Mechanism of Primer-Template-dependent Conversion of dNTP →
dNMP by T5 DNA Polymerase*

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T5 DNA polymerase catalyzes both 5' → 3' polymerization and 3' → 5' hydrolysis in a processive fashion. This knowledge has been utilized to obtain evidence indicating that the enzyme has a single primer-template binding site which can function as either polymerase or exonuclease, perhaps with the cooperation of additional or different side groups. Template-dependent conversion of dNTP → dNMP was observed with an excess of either primer-template or enzyme. With primer-template excess, practically all the enzymes were functional as polymerase; with enzyme excess, all primer-template were extended during the first cycle of catalysis. These observations suggest that turnover takes place at the points of chain growth. Evidence is also provided which demonstrates that the enzyme is capable of switching its direction of catalysis from 3' → 5' to 5' → 3' without leaving the primer-template. A clear correspondence between the relative amount of hydrolysis of a terminally labeled residue on the primer and the relative amount of turnover suggests that (a) the probability of hydrolysis of a given type of residue in contact with the "active site" is constant, and (b) during each turnover episode enzyme usually takes only one step in the 3' → 5' direction. A simple probabilistic model of turnover is discussed.

Several prokaryotic DNA polymerases have 3' → 5' exonuclease activity and "turnover" activity associated with them (1–5). Turnover here denotes the primer-template-dependent conversion of newly incorporated dNTPs to dNMPs during DNA replication. Such turnover activity is assumed to be due to the polymerase-associated 3' → 5' exonuclease (1, 6, 7). Several experimental data suggest that the turnover process plays a key role in fidelity of replication (1, 3, 8, 9). Although eukaryotic DNA polymerases seem, in general, to be devoid of such activity, it has been hypothesized that other proteins such as DNase III might perform this task in a similar manner (10, 11).

The laboratories of Bessman and Nossal have studied extensively the properties of turnover activities of various mutant and wild type T4 DNA polymerases (8, 9, 12–16). Several of these enzymes show correlated in vivo/in vitro properties. More specifically, in several instances, mutant polymerases which have a high turnover activity also show the anti-mutator phenotype and vice versa (9, 12, 17). These elegant studies have led recently to several proposals for mechanisms of base selection by DNA polymerases possessing 3' → 5' exonuclease activity (14, 18–20). However, one of the key assumptions for which there is no experimental evidence is that turnover and chain growth take place at the same primer end by a single enzyme. The idea of proofreading postulates that DNA polymerases choose dNTPs for DNA synthesis on the basis of hydrogen-bonding properties of complementary bases during the first step of selection; after the phosphodiester bond formation, enzyme enhances its selectivity by virtue of its ability to hydrolyze the incorporated residue. Whether the same polymerase itself can carry out a second step of discrimination by rejecting the incorporated residue after the first Michelian selection of dNTPs is open to question. As Kornberg (21) has emphasized, replicative errors should be removed during the process for a substantial reduction in the error load. Although it has been pointed out recently that there may be special biological mechanisms for the recognition of daughter strand vis à vis parental strand enabling error correction after replication, the fact remains that mutant polymerases can greatly influence the in vivo mutation rates (22–25).

We would like to point out specifically situations which can give rise to turnover unrelated to the point of chain growth. For example, some of the enzyme molecules might be defective and incapable of carrying out net replication, although they can carry out turnover. Gillin and Nossal (16) have described a mutant T4 DNA polymerase presumably defective in translocation subsequent to phosphodiester bond formation which shows similar properties. This mutant enzyme converts a sizable fraction of dNTPs to dNMPs during DNA replication in vitro. Such enzyme preparations may have a much larger fraction of enzyme molecules which are not capable of net primer elongation. It is widely recognized that minor structural differences may give rise to protein molecules which are drastically altered in their properties. A good case in point is the family of chymotrypsinogen proteins, in which the protein molecules assume several different conformations under similar conditions (26). Yeast hexokinase has also been shown to exist in multiple conformational states (27, 28). Since most DNA polymerases are rather large molecular weight proteins (~100,000 range), a greater caution in the interpretation of the data under consideration is called for.

