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Dye structure affects *Taq* DNA polymerase terminator selectivity

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ABSTRACT

All DNA sequencing methods have benefited from the development of new F667Y versions of *Taq* DNA polymerase. However, terminator chemistry methods show less uniform peak height patterns when compared to primer chemistry profiles suggesting that the dyes and/or their linker arms affect enzyme selectivity. We have measured elementary nucleotide rate and binding constants for representative rhodamine- and fluorescein-labeled terminators to determine how they interact with F667 versions of *Taq* Pol I. We have also developed a rapid gel-based selectivity assay that can be used to screen and to quantify dye–enzyme interactions with F667Y versions of the enzyme. Our results show that 6-TAMRA-ddTTP behaves like unlabeled ddTTP, while 6-FAM-ddTTP shows a 40-fold reduction in the rate constant for polymerization without affecting ground-state nucleotide binding. Detailed mechanism studies indicate that both isomers of different fluorescein dyes interfere with a conformational change step which the polymerase undergoes following nucleotide binding but only when these dyes are attached to pyrimidines. When these same dyes are attached to purines by the same propargylamino linker arm, they show no effect on enzyme selectivity. These studies suggest that it may be possible to develop fluorescein terminators for thermocycle DNA sequencing methods for polymerases that do not discriminate between deoxy- and dideoxynucleotides.

INTRODUCTION

Fluorescent DNA sequencing can be broadly classified into either primer chemistry or terminator chemistry methods depending on whether the dye resides on the primer or on the terminator. Each method has its advantages and disadvantages. New modified forms of *Taq* Pol I having the F667Y substitution do not discriminate between deoxy- and dideoxynucleotides (1). Hence, primer chemistry peak height patterns show remarkable uniformity (2) which makes base calling and heterozygote detection much more reliable (3). A major disadvantage for primer chemistry methods is that four separate reactions are necessary and each reaction requires a different dye-labeled primer. Consequently, primer chemistry methods do not easily lend themselves to the high throughput demands of genomic DNA sequencing, for example.

Terminator chemistry methods are more amenable to high throughput applications (4). Major advantages include the need for only a single extension reaction for each template and the fact that any primer may be used in the reaction. Although terminator chemistry methods have also benefited from the development of F667Y versions of *Taq* Pol I plus the use of improved dye-labeled terminators (5), the peak height patterns in terminator chemistry profiles are still far less uniform compared to the profiles for primer chemistry methods. In this report, we investigate the mechanistic basis for the non-uniform peak height patterns observed for dye-labeled terminators.

Historically, fluorescent dye-labeled terminator chemistry was made possible by the observation that a propargylamino linker arm attached to the 5 position of pyrimidines and to the 7-deaza position of purines still allowed for relatively high dye terminator incorporation (6). While this linker arm was generally successful, only certain dye types could be used for certain enzymes. One of the first dye sets developed for T7 DNA polymerase consisted of propargylamino linkages to fluorescein dyes (7). The dye terminators developed for *Taq* Pol I, however, were based on rhodamines because fluorescein terminators were only poorly incorporated (8). Consequently, rhodamine terminators have been the focus for a wide variety of thermocycle sequencing applications; however, the requirement for this particular dye structure causes problems for reaction ‘clean-up’ and for electrophoretic separations. The exocyclic amino groups on rhodamines generate zwitterionic structures which cause the fluorescent by-products produced during thermocycling to migrate in the sequence ladders unless they are removed either by very careful ethanol precipitation or by the use of expensive size exclusion columns. Rhodamine dyes also show a tendency to form or stabilize DNA secondary structures that result in electrophoretic migration artifacts. Therefore, dGTP must be replaced with dITP to minimize gel compression (9). The presence of dITP results in inefficient use of the polymerase because this nucleotide behaves as a competitive inhibitor and its incorporation requires suboptimal reaction temperatures (10).

