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Hepatitis C Virus Helicase/NTPase: an Efficient Expression System and New Inhibitors

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Abstract—A method has been developed for obtaining a full-length protein NS3 of hepatitis C virus with the yield of 6.5 mg/liter of cell culture, and conditions for measuring its NTPase and helicase activities have been optimized. The helicase reaction can proceed in two modes depending on the enzyme and substrate concentration ratio: it can be non-catalytic in the case of enzyme excess and catalytic in the case of tenfold substrate excess. In the latter case, helicase activity is coupled with NTPase and is stimulated by ATP. A number of NTP and inorganic pyrophosphate analogs were studied as substrates and/or inhibitors of NS3 NTPase activity, and it was found that the structure of nucleic base and ribose fragment of NTP molecule has a slight effect on its inhibitory (substrate) properties. Among the nucleotide derivatives, the most efficient inhibitor of NTPase activity is 2′-deoxythymidine 5′-phosphoryl-β,γ-hypophosphate, and among pyrophosphate analogs imidodiphosphate exhibited maximal inhibitory activity. These compounds were studied as inhibitors of the helicase reaction, and it was shown that imidodiphosphate efficiently inhibited the ATP-dependent helicase reaction and had almost no effect on the ATP-independent duplex unwinding. However, the inhibitory effect of 2′-deoxythymidine 5′-phosphoryl-β,γ-hypophosphate was insignificant in both cases, which is due to the possibility of helicase activation by this ATP analog.

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Key words: hepatitis C virus, protein NS3, NTPase, helicase, NTP analogs, inorganic pyrophosphate analogs

Hepatitis C virus (HCV) is a main etiologic agent causing the disease that was for a long time called "non-A non-B hepatitis" [1]. The disease caused by HCV includes an acute phase that in most cases is transformed into the chronic form. During chronic hepatitis, there is a high risk of developing liver cirrhosis and hepatocellular carcinoma.

Therapy of hepatitis C, which is based on the use of the interferon α preparations (Intron-A, Rebetron) and ribavirin (Virazole) [1, 2] is not very efficient. An intensive search for other potential anti-HCV agents for the last 15 years still did not give any significant results [2].

The HCV genome consists of the 9600 nucleotides long (+) chain RNA containing one open reading frame [3]. Translation and subsequent processing of the formed polypeptide result in 10 structural and non-structural proteins [3, 4]. Three non-structural proteins exhibit enzymatic activities. Protein NS5B is a RNA-dependent RNA polymerase, the key component of the virus replication complex. Protein NS2 within the NS2-NS3 polyprotein precursor forms an autoproteinase. Finally, NS3 is a multifunctional protein exhibiting three catalytic activities. Its N-terminal domain is a chymotrypsin-like zinc-binding serine proteinase carrying out processing of C-terminal non-structural HCV proteins [4]. The C-terminal domain of NS3 involving two thirds of the protein contains structural motifs characteristic of DEAD-box RNA helicases and exhibits helicase and polynucleotide-stimulated nucleoside triphosphatase activities [5, 6].

Protein NS3 is able to unwind double-stranded RNA and DNA as well as DNA–RNA heteroduplexes in the 3′→5′ direction using NTP or dNTP hydrolysis as a source of energy [5, 6]. NTPase and helicase activities exhibit different specificities towards the nucleoside

Abbreviations: HCV) hepatitis C virus; IPTG) isopropyl-1-thio-β-D-galactopyranoside; PMSF) phenylmethylsulfonyl fluoride.

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triphosphates structure, but no reliable regularities have been revealed between NTP structure and enzyme activity [7]. Very few inhibitors of NS3 helicase/NTPase have been described [8].

In this work, we present a system for expression and subsequent isolation of the full-length NS3 protein based on our plasmid vector described previously [9]. We have also optimized methods for determination of NTPase and helicase activities and investigated a number of NTP and PPi analogs as enzyme substrates and inhibitors.

MATERIALS AND METHODS

The following reagents were used in this work: bacto-tryptone, yeast extract, and bacto-agar (Difco, USA); Tris, Hepes, 2-mercaptoethanol, and DL-dithiothreitol (DTT) (Merck, Germany); glycerol, imidazole, Triton X-100, Nonidet P40, ammonium persulfate, phenylmethylsulfonyl fluoride (PMSF), and aprotinin (Sigma, USA); acrylamide and methylene-bis-acrylamide (Roth, Germany); EDTA (Serva, Germany); Coomassie brilliant blue R-250 (Bio-Rad, USA); tetramethylethylenediamine (Reanal, Hungary); isopropyl-1-thio-β-D-galactopyranoside (IPTG), 2′-deoxyribo- and 2′-ribonucleoside triphosphates (Promega, USA). Other reagents were of special or chemical purity (Reakhim, Russia). Enzymes of Sibenzyme (Russia), Promega (USA), and GE Healthcare (England) were used in gene engineering experiments. Synthesis of 2′-deoxythymidine-5′-O-α-phosphoryl-β,γ-hypophosphate (ppopT) is described in [10], and adenosine-5′-O-α-phosphoryl-β,γ-methylendiphosphonate (pCH_2popA) was synthesized as described in [11]. Adenosine triphosphate N^1-oxide (N^1-O-ATP) was synthesized from ATP as described earlier [12]. Inosine triphosphate N^1-hydroxide (N^1-OH-ITP) was obtained from N^1-O-ATP as described in [13].

