The Nucleotide Analog 2-Aminopurine as a Spectroscopic Probe of Nucleotide Incorporation by the Klenow Fragment of *Escherichia coli* Polymerase I and Bacteriophage T4 DNA Polymerase†

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ABSTRACT: The fluorescent properties and their sensitivity to the surrounding environment of the nucleotide analog 2-aminopurine (2-AP) have been well documented. In this paper we describe the use of 2-AP as a direct spectroscopic probe of the mechanism of nucleotide incorporation by *Escherichia coli* Pol I Klenow fragment (KF) and bacteriophage T4 DNA polymerase. The nucleotidyl transfer reaction may be monitored in real time by following the fluorescence of 2-AP, allowing the detection of transient intermediates along the reaction pathway that are inaccessible through traditional radioactive assays. Previous studies with Klenow fragment [Kuchta, R. D., Mizrahi, V., Benkovic, P. A., Johnson, K. A., & Benkovic, S. J. (1987) *Biochemistry 26*, 8410−8417] have revealed the presence of a nonchemical step prior to chemistry and have identified this conformational change as the rate-limiting step of correct nucleotide incorporation. During correct incorporation, phosphodiester bond formation occurs at a rate greater than the conformational change and has not been measured. However, during misinsertion, the rate of the chemical step becomes partially rate limiting and it becomes possible to detect both steps. We have successfully decoupled the chemical and conformational change steps for nucleotide insertion by KF using the misincorporation reaction, and we present direct spectroscopic evidence for an activated KF′-DNA-dNTP species following the conformational change step which features hydrogen bonding between the incoming and template bases. In addition, we have utilized these same experiments to demonstrate the existence of a similar nonchemical step in the mechanism of dNTP incorporation by bacteriophage T4 DNA polymerase. This study provides the first direct evidence of a conformational change for T4 polymerase and emphasizes the importance of this step in a general polymerase kinetic sequence.

DNA polymerases are a family of enzymes responsible for the faithful duplication of DNA, *in vivo*. Though individual polymerases differ in their size, structure, requirement of accessory proteins, and role in DNA replication, their fundamental purpose is to catalyze the nucleotidyl transfer reaction—the addition of dNTPs onto the end of the growing DNA chain. Polymerase-mediated primer extension has been the focus of several studies in recent years aimed at gaining insight into the high fidelity achieved by this group of enzymes. These studies include extensive kinetic characterization of the polymerization mechanism for *Escherichia coli* Pol I and Klenow fragment (McClure & Jovin, 1975; Bambara et al., 1976; Bryant et al., 1983; Mizrahi et al., 1985, 1986a,b; Kuchta et al., 1987, 1988; Eger et al., 1991; Dahlberg & Benkovic, 1991; Eger & Benkovic, 1992) and bacteriophage T4 and T7 DNA polymerases (Patel et al., 1991; Wong et al., 1991; Donlin et al., 1991; Capson et al., 1992). Recent reports from our laboratory (Kuchta et al., 1988; Dahlberg & Benkovic, 1991; Eger & Benkovic, 1992) have established a minimal kinetic mechanism governing both correct and incorrect nucleotide incorporation for the Klenow fragment of *E. coli* Pol I. A significant feature of the mechanism is the proposed conformational changes prior to and following the chemical step of incorporation. Evidence for these conformational changes was obtained through a series of rapid quench experiments utilizing traditional radioactive assays. However, this type of assay is limited by the fact that it only measures product formation and cannot directly detect the presence of transient intermediates.

In this paper we describe a fluorescence-based continuous assay utilizing the nucleotide analog 2-aminopurine (2-AP).[1] The relatively high intrinsic fluorescence of 2-AP coupled with its extreme sensitivity to the surrounding environment (Ward et al., 1969; Guest et al., 1991; Bloom et al., 1993, 1994; Hochstrasser et al., 1994; Raney et al., 1994) provide a unique look into discrete steps along the reaction pathway. The data presented clearly demonstrate the ability to directly detect the previously documented conformational change in Klenow fragment, and they provide the first evidence for the existence of such a step in the bacteriophage T4 DNA polymerase mechanism.

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[1] Abbreviations: Pol I, *E. coli* polymerase I; KF, Klenow fragment of pol I; KF exo⁻, (D335A, E357A) exonuclease deficient mutant of KF; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid, sodium salt; dNTP, deoxynucleoside 5′-triphosphate; T4 exo⁻, D219A exonuclease deficient mutant of bacteriophage T4 DNA polymerase.

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EXPERIMENTAL PROCEDURES

Materials. Radioactive nucleotides [α-^{32}P]dATP, [γ-^{32}P]-ATP, and [α-^{32}P]TTP were purchased from New England Nuclear. T4 polynucleotide kinase was supplied by United States Biochemical (USB). Unlabeled, ultrapure nucleotides were obtained from Pharmacia. The (*Sp*)-dATPαS was synthesized by Dr. Jin Tann Chen. All other materials were of the highest purity commercially available.

Klenow Fragment. The Klenow Fragment (KF) exo$^-$ (D355A,E357A) was purified according to published procedures (Derbyshire et al., 1988) and then further purified by column chromatography using a BioRex 70 (Bio-Rad) anion-exchange resin which had been equilibrated with 10 mM PIPES, pH 7.0, and 1 mM DTT (PD buffer). The protein was eluted with a 1-L linear gradient 0−1 M NaCl in PD buffer. Fractions containing KF exo$^-$ (determined by A_{278}) were combined, dialyzed against 50 mM HEPES, pH 7.4, and 1 mM DTT, diluted 1:1 with 100% ACS grade glycerol, and stored at −20 °C. The stock concentration of the KF exo$^-$ was determined by $\epsilon_{278} = 6.32 \times 10^4$ M^{-1} cm^{-1} (Setlow et al., 1972) and active site titration (see below). Both values were in close agreement.