Fluorescein-labeled terminators would actually be more desirable for thermocycle sequencing. Fluorescein terminators yield very predictable migration patterns in acrylamide gels even when dGTP is used and any extension by-products migrate faster than the primer bands in sequence ladders. However, as described above, *Taq* Pol I only poorly incorporates fluorescein terminators. To understand how these dyes interact with the *Taq* Pol I, we have measured elementary rate and binding constants for a representative

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rhodamine terminator (6-TAMRA-ddTTP) and a representative fluorescein terminator (6-FAM-ddTTP). This investigation also led to the development of a new assay for quickly screening dye structural effects on polymerase selectivity. Our results suggest that it may be possible to develop fluorescein-labeled terminators for specially modified forms of *Taq* Pol I.

MATERIALS AND METHODS

Enzymes

'KlenTaq,' a truncated form of *Thermophilus aquaticus* DNA polymerase I (11), was obtained from K. A. Johnson (Penn State University). This deletion removes the entire 5′→3′ nuclease region. A full-length form of *Taq* Pol I, AmpliTaq FS® (or G46D; F667Y), was a gift from D. H. Gelfand (Roche Molecular Systems, Alameda, CA). The G46D substitution renders the enzyme 5′→3′ nuclease deficient. The F667Y substitution improves 2′,3′-ddNTP incorporation (1).

Nucleotide triphosphates and other materials

All four ddNTPs were purchased as premixed solutions from Amersham Pharmacia Biotech. Dye-labeled nucleotides were synthesized and characterized by the Organic Synthesis Group at PE Applied Biosystems (Foster City, CA). The dye-labeled terminators used in this study can be obtained from the author by written request.

Synthetic oligodeoxyribonucleotides

The primer and template oligodeoxynucleotides listed in Table 1 were synthesized on an Applied Biosystems 380A DNA Synthesizer (DNA Synthesis Facility, PE Applied Biosystems) and purified using reverse phase HPLC. Each primer strand was synthesized and dye labeled using standard chemistries recommended for this instrument. Concentrations of the single-stranded oligodeoxynucleotide fragments were determined by UV absorbance. Duplex primer/template pairs were formed by annealing equimolar amounts of the dye-labeled 25mer primer with the appropriate 36mer template in a solution containing 5 mM Tris–HCl, 5 mM NaCl and 0.2 mM EDTA (pH 8.0 at 20°C) using the following temperature regimen: 5 min at 95°C, 10 min at 60°C and 15 min at room temperature. Duplex DNAs were stored at –20°C.