Inorganic pyrophosphate (1, Table 2) imidodiphosphate (3, Table 2), and phosphoformiate (10, Table 2) were obtained from Sigma, methylene pyrophosphate (2, Table 2) and bis-methylene triphosphate (4, Table 2) were provided through the courtesy of N. B. Tarusova (Engelhardt Institute of Molecular Biology (EIMB)), hypophosphate (5, Table 2) was synthesized as described in [14]. Other pyrophosphate analogs (compounds 6-9, Table 2) were provided through the courtesy of S. N. Mikhailov (EIMB).

Escherichia coli strains XL-1 Blue (Promega), Rosetta (DE3) (Novagen, USA), and M15 [pREP4] (Qiagen, USA) were used for the NS3 protein gene cloning and expression. Protein was isolated on Ni-NTA-agarose (Novagen) and heparin-agarose (Sigma) columns. Plasmid pCV-NS3 was provided through the courtesy of A. M. Atrazhev.

Gene cloning, NS3 protein expression and isolation. The gene of the full-length protein NS3 of genotype 1a, virus strain H77, was amplified by PCR from the plasmid pCV-NS3 using the following oligonucleotides (restriction sites are underlined, corresponding endonucleases are shown in parentheses):

NS3-For: 5′-AT<u>G AAT TC</u>G GCG CCC ATC ACG GCG TAC GCC CAG-3′ (*EcoR*I);

NS3-Rev: 5′-GTG <u>CTC GAG</u> GAA AGC TGG GTC TTA G-3′ (*Xho*I).

The isolated product was cloned into sites *EcoR*I and *Xho*I of the vector pET-21-2c described previously [9]. Primary structure of the obtained plasmid pET-21-2c-NS3 was confirmed by automatic sequencing (Genome Center, EIMB).

Plasmid pET-21-2c-NS3 was transformed into *E. coli* strain Rosetta (DE3), a single colony was suspended in 10 ml of LB medium (10 g/liter bacto-tryptone, 5 g/liter yeast extract, 0.17 M NaCl) containing glucose (10 mg/ml), ampicillin (150 μg/ml), and chloramphenicol (15 μg/ml). Cells were grown for 10-15 h at 37°C with stirring, then pelleted by centrifugation (4000*g*, 5 min), washed with LB medium, pelleted again, and resuspended in 250 ml of fresh LB medium containing ampicillin and chloramphenicol. The culture was grown at 37°C with stirring to optical density 0.5 (A_{550}), IPTG was added to final concentration 1 mM for induction, and then the culture was shaken for an additional 2.5 h at 22°C. Then cells were pelleted by centrifugation (4000*g*, 10 min) and stored at −70°C.

The cell pellet was thawed on ice and resuspended in 15 ml buffer A (20 mM Tris-HCl, pH 7.6, 500 mM NaCl, 10 mM 2-mercaptoethanol, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 1 mM PMSF, 10 μg/ml aprotinin). Cells were sonicated on ice (8 × 45 sec with 1.5 min intervals), and the suspension was centrifuged for 20 min at 10,000*g* at 4°C. The supernatant was applied onto a column with Ni-NTA agarose (15 × 40 mm), which was sequentially washed with buffer A containing 10, 30, 50, 200, and 500 mM imidazole (fractions of 6 ml). The eluate obtained at imidazole concentration of 200 mM was collected in fractions of 1.5 ml. Fractions containing the sought protein were identified by staining aliquots of 5-10 μl with Bradford reagent [15], pooled, dialyzed for 4 h against buffer B (20 mM Tris-HCl, pH 7.6, 350 mM NaCl, 5 mM 2-mercaptoethanol, 10% (v/v) glycerol), diluted with glycerol to final concentration of 40% (v/v), and stored in 200-μl aliquots at −20°C. Protein concentration was estimated by comparison with standard BSA solution after staining SDS-PAGE gels. In some experiments, protein was additionally purified and concentrated on a column with heparin-agarose. For this purpose, fractions obtained after chromatography on Ni-NTA agarose were combined and diluted with two volumes of buffer C (50 mM Tris-HCl, pH 7.0, 1 mM EDTA, 1 mM

2-mercaptoethanol, 5% (v/v) glycerol, 1% (v/v) Nonidet P-40) and applied onto a 4 × 20-mm column. The sorbent was sequentially washed with buffer C containing 200 and 400 mM KCl, and the protein was eluted with buffer C containing 600 mM KCl. Fractions with the highest amount of NS3 protein were dialyzed against buffer B containing 40% (v/v) glycerol and stored as described above.

Determination of NTPase activity of NS3 protein. The standard reaction mixture (10 μl) contained 0.2 μg NS3 protein, 0.5 μCi [γ-^{32}P]ATP, and 1-200 μM ATP in buffer D (50 mM Tris-HCl, pH 7.5, 25 mM NaCl, 2.5 mM $MgCl_2$, and 1.5 (v/v) glycerol). The reactions were carried out at 37°C, and products were analyzed after application of 1-μl aliquots on PEI cellulose plates and following chromatography in 0.375 M potassium phosphate buffer, pH 3.5, and visualization of radioactivity using a Packard Cyclone Storage Phosphor System (Packard BioScience Company, USA).

To study inhibitory properties of the modified NTP or pyrophosphate analogs, the compounds were added to a reaction mixture in the range of concentrations from 0 to 1000 μM.

To determine substrate properties of some analogs that were not available as radioactively labeled compounds, the substrate and reaction product were separated by HPLC. Standard reaction mixture (10 μl) contained 0.2 μg NS3 protein and 200 μM of the NTP analog in buffer D. Reaction was carried out at 37°C for 5-30 min. The products were separated on a Lichrosorb-NH_2 column (4 × 150 mm, 6 μ), with elution in a gradient of sodium phosphate concentrations (pH 5.5): 20 mM, 0-5 min; 20→1000 mM, 5-37 min; 1000 mM, 37-40 min. Substrate properties of the analogs were characterized by the ratio of their hydrolysis rates and the rate of natural substrate (ATP) conversion.

