Using 2-Aminopurine Fluorescence to Measure Incorporation of Incorrect Nucleotides by Wild Type and Mutant Bacteriophage T4 DNA Polymerases*

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The ability of wild type and mutant T4 DNA polymerases to discriminate in the utilization of the base analog 2-aminopurine (2AP) and the fluorescence of 2AP were used to determine how DNA polymerases distinguish between correct and incorrect nucleotides. Because T4 DNA polymerase incorporates dTMP opposite 2AP under single-turnover conditions, it was possible to compare directly the kinetic parameters for incorporation of dTMP opposite template 2AP to the parameters for incorporation of dTMP opposite template A without the complication of enzyme dissociation. The most significant difference detected was in the $k_p$ for dTTP, which was 10-fold higher for incorporation of dTMP opposite template 2AP (367 μm) than for incorporation of dTMP opposite template A (31 μm). In contrast, the dTMP incorporation rate was reduced only about 2-fold from about 318 s⁻¹ with template A to about 165 s⁻¹ for template 2AP. Discrimination is due to the high selectivity in the initial nucleotide-binding step. T4 DNA polymerase binding to DNA with 2AP in the template position induces formation of a nucleotide binding pocket that is preshaped to bind dTTP and to exclude other nucleotides. If nucleotide binding is hindered, initiation of the proofreading pathway acts as an error avoidance mechanism to prevent incorporation of incorrect nucleotides.

High fidelity DNA polymerases are responsible for replicating the genomic DNA of most organisms. Replication fidelity is achieved by accurate nucleotide incorporation, which has an error frequency of about $1 \times 10^{-6}$ (1), and by exonucleolytic proofreading, which preferentially removes incorrect versus correct nucleotides from the primer terminus (2, 3) and improves fidelity another 100-fold or more. Many organisms also have additional mismatch correction pathways that further improve fidelity another 100-fold. However, the largest single contributor to accurate DNA replication is the ability of the DNA polymerase to insert the correct nucleotide with high precision.

How do DNA polymerases discriminate between correct and incorrect nucleotides? Although hydrogen bonding appears to be an obvious mechanism for determining base pair specificity, this mechanism alone is not adequate. In the presence of water, free energy differences in hydrogen bonding between complementary and noncomplementary bases are not sufficient to produce the observed low nucleotide error frequency of $1 \times 10^{-6}$ (4). Instead, results from numerous studies are consistent with the proposal that DNA polymerases discriminate between inserting correct or incorrect nucleotides based on the geometry of the base pair. For example, the Klenow fragment of Escherichia coli DNA polymerase I was shown to incorporate an isosteric analog of thymidylate with accuracy, even though the base analog could not form any hydrogen bonds with the template base (5). Structural studies of nucleotide pre-incorporation complexes formed with several DNA polymerases suggest that DNA polymerases may assess the geometry of the newly forming base pair by “closing down” on the primer-terminal region to make several tight contacts that are in position to distinguish between correct and incorrect base pairs (6–9). The structural studies are consistent with kinetic studies in which an induced-fit mechanism is proposed to determine nucleotide selectivity (10–13).

Despite the above reports, it is still not clear how DNA polymerases distinguish between correct and incorrect nucleotides, because the high fidelity of most DNA polymerases dictates that it is difficult to “capture” a DNA polymerase in the act of inserting an incorrect nucleotide, even in reactions in which only the incorrect nucleotide is supplied. If the DNA polymerase dissociates ($k_{d,e}$) from the DNA substrate faster than an incorrect nucleotide is incorporated, then the apparent nucleotide misincorporation rate may be more reflective of multiple cycles of enzyme association and dissociation rather than nucleotide misincorporation (see discussion by Wong et al. (13)). There are at least three potential steps in which the DNA polymerase can assert specificity for the correct base pair (reviewed in Ref. 14): initial dNTP binding, post-binding selection by an induced-fit mechanism, and the chemical step of phosphodiester bond formation (see Fig. 1, Steps 2–4). Different DNA polymerases have been proposed to derive specificity in nucleotide incorporation from one or more of these steps (for examples see Refs. 10–13, 15–17) or from the sequential application of each step (18).

We report here that the technical problems in measuring incorporation of an incorrect nucleotide have been overcome for the bacteriophage T4 DNA polymerase by using the base analog 2-aminopurine (2AP).† Whereas in some studies the 2AP-T

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*‡ Supported CAPES (Brasilia-Brazil) and the Universidade Gama Filho, Rio de Janeiro, Brazil.
∥ Scientist of the Alberta Heritage Foundation for Medical Research.
§ Supported by an Alberta Heritage Foundation for Medical Research (to L. J. R.-K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
¶ Supported by an Alberta Heritage Foundation for Medical Research Grant MOP-14300 and the Alberta Heritage Scientist of the Alberta Heritage Foundation for Medical Research.

† The abbreviations used are: 2AP, 2-aminopurine; $E^{\text{en}}$D, enzyme/DNA complex with the primer terminus bound in the polymerase active center; $E^{\text{en}}$-dTTP-Mg$^{2+}$ complex, nucleotide pre-incorporation complex; DTT, dithiothreitol; HMDCTP, hydroxymethylcytosine deoxynucleoside triphosphosphate.
DNA Polymerase Nucleotide Incorporation

Enzymes—Purification and characterization of the exonuclease-deficient D112A/E114A T4 DNA polymerase (27) and the proofreading-defective, but exonuclease-proficient G255S-DNA polymerase (28) have been described. T4 DNA polymerase mutants with single amino acid substitutions in the conserved Motif A sequence, L412M and I417V, have also been described (29). The exonuclease-deficient forms of the Motif A mutants were constructed by standard in vitro mutagenesis procedures, and the mutant DNA polymerases were purified by standard methods (30).