Table 1. Oligodeoxynucleotides

5'-(Dye[a])-25/36AG: Single T Incorporation (followed by a C)

```
DYE-CCC TCG CAG CCG TCC AAC CAA CTC A
    GGG AGC GTC GGC AGG TTG GTT GAG TAG GTC TTG TTT
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5'-(Dye[a])-25/36AC: Single T Incorporation (followed by G)

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DYE-CCC TCG CAG CCG TCC AAC CAA CTC A
    GGG AGC GTC GGC AGG TTG GTT GAG TAC GTC TTG TTT
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[a]The dye selected for the 5′-end of the primer strand (the 'detection dye') depended upon the spectral characteristics of the dye on the in-coming terminator in each experiment as noted in the text. The underlined base indicates the next template position beyond the end of the primer.

Buffers

All experiments were carried out using 80 mM Tris–HCl and 2.4 mM $MgCl_2$ (pH 9.0 at 20°C) except where noted in the text. All reaction temperatures were 60°C.

Product analysis

The dye-labeled primer and product bands were resolved on 16% polyacrylamide/8 M urea denaturing gels and 'visualized' on a PE Biosystems Model 373 DNA Sequencer using 672 GeneScan® fragment analysis software for peak identification and relative fluorescence measurements. The amount of DNA in each product band was calculated by normalizing the product peak areas for each time point. The resulting curves were fitted to appropriate equations depending on the experiment (as described in the text) by regression analyses using curve fitting software purchased from Synergy Software (Reading, PA).

Measuring elementary rate constants

The nucleotide concentration dependence of the rate of 26mer product formation was measured under polymerase excess (500 nM) over DNA (100 nM) reaction conditions. Reaction times for each experiment varied depending on the type of nucleotide being tested as described in the text. Plots of product formation rates versus nucleotide concentrations yielded the maximum rate of phosphodiester bond formation, k_{pol}, and the equilibrium dissociation binding constant, K_d (12,13). The designations for the kinetic constants refer to the enzyme reaction steps represented in Figure 1.

Measurement of the dissociation rate of the E·DNA complex

By measuring the steady-state dissociation rate of the enzyme from an E·D_{dd} complex with or without the next correct dye-labeled nucleotide also present in the reaction mixture (under conditions where the chemistry step has been blocked), it was possible to infer whether or not dye/linker arm structure affects the ability of the polymerase to form a tighter binding or closed conformational (E*·D_{dd}·dye-Nuc) complex. A preincubated solution of polymerase (1 nM) and primer/template (1000 nM) was reacted with 2.4 mM Mg^{2+} and 400 µM ddT_1TP (the correct nucleotide for the first template position following the 3′-end of the primer strand or 'N_1') either alone or in the presence of 400 µM ddN_2TP (the next correct, *unlabeled* nucleotide) or the presence of 400 µM dye-ddN_2TP (next correct *dye-labeled* nucleotide) or 400 µM incorrect nucleotide (i.e. one incapable of forming a correct Watson–Crick base pair in the 'N_2' template position). Reaction temperatures were 60°C. Timed samples were removed as indicated in the figures and quenched in ice-cold 0.48 M EDTA (final concentration). These quenched samples were processed as described below for the selectivity assay to remove excess unincorporated nucleotides and samples were loaded onto 16% denaturing gels as described above. The steady-state ddTMP incorporation rate measured in the absence of the next correct nucleotide represented the rate of dissociation of the E·D_{dd} complex. The ddTTMP incorporation rate measured in the presence of the next correct nucleotide corresponded to the dissociation rate of the ternary E*·D_{dd}·ddN_2TP or ternary complex which represents the kinetic step immediately prior to the 'chemistry' or group transfer step in the reaction (14).

Selectivity assay format

