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Interaction of 5'-ribonucleoside monophosphates with Interferon α-2b

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Aim. To characterize by different approaches the interaction of Interferon α-2b (IFN α-2b) with cytidine 5'-monophophosphoric acid (5'-CMP), guanosine 5'-monophophosphoric acid (5'-GMP), uridine 5'-monophophosphoric acid (5'-UMP) and their disodium salts, in the presence and absence of D-mannitol. **Methods.** Spectroscopy (time-resolved, circular dichroism and fluorescence), isothermal nanocalorimetry. **Results.** Using IFN α-2b intrinsic fluorescence measurements we established that 5'-GMP and 5'-CMP interact with IFN α-2b stronger than their disodium salts while no difference of affinity was observed for 5'-UMP and its disodium salt. The presence of D-mannitol enhances the observed effects for 5'-GMP and 5'-CMP, but not for their disodium salts and all forms of 5'-UMP. Thermodynamic measurements reveal that the interaction of all tested 5'-ribonucleoside monophosphoric acids with IFN α-2b are exothermic, while of all disodium salt of 5'-ribonucleoside monophosphates are endothermic. The entropy factor TΔS was calculated to be negative in the presence of all 5'-ribonucleoside monophosphoric acids indicating a decrease of randomness in the system and positive in the presence of their disodium salts indicating an opposite effect. Circular dichroism measurements showed that 5'-ribonucleoside monophosphoric acids induce a decrease of the regular alpha helices content and increase the unstructured regions length in INF α-2b. In contrast, their disodium salts make rather stabilizing effect on the secondary structure elements of the protein. **Conclusions.** 5'-ribonucleoside monophosphoric acids used in this study interact with IFN α-2b in different way compared to their disodium salts. Acid forms of 5'-ribonucleoside monophosphates possess higher affinity to IFN α-2b and destabilize secondary structure elements of this protein. This may restrain a conformational mobility of IFN α-2b and as a consequence modify its functional activity. In contrast, disodium salts of 5'-ribonucleoside monophosphates display a weak affinity to IFN α-2b and stabilize its secondary structure organization.
Keywords: kinetics; mononucleosides; Interferon α-2b; protein-ligand interactions; titration.

Introduction

RNA molecules participate in a variety of interactions and perform numerous cellular functions by forming specific structures that enable RNA-RNA, RNA-DNA, or RNA-protein interactions. Recent progress in the study of such interactions has led to the development of technologies that create new substances for treating various diseases [1]. Sodium salt of

the total RNA preparations, mostly isolated from yeast, have long been used as therapeutic drug — with immunomodulatory properties [2]. The ability of yeast RNA sodium salt preparations to heal wounds has been reported [3]. In addition, the total yeast RNA preparation in the form of a free acid had an immunomodulatory activity, demonstrated an anti-inflammatory effect [4] and pronounced membrane-stabilizing effect with normalization of NO-synthetase activity and antioxidant activity *in vitro* and *in vivo* [5].

In previous studies, we have used the hydrolyzed total yeast RNA (free of DNA, polysaccharides and proteins) and its complex with D-mannitol. Mannitol is a hexatomic sugar alcohol that is widely used in pharmacology as a filler due to its ability to structure the water shell, to increase the solubility and stability of drugs [6].

Importantly, the yeast oligoribonucleotides preparation in the form of a free acid in the presence of D-mannitol (ORNs-D-M) possesses a wide range of antiviral activity [7]. ORNs-D-M has been shown to be efficient in both the prevention and the treatment of influenza infections in mice due to the inhibition of the neuraminidase activity [8]. In contrast, the sodium salt of yeast oligoribonucleotides preparation (ORNs-Na) possessed only immunomodulatory activity [4]. The mechanism of abovementioned anti-inflammatory, anti-rheumatic, and anti-viral action of the yeast oligoribonucleotides preparations remains unclear.

MALDI-TOF (matrix-assisted laser desorption/ionization-time-of-flight) mass spectrometry analysis of yeast oligoribonucleotides preparation revealed that the major fraction is composed of 2–7 base fragments and the minor one contains oligoribonucleotides of other sizes [9]. We suppose that the oligoribonucleotides can be further hydrolyzed to ribonucleoside monophosphates in the body of laboratory animals and that, in turn, can have a therapeutic effect.

It is known that ribonucleoside monophosphates may have an impact on the secondary structure of peptides. Ribonucleoside monophosphates was shown to bind the Ac-KA14K-NH2 peptide via both hydrophobic and electrostatic interactions [10].

In our previous study, we reported the effect of 5’-adenilic acid on the conformation and fluorescence properties of Interferon α-2b [11]. In this work we extended our study to other 5’-ribonucleoside monophosphates (5’-rNMPs), namely 5’-cytilic acid, 5’-uridilic acid and 5’-guanilic acid and their disodium salts and tested the interaction of 5’-CMP, 5’-GMP, and 5’-UMP in the absence and presence of D-mannitol with Interferon α-2b by measuring its intrinsic fluorescence, circular dichroism spectra and thermodynamic parameters of the reaction.

Materials and Methods

We used recombinant Interferon α-2b as a model cytokine kindly provided by Interpharmbiotek Kyiv, Ukraine. D-mannitol and 5’-rNMPs, 5’-rNMPs-Na_2 were from Sigma Aldrich, USA. 5’-rNMPs stock concentration was 3mM. Working *in vitro* concentration of 5’-rNMPs and 5’-rNMPs-Na_2 varied from 0 to 15 mM. Concentration of D-mannitol varied from 0 to 3 mM and was in excess over IFN α-2b. The recombinant INF α-2b used for measurements is a monomeric protein with a mo-

lecular mass of about 18 kDa as judged by SDS-PAGE [11] and about 20 kDa accoding to the analytical gel filtration in native conditions (data not shown). It has been reported that recombinant human IFN α-2b forms a homodimer in crystal where each monomer consists of five alpha helices connected by short loops [12].

