T4 DNA POLYMERASE HAS A LOWER APPARENT K_M FOR DEOXYNUCLEOSIDE TRIPHOSPHATES COMPLEMENTARY RATHER THAN NONCOMPLEMENTARY TO THE TEMPLATE

Frances D. Gillin* and Nancy G. Nossal

Laboratory of Biochemical Pharmacology
National Institute of Arthritis, Metabolism, and Digestive Diseases
National Institutes of Health, Bethesda, Maryland 20014

Received March 19,1975

SUMMARY: The apparent Michaelis constants (K_m) of the T4 phage-induced DNA polymerase for the complementary nucleotides, dATP (17 μM) and dTTP (6 μM), are much lower than those obtained with the noncomplementary nucleotides, dGTP (190 μM) or dCTP (1,600 μM) with the homopolymers poly dA · poly dT as template-primer. In control experiments with denatured salmon sperm DNA as a template-primer, the K_m values determined separately for each of the 4 dNTP were nearly identical (1.3 μM to 1.9 μM).

DNA polymerases copy DNA templates with a high degree of accuracy, but the means by which these enzymes achieve this specificity is not yet well understood. On the basis of studies suggesting that *Escherichia coli* polymerase I has a single site to which each of the 4 deoxynucleoside triphosphates (dNTP) binds, Kornberg (1) proposed a model in which the polymerases recognize correct Watson-Crick base pairing between incoming dNTP and template, rather than recognizing individual nucleotides. Genetic experiments led Freese and Freese to a similar model (2). Models in which the polymerase plays a more active role in base selection have been reviewed by Drake (3). In addition to mechanisms promoting specificity at the time a nucleotide is incorporated, accuracy is increased by the ability of the 3' to 5' exonuclease associated with many polymerases to remove mismatched residues (4-8).

We have begun to approach the problem of the interaction of the polymerase, DNA template, and nucleoside triphosphates by asking whether the kinetic constants for each deoxynucleoside triphosphate (dNTP) are different when the nucleotide is complementary as opposed to noncomplementary to the template employed. Equilibrium dialysis studies by Englund *et al.* (9) established that

* Recipient of a Special Fellowship 5F03 AM55286-02 from the National Institutes of Health.

the dissociation constants for each of the 4 dNTP for *E. coli* DNA polymerase I were similar in the absence of DNA and indicated that the 4 dNTP compete for a single binding site on the enzyme. In addition, Englund, Kelly, and Kornberg (10) have shown that this enzyme binds to DNA in the absence of dNTP or Mg^{2+}. Although Englund *et al.* (9) predicted that the presence of template might confer greater specificity upon the interaction of dNTP with enzyme, the equilibrium dialysis studies could not be carried out in the presence of DNA and Mg^{2+} since under these conditions the substrates would be consumed (1, 9).

The studies reported in this paper were carried out with the wild-type phage T4 DNA polymerase using the homopolymer pair, poly dA · poly dT, as the template-primer. Hershfield and Nossal (6) have shown that with this homopolymer pair, the T4 DNA polymerase catalyzes the incorporation of the complementary nucleotides dATP and dTTP into polymer, as well as the hydrolysis of newly incorporated dAMP and dTMP residues to free dAMP or dTMP by the 3' to 5' exonuclease activity of the enzyme ("turnover"). They have also described the poly dA · poly dT-dependent conversion of the noncomplementary dGTP and dCTP to free dGMP and dCMP by T4 DNA polymerase (6, 11). This is probably the net result of transient incorporation into polymer and rapid removal of the noncomplementary residues which have been shown to be especially susceptible to the exonuclease activity of T4 polymerase (4-8). However, the possibility that the noncomplementary dNTP are hydrolyzed to dNMP by the polymerase without being covalently attached to the DNA primer has not been excluded. For comparison, the apparent K_m's for each of the 4 dNTP with a complementary template have also been determined, using alkali-denatured salmon sperm DNA.

