Characterization of a Nucleotide Kinase Encoded by Bacteriophage T7*

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Running title: Nucleotide Kinase of Bacteriophage T7

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Background: Gene 1.7 of bacteriophage T7 encodes a nucleotide kinase

Results: Gp1.7 dodecamers catalyze the reversible dTMP and dGMP kinase reactions

Conclusion: Gp1.7 is a unique nucleotide kinase that does not require a divalent metal ion

Significance: The unique nucleotide kinase of bacteriophage T7 supplies dTDP and dGDP, respectively, for conversion to the nucleoside 5’-triphosphate and recycles the dTDP derived from helicase function to dTTP.

SUMMARY

Gene 1.7 protein is the only known nucleotide kinase encoded by bacteriophage T7. The enzyme phosphorylates dTMP and dGMP to dTDP and dGDP, respectively, in the presence of a phosphate donor. The phosphate donors are dTTP, dGTP, and rGTP as well as the thymidine and guanosine triphosphate analogs ddTTP, ddGTP, and dITP. The nucleotide kinase is found in solution as a 256-kDa complex consisting of approximately 12 monomers of the gene 1.7 protein. The two molecular weight forms co-purify as a complex but each form has nearly identical kinase activity. Although gene 1.7 protein does not require a metal ion for its kinase activity, the presence of Mg²⁺ in the reaction mixture results in either inhibition or stimulation of the rate of kinase reactions depending on the substrates used. Both the dTMP and dGMP kinase reactions are reversible. Neither dTDP nor dGDP is a phosphate acceptor of nucleoside triphosphate donors. Gene 1.7 protein exhibits two different equilibrium patterns toward deoxyguanosine and thymidine substrates. The Kₘ of 4.4 x 10⁻⁴ M obtained with dTTP for dTMP kinase is approximately 3-fold higher than that obtained with dGTP for dGMP kinase (1.3 x 10⁻⁴ M) indicating that a higher concentration of dTTP is required to saturate the enzyme. Inhibition studies indicate a competitive relationship between dGDP and both dGTP, dGMP, whereas dTDP appears to have a mixed type of inhibition of dTMP kinase. Studies suggest two functions of dTTP, as a phosphate donor and a positive effector of the dTMP kinase reaction.

Gene 1.7 of bacteriophage T7 came to our attention when we found that mutations in gene 1.7 rendered T7 growth on Escherichia coli resistant to exogenous dideoxythymidine (ddT) in the media (1). E. coli can grow in the presence of ddT up to 5 mM, whereas T7 fails to form plaques in the presence of 0.1 mM ddT. Rare ddT-resistant phage did appear, and essentially all of them had a mutation in gene 1.7. Although genetic data suggested that gene 1.7 protein (gp1.7) was involved in nucleotide metabolism it was not until the protein was purified that it was identified as a nucleotide kinase that phosphorylates both dTMP and dGMP to dTDP and dGDP, respectively (2). One of the interesting properties of the T7 nucleotide kinase is that it phosphorylates ddTMP with essentially the same efficiency as it does dTMP. We have shown that
the host *E. coli* thymidylate kinase (EC 2.7.4.9), whose activity on dTMP is comparable to the T7 gp1.7, discriminates against the use of ddTMP by more than 500-fold (2). This lack of specificity reveals the mechanism for selective inhibition of phage T7 growth by ddT (2); ddT enters *E. coli* cells and is phosphorylated to ddTMP by *E. coli* thymidine kinase (EC 2.7.1.21). The conversion of ddTMP to ddTDP by gp1.7 maintains this chain terminating nucleotide on the pathway to ddTTP, which is readily incorporated into T7 DNA by T7 DNA polymerase (3). Finally, the incorporation of ddTMP into DNA results in termination of the chain and thus inability of the phage genome to be successfully replicated.

In *E. coli* cells infected with bacteriophage T7, synthesis of T7 DNA occurs at a rapid rate. T7 DNA synthesis is initiated between 5 and 10 minutes after infection and reaches a maximum rate between 15 and 20 min after infection. The rate of T7 DNA synthesis is approximately 5 to 10-fold the pre-infection rate of *E. coli* DNA synthesis (4, 5). This rapid DNA synthesis requires a large pool of deoxynucleoside 5’-triphosphate (dNTP) precursors for T7 DNA polymerase. In addition, during DNA replication the unwinding of the DNA by the T7 helicase is fueled by the hydrolysis of dTTP (6). T7 has bypassed a rate limiting step in the synthesis of the dNTP precursors by using the deoxynucleoside monophosphates obtained from the breakdown of the host DNA (7, 8), thus bypassing the complex *de novo* reduction of ribonucleotides to deoxyribonucleotides. This degradation is accomplished by the combined action of the T7 gene 3 endonuclease (9, 10) and gene 6 exonuclease (11).

The identification of the T7 nucleotide kinase that phosphorylates dTMP (EC 2.7.4.9) and dGMP (EC 2.7.4.8) to the corresponding dTDP suggests that the *E. coli* nucleotide kinases are not sufficient to provide an adequate supply of dNTPs for the synthesis of T7 DNA. No other T7 phage-encoded nucleotide kinase has been identified. It would appear that the *E. coli* cytidylate kinase, CMK (EC 2.7.4.14), is responsible for the conversion of dCMP to dCDP in T7-infected cells since this enzyme is essential for T7 growth (12).

Aside from its interesting role in T7 DNA replication, the T7 nucleotide kinase is a fascinating enzyme. Gp1.7 does not share sequence homology with any known nucleotide kinase and there are no identifiable nucleotide binding motifs found in its protein sequence. A most unusual feature is its full activity in the absence of added metal ion (2). Gp1.7 exists as two molecular weight forms of 22-kDa and 18-kDa. The smaller form is missing the first 41 residues at the N-terminus found in the larger form as a result of an internal ribosome-binding site and an in-frame start codon within the coding sequence (2). The physical properties of the protein are also quite unusual. T7 gp1.7 is precipitated by the presence of even small amounts (50 mM) of NaCl. This curious property has facilitated its purification by sequential NaCl precipitation and resolubilization steps. This communication describes the further characterization of this unique nucleotide kinase with a focus on the distinct mechanisms by which the enzyme regulates deoxyguanosine and thymidine substrates.