We used the 5'-rNMPs mixes with D-mannitol in a weight ratio of 2.5:1 (5:1 in terms of molar concentration). Multivariate curve resolution — alternating least squares analysis of the IR spectra of RNA-ligand mixtures of the curves indicates that the maximum formation of the complex between RNA and mannitol, almost 100 %, occurs at the weight ratio of RNA: mannitol — 2.5:1 [6].

Quenching of Interferon α-2b intrinsic fluorescence by 5'-rNMPs

The ability of 5'-cytidine, 5'-guanosine, 5'-uridine monophosphates and their disodium salts in the presence and absence of D-mannitol to interact with Interferon α-2b was studied using the method of intrinsic fluorescence quenching. Fluorescence emission spectra were recorded in the 300–450 nm range at the excitation wavelength λ_{ex} = 295 nm using a Jasco FP-8200 spectrofluorometer with a 1 cm cuvette. For the measurements, IFN α-2b was dissolved in 50 mM Tris-HCl buffer (pH 7.5) at 37 °C. The scan speed was set to 200 nm/min, with an excitation bandwidth of 2.5 nm and an emission bandwidth of 2.5 nm. 5'-rNMPs stock concentration was 3mM. The final protein concentration was 1 μM in 2 mL (molar extinction coefficient 19200 $M^{-1} \times cm^{-1}$) and the final 5'-rNMPs titrant concentration was approximately 15 μM. The spectra of IFN α-2b during titration with 5'-rNMPs and 5'-rNMPs+D-M were normalized. A value of the maximum of the IFN α-2b emission intensity (F) was measured at λ_{em} = 336 nm. Fluorescence quenching was calculated as follows:

$Q = [1–(F/F_0)]x100\%$, where F is the value of the maximum of emission intensity of the protein in the presence of 5'-rNMPs, F_0 is the value of the maximum of emission intensity of the protein itself under the same conditions [13].

Influence of 5'-rNMPs and 5'-rNMPs-Na_2 in the presence and absence of D-mannitol on the IFN α-2b fluorescence lifetime.

The non-radiative interaction of different ligands with IFN α-2b was determined by time-resolved pulse spectroscopy (Horiba FluoroMax 4 Plus fluorometer, USA). Experimental parameters: scan range — 100 ns; number of channels — 4000; emission band gap — 2.5 nm; excitation wave — 295 nm; emission detection wave — 336 nm; optical path length — 1 cm. The lifetime is defined as the exponential regression of the number of emission signals versus time: $I(t) = \exp^{(-t/\tau)}$. The energy transfer efficiency is the ratio of the number of energy transfer events to the number of donor excitation events:

$E = 1–(\tau_D'/\tau_D)$, where τ_D' and τ_D are the fluorescence donor lifetimes in the presence and absence of the acceptor, respectively [14].

Calculation of dissociation constants of 5'-rNMPs and 5'-rNMPs-Na_2 in the absence and presence of D-mannitol to IFN α-2b.

Dissociation constants were calculated by performing a non-parametric regression of the

fluorescence quenching spectra of IFN α-2b obtained after its titration by different concentrations of ligands using Origin 8.0 (Origin Lab, USA).

The following formula was used to estimate the dissociation constant values:

$$\frac{F}{F0} = \left(0.5m - \sqrt{0.25m^2 - \frac{P}{D_0}}\right),$$

$$m = 1 + \left(\frac{P}{D_0}\right) + \left(\frac{K_d}{D_0}\right),$$

where D_0 is the concentration of the titrated substance, and P is protein concentration. F_0 — maximum fluorescence, F — observed fluorescence, K_d — dissociation constant, m — analytical function [15].

Estimation of thermodynamic parameters of interaction between 5'-ribonucleoside monophosphates and IFN α-2b by isothermal titration calorimetry.

We used the method described in [16] to perform isothermal titration nanocalorimetry (ITC) using a TA INSTRUMENTS Nano ITC Low Volume. The thermodynamic parameters of the interaction reaction, including changes of enthalpy, Gibbs free energy, and entropy, were calculated by measuring the changes of heat release or absorption during the binding process. A total of 25 injections were made with an interval of 300 seconds between each injection. The protein was pre-dialyzed against the buffer containing 50 mM Tris-HCl buffer (pH 7.5). The ligands have been prepared in degassed and de-ionized water. The same buffer was used in the reference cell. The following equation was applied to determine these reaction parameters:

$$\frac{dQ}{dn_L}(Z) = \frac{1}{2}\Delta H_b \left[1 + \left(1 - \frac{[L]}{n[M]} - \frac{1}{nK_b[M]}\right)\left(\left(1 + \frac{[L]}{n[M]} + \frac{1}{nK_b[M]}\right)^2 - \frac{4[L]}{n[M]}\right)^{-\frac{1}{2}}\right],$$

where the heat change plot (dQ/dn_L) is a function of concentration ($Z = [L]/[M]$, where $[L]$ and $[M]$ are the molar concentrations of the ligand and macromolecule, respectively). In this equation, the unknown parameters are ΔH [κJ/mol], K_b [mol^{-1}], and n — stereochemical index, which are the binding enthalpy (the energy involved in the association process), binding constant, affinity (affinity between two compounds) and reaction stoichiometry.

We used the type II variant of experiment (Fig. 1) when the ligand is added to the protein. The calorimeter records the change of the heat for 3 min for each of the 25 injections. The obtained experimental curves were fit in the nanoanalysis program to calculate ΔH and K_b (Multiple Non-Interacting Sites (MNIS) mathematical model).