MATERIALS AND METHODS

DNA Polymerase Assay. All DNA polymerase assay mixtures contained 5 mM NH_4HCO_3, pH 8.8, 0.1 mg per ml bovine serum albumin, 10 mM mercaptoethanol, 6.7 μM EDTA (6), and 3 mM $MgCl_2$, in a final volume of 15 μl. The poly dA and poly dT (Miles Laboratories) used as template-primer in the first series of experiments were each heated to 65° and quickly cooled to 0° in order to disrupt intrastrand hydrogen bonding. Each polymer was added to the reaction mixture at a final concentration of 0.3 mM nucleotide equivalents. The poly

dT was described by Miles to have an average sedimentation coefficient of 4.7S while that of the poly dA was 3S. End group analysis showed an average chain length of 400 for the poly dA.[1] Each reaction mixture contained the specified radioactive dNTP at concentrations which were varied from 3 μM to 550 μM and specific activities of 100 to 1,000 cpm/pmol. No other dNTP were present. [^{14}C]dATP, [^{32}P]dTTP, [^{32}P]dGTP, and [^{32}P]dCTP, from New England Nuclear Corporation and unlabeled dNTP from various commercial sources were purified if necessary (12). The reaction mixtures were warmed to 30° prior to addition of T4 DNA polymerase at a final concentration of 2 μg/ml with the complementary dATP or dTTP, or 2.9 μg/ml with the noncomplementary dGTP or dCTP. The T4 DNA polymerase used throughout this study is a homogeneous enzyme induced by the wild-type phage, whose preparation has been described (13, 14). Samples of 5 μl were taken after 5 minutes and 10 minutes for the complementary dNTP or after 20 minutes and 40 minutes for the noncomplementary dNTP, applied to PEI cellulose thin-layer plates (Brinkmann, Inc.), and chromatographed in order to separate the dNTP stably incorporated into polymer from that converted to free deoxynucleoside monophosphate and from unreacted dNTP (11).

DNA polymerase assays which utilized denatured sperm DNA (15) at a final concentration of 0.3 mM in nucleotide equivalents, as template-primer, contained three unlabeled dNTP, 100 μM each, and one ^{32}P labeled dNTP, whose concentration was varied from 1 μM to 150 μM, in addition to the components listed above. T4 DNA polymerase (1.5 μg/ml, final concentration) was added to the prewarmed reaction mixtures, and incubation at 30° was continued for 3½ minutes. Duplicate 5 μl samples were then applied to trichloroacetic acid impregnated GF/C filters, which were washed as described (14). The filter assay for acid insoluble product could be used with this template since less than 5% of the dNTP used is subsequently converted to dNMP.

The concentration of total Mg^{2+} was kept constant in both series of experiments and was optimal for the polymerase under these conditions. It was estimated from the association constant of Mg^{2+} and ATP (16) that approximately 90% of the dNTP existed as the dNTP · Mg^{2-} complex at all concentrations of dNTP and template-primer used. All determinations were made while the reactions were linear with time.

RESULTS AND DISCUSSION

The initial rates of incorporation and turnover of each deoxynucleoside triphosphate by T4 DNA polymerase with poly dA · poly dT as a function of nucleotide concentration are shown in Fig. 1. At the poly dA primer sites, dATP is turned over approximately 3 times as rapidly as it is stably incorporated, while 80% of the dTTP incorporated on the opposite strands remains in polymer. This difference between the incorporation patterns of the 2 complementary nucleotides may be due to differences in the length and distribution of gaps where each nucleotide can be incorporated with this homopolymer pair. Turnover is favored when the enzyme approaches the end of the template

[1] Gillin, F. D., and Nossal, N. G., manuscript in preparation.

