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Substrate properties of C′-methyl UTP derivatives in T7 RNA polymerase reactions. Evidence for N-type NTP conformation

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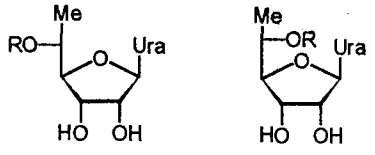
Abstract The number of synthetic UTP analogues containing methyl groups in different positions of the ribose moiety were tested as substrates for T7 RNA polymerase (T7 RNAP). Two of these compounds (containing substituents in the 5′ position) were shown to be weak substrates of T7 RNAP. 3′Me-UTP was neither substrate nor inhibitor of T7 RNAP while 2′Me-UTP was shown to terminate RNA chain synthesis. Conformational analysis of the analogues and parent nucleotide using the force-field method indicates that the allowed conformation of UTP during its incorporation into the growing RNA chain by T7 RNAP is limited to the χ angle range of 192–256° of N-type conformation.

Key words: T7 RNA polymerase; C′-methyl analogue of UTP; Transcription; RNA chain terminator; Conformational energy calculation; Substrate conformation

1. Introduction

Functionally competent analogues of nucleosides and nucleotides, namely C′-methylnucleosides and their phosphoric esters, are widely used for the study of different enzymes catalyzing the synthesis and decomposition of nucleic acids [1,2]. These compounds retain all the functionalities of natural compounds, including all the possible binding sites for the enzymes. This communication deals with the study of bacteriophage T7 RNA polymerase (T7 RNAP) with four UTP analogues, namely 1-(6-deoxy-β-D-allofuranosyl)uracil 5′-triphosphate (**1**, 5′aMe-UTP), 1-(6-deoxy-α-L-talofuranosyl)uracil 5′-triphosphate (**2**, 5′tMe-UTP), 1-(2′-C-methyl-β-D-ribofuranosyl)uracil 5′-triphosphate (**3**, 2′Me-UTP), and 1-(3′-C-methyl-β-D-ribofuranosyl)uracil 5′-triphosphate (**4**, 3′Me-UTP). Recently the same compounds were tested in *E. coli* RNA polymerase reaction [3] and corresponding dTTP derivatives with a number of DNA polymerases [4].

T7 RNAP is a suitable model for the study of the mechanism of transcription. The enzyme belongs to single-subunit nucleic acid polymerases superfamily and its three-dimensional structure closely resembles those of single-subunit DNA polymerases rather than multisubunit *E. coli* RNA polymerase [5]. In this connection the specificity of T7 RNAP toward the above-mentioned UTP analogues is of great interest.



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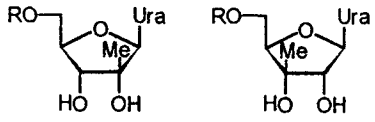
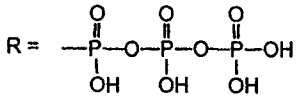


3 2'Me-UTP 4 3'Me-UTP



2. Materials and methods

T7 RNAP (200 000 U/mg) was purified as described earlier [6]. The activity of T7 RNAP was assayed according to [7] using plasmids pGEMT [8] or pTZ18R (USB) as substrates. The reaction was carried out for 15 min at 37°C in a mixture containing 40 mM Tris-HCl buffer (pH 7.8), 10 mM $MgCl_2$. Samples (10 μl) contained saturation concentrations (0.25 mM) of each GTP, ATP, CTP, and UTP, or one of the analogues tested, 500 000 cpm of [α-^{32}P]ATP and 0.05 μg of the enzyme. In some experiments the equimolar mixture of UTP and an analogue was used. To calculate the kinetic parameters of the reaction, variable concentrations of the analogue (5–200 μM) were used. The kinetic constants were calculated using the computer program Microcal Origin 3.5 (Microcal Software).