Using the obtained values of ΔH and K_b, the entropy factor $T\Delta S$ [kJ*mol] and total free Gibbs energy ΔG [kJ/mol] of interaction between INF α-2b and respective ligands were calculated according to the equations $\Delta G = -RTlnK_b$ and $T\Delta S = (\Delta H - \Delta G)$.

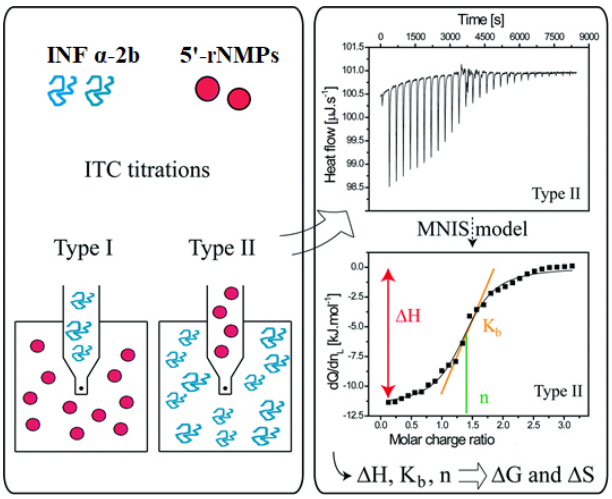

Fig. 1. A schema of the nanocalorimetry titration of INF by the 5'-rNMPs preparations [modified from 16]

Circular dichroism

We used CD spectroscopy using a Jasco J-815 to determine the effect of different forms of 5'-rNMPs on the secondary structure of IFN α-2b. To measure the CD spectra, IFN α-2b was dissolved in 50 mM Tris-HCl buffer (pH 7.5) at a temperature of 37 °C. A 3 mM stock solution of each ribonucleoside monophosphate with or without D-mannitol was prepared on the same buffer solution. The final protein concentration was 1 μM in 2 mL cuvette, and the final titrant concentration was approximately 15 μM. The scan speed was set to 200 nm/min, with an excitation bandwidth of 2.5 nm and an emission bandwidth of 2.5 nm. The scanning range was 260–195 nm with two passes and an accumulation of 2s at a scanning speed of 100 nm/min. The CD spectrum was converted from the [mdeg] units to molecular ellipticity $[\Theta]_{MRE}$ [deg·cm^2·dmol^{-1}] taking into account the protein concentration, the number of peptide bonds in the protein (165) and the optical path length of the cuvette (1 cm) using the service http://bestsel.elte.hu according to the following relationship:

$[\Theta]_{MRE} = \Theta/(10 \cdot c \cdot N_r \cdot l)$, where Θ is the measured ellipticity in [mdeg] units, c — stands for the molar concentration of the protein, N_r is the number of residues in the protein, and l is the pathlength in cm. We calculated the secondary structure elements: the content of α-helices, which were divided into regular middle parts and relaxed ends of the helices. Additionally, parallel and antiparallel beta-sheets were divided into three subgroups: left-handed, relaxed (with a rotation angle of up to 4° to the left and right), and right-turned, as well as unstructured areas [17].

Statistical processing of results

Experimental data from 10 independent experiments were statistically processed to calculate the mean (M), standard deviation (δ or ±SD), and standard error (m or ±SE). One-way analysis of variance (ANOVA) was used to determine the significance of differences between experiments. Statistical processing of results and graphs was performed using Origin Pro 8.0 software.

Results and Discussion

First, we tested the interaction between IFN α-2b and 5'-ribonucleoside monophosphates by the fluorescence quenching technique. If 5'-ribonucleoside monophosphates collide with the tryptophan residues of IFN α-2b it may result in a decrease of their fluorescence intensity that is a dynamic quenching. Dynamic quenching requires molecular contact between the fluorophore and quencher [13].

Addition of 5'-CMP to IFN α-2b causes 38% quenching of its intrinsic tryptophan flu-

