Using 2-Aminopurine Fluorescence To Detect Base Unstacking in the Template Strand during Nucleotide Incorporation by the Bacteriophage T4 DNA Polymerase†

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ABSTRACT: The fluorescence of the base analogue 2-aminopurine (2AP) was used to detect physical changes in the template strand during nucleotide incorporation by the bacteriophage T4 DNA polymerase. Fluorescent enzyme−DNA complexes were formed with 2AP placed in the template strand opposite the primer terminus (the *n* position) and placed one template position 5′ to the primer terminus (the $n + 1$ position). The fluorescence enhancement for 2AP at the *n* position was shown to be due to formation of the editing complex, which indicates that the 2AP-T terminal base pair is recognized primarily as a mismatch. 2AP fluorescence at the $n + 1$ position, however, was a reporter for DNA interactions in the polymerase active center that induce intrastrand base unstacking. T4 DNA polymerase produced base unstacking at the $n + 1$ position following formation of the phosphodiester bond. Thus, the increase in fluorescence intensity for 2AP at the $n + 1$ position could be used to measure the nucleotide incorporation rate in primer extension reactions in which 2AP was placed initially at the $n + 2$ position. Primer extension occurred at the rate of about 314 s^{-1}. The amount of base unstacking at the template $n + 1$ position was sensitive to the local DNA sequence. More base unstacking was detected for DNA substrates with an A-T base pair at the primer terminus compared to C-G or G-C base pairs. Since proofreading is also increased by A-T base pairs compared to G-C base pairs at the primer terminus, we propose that base unstacking may provide an opportunity for the DNA polymerase to reexamine the primer terminus.

DNA polymerases catalyze cycles of template-directed nucleotide incorporation. High-fidelity replication is achieved by the combination of accurate nucleotide incorporation and exonucleolytic proofreading (reviewed in ref *1*). These activities require several coordinated structural changes within the DNA polymerase−DNA complex, which include binding the correct dNTP, formation of the phosphodiester bond, dissociation of PP_i, and translocation of the enzyme to position the DNA template for incorporation of the next nucleotide (*2*−*6*; Figure 1). If the nucleotide incorporated is incorrect, the primer is usually not extended; instead, the primer terminus is separated from the template strand and transferred to the exonuclease active center where the incorrect nucleotide is removed (*2*−*5*, *7*−*12*; Figure 1).

To learn how DNA polymerases determine if a primer terminus is a substrate for continued nucleotide incorporation or for proofreading, the branch point in Figure 1, we have examined the nucleotide incorporation reaction catalyzed by the bacteriophage T4 DNA polymerase. While a comprehensive study of nucleotide incorporation by the T4 DNA polymerase has been done using a radioactive-based, rapid-quench assay (*5*), we have used the intrinsic fluorescence of the base analogue 2-aminopurine (2AP)[1] to detect reaction intermediates that cannot be observed in radioactive assays. 2AP fluorescence is a sensitive reporter of base-stacking interactions in DNA. A high level of fluorescence intensity is observed for the free 2AP nucleotide, but fluorescence intensity is quenched when 2AP resides in single- or double-stranded DNA (*7*, *13*). Enzyme interactions with 2AP-labeled DNA that disrupt 2AP base-stacking interactions, however, can produce large increases in fluorescence intensity (for examples see refs *7*−*11*, *14*, and *15*). An increase in fluorescence intensity has been reported for T4 DNA polymerase complexes formed with DNA labeled at various positions with 2AP in the template strand (*11*, *16*). We have characterized the fluorescent complexes formed with 2AP placed in the template *n* and $n + 1$ positions to determine the DNA polymerase−DNA interactions, and we have used changes in 2AP fluorescence intensity to measure nucleotide incorporation rates using rapid-mixing, stopped-flow techniques.

We report the first use of 2AP fluorescence-based assays to determine nucleotide incorporation rates in which 2AP is not a base-pairing partner. These studies provide information on the kinetics for incorporation of correct nucleotides by the bacteriophage T4 DNA polymerase. These studies also provide new information on physical changes in the template

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[1] Abbreviations: 2AP, 2-aminopurine; E^{pol}·D, binary DNA polymerase−DNA complex; E^{pol}·D·dNTP·Mg^{2+}, ternary DNA polymerase−DNA−nucleotide complex with Mg^{2+}.

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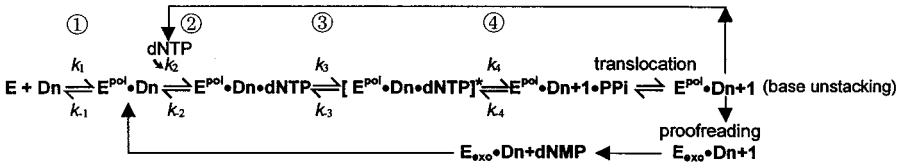

FIGURE 1: Nucleotide incorporation pathway adapted from refs *1*−*5*. Details are explained in the text.

strand during nucleotide incorporation, which may affect the decision by the DNA polymerase to continue primer elongation or to proofread.

MATERIALS AND METHODS

Enzymes. Purification and characterization of the wild-type and the exonuclease-deficient D112A/E114A T4 DNA polymerases have been described (*17*, *18*).

DNA Substrates. Oligonucleotides were synthesized using standard solid-state chemistry (*9*, *10*). Template and primer strands were annealed as described (*7*). The 3′-terminus of template strands was protected from DNA polymerase binding and exonuclease digestion by addition of a biotin attachment (BiotinTEG-CPG, Glen Research). The 3′-end modifications of the template strand ensured that all DNA polymerase−DNA interactions were directed to the primer terminus. Chain-terminated DNA substrates were synthesized in DNA polymerase reactions with ddGTP. Samples of the DNA substrates were labeled at the 5′-ends with ^{32}P by T4 polynucleotide kinase and [γ-^{32}P]ATP before and after reactions, and the products were run on denaturing polyacrylamide gels to verify the authenticity of the starting substrate, to verify primer elongation, and to verify that the substrates were not degraded during the reactions.