To analyze the run-off transcripts, the samples were incubated for 15–30 min, the reaction was stopped with 4 μl of sample mixture (1 ml formamide, 1 mg/ml of each bromophenol blue and xylenecyanol, and 20 mM EDTA, pH 8.0), heated for 10 min at 90°C and subjected to electrophoresis in 20% polyacrylamide gels containing 7.5 M urea according to [9].

The UTP analogues **1–4** were synthesized as described previously [3,10,11].

Calculations of conformational parameters were performed at fixed bond lengths and angles [12]. We used algorithms described earlier to calculate the amplitude of pseudorotation corresponding to the optimal form of a furanose cycle at the variable P angle [13]. Methyl group was considered a tetrahedron with standard C-H bond lengths and free rotation around C-C bond. Conformational energetic maps (influence of the pseudorotation angle P and glycoside angle χ on potential energy, kcal/mol) were calculated for 5′-phosphorylated uridine and its C′-methyl derivatives. The maps were created for free rotation around the exocyclic bond C4′-C5′ and C-O-P bond and were almost identical to the maps for uridine, UTP, and their C′-methyl derivatives. In calculations we used various sets of force pa-

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rameters. For all cases we obtained very similar energetically forbidden areas ($E_{analogue} - E_{natural\ nucleoside} > 5$ kcal/mol) caused by mutual interaction of the methyl group with the uracil base.

3. Results and discussion

First, the ability of analogues to substitute for UTP in T7 RNAP-catalyzed reaction was tested. The T7 RNAP's overall activity on the pGEMT template in the absence of UTP was about 15 and 7% for analogues **1** and **2**, respectively, and negligible for analogues **3** and **4** (Fig. 1). If the incubation mixture contained both UTP and an analogue, the contribution of **1** and **2** to the overall activity was additive, **3** strongly inhibited the RNA synthesis, and **4** showed no apparent effect on the reaction. For analogues **1** and **2**, the K_m and relative V_{max} values were calculated (Table 1). The binding parameters for analogues **1** and **2** are somewhat lower than that for UTP, demonstrating their high affinity to T7 RNAP. At the same time, the V_{max} values for analogues **1** and **2** decrease dramatically. Possibly, the methyl group placed in a close proximity to the reaction centre (i.e. the α-phosphate of UTP) does not prevent sufficient binding of the analogue; rather, it causes a sterical hindrance for the efficient processing of the reaction chemical step.

The inhibition of the T7 RNAP reaction by the analogue **3** may be caused by two factors. First, analogue **3** may be a true competent inhibitor of the reaction and, second, it may function as the RNA chain terminator. To resolve this alternative, analysis of the reaction products was carried out. PAAG electrophoresis patterns obtained with analogues **1–4** (Fig. 2) demonstrated the substantial quantities of abortive transcripts (2–10 nucleotides) typical for the T7 RNAP reaction [14]. Evidently, the full-length transcripts were formed in the presence of analogues **1** and **2**, but their yields were much lower than in the control. This observation suggests that analogues **1** and **2** are incorporated into the nascent RNA chain, although not as efficiently as UTP. No long RNA chains were synthesized with analogues **3** and **4**. 2′Me-UTP (**3**) is also incorporated into nascent RNA chain in the position +13 corresponding to the first U and efficiently terminates the transcript elongation. Consequently, analogue **3** is the **RNA** chain terminator but not a competent inhibitor of the T7 RNAP reaction. Finally, the pattern with analogue **4** is identical to that obtained with three NTPs (−UTP). Therefore, probably analogue **4** is neither the enzyme substrate nor its inhibitor.