Determination of NS3 helicase activity. To determine helicase activity, a duplex formed by the following oligonucleotides was used:

26: 3′-GTAGCTATAATTATGCTGAGTGATAT-5′;

34: 5′-CATCGATATTAATACGACTCACTATAGGAC-TCCA-3′.

Oligonucleotide 26 was phosphorylated with T4 polynucleotide kinase and [γ-^{32}P]ATP according to a standard technique [16]. Then 0.1 pmol/μl of ^{32}P-labeled and 0.9 pmol/μl of unlabeled oligonucleotide 26 were annealed with 1.1 pmol/μl of oligonucleotide 34 in 0.1 M NaCl by heating at 95°C for 3 min with subsequent cooling for 3 h. Final concentration of 26/34 duplex was 1 pmol/μl.

The standard reaction mixture for activity determination (10 μl) contained 0.25 μg NS3 protein, 0.1 μM duplex 26/34, 0-5 mM ATP, 0-5 mM $MgCl_2$ or other metal salts in buffer E (20 mM Hepes, pH 7.4, 2 mM DTT, and 0.1 mg/ml BSA). Reaction was carried out at 37°C for 2-60 min and stopped by addition of 1 μl 50% glycerol containing 20 mM EDTA and 0.5% SDS. The products were analyzed by electrophoresis in a 12% polyacrylamide gel in 0.1× TBE buffer containing 3 mM $MgCl_2$ at 4°C at 15 mA for 3-4 h. The electrode buffer was 0.5× TBE containing 3 mM $MgCl_2$. After electrophoresis, gels were vacuum-dried at 80°C, and radioactive bands were visualized using the Packard Cyclone Storage Phosphor System.

RESULTS AND DISCUSSION

Expression and isolation of NS3 protein. Although there is a significant number of works on expression and isolation of the full-length NS3 protein, there is still no efficient method for its production in *E. coli* cells. In most available works objects of investigation were the separate recombinant proteinase or helicase/NTPase domains partially retaining properties of the full-length protein. However, the problem concerning creation of a plasmid that would enable expression of the full-size soluble NS3 protein with a high yield is still unsolved (S. S. Patel, personal communication). Expression systems described to date differ significantly in yields of the recombinant protein, which can be explained by variability of the primary structure of the HCV protein genes of different genotypes and isolates (C. E. Cameron, personal communication). In "rich" nutrient media, sufficiently high yield of protein is often observed which, however, is concentrated in so-called "inclusion bodies"; some increase in solubility can be achieved using "poor" medium M9 with certain additives [17]. An alternative strategy consists of isolation of the protein from "inclusion bodies" under denaturing conditions with following renaturing in the presence of zwitterionic detergents [6]. Another approach for overcoming the problem of low protein yield is an expression of a chimeric protein with additional amino acid sequence at N-terminus of the polypeptide chain [18].

In most works, protein yield is not shown, though it could allow one to estimate the efficiency of expression systems and compare them. There are only data showing that chimeric protein pBAD-HisNS3 can be expressed as "inclusion bodies" with the yield of about 6 mg/liter [19], whereas the unmodified protein is also expressed in insoluble form with a yield of 2 mg/liter in LB medium [20] or with a yield of about 8 mg/liter in a modified medium M9 [17].

Thus, the creation of an efficient and simple plasmid for expression of the full-length protein NS3 without alterations at the N-terminus of the polypeptide chain and enabling production of soluble protein is a significant problem.

In this work we have applied an approach that we successfully used for expression of other non-structural

HCV proteins: NS5A and NS5B [9]. The gene for the full-length protein NS3 was inserted into our vector pET-21-2c, described previously, making it possible to obtain recombinant proteins with six histidine residues at the C-terminus of the polypeptide chain. The yield of the protein NS3 from the plasmid pET-21-2c-NS3 at 37°C with induction by 1 mM IPTG made up 14-15% of total cell protein. However, under these conditions the product was found entirely in inclusion bodies. To increase the solubility of the protein, an expression was carried out at lowered temperature (20-25°C) (Fig. 1a) for 2 h, which resulted in somewhat lower yield, but the entire product was soluble.

A soluble protein fraction was obtained by sonication of the cells in buffer A. In the absence of detergent and at a concentration of glycerol, NaCl, and β-mercaptoethanol, the yield of the soluble protein significantly decreased. We have also observed a high nonspecific sorption of contaminating proteins during protein isolation by affinity chromatography.

Further isolation of the NS3 protein was achieved by metal-affinity column chromatography on Ni-NTA-agarose (Fig. 1b). In this case, the protein yield made up 6.5 mg/liter of cell culture. The preparation was additionally purified by chromatography on a column with heparin-agarose.

Determination of helicase and NTPase activities of NS3 protein. The NTPase activity was determined by an ability to cleave the radioactive [γ-^{32}P]ATP with formation of labeled inorganic phosphate. The products were analyzed by TLC on PEI cellulose (Fig. 2a).

Linear accumulation of NTPase reaction products was observed for at least 40 min. Maximal NTPase activity of the protein was achieved at 37°C in the presence of 1.5-2 mM $MgCl_2$ (Fig. 3a) and 30-50 mM NaCl (data not shown). Addition of KCl resulted in a slight decrease in activity. Enzyme activity was 3-5-fold lower when magnesium ions were replaced by manganese, nickel, cobalt, copper, or zinc (Fig. 3a).