Steady-State Fluorescence Emission Spectra. Emission spectra 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 from 330 to 460 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. Enzyme titrations were performed to determine the enzyme:DNA ratio required to achieve maximum fluorescence intensity. Intrinsic protein fluorescence was subtracted from the reported spectra.

Pre-Steady-State Nucleotide Incorporation Reactions. Stopped-flow experiments were performed with the Applied Photophysics SX.18 MV spectrofluorometer. Excitation was at 310 nm; a 335 nm cutoff filter was used. The temperature in the sample-handling unit was maintained at 20.0 ± 0.5 °C.

To use 2AP fluorescence to measure primer elongation, 2AP was placed at the $n + 2$ position in the template strand. Primer extension produced an increase in fluorescence intensity. Primer extension reactions were initiated by mixing equal volumes of a solution of T4 DNA polymerase, DNA with 2AP at the $n + 2$ template position, and EDTA in buffer [25 mM HEPES (pH 7.6 or 6.5), 50 mM NaCl, 1 mM DTT] with a second solution containing Mg^{2+} and dNTP in the same buffer. The concentrations of reaction components after mixing were 500 nM T4 DNA polymerase, 200 nM $n + 2$ 2AP-labeled DNA, 1 mM DTT, 8 mM $MgCl_2$, 25 mM HEPES (pH 7.6 or 6.5), 0.25 mM EDTA, 50 mM NaCl, and variable concentrations of dNTP. Pre-steady-state rates for incorporation of a single nucleotide were determined by measuring the rate of increase in fluorescence intensity with time due to elongation of the primer to place 2AP at the template $n + 1$ position. Curves were fit to single- or double-exponential equations using the kinetic software package supplied by Applied Photophysics. Eight or more determinations were performed for each experiment, and the mean values were calculated.

Determination of the Rate of T4 DNA Polymerase Binding to DNA Labeled at the n + 1 Position with 2AP. The T4 DNA polymerase binding rate was determined in the reaction conditions used for primer elongation described above. One solution containing T4 DNA polymerase in buffer was mixed in the stopped-flow apparatus with an equal volume of a second solution containing the $n + 1$ 2AP-labeled DNA substrate. The concentrations of reaction components after mixing were 100−500 nM T4 DNA polymerase, 200 nM 2AP-labeled DNA, 1 mM DTT, 25 mM HEPES (pH 7.6 or 6.5), 0.25 mM EDTA, and 50 mM NaCl. Data were analyzed as explained above.

RESULTS AND DISCUSSION

Formation of Fluorescent DNA Polymerase−DNA Complexes. 2AP was placed in the template strand at several positions: base paired with the primer terminus (the n position), at the adjacent template 5′-position (the $n + 1$ position), and at the more distal $n + 2$ position (Table 1). Only weak fluorescence, about 700 counts/s, was detected for the unbound 2AP-labeled single- and double-stranded DNAs (Figure 2A, traces 1 and 2), as expected since 2AP fluorescence is quenched in DNA compared to the fluorescence of the free base or nucleotide (*7*, *13*). T4 DNA polymerase binding to the DNA substrates enhanced the fluorescence of 2AP to varying degrees. T4 DNA polymerase binding to single-stranded DNA produced about a 5-fold increase in fluorescence intensity relative to unbound 2AP-labeled DNA (Figure 2A, trace 3, ssDNA), but a 25-fold increase in fluorescence intensity was detected for T4 DNA polymerase binding to the $n + 1$ duplex DNA (Figure 2A, trace 4, $n + 1$). Only a small increase in fluorescence intensity, however, was detected for T4 DNA polymerase complexes formed with duplex DNA labeled at the $n + 2$ position with 2AP (Figure 2A, trace 5) and at more distal positions (data not shown). An approximate 11-fold increase in fluorescence intensity was detected for complexes formed with 2AP at the n position (Figure 2A, trace 6, n). The emission λ_{max} for the T4 DNA polymerase−DNA complexes was at 368 nm as observed for free 2AP nucleotide in solution (*7*). These polymerase−DNA binding experiments were done in the absence of Mg^{2+} to prevent the exonuclease reaction by the wild-type T4 DNA polymerase, but similar emission spectra were detected in the presence of Mg^{2+} with the exonuclease-deficient D112A/E114A DNA polymerase (data not shown).

Table 1: DNA Substrates Labeled at the *n*, *n* + 1, or *n* + 2 Position with 2AP[a]

DNA	Sequence
n	3' B-CCCTTCGTGCAGTAGCCATTA**P**AGATCGATGGTTT
	5' GGGAAGCACGTCATCGGTAATT
n(M)[b]	3' B-CCCTTCGTGCAGTAGCCATTA**P**AGATCGATGGTTT
	5' GGGAAGCACGTCATCGGTAAGG
n+1	3' B-CCCTTCGTGCAGTAGCCATTAA**P**GATCGATGGTTT
	5' GGGAAGCACGTCATCGGTAATT
n+1(M)	3' B-CCCTTCGTGCAGTAGCCATTAA**P**GATCGATGGTTT
	5' GGGAAGCACGTCATCGGTAAGG
n+2	3' B-CCCTTCGTGCAGTAGCCATTAAT**P**GTCGATGGTTT
	5' GGGAAGCACGTCATCGGTAATT
n+1(dd)[b]	3' B-CCCTTCGTGCAGTAGCCATTAATC**P**GCGATGGTTT
	5' GGGAAGCACGTCATCGGTAATTAG$_{dd}$
n+2(dd)	3' B-CCCTTCGTGCAGTAATTGCCGGCT**P**GCGATGGTTT
	5' GGGAAGCACGTCATTAACGGCCG$_{dd}$

[a] The base analogue 2-aminopurine (2AP) is depicted as a large, bold **P**. The B indicates a biotin at the 3′-end of the template strand. [b] The symbols M and dd indicate mismatched and dideoxy-terminated primer termini, respectively.