Primer-template configurations which do not permit chain elongation may still cause turnover unrelated to the points of replication. This situation could arise due to a block in the template, for instance, at a 6-methyladenine or apurinic/apyrimidinic site (29, 30). Such a block may cause enzyme "idling," and enzyme may simply stay at one place and continuously produce dNMPs. If the size of a replicable region is small,
such as near the end of a single-stranded template or a short gap in the DNA, turnover might simply be caused by the inability of the enzyme to carry out further replication dt, 32). It has been demonstrated by Englund (7) that T4 polymerase can carry out turnover under conditions at which substantial net replication does not occur. With T5 DNA polymerase, we, too, have observed turnover without net DNA synthesis. 1 Successive utilization of a given primer-template by 2 enzyme molecules which act as polymerase or exonuclease can also produce free dNMPs by the hydrolysis of newly synthesized DNA. This problem is particularly important with enzymes which are nonprocessive (33).

With these possibilities in mind, we sought experimental evidence to determine whether turnover is indeed "spatio-temporally" co-extensive with replication. For these experiments, several properties of T5 DNA polymerase were used to advantage. We found that T5 DNA polymerase is most processive in chain elongation process among the polymerases we have tested (33). Its exonuclease is also processive (34). In the absence of Mg2+, T5 DNA polymerase preferentially forms a complex with 3'-OH terminus of a primer template and it can act as either polymerase or exonuclease depending on the conditions (this report). From our results, we conclude that turnover and elongation at a 3'-OH terminus of a primer can be catalyzed by the same enzyme.

**EXPERIMENTAL PROCEDURES**

**Materials**—T5-induced DNA polymerase was purified to homogeneity by the method of Fujimura and Roop (36). This homogeneous enzyme preparation is devoid of any detectable amount of endonuclease or 5'→3' exonuclease activity when assayed with [3H]PM2 DNA and [5'32P]poly(dA), respectively. Poly(dA)zz, oligo(dT)a0.0,[3H](dT)z, and oligo(dT)a0.0,[3H](d'T)z were synthesized according to the procedure of Bollum (37) with oligo(dA)a0.5, oligo(dT)a0.0, and oligo(dT)a0.0 as initiators. The specific activity of dTTP in the reaction mixture was 1000 to 2000 cpm/pmol. The concentrations of pyrimidine nucleotides and oligonucleotides were determined from published extinction coefficients (38). Poly(dA)a5 was characterized by the polyonucleotide kinase method of Richardson and by alkaline sucrose density gradient analysis (39, 40). Oligo(dT)a0.0,[3H](dT)z was characterized by the micrococal nuclease-spleen phosphodiesterase method described by us earlier (33). This analysis showed that >85% of the radioactivity was terminal. [5'→3'P]poly(dA)a5 was synthesized as described by Raue et al. (41) by use of [γ→32P]ATP (specific activity, ~1000 cpm/ pmol).

[3H]dTTP (Schwarz/Mann) was purified according to the procedure of Smith and Khorana (42) to reduce the background dTTP level, which varied from one lot to another and was as high as 15% in some preparations. This was always necessary for measurement of turnover during very brief incubations.

**Polymerase Assay**—Each 0.3 ml of the reaction mixture for polymerase assay contained the following: 33 μM dTTP, Tris-HCl (67 mm, pH 8.6), 17 mm dithiothreitol, and the indicated amounts of poly(dA)-[3H]oligo(dT). The enzyme was diluted in 0.02 M Tris-HCl (pH 8.6) containing 0.1% bovine serum albumin, 2 nm dithiothreitol, and 20% glycerol. The reaction was initiated by addition of Mg2+ (final concentration, 6.6 mM) at a stated temperature. The reaction was terminated by the addition of 2 ml of ice-cold (5%) trichloroacetic acid containing 0.01 M Na3PO4; 5 μg of denatured calf thymus DNA were added as carrier. The acid-insoluble material was collected on a Whatman glass-fiber filter (GF/C), washed twice with 2 ml of ice-cold 5% trichloroacetic acid with 0.01 M Na3PO4 and four times with 5 ml of ice-cold (0.1 N HCl, and dried. The radioactivity was counted in a toluene scintillator (4 g of 2,5-bis[5'-2-(5-phenylbenzoxazolyl)]thiophene liter of toluene). When annealed to poly(dA)6, in the presence of calf thymus DNA, to the incubation mixture. For polymerization carried out without calf thymus DNA, the rate-limiting step during the polymerization reaction is shown (39). Although catalytically silent, this complex is well poised for action both as polymerase and as exonuclease when the required substrates and/or cofactors are supplied (34, 46). This observation indicated to us that a common intermediate might function as exonuclease or polymerase depending on the conditions. To determine whether this is true, we formed such an intermediate by preincubating 1 pmol of enzyme with 10 pmol of poly(dA)5 oligo(dT)a0.0,[3H](d'T)z. Then for hydrolysis (the first mixture), the reaction was started by addition of Mg2+ and an excess of challenger substrate, denatured calf thymus DNA, to the incubation mixture. For polymerization (the second mixture), the above ingredients and dTTP (33 μM) were added. These conditions allow one round of processive hydrolysis or synthesis of oligo(dT), respectively.