A substrate selectivity assay was developed to measure any preference that a given enzyme showed for an unlabeled terminator over a dye-labeled terminator. This was a true selectivity assay since both types of terminators were present in the same reaction mixture and were, of course, able to compete for the same active site under the same reaction conditions. The kinetic steps involved in determining a selectivity ratio are presented in the pathway shown in Figure 1. The polymerase, E, forms an enzyme·DNA complex ($E \cdot D_n$) with an equilibrium dissociation constant K_D. This binary complex combines with the next correct in-coming nucleotide to form a 'ground-state' ternary complex ($E \cdot D_n \cdot N_n$) with a nucleotide dissociation constant, K_d. This 'open' structure then undergoes a conformational change (governed by the rate constant k_{pol}) to form an active or 'closed' structure ($E^* \cdot D_n \cdot N_n$). The formation of the closed complex aligns the reactive groups for a rapid group transfer reaction or chemistry step (not shown). Eventually, the product complex ($E \cdot D_{n+1}$) is formed following release of inorganic pyrophosphate (PP_i). The equation applies to any pair of substrates that compete for the same active site (15).

Since dye-labeled terminators have higher molecular weights than their unlabeled counterparts, two different types of 26mers were produced with regard to gel mobilities: the 26mer product resulting from incorporation of the unlabeled terminator migrated as expected for a 26mer fragment; however, the other 26mer which was the product of dye-labeled terminator incorporation migrated more slowly behaving as an 'apparent 27mer'. Consequently, there were typically three bands in each gel lane: unreacted 25mer primer; 26mer (or ddNMP product); '27mer' (or dye-ddNMP product). The relative fluorescence in each of the bands was measured as described above. The ratio of normalized fluorescence in the 26mer band divided by the normalized signal in the 'apparent 27mer' band represented the selectivity ratio or the preference that the enzyme showed for the unlabeled terminator over the corresponding dye-labeled terminator. A selectivity ratio of *unity* was interpreted to mean that the enzyme could not distinguish between an unlabeled terminator and the same molecule containing a dye. A selectivity ratio greater than one was interpreted to mean that the enzyme preferred the unlabeled over the dye-labeled terminator.

During the development of this assay, our early experiments utilized a 5′-^{32}P-labeled primer. However, to avoid having to resynthesize radioactive primers, we chose to use 5′-dye-labeled primers. Since a dye was present on the 5′-end of the primer for detection and quantitation purposes, it was necessary to correct for any energy transfer between the detection dye and the dye on the newly incorporated terminator on the 3′-end of the 'apparent 27mers' in the gel results (16). It was possible to empirically determine an energy transfer correction factor by using standards on the same gel. For example, to measure 6-FAM-ddNTP incorporation, it was necessary to use 5′-TAMRA-labeled primers; 6-FAM (λ_{max} emission = 522 nm) transfers energy to TAMRA (λ_{max} excitation = 560 nm). Therefore, the raw TAMRA signals in each 'apparent 27mer' band were artificially high due to the presence of a FAM moiety on the same molecule and had to be adjusted. In this example, the correction factor was determined by loading a separate gel lane with a sample containing equal moles of 5′-TAMRA-25mer (detection dye only or 'single-dye species') plus 5′-TAMRA-26-FAM-labeled fragment

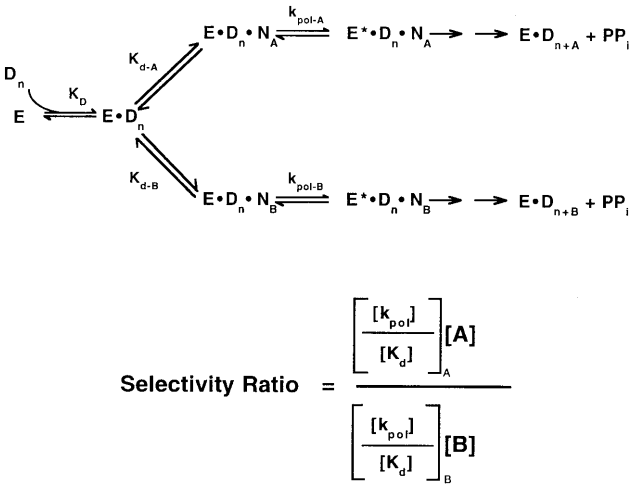

Figure 1. Kinetic pathway model for competing nucleotides. The figure shows part of the kinetic mechanism that is thought to be common to all Pol I-type enzymes. In the case of a DNA sequencing reaction, substrate A would be a dye-labeled terminator with concentration [A] whereas substrate B would be an extendible base or 2′-dNTP represented by concentration [B]. Since both molecules compete for the same active site, the preference or 'selectivity ratio' that the enzyme shows for one over the other is given by a ratio of rate and binding constants as shown in the equation.