Because T4 DNA polymerase may form complexes in which the primer terminus is bound in either the polymerase or exonuclease active centers, the next experiments were designed to determine if the fluorescence enhancement detected for the polymerase−DNA complexes was due to polymerase- or exonuclease-binding modes. Binding in the exonuclease active center can be forced by using DNA substrates with preformed mismatched primer termini (*7*, *8*). Mismatched (M) counterparts to the *n* and *n* + 1 DNA substrates were constructed (Table 1). Substantially less fluorescence was detected for polymerase−DNA complexes formed with DNA labeled at the *n* + 1 position with 2AP if the primer terminus was mismatched [Figure 2B, *n* + 1 (mismatched)] than when matched [Figure 2B, *n* + 1 (matched)]. Thus, the high level of fluorescence detected for 2AP at the *n* + 1 position with the matched primer terminus appears to be due primarily to a polymerase-binding mode [E^{pol}·D], which is prevented by a mismatched primer terminus. In contrast, a slightly higher level of fluorescence intensity was detected for DNA labeled at the *n* position with 2AP if the primer terminus was mismatched [Figure 2B, *n* (mismatched)] than when matched [Figure 2B, *n* (matched)]. 2AP fluorescence at the *n* position thus appears to be due primarily to an exonuclease-binding mode [E_{exo}·D], a result that was expected since the 2AP-T primer terminus is recognized primarily by the T4 DNA polymerase as a mismatch and is subjected to exonucleolytic proofreading (*19*; reviewed in ref *20*).

Characterization of the Fluorescent n + 1 Complex. The fluorescent *n* + 1 complexes formed with the T4 DNA polymerase are complexes in which the primer terminus is bound in the polymerase active center, as discussed above, but two distinct polymerase conformations have been observed in structural studies in which base stacking in the template strand is disturbed. A "closed" conformation has been detected for structures of several DNA polymerase

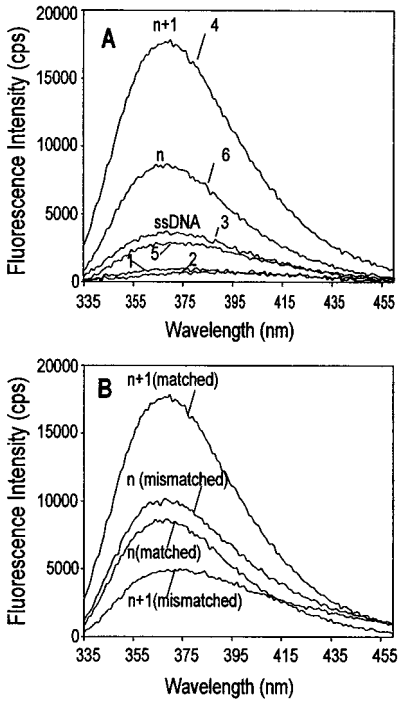

FIGURE 2: Fluorescence emission spectra for 2AP in different environments in the template strand. The DNA substrates are described in Table 1. Titrations were done to determine the optimal fluorescence intensity for the enzyme−DNA complexes, which was observed at 500 nM wild-type or D112A/E114A T4 DNA polymerase with 200 nM DNA. The buffer contained 25 mM HEPES (pH 7.6), 50 mM NaCl, 1 mM DTT, and 0.5 mM EDTA. Excitation was at 310 nm. The data were corrected for intrinsic protein fluorescence. Panel A: Traces 1 and 2 are the single- and double-stranded DNAs with 2AP at the *n* + 1 position, respectively, in the absence of T4 DNA polymerase. Trace 3 is of T4 DNA polymerase bound to single-stranded DNA labeled at the *n* + 1 position with 2AP. Trace 4 is of T4 DNA polymerase bound to double-stranded DNA labeled at the *n* + 1 position with 2AP, and trace 5 is also with double-stranded DNA, but 2AP is at the *n* + 2 position. Trace 6 is of T4 DNA polymerase bound to double-stranded DNA with 2AP at the base pairing *n* position in the template strand. Panel B: The emission spectra for T4 DNA polymerase bound to the *n* + 1 and *n* matched and mismatched DNA substrates, which are described in Table 1.

prenucleotide incorporation complexes in which tight contacts are made between the DNA polymerase and the DNA in the primer-terminal region and the incoming nucleotide. The DNA in closed structures may be stretched, and/or a sharp bend in the template strand is observed at the *n* + 2 position (*21*−*26*). Either DNA strand stretching or bending could produce base unstacking and increase 2AP fluorescence. An "open" conformation has also been detected, and as the name suggests, the DNA primer-terminal region fits more loosely in the polymerase active center of open complexes compared to closed complexes. Base unstacking at the *n* + 1 position is observed in some open complexes of the large fragment of the *Taq* DNA polymerase; the base at the *n* + 1 position is displaced due to insertion of a conserved Tyr residue (*25*; Figure 3).