**Results**—The results were as expected (Fig. 1). In the first mixture, the enzyme in complex with poly(dA)-oligo(dT) functioned as exonuclease and 1 pmol of dTTP was released. Excess calf thymus DNA effectively prevented further hydrolysis of other oligo(dT). In the second mixture, the enzyme in the complex functioned as polymerase, and 1 pmol of dTTP became acid-insoluble due to chain elongation of these primers. Again, calf thymus DNA prevented elongation of other primers. The second phase of the hydrolytic reaction is slower because it includes the hydrolysis of unlabeled part of the substrate, and the rate-limiting step during the polymerization reaction is the release of enzyme at the end of the template, as shown previously (46). It was noted that one set of controls, denatured calf thymus DNA was added prior to addition of poly(dA)-oligo(dT). Thus, enzymes were in complex with nonradioactive substrate, and no action on [3H]oligo(dT) was observed. It seems likely that a common "enzyme-primer-template com-
Fig. 1. Hydrolysis (exonuclease) and trichloroacetic acid (TCA) insolubilization (polymerase) of labeled dTMP residues of poly(dA)-oligo(dT)·[14C]dTMP (1:1, 3' end basis) by T5 DNA polymerase under conditions for polymerase and exonuclease reactions described under "Experimental Procedures." Ten picomoles of primer and 1.0 pmol of enzyme were used for each time point. Enzyme was preincubated with the labeled substrate for 5 min without Mg++. Reaction was started by the addition of Mg++. (final concentration, 6.6 mM) (□, □) or Mg++ and 20 µg of denatured calf thymus DNA (○, ●). Calf thymus DNA was added before poly(dA)-oligo(dT) (□, □) and Mg++ was added at 0 min. Circles and open triangle show exonuclease reactions, and squares and filled triangle show polymerase reactions. In the case of polymerase assay, dTTP (33 µM) was also added with Mg++ at 0 min. Temperature of incubation was 18°C. For the polymerization, the reaction was stopped with acid. Amounts of primer-oligo(dT)·[14C]dTMP acid-insolubilized indicate chain elongation. For hydrolysis, the reaction was stopped and spotted on DE81. Loss of oligo(dT)·[14C]dTMP adsorbable indicates hydrolysis to dTMP.

Fig. 2. Titration of exonuclease and polymerase activities obtained at various enzyme concentrations. Poly(dA)·oligo(dT)·[14C]dTMP concentration was 25 nM. Reactions were carried out as in Fig. 1, with Mg++, denatured calf thymus DNA (20 µg) and dTTP (33 µM), only for polymerase reaction being added at 0 min. The reaction was allowed to proceed for 5 min. The data were fitted to the following equation for the computation of $K_{dissoc}$, the dissociation constant of enzyme-primer-template complex (see "Appendix" for derivation):

$$
[ES] = -\sqrt{0.25(K_{dissoc} + [S_{total}] + [E_{total}])^2 - [E_{total}][S_{total}]} + 0.5(K_{dissoc} + [S_{total}] + [E_{total}])
$$

[ES], [S_{total}], and [E_{total}] are the concentrations of the complex, primer-template, and enzyme. The dashed line shows a theoretical curve with $K_{dissoc} = 0$. The solid line is computed with $K_{dissoc} = 10.0$ nM. ○, Exonuclease activity; ●, polymerase activity.