EXPERIMENTAL PROCEDURES

Materials

Enzymes—Purification and characterization of the exonuclease-deficient D112A/E114A T4 DNA polymerase (27) and the proofreading-defective, but exonuclease-proficient G255S-DNA polymerase (28) have been described. T4 DNA polymerase mutants with single amino acid substitutions in the conserved Motif A sequence, L412M and I417V, have also been described (29). The exonuclease-deficient forms of the Motif A mutants were constructed by standard in vitro mutagenesis procedures, and the mutant DNA polymerases were purified by standard methods (30).

DNA Substrates—Oligonucleotides (see Table I) were synthesized using solid-state chemistry as described previously (31). The 3′ terminus of the template strand was protected from DNA polymerase binding by 32P end-labeling of the primer terminus in the polymerase active center of the L412M-mutated T4 DNA polymerase by a biotin attachment (Biotin-TEG-CPG, Glen Research). The biotin modification at the 3′-end of the template strand ensured that DNA polymerase-DNA interactions were directed to the primer terminus as determined by enzyme titration. All DNAs were purified by polyacrylamide gel electrophoresis. The primer and template DNAs were annealed in buffer containing 25 mM HEPES (pH 7.6), 50 mM NaCl, 1 mM DTT, and 0.5 mM EDTA. Intrinsic protein fluorescence was subtracted.

Methods

Steady-state Fluorescence Emission Experiments—Emission data for 2AP-DNAs and polymerase-DNA complexes were obtained with a Photon Technology International scanning spectrofluorometer. Samples were excited at 310 nm, and fluorescence emission data were collected at 368 nm. Solutions contained 200 nM 2AP-labeled DNA, 500 nM T4 DNA polymerase, 25 mM HEPES (pH 7.6), 50 mM NaCl, 1 mM DTT, and 0.5 mM EDTA. Intrinsic protein fluorescence was subtracted.

RESULTS

We have shown that when T4 DNA polymerase binds to a DNA substrate labeled with 2AP at the n+1 template position (Fig. 1, Step 1), up to a 25-fold increase in fluorescence intensity is produced due to enzyme-induced base unstacking at the n+1 position (25). In contrast, T4 DNA polymerase binding to DNA templates labeled with 2AP at the more distal n+2 position (Table I) produces only a small, less than 4-fold increase in fluorescence intensity (25). Thus, nucleotide incorporation by the T4 DNA polymerase can be observed as an increase in...
to achieve maximal DNA polymerase binding as determined by fluorescence measurements in the absence of the biotin attachment.

**Incorporation of dTMP Opposite Template A—**Incorporation of dTMP opposite template A was determined with DNA labeled initially at the n+2 position with 2AP (Table I). The rate of increase in fluorescence intensity produced by primer elongation was measured as a function of dTTP concentration. A solution of preformed enzyme-DNA complexes (1 μM DNA polymerase + 400 nM n+2 DNA) in buffer with 0.5 mM EDTA was mixed in the stopped-flow apparatus with a second solution containing Mg²⁺ and different concentrations of dTTP. After mixing, the concentrations of reactants were 200 nM n+2 DNA substrate (Table I), 500 n n exonuclease-deficient D112A/E114A-DNA polymerase; 25 mM HEPES (pH 7.6), 50 mM NaCl, 1 mM DTT, 8 mM Mg²⁺, and dTTP from 5 to 200 μM. The apparent nucleotide incorporation rates (k_app) were plotted against dTTP concentration (Fig. 3). The data were fit to the equation, 

\[
K_d = k_{pol}[dTTP]/(K_d + [dTTP])
\]

The K_d for dTTP with template A (K_d(TMP-2AP)) is 31 ± 4 μM and the k_pol is 318 ± 13 s⁻¹. These values are similar to the kinetic parameters determined for the incorporation of dAMP opposite template T also measured by the fluorescence assay, but with another n+2 DNA substrate (Table I); K_d(TMP-T) = 16 ± 3 μM and k_pol = 314 ± 18 s⁻¹ (25). Similar kinetic parameters were also obtained for the incorporation of dGMP opposite template C (data not shown). The nucleotide incorporation rates determined by the fluorescence assay, about 314–318 s⁻¹, are slower than the rate of 400 s⁻¹ observed for incorporation of dAMP opposite template T by a radioactive-based, rapid quench assay (34). However, nucleotide incorporation reactions were done at pH 8.8 in the rapid quench experiments, but at pH 7.6 for the fluorescence assay. Because higher pH stimulates nucleotide incorporation by the T4 DNA polymerase (35), the higher apparent nucleotide incorporation rates measured in the rapid quench experiments may reflect differences in the pH of the reaction.

**Determination of the Equilibrium Dissociation Constant (K_d) for dTTP Binding Opposite Template 2AP with Chain-terminated DNA Substrates—**The K_d for dTTP binding opposite template 2AP was first determined with nonextendable primers to study nucleotide binding in the absence of incorporation. Because Mg²⁺ is required to detect dTTP binding opposite template 2AP (25), nucleotide incorporation would normally take place unless prevented. A solution of preformed fluorescent enzyme-DNA complexes was made with 200 nM of the ddAMP-terminated n+1 DNA substrate (Table I), 500 mM exonuclease-deficient D112A/E114A-DNA polymerase, 25 mM HEPES (pH 7.6), 50 mM NaCl, 1 mM DTT, and 8 mM Mg²⁺ and titrated with increasing concentrations of dTTP. The decrease in fluorescence intensity (% Quench) of the Epol-DdTTP-Mg²⁺ complexes as a function of dTTP concentration is plotted in Fig. 4. The K_d for dTTP binding opposite template 2AP with the dideoxy-terminated primer [K_d(ddideoxy-terminated)] was determined to be 34 ± 3 μM by fitting the data to the hyperbolic equation, 

