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Conformational, Fluorescence and Energy Parameters of Interferon α2b with Different Forms of Oligoribonucleotides and Adenosine Monophosphate †

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Abstract: (1) Background: It is known that RNA in Na+ salt form has only immunomodulatory activity, and in acid form additionally acquires anti-inflammatory activity and in combination with D-mannitol acquires an even broader antiviral effect. The study aimed to study the ability AMP and ORN in acid and salt form and combination with D-mannitol to affect the conformation and fluorescence of interferon (INF) α-2b and to determine the energy parameters of these interactions; (2) Methods: spectroscopy (time pulsed and fluorescence), isothermal nanocalorimetry; (3) Results: AMP and ORN in acid form and complex with D-mannitol bind more strongly to interferon α-2b than salt analogues. In the interaction of interferon α-2b and acid AMP and especially in complex with D-mannitol, the reaction occurs exothermically and change in conformational mobility INF by increasing the content of disordered regions. When INF α-2b interacts with salt AMP, the reaction occurs endothermically, and probably salt form increases the conformational stiffness of INF α-2b. The greater efficiency of nonradiative energy transfer from INF α-2b to acid AMP has been shown, due to the closer distance between molecules; and (4) Conclusions: AMP in acid form interacts more actively and increases the conformational mobility of INF, at a more relative distance and with less Gibbs energy compared to the salt form, which probably causes the appearance of additional biological properties of acidic AMP.

Keywords: oligonucleotides; interferon α2b; lifetime of fluorescence; isothermal titration calorimetry

1. Introduction

Drugs based on oligoribonucleotides from yeast RNA have a reasonably low solubility in the range of 0.5–3 mg/mL. Our previous studies have shown that ORN has anti-inflammatory properties, and ORN with D-mannitol has anti-inflammatory and antiviral properties. The mechanism of this phenomenon is unclear. D-mannitol itself does not have these activities, but increases the solubility by 1.5–2 times and stabilises the structure of the ORN, which probably leads to new biological effects. In the course of our research, we found that D-mannitol affects the binding of ORN to interferon and the thermodynamic and conformational parameters of this interaction [1–4].

Interactions of protein nucleic acids play a decisive role in many biological processes. RNA-based drugs that can bind and affect the work of epigenetic regulators and transcriptional proteins

through interaction, with regulatory domains, can be used as safe analogs. In the previous work we showed, the combination of oligonucleotides with alcohol sugar D-mannitol leads to changes in their biological activity and efficiency. ORN increase interferon production and stimulate non-specific antivirus protection, but the molecular mechanism of its action is still unclear. Interactions of protein and nucleic acids play a decisive role in many biological processes. RNA-based drugs that can bind and affect the work of epigenetic regulators and transcriptional proteins through interaction, with regulatory domains, can be used as safe analogues. We studied the ability of yeast RNA (ORN), yeast RNANa salt (ORN Na), and yeast ORN: D-mannitol complex (ORN: D-M) and AMP to effect on fluorescence quenching and conformational changes of Interferon α2b. To determine the energy parameters of protein-ligand interactions, we use isothermal titration nanocalorimetry Nano ITC.

2. Experiments

The integrity and molecular weight of interferon α-2b established by electrophoresis, and it was equal to about 18.2 kDa (Figure 1).

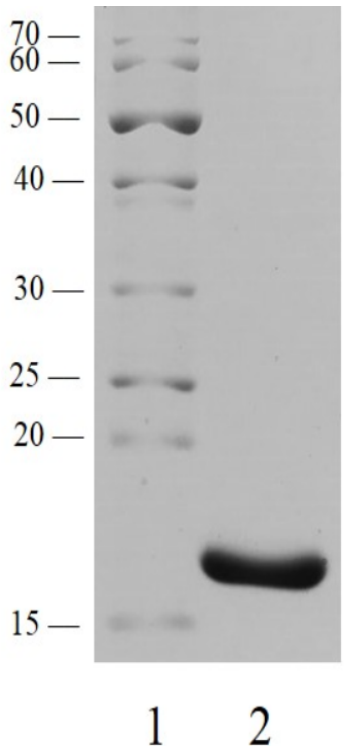

Figure 1. Gel electrophoresis of interferon α-2b: **1**—a mixture of marker proteins "PageRuler Unstained Protein Ladder"; **2**—interferon α-2b ~ 18.2 kDa.