According to data in the literature, NTPase activity of NS3 is stimulated in the presence of RNA [6]. We analyzed the dependence of activity on structure of synthetic polynucleotides. In this case, in the presence of a single-stranded polyadenylate or polyuridylate, approximately doubling of ATPase activity was observed, and addition of polyuridylate resulted in some lowering of Michaelis constant for the ATP substrate (K_m = 256 ± 40 and 163 ± 25 μM, respectively). However, simultaneous addition of poly(A) and poly(U) (i.e. formation of dsRNA) only slightly stimulated the reaction; addition of 26/34 duplex used for measurement of NS3 helicase activity caused a significant enhancement of NTPase activity (Fig. 3b) despite the fact that this duplex is mainly double-stranded and contains only an 8-membered single-stranded fragment. Thus, the question is still open what is in this case the main stimulating factor, the presence of a partial

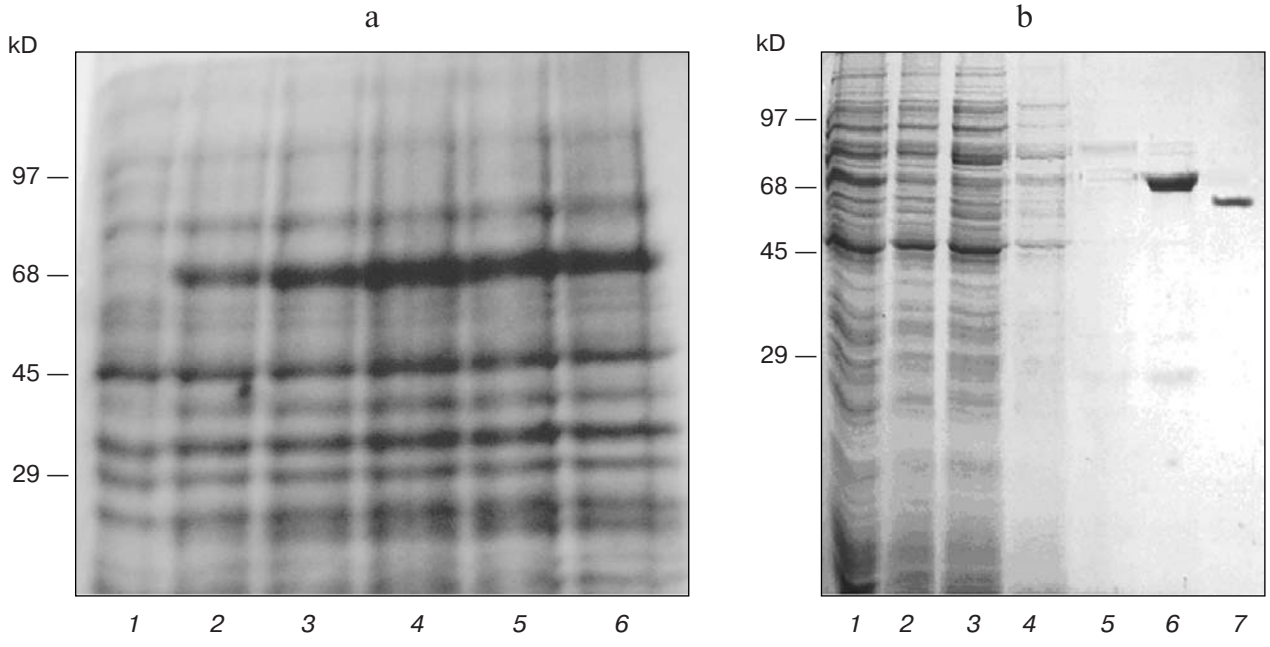


Fig. 1. a) Kinetics of NS3 protein accumulation upon its expression in *E. coli* from plasmid pET-21-2c-NS3 at 25°C for 1-5 h (lanes *2-6*, respectively). Lane *1* corresponds to a cell lysate before induction of NS3 protein. b) Isolation of NS3 protein by chromatography on a Ni-NTA-agarose column. Lysate containing the target protein (*1*) was applied onto the column that was washed by buffer A (*2*), then by buffer A containing 10 (*3*), 30 (*4*), and 50 mM (*5*) imidazole. The protein was eluted with buffer A containing 200 mM imidazole (*6*). Lane *7* corresponds to BSA.

