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# Substrate Specificity of T5 Bacteriophage Deoxyribonucleoside Monophosphate Kinase and Its Application for the Synthesis of [α-$^{32}$P]d/rNTP

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**Abstract**—Bacteriophage T5 deoxynucleoside monophosphate kinase (dNMP kinase, EC 2.7.4.13) is shown to catalyze the phosphorylation of both $d_2$CMP and ribonucleotides AMP, GMP, and CMP, but does not phosphorylate UMP. For natural acceptors of the phosphoryl group, $k_m$ and $k_{cat}$ were found. The applicability of T5 dNMP kinase as a universal enzyme capable of the phosphorylation of labelled r/dNMP was shown for the synthesis of [α-$^{32}$P]rNTP and [α-$^{32}$P]dNTP.

*Key words: T5 bacteriophage deoxyribonucleoside monophosphate kinase, [α-$^{32}$P]rNTP and [α-$^{32}$P]dNTP, synthesis*

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## INTRODUCTION

The phosphorylation of 5' nucleotides to the corresponding nucleoside 5' diphosphates plays a key role in the process of the intracellular metabolism of precursors of nucleic acid biosynthesis. This process is catalyzed by specific enzymes called nucleoside monophosphate kinases (ATP: nucleoside 5'-monophosphate transferase (NMP kinases).[2] Over 50 years of intense study, much information on the properties of these enzymes, their mechanism of action, and their potential use in various areas has been accumulated. This information was systematized in several reviews [1, 2].

As a rule, bacterial and eukaryotic NMP kinases are highly specific to the heterocyclic base of the substrate, whereas the specificity to sugar is expressed to a much lesser degree.

In *E. coli* cells, five nucleoside monophosphate kinases were found: adenylate kinase (EC 2.7.4.11), thymidylate kinase (EC 2.7.4.9), guanylate kinase (EC 2.7.4.8), cytidylate kinase, and uridylate kinase (EC 2.7.4.14) [3, 4]. Human tissues contain thymidylate kinase, cytidylate–uridylate kinase, five isoforms of adenylate kinase, and several guanylate kinases [5].

Enzymes encoded by bacteriophage genomes occupy a special place among NMP kinases. For the provision of the need for nucleotides, which increase in the presence of bacteriophages, first of all, in thymidyl-based nucleotides, many bacteriophages generate the synthesis of their own monophosphate kinases. Due to a small genome size, phages cannot "afford" the encoding of the four enzymes. Therefore, phage monophosphate kinases often manifest a rather wide substrate specificity, which, as a rule, correlates with the nucleotide composition of phage DNA. In particular, bacteriophages T2, T4, and T6, whose DNA contains hydrohymethylated cytidine, produce dNMP kinases (EC 2.7.4.12) that can phosphorylate hydrohymethylated (but not common) dCMP along with dGMP and dTMP [6, 7].

T5 bacteriophage can also induce the synthesis of dNMP kinase (EC 2.7.4.13). This enzyme is active towards all four canonical substrates dAMP, dCMP, dGMP, and dTMP. Its wide substrate specificity can be explained by an increased need for dNMP induced by infection. This specificity is especially low for T5 phage because it does not use a pool of cell nucleotides for the synthesis of its own DNA [8].

Bessman's group was the first to isolate and characterize dNMP kinase from *E. coli* infected with T5 [9]. Unfortunately, as the enzyme content was very small,

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[2] Abbreviations: $d_2$CMP, 2',3'-dideoxycytidine 5'-phosphate; PEI, polyethyleneimine; PEP, phosphoenolpyruvate.

Kinetic characteristics of T5 bacteriophage dNMP kinase in the reactions with different phosphoryl acceptors and ATP as a phosphoryl donor determined by the spectrophotometrical method

| Substrate | $K_m$, mM | $k_{cat}$, $c^{-1}$ |
|---|---|---|
| dAMP | 0.275 | 58 |
| rAMP | 0.367 | 31 |
| dGMP | 0.267 | 43.2 |
| rGMP | 0.81 | 0.29 |
| dCMP | 0.037 | 24.7 |
| rCMP | 0.39 | 5.6 |
| dTMP | 0.190 | 38.7 |
| dUMP | 4.2 | 10 |
| rATP (c dAMP) | 0.042 | 58 |

the authors failed to purify the enzyme to a homogenous state. However, they determined the main parameters: optimum pH, potential substrates and the affinity to them, and the dependence of activity versus the ions of various bivalent metals.