**EXPERIMENTAL PROCEDURES**

**Purification of T7 Nucleotide Kinase by Ni-NTA Affinity Chromatography** – Full-length gene 1.7 protein (gp1.7) fused with a 6-his tag at its N-terminus was expressed as previously described (2). Cells from 6 liters of culture were lysed by sonication in binding buffer (50 mM Tris-Cl pH 7.5, 1 mM PMSF, 10 mM β-mercaptoethanol, 0.1% Tween 20, 10% glycerol), followed by centrifugation. To the supernatant following components were added: 300 mM NaCl, 50 mM imidazole and 3 ml of Ni-NTA agarose resin. Note that in the presence of 300 mM NaCl, gp1.7/6his binds tightly to Ni-NTA even in the presence of 50 mM imidazole, thus minimizing nonspecific binding. After allowing binding to proceed for 2 hrs at 4 °C, the mixture was poured into a column (10 x 1.5 cm). The column was washed sequentially with: (i) 100 ml of binding buffer containing 300 mM NaCl and 50 mM imidazole; (ii) 50 ml of binding buffer containing 300 mM NaCl and 100 mM imidazole; (iii) 100 ml of a linear gradient of NaCl (300 to 0 mM) in buffer containing 20 mM Tris-Cl pH 7.5, 10 mM β-mercaptoethanol, 0.1% Tween 20, 10% glycerol, followed by centrifugation. To the supernatant following components were added: 300 mM NaCl, 50 mM imidazole and 3 ml of Ni-NTA agarose resin. Note that in the presence of 300 mM NaCl, gp1.7/6his binds tightly to Ni-NTA even in the presence of 50 mM imidazole, thus minimizes nonspecific binding. After allowing binding to proceed for 2 hrs at 4 °C, the mixture was poured into a column (10 x 1.5 cm). The column was washed sequentially with: (i) 100 ml of binding buffer containing 300 mM NaCl and 50 mM imidazole; (ii) 50 ml of binding buffer containing 300 mM NaCl and 100 mM imidazole; (iii) 100 ml of a linear gradient of NaCl (300 to 0 mM) in buffer containing 20 mM Tris-Cl pH 7.5, 10 mM β-mercaptoethanol; and (iv) finally with 50 ml of buffer containing 20 mM Tris-Cl pH 7.5, 10 mM β-mercaptoethanol. Bound protein was eluted by steps of 0.1, 0.3 and 0.5 M imidazole in buffer containing 20 mM Tris-Cl pH 7.5 and 10 mM β-
mercaptoethanol. The protein was further purified by gel filtration using Sephacryl S-400 HR column as previously described (2).

Purification of gp1.7/6his under denaturing conditions followed by renaturation of the protein on the column was carried out using conditions modified from those previously described (13). After binding step, the resin was slowly washed with the following solutions: (i) 10 column volumes (cv) of 8 M urea, 20 mM Tris-Cl, pH 8, 10 mM β-mercaptoethanol; (ii) 10-cv of Buffer A (20 mM Tris-Cl, pH 8, 100 mM NaCl, 10 mM β-cyclodextrin and 10 mM imidazole); and (iii) 10-cv of Buffer B (20 mM Tris-Cl, pH 7.5 and 10 mM β-mercaptoethanol). Protein was eluted with the Buffer B containing 0.3 M imidazole.

Molecular Weight Determination – The native molecular weight of purified gp1.7 was determined by multiple-angle light scattering (MALS). Purified gp1.7 was flowed through a Sephadex-75 gel filtration column using an AKTA-FPLC system (GE Healthcare) connected online to a MALS system, DAWN® HELEOS™ II (Wyatt Technology Corporation). Operation of the system and interpretation of the results was followed as in the manufacturer’s instructions. The purified gp1.7 was also examined by electron microscopy (EM). The EM samples were prepared by re-suspending the protein in buffer containing 20 mM Tris-Cl pH 7.5, 10 mM β-mercaptoethanol, and 50 μM dTMP. Samples were adsorbed to glow-discharged, carbon-coated EM grids and stained with 0.75% (w/v) uranyl formate solution as described previously (14). Images were collected using Tecnai™ G³ Spirit Bio TWIN electron microscope (FEI, Eindhoven, Netherlands) at an acceleration voltage of 80 kV under different magnifications.

Preparation of [3H]-dTDP and [3H]-dGDP – Radioactive materials [3H]-dTMP, [3H]-ddTMP and [3H]-dGMP used in this study were purchased from Moravek Biochemicals Inc., CA, U.S.A. We prepared [3H]-dTDP from [3H]-dTMP. A reaction mixture (500 μl) contained 100 mM Tris-Cl (pH 7.5), 10 mM MgCl₂, 5 mM DTT, 1 mM ATP, 50 μCi [3H]-dTDP and 500 ng of E. coli thymidylate kinase, which was purified as previously described (2). After incubation at 37 °C for 30 min, the reaction was stopped by heating at 95 °C for 3 min. The mixture was diluted two times with water and applied to a DEAE DE52 cellulose column (1.6 x 2 cm) pre-equilibrated with 50 mM NH₄HCO₃ pH 7.8. Column was washed with 10 ml of 50 mM NH₄HCO₃ pH 7.8. Bound [3H]-dTDP was eluted by a 100 ml linear gradient (50 to 300 mM) of NH₄HCO₃ pH 7.8. 1 ml fractions were collected and an aliquot was checked for radioactivity.

[3H]-dGDP was prepared by a similar procedure except that the conversion of [3H]-dGMP to [3H]-dGDP was carried out using T7 gp1.7. The products of [3H]-dTDP and [3H]-dGDP were determined to be more than 99% pure as measured by PEI Cellulose thin layer chromatography (TLC).

Nucleotide Kinase Assay – Assays for T7 gp1.7 dTMP kinase, unless otherwise indicated, measured the conversion of [3H]-dTMP to [3H]-dTDP with dTTP as the phosphate donor. Similarly, dGMP kinase measures the conversion of [3H]-dGMP to [3H]-dGDP with dGTP as the phosphate donor. The standard dTMP kinase reaction mixture contained 100 mM Tris-Cl pH 7.5, 5 mM DTT, 0.1 mM [H³]-dTMP (~10 cmp/pmole), 5 mM dTTP and the indicated amounts of gp1.7. Reactions were carried out at 37 °C for the indicated times and terminated by heating the mixture at 95 °C for 3 min. The mixture was separated along with a marker containing 1 μg of each unlabeled dTMP, dTDP and dTTP by PEI cellulose TLC in the solvent consisting of 0.5 N LiCl and 2 N acetic acid. In this solvent dTMP, dTDP, and dTTP migrate with Rf values of 0.8, 0.4 and 0.04, respectively. The nucleotides were localized under UV light at 254 nm, cut out and radioactivity was determined by liquid scintillation counting. dGMP kinase assays were performed in an identical procedure with deoxyguanosine substrates.

Reversibility of the dTMP kinase reaction was determined by measuring the amount of [3H]-dTTP and [3H]-dTDP formed in reactions containing only [3H]-dTDP. Similarly, reversibility of dGMP kinase reactions measured the formation of [3H]-DGTP and [3H]-dGDP in reactions containing only [3H]-dGDP.

Inductively Coupled Plasma Mass Spectroscopy (ICP-MS) Analysis of Metal Content – ICP-MS analysis was performed at Trace Metal...
Laboratory, Harvard School of Public Health, Boston, MA based on the procedure previously described (15). Three different forms of gp1.7 were analyzed: the wild-type enzyme (a complex of the two molecular weight forms), the 22-kDa species, and the 18-kDa species. These protein samples did not contain a histidine tag and were purified by salting in/out as described (2).

RESULTS

Co-purification of Two Molecular Weight Forms and General Properties of Gp1.7 – Gp1.7 exists in two molecular weight forms of 18-kDa and 22-kDa. The smaller form that lacks the first 41 amino terminal residues arises as a result of an internal ribosome-binding site and in-frame initiation site (2). T7 gp1.7 has the interesting property of precipitating in the presence of even low concentrations of NaCl. We have previously reported the co-purification of the two forms of gp1.7 by serial salting in/out with NaCl (2). However, this method is time consuming and results in considerable losses with each precipitation and resolubilization. In this study we describe an effective and simpler method to purify this enzyme. We overproduced gp1.7 fused to a 6-his tag at its N-terminus and purified the enzyme using Ni-NTA affinity chromatography and gel filtration (see Experimental Procedures). By attaching the 6-his tag to the N-terminus of the full-length gp1.7, we also anticipated that Ni-NTA affinity chromatography would result in the purification of only the 22-kDa form; since the 18-kDa form of gp1.7 does not contain the his tag and thus in theory would not bind to the Ni-NTA resin. Surprisingly, the 18-kDa species co-purified on the Ni-NTA affinity with the 22-kDa gp1.7 (Fig. 1A, lanes 3 and 4). When gp1.7 was purified under denaturing condition using Ni-NTA affinity, no 18-kDa species was detected (Fig. 1A, lane 5 and 6). This result indicates that the two species interact physically to form a complex; the 18-kDa species binds to the 22-kDa/his tag protein and thus co-purifies with it.