(equivalent to the two-dye 'apparent 27mer' or 'two-dye species'). The ratio of the relative fluorescence signals in the two-dye band over the single-dye band provided a correction factor for dye-to-dye energy transfer.

While a TAMRA/FAM combination caused an enhancement of the TAMRA signal (by 1.6-fold), other dye combinations caused quenching of the 5′ detection dye signal. Nevertheless, in each case, it was still possible to generate suitable correction factors in the same manner as described above.

Selectivity assays were conducted under steady-state reaction conditions, namely 1000 nM primer/template, 1 nM enzyme, 400 µM each nucleotide (except where noted in the text), 2.4 mM Mg^{2+} at 60°C. Timed samples were removed and quenched in ice-cold 0.48 M EDTA (final concentration). To remove excess unincorporated dye-labeled nucleotide, an appropriate aliquot of the quenched sample was transferred to 250 µl lithium/tRNA precipitation solution (0.8 M LiCl plus 0.2 µg/µl *Escherichia coli* tRNA carrier) and 750 µl 95% ethanol. Total nucleic acids were precipitated on ice for a minimum of 20 min and then pelleted. Pellets were washed with 70% ethanol, air dried, and dissolved in 50% formamide for loading on 16% polyacrylamide/8 M urea denaturing gels (12).

RESULTS AND DISCUSSION

Measuring dye effects on the nucleotide rate and binding constants for KlenTaq

Each 'rung' in a sequencing 'ladder' represents the incorporation of a terminator rather than an extendible base. Since both of these molecules must compete for the same active site at each template position, the probability of a termination event versus an extension event is governed by enzyme selectivity as defined by the equation in Figure 1 (15). Since the nucleotide pool sizes do not change substantially during thermocycling, uneven peak heights

Table 2. Rate and binding constants for dye-labeled terminators for KlenTaq

	k_{pol} (sec^{-1})	K_d (μM)	k_{pol}/K_d ($M^{-1} \bullet s^{-1}$)	Predicted Selectivity substrate /terminator	Predicted Selectivity ddTTP /dye-ddTTP
Unlabeled-Nucleotides					
dTTP	31 ± 3	60 ± 16	5.2×10^5	-	-
ddTTP	0.011 ± 0.000	66 ± 8	170	3.1×10^3	-
Rhodamine-ddTTP					
TAMRA-ddTTP	0.006 ± 0.000	50 ± 14	120	4.3×10^3	1.4
Fluorescein-ddTTP					
FAM-ddTTP	0.00027 ± 0.00005	70 ± 4	4	1.3×10^5	43

indicate sequence context-dependent changes in terminator rate and binding constants which must be due to differential interactions between the dyes and the polymerase.

Figure 1 also shows the relevant kinetic steps in the mechanism which is thought to be common to all Pol I-type enzymes (17,18). In this model, the polymerase first binds to DNA. The binary complex in turn binds an in-coming nucleotide (either a dye-labeled terminator or an extendible base) to form an open ternary complex. Correct nucleotide binding is followed by a rapid conformational change in the polymerase to form a 'closed' complex that aligns the reactive groups in the active site for an even faster group transfer reaction (19,20).

To be consistent with our previous kinetic studies on terminator structural effects (12), we made our initial measurements using KlenTaq. F667 versions of *Taq* Pol I like KlenTaq show a strong bias against 2′,3′-ddNTP incorporation. As shown in Table 2, KlenTaq would be expected to strongly favor incorporation of 2′-dTTP over unlabeled 2′,3′-ddTTP by approximately 3100-fold as predicted by the calculated substrate/terminator selectivity ratio. 6-TAMRA attached to ddTTP via a propargylamino linker arm (Fig. 2) has little effect on the nucleotide binding constant compared to unlabeled ddTTP (66 ± 8 μM for ddTTP versus 50 ± 14 M for 6-TAMRA-ddTTP) and shows only a small reduction in the rate constant (0.011 ± 0.0005 s^{-1} for ddTTP versus 0.006 ± 0.0001 s^{-1} for 6-TAMRA-ddTTP). This suggests that rhodamine dyes interact minimally behaving as if they are 'transparent' to the polymerase (calculated selectivity value approximately 1). On the other hand, 6-FAM attached in the same manner as 6-TAMRA does not affect the nucleotide binding constant but shows a 40-fold reduction in the rate constant compared to unlabeled ddTTP suggesting that fluorescein dyes exacerbate the strong bias that unmodified forms of *Taq* DNA polymerase already show against 2′,3′-ddNTPs (calculated selectivity value approximately 43).