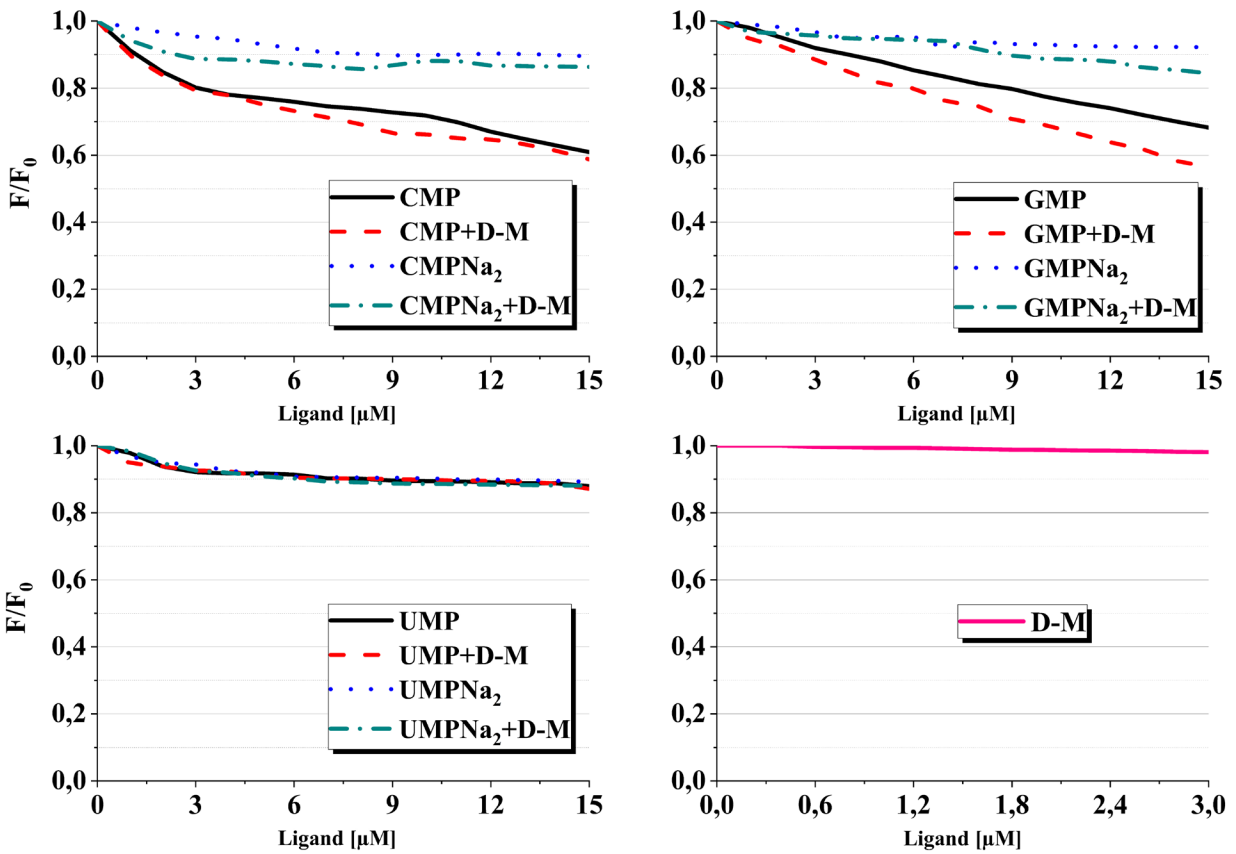

Fig. 2. Quenching of IFN α-2b tryptophan fluorescence by increasing concentrations of 5'-cytidine, 5'-guanosine, and 5'-uridine monophosphates, their disodium salts in the absence and presence of D-mannitol. F/F_0 — the ratio of interferon fluorescence in the presence of ligand to its fluorescence without ligand

orescence at a 15 mM concentration, while addition of 5'-CMP with D-mannitol (5'-CMP+D-M) – 42% quenching. Disodium salt form 5'-$CMPNa_2$ alone and in the presence of D-mannitol has a smaller effect on the IFN α–2b fluorescence, 11% and 18%, respectively (Fig. 2). D-mannitol alone does not show any quenching affect (Fig. 2).

Similarly, 5'-GMP and 5'-GMP+D-M addition quenches the IFN α-2b tryptophan fluorescence on 32% and 45%, respectively, while $GMPNa_2$ and 5'-$GMPNa_2$+D-M addition results in 8% and 16% quenching, respectively (Fig. 2).

In contrast, the effect of 5'-UMP, 5'-UMP+D-M, 5'-$UMPNa_2$ and 5'-$UMPNa_2$+D-M on the IFN α-2b intrinsic tryptophan fluorescence is indistinguishable. All of them quench the IFN α-2b fluorescence on approximately 10% (Fig. 2).

Of note, previously published results for 5'-AMP, 5'-AMP+D-M, $AMPNa_2$ and 5'-$AMPNa_2$+D-M demonstrated the quenching effect on INF α-2b fluorescence as 22%, 26%, 8% and 10%, respectively [11].

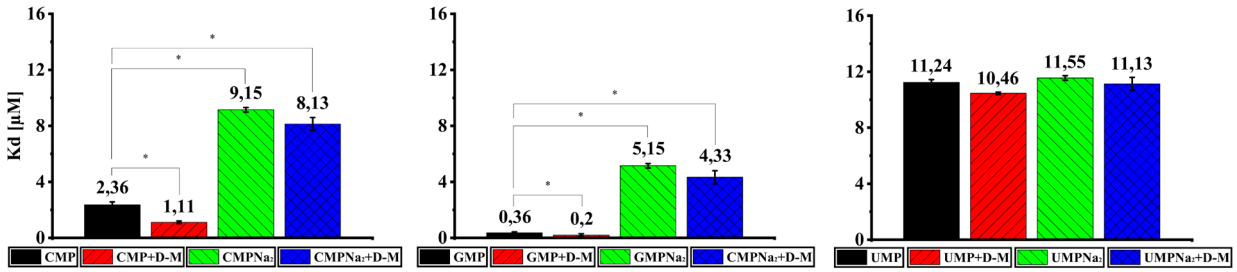

Fig. 3. Deduced dissociation constants for the interaction reaction of IFN α-2b with 5'-ribonucleoside monophosphates, their disodium salts, in the absence and presence of D-mannitol. * — statistically significant difference, $P \leq 0.05$.

Thus, among 5'-ribonucleoside monophosphates tested (this study and previously published [11]). we observed a significant quenching of INF α-2b fluorescence in the presence of guanilic, citidilic and adenilic acids. Addition of D-mannitol slightly enhanced this effect (Fig. 2). In contrast, disodium salt of mentioned above acids as well as 5'-UMP and 5'-$UMPNa_2$ had much smaller effect on INF α-2b tryptophan fluorescence (Fig. 2).

From the obtained quenching curves, we deduced dissociation constants as a measure of strength of the interaction between different 5'-ribonucleoside monophosphates and IFN α-2b (Fig. 3). 5'-CMP has about 4-times higher affinity to IFN α-2b than 5'-$CMPNa_2$. The affinity of 5'-CMP is slightly increases in the presence of D-mannitol, the Kd ratio 5'-$CMPNa_2$+D-M and 5'-CMP+D-M is equal to 5.3. 5'-GMP interacts with IFN α-2b about seven times stronger than 5'-CMP. In the presence of D-mannitol this ratio remains the same. Comparison of 5'-GMP with its disodium salt reveals that the guanilic acid has about 15-fold higher affinity compared to 5'-$GMPNa_2$. In the presence of D-mannitol the affinity of the guanilic acid to IFN α-2b increases to 20-fold over it's disodium salt. All forms of 5'-uridine monophosphates used in the experiment have similar affinity for IFN α-2b, Kd values were estimated to be in the range from 10.46 to 11.55 mM. This is also comparable to the Kd value of 5'-$CMPNa_2$ (Fig. 3).