Purification of gp1.7 using his tag followed by gel-filtration resulted in nearly homogeneous gp1.7 as seen on SDS-PAGE stained with coomassie blue (Fig. 1A, lane 4). Separate experiments showed that gp1.7/his tag and wild-type gp1.7 purified by serial NaCl salting in/out exhibit the same specific activity for conversion of dTMP to dTDP (data not shown). Fig. 1B shows that the 18-kDa and 22-kDa species each purified alone and wild-type (wt) gp1.7 containing an equimolar mixture of the two species had essentially identical kinase activities with respect to the conversion of dTMP to dTDP. Therefore, wild-type gp1.7 with his tag, unless otherwise indicated, was used for routine assays described in this study.

Gp1.7 exhibits an optimum activity in the pH range of 7 to 7.5 in Tris-Cl buffer (Fig. 2A). The sensitivity of the kinase to temperature is shown in Fig. 2B. In this experiment the enzyme was incubated at the indicated temperatures for 5 and 10 min prior to immediately assaying in the standard reaction at 37 °C for 2 min. The activity is nearly unaffected by temperatures up to 40 °C but rapidly declines at higher temperatures; ~50% activity remains after 10 min at 45 °C, while < 1% of the activity remains after incubation for 10 min at 65 °C.

The solubility of gp1.7 in varying concentrations of NaCl and KCl is presented in Fig. 2C. In this experiment increasing amounts of either salt were added to a solution containing gp1.7. The precipitated gp1.7 was removed by centrifugation and the supernatant were collected and used for the assay. The % of activity reflects the amount of soluble gp1.7 remaining in supernatant as compared with no salt treatment (no precipitation). Approximately 70% of gp1.7 precipitates at 50 mM NaCl, while essentially all (> 95%) is precipitated by the presence of 100 mM NaCl as measured by the activity remaining in the supernatant after centrifugation. Essentially identical results were obtained with KCl (Fig. 2C). In each case all of the activity not in the supernatant can be recovered from the pellet by dissolving the protein in buffer lacking NaCl. Interestingly, although both NaCl and KCl precipitate gp1.7 neither salt is inhibitory to kinase activity when added to the standard reaction mixture up to 500 mM (Fig. 2D).

Based on these properties, the standard assays of gp1.7 in this study were carried out at 37 °C in Tris-Cl buffer pH 7.5 without salt.

T7 Gp1.7 Forms Dodecamers – The purification profile discussed above suggests that gp1.7 interacts with itself to form oligomers. The Stokes radius of gp1.7 was analyzed in the absence of NaCl by gel-filtration on HiPrep 26/60
Sephacryl S-200HR (fraction range: 5 - 250 kDa) using an AKTA-FPLC. Results presented in Fig. 3A show that gp1.7 elutes in the excluded volume in the same position as apoferritin (449-kDa) and thyroglobulin (669-kDa).

We further analyzed gp1.7 by electron microscopy (Fig. 3B). The majority of particles have a globular shape with an average diameter of 25.6 ± 0.8 nm, much larger than the expected diameter of a gp1.7 monomer. However, the resolution is not sufficient to determine the stoichiometry of the gp1.7 complexes.

We used multiple-angle light scattering (MALS) to obtain a more precise estimate of the molecular mass of gp1.7. The preparation of gp1.7 contains equimolar mixture of the two molecular weight forms, as shown in Fig. 1 lane 4. This analysis estimates the molecular mass of gp1.7 to be 256 kDa, equivalent to a complex containing approximately 12.2 monomers of gp1.7 based on equal amounts of the two molecular weight forms in the complex. Consistent with this result, a comparable MALS analysis of the 22-kDa gp1.7/his tag alone (~24 kDa including the his-tag) estimated the molecular mass to be 273 kDa, equivalent to approximately 11.3 monomers of the 22-kDa gp1.7/his tag. These results, taken together suggest that the gp1.7 exists predominantly as dodecamer.

Gp1.7 Activity Does Not Require Divalent Cations – We have previously shown that T7 gp1.7 has kinase activity in the absence of added divalent cations, even in the presence of the chelating agent EDTA (2). One possibility is that a divalent cation is required but that it is tightly bound to gp1.7 and not accessible to chelating agent. To explore this possibility, we analyzed the metal content of purified gp1.7 by ICP-MS. Three different preparations of gp1.7 were analyzed: the 22-kDa, the 18-kDa and the wild-type species consisting of both molecular weight forms. In each case the proteins did not contain his tag and were purified by serial salting in/out (2). Initially, 21 divalent cations were screened. Based on these results, the four cations that gave the strongest signals were further analyzed for their content (Table 1). Mg$^{2+}$, the divalent cation most commonly used in kinase reactions, is found in only a 1 to 30 molar ratio with gp1.7 monomers. Only zinc was present at a significant level and only in the 22-kDa and wild-type species; the highest level of this metal was ~ 0.3 moles per mole of protein (Table 1).

Gp1.7 has nine cysteines. Seven of which are located within the first 79 amino terminal residues. These cysteines are found in two sequences: 21-CX$_2$CX$_{10}$C-35 and 67-CX$_3$CX$_4$CX$_2$C-79 that are putative zinc-binding motifs (16). Therefore it is likely that these cysteines account for binding the detected zinc. Consistent with this interpretation, the 18-kDa gp1.7 that lacks the first 41 amino terminal residues contains 10-fold less zinc: 0.04 moles per mole of gp1.7 monomer (Table 1). However, the 18-kDa protein has the same kinase activity as does the 22-kDa species (Fig. 1B). Furthermore, deletion genetic mapping has shown that the N-terminal half of gp1.7 is not required for conferring sensitivity of T7 phage to dideoxynucleoside (1, 2). We have also purified gp1.7 under denaturing condition followed by renaturation in order to release any tightly bound metal. The renatured gp1.7 has approximately 60% of the kinase specific activity as the protein purified under native conditions (data not shown).

These results taken together strongly suggest that no divalent cations are required for activity of T7 gp1.7.

Phosphate Acceptor and Donor Specificity – In the experiments presented in Table 2, we have examined the ability of a number of nucleoside monophosphates to serve as acceptors of the phosphate from dTTP in the presence and absence of MgCl$_2$. Gp1.7 specifically phosphorylates dTMP, ddTMP and dGMP in the presence or absence of Mg$^{2+}$. Surprisingly, dUMP, an effective phosphate acceptor of known thymidylate kinases (17-19), is only a poor substrate for gp1.7. The activity with dUMP is only 1% and 10% of the activity of that observed with dTMP in the presence and absence of Mg$^{2+}$ respectively. Other analogues, including thymidine, dideoxynucleosides, and 3’-azido 3’-dideoxynucleoside 5’-monophosphate (AZT), are not phosphate acceptors from dTTP. It is noteworthy that Mg$^{2+}$ reduces the reaction with dGMP by approximately 40% but stimulates slightly the ability of dTMP and ddTMP to accept phosphate.