T4 D219A Exonuclease-Deficient Polymerase. The exonuclease-deficient mutant of T4 DNA polymerase (D219A) was purified as described previously (Frey et al., 1993) and stored at −70 °C. The concentration of the enzyme stock was determined by active site titration (Capson et al., 1992).

Oligonucleotides. The 13-mer and 20-mer oligonucleotides (see Figure 1) were supplied by Operon Technologies. An identical 20-mer except for the substitution of 2-aminopurine (2-AP) for thymine at position 7 was synthesized using the phosphoramidite method and will be described elsewhere. All oligonucleotide single strands (ss) were subjected to Hoefer gel purification as previously described (Capson et al., 1992) except that single strands were suspended in 25 mM Tris−acetate, pH 7.5, after desalting. DNA duplexes (13/20-mer and 13/20-AP) were purified on 3-mm non-denaturing gels (20% acrylamide/1X TBE/no urea), recovered as previously described (Capson et al., 1992), and quantitated as described below.

Enzyme Assays. Klenow Fragment assays were carried out in 50 mM Tris-HCl, pH 7.5. T4 polymerase assays were done in a buffer system consisting of 50 mM Tris−acetate, pH 7.5, 60 mM KOAc, and 10 mM 2-mercaptoethanol. All reactions were carried out at 20 °C. The rapid quench experiments were performed on the instrument described by Johnson (1986). Fluorescence assays were done using an Applied Photophysics stopped flow spectrometer with an excitation wavelength of 310 nm and a band-pass of 10 nm. Emission was monitored by using a 330-nm cutoff filter. All concentrations are initial concentrations unless otherwise noted.

Gel Electrophoresis. For radioactive assays, a 10-μL aliquot was removed at each time point and combined with 10 μL of gel load buffer (90% deionized formamide, 1× TBE, 0.25% bromophenol blue, and 0.25% xylene cyanol). Samples (7.5 μL) were then separated on denaturing gels (20% acrylamide/8 M urea). Products were visualized and quantitated using the Molecular Dynamics PhosphorImager and ImageQuant software version 3.3.

Incorporation of dTTP by KF exo$^-$. The 13/20-AP substrate (0.75 μM) was preincubated with an excess of KF exo$^-$ (3 μM) in one syringe of the rapid quench device, and then mixed with an equal volume of a solution containing $MgCl_2$ (20 mM) and correct nucleotide, dTTP (80 μM). The mixture was allowed to react for varying amounts of time (5−2000 ms) before being quenched with EDTA (0.5 M, pH 8.0). Products were separated and visualized as described above, and the quantities of each were calculated by determining the relative amounts of 13-mer and 14-mer, applying a 25% correction factor (vide infra), and multiplying by the DNA concentration (μM).

Stopped Flow Fluorescence Assay of Polymerization by KF exo$^-$. Excess KF exo$^-$ (6 μM) was incubated with 13/20-AP substrate (1.5 μM), and the reaction was initiated by mixing with an equal volume of a solution containing $MgCl_2$ (20 mM) and dTTP (80 μM). Quenching of 2-AP fluorescence was seen as an increase in signal voltage and was converted to concentration of DNA product by the factor illustrated in the caption of Figure 3. Multiple time courses were averaged (4−6 runs) to ensure proper signal to noise.

Rapid Quench Assay To Monitor the Incorporation of dTTP by T4 D219A Polymerase. The concentrations used for this assay were identical to that used in the KF exo$^-$ assay. Polymerization was initiated by mixing equal volumes of the polymerase/DNA solution (E·D) and Mg^{2+}·dTTP solution. The reaction was terminated at various times by the addition of EDTA (0.5 M, pH 8.0). Products were analyzed and corrected as described above.

Polymerization on 13/20-AP Substrate by T4 D219A Polymerase Followed by Fluorescence. Conditions for this experiment were the same as those used in the KF exo$^-$ fluorescence assay. Reactions were initiated in the stopped flow instrument by mixing equal volumes of the E·D and Mg^{2+}·dTTP solutions. The data from seven runs were averaged, and the fluorescence signal was converted to DNA concentration by the factor described in Figure 3.

Stopped Flow Fluorescence Assay of the Misincorporation of dATP opposite Template 2-AP by KF exo$^-$. Excess KF exo$^-$ (6 or 8 μM) was preequilibrated with the 13/20-AP substrate (1.5 or 2 μM) in one syringe of the stopped flow device and pushed against an equal volume of $MgCl_2$ (20 mM) and incorrect nucleotide dATP (80 μM) from a second syringe. The data shown are an average of at least four consecutive runs.

Radioactive Gel Assay of Misincorporation of dATP by KF exo$^-$. In a total reaction volume of 220 μL, KF exo$^-$ (3 μM) was incubated with 13/20-AP (0.75 μM) in assay buffer. The reaction was initiated by the addition of $MgCl_2$·dATP (final concentrations of 10 mM and 40 μM, respectively), and the time course was followed by removing 5-μL aliquots and quenching into 5 μL of EDTA (0.5 M, pH 8.0) at variable time intervals. Load buffer (10 μL) was added to each time point aliquot, and the products were separated and quantitated as described above.

Misincorporation of dATP opposite Template 2-AP by T4 D219A Followed by Fluorescence. An excess of T4 D219A polymerase (6 μM) was preincubated with 13/20-AP substrate (1.5 μM), and the reaction was initiated in the stopped flow system by mixing with an equal volume of a solution containing $Mg(OAc)_2$ (20 mM) and dATP or dATPαS (1 mM).