The development of the gene engineering technique supported the identification and cloning of the *dnk* gene encoding T5 dNMP kinase, implemented its expression in *E. coli*, and obtained considerable amounts of the highly purified enzyme [10]. In this work, we report the results of studies on the substrate specificity of T5 dNMP kinase and the use of this enzyme for the synthesis of [α-$^{32}$P]dNTP and [α-$^{32}$P]rNTP.

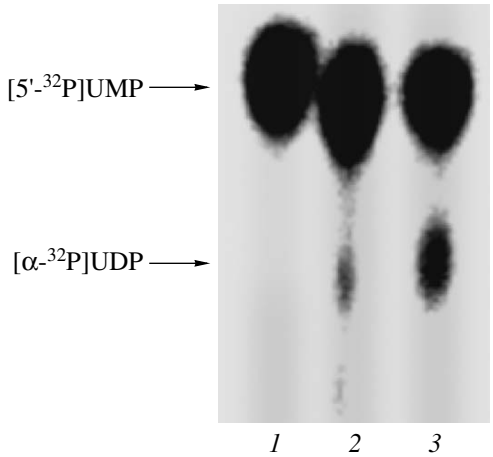



**Fig. 1.** An autoradiograph of a chromatogram on PEI cellulose of [α-$^{32}$P]UMP phosphorylation catalyzed by T5 dNMP kinase after incubation for 0 (*1*, control), 15 (*2*), and 60 min (*3*). The portion of [α-$^{32}$P]UDP in aliquot *3* determined using a phosphorimager was 10% of the total activity of the reaction mixture.

## RESULTS AND DISCUSSION

The unusually wide substrate specificity of T5 dNMP kinase towards phosphoryl group acceptors makes this enzyme very attractive for application in the enzymatic syntheses of nucleoside 5'-triphosphates. Evidently, all natural dNMP can be phosphorylated with T5 dNMP kinase. This potential was realized in early studies [11]. However, the phosphorylation of ribonucleotides with this enzyme was not studied in detail.

We studied the kinetic properties of T5 dNMP kinase for the phosphorylation of various substrates by the spectrophotometrical determination of Michaelis constants ($k_m$) and catalytic constants ($k_{cat}$) [12]. As is seen in the table, the enzyme as a phosphoryl group acceptor demonstrated the maximal affinity to dCMP ($k_m$ 0.037). It is noteworthy that the $k_m$ and $k_{cat}$ values for the process of phosphorylation of all natural dNMP were similar and only varied by a few times. At the same time, the kinetic parameters of ribonucleotide phosphorylation dramatically differed: $k_m$ of AMP, GMP, and CMP reactions differed by three times, whereas $k_{cat}$ differed by 100 times. It is noteworthy that the pH optima for dAMP–AMP and dCMP–CMP pairs were the same and were close to a value of 7.0 (data not shown).

The data on UMP phosphorylation are not given in the table, since the enzyme activity with UMP as an acceptor of the phosphoryl group could not be measured by optical methods. For the determination of the applicability of T5 dNMP kinase to be used for UMP phosphoryration, the substrates labeled with phosphorus-32 with a high specific activity were used (see the Experimental section). The use of substrates labeled with phosphorus-32 essentially increased the sensitivity of the method both for the case of [[γ-$^{32}$P]ATP as a phosphoryl donor and for the case of [5'-$^{32}$P]UMP as its acceptor. As UMP phosphorylation turned out to be very ineffective, the testing of [[γ-$^{32}$P]ATP as a donor yielded ambiguous results. Therefore, the activity of T5 dNMP kinase was tested using [5'-$^{32}$P]UMP with a specific activity of 3000 Ci/mmol (Fig. 1). In this case, the rate of UMP phosphorylation was 1 pmol/min. The reaction mixture contained 0.4 U T5 dNMP kinase (see the Experimental section). When considering that a 0.4-U enzyme catalyzes the conversion of 0.4 μmol dCMP per minute, one can conclude that the efficacy of UMP phosphorylation is extremely low. A direct comparison of UMP and dCMP phosphorylation rates is inconsistent, because the concentrations of substrate–acceptor phosphoryl groups are incomparable (4 μmol and 1 mM, respectively, see the Experimental section). However, one must accept that the $10^4$- to $10^5$-fold difference in the reaction rates is very significant.