To determine if the fluorescence observed for 2AP at the *n* + 1 position is due to formation of a closed or open structure, T4 DNA polymerase complexes were formed with chain-terminated DNA substrates as used for the structural studies (Table 1). Complexes were formed with the exonuclease-deficient D112A/E114A T4 DNA polymerase in order

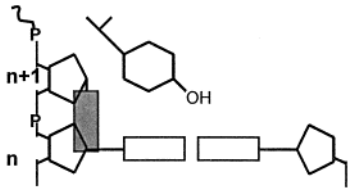

FIGURE 3: Base unstacking at the $n + 1$ position for the open complex of the large fragment of the *Taq* DNA pol I bound to DNA (*25*). Insertion of a conserved Tyr residue displaces the base at the $n + 1$ position.

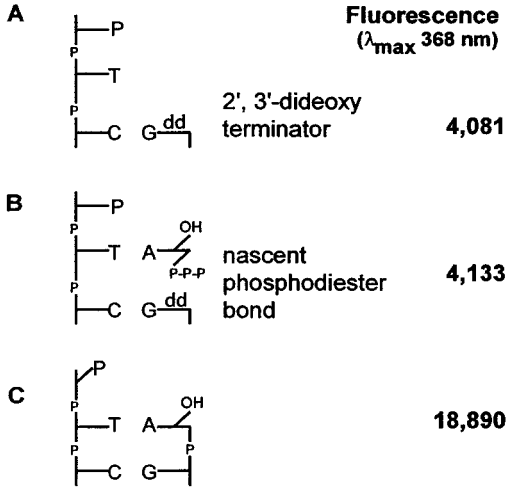

FIGURE 4: Base unstacking at the $n + 1$ template position detected by 2AP fluorescence. The primer-terminal region of the ddGMP chain-terminated $n + 2$ DNA substrate is illustrated; the full structure is given in Table 1. DNA polymerase−DNA complexes were formed with 200 nM chain-terminated $n + 2$ DNA substrate, 500 nM exonuclease-deficient D112A/E114A DNA polymerase, 25 mM HEPES (pH 7.6), 50 mM NaCl, 1 mM DTT, and 8 mM Mg^{2+}. The complexes were excited at 310 nm. The fluorescence intensity values are given for the emission peak at 368 nm. 2AP is illustrated as a **P**. In panel A, little 2AP fluorescence was detected for the complex with 2AP at the $n + 2$ position. In panel B, dATP at 100 μM or 1 mM was added to form the nucleotide preincorporation complex. The DNA substrate used in panel C was produced by adding 100 μM dATP to a DNA substrate similar to the one shown in panel A but having an extendable primer terminus. Addition of dATP resulted in elongation of the primer terminus to place 2AP at the $n + 1$ position. 2AP is illustrated in a base-unstacked conformation for the fluorescent complexes.

to protect the primer strand from exonuclease degradation in the presence of Mg^{2+}. As a further control, the DNA substrates were labeled with ^{32}P following the fluorescence measurements, and gels were run to confirm that the DNA substrates remained intact throughout the experiment.

E^{pol}·D·Mg^{2+} complexes were first formed with duplex DNA labeled at the $n + 2$ position with 2AP (Figure 4A). Only a small increase in fluorescence intensity was detected for these complexes as observed for complexes formed with the $n + 2$ DNA substrate with an extendible primer terminus (Figure 2A, trace 5). No further increase in fluorescence intensity was detected when dATP was added to form E^{pol}·D·dATP·Mg^{2+} complexes (Figure 4B), even with 1 mM dATP. A high level of fluorescence intensity was observed when dATP was added to complexes with an extendible primer terminus, and the primer was extended to place 2AP at the $n + 1$ position (Figure 4C). Since template strand bending at the $n + 2$ position is observed in structures of prenucleotide incorporation complexes (*21*−*26*), strand

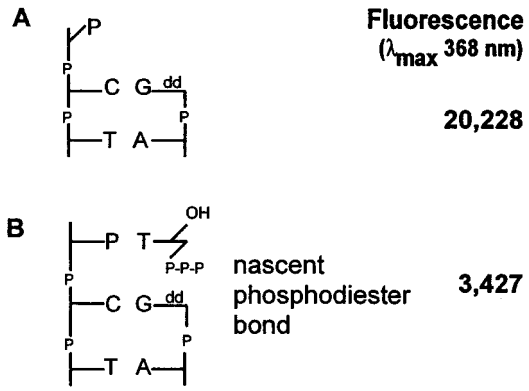

FIGURE 5: Formation of fluorescent open binary DNA polymerase complexes and quenched prenucleotide incorporation complexes. The primer-terminal region of the ddGMP chain-terminated $n + 1$ DNA is illustrated; the full structure is given in Table 1. Conditions were the same as described for Figure 4. Panel A: the fluorescent $n + 1$ complex. 2AP is illustrated in a base-unstacked conformation. Panel B: addition of 200 μM dTTP·Mg^{2+} produced fluorescence quenching by restoring base-stacking interactions in forming the nascent 2AP-T base pair.

bending was expected for the E^{pol}·D·dATP·Mg^{2+} complex diagramed in Figure 4B. However, if there is strand bending, this bending is reported by only a small increase in 2AP fluorescence intensity at the $n + 2$ position. An attempt was made to increase 2AP fluorescence at the $n + 2$ position for the prenucleotide incorporation complex described in Figure 4B by using a DNA template strand with a shorter 5′ overhang, as used in the structural studies, but no further increase in fluorescence intensity with the shorter substrate was observed.