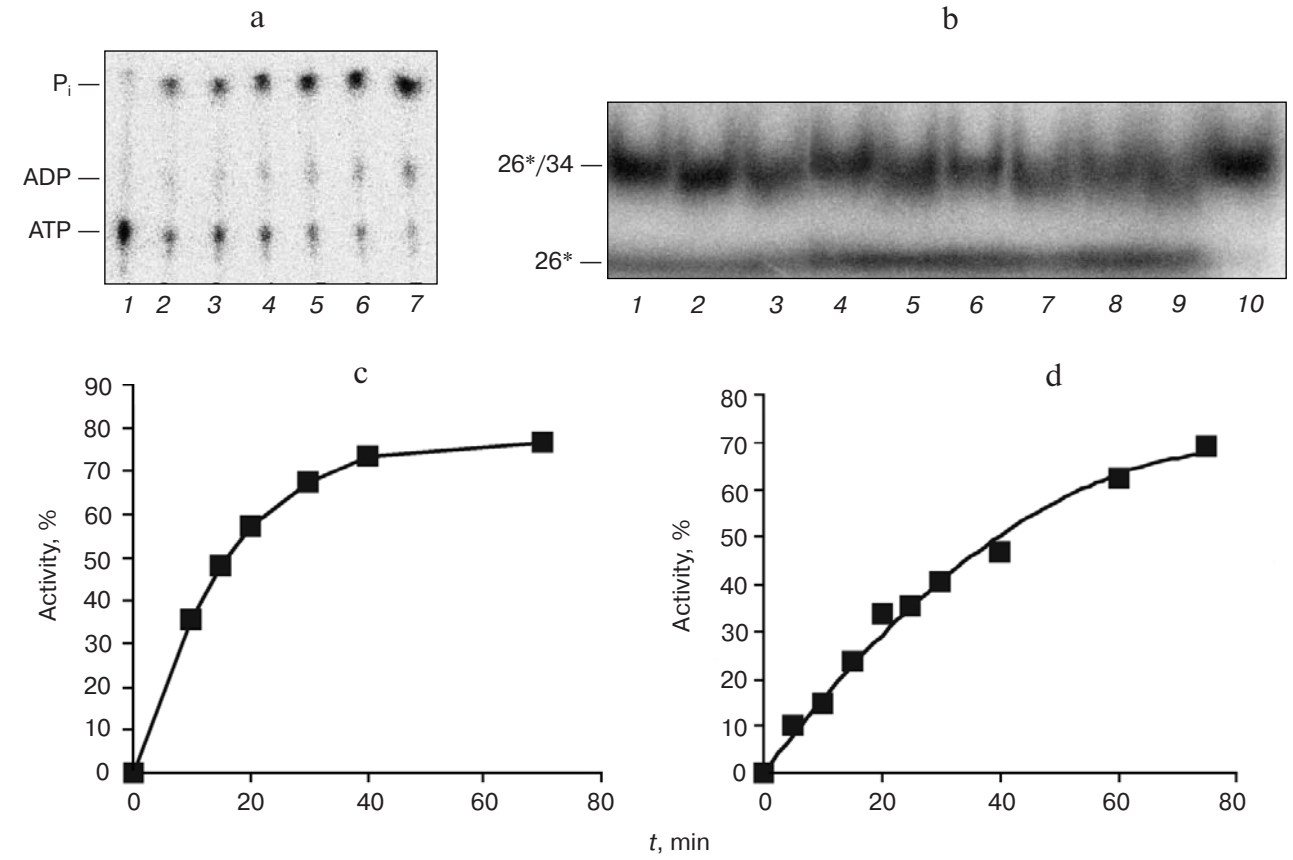


Fig. 2. a) Autoradiograph of PEI-cellulose plate after separation of products of NS3 NTPase reaction. Lanes: *1*) reaction mixture before enzyme addition; *2-7*) reaction mixtures after incubation with the enzyme for 10, 15, 20, 30, 40, and 70 min, respectively. b) Autoradiography of helicase reaction products. Lanes: *10*) an unwound duplex; *1-9*) reaction mixtures after incubation with the enzyme for 5, 10, 15, 20, 25, 30, 40, 60, and 75 min, respectively. Band intensity was determined using the Packard Cyclone Storage Phosphor System, and time dependence of activity (shown at the bottom) was calculated. c, d**)** Kinetic curves of accumulation of NTPase and helicase reaction products, respectively.

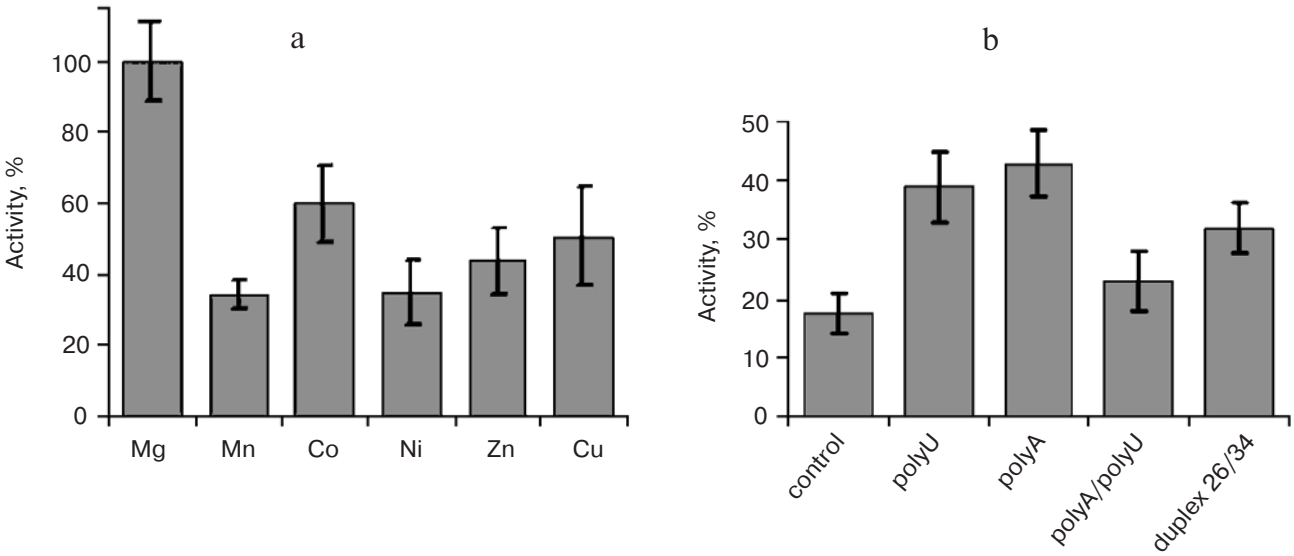


Fig. 3. a) Dependence of NS3 protein NTPase activity on the nature of bivalent metals used under concentrations favorable for maximal enzyme activity. Activity in the presence of Mg^{2+} was taken as 100%. b) NS3 NTPase activity stimulation in the presence of oligo- and polynucleotides.