Radioactive Assay of T4 D219A Polymerase Misincorporation opposite Template 2-AP. 13/20-AP (0.75 μM) and T4 D219A polymerase (3 μM) were preequilibrated in a total

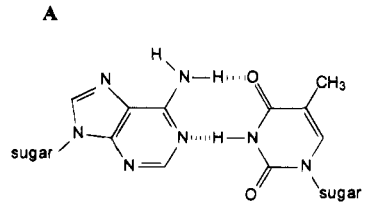
reaction volume of 200 μL. The reaction was initiated by the addition of $Mg(OAc)_2$·dATP or $Mg(OAc)_2$·dATPαS (final concentrations of 10 mM and 500 μM, respectively), and the progress was followed by quenching 5-μL aliquots into 5 μL of EDTA (0.5 M, pH 8.0) at various times. Load buffer (10 μL) was added to each time point aliquot, and the products analyzed as stated above.

Determination of Active 13/20-AP Concentration. An accurate concentration of extendible ends was determined by the incorporation of [α-^{32}P]TMP under conditions of excess KF exo$^-$ (5 μM) over 13/20-AP substrate (varying concentrations), 10 μM dTTP (10 000 cpm/pmol), 5 mM $MgCl_2$, and 50 mM Tris-HCl, pH 7.5, in a total volume of 50 μL. Reactions were initiated by the addition of enzyme and were quenched after 10 or 60 s with either 0.1 M EDTA (20 μL) or phenol/chloroform (50 μL). Water (20 μL) was added to those reactions quenched by extraction. The amount of incorporated [α-^{32}P]TMP was determined by filter binding assay (Bryant et al., 1983), and the stock concentrations was calculated. To be sure that all 13/20-AP had been utilized, the extracted reaction samples (10 pmol) were 5′-^{32}P-end-labeled using standard protocols, separated on a 20% acrylamide denaturing gel, and visualized using the Molecular Dynamics PhosphorImager (data not shown). Through multiple trials it was found that approximately 25% of the 13-mer primer remained unextended up to 60 s.[2] Although the exact identity of this species is not known, it is not believed to interfere with the fluorescence experiments at hand because, upon conversion of the fluorescence data to DNA concentration, a full inventory of DNA product is obtained. However, the presence of this labeled, nonextendible 13-mer in the rapid quench assays interferes with the calculation of product formation (recall that the concentration of product is described by 14-mer cpm/(13-mer cpm + 14-mer cpm = rel%). Therefore, for the rapid quench assays, all data has been corrected for the unused 25% of 13-mer.

RESULTS

Kinetics of Incorporation of dTTP opposite Template 2-Aminopurine by KF exo$^-$. The DNA duplex structure remains relatively undisturbed when the nucleotide analog 2-aminopurine forms a Watson–Crick type base pair with thymine (Sowers et al., 1986). However, recent experiments by Bloom et al. (1993) suggested an alteration of the mechanism of incorporation by KF exo$^-$ when the 2-aminopurine 2′-deoxyribonucleoside 5′-triphosphate (2-APTP) was used (i.e., no burst of stoichiometric incorporation). Therefore, we wished to examine the kinetics of incorporation of dTTP opposite template 2-AP to determine what differences, if any, are manifest.

A 20-mer oligonucleotide with 2-AP at the 7th position was synthesized and annealed to a 13-mer primer, providing the primer/template (P/T) system shown in Figure 1. Initial rapid quench experiments to examine the pre-steady-state and steady-state rates of incorporation of a correct deoxynucleotide opposite template 2-AP by KF exo$^-$ revealed an initial burst phase followed by a second slower phase (M.



B

13/20-mer substrate:

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5' - TCGCAGCCGTCCA
3' - AGCGTCGGCAGGTTCCCAAA
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13/20-AP substrate:

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5' - TCGCAGCCGTCCA
3' - AGCGTCGGCAGGTAPCCCAAA
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FIGURE 1: (A) Watson–Crick type base pairing for the normal deoxyadenosine–thymidine and 2-aminopurine deoxynucleoside–thymidine base pairs. (B) Duplex DNA sequences. The numbers indicate the lengths of the primer/template. The two substrates differ only in the substitution of the nucleotide analog 2-aminopurine for the thymine at position 7 of the template strand (bold).

W. Frey unpublished results) reminiscent of previous experiments with the normal 13/20-mer substrate (Kuchta et al., 1987; Dahlberg & Benkovic, 1991; Eger & Benkovic, 1992). To measure the burst rate of incorporation more accurately, an experiment with excess enzyme over 13/20-AP substrate (single-turnover conditions) was done. A solution containing KF exo$^-$ (3 μM) and 13/20-AP (0.75 μM) in assay buffer was mixed with an equal volume of a solution containing $MgCl_2$ (20 mM) and dTTP (80 μM). The resulting time course is shown in Figure 2. The data were fit to a single exponential, and the burst rate constant was found to be on the order of 7.4 s^{-1} (Table 1).

Stopped Flow Fluorescence Assay of Incorporation of dTTP. The sensitivity of 2-AP to its environment, and its change in fluorescence upon going from single-stranded to duplex DNA have been well documented (Bloom et al., 1993, 1994; Ward et al., 1969; Guest et al., 1991; Hochstrasser et al., 1994; Raney et al., 1994). These properties, along with its similarity to the naturally occurring deoxyadenosine, foretell its use as a spectroscopic probe of polymerase action. The polymerization reaction was initiated in the stopped flow instrument by mixing equal quantities of an E·D solution (KF exo$^-$, 6 μM; 13/20-AP, 1.5 μM) and a Mg^{2+}·dTTP solution (20 mM $MgCl_2$; 80 μM dTTP). The reaction was monitored by the quenching of 2-AP fluorescence as the 13/20-AP was converted to 14/20-AP. The data shown in Figure 3 were converted to DNA concentration and fit to a single exponential, yielding a burst rate constant of 7.7 s^{-1} (Table 1). This value is in close agreement with that determined from the radioactive gel assay and indicates that the same step is being measured in both assays.