Fig. 3. Kinetics of dissociation of enzyme-primer-template complex by polymerase and exonuclease assay. Preincubation was started as in Fig. 1, except that for each time point, 5 pmol of primer-template and 2 pmol of enzyme was used. At 0 min, calf thymus DNA was added. Thereafter, at indicated times, Mg++ or Mg++ and dTTP were added; reactions were allowed to continue for 5 min. This experiment measures the amount of enzyme remaining complexed with poly(dA)·oligo(dT)·[14C]dTMP at various times after addition of denatured calf thymus DNA. ○, exonuclease activity; ●, polymerase activity.
Proofreading Mechanism

is taken into account, the agreement between the two sets of values becomes even better.

We would like to re-emphasize here that under the conditions chosen for this experiment the values represent only one cycle of processive polymerization or hydrolysis with the primer-template in question as substrate. These results therefore indicate that the complex formed with the primer-template in the absence of Mg\(^{2+}\) is probably the same for both reactions.

Dissociation Kinetics of the Complex—Another piece of evidence which supports our view that for both exonuclease and polymerase functions of the enzyme a common intermediate with the DNA substrate is formed was provided by the study of dissociation kinetics of the complex. Enzyme was preincubated with terminally labeled primer-template. At \( t = 0 \), excess unlabeled denatured calf thymus DNA was added to the reaction mixture. The reaction was started at various times thereafter by the addition of Mg\(^{2+}\) for hydrolysis or Mg\(^{2+}\) and dTTP for polymerization. The amount of primer solubilized or extended was equal to the amount of enzyme to the reaction mixture. The reaction was started at various Mg\(^{2+}\) and dTTP concentrations for polymerization. The amount of primer solubilized or extended was equal to the amount of enzyme which was still bound at the original primer-template. Fig. 3 shows that both assays gave results which showed a monoeponential decay of the complex, with a rate of dissociation of 5.78 × 10\(^{-4}\) s\(^{-1}\).

Turnover with Excess Enzymes or Excess Primer-Tem-

| Enzyme primer | Time | Incorporation | Turnover
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* Average of two separate estimations.

Fig. 5. Mg\(^{2+}\) dependence of turnover and hydrolysis of 3'-terminal residue of the primer. Turnover and hydrolysis of the terminal residue were determined by use of poly(dA)\(\overline{\text{a}}\) oligo(dT)\(\text{a}0\), and poly(dA)\(\overline{\text{a}}\) oligo(dT)\(\text{a}0\)\(\text{3H}\) dT\(\text{T}\), respectively. Conditions for turnover reaction were as described for Table 1, and conditions for hydrolysis were as described for Fig. 1. Temperature of incubation was 22°C. For turnover (C), and hydrolysis (A), times of incubation were 15 s and 2 min, respectively. The results are expressed as percent of incorporation + turnover and (incorporation + hydrolysis) in the two cases. The additional point at 6.6 mM Mg\(^{2+}\) was obtained with T\(\text{t}5\text{ts}53\) polymerase, which shows ts phenotype at 43°C (C, ▲) (49).

Table I
Extent of turnover

For each time point, 1 pmol of poly(dA)\(\overline{\text{a}}\) oligo(dT)\(\text{a}0\) primer-template was used in a reaction volume of 50 µl. Specific activity of [\(\text{3H}\) dTTP was ~3000 cpm/pmol. Amounts of competing calf thymus DNA were as in Fig. 1. In all cases, 5 µl were analyzed for measurement of incorporation and turnover. Temperature of incubation, 22°C; blank for turnover measurement, 40 ± 8.

Turnover—Having obtained this knowledge, we carried out turnover experiments under conditions of enzyme excess or primer-template excess. As is shown in Table I, under both conditions, similar turnover/(incorporation + turnover) ratios were obtained. In these experiments, measurements were made during the linear phase of the reaction. This is particularly important since T5 polymerase shows biphasic kinetics of synthesis due to the formation of a dead-end complex at the 5' end of the template (46). The dead-end complex carried out turnover but has no net incorporation.1

Comparison of Hydrolysis of Terminal Residue, in the Presence of dNTP, and Turnover—Although most of the terminal residues at the 3'-OH end of the primer molecules utilized by the enzyme are incorporated into the product in the presence of dNTPs, a small but significant number are hydrolyzed. The relative amount of hydrolysis remains constant as the reaction proceeds (Fig. 4). When 5'-32P-labeled primers are used, no detectable hydrolysis is observed. The ratio of terminal residues hydrolyzed/(hydrolyzed + incorporated) is close to the turnover/(incorporation + turnover) ratio (compare Table I and Fig. 4). This evidence indicates that every terminal residue in contact with the active site of the enzyme has a finite chance of being hydrolyzed in the presence of dNTPs. In the absence of dNTPs, of course, this value will approach 1.