Measuring dye effects on AmpliTaq FS selectivity

To determine if the behavior exhibited by 6-FAM-ddTTP was peculiar to this dye terminator structure or if it is, in fact, a general property of fluorescein dyes, we developed a rapid gel-based selectivity assay as described in Materials and Methods using AmpliTaq FS. Because it is an F667Y version of *Taq* Pol I, AmpliTaq FS does not show a strong bias against terminator

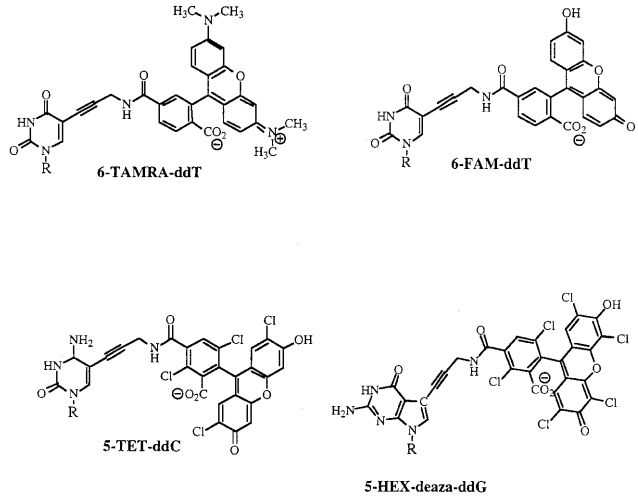

Figure 2. Dye-labeled terminator structures. The figure shows the structures for 6-TAMRA-ddTTP, 6-FAM-ddTTP, 5-TET-ddCTP and 5-HEX-deaza-ddGTTP, where R stands for 2′,3′-dideoxyribose-5′-triphosphate. Each dye consists of an 'upper' or xanthene triple ring structure plus a 'bottom' or benzoic acid ring. The propargylamino linker arm is attached to the bottom ring of the dye at one of two positions as indicated which defines the isomers examined in this study.

incorporation (1) which made it possible to investigate the kinetic effects of these dye and linker arm combinations independently from 'terminator structural effects'. AmpliTaq FS has also become the polymerase of choice for high throughput applications and is widely used in DNA sequencing (21,22) and fragment analyses (3).

Experimentally determined selectivity values for several different dye and base combinations are listed in Table 3 which shows that the actual value for unlabeled ddTTP over 6-TAMRA-ddTTP was indeed as predicted. The actual value measured for ddTTP over 6-FAM-ddTTP was 50 times which agrees well with the calculated value of 43 times for KlenTaq.

The 'fluorescein dye effect' depended on the type of base involved. The purines 6-FAM-ddATP, 5-TET-ddGTP and 5-HEX-ddGTP showed selectivity values of 1 behaving as if these dyes and their linkers were also transparent to AmpliTaq FS. Since the dyes tested represented a range of structures as well as both isomers, these data suggest that many different fluorescein dyes

may be accommodated by AmpliTaq FS without significantly affecting incorporation when a propargylamino linker arm is used to attach either isomer to a purine.

The situation for the same fluorescein dyes on pyrimidines was quite different. All combinations showed selectivity values much greater than 1 indicating that AmpliTaq FS shows a very strong preference for an unlabeled pyrimidine terminator over its fluorescein-labeled counterpart. The dyes in this study are composed of two ring systems, a xanthene (or 'upper') triple ring structure plus a benzoic acid (or 'bottom') ring as shown in Figure 2. The selectivity ratios listed in Table 3 appear to roughly correlate with the size and the complexity of the ring structures. The highest bias indicating the highest interaction with the polymerase was shown by 5-HEX-ddCTP (800:1). This dye has four chlorine atoms in its upper ring system plus it has a dichloro bottom ring making it the largest dye structure tested. The selectivity ratio measured for unlabeled ddCTP over 5-TET-ddCTP was 170:1. TET has two less chlorine atoms in its upper ring making it smaller than HEX and it was intermediate in selectivity bias. 6-FAM-ddCTP and 6-FAM-ddTTP showed the lowest selectivity ratios. FAM represents the smallest and least complex dye. One explanation for this unusual behavior may be that the linker arm projects away from pyrimidines and purines at different angles.

Table 3. AmpliTaq FS selectivity assay results

	Selectivity Ratio (ddNTP / Dye-ddNTP)
6-TAMRA-ddTTP	1x
Fluorescein Labeled Purines	
6-FAM-ddATP	1x
5-TET-ddGTP	1x
5-HEX-ddGTP	1x
Fluorescein Labeled Pyrimidines	
6-FAM-ddTTP	50x
6-FAM-ddCTP	60x
5-TET-ddCTP	170x
6-HEX-ddCTP	800x

Dye effects on the pre-steady-state burst for AmpliTaq FS