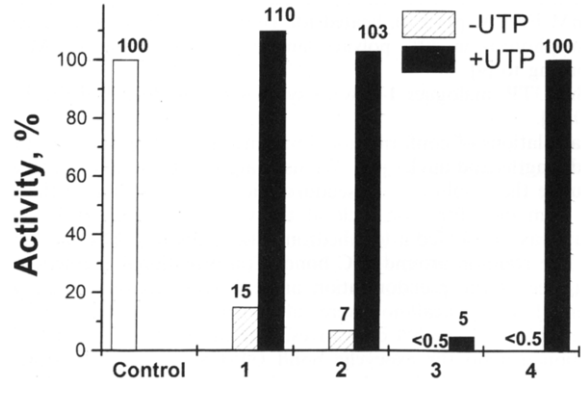

Fig. 1. Relative activities of T7 RNAP with analogues **1–4**.

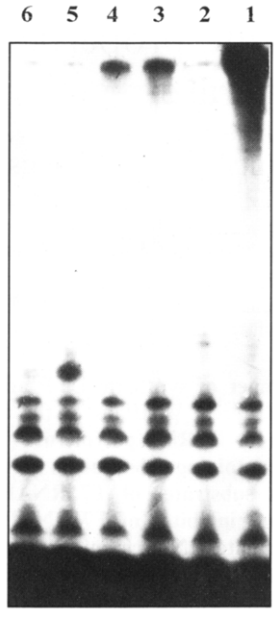

Fig. 2. In vitro transcription by T7 RNAP in the presence of analogues **1–4** (pGEMT was used as a template). All samples contained 5×10^5 cpm of [α-^{32}P]ATP. 250 μM of each GTP, ATP, CTP, and UTP or analogues. Lanes: 1, +UTP (control); 2, −UTP; 3, +**1**; 4, +**2**; 5, +**3**; 6, +**4**.

To evaluate the kinetic parameters of analogue **3** incorporation into the RNA chain the reaction rate versus analogue **3** concentration was plotted. In these experiments the plasmid pTZ18R (USB) was used as a template. The sequence immediately downstream from the T7 promoter in this plasmid is GGGAAUUC... The bands corresponding to the terminated RNA were cut out from the gel and their radioactivities were counted. To compare the parameters of UTP and analogue **3** incorporation, the assay mixture omitting CTP was used as a control. The kinetic constants obtained are presented in Table 1. It should be noted that these parameters cannot be compared with that obtained for the 'overall' reaction: in the first case these parameters reflect the multitude of UTP incorporation in a long transcript, while in the second we deal with a sole chemical step.

Previously, it was demonstrated that analogues **1**, **2** [3,15] and **3** [16] were incorporated into a growing RNA chain with different efficiency, while **4** [3] acted as a terminator in the reaction catalyzed by *E. coli* RNA polymerase. Evidently, the results with analogues **1** and **2** presented here coincide with those with *E. coli* enzyme, while essential differences occur for analogues **3** and **4**. This is probably due to different requirements of the NTP-binding sites in these polymerases for the substrate structure and/or conformation.

The introduction of a methyl group into the nucleoside molecule may change the reactivity of the neighboring hydroxyl groups and analogue conformation. The presence of voluminous groups may result in steric hindrance caused by intermolecular and intramolecular collisions when the substrate is fixed in a certain conformation in the enzyme-substrate complex. The detailed analysis of C′-methylnucleoside conformation in solution and crystal was published earlier [2].

To detect intramolecular contacts between methyl group and heterocyclic base, conformational analysis of nucleotides and their analogues using the force-field method in the 'rigid'