[2] In the misincorporation reactions followed by radioactivity, with time points > 10 min, the unused 13-mer primer described above was slowly utilized, perhaps as the polymerase slowly bypassed a compromised site (apurinic).

Table 1: Rate Constants Determined by Fluorescence or Radioactive Assays for Nucleotide Incorporation

enzyme	fluorescence assay (s^{-1}) 1st phase	fluorescence assay (s^{-1}) 2nd phase	radioactive assay (s^{-1})
KF exo$^-$			
correct incorporation		7.7 ± 0.1	7.4 ± 0.7
incorrect incorporation	0.17 ± 0.08	$0.0025 \pm 1.5 \times 10^{-4}$	$0.0025 \pm 2 \times 10^{-4}$
T4 exo$^-$			
correct incorporation		228 ± 5	220 ± 10
incorrect incorporation (dATP)		$0.02 \pm 6 \times 10^{-4}$	$0.021 \pm 5 \times 10^{-4}$
incorrect incorporation (dATPαS)	0.49 ± 0.02	$0.010 \pm 5.2 \times 10^{-4}$	$0.010 + 4 \times 10^{-4}$

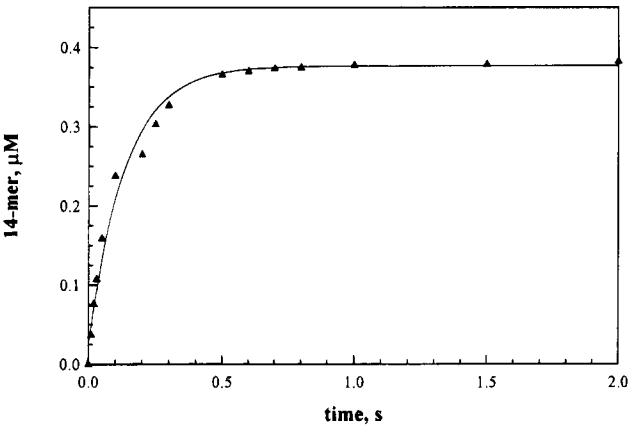


FIGURE 2: Incorporation of dTTP opposite template 2-AP as determined by rapid quench kinetics. Excess KF exo$^-$ (3 μM) was incubated with 13/20-AP (0.75 μM), and polymerization was initiated in the rapid quench instrument by mixing an aliquot of this solution with an equal volume of a solution containing $MgCl_2$ (20 mM) and dTTP (80 μM). The reaction was quenched at various times by the addition of 0.5 M EDTA, pH 8.0 (final concn = 0.35 M EDTA). Products were separated and analyzed as described in Experimental Procedures. Concentrations are initial concentrations unless otherwise noted. The data were fit to a single exponential with a rate constant of 7.4 s^{-1}.

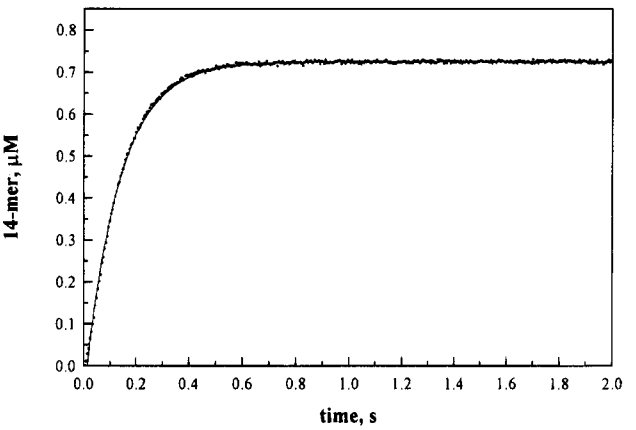


FIGURE 3: Stopped flow fluorescence assay of polymerization by KF exo$^-$. Excess KF exo$^-$ (6 μM) was preincubated with 1.5 μM 13/20-AP substrate (ED solution). Reactions were initiated by mixing an aliquot of the ED solution with an equal volume of a solution containing $MgCl_2$ (20 mM) and dTTP (80 μM). The data shown are an average of six consecutive runs and have been converted to DNA concentration using the factor DNA (μM) = $(\Phi + \Phi_0)/\Delta\Phi$XDNA (initial 13/20-mer DNA concentration), where Φ = fluorescence intensity at time t, Φ_0 is the initial fluorescence intensity, and $\Delta\Phi$ is the total change in fluoresence intensity. The data were fit to a single exponential, and the rate constant was determined to be 7.7 s^{-1} (Table 1). Unlike the radioactive assays, the DNA concentration is not corrected for the 25% unused material described in Experimental Procedures because it is not used by the polymerase and does not interfere with the calculation of product formation.

Rapid Quench Assay of dTTP Incorporation by T4 D219A pol. With the availability of the 13/20-AP substrate, another polymerase, T4 D219A pol (T4 exo$^-$), was examined in an attempt to further detail the kinetic mechanism of dNTP incorporation (see Discussion). Preliminary rapid quench experiments revealed biphasic kinetics for nucleotide incorporation opposite 2-AP by T4 exo$^-$ (M. W. Frey, unpublished results), similar to that reported previously (Frey et al., 1993). Conditions for the excess enzyme experiment were identical to those used in the KF exo$^-$ assay, and the time points were taken on the rapid quench instrument. The corrected data are shown in Figure 4. A computer fit to a single exponential provided a rate constant of 220 s^{-1}.