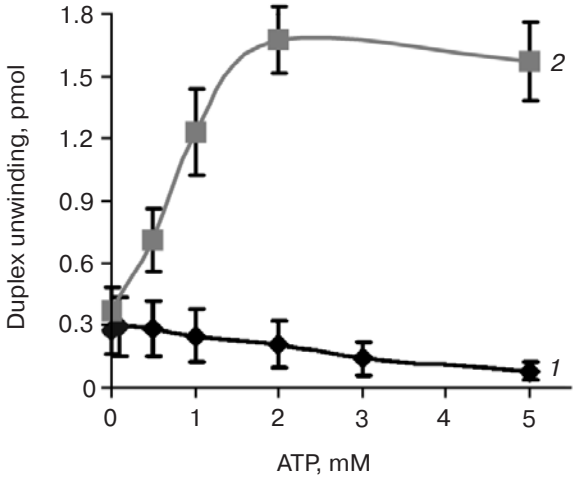


Fig. 4. ATP dependence of the helicase reaction catalyzed by NS3 at the enzyme/substrate ratio 10 : 1 (*1*) and 1 : 10 (*2*).

single-stranded structure and/or simultaneous helicase reaction.

As shown in the literature, helicase of HCV NS3 is able to unwind DNA–DNA, RNA–RNA, and DNA–RNA duplexes [21]. The only prerequisite for exhibition of substrate properties is the presence of 3′ single-stranded region. Owing to this, we used a standard approach for helicase activity determination. A short strand of DNA–DNA duplex was labeled using [γ-^{32}P]ATP, and the reaction products were analyzed by electrophoresis in 12% polyacrylamide gel at 4°C under non-denaturing conditions. A typical electrophoretic pattern is shown in Fig. 2b. Since the HCV protein NS3 is an NTP-dependent helicase [21], the reaction was carried out at different ATP concentrations. However, in the case of enzyme/duplex ratio close to stoichiometric (non-catalytic conditions), the enzyme worked efficiently in the absence of ATP as well, whereas at the ATP concentration above 1 mM inhibition was observed (Fig. 4). These results correlated with our previous data [20]. We have also shown that neither ATP nor Mg^{2+} is required for the helicase reaction. The data allow us to conclude that bivalent metal ions are not necessary for the helicase reaction, but are important only for ATP hydrolysis. At a lower enzyme concentration and increased concentration of DNA duplex, the helicase reaction is metal- and ATP-dependent (Fig. 4).

Investigations of the modified NTP as inhibitors/substrates of NS3 NTPase activity. There are not many data in the literature concerning the HCV NTPase specificity towards NTP derivatives. Substrate properties of "canonical" rNTP and dNTP [6] and inhibitory characteristics of their derivatives [7] are described. The NTPase reaction was inhibited with different efficiency by 2′-ribo-, 2′-deoxyribo-, 2′,3′-dideoxy-, and 2′,3′-dideoxy-2′,3′-didehydroribonucleoside-5′-triphosphates as well as by their L-isomers.

In this work, we have studied the inhibition of the NTPase reaction by some new NTP analogs (Table 1). The highest activity was observed for dTTP analog ppopT with β-γ-pyrophosphate fragment replaced by a truncated hypophosphate (K_i = 97.2 μM). It is notable that this compound is a substrate for HIV-1 reverse transcriptase and its activity is comparable with that of natural dTTP [10]. A non-hydrolyzable ATP analog β,γ-methylene-ATP (pCH_2popA), not able to serve as the NTPase substrate, slightly inhibits the enzyme (K_i > 1 mM), whereas pNHpopA was an efficient inhibitor like N^1-oxides: N^1-O-ATP and N^1-OH-ITP. Nucleotides like AMP and ADP, containing no triphosphates fragment, had no appreciable effect on the reaction. In addition to the abovementioned nucleotide analogs, triazavirin [22], a highly efficient inhibitor of influenza virus replication, was also tested. On one hand, the possibilities of using this compound against other viral infections are still at the stage of a comprehensive investigation; on the other hand, the mechanism of triazavirin antiviral activity is unknown. Therefore, its inclusion into the series of the studied inhibitors of NS3 activity should test the possibility that the compound may inhibit HCV replication by modulating activity of one of the key viral proteins. However, this compound appeared to be almost inactive as inhibitor of NS3 NTPase activity (Table 1).

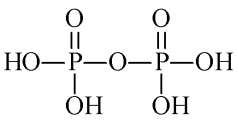
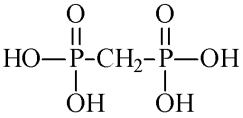
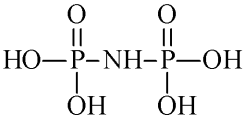
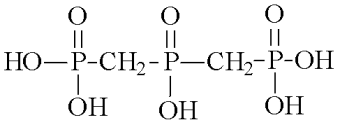
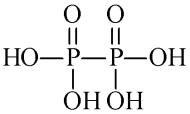
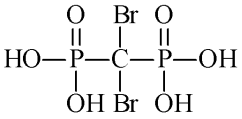
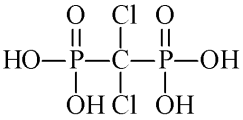
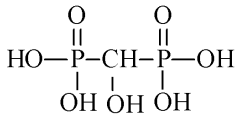
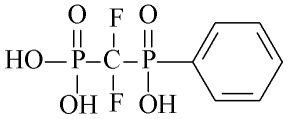
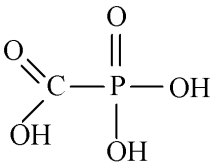
A number of studied compounds containing unmodified β,γ-phosphoanhydride fragment can, along with

Table 1. Inhibition of NTPase activity of NS3 protein by NTP derivatives

Compound	K_i, μM
AMP	> 5000
ADP	1300
2'-dATP	291
3'-dATP	141
2',3'-ddATP	116.5
N^1-O-ATP	205
N^1-OH-ITP	109
UTP	1460
3'-dUTP	260
GTP	576
2'-dGTP	277
3'-dGTP	443
2',3'-ddGTP	721
2'-dTTP	116
2',3'-ddTTP	298
ppopT	97.2
pCH_2popA	>1000
pNHpopA	145
Triazavirin*	>1000

* See the text.