We analyse the molecular weight and purity of interferon α-2b were from gel electrophoresis. We are using 10% polyacrylamide gel under denaturing conditions with addition SDS by Lemley method [5]. Standard solution of a mixture of marker proteins with a known molecular weight "PageRuler Unstained Protein Ladder" manufactured by "Thermo Fischer Scientific" (USA) and ten μL of the test solution (protein) at a concentration of 50 μg/μL was applied to the appropriate cells and added to the next well.

Materials Interpharmbiotek Kyiv, Ukraine kindly provided the recombinant interferon α-2b. D-mannitol used from Sigma Aldrich, St. Louis, MO, USA, total acid yeast RNA preparation provided by SC "BioCell", Kyiv, Ukraine. The work was performed on the acid form and RNA complex with D-mannitol in a weight ratio of 2.5:1, as well as on the sodium salt of total yeast RNANa and the salt RNA complex with D-mannitol. The rate of 2.5 to 1 is because as a result of previous experiments conducted in our laboratory with MCR-ALS analysis of mixtures of RNA with sugars, it found that it is at this ratio of components between them forme a complex [6].

The method of fluorescence spectroscopy used to study the interaction of different forms of RNA with interferon α-2b. Fluorescence spectra were recorded in the range of 300–450 nm on a spectrofluorimeter Jasco FP-8200 at room temperature using a cuvette 1 cm To measure the spectra of interferon α-2b (IFN α-2b) was dissolved in 50 mm Tris-HCl buffer (pH–7, 5) at a temperature t = 25 °C; scanning speed—200 nm/min; excitation slit width—2.5 nm; radiation slit width—2.5 nm; the final protein concentration is one μm; the final concentration of titrants ≈ ten μm [7]. We performed the experiment in 50 mM Tris-HCl buffer (pH 7.5) (RNA in the buffer does not convert to tris salt and remains stable because HCl is a more vital acid). The spectra of interferon α-2b in the titration of RNA and RNA: D-mannitol complex normalised. In this study, we calculated the protein binding constant of the ligands by nonparametric regression of the dependence of tryptophan autofluorescence of the protein on the concentration of the ligand.

$$m = 1 + \left(\frac{P}{D_0}\right) + \left(\frac{K_d}{D_0}\right)$$
$$A = A_f - (A_f - A_b) \times \left(0.5m - \sqrt{0.25m^2 - \frac{P}{D_0}}\right) \quad (1)$$

where D0 is the conc. titrated substance, *P*-fluorescence value. Af-maximum fluorescence, Ab-minimum fluorescence. The spectra of interferon α-2b and RNA and its RNA: D-mannitol complex normalised.

The energy parameters of the ligand-protein interaction determined by isothermal titrating nanocalorimetry. The number of injections—25; the interval between injections—300 s. The protein was pre-dialyzed against buffer. There is a buffer in the comparison cell. We performed isothermal titration nanocalorimetry according to the method presented in [8] in 50 mm Tris-HCl buffer (pH 7.5). By measuring the changes in heat release or absorption during the binding process, we calculated the thermodynamic parameters of the interaction reaction (enthalpy changes, Gibbs free energy and entropy).

The efficiency of energy interaction of Foster ligands with protein (Horiba FluoroMax 4 Plus fluorometer, USA) determined by time division pulse spectroscopy. Experiment parameters: scanning range - 100ns; number of channels—4000; radiation gap—2.5 nm; excitation wave—295 nm; emission detection wave—337 nm; the length of the optical track is 1 cm. Lifetime defined as the exponential regression of the number of emission signals from the time: I (t) = exp (-t/τ). Energy transfer efficiency is the ratio of the number of energy transfer events to the number of donor excitation events: E = 1- (τD′/τD), where τD′ and τD are the lifetime of the fluorescence donor, in the presence and absence of the acceptor, respectively.

3. Results

Interactions of protein and nucleic acids play a decisive role in many biological processes. RNA-based drugs that can bind and affect the work of epigenetic regulators and transcriptional proteins through interaction, with regulatory domains, can be used as safe analogs. We studied the ability of yeast RNA (ORN), yeast RNANa salt (ORN Na), and yeast ORN:D-mannitol complex (ORN:D-M) and AMP to effect on fluorescence quenching and conformational changes of Interferon α2b. To determine the energy parameters of protein ligand interactions, we used isothermal titration nanocalorimetry Nano ITC.