Various nucleoside triphosphates were also examined for their ability to donate the phosphate to dTMP and dGMP in the presence and absence of Mg$^{2+}$ (Table 3). Gp1.7 uses rGTP, dGTP, dITP, dTTP, dUTP, ddGTP and ddTTP as phosphate donors.
Donors. The other nucleoside triphosphates tested gave less than 1% of the activity obtained with dTTP. Again, in spite of the nonessential nature of Mg\(^{2+}\), the addition of Mg\(^{2+}\) either stimulates or inhibits the kinase activity of gp1.7 depending on the donor (Table 3). The presence of Mg\(^{2+}\) stimulates significantly the phosphorylation of dTMP by most of active donors. Indeed a 3.5-fold stimulation is observed with the phosphorylation of dTMP by dGTP making it the best phosphate donor. Mg\(^{2+}\) also stimulates the phosphorylation of dGMP with dGTP and ddTTP as the donors. In particular, the ability of ddGTP to donate phosphate to both dGMP and dTMP is very low in the absence of Mg\(^{2+}\) but is increased by 20 and 30-fold, respectively by the presence of Mg\(^{2+}\). The presence of Mg\(^{2+}\) inhibits the phosphorylation of dGMP by the donors, dITP, GTP, dTTP, and dUTP by 35, 35, 40, and 60%, respectively.

In view of these differing effects of Mg\(^{2+}\) on the kinase reaction we have examined the effect of Mg\(^{2+}\) concentration on the four acceptor/donor combinations of thymidine and deoxyguanosine nucleotides (Fig. 4). In the absence of Mg\(^{2+}\), dGMP/ddTTP is the most active combination. The initial rate of 13 pmol/ng/min obtained with this combination is almost 4-fold higher than the dTMP/ddTTP combination which gives the lowest initial rate (3 pmol/ng/min). Mg\(^{2+}\) stimulates the initial rate of the reaction containing either dTMP or ddTTP up to 5 mM. Conceivably, the greatest stimulation (> 3-fold) is obtained with combination dTMP/ddTTP. The reaction rates of dTMP/ddTTP and dGMP/ddGTP were also increased by 30 and 90%, respectively, by the presence of 5 mM Mg\(^{2+}\). In sharp contrast, the rate of the reaction containing dGMP/ddGTP decreased by 40% at this concentration of Mg\(^{2+}\) (Fig. 4).

Competitive Inhibition of dTMP by dGMP – We examined the inhibitory effect of dGMP on the conversion of \([^3H]\)-dTMP to \([^3H]\)-dTDP with ddTTP as donor (Fig. 5). A Lineweaver-Burk plot (20) of the inhibition of the phosphorylation of dTMP by dGMP shows that the curves intersect on the Y-axis, indicating dGMP acts as a competitive inhibitor of dTMP (Fig. 5A). Likewise, a competitive relationship was detected between the two phosphate donors, dGTP and ddTTP (Fig. 5B). These results indicate a competitive binding site for dTMP and dGMP and likewise a binding site for ddTTP and ddGTP.

Stoichiometry of dTMP and dGMP Kinase Reactions – We determined the stoichiometry of nucleoside mono-, di-, and triphosphates in the gp1.7 dTMP and dGMP kinase reactions. For each kinase reaction, two parallel mixtures containing equivalent concentration (0.1 mM) of acceptor and donor were prepared. One mixture contained \([^3H]\)-labeled acceptor (dTMP or dGMP) while the other mixture contained \([^3H]\)-labeled donor (dTTP or ddGTP). Changes in the amount of reactants and products over time were measured by TLC. The radioactive components found after the reaction had reached equilibrium (120 min) are shown in Table 4. The results show that the amount of dTDP formed in the reaction corresponds to the total amount of dTMP and dTTP lost (Table 4, i). Similarly, dGDP formed corresponds to the total amount of dGMP and dGTP lost (Table 4, ii). From these data, we conclude that the stoichiometry of the kinase reactions is represented by the equations:

\[
\text{dTMP} + \text{dTTP} \rightleftharpoons 2 \text{dTDP}
\]

\[
\text{dGMP} + \text{dGTP} \rightleftharpoons 2 \text{dGDP}
\]

Interestingly, the results in Table 4 reveal two distinguishable equilibrium patterns between dTMP and dGMP kinase reaction. The dTMP kinase reaction reached equilibrium even though the dTTP and dTMP were still in considerable 2-fold excess over the amount of dTDP formed (Table 4, i). In contrast, the dGMP kinase reaction reached equilibrium only after the dGDP concentration was considerably 2-fold higher than dGMP and dGTP (Table 4, ii, column 3). The apparent equilibrium constants ($K_{eq}$) for the dTMP and dGMP kinase reactions derived from these experiments are 0.07 and 0.85, respectively. The results imply that the product dTDP has a strong inhibition on its synthesis. The inhibition can be overcome by increasing the concentration of phosphate donor. The experiment presented in Fig. 6A shows an exponential conversion of dTMP to dTDP in a reaction containing 0.1 mM \([^3H]\)-dTMP and a 25-fold excess of dTTP. After 120 min of incubation when the reaction had reached equilibrium, approximately 85% of the available \([^3H]\)-dTTP (2,000 pmols) was phosphorylated to yield \([^3H]\)-dTDP (~ 75%) and \([^3H]\)-dTTP (~ 10%). The conversion of \([^3H]\)-dTDP to \([^3H]\)-dTTP appears not to be proportional to the amount of \([^3H]\)-dTDP synthesized and ceases when the conversion \([^3H]\)-dTDP to \([^3H]\)-dTDP ceases (Fig.
In contrast when the dGMP kinase reaction contained dGTP at a 25-fold excess over \(^{3}\)H-dGMP (0.1 mM), the \(^{3}\)H-dGMP was quickly converted to \(^{3}\)H-dGDP, followed by conversion to \(^{3}\)H-dGTP (Fig 6B); approximately 97% of available \(^{3}\)H-dGMP was phosphorylated to nearly equal amounts of \(^{3}\)H-dGDP (~51%) and \(^{3}\)H-dGTP (~46%) after the 120 min incubation period. The results imply that both dGMP and dTMP kinases catalyze the reversible reaction. The differences in amount of \(^{3}\)H-dGTP and \(^{3}\)H-dTTP obtained could also suggest that newly synthesized \(^{3}\)H-dGDP is an active phosphate acceptor from dGTP, whereas newly synthesized \(^{3}\)H-dTDP is not a phosphate acceptor from dTTP in the further NDP kinase step. However, this model does not explain the inability of gp1.7 to convert the remaining \(^{3}\)H-dGDP to \(^{3}\)H-dGTP even when dGTP is present in excess.

Reversibility of the Gp1.7 Kinase Reactions – In order to confirm that the dTMP and dGMP kinase reactions are truly reversible, we carried out reactions in which \(^{3}\)H-dTDP or \(^{3}\)H-dGDP were the only substrates. Under these conditions, \(^{3}\)H-dTTP and \(^{3}\)H-dTMP were produced equally in the reaction containing only \(^{3}\)H-dTDP at the outset, while \(^{3}\)H-dGTP and \(^{3}\)H-dGMP were produced equally in the reaction containing only \(^{3}\)H-dGDP at the outset (Fig. 7A). The ratio of the nucleotides found at equilibrium in the dGMP reverse kinase reaction were similar to those obtained with the dGMP forward kinase reaction; approximately 50% of the available \(^{3}\)H-dGDP was converted to \(^{3}\)H-dGTP and \(^{3}\)H-dGMP, resulting in a two-fold of excess of \(^{3}\)H-dGDP over \(^{3}\)H-dGTP (Table 5). The conversion of \(^{3}\)H-dTDP to \(^{3}\)H-dTTP and \(^{3}\)H-dTMP occurs at a rate approximately five-fold slower than that observed with \(^{3}\)H-dGTP and \(^{3}\)H-dGMP. Additionally, the extent of synthesis of \(^{3}\)H-dTTP and \(^{3}\)H-dTMP at equilibrium is considerably lower than that observed with \(^{3}\)H-dGTP and \(^{3}\)H-dGMP; the reversibility of the dTMP kinase reaction essentially stops when approximately 30% of the available \(^{3}\)H-dTDP has been converted to \(^{3}\)H-dTTP and \(^{3}\)H-dTMP, resulting in more than a three-fold excess of \(^{3}\)H-dTDP over \(^{3}\)H-dTTP and \(^{3}\)H-dTMP (Table 5).