\[
q = (q_d)[dTTP]/(K_d + [dTTP])
\]

where q is the observed fluorescence quench at various dTTP concentrations and Q is the maximum quench observed at saturating dTTP. Surprisingly, the K_d for dTTP binding opposite template 2AP with the dideoxy-terminated primer (34 μM) is indistinguishable from the K_d determined for dTTP in reactions in which dTMP was incorporated opposite template A (K_d(TMP-2AP) = 31 μM) (Fig. 3). This result was unexpected because the T4 DNA polymerase discriminates in the utilization of 2AP (19–21). However, there is the possibility that the dideoxy-terminated DNA substrate decreases the ability of the T4 DNA polymerase to discriminate against T-2AP.
The base analog 2-aminopurine (2AP) is depicted as a bold P in the sequences. The "B" indicates biotin at the end of a 15-atom spacer arm attached to the 3'-end of the template stand. The "dd" indicates the presence of the 2',3'-dideoxy chain-terminator at the primer terminus. The "3'd" indicates the presence of the 3'-deoxy adenosine, which is also a terminator. Two n + 2 DNA substrates were used in this study. For the first n + 2 DNA substrate, addition of dTTP and Mg^{2+} results in the incorporation of two successive dTMP residues. For the second n + 2 DNA, addition of dATP and Mg^{2+} results in the incorporation of a single dAMP.

| DNA substrate | Template | Primer |
|---------------|----------|--------|
| n+1 ( incorporation of dTMP) | 3' B--CCTTTCGTCAGTAGACATTAA5GCATCGATGTTT | 5' GGGAAACAGTCTACGTTAATT |
| n+2 ( incorporation of dTMP) | 3' B--CCTTTCGTCAGTAGACATTAA5GCATCGATGTTT | 5' GGGAAACAGTCTACGTTAATT |
| n+2 ( incorporation of dAMP) | 3' B--CCTTTCGTCAGTAGACATTAA5GATCGATGTTT | 5' GGGAAACAGTCTACGTTAATT |
| n+1 (dd) | 3' B--CCTTTCGTCAGTAGACATTAA5GATCGATGTTT | 5' GGGAAACAGTCTACGTTAATT |
| n+1 (3’d) | 3' B--CCTTTCGTCAGTAGACATTAA5GATCGATGTTT | 5' GGGAAACAGTCTACGTTAATT |

**Table I**

**DNA substrates labeled at the n+1 or n+2 position with 2AP**

The base analog 2-aminopurine (2AP) was immediately observed (Fig. 5, data not shown). Thus, the presence of the 2AP base-stacking interactions.

**FIG. 2.** Dynamics of nucleotide incorporation. The sequential incorporation of two nucleotides was observed by changes in fluorescence intensity for DNA labeled with 2AP initially at the n+2 template position (Table I). An increase in fluorescence intensity was observed for incorporation of dTMP opposite template A as the primer was extended, which moved 2AP initially in the n+2 position to the n+1 position. The increase in fluorescence intensity was followed by a decrease due to incorporation of a second dTMP opposite template 2AP, which restored 2AP base stacking interactions and quenched 2AP fluorescence.

**FIG. 3.** Pre-steady-state kinetics of dTMP incorporation opposite template A. Incorporation of dTMP opposite template 2AP was determined by measuring the rate of increase in fluorescence intensity for 2AP at the base pairing (n+1) position, we attempted to measure dTTP-Mg^{2+} binding opposite template 2AP in the stopped-flow apparatus. One syringe contained preformed fluorescent n+1 complexes made with 400 nM of the chain-terminated n+1 DNA substrate (Table I), 1 μM exonuclease-deficient D112A/E114A-DNA polymerase, 25 mM HEPES (pH 7.6), 50 mM NaCl, 0.5 mM EDTA, and 1 mM DTT. For the first experiment, the second syringe contained buffer (25 mM HEPES (pH 7.6), 50 mM NaCl, 1 mM DTT) and 16 mM Mg^{2+}. Rapid mixing of equal volumes of the two solutions produced the trace for the level of fluorescence for the E_{pol-D-Mg^{2+}} complexes formed with a final concentration of 200 nM chain-terminated n+1 DNA and 500 nM enzyme (Fig. 5, trace 1). When the experiment was repeated with 400 μM dTTP added to the second syringe, to give a final concentration of 200 μM dTTP after mixing, a lower level of fluorescence intensity was immediately observed (Fig. 5, trace 2). The lower initial level of fluorescence intensity detected by the addition of dTTP-Mg^{2+} indicates that dTTP-Mg^{2+} binding produced a rapid quench in fluorescence intensity within the dead-time of the stopped-flow apparatus. No further decrease in fluorescence intensity was observed after 10 ms.
The rapid initial quench in fluorescence intensity produced by dTTP-Mg\(^{2+}\) binding to the fluorescent complexes with 2AP at the n+1 position was not caused by random collisional quenching. Only a small decrease in fluorescence intensity was observed by the addition of 200 \(\mu\)M dCTP-Mg\(^{2+}\), <10% the quench observed for 200 \(\mu\)M dTTP (data presented in a later section). If nonspecific nucleotide binding to the fluorescent n+1 complexes can take place, then these interactions do not quench 2AP fluorescence as efficiently as does specific nucleotide binding.

