suggested that the interaction of 2'-5'A_3 with the human *apo*-S100A1 protein is biologically relevant, but the specificity is not very high.

A comparison of the obtained results with known high-resolution 3D structure of the human apo-S100A1 [27, 28] gave us an idea about structural alterations caused by 2'-5'A_3 binding (Fig. 4). It turned out, that positions of the residues with higher csp values referred to the Ca^{2+}-binding loops, which constituted the central part of the EF-hand domains. Majority of these signals came from the N-terminal part of the Ca^{2+}-binding motif (His18, Lys21, As 24, Lys25, Lys30), which was characterized by strong dependence on tiny changes in experimental conditions such as temperature, pH or/ and ionic strength of solution. The amino acid residues within the C-terminal region of the protein globule demonstrated lower csp values upon the interaction with 2'-5'A_3-nat oligoadenylates: only Val69 and Gln72 revealed notable csp values (Fig. 4). We suggest that even tiny structural alterations in those regions of the protein globule may cause changes in its Ca^{2+}-affinity.

The linker region (Glu40-Val51), in addition to C-terminal helix IV, constitutes another important region of S100A1 globule, which is mostly responsible for the interaction with target proteins/peptides. For instance, amino acid residues within the linker region are responsible for the hydrophobic contacts formation in case of interaction between S100A1 protein and Ryanodine receptor [31]. Another example is the complex between S100A1 and TRTK12 peptide, derived from actin-capping CapZ protein, which is formed via the interactions with amino acids within the «hinge» region (Phe44, Leu45, Lys49) and helix IV (Ala84, Cys85, Phe88) [32].

We have recently shown that central position in the linker region is being held by Phe44, which forms the so-called thiol-aromatic switch that controls hydrophobic interactions within this part of the protein globule (linker-helix IV) [28]. Some of the amino acid residues, located in close proximity to Phe44, demonstrated high csp values in a response to native 2'-5'A_3 binding, namely Thr39, Glu40, and Phe44 itself (Fig. 3, *A*).

As it has been previously noted, specific structural changes at the position of Phe44 further propagated to the whole linker [33]. As a result, notable alterations occurred anywhere within the S100A1 globule. In particular, the conformational changes might lead to α-helix shortening by 3–4 residues (one turn) within either the linker region [28, 33] or helix IV [25, 34]. It has recently been shown in our group, that similar structural modifications caused the increase of Ca^{2+} affinity and changes in cooperativity of Ca^{2+}-binding process [28]. These effects might explain small fluctuations within the S100A1 α-helical content upon binding natural 2'-5'A_3 oligoadenylates and its epoxy derivative (Table).

As has been previously noted, the linker region together with helix IV contained several residues strongly conserved throughout the whole S100 family of proteins [28]. Comparison of the S100A1 epitops involved in the interaction process with natural 2'-5'A_3 revealed that at least some of them formed contacts with the target proteins [31, 32]. This supports an idea that oligoadenylates might regulate interactions of Ca-signaling proteins within S100 family with their targets in the similar fashion.

It worth mentioning, that our experimental data demonstrated the impact of 2'-5'A_3 on the homodimeric interface within S100A1 globule. Some residues exhibited higher csp values (Ser2, Glu3, Ala7, Tyr74, Thr82, Asn 87, and Glu91), were involved in the formation of hydrophobic intersubunit contacts between helices I/I' and IV/IV' (Fig. 4). Considering a low value of solvent-accessible surface area of this particular region of the protein globule, we suppose that 2'-5'A_3 does not directly bind to residues within intersubunit interface, but rather transmits the effect to homodimeric surface upon binding to Ca^{2+}-binding loop or/and linker region. The similar effect has previously been reported for structurally similar S100B protein upon binding Ca^{2+} ions [35]. The tiny effect of 2'-5'A_3 binding to the *apo*-S100A1 homodimer interface might serve as an additional natural element, involved in the regulation of Ca^{2+} transduction pathway.

Conclusions. In our study we have demonstrated that 2'-5'A_3 can act as a compound, capable of altering the secondary structure of the human *apo*-S100A1 protein. Addition of both natural and epoxy-modified oligoadenylates triggered the decrease of its helical structure nearly to the same extent (around 6 %). We believe that the changes within its helical structure might trigger functional Ca^{2+} signal transduction regulation through tuning the function of human S100A1 protein. The presented experimental data obtained by CD and NMR spectroscopy exhibits tiny conformational alterations observed within the most important regions of the human *apo*-S100A1 globule caused by natural 2'-5'A_3 and its epoxy-modified derivative.

Our results suggested a possible implication of oligoadenylates in the regulation of the of S100A1 protein functioning.

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Вивчення впливу дефосфорильованих 2'-5' олігоаденілатів на конформацію білка *apo*-S100A1 методами гетероядерного ЯМР та кругового дихроїзму

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Резюме

Низькомолекулярні медіатори природного походження – 2'-5' олігоаденілати – відіграють важливу роль в антивірусному механізмі, пов'язаному з інтерфероном, вони причетні до росту клітин, апоптозу та інших важливих процесів, що протікають у клітині. Зважаючи на значну концентрацію 2'-5' олігоаденілатів всередині живої клітини, можна припустити, що вони слугують додатковими біологічно активними субстратами і здатні регулювати функціонування білка S100A1 in vivo. ***Мета****. Пошук доказів можливості взаємодії 2'-5' олігоаденілатів з апо-формою білка S100A1 людини.* ***Методи****. Використано методи ЯМР і КД.* ***Результати****. Отримані дані вказують на те, що внаслідок взаємодії між 2'-5' олігоаденілатами та S100A1 відбуваються перебудови у вторинній структурі останнього. Крім того, вдалося визначити, які саме амінокислотні залишки беруть участь у цій взаємодії.* ***Висновки****. Ймовірно, 2'-5' олігоаденілати є додатковими елементами складної системи регуляції процесів, опосередкованих іонами Ca^{2+}.*

Ключові слова: 2'-5' олігоаденілати, S100A1.

Изучение влияния дефосфорилированных 2'-5' олигоаденилатов на конформацию белка *apo*-S100A1 методами гетероядерного ЯМР и кругового дихроизма

А. Ю. Скоробогатов, Д. Н. Ложко, И. Ю. Жуков, А. В. Козлов, З. Ю. Ткачук

Резюме

Низкомолекулярные медиаторы природного происхождения – 2'-5' олигоаденилаты – играют важную роль в антивирусном механизме, связанном с интерфероном, они причастны к росту клеток, апоптозу и другим важным процессам, происходящим в клетке. Учитывая концентрацию 2'-5' олигоаденилатов внутри живой клетки, можно предположить, что они служат дополнительными био логически активными соединениями, способными регулировать функционирование белка S100A1 in vivo. ***Цель****. Поиск доказательств возможности взаимодействия 2'-5' олигоаденилатов с апо-формой белка S100A1 человека.* ***Методы****. Использованы методы ЯМР и КД.* ***Результаты****. Полученные данные указывают на то, что в итоге взаимодействия между 2'-5' олигоаденилатами и белком S100A1 происходят изменения вторичной структуры последнего. Кроме того, удалось определить аминокислотные остатки, непосредственно участвующие в этом взаимодействии.* ***Выводы****. Вероятно, что 2'-5' олигоаденилаты являются дополнительными элемен тами сложной системы, регулирующей процессы, опосредованные ионами Ca^{2+}.*

Ключевые слова: 2'-5' олигоаденилаты, S100A1.

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