We examined the ability of the nucleoside triphosphate products dTTP and dGTP to serve as phosphate donors to dTDP and dGDP, respectively (Fig. 7B). An increasing amount (0 to 1 mM) of dGTP and dTTP was correspondingly added to the reactions containing 0.1 mM \(^{3}\)H-dGMP and \(^{3}\)H-dTDP. The rate of conversion of \(^{3}\)H-dGDP to \(^{3}\)H-dGTP was inhibited approximately 80% by dGTP at 1 mM. At this concentration (1mM), dTTP inhibits the rate of conversion of \(^{3}\)H-dTDP to \(^{3}\)H-dTTP by almost 50%. The results taken together indicate that neither dTDP nor dGDP actively serve as phosphate acceptors by nucleoside triphosphates.

Product Inhibition of dTMP and dGMP Kinase Reactions – We examined the inhibitory effect of both dTDP and dGDP on its synthesis in the forward kinase reactions. In the experiments shown in Fig. 8A, increasing amounts (0 – 0.4 mM) of dGDP (8A) were added to the kinase reactions containing 0.1 mM \(^{3}\)H-dGMP and 2.5 mM dGTP (empty circles) or 2.5 mM dGMP and 0.1 mM \(^{3}\)H-dGTP (filled circles). Analogous experiments were carried out with the dTMP kinase reaction in Fig. 8B. In both instances the formation of \(^{3}\)H-dGDP or \(^{3}\)H-dTDP was measured.

dGDP is a potent inhibitor of the conversion of \(^{3}\)H-dGMP to \(^{3}\)H-dGDP when either dGMP or dGTP are present in excess (Fig. 8A). In both cases the rate of \(^{3}\)H-dGDP formation was inhibited approximately 84% by 0.4 mM dGDP. Surprisingly, inhibition of dTMP kinase by dTDP was considerably weaker than that obtained with dGDP on the dGMP kinase reaction (Fig. 8B). The rate of \(^{3}\)H-dTDP formation in the reaction containing 0.1 mM \(^{3}\)H-dTMP and 2.5 mM dTTP was inhibited only 20% by 0.4 mM dTDP (Fig. 8B, empty triangles). Inhibition by dTDP on the rate of conversion of \(^{3}\)H-dTTP to \(^{3}\)H-dTDP is 2-fold greater in the reaction containing a 25-fold excess of dTMP over \(^{3}\)H-dTTP (0.1 mM) (Fig. 8B, filled triangles). These results suggest that the presence of high dTTP prevents dTDP from binding to dTMP site.

Lineweaver-Burk plots (20) indicate a competitive relationship between dGDP and dGMP (Fig. 9A) as well as dGTP (data not shown). However Lineweaver-Burk plots (Fig. 9B) of the reciprocals of the initial rates against the reciprocals of dTMP concentration in the presence of dTDP as an inhibitor shows the intersection of the curves does not fall on either
axis indicating a mixed type of inhibition of dTMP kinase by dTDP.

**Effect of dTTP and dGTP Concentration on the Reaction**  - The influence of dTTP and dGTP concentration on the rate of reaction are presented as a Michaelis-Menten plot in Fig. 10. At low concentrations (Fig 10, inset) of dTTP the rate of the dTMP kinase reactions is not proportional to the donor concentration. On the other hand, the rate of the dGMP kinase reaction is proportional at even low concentrations of dGTP. The apparent Michaelis-Menten constants, Km calculated for dTTP and dGTP are 4.4 x 10^{-4} M and 1.3 x 10^{-4} M, respectively. These results suggest that unusual high dTTP is required to saturate the enzyme as compared to dGTP.

**DISCUSSION**

T7 gp1.7 was originally identified as a thymidylate kinase (EC 2.7.4.9) based on its ability to phosphorylate dTMP to dTDP (2). There are fundamental differences in the properties of this enzyme from those of other known thymidylate kinases found in *E. coli* (19), yeast (17), and mouse (18). First, it does not share sequence homology with these other thymidylate kinases and does not contain any of the identifiable motifs found in nucleotide binding domains. Second, the substrate specificity for both phosphate acceptor and donors of gp1.7 is different from that of any other known thymidylate kinase: it phosphorlates dGMP as well as dTMP, while it uses dTTP and dGTP as phosphate donors, in spite of their structural differences. Finally, the lack of any requirement for a divalent cation for catalytic activity is unique among all known thymidylate kinases (17-19).

In addition to its unique enzymatic properties, the physical properties of gp1.7 are also intriguing. Its salt precipitated property proved an asset in protein purification by serially salting in/out (2). The insolubility of the protein in the presence of salt is puzzling since its activity is not affected by the presence of NaCl or KCl up to 500 mM. One interpretation is that the salt promotes association of gp1.7 molecules to form a large matrix but does not affect the functional conformation of the enzyme. Indeed, gp1.7 is found as a large complex in solution even in the absence of salt. We estimate by MALS analysis that these large complexes each contain twelve monomers of gp1.7. This oligomerization could explain the low solubility of gp1.7 if, in the presence of salt, larger complexes lead to aggregation. The oligomerization of gp1.7 into dodecamers also explains why the two forms of gp1.7 co-purify by Ni-NTA chromatography even when only the large form has his tag attached.

In light of the fact that all other known nucleotide kinases have a strict requirement for a divalent cation for activity we wanted to examine for the presence of a sequestered metal ion. An analysis of the metal content of the purified enzyme showed that only Zn^{2+} was present at a small, but significant level, in the 22-kDa and the wild-type species. Full-length gp1.7 (22-kDa) has 9 cysteine residues arranged in two putative zinc-binding motifs: 21-CX_{2}CX_{10}C-35 and a 67-CX_{2}CX_{3}CX_{2}C-79 (21) within the N-terminal half. Results suggest that at least the first three cysteines are critical for binding to zinc, since the 18-kDa gp1.7 lacking the N-terminal 41 amino acid residues, binds considerably less zinc. The first 41 amino acids of gp1.7, however, are not required for conferring sensitivity of phage T7 to ddT in vivo (2) or for kinase activity of the purified 18-kDa protein in vitro (Fig. 1B). Since there are no other metal ions present at a significant level we conclude that no divalent cation is required for the kinase activity of gp1.7.

Interestingly, in spite of not requiring Mg^{2+} for activity the presence of Mg^{2+} does have either stimulatory or inhibitory effect, depending on the acceptor/donor combination. In general, the presence of Mg^{2+} stimulates activity when the acceptor/donor combination contains either dTMP or dGTP, and inhibits activity when the acceptor is dGMP in combination with donor dTTP, dITP, rGTP, or dUTP. Since Mg^{2+} complexes directly with the phosphate groups of the nucleotide substrates, these complexes likely interact with the enzyme differently than the nucleotides without any bound metal ions.