Previously published experiments on IFN α-2b interaction with different forms of 5'-AMP revealed that Kd for 5'-AMP alone was equal to 1.53 ± 0.41 μM, while in the presence of D-mannitol (5'-AMP+D-M) – 0.94 ± 0.09 μM. In the case of 5'-$AMPNa_2$ and 5'-$AMPNa_2$+D-M, the Kd was 8.14 ± 0.19 μM and 7.1 ± 0.73 μM, respectively [11]. Therefore, the affinity of IFN α-2b to the adenylic acid is 5.3-fold higher than to $AMPNa_2$, in the presence of D-mannitol this affinity increases to 7.6-fold. Of note, the affinity of 5'-AMP to IFN α-2b is comparable to that of 5'-CMP measured in this study. The highest affinity for IFN α-2b was observed for different forms of 5'-GMP and the lowest — for 5'-UMP.

Thus, we found here that the binding affinity of different 5'-ribonucleoside monophosphates to IFN α-2b (Kd ranges from 0.2 to 11.55 μM) corresponds to the affinity of the transient protein-protein and protein-ligand interactions frequently observed in signal

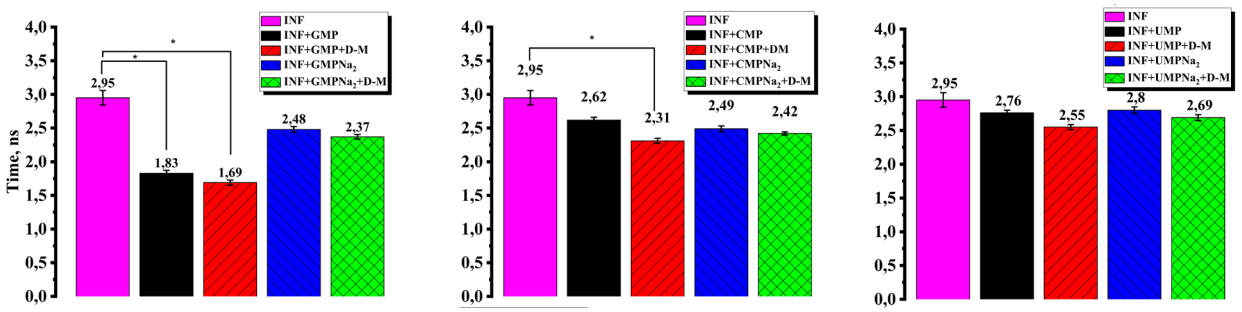

Fig. 4. INF α-2b tryptophan fluorescence lifetime in the presence of 5'-rNMPs acids, their disodium salts, and D-mannitol. * — statistically significant difference compared to IFN α-2b as a control, P≤0.05

transduction and membrane trafficking in the cell [18].

Next, we measured the IFN α-2b tryptophan fluorescence lifetime in the presence of different forms of 5'-rNMPs with or without D-mannitol (Fig. 4). This approach is useful for detecting changes in the physicochemical environment of the fluorophore, specifically the tryptophan residues of IFN α-2b.

Addition of the 5'-GMP acid alone and with D-mannitol to IFN α-2b shortened its fluorescence lifetime 1.6 and 1.7-fold, respectively. A statistically significant decrease of the fluorescence lifetime was also observed in the presence of 5'-CMP+D-M (Fig. 4). The 5'-uridilic acid alone or in the presence of D-mannitol did not affect the IFN α-2b tryptophan fluorescence lifetime (Fig. 4). Previously, we demonstrated that 5'-AMP and 5'-AMP+D-M reduced the IFN α-2b tryptophan fluorescence lifetime to 2.01 and 1.92 ns [11]. A presence of D-mannitol alone did not have any significant impact [11]. It should be noted that in contrast to 5'-rNMPs acids their disodium salts alone or in presence of D-mannitol did not have statistically significant effect on the IFN α-2b tryptophan fluorescence lifetime (Fig. 4 and [11]).

Thus, we conclude that 5'-guanilic acid alone and 5'-guanilic and 5'-cytidilic acids in the presence of D-mannitol influenced the physicochemical environment of the IFN α-2b tryptophan residues in the way that reduced their fluorescence lifetime.

Additionally, we performed the Förster energy transfer (FRET) measurements between IFN α-2b and different forms of 5'-ribonucleoside monophosphates (Fig. 5). The efficiency of FRET is inversely proportional to the sixth power of the distance between the donor and acceptor molecules making this technique extremely sensitive to small changes in distance between them.

As a general tendency, the FRET efficiency measured for 5'-nucleoside monophosphoric acids in this study are two times higher than for their respective disodium salts. This effect does not depend on the presence of D-mannitol (Fig. 5). When IFN α-2b interacted with 5'-AMP and 5'-AMP+D-M, the energy transfer efficiency was 0.2 and 0.22, respectively, 5'-AMPNa_2 and 5'-AMPNa_2: D-M 0.07 and 0.16. Similar result was obtained for different forms of 5'-AMP previously published in [11]. Indeed, energy transfer efficiency from IFN α-2b to 5'-AMP and 5'-AMP+D-M was esti-