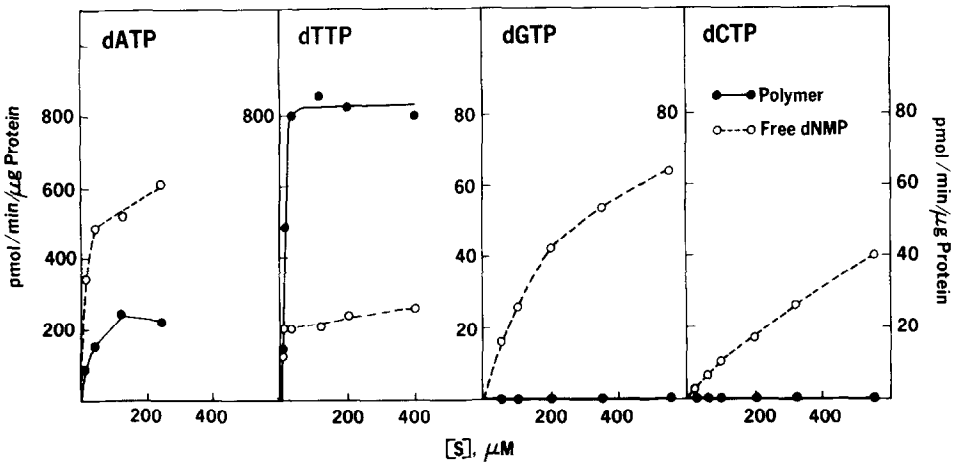

FIG. 1. *Initial rate of utilization of of a single deoxyribonucleoside triphosphate by phage T4 DNA polymerase with poly dA · poly dT as template-primer as a function of nucleotide concentration.* The solid lines and circles represent nucleotide stably incorporated into polymer. The dashed lines and open circles represent DNA-dependent conversion of deoxynucleoside triphosphate to free deoxynucleoside monophosphate. The DNA polymerase assay is described under *Materials and Methods*.

available for copying (17). These 2 complementary nucleotides are incorporated and turned over by the polymerase at equivalent rates with the alternating polymer, poly [d(A-T)], as the template-primer, where the primer sites for the 2 nucleotides are likely to be equivalent (data not shown).

The noncomplementary nucleotides dGTP and dCTP are converted to deoxynucleoside monophosphates by the polymerase, but stable incorporation is not observed with the specific radioactivities used in these studies (Fig. 1 and reference 11). The rate of formation of dGMP or dCMP approximates the total rate of utilization of the noncomplementary nucleotides and is not limited by the rate at which the polymerase can hydrolyze noncomplementary residues. In separate experiments, with poly dT plus poly dA containing a dG residue at the 3' terminus, the rate of removal of the terminal dGMP was 5 times more rapid than the rate of turnover of dGTP with poly dA · poly dT, when both reactions were measured under the conditions given in Fig. 1.[1] The addition of an equal concentration of dATP inhibits the utilization of dGTP or dCTP by 75%

TABLE 1: *Apparent K_m for Each Deoxynucleoside Triphosphate*

	Template-Primer	
	Poly dA · Poly dT [a]	Denatured Sperm DNA
	apparent K_m (μM)	
dATP	17 (± 3)	1.3 (± 0.3)
dTTP	6 (± 1)	1.8 (± 0.2)
dGTP	190 (± 10)	1.3 (± 0.5)
dCTP	1,600 (± 400)	1.9 (± 0.3)
	V_{max} (pmol/min/μg protein) [b]	
dATP	800 (± 60)	50 (± 0.1)
dTTP	1,120 (± 44)	54 (± 0.1)
dGTP	80 (± 2)	60 (± 0.4)
dCTP	160 (± 32)	60 (± 0.2)

The kinetic constants K_m and V_{max} were determined by fitting data from DNA polymerase assays to Equation 1 by means of an interactive curve fitting program, MLAB, developed at the National Institutes of Health, and running on a PDP-10 digital computer (18). S is the initial substrate concentration and v is the initial velocity.

$$v = \frac{V_{max}\, S}{K_m + S} \qquad \text{(Equation 1)}$$

[a] With poly dA · poly dT as template-primer, dATP and dTTP are each both stably incorporated into polymer and turned over to free deoxyribonucleoside monophosphate (Fig. 1). The kinetic constants in this table were each calculated from the sum of incorporation and turnover reactions. The constants, calculated for turnover and stable incorporation separately, are shown in Table 2.

[b] The maximal velocity refers to the rate of utilization of the single specified dNTP. Where sperm DNA was the template-primer, the other 3 appropriate cold dNTP were also present. Therefore, the actual V_{max} of the total reaction would be the sum of the V_{max} values shown for the 4 individual dNTP.

TABLE 2: *Apparent K_m for Stable Incorporation or Turnover of dATP or dTTP with Poly dA · Poly dT*

	Stable Incorporation	Turnover
	apparent K_m (μM)	
dATP	34 (± 4)	12 (± 2)
dTTP	8 (± 3)	2 (± 1)
	V_{max} (pmol/min/μg protein)	
dATP	280 (± 10)	620 (± 26)
dTTP	890 (± 59)	240 (± 12)

Kinetic constants were determined by computer (as in Table 1) from data shown in Fig. 1.

to 100%, while addition of the same amount of dTTP does not inhibit at all. This specificity of competition is consistent with the idea that both dGTP and dCTP may be utilized instead of dATP at the 3' OH terminus of the poly dA strand under the conditions employed.