In contrast to the relatively small increase in fluorescence intensity observed for T4 DNA polymerase complexes formed with DNA labeled with 2AP at the $n + 2$ position (Figures 2A, trace 5, 4A, and 4B), a large increase in fluorescence intensity was observed when complexes were formed when 2AP was placed at the $n + 1$ position adjacent to a chain-terminated primer terminus (Figure 5A), as observed for the fluorescent $n + 1$ complexes formed in Figure 2A, trace 4. Thus, T4 DNA polymerase can bind to chain-terminated DNA substrates, but base unstacking reported by 2AP fluorescence is specific for the $n + 1$ position. These observations are most consistent with the open structure observed for the *Taq* DNA polymerase in which the base at the $n + 1$ position is displaced (Figure 3). The relatively large increase in fluorescence intensity detected for complexes formed with 2AP at the $n + 1$ position (Figures 2−4) is consistent with a severe displacement of the $n + 1$ template base. For comparison, a 14-fold increase in fluorescence emission intensity is observed when the *Eco*RI DNA adenine methylase binds DNA labeled with 2AP at the substrate position and produces DNA bending and apparent nucleotide flipping (*14*, *15*).

A sharp decrease in fluorescence intensity was observed when dTTP·Mg^{2+} was bound opposite template 2AP to form the prenucleotide incorporation complex (Figure 5B). This decrease was not detected in the absence of Mg^{2+} or for dCTP·Mg^{2+} (data not shown). Thus, this complex appears to a true prenucleotide incorporation complex and a closed structure.

Determination of the Rate of dAMP Incorporation Opposite Template T at pH 7.6 and 6.5. For duplex DNAs

labeled initially with 2AP at the $n + 2$ position (Table 1), primer elongation by one nucleotide produced an increase in fluorescence intensity due to formation of fluorescent $n + 1$ complexes (Figure 4C). Since 2AP base unstacking occurs *after* formation of the phosphodiester bond (Figures 4 and 5), the rate of increase in 2AP fluorescence reports on the *overall* nucleotide incorporation reaction (Figure 1). A solution containing E^{pol}·D complexes, formed with the exonuclease-deficient D112A/E114A DNA polymerase and the $n + 2$ DNA substrate (Table 1), was mixed in the stopped-flow apparatus with a second solution containing variable concentrations of dATP·Mg^{2+}. The buffer was 25 mM HEPES (pH 7.6), 50 mM NaCl, and 1 mM DTT. The increase in fluorescence intensity was biphasic; a representative reaction with 100 μM dATP is shown (Figure 6A). The best curve fit for this reaction was to the double-exponential rate equation to give a major rate of 257 ± 8 s^{-1} (amplitude 0.93) plus a minor rate of 18 ± 8 s^{-1} (amplitude 0.07). The major rate or "burst phase" is the rate of dAMP incorporation for the first enzyme turnover. The burst rates were determined for several dATP concentrations, and the data were fit to the hyperbolic equation $k_{obs} = k_{pol}[dATP]/(K_d + [dATP])$ (Figure 6B), yielding values of 16.2 ± 2.8 μM for K_d and 314 ± 18.1 s^{-1} for k_{pol}. The fluorescence experiments were repeated at pH 6.5, since low pH is reported to decrease the rate of nucleotide incorporation catalyzed by the T4 DNA polymerase (*27*). The nucleotide incorporation rate was reduced 2.5-fold to 126 ± 3.4 s^{-1}, and the K_d for dATP was reduced a smaller extent to 10 ± 1 μM (Figure 6C) compared to reactions at pH 7.6 (Figure 6B).

The apparent rate for nucleotide incorporation determined in the fluorescence assay at pH 7.6, 314 s^{-1}, is slower than the 400 s^{-1} rate determined at pH 7.5 by a rapid-quench, radioactive-based assay (*5*). The differences in rates may be due to experimental conditions, or the fluorescence assay may be measuring a slower rate-limiting step that could not be detected in the radioactive assay. For example, translocation and base unstacking at the $n + 1$ position follow product formation (Figure 1). To determine if the conformational change that is required to produce base unstacking at the $n + 1$ position could be rate limiting, DNA polymerase−DNA association rates were measured by 2AP fluorescence.

Determination of the Rate of T4 DNA Polymerase Binding to DNA Labeled at the n + 1 Position with 2AP. T4 DNA polymerase binding to DNA labeled with 2AP at the $n + 1$ position produces an increase in fluorescence intensity, which can be described by the reaction:

$$E + D \overset{k_{assoc}}{\rightleftharpoons} [E^{pol}\cdot D^{n+1\ stacked} \leftrightarrows E^{pol}\cdot D^{n+1\ unstacked}]$$

The $E^{pol}\cdot D^{n+1\ unstacked}$ complex is fluorescent. Thus, the increase in fluorescence intensity measures enzyme−DNA association or base unstacking, whichever reaction is rate limiting.

The rate of T4 DNA polymerase binding to DNA was determined in the stopped-flow apparatus by mixing a solution containing the exonuclease-deficient D112A/E114A DNA polymerase in pH 7.6 buffer with a second solution containing 200 nM $n + 1$ DNA substrate (Table 1). Mixing resulted in an increase in fluorescence intensity with time due to formation of fluorescent $n + 1$ complexes (Figure 7). The rate of increase was biphasic, and curve fitting to the double-exponential rate equation yielded a major rate, average of multiple reactions, of 307 ± 9.1 s^{-1} (amplitude 0.7) and a minor rate of 42.3 ± 1.8 s^{-1} (amplitude 0.3). The rate of increase in fluorescence intensity was independent of enzyme concentration between 100 and 500 nM. The experiment was repeated at pH 6.5, but the lower pH did not change the rate.