Both dTMP and dGMP kinase reactions are reversible. We have shown that there are distinct regulatory controls toward thymidine and deoxyguanosine substrates by gp1.7. dGDP is a competitive inhibitor of both dGMP and dGTP.
The results suggest that nucleotide binding sites for dGDP are identical or overlap with that of dGTP and dGMP respectively. The $K_{eq}$ of 0.85 for dGMP obtained with gp1.7 is similar to that found for AMP in the *E. coli* adenylate kinase (AMK) reaction. *E. coli* AMK catalyzes the conversion of AMP to ADP with ATP as donor (24). X-ray crystallography and NMR studies of *E. coli* AMK also revealed two nucleotide binding sites, one for ATP or ADP and the other for AMP or ADP (23, 24). In contrast, the $K_{eq}$ of 0.07 for dTMP obtained with gp1.7 is much lower than that for dGMP. This finding suggests that product dTDP gives strong inhibition and that a high concentration of dTTP is required to produce dTDP. Inhibition studies however show that dTDP is only a weak inhibitor of dTMP. Lineweaver-Burk plot analysis indicates a mixed type of inhibition by dTDP. These results, taken together, could be interpreted to mean that the enzyme complexes have separate binding sites for dTTP and dTDP as donor. This interpretation is supported by the observation that dTDP has no inhibitory effect on dGTP as donor (data not shown).

We hypothesize that binding of dTDP to the enzyme (dTDP-site) prevents the dissociation of the products dTDP from the enzyme complex, thus interfering with the binding of both dTTP and dTMP as donor and acceptor, respectively. Therefore to shift the equilibrium of dTMP kinase reaction in a forward direction in the presence of dTDP, dTTP has to serve not only as a donor but a competitor with dTDP for the dTDP-site, i.e. more dTTP is required. Consequently, binding of dTTP to the dTDP-site would prevent the binding of dTDP, but dTDP to bind to acceptor site of the enzyme. In support of this hypothesis, dTTP and dTMP are present 2-fold higher than dTDP at equilibrium in a dTMP kinase reaction that contained equivalent amount of dTTP and dTMP at the outset (Table 4, i). The apparent Km obtained with dTTP for dTMP kinase is 3-fold higher than that obtained for dGTP in the dGMP kinase reaction, indicating that an unusual high concentration of dTTP is required to saturate the enzyme. Additionally, the model can also explain why only a small amount of [3H]-dTTP can be produced at equilibrium in a reaction containing excess dTTP. Gp1.7 however does not display a clear sigmoidal dependence on low concentrations of dTTP in the dTMP kinase reaction, a property typical for homotropic allosteric modulators with positive cooperative binding (25). One possible explanation is that dTTP acts as activator of dTMP kinase only when dTDP is present.

These studies suggest that the mechanism of gp1.7 might likewise be quite complex in vivo. However, in T7 infected cells, synthesis of DNA occurs at a rapid rate, perhaps 5 to 10-fold pre-infection (4, 5). Therefore the nucleoside triphosphates like dTTP and dGTP would be rapidly converted to the corresponding nucleoside triphosphates by the highly active host nucleoside diphosphate kinase (NDK, EC 2.7.4.6). We have previously shown that dTMP is exponentially converted to dTTP by the joint action of T7 gp1.7 and *E. coli* NDK using ATP as the ultimate phosphate donor (2). Note that in addition to being a precursor for DNA synthesis dTTP is also used by the T7 gene 4 helicase to provide energy for the unwinding of DNA (27). The product of dTTP hydrolysis in the helicase reaction is dTDP. The amount of dTDP formed by helicase function is considerable since the hydrolysis of dTTP is required for the unwinding of just a few nucleotides. Thus, if the dTDP accumulated appreciably, perhaps by compartmentalization, then it could be recycled by gp1.7 as dTTP to the helicase. Furthermore, dGTP is a substrate of *E. coli* dGTPase, an enzyme that degrades dGTP to guanosine and tripolyphosphate (28). Thus one function of gp1.7 may be to maintain the appropriate balance of dNTPs that is critical for DNA replication fidelity (29). Perhaps, the delay in the onset of maximal DNA synthesis in the absence of gp1.7 (1) reflects the an imbalance of the nucleoside triphosphate pool.

Finally, it is puzzling that gp1.7 does not share any sequence homology to other known nucleotide kinases. This lack of homology suggests that gp1.7 functions by a novel mechanism. It is tempting to speculate that the dodecamer plays a functional role in the metal–independent reaction, perhaps forming a protein based compartment (30), in which the critical residues from monomer are either components of the catalytic interfaces or involved in subunit assembly. We have shown that even a single amino acid alteration in the C-terminal half of gp1.7 renders T7 phage resistant.
to ddT (1). The crystal structure of this remarkable enzyme should provide insight into the molecular basis of its catalytic activity.
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FOOTNOTES

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3The abbreviations are used: wt, wild-type; gp1.7, T7 nucleotide kinase; gp5, T7 DNA polymerase; dNTPs, deoxynucleoside 5'-triphosphates; TLC, thin layer chromatography; MALS, multiple angles light scattering.

FIGURE LEGENDS

FIGURE 1. Purification and comparison of kinase activity of the two molecular weight forms of gp1.7. A. Expression and purification gene 1.7 protein. Overproduced gp1.7/his-tag was purified as described under “Experimental Procedures”. Lane 1 and 2, lysate from uninduced and induced cells, respectively; lanes 3 and 4, purified gp1.7 after Ni-NTA affinity chromatography and gel filtration, respectively; lane 5, gp1.7 that was denatured, purified using Ni-NTA affinity chromatography, and renatured; lane 6, the renatured protein following by gel filtration. B. Comparison of kinase activity of wild-type (○), 22-kDa (●) and 18-kDa (▲) gp1.7. Each gp1.7 species was purified by salting in/out as described previously (2). Reaction mixtures (200 µl containing 0.1 mM [3H]-dTMP, 5 mM of dTTP, and 500 ng of the indicated species of gp1.7 were incubated at 37 °C. At the indicated times, 20 µl aliquots were removed. The [3H]-dTDP formed was determined by Pei Cellulose TLC.

FIGURE 2. General properties of gp1.7. A. Determination of the pH optima. dTMP kinase reactions were carried out in Tris-Cl buffers ranging in pH from 6 to 9. B. Effect of temperature on dTMP kinase activity of gp1.7. 50 ng of gp1.7 was incubated in 20 mM Tris-Cl pH 7.5, 1 mM DTT at the indicated temperatures for 5 (●) or 10 (▲) minutes prior to the addition to the standard reaction mixtures. Reactions were carried out at 37 °C for 2 min. Activities are compared with a standard reaction carried out without prior incubation. C. Precipitation of gp1.7 by NaCl (●) and KCl (▲). Varying concentrations (0 to 750 mM) of either salt was added to the cell lysate containing overexpressed gp1.7 and then incubated on ice for 1 hr. Samples were centrifuged at 14,000 rpm for 30 min using a microcentrifuge. Supernatants were collected and used for kinase assay in the absence of Mg2+. Activities were compared with a sample that was treated under identical conditions without salt. D. Effect of NaCl (●) and KCl (▲) on kinase activity of purified gp1.7. The indicated concentrations of NaCl or KCl were added to the reaction mixtures containing 50 ng of purified gp1.7. Reactions were carried out at 37 °C for 2 min.