As a whole, the phenomenon of T5 dNMP kinase specificity towards natural dNMP is unique: the kinetic parameters of the phosphorylation of dCMP–CMP or dAMP–AMP pairs are close; whereas $k_{cat}$ for the

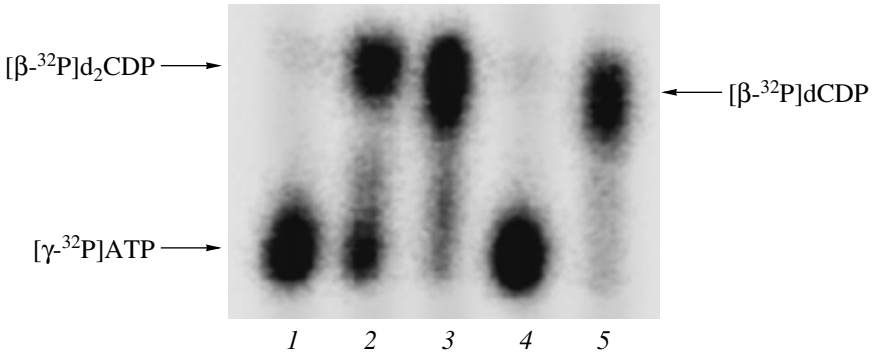
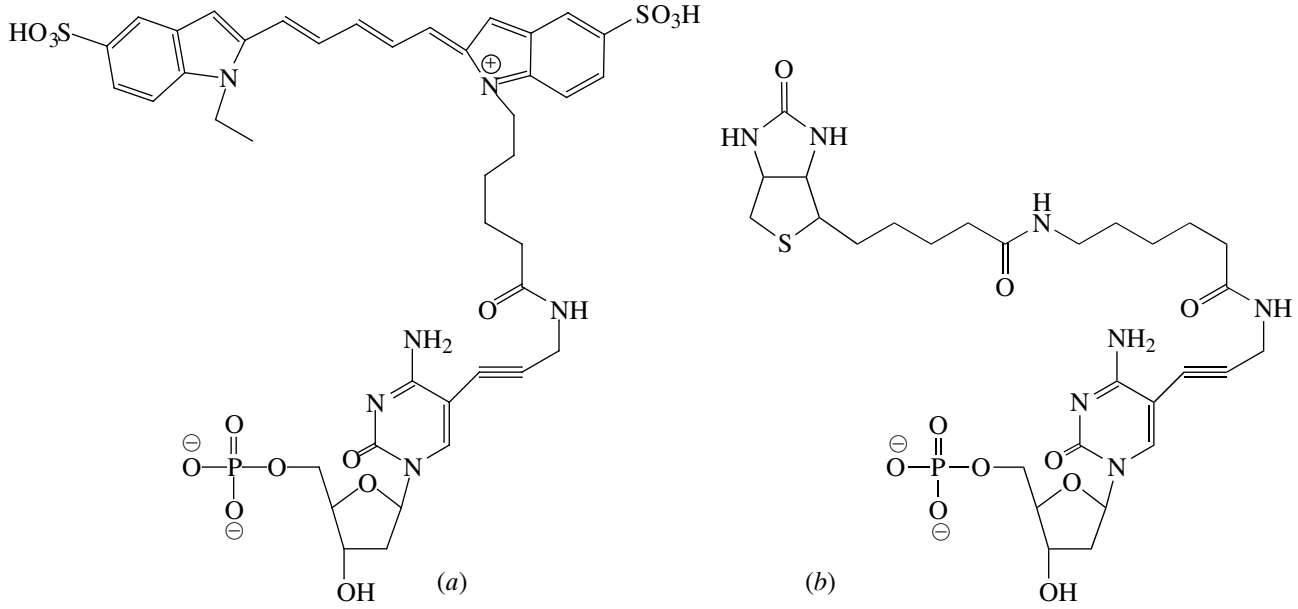



**Fig. 2.** A TLC autoradiograph on PEI cellulose of aliquots of $d_2$CMP and dCMP phosphorylation catalyzed by T5 dNMP kinase (*2* and *3*, respectively) and *E. coli* CMP kinase (*4* and *5*, respectively); *1,* [γ-$^{32}$P]ATP (control).

dGMP–GMP pair differs by 200 times, and for the dUMP–UMP pair, by more than $10^4$ times.

It is difficult enough to understand the reason for such dramatic differences in the efficacies of dUMP and UMP phosphorylation in view of the phosphorylation potential towards other natural nucleotides. One can assume that these crucial differences result from the fact that UMP phosphorylation may be involved in the regulation of the synthesis of the ribonucleoside triphosphate pool in a phage-infected cell. However, the molecular mechanism of such a specificity cannot be comprehended without deep insight into the structure of the active site of the enzyme and its interaction with the substrates. Similar specificity was found for neither of the known NMP kinases isolated from bacterial, plant, or animal cells because the specificity of these enzymes towards phosphoryl group acceptors is determined by the nucleotide heterocyclic base and, to a lesser degree, the carbohydrate moiety of the molecule [1].