Fluorescence Assay of Polymerization by T4 D219A pol. Again, the conditions used for the KF exo$^-$ enzyme were applied to the T4 exo$^-$ and 13/20-AP experiment. T4 D219A (6 μM) was preincubated in the presence of 13/20-AP substrate (1.5 μM) in one syringe of the stopped flow instrument. The reaction was initiated by mixing an aliquot of the E·D solution with an equal volume of a Mg^{2+}·dTTP solution (20 and 80 μM, respectively) from a second syringe. The progress of the reaction was followed by the quenching of the 2-AP fluorescence, and the resulting change in Φ data converted to DNA concentration. The data and its fit to a single exponential are shown in Figure 5. The rate constant determined from the fluorescence assay, 228 s^{-1}, is in close agreement with that of the rapid quench gel electrophoresis assay.

Examination of the Misincorporation of dATP opposite Template 2-AP by Both Fluorescence and Radioactive Gel Assays. A tool for the further dissection of the polymerization mechanism is the misincorporation reaction. The misincorporation of dATP opposite template 2-AP in the 13/20-AP substrate by both polymerases (KF exo$^-$ and T4 D219A pol) was evaluated by both fluorescence and radioactive assays. The data from two representative time courses are shown in Figures 6 and 7. The raw fluorescence signal for the misincorporation of dATP opposite 2-AP by KF exo$^-$ is shown in the inset of Figure 6. An interesting feature of this time course is its apparent biphasic nature. The experiment, which is carried out under single-turnover conditions (excess enzyme over 13/20-AP substrate), is expected to fit a single exponential. However, the data are best described by a double exponential (inset, Figure 6). The identities of these two phases will be discussed below.

In contrast, the radioactive gel electrophoresis assay time course for the misincorporation of dATP opposite 2-AP by KF exo$^-$ (Figure 6, ●) is not biphasic, and it is best fit to a single exponential with a misincorporation rate constant of ca. 0.002 s^{-1} (Table 1). The second phase of the stopped flow fluorescence data which has been converted to DNA (μM) is overlayed with the gel assay data in Figure 6. The

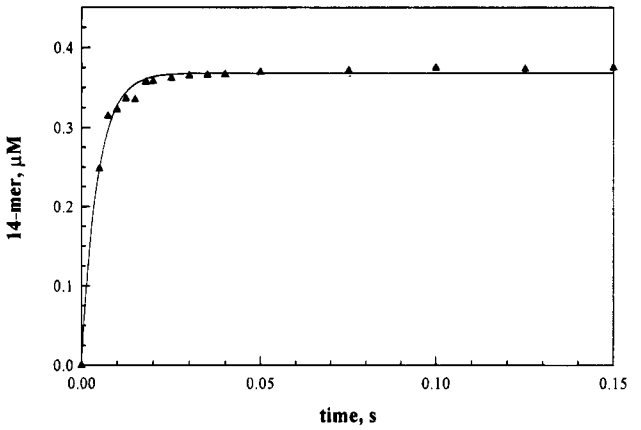


FIGURE 4: Rapid quench determination of the incorporation of dTTP by T4 D219A (T4 exo$^-$). Concentrations were identical with that used for the KF exo$^-$ (Figure 2). The reaction was initiated by mixing equal volumes of the two solutions and quenched at various times (5−150 ms) by the addition of 0.5 M EDTA, pH 8.0. (final concn = 0.35 M EDTA). Products were analyzed as described in Experimental Procedures. The resulting time course was fit to a single exponential, yielding a rate constant of 220 s^{-1} (Table 1).

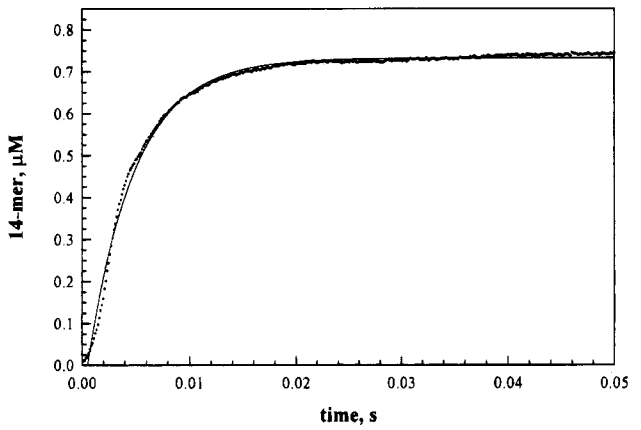


FIGURE 5: Fluorescence analysis of the polymerization by T4 D219A. Conditions were the same as those used for the KF exo$^-$ fluorescence assay. Reactions were initiated in the stopped flow by mixing equal volumes of an ED solution (6 μM T4D219A, 1.5 μM 13/20-AP) and a Mg^{2+}•dTTP solution (20 mM $Mg(OAc)_2$; 80 μM dTTP). The data shown are an average of seven runs, and fluorescence was converted to DNA (μM) by the factor illustrated in the caption of Figure 3. The data were fit to a single exponential, the rate constant of 228 s^{-1} is shown in Table 1.

data for both experiments are nearly identical, and the respective rate constants are equal within error (Table 1).

Similar experiments were carried out using the T4 exo$^-$ enzyme and the 13/20-AP DNA substrate. For those assays utilizing dATP, the reaction profile for both the fluorescence and radioactive assays (data not shown) is best described by a single exponential, unlike the results obtained for the KF exo$^-$ enzyme. However, when the α-phosphorothioate-substituted dATP is employed, the biphasic nature of the misincorporation reaction is again evident in the fluorescence-based assay (inset, Figure 7) but is absent from the radioactive assay (Figure 7, ●). The fluorescence data are best fit by a double exponential, and the exponential component describing the second phase overlays with the radiolabeled assay in Figure 7. The rate constants derived from the overlayed phases are nearly identical (0.01 s^{-1}) as shown in Table 1.