Fig. 1 shows that both incorporation and turnover of dATP and dTTP are saturated at lower substrate concentrations than are necessary for saturation of turnover of dGTP or dCTP. The apparent Michaelis constants (K_m) calculated for the total utilization (stable incorporation plus turnover, Table 1) or calculated separately for turnover and stable incorporation of dATP and dTTP (Table 2) are strikingly lower than the apparent K_m for utilization of dGTP or dCTP (Table 1). The maximal velocities (V_{max}) of the reactions with the complementary dNTP are greater than for the turnover of the noncomplementary dNTP (Tables 1 and 2). It is interesting that although the V_{max} for dCTP is 2-fold greater than that of dGTP (Table 1), the apparent K_m for dCTP is approximately 8 times greater than that of dGTP.

The apparent Michaelis constants of the 4 nucleotides were also compared using a DNA template-primer to which all are complementary. In separate experiments, the concentration of a single radioactive dNTP was varied in the presence of an excess of the appropriate 3 nonradioactive dNTP, using denatured salmon sperm DNA as the template. This insured that the rate of the reaction being measured with constant enzyme concentration was determined by the availability of a single dNTP. The apparent Michaelis constants and the maximal velocities computed for each of the 4 dNTP are nearly identical (Table 1).

We have also determined the apparent K_m of T4 DNA polymerase for poly dA · poly dT in its reaction with excess dATP or dGTP (data not shown). In contrast to the present study, the differences observed in the K_m for polymer in the reaction with the complementary versus noncomplementary nucleotide are small: 5 ± 2 μM and 12 ± 4 μM nucleotide equivalents, respectively.

The kinetic experiments reported here are only a first step in assessing the role of the polymerase enzyme in achieving fidelity of replication. They indicate that T4 DNA polymerase interacts with the template molecule in such a way that reactions with correct or complementary dNTP are saturated at much lower substrate concentrations than are necessary for saturation of reactions with wrong or noncomplementary dNTP.

ACKNOWLEDGMENT

We would like to thank Dr. Claude B. Klee for helpful discussions.

REFERENCES

1. Kornberg, A. (1969) *Science*, 163, 1410-1418.
2. Freese, E. B., and Freese, E. (1967) *Proc. Nat. Acad. Sci. USA*, 57, 650-657.
3. Drake, J. W. (1970) *The Molecular Basis of Mutation*, Holden-Day, Inc., San Francisco, California.
4. Goulian, M., Lucas, Z. J., and Kornberg, A. (1968) *J. Biol. Chem.*, 243, 627-638.
5. Englund, P. T. (1971) *J. Biol. Chem.*, 246, 3269-3276.
6. Hershfield, M. S., and Nossal, N. G. (1972) *J. Biol. Chem.*, 247, 3393-3404.
7. Brutlag, D., and Kornberg, A. (1972) *J. Biol. Chem.*, 247, 241-248.
8. Muzyczka, N., Poland, R. L., and Bessman, M. J. (1972) *J. Biol. Chem.*, 247, 7116-7122.

9. Englund, P. T., Huberman, J. A., Jovin, T. M., and Kornberg, A. (1969) *J. Biol. Chem.*, 244, 3038-3044.
10. Englund, P. T., Kelly, R. B., and Kornberg, A. (1969) *J. Biol. Chem.*, 244, 3045-3052.
11. Hershfield, M. S. (1973) *J. Biol. Chem.*, 248, 1417-1423.
12. Smith M., and Khorana, H. G. (1963) *Methods Enzymol.*, 6, 645-669.
13. Nossal, N. G., and Hershfield, M. S. (1971) *J. Biol. Chem.*, 246, 5414-5426.
14. Nossal, N. G. (1974) *J. Biol. Chem.*, 249, 5668-5676.
15. Studier, F. W. (1969) *J. Mol. Biol.*, 41, 189-197.
16. Phillips, R. (1966) *Chem. Rev.*, 66, 501-527.
17. Nossal, N. G., and Hershfield, M. S. (1973) *in* Wells, R. D., and Inman, R. B. (eds.): *DNA Synthesis in Vitro*, pp. 47-62, University Park Press, Baltimore, Maryland.
18. Knott, G. D., and Reece, D. K. (1972) in *Proceedings of the ONLINE '72 International Conference*, Vol. 1, pp. 497-520, Brunel University, England.