To determine whether these two ratios remain similar under different reaction conditions, we compared them at various Mg\(^{2+}\) concentrations. As shown in Fig. 5, the turnover/(incorporation + turnover) ratio increased by as much as 2.5-fold when the Mg\(^{2+}\) concentration was dropped from 12.0 to 1.00 mM. The hydrolysis/(incorporation + hydrolysis) ratio also showed a similar Mg\(^{2+}\) dependence. A mutant T5 polymerase (ts53) showed comparatively higher values for both the parameters.

 Interruption of Primer Hydrolysis by Addition of dNTPs—As was shown earlier, when excess enzyme is supplied practically all the primer molecules can be processively hydrolyzed by T5 polymerase. This is true for poly(dA)\(\overline{\text{a}}\) oligo(dT)\(\text{a}0\)\(\text{3H}\) dT\(\text{T}\) substrate also. In a reaction carried out with this
substrate, in the presence of competing polymer, the processive hydrolysis of the substrate could be halted within a minute by the addition of dTTP (Fig. 6A). When the trichloroacetic acid-insoluble fraction was examined, it became clear that the residues which escaped hydrolysis were incorporated into the product (Fig. 6B). This result indicates that the enzyme formed a complex with primer-template which was catalyzing $3' \rightarrow 5'$ hydrolysis could switch its direction and catalyze $5' \rightarrow 3'$ polymerization, without leaving the primer-template, as soon as dNTPs were made available. A simple conclusion which can be drawn from this result is that exonuclease and polymerase active sites are intimately related. We suggest that during turnover the catalytic switch takes place in the other direction.

**DISCUSSION**

A number of papers describing some elegant work have been written on the possible role of turnover in the reduction of replicative errors (1, 3, 8, 9, 12-20). A tacit assumption implied in these discussions, namely, that turnover takes place at the point of chain growth, has not been critically evaluated so far. Subtle variations notwithstanding, the essence of the proofreading idea is that incorporated residues can be rejected by the same enzyme. We therefore sought and obtained evidence indicating that during the early phase of primer elongation by T5 DNA polymerase, the primer-template-dependent conversion of dNTP to dNMP takes place at the point of chain growth. Thus, under conditions at which practically all the enzyme molecules are involved in primer extension, turnover of newly incorporated residue was observed. The turnover/polymerization ratios obtained were quite comparable to the ratio obtained when excess enzyme was supplied. Under the conditions of excess enzyme, most of the primer molecules are processively elongated during one cycle of synthesis. This evidence indicates that the enzyme can reject the newly incorporated residues during chain growth.

The identity of the binding isotherms obtained by measurement of the amount of processive hydrolysis or primer elongation indicates that the enzyme has a single primer-template binding site. This suggestion is further strengthened by the finding of similar dissociation kinetics in these two cases. It seems that the primer-template binding site can function as either exonuclease or polymerase, perhaps employing additional regions and/or active groups in the vicinity during catalysis which may be different in these two situations. Although Englund et al. (50) showed that *Escherichia coli* DNA polymerase I has a single DNA binding site, it is not clear whether this binding was functional.