FIGURE 3. Oligomerization of gp1.7. A. Gel filtration of gp1.7. Purified gp1.7 was passed through a HiPrep 26/60 Sephacryl S-200HR column (FR: 5 to 250 kDa) using an AKTA-FPLC system. The elution profile of gp1.7 was compared with that of known molecular weight protein markers (Sigma) as indicated. B. Visualization of gp1.7 by electron microscopy. Samples of purified wt gp1.7 containing both molecular weight forms were prepared for electron microscopy as described in “Experimental Procedures”. The images were collected at an acceleration voltage of 80 kV and a magnification of 98,000.
FIGURE 4. Effect of Mg\(^{2+}\) concentration on the initial rate of different acceptor/donor combinations. Reaction mixtures (20 µl) contained 50 ng gp1.7, 0 to 10 mM Mg\(^{2+}\) and the indicated acceptor/donor combinations: [\(^{3}\)H]-dTMP/dTTP (▲), [\(^{3}\)H]-dTMP/dGTP (●), [\(^{3}\)H]-dGMP/dGTP (● ●), and [\(^{3}\)H]-dGMP/dTTP (▲). Reaction mixtures were incubated at 37 °C for 1 min. The amount of [\(^{3}\)H]-dTDP and [\(^{3}\)H]-dGDP formed was determined.

FIGURE 5. Competitive inhibition of dTMP and dTTP by dGMP and dGTP, respectively. Lineweaver-Burk plot (20) of the reciprocal of the initial velocity (nmoles/50 ng/min) is plotted versus the reciprocal of dTMP (A) and dTTP (B) concentration (mM). The reaction was carried out at 37 °C for 1 min. The amount of [\(^{3}\)H]-dTDP formed was measured by Pei Cellulose TLC. A. Reaction mixtures (20 µl) contained 5 mM dTTP and various concentrations of [\(^{3}\)H]-dTMP in the presence of 0 (○), 0.15 (▲) and 0.3 (●) mM of dGMP. B. Reaction mixtures (20 µl) contained 2.5 mM dTMP and various concentrations of [\(^{3}\)H]-dTTP in the presence of 0 (○), 0.15 (▲) and 0.3 (●) mM of dGTP.

FIGURE 6. Time kinetics of the dTMP and dGMP kinase reactions. A. dTMP kinase reaction. The reaction mixtures (200 µl) contained 0.1 mM [\(^{3}\)H]-dTMP, 2.5 mM dTTP and 500 ng of gp1.7. Reactions were incubated at 37 °C. At the indicated times, 20 µl aliquots were removed and the loss of [\(^{3}\)H]-dTMP (-) and the production of [\(^{3}\)H]-dTDP (●) and [\(^{3}\)H]-dTTP (▲) were determined by Pei Cellulose TLC. B. dGMP kinase reaction. The identical experimental procedures as in A were carried with corresponding deoxyguanosine substrates. (-) [\(^{3}\)H]-dGMP lost; (●) [\(^{3}\)H]-dGDP produced; (▲) [\(^{3}\)H]-dGTP produced.

FIGURE 7. Reversibility of the dTMP and dGMP kinase reactions. A. dTMP and dGMP reverse kinase assays. Reaction mixtures (200 µl) contained 500 ng of gp1.7 and either 0.1 mM [\(^{3}\)H]-dTDP or 0.1 mM [\(^{3}\)H]-dGDP as the only substrate were incubated at 37 °C. At the indicated times, 20 µl aliquots were removed. The products [\(^{3}\)H]-dTMP (■) and [\(^{3}\)H]-dTTP (●) or [\(^{3}\)H]-dGMP (□) and [\(^{3}\)H]-dGTP (○) were determined by Pei Cellulose TLC. B. Inhibition of the reversibility of the kinase reactions by immediate triphosphate products. Various concentrations (0-1 mM) of dTTP (▲) and dGTP (△) were added to dTMP and dGMP reverse reaction as described in A, respectively. After incubation at 37 °C for 1 min, the amounts of [\(^{3}\)H]-dTTP and [\(^{3}\)H]-dGTP formed were measured, and the activities are presented as a percentage of that obtained with reactions that contained no added nucleoside triphosphate.

FIGURE 8. Inhibition of dTMP and dGMP kinase by immediate products. A. Inhibition of dGMP kinase by dGDP. Increasing (0 - 0.4 mM) amounts of dGDP were added to reaction contained 0.1 mM [\(^{3}\)H]-dGMP/2.5 mM dGTP (○) and 2.5 mM dGMP/0.1 mM [\(^{3}\)H]-dGTP (●). Reactions were carried out at 37 °C for 1 min. Activity was determined by measuring the amount of [\(^{3}\)H]-dGDP formed and is presented as a percentage of that obtained with no added exogenous dGDP. B. Inhibition of dTMP kinase by dTDP. An identical experiment procedure in A was performed except that deoxyguanosine substrates were replaced by corresponding thymidine substrates. (△) Reactions contained 0.1 mM [\(^{3}\)H]-dTDP/2.5 mM dTTP and (▲) 2.5 mM dTMP/0.1 mM [\(^{3}\)H]-dTTP.

FIGURE 9. Type of inhibition by dGDP and dTDP. A and B: Lineweaver-Burk (20) plot of reciprocal of the initial velocity (nmoles/50ng/min) of kinase reaction versus the reciprocal of substrate concentration (mM) in the presence of inhibitor. A. Reactions (20 µl) contained 5 mM dGTP, various concentrations of [\(^{3}\)H]-dGMP and in the presence of 0 (○), 0.2 (▲) and 0.4 (●) mM of dGDP. B. Reactions mixture (20 µl) contained 5 mM dTTP, various concentration of [\(^{3}\)H]-dTMP in the presence of 0 (○), 0.2 (▲) and 0.4 (●) mM of dTDP. Kinase activities were determined by amount of [\(^{3}\)H]-dGDP and [\(^{3}\)H]-dTDP formed as described in “Experimental Procedures”.

FIGURE 10. Dependence of nucleoside triphosphate donor concentration on the initial rate. The reaction mixtures (20 µl) contained 0.25 mM of [\(^{3}\)H]-dTMP or [\(^{3}\)H]-dGMP, the indicated amount of dTTP.
and dGTP, respectively and 50 ng of gp1.7. The reaction was incubated at 37 °C for 1 min. The amount of [³H]-dTDP (●) and [³H]-dGDP (○) formed was measured by TLC as described in “Experimental Procedures”. Km was determined using GraphPad Prism 5.03 (GraphPad Software, Inc.). Inset, comparison of initial rates at low concentration of phosphate donor. These data are taken from phosphate donors < 0.2 mM shown within the boxes.