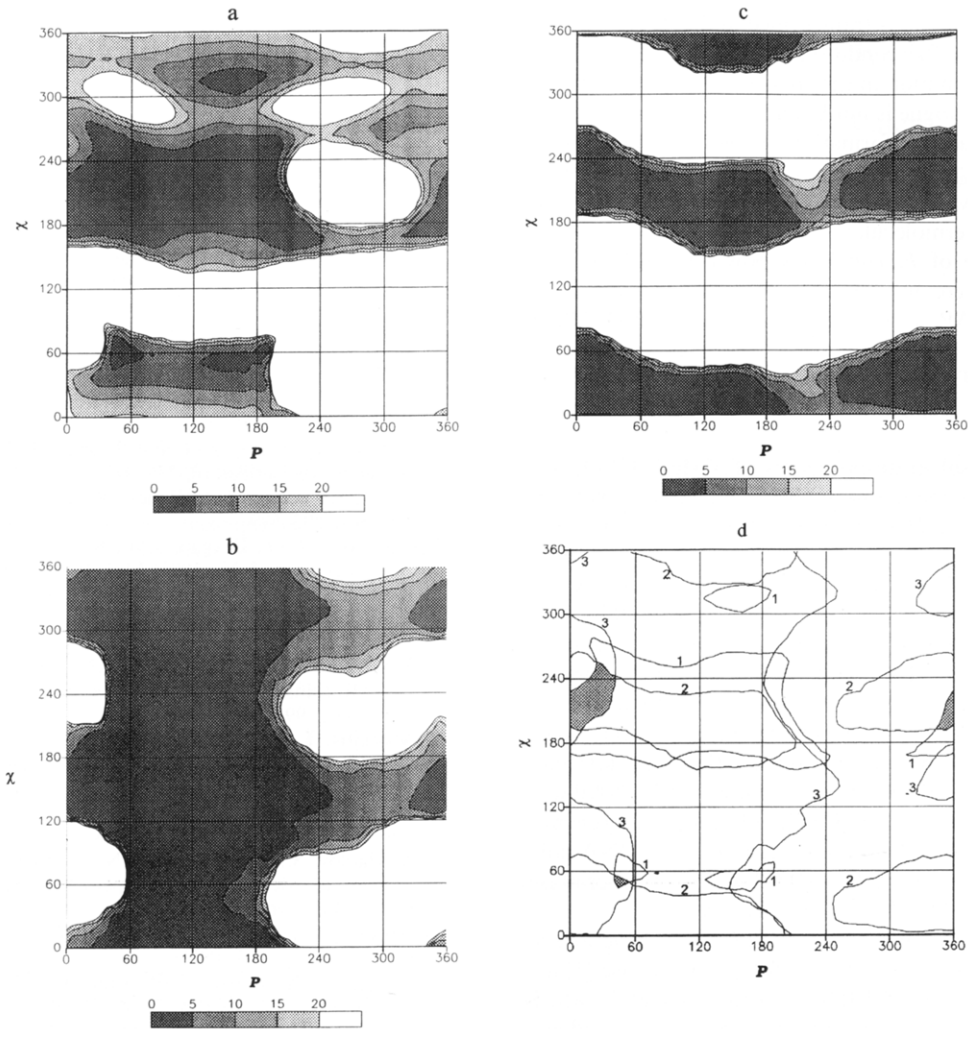

Fig. 3. Conformation energy maps in the χ-P space. Isoenergy contours are drawn at 5 kcal/mol intervals. The first contour corresponds to 5 kcal/mol relative to global minimum. a: Potential energy (E) for natural UMP. b: $E_{2'Me-UMP}-E_{UMP}$. c: $E_{3'Me-UMP}-E_{UMP}$. d: Isoenergy contours corresponding to 5 kcal/mol relative to global minimum: 1, UMP; 2, $E_{2'Me-UMP}-E_{UMP}$; 3, $E_{3'Me-UMP}-E_{UMP}$.

bond length approximation was carried out. The energetically forbidden conformation was defined as a conformation fitting the following condition: $E_{analogue}-E_{natural\ nucleoside} > 5$ kcal/mol. The conformational energy maps (the χ-P dependence versus the potential energy E, free rotation round the C4′-C5′ bond) are nearly identical for **1** and **2** (data not shown) and natural UMP (Fig. 3a). Differences appear when the methyl group in an analogue is arranged above the furanose cycle [17]. Only *N*-conformers of 3′Me-UTP (**4**) have energetically forbidden conformations, whereas no intramolecular collisions occur in *S*-conformers (Fig. 3b). The energy barrier of the *syn-anti* conversion is noticeably higher when a methyl group is introduced in the 2′ position (Fig. 3c). Allowed and forbidden conformations due to intramolecular contacts may be clearly seen from the $E_{analogue}-E_{natural\ nucleoside}$ energy maps in which energetic barriers are rather steep (Fig. 3b,c). These intramolecular contacts of the methyl group with the heterocyclic base should be considered during analysis of enzymatic transformations of C′-methylnucleosides and their phosphoric esters.