Unfortunately, our attempts to phosphorylate 5-substituted derivatives of cytidyl 5'-nucleotides failed. Even with the great excess of the enzyme phosphorylation of 5-substituted (biotinylated and fluorescent) dCMP derivatives (see the structures) with the use of [[γ-$^{32}$P]ATP as a phosphoryl donor, the phosphoryl was not detected. At the same time, unlike *E. coli* CMP kinase, T5 dNMP kinase effectively phosphorylated 2',3'-dideoxycytidine 5'-phosphate (Fig. 2). This property of T5 dNMP kinase is especially interesting in view of the potential technological use of the enzyme for ddNTP syntheses. We plan to study these opportunities in the future.




Structures of 5-substituted 2'-deoxycytidine 5-phosphate derivatives: (*a*) a fluorescent derivative containing a CY-5 dye and (*b*) biotinyl derivative.

Taking into consideration the capacity of T5 dNMP kinase to phosphorylate both dNMP and rNMP, we used the enzyme for the preparative syntheses of [5'-$^{32}$P]dATP and [5'-$^{32}$P]rATP. The synthetic conditions for [5'-$^{32}$P]dATP and [5'-$^{32}$P]rATP from the corresponding [5'-$^{32}$P]monophosphates are described in the Experimental section. The two-step phosphorylation, namely, T5 dNMP kinase-catalyzed phosphorylation followed by a reaction with piruvate kinase and PEP, was performed in situ without the isolation of intermediate products. We synthesized [5'-$^{32}$P]dATP, [5'-$^{32}$P]dGTP, [5'-$^{32}$P]dCTP, and [5'-$^{32}$P]TTP, as well as [5'-$^{32}$P]ATP, [5'-$^{32}$P]GTP and [5'-$^{32}$P]CTP, in 80–90% yields and specific activities of 3–4 kCi/mmol. The attempt to use T5 dNMP kinase for the preparative phosphorylation of [5'-$^{32}$P]UMP failed. A reasonable product yield was not achieved even in the presence of considerable excesses of the enzyme after several hours of incubation.

It is noteworthy that the synthesis catalyzed by T5 dNMP kinase and piruvate kinase was performed in the presence of a small excess of ATP and a significant excess of the enzymes and PEP. Such a ratio of the components in the reaction mixture is characteristic of the enzymatic synthesis of phosphorus-labeled compounds. It enables successive NMP phosphorylation by the common mixing of all reagents in a reaction container, a reduction of the reaction time, and the simplification of the chromatographic purification of the product. The target product was isolated from the reaction mixture by reverse-phase HPLC in ion-pair mode. The use of [5'-$^{32}$P]ATP as a donor of the phosphoryl group in the phosphorylation of dATP allowed us to maintain the molar activity of [5'-$^{32}$P]ATP.

Thus, by using T5 dNMP kinase for the synthesis of nucleoside 5'-triphosphates labeled with phosphorus-32 at the α position, we simplified the synthesis with the retention of high product yields. Apparently, the obtained results can be used for the synthesis of similar compounds labeled with phosphorus-33. In general, the technological approach of the replacement of low-specific *E. coli* NMP kinases by a "universal" enzyme capable of effectively phosphorylating nucleoside 5'-monophosphates is promising and, when properly developed, can expand into other technological processes where d/rNMP need phosphorylation.

## EXPERIMENTAL

*Materials.* Tris, phosphoenol piruvate (PEP), NADH (reduced), piruvate kinase, and lactate dehydrogenase containing piruvate kinase were from Sigma; plates with PEI cellulose were from Merck; [γ-$^{32}$P]ATP (radioactive concentration of 10 mCi/ml, molar activity of 4000 Ci/mmol) was purchased from TsKP Phosphor, Russian Academy of Sciences. $d_2$CMP was synthesized from 2',3'-dideoxycytidine and phosphorus oxychloride as described in [13]. All [5'-$^{32}$P]rNMP and [5'-$^{32}$P]dNMP were prepared as described in [14] with a molar activity of 3000 Ci/mmol. *E. coli* CMP kinase was isolated according to [4] with a specific activity of 4 U/mg. T5 dNTP kinase was obtained as described in [10].