Hydrolysis of the 3'-OH terminal residue of the primer in the presence of dNTPs was comparable to the relative amount of turnover under similar conditions. Our observation is consistent with the probabilistic explanation for turnover which is proposed by Galas and Branscomb (19). According to this model, during catalysis as polymerase or exonuclease after each step, a common intermediate is formed in a cyclic fashion which functions as exonuclease in the absence of proper dNTPs. In the presence of dNTPs, however, the choice of enzyme in this complex becomes binary. It can act as either exonuclease or polymerase. Thus, in the presence of dNTPs every terminal residue in contact with the active site has a fixed, finite, macroscopic probability of being hydrolyzed. This value is small when the terminal residue is H-bonded in a proper fashion (Watson-Crick sense) (19, 20). When an improper residue is located at the 3'-OH end of the primer, the probability of hydrolysis is $=1.0$. The correspondence between the amount of relative hydrolysis of the terminal residue and relative turnover also indicates that during turnover enzyme takes only one step in the $3' \rightarrow 5'$ direction before switching its direction to $5' \rightarrow 3'$ polymerization again. If the enzyme took on the average $n$ steps in the $3' \rightarrow 5'$ direction during each turnover episode, the fractional amount of turnover would be $=n$ times higher than the relative amount of hydrolysis of the terminal residue. We have shown elsewhere that primer-templates with mismatched residues at the 3' end are extended by T5 enzyme only after the improper terminal residue(s) are removed (34).

The amount of Mg$^{2+}$ added in the incubation mixture seems to have an effect on the possibility of hydrolysis of a terminal residue in the presence of dNTPs. At higher Mg$^{2+}$ concentration, the amount of turnover is reduced, as is the hydrolysis of the terminal residue of the primer in contact with the active site. Although at present we cannot be sure what factors influence the decision of the enzyme to hydrolyze a given residue, structural destabilization of the 3' end comes to mind (1, 20, 21). It is well known that the primer-template complexes used in this study have a higher melting temperature at increased Mg$^{2+}$ concentration (38).

The marked reduction of hydrolysis of terminal residues located in the active site of the enzyme in the presence of dNTPs could be due to the stabilization of the 3'-OH terminal residue by the incoming triphosphate, in addition to the fact that after the polymerization step this residue is no longer terminal (20). Stacking interaction between the 2 residues may be an important stabilizing force. We have obtained evidence indicating that the binding of a triphosphate to the enzyme substantially lowers the rate of hydrolysis. Thus, CTP and GTP are good noncompetitive inhibitors of the hydrolysis of duplex DNA substrates in which these dNTPs can bind to the enzyme in a template-directed manner (51).

The suggestion that the enzyme has a single primer-template binding site implies that the exonuclease and polymerase active sites are very intimately related if not identical. This is particularly interesting since it appears that the enzyme can be induced to switch the direction of catalysis from $3' \rightarrow 5'$ hydrolysis to $5' \rightarrow 3'$ polymerization by the addition of dNTPs without dissociation from the template. Finally, the remarkable preference of the T5 polymerase for 3'-OH ends of the primer is quite atypical of DNA polymerases (6, 35, 47, 48) as has been previously emphasized. In fact, perhaps this is the
reason gene D5 product of bacteriophage T5, which is a DNA-binding protein (52), shows no stimulation of T5 DNA polymerase with denatured DNA as substrate, even though T4 DNA polymerase is stimulated as much as 10-fold with the same substrate.

On the basis of theory of evolution, it would be natural to think that the basic mechanism of DNA polymerase from closely related organisms is the same. Thus, even though it is the unique properties of T5 DNA polymerase that enable us to do the experiments reported herein, the conclusion that the turnover takes place at the point of chain growth with the same enzyme that is involved in polymerization is probably applicable to all prokaryotic DNA polymerases with associated 3' → 5' exonuclease. The difference in properties among prokaryotic DNA polymerase is quantitative and not qualitative. One of the most nonprocessive DNA polymerases is that of T4 (33). However, in complex with other proteins involved in replication, it does become processive (53). It will be of interest to test our conclusion with T4 DNA replication complex.

APPENDIX

Derivation of the equation used to compute dissociation constant ($K_D$)

\[ E + S \rightleftharpoons ES \]

Where $E$ is enzyme, $S$ is primer-template, and $ES$ is enzyme-primer-template complex

\[ K_D = \frac{[E][S]}{[ES]} = \frac{[E] - [ES][S] - ES}{[ES]} \]

Rearranging this to a quadratic equation and solving for [ES]

\[ [ES] = \frac{[E] + S + K_D}{2} \pm \sqrt{\frac{[E] + S + K_D}{4} - 4[ES]} \]

(+) root of this leads to $[ES] > [ES]$ which is absurd. Therefore, (-) root was used for computation.

REFERENCES