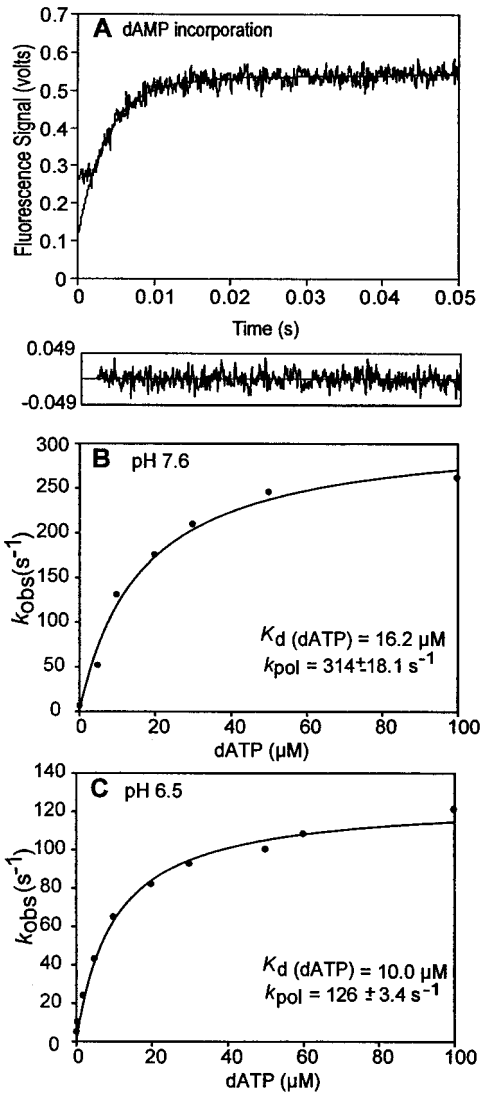

FIGURE 6: Pre-steady-state kinetics of dAMP incorporation opposite template T. A solution of 400 nM $n + 2$ DNA substrate (Table 1), 1 μM exonuclease-deficient D112A/E114A DNA polymerase, 25 mM HEPES (pH 7.6), 50 mM NaCl, 1 mM DTT, and 0.5 mM EDTA was mixed with a second solution containing a variable concentration of dATP, 25 mM HEPES (pH 7.6), 50 mM NaCl, 1 mM DTT, and 16 mM Mg^{2+} in the stopped-flow apparatus. Representative data are shown in panel A for dATP at a final concentration of 100 μM. The increase in fluorescence intensity was fit to a double-exponential rate equation to give the faster burst rate, which represents the first turnover of the enzyme (about 257 s^{-1}), and a slower minor rate, which is due to multiple turnover reactions (about 18 s^{-1}). The residuals are shown. Multiple runs at several dATP concentrations were performed in order to determine k_{pol} and K_d at pH 7.6 (panel B) and pH 6.5 (panel C) as described in the text.

Since the apparent nucleotide incorporation rate of 314 s^{-1} is similar to the enzyme−DNA association rate, 307 s^{-1}, both rates may be measuring the same rate-limiting step, base unstacking. However, these experiments do not exclude the

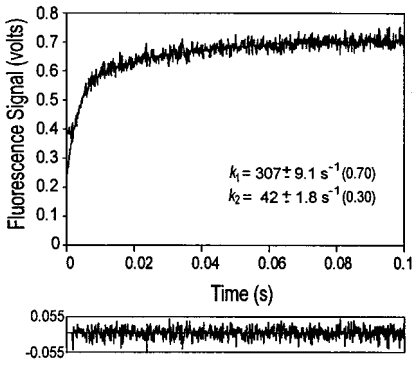

FIGURE 7: Rate for T4 DNA polymerase base unstacking at the $n + 1$ position. A solution of 400 nM $n + 1$ DNA substrate (Table 1) was mixed in the stopped-flow apparatus with a second solution containing from 200 to 1000 nM exonuclease-deficient T4 DNA polymerase in either pH 7.6 or pH 6.5 buffer. A representative reaction for 500 nM enzyme at pH 7.6 is shown. The best fit was obtained with a double-exponential equation, with a major rate (k_1) of about 307 s^{-1} and a minor rate (k_2) of about 42 s^{-1}. The rates were independent of enzyme concentration and pH.

Table 2: Effect of DNA Sequence on the Fluorescence Intensity of 2AP at the $n + 1$ Position in T4 DNA Polymerase−DNA Complexes[a]

DNA substrate	Sequence	Fluorescence emission intensity (λ_{max} 368 nm) (cps)
I	3′ B-CCCTTCGTGCAGTAGCCATTAA**P**GATCGATGGTTT 5′ GGGAAGCACGTCATCGGTAATT	28,600
II	3′ B-CCCTTCGTGCAGTAGCCATTAAC**P**GATCGATGGTTT 5′ GGGAAGCACGTCATCGGTAATTG	24,630
III	3′ B-CCCTTCGTGCAGTAGCCGCCGA**P**GATCGATGGTTT 5′ GGGAAGCACGTCATCGGCGGCT	22,260
IV	3′ B-CCCTTCGTGCAGTAGCCGCCGC**P**GATCGATGGTTT 5′ GGGAAGCACGTCATCGGCGGCG	13,330
V	3′ B-CCCTTCGTGCAGTAGCCGCCGG**P**GATCGATGGTTT 5′ GGGAAGCACGTCATCGGCGGCC	5,500

[a] Polymerase−DNA complexes were formed with the exonuclease-deficient D112A/E114A DNA polymerase. The DNA polymerase was present at 500 nM, and the DNA substrate was present at 200 nM. The excitation wavelenght was 310 nm. 2AP is indicated by a bold **P**. The sequences of interest are underlined.

possibility that the 307 s^{-1} rate measures the rate of enzyme−DNA association and base unstacking is more rapid. If the second possibility were true, then it would be coincidence that the apparent nucleotide incorporation and enzyme−DNA association rates were similar. Another way to examine base stacking is to determine the amount of DNA polymerase induced base unstacking with different DNA substrates.