Table 1
Kinetic parameters of analogues **1–3** in T7 RNAP-catalyzed reaction

NTP	K_m (μM)	V_{max} (rel. units)
'Overall' reaction		
UTP (control)	27.8 ± 5.0	1.00
5′aMe-UTP (**1**)	6.0 ± 1.5	0.18
5′tMe-UTP (**2**)	10.0 ± 1.8	0.07
RNA chain termination		
UTP (control)	57 ± 17	1.00
2′Me-UTP (**3**)	2.1 ± 0.53	0.20

The methyl group substitution for protons in ribose residue results in the appearance of nucleoside analogues having all functional groups of natural compounds, e.g. all possible binding sites for the enzymes of nucleic acid biosynthesis. As a result, comparable binding constants for analogues and natural compounds can be expected. This was confirmed by a number of data [2]. However, in some cases a significant decrease of the analogue affinity to the enzyme was observed, which may be due to the intra- or intermolecular encounter of the voluminous methyl group with the heterocyclic base or the protein amino acid residues during enzyme-substrate complex formation [1,2].

Two extreme cases of the proposed approach may be con-

sidered: (1) the analogue is transformed and is well bound to the enzyme, so that the condition $E_{analogue} \approx E_{natural\ nucleoside}$ for substrate conformation in the enzyme-substrate complex is satisfied; (2) the analogue is not bound to the enzyme, then an appropriate substrate conformation should be sought in the region where $E_{analogue} \gg E_{natural\ nucleoside}$, provided that the introduction of the voluminous methyl group causes intramolecular, but not intermolecular steric hindrance.

Like in the case of *E. coli* RNA polymerase [3,15], analogues **1** and **2** were incorporated into the growing RNA chain by T7 RNAP at different efficiency. 2′Me-UTP **(3)** acts as an RNA chain terminator, i.e. it is also the T7 RNAP substrate. Therefore, the conformations of **1–3** in the enzyme-substrate complexes do not acquire any forbidden states.

The absence of substrate properties of 3′Me-UTP **(4)** may be attributed to the methyl group steric interactions with the enzyme amino acid residues or, more likely, to intramolecular interactions in the substrate. The finding that 3′Me-UTP is not able to act as a substrate in the reactions of RNA synthesis may be associated with steric hindrances within the χ angle range from 200° to 290° in *anti*-conformation in the *N*-family of conformers. It should be noted that 3′-C-methyl derivatives of TTP cannot be incorporated into a growing DNA chain by different DNA polymerases and reverse transcriptases [4]. These observations agree with recent data indicating that the three-dimensional structure of T7 RNAP and, especially, its template and NTP binding sites closely resemble those of simple DNA polymerases (such as *E. coli* DNA polymerase I Klenow fragment and HIV-1 reverse transcriptase) rather than that of *E. coli* RNA polymerase [5].

2′Me-UTP **(3)** can substitute for UTP in the reactions of RNA synthesis catalyzed by *E. coli* RNA polymerase [3] and is a chain terminator for T7 RNAP. This fact may be attributed to the conformational differences of nucleoside residue in NTP and the 3′ end of the growing RNA chain. Taking into consideration the above-mentioned conclusions, the allowed conformation of UTP during its incorporation in the primer 3′ end by T7 RNAP may be deduced from the energetically allowed conformations of natural UTP and 2′Me-UTP **(3)** and forbidden conformations for 3′Me-UTP **(4)** (Fig. 3d) and it is limited to the χ angle range of 192–256° (the dotted space in Fig. 3d) of the *N*-type.