Table 2. Inhibition of NTPase activity of NS3 protein by pyrophosphate analogs

No.	Compound	K_i, μM
1		20
2		180
3		12
4		>>1000
5		640
6		1200
7		1700
8		1300
9		800
10		>>1000

inhibitory effect, serve as enzyme substrates, so experimentally determined K_i values are not considered as real inhibition constants. However, such "apparent K_i" can serve as comparative parameters for estimation of analog affinity to the enzyme. To characterize substrate properties of NTP analogs we have analyzed reaction mixture by HPLC and determined the rate of triphosphates hydrolysis relative to control ATP substrate. Analysis of the results (Fig. 5 and Table 1) indicate that the nature of nucleoside fragment of NTP molecule plays a very insignificant role in substrate binding, whereas the triphosphate fragment of the molecule makes the main contribution to the nucleotide recognition and correct arrangement in the active center.

Keeping in mind the hypothesis expressed above, we tested a series of inorganic pyrophosphate analogs as inhibitors of NTPase activity (Table 2). Analysis of results shown in this table revealed a number of interesting mechanisms. The most efficient NTPase inhibitors are natural pyrophosphate 1 and its analog imidodiphosphate 3, whose steric characteristics are close to those of pyrophosphate 1. Triphosphate analog 4 has almost no effect on the enzyme, i.e. it does not bind in the active center. Bulky substituents between phosphorus atoms of pyrophosphate analogs (compounds 6-8) have negative effect on inhibitory activity, whereas a phenyl residue located at one of the phosphorus atoms in compound 9 slightly influences the binding of the compound to the enzyme. It is interesting that "shortened" hypophosphate 5 appeared to be a weak NTPase inhibitor, whereas 2′dTTP analog, containing the hypophosphorous acid residue in β,γ-positions (ppopT), was, on the contrary, a more powerful inhibitor compared to other inhibitors of nucleotide nature (Table 1). Weak inhibition of NTPase reaction by hypophosphate can be explained by the absence of nucleoside fragment that can contribute to correct orientation of the molecule and/or enhance its interaction with the protein.

We have included into the series of compounds under investigation phosphoformate 10 known as an inhibitor of a number of DNA polymerases, which is considered as a pyrophosphate analog (for example, [23]). However, in the case of NTPase of NS3 protein this compound cannot play this role and has little inhibitory effect.

We have shown for most efficient inhibitors—pyrophosphate analogs—that NTPase inhibition has a miscellaneous character, i.e. both K_m and V_{max} values are changed (Fig. 6). This is probably due to the possibility of inhibitor interaction with the enzyme at the binding site of the nucleotide triphosphates fragment. In this case, the nucleoside part of the NTP molecule retains the ability to interact with the enzyme, but due to incorrect position of triphosphates, the NTPase reaction is impossible.

Compounds that exhibited the most interesting properties upon inhibition of NTPase reaction, namely, imidodiphosphate and 2′-deoxythymidine 5′-phosphoryl-

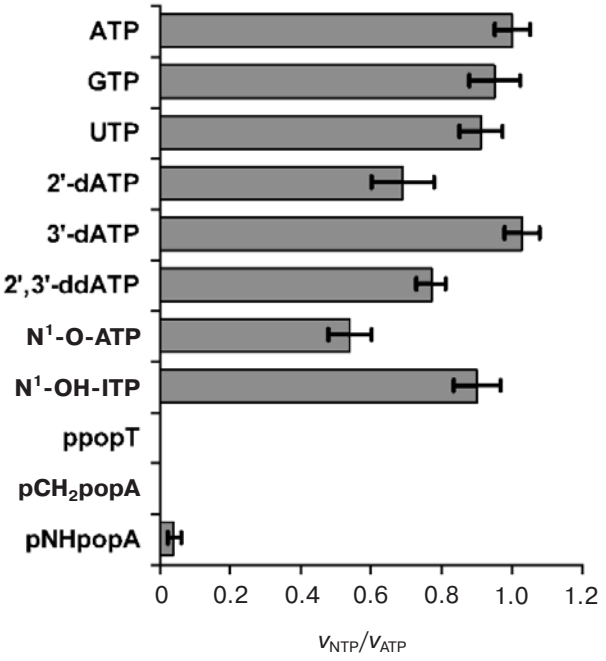


Fig. 5. Substrate properties of NTP derivatives hydrolyzed in NTPase reaction catalyzed by NS3.