Incorporation of dTMP Opposite Template 2AP—The pre-steady-state nucleotide incorporation rate for dTMP opposite template 2AP was determined in the stopped-flow apparatus by using the same conditions that were used for dTTP binding with the dideoxy-terminated DNA substrate, except that the primer terminus was extendable. In a representative set of experiments, one syringe of the stopped-flow apparatus contained 400 nM extendable n+1 DNA substrate (Table 1), 1 \(\mu\)M exonuclease-deficient D112A/E114A-DNA polymerase, 25 mM HEPES (pH 7.6), 50 mM NaCl, 1 mM DTT, and 16 mM Mg\(^{2+}\). Rapid mixing of equal volumes of solutions from both syringes produced the trace for the level of fluorescence for the \(E^{\text{pol}}\)-d-Mg\(^{2+}\) complexes formed with a final concentration of 200 nM n+1 DNA and 500 nM enzyme (Fig. 6, trace 1). When the experiment was repeated with 400 \(\mu\)M dTTP added to the second syringe to give a final concentration of 200 \(\mu\)M dTTP, fluorescence intensity was observed to decrease by at least three different rates (Fig. 6, trace 2).

As observed with the dideoxy-terminated primer (Fig. 5), dTTP binding opposite template 2AP produced an initial very rapid decrease in fluorescence intensity within the dead-time of the instrument. The extent of the initial rapid decrease was dependent on dTTP concentration, but the amount of quench was less than the amount observed with the dideoxy-terminated DNA substrate. Although about 25% quench in fluorescence intensity was observed in the initial rapid phase of the reaction with the extendable primer and 200 \(\mu\)M dTTP (Fig. 6, trace 2), only 10 \(\mu\)M dTTP was required to reach the same extent of quench with the dideoxy-terminated DNA substrate (Fig. 4). The \(K_d\) for dTTP binding opposite template 2AP with the extendable primer \([K_{d\text{, dTTP-2AP, fast phase}}]\) was determined by plotting the amount of quench as a function of dTTP concentration. The data were fit to the hyperbolic equation, \(q = [Q(dTTP)/(K_{d\textвел}} + [dTTP])\). The apparent \(K_{d\text}d\) for dTTP binding opposite template 2AP with the extendable primer was 358 \(\pm\) 76 \(\mu\)M (Fig. 7), which is about 10-fold higher than the \(K_{d}\) determined for dTTP binding opposite template 2AP with the dideoxy-terminated primer (Fig. 4). The 10-fold lower \(K_{d}\) for dTTP binding opposite template 2AP with the dideoxy-terminated DNA substrate indicates that the dideoxy-terminated primer reduces the ability of the T4 DNA polymerase to discriminate against formation of dTTP-2AP base pairs.

A further decline in fluorescence intensity was observed with the extendable primer (Fig. 6, trace 2) that did not occur with the chain-terminated DNA substrate and, thus, is attributed to dTTP incorporation. The rate of decline in fluorescence intensity was dependent on dTTP concentration and biphasic kinet-
The determination of the $K_d$ for dTTP binding opposite template 2AP from the initial rapid quench phase of the nucleotide incorporation reaction. Incorporation of dTMP opposite template 2AP produces a rapid initial decrease in fluorescence intensity that is dependent on dTTP concentration as illustrated in Fig. 6. The amount of quench was plotted as a function of dTTP concentration and the data were fit to the hyperbolic equation, $q = Q([dTTP]/(K_d + [dTTP]))$.

The major rate is the rate of dTMP incorporation, and the second rate is enzyme dissociation as demonstrated by experiments with heparin, which traps T4 DNA polymerase that dissociates from the DNA substrate and prevents rebinding (Table II). Reactions were initiated in the stopped-flow by mixing equal volumes of a solution of T4 DNA polymerase, DNA, and EDTA in buffer (25 mM HEPES (pH 7.6), 50 mM NaCl, 1 mM DTT) with a second solution containing buffer, dTTP, Mg$^{2+}$, and heparin. After mixing, the final concentrations of reactants were 500 nM T4 DNA polymerase, 200 nM 2AP-labeled DNA, 1 mM DTT, 8 mM MgCl$_2$, 25 mM HEPES (pH 7.6), 0.5 mM EDTA, 50 mM NaCl, 200 μM dTTP, and 10 μg/ml heparin. Similar biphasic kinetics for the decrease in fluorescence intensity were observed in the presence of heparin as observed in the absence with a major rate of 50 ± 3 s$^{-1}$ (amplitude 0.9) and a minor rate of 6.5 ± 0.7 s$^{-1}$ (amplitude 0.15). Similar values were obtained from repeat reactions.

For incorporation of dTMP opposite template 2AP, the $K_d$ for dTTP was determined using the same conditions, but without dTTP. Two rates were again observed (Table II) with a major rate of 5.8 ± 0.1 s$^{-1}$ (amplitude 0.7), which corresponds to the slower rate observed in reactions with dTTP. The apparent dissociation rate of about 6 to 7 s$^{-1}$ matches the dissociation rate of 6.5 s$^{-1}$ reported for the T4 DNA polymerase (34). Thus, the slower rate of 7.3 s$^{-1}$ detected for the incorporation of dTMP opposite template 2AP in the absence of heparin (Table II) is due to enzyme dissociation followed by reformation of the enzyme-DNA complex and subsequent nucleotide binding and incorporation.

Kinetic parameters for dTMP incorporation opposite template 2AP were determined by measuring the rate of fluorescence decline at several dTTP concentrations. The data were fit to the hyperbolic equation, $k_{obs} = k_{pol}[dTTP]/(K_d + [dTTP])$, yielding values of 386 ± 36 μM s$^{-1}$ for $K_d$ and 165 ± 5 s$^{-1}$ for $k_{pol}$ (Fig. 8). The $K_d$ for dTTP determined by dTMP incorporation opposite template 2AP is indistinguishable from the apparent $K_d$ for dTTP binding (358 μM) determined from the amount of quench observed in the initial rapid phase of the reaction (Fig. 7). Thus, T4 DNA polymerase discriminates in the binding of dTTP opposite template 2AP. The $K_d$ for dTTP determined by incorporation of dTMP opposite template 2AP is 367 μM, but the $K_d$ for dTTP of incorporation of dTMP opposite template A is just 31 μM (Fig. 3). Discrimination, however, requires the presence of the correct primer terminus, because the $K_d$ determined for binding dTTP opposite template 2AP with the deoxyterminated DNA substrate is only 34 μM (Fig. 4).