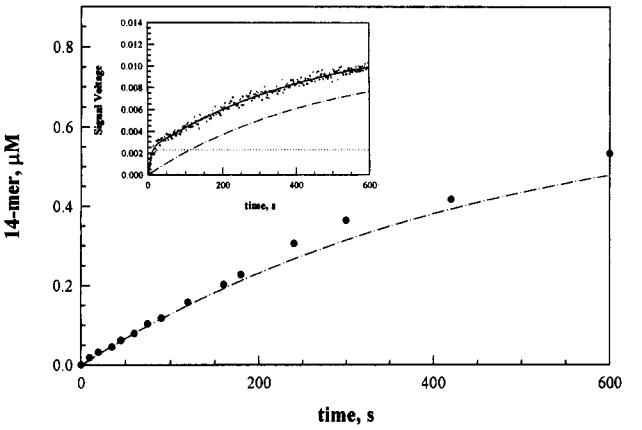


FIGURE 6: (Inset) Stopped-flow fluorescence assay of the misincorporation of dATP opposite template 2-AP by KF exo$^-$. Excess KF exo$^-$ (6 μM) was incubated with 13/20-AP substrate (1.5 μM) and pushed against an equal volume of Mg^{2+}•dATP solution (20 mM and 80 μM, respectively) in the stopped flow. The resulting time course was fit to the sum of two single exponentials. The identity of the initial burst is discussed in the text. The second phase of the fluorescence experiment was converted to DNA (μM) (•—•) and overlayed with the data from the radioactive gel assay data (●), indicating the second phase of the fluorescence assay time course is representative of product formation. Rate constants for both are on the order of 0.002 ± 0.0003 s^{-1} and are identical within error.

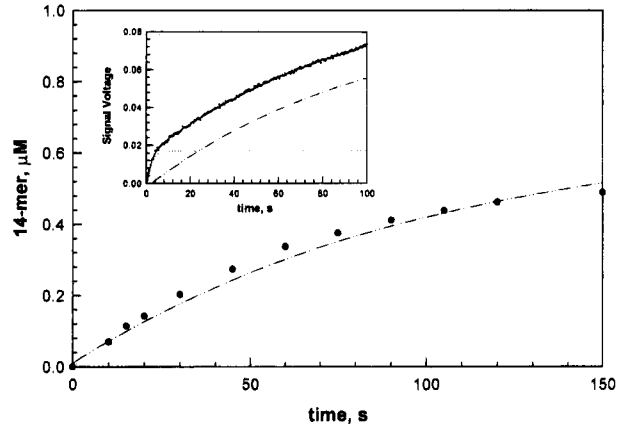
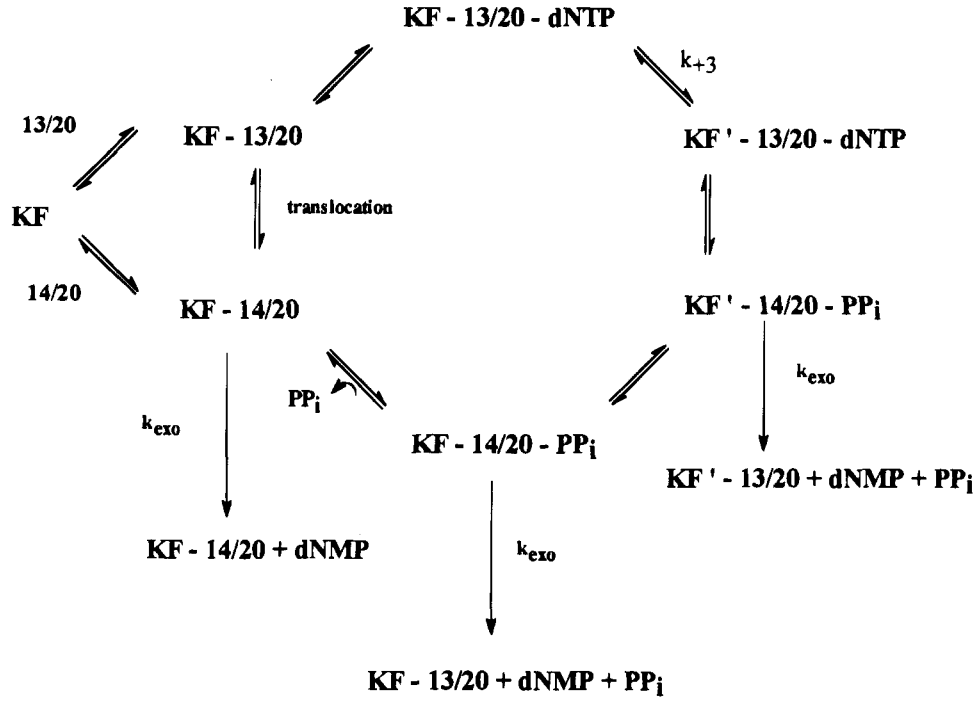


FIGURE 7: (Inset) Stopped flow fluorescence assay of the misinsertion of dATPαS opposite template 2-AP by T4 exo$^-$. Excess T4 exo$^-$ (6 μM) and 13/20-AP (1.5 μM) were preincubated in one syringe, and the reaction was initiated in the stopped flow by mixing with an equal volume of a Mg^{2+}•dATPαS solution from another syringe. The resulting biphasic time course was fit to the sum of two single-exponential functions. The second component of the fluorescence experiment was converted to DNA (μM) as described in the caption of Figure 3 (•—•) and is overlayed with the radioactive assay data (●). The rate constants derived for both are equal within error and are shown in Table 1.