It is shown that when using ORN and ORN:D-M quenching of the fluorescent INF were 25% and 28%, AMP and AMP: DM—15 and 21%. Quenching INF fluorescence in the titration of ORNsNa and ORNsNa: DM was 16% and 17%, AMPNa and AMPNa: DM - 8% and 10% (Figure 2).

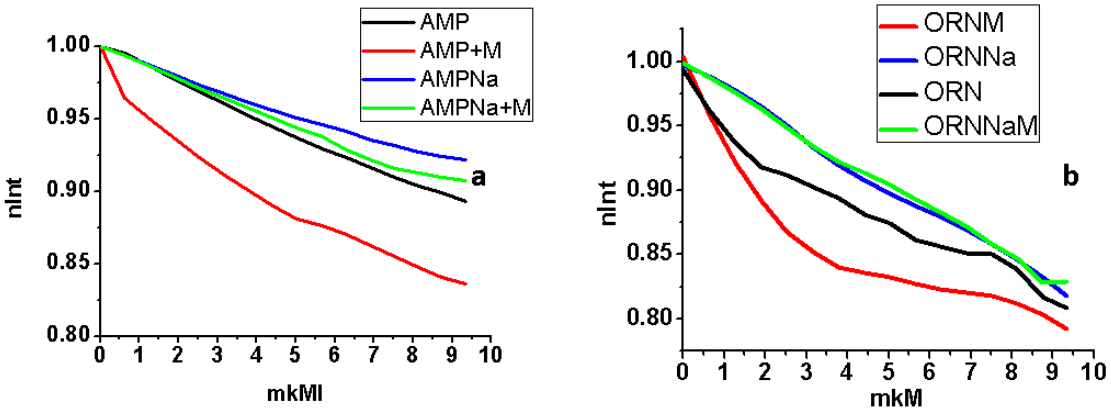

Figure 2. Dependence of quenching of IFN emission intensity α-2b λem = 336 nm when light-excited Xe lamps λex = 295 nm on the concentration of (**a**) AMP; (**b**) ORN ligands and their complexes with D-mannitol.

The dissociation constant (Figure 3) Kd = 1.11 ± 0.09 μM μM in the fluorescence quenching interaction between INF and ORNs-D-M. The dissociation constant between IFN and ORNs is Kd = 2.36 ± 0.47 μM, between INF and ORNsNa—Kd = 9.15 ± 0.16 μM and INF and ORNsNa-D-M Kd = 8.13 ± 0.46 μM. The dissociation constant Kd = 0.94 ± 0.09 μM μM in the fluorescence quenching interaction between INF and AMP-D-M. The dissociation constant between IFN and AMP is Kd = 1.53 ± 0.41 μM, between INF and AMPNa—Kd = 7.1 ± 0.19 μM and INF and AMPNa-D-M Kd = 8.14 ± 0.73 μM.

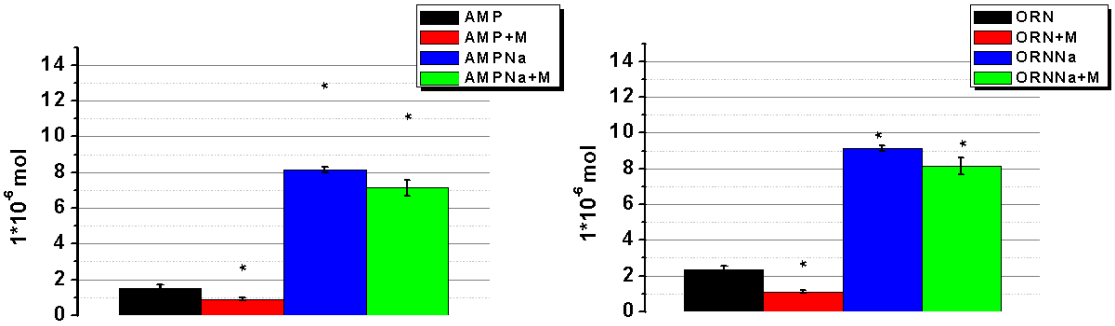

Figure 3. The binding constant of interferon α-2b to (**a**) AMP and (**b**) oligonucleotides.

INF has a life-time of 2.95 ns (Figure 4). When interacting with ORN and ORN: D-M INF has fluorescence time of 2.37 and 2.32 ns, respectively, AMP and AMP: D-M 2.01 and 1.92 ns. When interacting with ORNsNa and ORNsNa: D-M INF has a fluorescence time of 2.73 and 2.49 ns, respectively, AMPNa and AMPNa: D-M 2.31 and 2.43 ns.