Nucleotide Incorporation by Mutant DNA Polymerases with the L412M or I417V Amino Acid Substitutions in the Polymerase Active Center—The above experiments were repeated for mutant DNA polymerases with the L412M or I417V amino acid substitutions in the polymerase active center. The L412M substitution decreases the fidelity of DNA replication and the I417V substitution increases fidelity (29). The L412M- and I417V-DNA polymerases were also made exonuclease-deficient by the D112A/E114A substitutions.

In a series of representative experiments with 200 μM dTTP, with or without heparin, the exonuclease-deficient L412M-DNA polymerase incorporated dTMP opposite template 2AP at a faster rate, >130 s$^{-1}$ (Table II), than detected for the exonuclease-deficient enzyme with the wild type polymerase active center, about 50 s$^{-1}$ (Table II). Biphasic kinetics were observed for the exonuclease-deficient L412M-DNA polymerase with the major rate corresponding to the dTMP incorporation rate and the slower rate corresponding to enzyme dissociation. Biphasic kinetics were also observed for enzyme dissociation in the absence of dTTP; the major rate was 2.4 ± 0.1 s$^{-1}$ (Table II). Kinetic parameters for incorporation of dTMP opposite template 2AP by the exonuclease-deficient L412M-DNA polymerase, $K_d^{dTMP-2AP}$ ~ 252 μM and $k_{pol}$ ~ 358 s$^{-1}$ (Table III), were determined as described for the exonuclease-deficient T4 DNA polymerase with the wild type polymerase active center.

Experiments with the exonuclease-deficient I417V-DNA polymerase illustrate why it is important to demonstrate that single-turnover conditions are established. Single rather than biphasic kinetics were observed for the incorporation of dTMP opposite template 2AP with 200 μM dTTP, but the apparent nucleotide incorporation rate was just 2 s$^{-1}$ (Table II). Furthermore, the rate of decrease in fluorescence intensity increased to >40 s$^{-1}$ in the presence of the heparin trap, irrespective of whether dTTP was present or not (Table II), which indicates that dTMP incorporation opposite template 2AP is slower than enzyme dissociation. Thus, the apparent dTMP incorporation rate of 2 s$^{-1}$ for the exonuclease-deficient I417V-DNA polymerase occurs during multiple enzyme dissociation-association events. Strong discrimination against template 2AP by the I417V-DNA polymerase was also observed for dTTP binding opposite template 2AP with the deoxyterminated DNA substrate. The I417V substitution decreased the ability of the DNA polymerase to bind dTTP opposite template 2AP, $K_d$ for incorporation of dAMP opposite template $T$ was 196 μM, compared with the L412M substitution, $K_d$ = 12.5 μM (Table III). From these observations, we conclude that the I417V substitution significantly decreases the ability of the DNA polymerase to utilize 2AP as a templating base.

In reactions in which 2AP was not a base-pairing partner, however, the exonuclease-deficient I417V-DNA polymerase incorporated nucleotides at the same rate as determined for the exonuclease-deficient L412M-DNA polymerase, 189 compared with 177 s$^{-1}$, for the incorporation of dAMP opposite template T (Table III). $K_d$ values for incorporation of dAMP opposite template T were also determined; the highest value, 31 μM, was observed for the exonuclease-deficient I417V-DNA polymerase, and the lowest value, 11 μM, was observed for the exonuclease-deficient L412M-DNA polymerase (Table III).

Incorporation of dCMP Opposite Template 2AP in Vitro—T4 DNA polymerase discriminates in the incorporation of dTMP opposite 2AP, but much greater discrimination is observed for incorporation of dCMP (36). The $K_d$ for binding dCTP opposite
2AP, determined with the dideoxy-terminated DNA substrate, was 3.3 mM (Fig. 9A), which is about 100-fold higher than the $K_d$ for dTTP binding opposite template 2AP, about 34 μM (Fig. 4). Thus, even though the dideoxy-terminated primer decreases discrimination in binding dTTP opposite template 2AP compared with the extendable primer (Figs. 4 and 7), strong discrimination is still exerted against dCTP binding opposite template 2AP.

The dCMP misincorporation rate opposite template 2AP was determined with the same n + 1 DNA substrate used to measure the kinetics of dTTP incorporation, but single-turnover conditions could not be established. A single rate of decline in fluorescence intensity was observed and there was no initial rapid decrease in fluorescence intensity as observed in reactions with dTTP, even at the highest concentration of dCTP tested (200 μM). The ability of dCTP to inhibit the incorporation of dTMP opposite template 2AP was also tested by adding dCTP to reactions with the exonuclease-deficient T4 DNA polymerase and 10 μM dTTP. No inhibition of dTMP incorporation opposite template 2AP was observed at concentrations up to 5 mM, the highest dCTP concentration tested (data not shown).

![Graph showing $k_{pol}$ vs dTTP concentration](image)

**Fig. 8. dTTP concentration dependence on rate for incorporation of dTMP opposite template 2AP.** Nucleotide incorporation rates were determined as described in Fig. 6 for several dTTP concentrations. The data were fit to the hyperbolic equation, $k_{inc} = k_{pol}[dTTP]/(K_d + [dTTP])$.