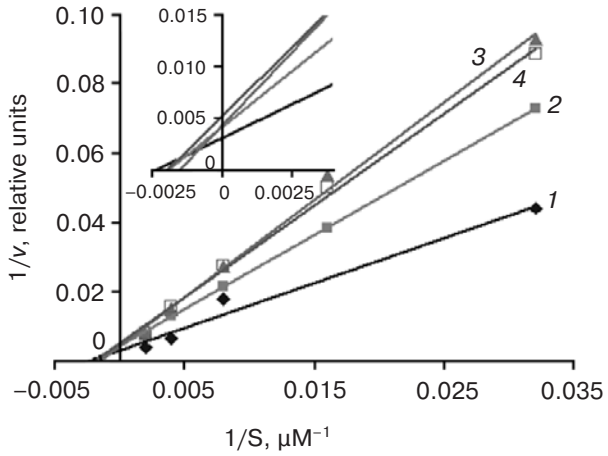


Fig. 6. Determination of kinetic mechanism of the NS3 protein ATPase inhibition by analogs of inorganic pyrophosphate without inhibitor (*1*), in the presence of 100 μM POP (*2*), PNP (*3*), and PP (*4*).

β,γ-hypophosphate (ppopT), were studied as potential inhibitors of NS3 helicase activity. It was shown that imidodiphosphate inhibited the helicase reaction at a concentration below 1 mM, and when the latter increased, residual activity did not change and made up about 30% in "catalytic conditions" (ATP-dependent) and 77% in "non-catalytic conditions" (ATP-independent) (Fig. 7). Probably in both cases "non-catalytic" and ATP-dependent components contribute to the measured activity, and the inhibitory effect of imidodiphosphate (as inhibitor of NTPase activity) is exhibited only towards the latter: in the case of enzyme deficiency catalytic conditions of reaction are prevalent (for 70%) and inhibitory effect, respectively, increases. Similarly, in the case of reaction with enzyme excess the contribution of ATP-dependent activity does not exceed 20-25%, and respectively, maximal extent of reaction inhibition reaches the same value.

However, we did not find a similar effect during investigation of inhibitory effect of 2′-deoxythymidine 5′-phosphoryl-β,γ-hypophosphate (ppopT): inhibition of helicase activity was insignificant and independent of the reaction character (data not shown). Earlier Levin et al. [24] showed that the NS3 helicase activation is achieved due to the protein conformational transformations upon binding ATP (or its analog), and after ATP hydrolysis this cofactor is released from the allosteric center, which makes possible the subsequent reinitiation of the reaction. We supposed that 2′-deoxythymidine 5′-phosphoryl-β,γ-hypophosphate (ppopT) can interact with the ATP-binding center and partially activate the helicase reaction. Then the insignificant effect of this inhibitor can be explained by a superposition of two processes: Activation "instead of ATP", on one hand, and inhibition of repetitive catalytic acts due to impossibility of the NTP-binding center release, on the other. To check this hypothesis, we carried out the helicase reaction under catalytic conditions with ATP replacement in the reaction mixture by 2′-deoxythymidine 5′-phosphoryl-β,γ-hypophosphate (ppopT). It is seen in Fig. 8 that this NTP analog indeed activates the helicase reaction under ATP-dependent conditions with efficiency almost equal to that for natural cofactor ATP.

Thus, we have developed a method for production of the soluble full-length recombinant HCV NS3 protein

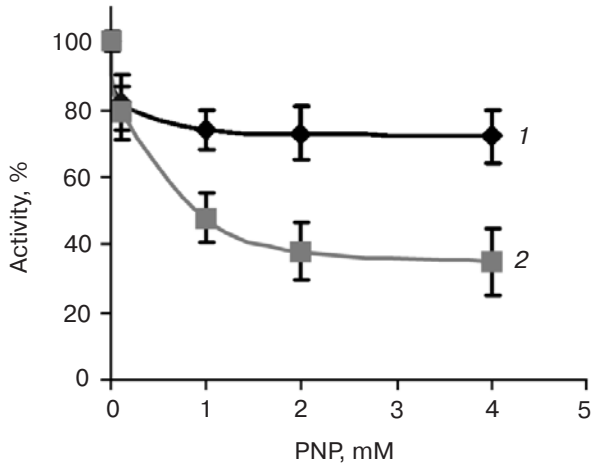


Fig. 7. Inhibition of the helicase reaction by imidodiphosphate in non-catalytic (*1*) and catalytic (*2*) conditions.

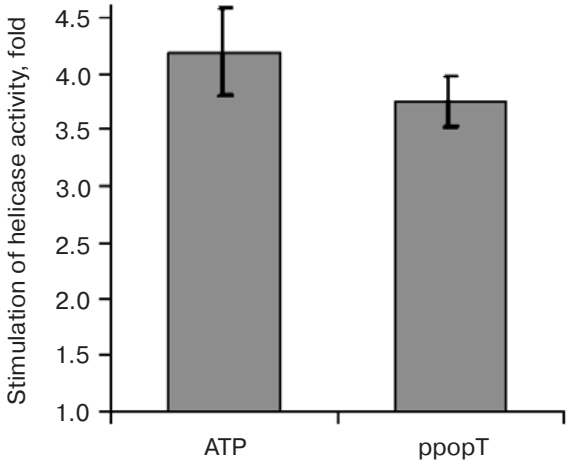


Fig. 8. Stimulation of NS3 protein helicase activity in the presence of natural ATP and its non-hydrolyzable analog ppopT. The reaction efficiency in the absence of ATP (non-catalytic mode) was taken as the unit activity.

with the yield exceeding 6 mg/liter of cell culture, optimized conditions for determination of both enzyme activities (NTPase and helicase) of this protein, and established some features of these reactions. The effect on these enzymatic activities of a number of nucleotide derivatives and inorganic pyrophosphate analogs was investigated. It was shown for nucleotide analogs that the decisive role in their interaction with NS3 belongs to the triphosphate fragment of the molecule, whereas the enzyme specificity towards the nucleic base and ribose fragment is very low. It was shown for the first time that inorganic pyrophosphate and its analogs can play the role of inhibitors of NS3 enzyme activities, and imidodiphosphate is the most active in this series.

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