Thus, ORN, and especially ORN:D-M and AMP:D-M leads to a change in the conformational mobility of interferon α-2b by increasing the content of disordered regions. At the same time, salt analogues increase the number of structured secondary elements, such as α-helices, β turns and β antiparallel sheets and probably increase the conformational stiffness of interferon α-2b. The results of the study of enthalpy changes in the titration of interferon α-2b acid form of ORN and ORN:D-M was −63.28 kJ/mol and −96.61 kJ/mol, respectively, and for the ORNNa and ORNNa:D-M respectively 4516 and 5139 kJ/mol. The change in entropy when adding the ORN to interferon α-2b was −38.72 and in the case of the ORN:D-M −63.53 kJ/mol*K, respectively. The change in entropy when adding the ORNNa to interferon α-2b was 17.05 kJ/mol*K, and the ORNNa:D-M, respectively, 17.58 kJ/mol*K. A similar pattern demonstrated when studying the change in Gibbs energy during titration of interferon α-2b with ORNORN and ORN:D-M and it was −24.56 and −33.07 kJ/mol, respectively. And when titrated with ORNNa and its ORNNa:D-M, respectively −12.9 and −12.43 kJ/mol.

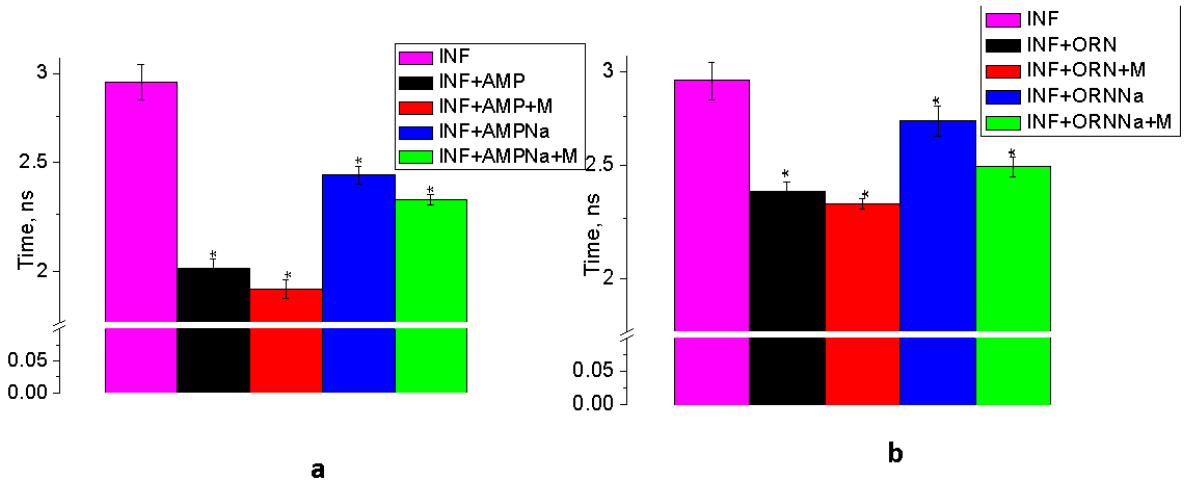

Figure 4. The fluorescence lifetime of interferon α-2b with (**a**) AMP and (**b**) ORN.