DISCUSSION

The kinetic mechanism by which DNA polymerases carry out nucleotide incorporation has been the subject of intense study for the past decade [reviewed in Johnson (1993), Echols & Goodman (1991), Wang (1991), Young et al. (1992), Kornberg and Baker, (1992), McHenry (1991), Richardson et al. (1987), and Carroll and Benkovic (1990)]. Several researchers have attempted to elucidate the polymerization reaction pathway through the use of pre-steady-state and steady-state kinetic analyses which utilize radiolabeled substrates as a means of observing the progress of a given reaction. These experiments have provided great

Scheme 1



insight into the discrete steps along the polymerization pathway for several polymerases (Kuchta et al., 1988; Patel et al., 1991; Wong et al., 1991; Eger & Benkovic, 1992; Capson et al., 1992). However, there is a limitation to the radioactive gel assay, which only measures product formation and does not detect transient intermediates along the reaction pathway. In this paper we describe the use of the nucleotide analog 2-AP as a fluorescent probe of polymerase action. Previous reports have demonstrated that the kinetic mechanisms of nucleotide incorporation by KF exo$^-$ and T4 D219A polymerase (T4 exo$^-$) remain unchanged compared to the wild-type counterparts (Kuchta et al., 1988; Frey et al., 1993). The mutants were employed in the present study to avoid the complicating effects of exonuclease activity.

The DNA template strand was synthesized substituting 2-aminopurine 2′-deoxyribonucleoside at position 7, opposite the point of nucleotide insertion. The sensitivity of 2-AP fluorescence to its surrounding environment (Ward et al., 1969; Guest et al., 1991; Bloom et al., 1993, 1994; Hochstrasser et al., 1994; Raney et al., 1994) provides a spectroscopic handle with which to evaluate nucleotide incorporation. Since the fluorescence can be monitored continuously during the course of the reaction, the possibility exists of detecting intermediate species. Previous studies in our laboratory have been aimed at delineating the kinetic mechanism of nucleotide incorporation by the Klenow fragment of Pol I (Kuchta et al., 1987, 1988; Dahlberg & Benkovic, 1991; Eger & Benkovic, 1992) and bacteriophage T4 DNA polymerase (Capson et al., 1992; Frey et al., 1993). For the Klenow fragment, the minimal kinetic sequence is shown in Scheme 1. Of note is the rate-limiting step of processive synthesis, 50 s^{-1}, designated k_{+3}. This step has been assigned to a conformational change preceding chemistry for both correct (Kuchta et al., 1988; Dahlberg & Benkovic, 1991) and incorrect incorporation (Eger & Benkovic, 1992). Experiments presented here directly illustrate the presence of such a conformational change preceding the chemical step of phosphodiester bond formation, and permit comment on the nature of the KF′-13/20-AP-dNTP species.

Recent work by Bloom and co-workers described fluorescence-based pre-steady-state measurements of the insertion of 2-aminopurine 2′-deoxyribonucleoside 5′-triphosphate (dAPTP) into duplex DNA by Klenow fragment. However, no burst of nucleotide incorporation was observed in these experiments, making it difficult to interpret the origin of the fluorescence transients. In contrast, our preliminary experiments with 2-AP present in the template strand of the DNA substrate exhibited biphasic kinetics under conditions of limiting KF exo$^-$ (M. W. Frey, unpublished results). Studies were then undertaken to identify and measure the processes being observed by comparing the fluorescence and rapid quench kinetics assays.

The 13/20-AP substrate was used in the traditional radioactive rapid quench assay in order to compare the burst rate of nucleotide incorporation into the 13/20-AP substrate with that of the standard 13/20-mer (50 s^{-1}). Under conditions of excess KF exo$^-$ (to avoid the complicating slow step of DNA dissociation) the rate constant for dTTP (correct) incorporation was found to be ca. 7 s^{-1}, approximately 7-fold slower than that on the normal 13/20-mer. The stopped flow fluorescence assay, in which the quenching of the intrinsic fluorescence of the 2-AP moiety was monitored, yielded, within error, a similar rate constant. In view of previous evidence, it appears that the quenching of AP fluorescence is associated with a conformational change followed by a rapid phosphoryl bond formation.

Previous studies have shown that the 2-aminopurine is more soluble than the naturally occurring purines (Albert & Brown, 1954); 10 times more soluble than adenine, and 1000 times more soluble than guanine. The exclusion of water from the active site has been postulated (Petruska et al., 1988) to magnify the differences in free energy between correct and incorrect base pairs and to account for the high fidelity observed for most polymerases. The 7-fold decrease in the

observed rate here may reflect the exclusion of additional water molecules from the polymerase active site during incorporation opposite 2-AP. In turn, the conformational change may be responsible for the removal of water from the active site, and the presence of additional water slows the conformational change with respect to the 13/20-AP substrate.

To obtain further direct evidence for the proposed conformational change, a misincorporation reaction was employed. Experiments by Eger and Benkovic (1992) had previously demonstrated that the chemical step in the misincorporation process was slowed as compared to that in correct incorporation. For correct incorporation, chemistry occurs at a rate greater than the conformational change step, and the two processes are not distinguished. However, during misinsertion, where the rate of the chemical step is rate limiting, it may be possible to observe both chemistry and conformational change steps using both assay methods.

The misincorporation of dATP opposite template 2-AP was evaluated using both the fluorescence- and the radioactivity-based assays under conditions of excess enzyme. In the fluoresence experiment a double-exponential trace was acquired (Figure 6, inset). The radioactive gel electrophoresis assay, which measures the formation of 14(A)/20-AP mismatched products at all points along the reaction pathway (Figure 6, ●), is *not* biphasic, and it clearly matches the second component of the stopped flow fluorescence assay. Because no formation of a DNA product is associated with the initial phase found in the fluorescence assay, we believe this phase is representative of a change in the KF-13/20-AP fluorescence prior to product formation and after nucleotide binding. [Kinetic simulations were carried out using Scheme 1 and the rate constants reported previously (Eger & Benkovic, 1992) to demonstrate that the partial quenching of fluorescence associated with the initial phase was not due to simple collisional quenching by dATP of the ED species. This quenching would occur at the diffusion-limited rate of dATP binding, much faster than the observed rate of the initial fluorescence phase.] We therefore conclude that the partial quenching of 2-AP fluorescence observed in the misincorporation of dATP is indicative of an enzyme conformational change that aligns the incoming nucleotide and DNA substrate with partial hydrogen bonding in a position poised for phosphodiester bond formation. This configuration presumably facilitates interactions between the electronically excited AP chromophore and neighboring DNA bases, resulting in the observed fluorescence quenching. The rate constant for the initial phase (0.17 s^{-1}, Table 1) is somewhat slower than that measured for the conformational change during correct incorporation and may be explained by an overall slowing of the reaction as a result of the formation of an incorrectly base paired product.