Incorporation of dCMP Opposite Template 2AP in Vivo—
Although dCTP does not appear to compete with dTTP for nucleotide incorporation opposite template 2AP in vitro, 2AP is mutagenic for T4 DNA replication in vivo. Reversion frequencies for an rII ochre mutant were determined in the presence of 2AP for the wild type, L412M- and I417V-DNA polymerases (Table IV). Because only A-T base pairs are present in an ochre codon, TAA, the 2AP nucleotide is incorporated opposite template T. In the next round of replication, 2AP may template the incorporation of either dTMP or dCMP as in the in vitro assays, but only the incorporation of dCMP is mutagenic. The L412M substitution increased the rII reversion frequency in the presence of 2AP > 7-fold compared with the wild type T4 DNA polymerase and > 60-fold compared with the I417V-DNA polymerase. The higher level of mutagenesis produced by the L412M substitution may reflect either increased ability of the L412M-DNA polymerase to incorporate the 2AP nucleotide opposite template T and/or increased ability to incorporate dCMP opposite template 2AP. The I417V substitution, on the other hand, likely discriminates in the initial incorporation of d2APMP, because the in vitro studies indicate that the I417V substitution substantially increases the ability of the DNA polymerase to discriminate in the utilization of 2AP.

**The Role of Amino Acids in the Polymerase Active Center in Stabilizing the Primer in the Polymerase Active Center**—The experiments with heparin (Table II) indicate that the L412M substitution decreases the dissociation rate of enzyme-DNA complexes, whereas the I417V substitution increases the dissociation rate. During DNA replication in vivo, however, the DNA polymerase is clamped to the DNA template and does not dissociate; however, release of DNA binding in the polymerase active center must occur to transfer the 3′-end of the primer strand from the polymerase to the exonuclease active center for proofreading. Thus, the relatively slow rate of dissociation produced by the L412M substitution may indicate a decreased rate for initiation of strand transfer for proofreading. The faster dissociation rate produced by the I417V substitution may indicate an increased rate for initiation of proofreading. Although this proposal has been tested in a radioactive-based assay (29), the fluorescence of 2AP in enzyme-DNA complexes provides greater sensitivity.

**Table II. Incorporation of dTMP opposite template 2AP in the absence and presence of heparin**

| Kinetic parameters | DNA polymerase |
|--------------------|----------------|
| $k_{inc}$ | Exo$^+$ | I412M-Exo$^+$ | I417V-Exo$^+$ |
| $k_{inc}$/H18528 | $51.1 \pm 2.2 (8.5) 7.3 \pm 0.5 (15)$ | $138.3 \pm 6.7 (75) 15.0 \pm 3.2 (25)$ | $2.0 \pm 0.1$ |
| $k_{inc}$/H11002 | $50.0 \pm 3.0 (90) 6.5 \pm 1.7 (10)$ | $132.4 \pm 6.8 (70) 21.0 \pm 1.6 (30)$ | $47.5 \pm 4.0 (80) 8.6 \pm 1.0 (20)$ |
| $k_{off}$/H18528 | $19.1 \pm 1.3 (30) 5.8 \pm 0.1 (70)$ | $16.5 \pm 1.1 (25) 2.4 \pm 0.1 (75)$ | $44.0 \pm 2.1 (60) 7.1 \pm 0.6 (40)$ |

In vivo incorporation of dTMP opposite template 2AP was observed at concentrations up to 5 mM, with a second solution of Mg$^{2+}$/dTTP or Mg$^{2+}$/dTTP/heparin or Mg$^{2+}$/heparin. The data were fit to single or double rate equations. The amplitudes of each phase are indicated in parentheses.

**Incorporation of dCMP Opposite Template 2AP in Vivo**—Although dCTP does not appear to compete with dTTP for incorporation of dCMP opposite template 2AP in vitro, dCTP is mutagenic for T4 DNA replication in vivo. Reversion frequencies for an rII ochre mutant were determined in the presence of 2AP for the wild type, L412M- and I417V-DNA polymerases (Table IV). Because only A-T base pairs are present in an ochre codon, TAA, the 2AP nucleotide is incorporated opposite template T. In the next round of replication, 2AP may template the incorporation of either dTMP or dCMP as in the in vitro assays, but only the incorporation of dCMP is mutagenic. The L412M substitution increased the rII reversion frequency in the presence of 2AP > 7-fold compared with the wild type T4 DNA polymerase and > 60-fold compared with the I417V-DNA polymerase. The higher level of mutagenesis produced by the L412M substitution may reflect either increased ability of the L412M-DNA polymerase to incorporate the 2AP nucleotide opposite template T and/or increased ability to incorporate dCMP opposite template 2AP. The I417V substitution, on the other hand, likely discriminates in the initial incorporation of d2APMP, because the in vitro studies indicate that the I417V substitution substantially increases the ability of the DNA polymerase to discriminate in the utilization of 2AP.
The G255S-DNA polymerase was studied, because both the G255S- and L412M-DNA polymerases were isolated in a genetic study designed to identify active site switching mutants, mutants that are defective in moving the primer end from the polymerase to the exonuclease active center for proofreading (37). The G255S substitution is in the loop of a prominent beta hairpin structure located in the exonuclease domain, but separate from the exonuclease active center. The G255S-DNA polymerase can degrade single-stranded DNA, but cannot readily degrade duplex DNA, which accounts for the strong mutator phenotype observed in vivo (37). We demonstrated using 2AP fluorescence assays that the G255S-DNA polymerase is defective in separating the primer strand from the template strand, which is needed to form the editing complex (28). From these studies, we proposed that the hairpin structure may act as a wedge between the separated primer and template strands (28), and this proposal was later supported by structural studies (38). If the G255S substitution also interferes with conformational changes that lead to enzyme dissociation in the absence of the clamp, then the G255S substitution should reduce the $k_{off}$ rate. The G255S-DNA polymerase dissociates at the rate of 1.7 s$^{-1}$, which is about 3-fold slower than the rate observed for the wild type T4 DNA polymerase (Table V). Thus, release of DNA binding in the polymerase active center in preparation for proofreading is sensitive to amino acid residues Leu-412 and Ile-417 in the polymerase active center, and to the G255S-hairpin structure in the exonuclease domain. The implications of these findings on the fidelity of DNA replication are presented under “Discussion.”