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References

[1] Mikhailov, S.N. (1994) Nucleic Acids Symposium Ser. 31, 281–282.
[2] Mikhailov, S.N., Lysov, Yu.P., Moiseev, G.P., Yakovlev, G.I., Mikhailopulo, I.A. and Beabealashvilli, R.Sh. (1995) In: Modern Enzymology: Problems and Trends (Kurganov, B.I., Kochetkov, S.N. and Tishkov, V.I., Eds.), pp. 293–304. Nova Science, Moscow.
[3] Savochkina, L.P., Sviriyeva, T.V., Beigelman, L.N., Padyukova, N.Sh., Kuznetsov, D.A., Lysov, Yu.P., Mikhailov, S.N. and Beabealashvilli, R.Sh. (1989) Mol. Biol. (USSR), 23, 1700–1710.
[4] Mikhailov, S.N., Padyukova, N.Sh., Lysov, Yu.P., Savochkina, L.P., Chidgeavadze, Z.G. and Beabealashvilli, R.Sh. (1991) Nucleosides Nucleotides 10, 339–343.
[5] Sousa, R., Chung, Y.J., Rose, J.R. and Wang, B.C. (1993) Nature 364, 593–599.
[6] Rechinsky, V.O., Dragan, S.M., Kostyuk, D.A., Lyakhov, D.L. and Kochetkov, S.N. (1991) Mol. Biol. (USSR) 25, 1588–1593.
[7] Kostyuk, D.A., Dragan, S.M., Lyakhov, D.L., Rechinsky, V.O., Tunitskaya, V.L., Chernov, B.K. and Kochetkov, S.N. (1995) FEBS Lett. 369, 165–168.
[8] Tunitskaya, V.L., Akbarov, A.Kh., Luchin, S.V., Memelova, L.V., Rechinsky, V.O. and Kochetkov, S.N. (1990) Eur. J. Biochem. 191, 99–103.
[9] Maniatis, T., Fritsch, E.E. and Sambrook, J. (1989) Molecular Cloning. A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[10] Karpeisky, M.Ya., Mikhailov, S.N. and Padyukova, N.Sh. (1990) Bioorgan. Khim. (USSR) 8, 933–939.
[11] Mikhailov, S.N., Beigelman, L.N., Gurskaya, G.V., Padyukova, N.Sh., Yakovlev, G.I. and Karpeisky, M.Ya. (1983) Carbohydr. Res. 124, 75–96.
[12] Arnott, S. and Hukins, D.W.L. (1972) Biochem. Biophys. Res. Commun. 47, 1504–1509.
[13] Zhurkin, V.B., Lysov, Yu.P., Florentiev, V.L. and Ivanov, V.I. (1982) Nucleic Acids Res. 5, 1811–1830.
[14] Martin, C.T. and Coleman, J.E. (1987) Biochemistry 26, 2690–2696.
[15] Aivazashvilli, V.A., Mikhailov, S.N., Padyukova, N.Sh., Beabealashvilli, R.Sh. and Karpeisky, M.Ya. (1987) Mol. Biol. (USSR) 21, 1080–1091.
[16] Aivazashvilli, V.A., Mikhailov, S.N., Padyukova, N.Sh., Karpeisky, M.Ya. and Beabealashvilli, R.Sh. (1986) Bioorgan. Khim. (USSR) 12, 708–710.
[17] Mikhailov, S.N., Meshkov, S.V., Kuznetsov, D.A., Lysov, Yu.P., Gorelik, E.Sh.-B., Fomitcheva, M.V., Beigelman, L.N. and Padyukova, N.Sh. (1989) Bioorgan. Khim (USSR) 15, 969–975.