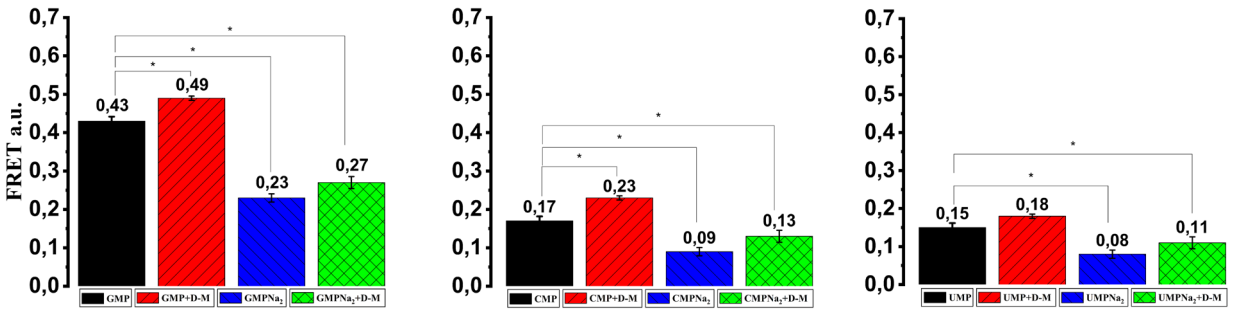

Fig. 5. Efficiency of Förster resonance energy transfer between Interferon α-2b and 5'-rNMPs acids, their disodium salts in the presence and absence of D-mannitol. * — statistically significant difference $P \leq 0.05$

mated to be 0.2 and 0.22, respectively, while for 5'-$AMPNa_2$ and 5'-$AMPNa_2$+D-M — 0.07 and 0.16, respectively.

Using pulse time-resolved spectroscopy, we have shown that the efficiency of the non-radiative energy transfer from Interferon to 5'-rNMPs is higher in the acidic form, due to the closer distance between the molecules and the higher Förster transfer rate. Thus, using pulse time-resolved spectroscopy we demonstrated that the efficiency of the non-radiative energy transfer from Interferon α-2b to 5'-rNMPs acids are about two times higher than for their disodium salts. Consequently, it is reasonable to assume that the distance between the fluorophores on INF α-2b and 5'-rNMPs acids is shorter compared to their respective disodium salts.

The interaction between protein and ligand can be also studied by the thermodynamic approach. The thermodynamic parameters of the interaction reaction between IFN α-2b and different forms of 5'-nucleoside monophosphates, including changes in enthalpy, Gibbs free energy, and entropy, can be determined by measuring the changes in heat release or absorption by ITC. The titration of IFN α-2b by different forms of 5'-rNMPs indicates that the interaction reactions between the protein and 5'-rNMPs acids are exothermic, while between the respective disodium salts are endothermic (Fig. 6). The change of the enthalpy value is decreasing in the row 5'-GMP > 5'-CMP > 5'-UMP without or with D-mannitol, while the change of the enthalpy value for the respective disodium salts is varying in the range 0.24–0.59 kJ/mol for 5'-$rUMPNa_2$ and 0.45–0.94 kJ/mol for 5'-$rUMPNa_2$+D-M (Fig. 6). The thermodynamic measurements for IFN α-2b with different forms of 5'-AMP have been reported earlier [11]. Titration of IFN α-2b by 5'-AMP acid alone and in the presence of D-mannitol resulted in the enthalpy change –10.7 and –12.8 kJ/mol, respectively. In contrast, the enthalpy change caused by 5'-$AMPNa_2$ and 5'-$AMPNa_2$+D-M was 0.65 and 0.92 kJ/mol, respectively [11]. These values are very similar for those obtained for different forms of 5'-GMP (Fig. 6). A presence of D-mannitol in the mixture with IFN α-2b did not result in the enthalpy change significant for detection [11].

It worth noting that the entropy change of the system depends on wither 5'-ribonucleoside monophosphoric acids or their disodium salts are added to IFN α-2b. 5'-rNMPs acids