**DISCUSSION**

The challenge in determining mechanisms that ensure high fidelity DNA replication is to develop assays with sufficient sensitivity to detect very rare events or, alternatively, to use chemically modified substrates that invoke discrimination but do not substantially reduce utilization. For reactions with the highly accurate T4 DNA polymerase, the base analog 2AP produces modest discrimination and also serves as a fluorescent reporter for the nucleotide incorporation reaction. Several important conclusions can be derived from experiments in which DNA labeled with 2AP is replicated by wild type and

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**TABLE III**

Comparison of nucleotide incorporation kinetic parameters by accurate and less accurate T4 DNA polymerases

| Parameters | Exo$^a$ | L412M-Exo | 1417V-Exo |
|------------|--------|------------|------------|
| $K_d(RIP)$ (dATP-T) (µM) | 34 ± 3 | 3300 ± 400 | 125 ± 0.6 |
| $k_{cat}$ (µM) | 16 ± 3 | 31 ± 4 | 367 ± 36 |
| $K_d(RIP)$ (dATP-2AP) (µM) | 314 ± 18 | 318 ± 13 | 165 ± 5 |
| $k_{cat}/K_d(RIP)$ (dATP-2AP) (µM$^{-1}$s$^{-1}$) | 10.3 ± 0.45 | 0.0004 | 16 ± 1.4 |

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**TABLE IV**

2AP-induced mutagenesis

| Phage | 2AP (1 mg/ml) | Relative |
|-------|---------------|----------|
| WT | 62 | 1 |
| L412M | 476 | 7.7 |
| I417V | 7 | 0.1 |

**TABLE V**

Dissociation of $E^{ΔC-D}$ complexes

| DNA polymerase | $k_{off}$ (s$^{-1}$) |
|----------------|---------------------|
| WT | 5.6 ± 0.2 |
| G255S | 1.7 ± 0.1 |
mutant T4 DNA polymerases.

One of the most important conclusions is that the accurate, initial selection of the correct nucleotide is overwhelmingly the most important determinant of replication fidelity by the T4 DNA polymerase. Because the T4 DNA polymerase incorporates dTMP opposite 2AP under pre-steady-state conditions (Table II), it is possible to compare directly the kinetic parameters for incorporation of dTMP opposite template 2AP to the parameters for incorporation of dTMP opposite template A without the complication of enzyme dissociation. The most significant difference detected was in the $K_d$ for dTTP, which was 10-fold higher for incorporation of dTMP opposite template 2AP ($367 \mu M$) than for incorporation of dTMP opposite template A ($31 \mu M$) (Table III). In contrast, the dTMP incorporation rate was reduced only about 2-fold from about 318 s$^{-1}$ with template A to about 165 s$^{-1}$ for template 2AP (Table III). Thus, the lower efficiency, $k_{cat}/K_m$, for incorporation of dTMP opposite template 2AP of 0.45 compared with 10.3 for incorporation of dTMP opposite template A (Table III) is caused primarily by the reduced ability of the T4 DNA polymerase to bind dTTP opposite template 2AP.

Although the importance of substrate discrimination in DNA replication fidelity has been demonstrated for other DNA polymerases such as the T7 DNA polymerase (13), there is a question of when the discrimination is exerted. For the proposed induced-fit mechanism (13), a rate-limiting conformational change is proposed to occur immediately before chemistry. Step 3 in Fig. 1. In this model (13), an incorrect nucleotide can bind, but the presumed initial “loose” binding serves only to collect together the substrates, DNA, and dNTP. Discrimination occurs if the bound nucleotide cannot trigger the conformational change. In terms of structural studies, DNA polymerases bind nucleotides in the “open” conformation and then form a “closed” conformation to assess the accuracy of the newly forming base pair.

The use of 2AP as the template base provides the means to test this model. When a solution of preformed fluorescent enzyme-DNA complexes is mixed with a second solution of dTTP and Mg$^{2+}$ in the stopped-flow apparatus, an initial rapid decrease in fluorescence intensity is observed (Fig. 6). Because dTTP binding is rapid, within the dead-time of the instrument, binding is not likely to involve large conformational changes, which suggests that nucleotides are bound initially in the open enzyme-DNA conformation.

The dissociation rate is only 2-fold slower than the rate for incorporation of dTMP opposite template A, 165 s$^{-1}$, is only 2-fold slower than the rate for incorporation of dTMP opposite template A, which suggests that nucleotides are bound initially in the open enzyme-DNA conformation.