Sequence Effects on Base Unstacking at the n + 1 Position. Base unstacking is predicted to be sensitive to the DNA sequence since strong base-stacking interactions between bases flanking the $n + 1$ base will impede unstacking. Fluorescent $n + 1$ complexes were formed with several DNA substrates that differ in the A+T or G+C richness in the primer-terminal region and with DNA substrates that differed only in the terminal base pair (Table 2). The most A+T-rich DNAs are listed first (DNA substrates I and II) and are followed by DNAs with increasing G+C richness (DNA substrates III−V).

There are at least three possible DNA polymerase−DNA complexes formed: an editing complex [E_{exo}·DNA] and two polymerase complexes, E^{pol}·$D^{n+1\ stacked}$ and E^{pol}·$D^{n+1\ unstacked}$, in which the base at the $n + 1$ position is either stacked or unstacked. If 2AP is at the $n + 1$ position, a large increase in fluorescence intensity is detected for the unstacked species (Figures 2, 4C, and 5A). The editing complex, E_{exo}·DNA, has much less fluorescence intensity as shown for editing complexes formed with a DNA substrate with a mismatched primer terminus and labeled with 2AP at the $n + 1$ position (Figure 2B); less than 5000 counts/s were observed (Figure 2B). However, for the DNA substrates compared in Table 2, the vast majority of enzyme−DNA complexes formed are predicted to be complexes in which the primer is bound in the polymerase active center since there are no mismatches. Thus, the fluorescence intensity values reported in Table 2 are due mostly to complexes in which the primer is bound in the polymerase active center.

Two DNA sequence effects on the level of DNA polymerase-induced base unstacking at the $n + 1$ position can be observed from the data presented in Table 2. First, the expected base-stacking interactions were observed. For example, DNA substrates IV and V differ only in whether a cytosine (C) is at the template *n* position adjacent to 2AP at the $n + 1$ position (DNA substrate IV) or if a guanine (G) is at the *n* position (DNA substrate V). Fewer fluorescent $n + 1$ complexes were detected if guanine was in the adjacent *n* position (DNA substrate V), which is consistent with the greater ability of guanine to base stack with adjacent bases and, thus, to hinder base unstacking.

The second DNA sequence effect was that A+T richness promotes base unstacking at the $n + 1$ position (Table 2). The highest levels of fluorescence intensity were for the A+T-rich DNA substrates I−III. Although DNA substrates III and IV differ only at the primer terminus, an A-T base pair for DNA substrate III and a C-G base pair for DNA substrate IV, more fluorescent $n + 1$ complexes were formed for DNA substrate III, 22260 counts/s, compared to 13330 counts/s for DNA substrate IV. More fluorescent $n + 1$ complexes are also formed with the relatively A+T-rich DNA substrate II (24630 counts/s) compared to the relatively G+C-rich DNA substrate IV (13330 counts/s). Both DNA substrates II and IV have a terminal C-G base pair with 2AP flanked by C in the *n* position and by G in the $n + 2$ position; thus, base-stacking interactions do not appear to be a factor in this case.

SUMMARY

Our studies provide evidence for base unstacking at the $n + 1$ position in the template strand by the T4 DNA polymerase. While previous studies (*16*) demonstrated that T4 DNA polymerase can form fluorescent $n + 1$ complexes, we have extended these studies to show that base unstacking at the $n + 1$ position occurs after formation of the phosphodiester bond (Figures 4 and 5). We have exploited base unstacking at the $n + 1$ position with 2AP placed initially at the $n + 2$ position in the template strand to measure the rate of primer elongation to place 2AP in the fluorescence-reporting $n + 1$ position. Thus, 2AP is not a base-pairing partner, which means that nucleotide incorporation for correct nucleotides can be measured. The apparent

rate of 314 s^{-1} was determined at pH 7.6, but a lower rate of 126 s^{-1} was observed at pH 6.5, which confirms a previous report that low pH reduces the nucleotide incorporation rate (*27*).

Why is there base unstacking at the $n + 1$ position following the chemistry step? The DNA polymerase forms a tightly closed complex before formation of the phosphodiester bond, as shown in several structures of DNA polymerase prenucleotide incorporation complexes (*21*−*26*), but the DNA polymerase may need to relax its tight grip following formation of the phosphodiester bond in order to translocate (*23*). Thus, base unstacking at the $n + 1$ position may reflect a looser complex as detected for some open complexes of the *Taq* DNA polymerase (*25*; Figure 3).

Base unstacking also occurs at a potential branch point in the nucleotide incorporation pathway, when the DNA polymerase has to "decide" whether to catalyze another cycle of nucleotide incorporation or to initiate the exonucleolytic proofreading pathway (Figure 1). If the DNA polymerase relaxes its tight grip following formation of the phosphodiester bond, this action would provide an opportunity for the DNA polymerase to reevaluate the accuracy of the newly formed terminal base pair. Incorrect nucleotides are not as readily incorporated as correct nucleotides, but if a wrong nucleotide should be incorporated, the DNA polymerase requires a mechanism to halt further primer extension. Thus, while base unstacking may reflect a looser DNA polymerase grip on the DNA, there is also the possibility that base unstacking has a purpose. One possibility is that base unstacking may also result when the DNA polymerase incorporates a wrong nucleotide and may lead to production of a complex that is not active for continued nucleotide incorporation. DNA polymerases have substantially reduced ability to extend a mismatched primer terminus, and the slower rate of primer elongation provides an opportunity for the DNA polymerase to initiate proofreading (*8*, *9*, *28*). Inactive complexes are also formed during nucleotide incorporation by RNA polymerases and are thought to provide an opportunity for RNA polymerases to proofread or to terminate transcription (*29*).