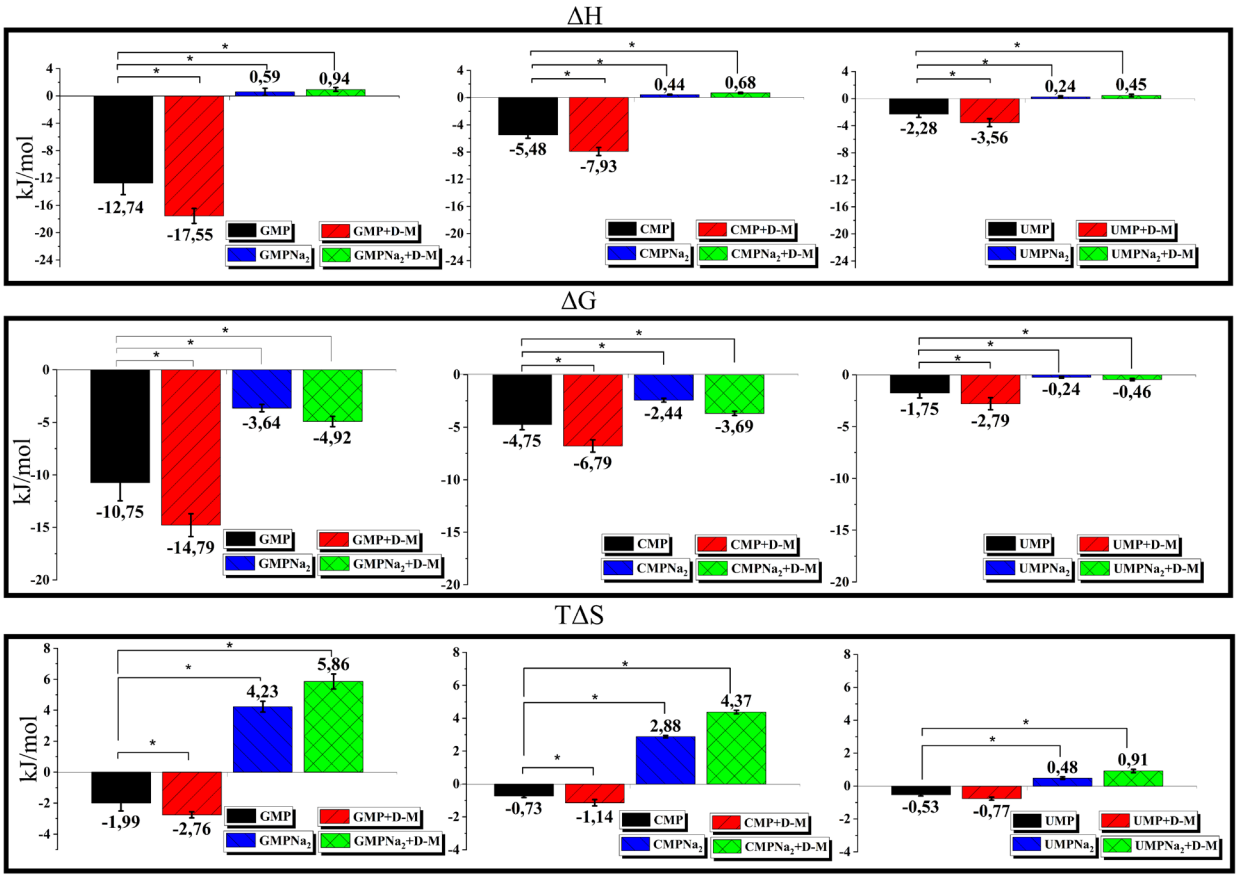

Fig. 6. Thermodynamic parameters of the interaction between Interferon α-2b and 5'-rNMPs acids, their disodium salts in the absence and presence of D-mannitol. * — statistically significant difference, P≤0.05

without or with D-mannitol induce negative change of entropy, while 5'-rNMPs-Na_2 alone or in the presence of D-mannitol have an opposite effect (Fig. 6). Similar observations were made for 5'-AMP and 5'-AMP+D-M for which the change of entropy was estimated to be –1.59 and –1.93 kJ/mol, respectively, while for 5'-$AMPNa_2$ and 5'-$AMPNa_2$+D-M — 1.59 and 3.07 kJ/mol, respectively [11].

The titration of Interferon α-2b with 5'-rNMPs indicates an exothermic reaction between the protein and acid ligands and an endothermic reaction with disodium salt. These results suggest that 5'-rNMPs can bind multiple times to different binding sites on the protein molecule. The literature indicates that protein-ligand interaction results in an exothermic reaction that increases Sendai virus N_{TAIL} activity, while an endothermic reaction inhibits it [19].

Most probably, 5'-rNMPs acids decrease randomness in the system by enhancing a structural rigidity of IFN α-2b, in contrast, a disodium salt of 5'-rNMPs increases randomness that may enhance IFN α-2b conformational flexibility.

Importantly, the ΔG value calculated for the interaction reactions with all forms of 5'-rNMPs used in this study (Fig. 6) and previously published [11] are negative, indicating that all these reactions proceed spontaneously. However, the ΔG values differ depending on the nature and form of 5'-nucleoside monophosphates mixed with IFN α-2b. The biggest change of free Gibbs energy was observed when 5'-GMP acid (Fig. 6) and 5'-AMP acid (ΔG = –9.11 k J/mol for 5'-AMP alone and –10.87 kJ/mol for 5'-AMP+D-M [11]) was added to IFN α-2b. On the contrary, their disodium salts have much smaller effect: ΔG = –3.46 kJ/mol for 5'-GMPNa_2 alone and –4.92 kJ/mol for 5'-GMPNa_2+D-M, as well as ΔG = –0.94 kJ/mol for 5'-AMPNa_2 alone and –2.16 kJ/mol for 5'-AMPNa_2+D-M [11]. The same tendency was observed for other 5'-ribonucleoside monophosphates: the change of free Gibbs energy was bigger for the acid forms than for their respective disodium salts. Besides, the 5'-CMP acid and its disodium salt caused a smaller change of the ΔG value compared to 5'-AMP while different forms of 5'-UMP — the smallest change among all

Table 1. **Influence of different forms of 5'-rNMPs on the content of secondary structure elements (in %) in Interferon α-2b**

	α-helixes regular	α-helixes diffuse	antiparallel β sheets right-handed	antiparallel β sheets relaxed	antiparallel β sheets left-turn	parallel β sheets	β turns	unorganized structure
INF	26,6	11,0	0,0	8,1	12,2	0,0	6,8	35,3
INF+GMP	15,3	10,2	0,0	8,6	0,0	0,0	7,1	58,8
INF+GMP+D-M	16,4	9,7	0,5	7,5	0,0	0,0	6,7	59,2
INF+GMPNa_2	30,0	11,5	1,5	9,2	10,1	2,4	11,0	24,3
INF+GMPNa_2+D-M	31,4	15,1	2,1	10,2	8,4	0,7	10,5	21,6
INF+CMP	16,0	10,5	0,0	8,4	0,0	0,0	7,8	57,3
INF+CMP+D-M	17,3	10,4	0,5	6,5	0,0	0,0	7,1	58,2
INF+CMPNa_2	32,0	13,5	2,3	9,2	14,1	1,5	11,1	16,3
INF+CMPNa_2+D-M	32,5	15,1	2,9	11,1	10,4	1,1	10,7	16,2
INF+UMP	22,6	10,9	0,0	8,2	0,0	0,1	8,0	50,2
INF+UMP+D-M	17,8	11,4	0,0	8,5	0,0	0,0	7,5	54,8
INF+UMPNa_2	33,0	12,6	1,3	9,2	13,1	2,6	10,2	18,0
INF+UMPNa_2+D-M	31,5	15,1	1,7	11,1	11,8	1,4	10,3	17,1

5’-ribonucleoside monophosphates tested (Fig. 6). This result indicates that purines interact stronger with IFN α-2b than pyrimidines. This is true for either their acid or disodium salt forms. The presence of D-mannitol positively influences the interaction of all tested 5’-ribonucleoside monophosphates with IFN α-2b (Fig. 6). This is in agreement with the fluorescence quenching experiments and the FRET measurements described above (Fig. 3–5).