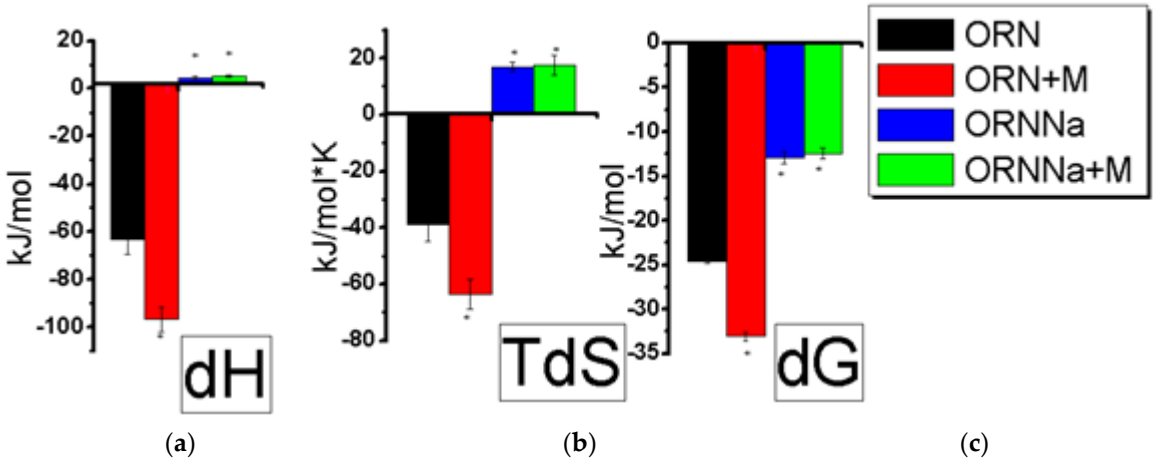

Figure 5. Changes in energy parameters during the interaction of Interferon α-2b with oligoribonucleotides. It is shown that positive energy indicators accompany the reaction of the interaction between interferon and acidic forms of RNA and their complex with D-mannitol, and with salt forms of RNA and their complexes with D-mannitol - opposing arrows. The addition of D-mannitol to RNA in both cases enhances the action of these ligands. (**a**) enthalpy; (**b**) entropy; (**c**) Gibbs energy.

These results of studying the effects of thermodynamics of different forms of RNA and their complexes with D-mannitol in the titration of interferon α-2b may indicate different sites of binding of different forms of ORN to protein, as well as other modes of binding and various types of conformational changes in the protein.

4. Discussion

The obtained titration curves of interferon α-2b from AMP indicate that the reaction of the interaction between protein and acid ligands occurs exothermically, and with salt—endothermically. These results can explain by the ability of AMP to bind multiple times to different binding sites on a molecule of a given protein. It is knowing from the literature that during protein-ligand interaction, the exothermic reaction is accompanied by an increase in protein activity, and the endothermic—by inhibition [9]. Thus, in our case, the same AMP compound, depending on the form, can affect the conformation of the protein in different ways. When interferon α-2b interacts with acidic conditions of RNA, heat is released, and protons exchanged from the acid form to the protein. When it interacts with salt forms, heat is absorbed, and protons transferred from the protein to the AMP. Thus, by reducing the free binding energy between the acidic condition of AMP and its complex with D-

mannitol by optimising the enthalpic contribution, and this can significantly improve the pharmacological properties of interferon α-2b. However, this fact can argue that the use of other preparations of RNA and DNA in acid form and especially in combination with D-mannitol can optimise their affinity for interaction with target proteins and thus discover new and improve known pharmacological properties.

The release or absorption of heat by the interaction of various forms of yeast RNA and its complexes with D-mannitol may indicate a change in the secondary structure of interferon α-2b. To confirm the assumption of a change in the conformation of this protein, we studied the secondary structure of interferon α-2b under the action of various forms of RNA by circular dichroism. It is showing that RNA in acid form, and especially in complex with D-mannitol, leads to a change in conformational mobility due to an increase in the content of disordered regions. At the same time, salt analogues of RNA increase the number of structured secondary elements and possibly increase the conformational stiffness of interferon α-2b.

The most active quenching and decrease lifetime of fluorescence INF, when titrated with AMPs and ORN, was obtained using acid forms in combination with mannitol. And when titrated INF saline forms slightly different from control. Spectra of circular dichroism show the decrease in the structure of the number of secondary elements when interacting between INF and acidic forms nucleotides. An increase in the content of secondary structure in the interaction between INF and salt forms ligands. The ITC curves titration indicate that the reaction between protein and acidic ligands is exothermic. And between INF with saline ligands endothermically. Exothermic protein-ligand interaction increases the conformational mobility of the protein and endothermic decrease.

The ORNs have the advantage of interacting with proteins, unlike salt ORNs and AMPs, because they have a stronger binding. Thus, we assume the same compound in various forms may act as an inhibitor and activator for the protein.

5. Conclusions

- AMP and ORN in acidic form and combination with mannitol bind more strongly to interferon α-2b than to saline analogs;
- In the interaction of interferon and AMP and ORN in acidic form, the reaction is exothermic. The ITC curve is endothermic between interferon and ORN and AMP in salt form;
- AMP and acidic ORN, and especially in combination with mannitol, lead to a change in conformational mobility by increasing the content of disordered sites. At the same time, salt analogs increase the number of structured secondary elements and probably increase the conformational rigidity of interferon;
- Pulse-time spectroscopy shows a higher efficiency of non-radiative energy transfer from interferon to AMP and ORN in acidic form, due to the closer distance between molecules and the higher Foster transfer rate.

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