**TABLE 1**

**Metal content of T7 gene 1.7 protein**

The metal content of three preparations of gp1.7 that were purified using serial salting in/out (2) were analyzed using ICP-MS based on the procedure previously described (15). Wild type gp1.7 contains a mixture of the 22-kDa and 18-kDa forms.

| GP1.7 species | [Mg²⁺] / [Protein] (mole/mole) | [Zn²⁺] / [Protein] (mole/mole) | [Cu²⁺] / [Protein] (mole/mole) | [Ni²⁺] / [Protein] (mole/mole) |
|---------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| wild type     | 0.03                            | 0.2                             | 0.01                            | <0.001                          |
| 22-kDa        | 0.03                            | 0.3                             | 0.005                           | <0.001                          |
| 18-kDa        | 0.03                            | 0.04                            | 0.01                            | <0.001                          |
TABLE 2
Phosphate acceptor specificity and the effect of Mg$^{2+}$
Various nucleoside monophosphates were examined for their ability to accept phosphate from dTTP. Standard reaction mixtures (20 µl) containing 2.5 mM $[^{3}H]$-dTTP, 0.1 mM of the potential phosphate acceptors and 50 ng of gp1.7 were incubated at 37 °C for 3 min. Assays were performed in the presence or absence of 10 mM Mg$^{2+}$. Activity was determined by measuring the conversion of $[^{3}H]$-dTTP to $[^{3}H]$-dTDP by Pei Cellulose TLC as described in “Experimental Procedures”.

| Acceptor | Specific activity (pmols/ 50ng/ min) |
|----------|-----------------------------------|
|          | - Mg$^{2+}$ | + Mg$^{2+}$ |
| dAMP     | <1          | <1          |
| dCMP     | <1          | <1          |
| dGMP     | 1,037       | 629         |
| dTMP     | 440         | 713         |
| dUMP     | 45          | 8           |
| ddTMP    | 530         | 677         |
| AMP      | <1          | <1          |
| CMP      | <4          | <7          |
| GMP      | <2          | <2          |
| UMP      | <1          | <1          |
| AZT      | <4          | <1          |
| dT       | <1          | <1          |
| ddT      | <1          | <1          |

TABLE 3
Phosphate donor specificity and the effect of Mg$^{2+}$
Various nucleoside triphosphates were examined for their ability to donate phosphate to dTMP and dGMP. Reaction mixtures (20 µl) containing 0.2 mM (4,000 pmols) of either $[^{3}H]$-dTMP or $[^{3}H]$-dGMP, 5 mM of the indicated nucleoside triphosphates and 50 ng of gp1.7 were incubated at 37 °C for 3 min, in the presence or absence of 10 mM Mg$^{2+}$. Activity was determined by measuring the amount of $[^{3}H]$-dGDP and $[^{3}H]$-dTDP formed as described in “Experimental Procedures”.

| Donor   | $[^{3}H]$-dGMP $\rightarrow$ $[^{3}H]$-dGDP (pmol/50 ng/ min) | $[^{3}H]$-dTMP $\rightarrow$ $[^{3}H]$-dTDP (pmol/50 ng/ min) |
|---------|---------------------------------------------------------------|---------------------------------------------------------------|
|         | - Mg$^{2+}$ | + Mg$^{2+}$ | - Mg$^{2+}$ | + Mg$^{2+}$ |
| ATP     | -           | -           | -           | -           |
| CTP     | 13          | 9.5         | 12.1        | 10.2        |
| GTP     | 277         | 165         | 169.2       | 220.3       |
| UTP     | 5.5         | 7.4         | <1          | <1          |
| dATP    | <1          | <1          | <1          | <1          |
| dCTP    | 2.3         | 2.1         | <1          | <1          |
| dGTP    | 544         | 906         | 243         | 843         |
| dTMP    | 974         | 584         | 545         | 710         |
| dUTP    | 170         | 70          | 81          | 69          |
| ddTTP   | 901         | 574         | 292         | 439         |
| ddGTP   | 17          | 507         | 14          | 286         |
| ddTTP   | 208         | 496         | 55          | 290         |
TABLE 4
Stoichiometry of gp1.7 kinase reactions with dTMP and dGMP substrates
Two set of reaction mixtures (200 µl), one containing 0.1 mM [3H]-dTMP/ 0.1 mM dTTP, and the other containing 0.1 mM dTMP/ 0.1 mM [3H]-dTTP, were prepared for analysis of stoichiometry of dTMP kinase reactions (i). Each reaction mixture contained 500 ng of gp1.7. The analogous experiments were carried out for dGMP kinase reactions (ii). Experiments were performed at 37 °C. The final amount of nucleoside mono-, di-, and triphosphate found in the reactions was a sum of each [3H]-labeled nucleotides found in parallel reactions over the time course. The data shown in the Table are the pmols of each component found in a 20 µl aliquot of the reaction mixtures after incubating for 120 min, an amount of time sufficient for the reaction to reach equilibrium.

| Experiments | At zero time | At equilibrium |
|-------------|--------------|----------------|
|             | pmoles       | pmoles         |
| i) 0.1 mM dTMP, 0.1 mM dTTP |              |                |
| dTMP        | 2,000        | 1,600          |
| dTDP        | 0            | 830            |
| dTTP        | 2,000        | 1,500          |
| ii) 0.1 mM dGMP, 0.1 mM dGTP |              |                |
| dGMP        | 2,000        | 1,010          |
| dGDP        | 0            | 1,900          |
| dGTP        | 2,000        | 980            |

TABLE 5
Reversibility of dTMP and dGMP kinase reactions
The standard kinase reaction mixtures (200 µl) containing either 0.1 mM [3H]-dTDP (i) or 0.1 mM [3H]-dGDP (ii) and 500 ng of gp1.7 were carried out at 37 °C. The change in [3H]-labeled nucleotides were determined by Pei Cellulose TLC. Results in the Table are the component found in 20 µl aliquot after 180 min incubation period when reactions reached equilibrium.

| Reactions | At zero time | At equilibrium |
|-----------|--------------|----------------|
|           | pmoles       | pmoles         |
| i) 0.1 mM [3H]-dTDP |              |                |
| [3H]-dTMP  | 0            | 365            |
| [3H]-dTDP  | 2,000        | 1,200          |
| [3H]-dTTP  | 0            | 350            |
| ii) 0.1 mM [3H]-dGDP |              |                |
| [3H]-dGMP  | 0            | 470            |
| [3H]-dGDP  | 2,000        | 980            |
| [3H]-dGTP  | 0            | 465            |
Figure 1

Panel A: SDS-PAGE gel showing protein bands of different molecular weights under various conditions.

Panel B: Graph showing the dTDP production over time. The graph includes two sets of data: one for a 22-kDa protein and another for an 18-kDa protein. The data points are marked with error bars.
Figure 2

A. pH dependence of dTDP activity.

B. Temperature dependence of enzyme activity.

C. Effect of NaCl and KCl on enzyme activity.

D. Effect of NaCl and KCl on dTDP activity.
Figure 3

A

- Apoferritin (669 kDa)
- Thyroglobulin (449 kDa)
- β-amylase (200 kDa)
- Alcohol dehydrogenase (150 kDa)
- Albumin (66 kDa)
- Anhydrase (29 kDa)

B

Scale: 20 nm
Figure 4

![Graph showing initial rate (pmole/min) vs. Mg²⁺ (mM) for different nucleotides: dGMP, dGTP, dTMP, and dTTP.](image-url)
Figure 5

Panel A: Graph showing the relationship between $1/V$ and $1/d$TMP (mM). The graph includes three curves for different concentrations of dGMP: 0 mM, 0.15 mM, and 0.3 mM.

Panel B: Graph showing the relationship between $1/V$ and $1/d$TTP (mM). The graph includes two curves for different concentrations of dGTP: 0 mM and 0.15 mM.
Figure 6

(A) Available [3H]-dTMP

[3H]-dTDP + [3H]-dTTP

[3H]-dTDP

[3H]-dTTP

Time (min)

pmoles

(B) Available [3H]-dGMP

[3H]-dGDP + [3H]-dGTP

[3H]-dGDP

[3H]-dGTP

Time (min)
Figure 7
Figure 8
Figure 9
Figure 10

![Graph showing initial rate (pmole/min) vs. dTTP or dGTP (mM) for dGTP/dGMP and dTTP/dTMP.](image)
Characterization of a Nucleotide Kinase Encoded by Bacteriophage T7
Ngoc Q. Tran, Stanley Tabor, Chitra J. Amarasiriwardena, Arkadiusz W. Kulczyk and Charles C. Richardson

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