It worth noting that hydrogen bonding and van der Waals interactions are the main contributors to favorable enthalpy interactions, ΔH. Unfavorable enthalpy interactions, on the other hand, arise from the desolation of polar groups. Favorable entropic contributions, $T\Delta S$, result from the desolvation of nonpolar and polar amino acids, while unfavorable entropic contributions arise from the structuring of residues in the protein or the ligand itself [20].

To get a deeper insight into the interaction mechanism between different 5’-rNMPs and Interferon α-2b we performed circular dichroism (CD) measurements (Table 1).

Previously, we used this method to measure the effect of various forms of 5’-AMP and oligoribonucleotides on the INF α-2b secondary structure [11]. Indeed, both oligoribonucleotide mixture and 5’-AMP in the absence or presence of D-mannitol modified the secondary structure of INF α-2b by increasing unstructured regions or decreasing number of structured secondary elements [11].

In this study, we demonstrate that all 5’-ribonucleoside monophosphoric acids reduce the content of regular alpha helices and antiparallel beta sheets, leading to an increase of the unstructured regions in INF α-2b by more than 20%. On the contrary, their disodium salts lead to an increase in the content of structured element i.e. α-helices and β turns and a decrease of unstructured regions. It can be assumed that 5’-nucleoside monophosphoric acids destabilize and break intramolecular hydrogen bonds in α-helices, leading to their unwinding and the formation of unstructured sites. In this case, hydrogen bonds can form between 5’-ribonucleoside monophosphoric acids and respective amino acid residues of unstructured regions. As a result, enthalpy increases and entropy decreases. A decrease of entropy may reflect an increase of the INF α-2b structural rigidity. In contrast, after addition 5’-rNMPs-Na_2 to INF α-2b a heat is absorbing and entropy is increasing in the system (Fig. 6). This suggests that a conformational flexibility of INF α-2b may increase in these conditions.

Conclusion

We established that 5’-ribonucleoside monophosphoric acids used in this study interact with IFN α-2b in different ways compared to their disodium salts. Acid forms of 5’-ribonucleoside monophosphates possess higher affinity to IFN α-2b as judged by its intrinsic tryptophan fluorescence measurements and destabilize secondary structure elements of this protein as judged by CD measurements. This may restrain a conformational mobility of IFN α-2b and as a consequence modify its functional activity. In contrast, disodium salts of 5’-ribonucleoside monophosphates display a weak affinity to IFN α-2b and to some extent stabilize its secondary structure organization. The result of thermodynamic experiments correlates well with the spectroscopic measurements.

Conflicts of Interest

The authors declare no conflict of interest.

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Взаємодія 5’– рибонуклеозид монофосфатів з Інтерфероном α-2b

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Мета. Охарактеризувати взаємодію інтерферону α-2b (ІФН α-2b) з цитидин-5’-монофосфорною кислотою (5’-ЦМФ), гуанозин-5’-монофосфорною кислотою (5’-ГМФ), уридин-5’-монофосфорною кислотою (5’-УМФ) та їхніми динатрієвими солями у присутності та за відсутності D-манітолу за допомогою різних підходів. **Методи.** спектроскопія (часова, кругового дихроїзму та флуоресценція), ізотермічна нанокалориметрія. **Результати.** За допомогою вимірювання власної флуоресценції ІФН α-2b встановлено, що 5’-ГМФ і 5’-ЦМФ взаємодіють з ІФН α-2b сильніше, ніж їхні динатрієві солі, тоді як для 5’-УМФ і його натрієвої солі різниці в афінності не спостерігали. Присутність D-манітолу посилює спостережувані ефекти для 5’-ГМФ і 5’-ЦМФ, але не для їхніх динатрієвих солей і всіх форм 5’-УМФ. Термодинамічні вимірювання показали, що взаємодія всіх досліджених 5’-рибонуклеозидмонофосфорних кислот з ІФН α-2b є екзотермічною, тоді як всі натрієві солі 5’-рибонуклеозид монофосфатів є ендотермічними. Ентропійна компонента TΔS виявилася від'ємною у присутності всіх 5’-рибонуклеозид монофосфорних кислот, що свідчить про зменшення хаотичності в системі, і додатньою у присутності їхніх натрієвих солей, що вказує на протилежний ефект. Вимірювання кругового дихроїзму показало, що 5’-рибонуклеозидмонофосфорні кислоти індукують зменшення вмісту регулярних альфа-спіралей і збільшення довжини неструктурованих ділянок в INF α-2b. На противагу, їхні динатрієві солі чинять радше стабілізуючий вплив на елементи вторинної структури цього білка. **Висновки.** Використані в роботі 5’-рибонуклеозидні монофосфорні кислоти по-різному взаємодіють з ІФН α-2b порівняно з їхніми натрієвими солями. Кислотні форми 5’-рибонуклеозид монофосфатів мають вищу афінність до ІФН α-2b і дестабілізують елементи вторинної структури цього білка. Це може обмежувати конформаційну рухливість ІФН α-2b і, як наслідок, змінювати його функціональну активність. На противагу цьому, натрієві солі 5’-рибонуклеозидмонофосфатів виявляють слабку спорідненість до ІФН α-2b і стабілізують його вторинну структурну організацію.

Ключові слова: кінетика; мононуклеотиди; інтерферон α-2b; білок-лігандні взаємодії; титрування.

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