Having demonstrated the ability to detect the previously documented conformational change of KF during nucleotide insertion, we attempted to identify a similar step for the bacteriophage T4 DNA polymerase. Previous attempts (Capson et al., 1992) to obtain support for a conformational change in the T4 DNA polymerase mechanism were unsuccessful. Experiments to examine the incorporation of a correct nucleotide into the 13/20-AP substrate were carried out with the T4 exo$^-$ as described for the KF exo$^-$. The fluorescence and radioactive gel assays yielded rate constants of 220 and 228 s^{-1}, respectively (Figures 4 and 5), a factor of 2 slower than that measured for the normal 13/20-mer. The misincorporation reaction was again utilized to decouple chemistry and a conformational change. The experiment was first attempted using dATP (data not shown). Unlike the results obtained with KF exo$^-$, the fluorescence assay was not biphasic and was best described by a single-exponential fit with a rate constant of 0.02 s^{-1}. The rate of misincorporated product formation is an order of magnitude faster than that measured for the KF exo$^-$, suggesting that the failure to detect a conformational change with T4 polymerase is due to the intrinsically higher rate of phosphodiester bond formation by this enzyme.

Table 2: Rates of Correct and Incorrect Nucleotide Incorporation by KF exo$^-$ and T4 Polymerase exo$^-$

enzyme	substrate	nucleotide	rate (s^{-1})	ratio
KF exo$^-$	13/20-mer (correct)	dATP	50[a]	
	9/20-mer (incorrect)	dATP	0.025[a]	2×10^3
	13/20-AP (correct)	dTTP	7.4	
	13/20-AP (incorrect)	dATP	0.0025	2.8×10^3
T4 exo$^-$	13/20-mer (correct)	dATP	400[b]	
	13/20-mer (correct)	dATPαS	200[b]	
	14/20-mer[c] (incorrect)	dATP	0.028	1.4×10^4
	13/20-AP (correct)	dTTP	228	
	13/20-AP (incorrect)	dATP	0.020	1.1×10^4
	13/20-AP (incorrect)	dATPαS	0.010	

[a] Data are from Eger and Benkovic (1992). [b] Data are from Capson et al. (1992). [c] The sequence of this 14/20-mer is

TGACGCACGTTGTC
ACTGCGTGCAACAGACTACG

(M. W. Frey, unpublished observations).

In an attempt to further slow the rate of chemistry, the phosphorothioate analog, dATPαS, was employed. The results shown in Figure 7 are very similar to those observed for the misincorporation by the KF exo$^-$. The fluorescence assay is again biphasic with the actual product formation (0.01 s^{-1}) being described by the second-exponential component, much like that seen for the KF exo$^-$, the 2-fold reduction in rate with dATPαS compared to dATP being the same as that previously determined for correct incorporation by T4 DNA polymerase (Capson et al., 1992). The initial phase of AP fluorescence quenching (0.49 s^{-1}, Table 1), is the first direct evidence for the existence of a conformational change in the kinetic mechanism of nucleotide incorporation by T4 DNA polymerase. Consequently, the kinetic step observed for the correct incorporation by T4 polymerase may also represent a conformational change prior to chemistry. A summary of the direct comparison of rate constants for correct and incorrect nucleotide incorporation on both substrates by both enzymes is presented (Table 2). The ratios for correct and incorrect insertion for both substrates are nearly identical, suggesting that no overall change in mechanism has occurred.

CONCLUSIONS

In this paper we have demonstrated the usefulness of the nucleotide analog 2-AP as a fluorescent probe of polymerase mechanism. A comparison of the ratios of correct vs. incorrect nucleotide incorporation on either 13/20-mer or 13/20-AP substrates for both polymerases indicates that no overall change in mechanism has occurred when 2-AP is substituted for thymine in the template. Rates measured with either the traditional radioactive assay or the fluorescence-

based assay are nearly identical. By monitoring the misinsertion reaction, we have detected the presence of an intermediate species that exists prior to phosphodiester bond formation, providing direct spectroscopic evidence for the proposed KF′-DNA-dNTP species. Furthermore, examination of T4 DNA polymerase by these methods has provided the first direct evidence of a previously undetected conformational change prior to chemistry and after nucleotide binding. The previous identification of a similar conformational change with the Klenow fragment (Eger & Benkovic, 1992; Dahlberg & Benkovic, 1991; Kuchta et al., 1988) as well as with T7 DNA polymerase (Wong et al., 1991) attests to the importance of this conformational change as a pivotal element in polymerase fidelity. This step, whether viewed as part of an induced fit mechanism for polymerase fidelity or as a necessary feature of nucleotide incorporation, acts as a discriminatory element in polymerase fidelity owing to its sensitivity to the nature of the nucleotide (complementary or noncomplementary to template strand). Differences in the rates of the conformational change step and in the chemical addition of nucleotides to the primer that have been observed for the KF exo$^-$ and T4 D219A polymerases may simply reflect the differing roles of these two polymerases in the cell, where the former serves as a repair enzyme and the latter as a replicative one.

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