Nucleotide binding is highly selective with strong discrimination against dCTP (Fig. 9). More than H-bonding or spatial interactions between 2AP in the template strand and the incoming nucleotide are required, because the decrease in 2AP fluorescence intensity requires Mg$^{2+}$, which suggests that interactions are made between Mg$^{2+}$ ions bound in the polymerase active center and the phosphate groups of the incoming nucleotide. There are also critical interactions between the sugar at the primer terminus and the incoming nucleotide. An OH group at the 2$^\prime$ position of the terminal sugar prevents dTTP binding, whereas the deoxy-terminated primer allows binding, but reduces discrimination in binding dTTP opposite template 2AP compared with dTTP binding with the correct primer terminus (Table III). Because the entire structure of the incoming nucleotide is assessed in the initial binding step, several features of the nucleotide-binding pocket that are required for nucleotide selectivity appear to be already formed. These observations argue strongly that when T4 DNA polymerase binds DNA with 2AP in the template position that formation of the enzyme-DNA complex produces a nucleotide-binding pocket already preformed to bind dTTP and to exclude other nucleotides. Structural studies of the RB69 DNA polymerase (12), a close relative of the T4 DNA polymerase, are consistent with the proposal that a nucleotide-binding pocket is present in the open enzyme-DNA conformation (39). Superposition of the RB69 DNA polymerase prenucleotide incorporation complex in the closed conformation with the apoenzyme structure shows that residue Y146, which acts as a “sugar gate” for rNTP/dNTP selection, occupies nearly the same position in both structures (39).

Because studies presented here indicate that the T4 DNA polymerase exerts high selectivity in the initial dNTP-binding step, is discrimination also exerted at subsequent steps in the nucleotide incorporation pathway? Structural studies indicate that the RB69 DNA polymerase does form a closed complex upon binding the correct nucleotide; the fingers domain rotates about 60$^\circ$ and moves 30 Å closer to the palm domain (9). Thus, binding of the correct nucleotide may trigger a rate-limiting conformational change, but the rate for incorporation of dTMP opposite template 2AP, 165 s$^{-1}$, is only 2-fold slower than the rate for incorporation of dTMP opposite template A (Table III), which indicates that this step provides little discrimination.

Another course of events takes place, however, in reactions in which dCMP is incorporated opposite template 2AP. The apparent dCMP incorporation rate determined under steady-state conditions of 2.3 s$^{-1}$ (Table III) is slower than the dissociation rate of about 6 s$^{-1}$ (Table II). Thus, enzyme dissociation is more likely to occur than dCMP incorporation. However, because the T4 DNA polymerase is normally clamped to the DNA during DNA replication in vivo, the dissociation rates reported in this study (Table II) actually indicate the rate for initiation of the proofreading pathway. Although the L412M and G255S substitutions reduce the dissociation rate (Tables II and IV) and also reduce proofreading (28, 29), the I417V substitution increases the dissociation rate (Table II) and increases proofreading (28). Thus, in reactions in which dCMP is incorporated opposite template 2AP, initiation of the proofreading pathway is in competition with dCMP incorporation and is faster, which suggests that initiation of the proofreading pathway can reduce incorporation of incorrect nucleotides. We propose that initiation of the proofreading pathway is an important error avoidance mechanism, which is in addition to the well-documented role of proofreading in removing incorrect nucleotides at the primer terminus. For DNA polymerases that do not have proofreading activity, dissociation from the DNA substrate also has the potential to prevent incorporation of an incorrect nucleotide if dissociation is faster than the misincorporation rate. Because DNA polymerase concentrations in the cell are considerably lower than used in many in vitro assays, rapid rebinding will not necessarily occur, which means that dissociation can also provide an error avoidance mechanism.

Given the strong discrimination by the T4 DNA polymerase against binding dCTP opposite template 2AP, it is difficult to understand how any dCMP is incorporated opposite template 2AP, which is required for 2AP mutagenesis. The concentrations of dTTP and hydroxymethylcytosine deoxynucleoside triphosphate, HMdCTP (T4 does not have dCTP), in T4-infected bacteria are both at about 200 μM (40), but in vitro, dCTP at 5 mM was not observed to compete against incorporation of dTMP opposite template 2AP with dTTP at 10 μM. Dissociation or initiation of the proofreading pathway also prevailed when dCTP was the only nucleotide supplied (Fig. 9), which indicates that, even if there were a large nucleotide pool imbalance with HMdCTP at a much higher concentration than dTTP, HMdCTP was not expected to compete successfully against dTTP. Yet, 2AP is mutagenic in vivo (Table IV), Because 2AP mispairs with cytosine more frequently when 2AP is in the template position rather than when it serves as a deoxyribonucleoside
triphasate substrate (41), the first step in 2AP mutagenesis is likely incorporation of d2APMP opposite template T. Incorporated 2AP then templates the incorporation of dTMP or dCMP. The probability of incorporation hydroxymethylcytosine nucleotide opposite template 2AP is reported to be about 2% per round of replication with the wild type, proofreading-proficient T4 DNA polymerase (41). Because proofreading is expected to correct many if not most C-2AP mismatches, 2% is an underestimate of the true misincorporation rate. Because there is more apparent discrimination against incorporation of dCMP in vitro compared with in vivo, incorporation of dCMP opposite template 2AP in vivo may not occur under typical replication conditions. Instead, apparent nucleotide misincorporation may arise by infrequent formation of aberrant replication conditions. Thus, one may expect that residues L412 and I417 make contacts with the DNA, but available structural studies of the polymerase active center and the I417V substitution decreased replication fidelity by increasing nucleotide misincorporation and by increasing the ability of mutant DNA polymerases to extend a mismatched primer terminus (44). The Motif A sequence is conserved in all polymerases, and thus, this motif is presumed to provide an essential function. The biochemical basis for these properties detected for the L412M-DNA polymerase appears to be due to the increased affinity of the mutant for binding DNA in the polymerase active center (Table II). Another interesting point is that another mutant DNA polymerase, the T4 L412I-DNA polymerase, has increased replication fidelity (29). Thus, conservative amino acid substitutions for L412 in the Motif A sequence of the T4 DNA polymerase, L412M and L412I, have profound effects on DNA replication fidelity, which provides a glimpse of the exquisite fine-tuning of the polymerase active center.

Acknowledgments—We thank Linda B. Bloom and members of the L. R.-K. laboratory for helpful comments on the manuscript.

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