### *Determination of the Activities of Nucleoside Monophosphate Kinases*

*The enzymatic activity of T5 dNMP kinase was determined using two methods*. 1) Spectrophotometrically by the oxidation of NADH. Constants $K_m$ and $k_{cat}$ were determined as described in [9]. The final reaction mixture 1 ml in volume contained 50 mM Tris-HCl (pH 7.5), 80 mM KC1, 8 mM $MgCl_2$, 2 mM EDTA, 0.8 mM PEP, 0.2 mM ATP, 0.1 mM NADH, 2 mM NMP or dNMP, 20 U lactate dehydrogenase containing piruvate kinase, and the tested enzyme (0.02–0.2 U). The reaction was performed in an acryl cuvette at 25°C for 3–5 min. The rate of NADH oxidation was measured spectrophotometrically by decreasing the optical absorption at 340 nm. 1 U was taken as the enzyme amount capable of catalyzing the conversion of 1 μmol of dCMP per minute at 25°C. 2) By the phosphorylation of nucleotides with [γ-$^{32}$P]ATP as a donor of a phosphoryl group. The reaction mixture 25 μl in volume contained a 50 mM Tris–HCl buffer (pH 7.6), 5 mM $MgCl_2$, 1 mM nucleoside 5'-monophosphate, 0.1 M KCl, 0.1 mM ATP, 1 μCi [γ-$^{32}$P]ATP, and 0.05 U of the tested enzyme. The mixture was incubated at 37°C. Aliquots (0.5 μl) were taken out in varied time intervals and loaded on PEI cellulose plates. TLC was performed in 0.5 M $KH_2PO_4$. After the chromatography, the plate was dried and the products on PEI cellulose plates were visualized using a Packard Cyclone Storage Phosphor System.

*The activity of T5 dNMP kinase in the reaction with UMP.* The reaction mixture 25 μl in volume contained a 50-mM Tris–HCl buffer (pH 7.6), 5 mM $MgCl_2$, 0.1 M KCl, 0.1 mM ATP, 300 μCi [5'-$^{32}$P]ATP, and 0.4 U T5 dNMP kinase. The mixture was incubated at 37°C. Aliquots (0.5 μl) were taken out at 20 and 60 min and loaded on PEI cellulose plates. TLC was performed in 0.5 M KCl. The products on PEI cellulose plates were visualized using a Packard Cyclone Storage Phosphor System.

*Synthesis of [α-$^{32}$P]dCTP.* A mixture of [5'-$^{32}$P]dCTP (10 mCi, 2.5–3 nmol), 5 U T5 dNMP kinase, and 5 U piruvate kinase was added to the reaction mixture (100 μl) containing a 50-mM Tris–HCl buffer (pH 8.0), 5 mM $MgCl_2$, 0.2 M KCl, 0.05 mM ATP, 5 mM dithiothreitol, and 5 mM PEP. The mixture was incubated at 37°C for 30 min. For the determination of the reaction yield, an aliquot (0.2–0.3 μl) was analyzed by TLC on a PEI cellulose plate in a 0.5-M potassium phosphate buffer (pH 4.0). The plate was dried and visualized using a phosphorimager or autoradiography. The product yield was 90% relative to radioactivity. The target product was isolated by reverse-phase HPLC in ion-pair mode on a C-18 column in a gradient of ethanol in 50 mM triethylammonium bicarbonate. The final yield

of [α-$^{32}$P]dCTP was 7.5 mCi (75% per the starting compound).

*Synthesis of [α-$^{32}$P]ATP.* A mixture of [5'-$^{32}$P]AMP (5 mCi, 1.5–2 nmol), 5 U T5 dNMP kinase, and 5 U piruvate kinase was added to the reaction mixture (100 μl) containing a 50-mM Tris–HCl buffer (pH 8.0), 5 mM $MgCl_2$, 0.2 M KCl, 5 mM dithiothreitol, 0.1 mM dATP, and 5 mM PEP. The mixture was incubated at 37°C for 30 min. For determination of the reaction yield, an aliquot (0.2–0.3 μl) was analyzed by TLC on a PEI cellulose plate in a 0.5-M potassium phosphate buffer (pH 4.0). The plate was dried and visualized using a phosphorimager or autoradiography. The product yield was 90% by radioactivity. The target product was isolated by reverse-phase HPLC in ion-pair mode on a C-18 column in a gradient of ethanol in 50 mM triethylammonium bicarbonate. The final yield of [α-$^{32}$P]ATP was 4.5 mCi (90% per the starting compound).

## CONCLUSIONS

The enzyme can effectively phosphorylate AMP, dAMP, GMP, dGMP, CMP, dCMP, $d_2$CMP, TMP, and dUMP, but not UMP, and can be used for the preparative synthesis of the corresponding nucleoside 5'-triphosphates labelled with radioactive isotopes.

## ACKNOWLEDGMENTS

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