The effect of DNA sequence on formation of fluorescent $n + 1$ complexes (Table 2) is consistent with the possibility that base unstacking at the $n + 1$ position in the polymerase active center may be a precursor to proofreading. A+T richness in the primer-terminal region increases both the likelihood of inducing base unstacking at the $n + 1$ position when the DNA is bound in the polymerase active center (Table 2) and the likelihood of forming editing complexes for proofreading (*7*, *8*, *19*, *20*). If the T4 DNA polymerase produces base unstacking at the $n + 1$ position by insertion of a conserved Tyr residue, as observed for the *Taq* DNA polymerase (*25*; Figure 3), and if the base-unstacked complex is a precursor to proofreading, then removal of the Tyr residue is predicted to reduce proofreading. This experiment was performed in vivo for the RB69 DNA polymerase (*30*). An Ala substitution for the conserved Tyr residue produced a mutant DNA polymerase with a strong mutator phenotype for base substitution mutations, but not for frameshifts, which is the phenotype expected if misinserted nucleotides are not proofread. The mutant DNA polymerase, however, may instead have reduced fidelity for nucleotide incorporation, or there may be defects both in nucleotide incorporation and in proofreading. Additional 2AP fluorescence studies and further structural studies will provide the means to test these proposals.

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REFERENCES

1. Kornberg, A., and Baker, T. A. (1992) *DNA Replication*, Freeman, New York.
2. Kuchta, R. D., Mizrahi, V., Benkovic, P. A., Johnson, K. A., and Benkovic, S. J. (1987) *Biochemistry 26*, 8410−8417.
3. Wong, I., Patel, S. S., and Johnson, K. A. (1991) *Biochemistry 30*, 526−537.
4. Johnson, K. A. (1993) *Annu. Rev. Biochem. 62*, 685−713.
5. Capson, T. L., Peliska, J. A., Kaboord, B. F., Frey, M. W., Lively, C., Dahlberg, M., and Benkovic, S. J. (1992) *Biochemistry 31*, 10984−10994.
6. Arndt, J. W., Gong, W., Zhong, X., Showalter, A. K., Liu, J., Dunlap, C. A., Lin, Z., Paxson, C., Tsai, M.-D., and Chan, M. K. (2001) *Biochemistry 40*, 5368−5375.
7. Bloom, L. B., Otto, M. R., Eritja, R., Reha-Krantz, L. J., Goodman, M. F., and Beechem, J. M. (1994) *Biochemistry 33*, 7576−7586.
8. Marquez, L. A., and Reha-Krantz, L. J. (1996) *J. Biol. Chem. 271*, 28903−28911.
9. Baker, R. P., and Reha-Krantz, L. J. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 3507−3512.
10. Reha-Krantz, L. J., Marquez, L. A., Elisseeva, E., Baker, R. P., Bloom, L. B., Dunford, H. B., and Goodman, M. F. (1998) *J. Biol. Chem. 273*, 22969−22976.
11. Beechem, J. M., Otto, M., Bloom, L. B., Eritja, R., Reha-Krantz, L. J., and Goodman, M. F. (1998) *Biochemistry 37*, 10144−10155.
12. Reha-Krantz, L. J. (2000) *Genetics 148*, 1551−1557.
13. Ward, D. C., Reich, E., and Stryer, L. (1969) *J. Biol. Chem. 244*, 1228−1237.
14. Allan, B. W., Beechem, J. M., Lindstrom, W. M., and Reich, N. O. (1998) *J. Biol. Chem. 273*, 2368−2373.
15. Allan, B. W., Garcia, R., Maegley, K., Mort, J., Wong, D., Lindstrom, W., Beechem, J. M., and Reich, N. O. (1999) *J. Biol. Chem. 274*, 19269−19275.
16. Frey, M. W., Sowers, L. C., Millar, D. P., and Benkovic, S. J. (1995) *Biochemistry 34*, 9185−9192.
17. Reha-Krantz, L. J., Nonay, R. L., and Stocki, S. (1993) *J. Virol. 67*, 60−66.
18. Reha-Krantz, L. J., and Nonay, R. L. (1993) *J. Biol. Chem. 268*, 27100−27108.
19. Bessman, M. J., and Reha-Krantz, L. J. (1977) *J. Mol. Biol. 116*, 115−123.
20. Goodman, M. R., Creighton, S., Bloom, L. B., and Petruska, J. (1993) *Crit. Rev. Biochem. Mol. Biol. 28*, 83−126.
21. Pelletier, H., Sawaya, M. R., Kumar, A., Wilson, S. H., and Kraut, J. (1994) *Science 264*, 1891−1903.
22. Sawaya, M. R., Prasad, R., Wilson, S. H., Kraut, J., and Pelletier, H. (1997) *Biochemistry 36*, 11205−11215.
23. Doublié, S., Tabor, S., Long, A. M., Richardson, C. C., and Ellenberger, T. (1998) *Nature 391*, 251−258.
24. Kiefer, J. R., Mao, C., Braman, J. C., and Beese, L. S. (1998) *Nature 391*, 304−307.
25. Li, Y., Korolev, S., and Waksman, G. (1998) *EMBO J. 17*, 7514−7525.
26. Franklin, M. C., Wang, J., and Steitz, T. (2001) *Cell 105*, 657−667.

27. Lo, K.-Y., and Bessman, M. J. (1976) *J. Biol. Chem. 251*, 2475−2479.
28. Donlin, M. J., Patel, S. S., and Johnson, K. A. (1991) *Biochemistry 30*, 538−546.
29. Erie, D. A., Hajiseyedjavadi, O., Young, M. C., and von Hippel, P. H. (1993) *Science 262*, 867−873.
30. Bebenek, A., Dressman, H. K., Carver, G. T., Ng, S.-S., Petrov, V., Yang, G., Konigsberg, W. H., Karam, J. D., and Drake, J. W. (2001) *J. Biol. Chem. 276*, 10387−10397.

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