The fact that the nucleotide dissociation constants for unlabeled ddTTP and 6-FAM-ddTTP were the same (within experimental error) as shown in Table 2 but 6-FAM-ddTTP showed a much lower rate constant indicated that the rate limiting step or 'fluorescein dye effect' must occur following initial nucleotide binding. Therefore, our first mechanism measurements involved determining whether or not dye-labeled terminators showed pre-steady-state burst curves under single nucleotide incorporation conditions. Pol I-type enzymes typically show burst kinetic patterns for 2′-dNTP incorporation (13) meaning that the rate limiting step during polymerization must occur *after* the chemistry step in the reaction. On the other hand, if the time course does not show a burst, the rate limiting step must occur *before or during* the chemistry step. As shown in Figure 3A, the time course for 6-TAMRA-ddTTP incorporation by AmpliTaq FS did indeed follow a biphasic curve. In this case, the rate of a 25/36mer elongating to a 26/36mer was measured under DNA excess conditions in order to observe the first and subsequent turnovers of this enzyme. The plot shows an exponential phase representing a rapid, first turnover of the preformed $E{\cdot}D_n$ complex with a rate of $60 \pm 12\ s^{-1}$ and a burst amplitude of 100 ± 10 nM. Subsequent incorporation events occurred at a slower rate of $7 \pm 1\ s^{-1}$. Independent measurements of the enzyme off rate using an 'enzyme-trap' approach (13) showed that the slower phase was due to the slow rate of dissociation of the polymerase from the $E{\cdot}D_{n+1}$ complex (data not shown).

While the plot for of 6-TAMRA-ddTTP incorporation followed a burst kinetic pattern, the curve for 6-FAM-ddTTP did not. As shown in Figure 3B, 6-FAM-ddTTP incorporation was better fitted by a smooth, single exponential curve with an amplitude of 300 ± 8 nM and an initial rate of $8 \pm 1\ s^{-1}$. The calculated ampitude is much greater than the actual enzyme concentration indicating the absence of a pre-steady-state burst. Therefore, the rate limiting step for 6-FAM-ddTTP incorporation must occur at or before the chemistry step in the reaction. To determine which or if both the conformational change and chemistry steps were involved in this 'fluorescein effect', it was necessary to measure dye effects on the rate of dissociation of the conformationally closed ternary complex.

Dye effects on the conformational change step

By measuring the steady-state turnover rate of the Enz*·DNA·Nuc under conditions where the chemistry step has been blocked, it is possible to investigate the conformational change step in polymerase reactions (12,14). We have applied this assay to kinetically probe dye effects on the conformational change step of AmpliTaq FS. These experiments consisted of measuring the steady-state rate of incorporation of ddTTP in the absence or in the presence of the next correct terminator (with and without a fluorescein dye). This was accomplished by reacting a solution containing enzyme (1 nM) plus excess 5′-dye-labeled primer/template (1000 nM) with ddTTP alone (400 μM) or in the presence of one of the other nucleotides (each 400 μM) as shown in Figure 4. Measuring the steady-state turnover rate in the presence of an additional nucleotide that cannot form a Watson–Crick base pair in the next template position served as a control. In these experiments, ddTTP was used as the 'first' nucleotide to be consistent with published results for F667 versions of *Taq* Pol I (12).

As shown in Figure 4A, AmpliTaq FS incorporated unlabeled ddTTP alone with a steady-state rate of $1.5 \pm 0.06\ s^{-1}$. This rate represents the $E{\cdot}D_{ddT}$ dissociation rate which was unaffected by the presence of an *incorrect* base for the next template position or in this case ddCTP ($1.2 \pm 0.04\ s^{-1}$). We interpret this to mean that an $E{\cdot}D_{ddT}{\cdot}$ddCTP (or open) complex could not undergo a conformational change to form an $E^*{\cdot}D_{ddT}{\cdot}$ddCTP (or closed) complex because ddCTP could not form a correct Watson–Crick base pair in the n + 1 template position. However, when the next correct terminator was also present in the reaction mixture, the steady-state turnover rate was reduced by a factor of approximately 10-fold for either unlabeled ddGTP ($0.15 \pm 0.01\ s^{-1}$) or for 6-HEX-ddGTP ($0.20 \pm 0.01\ s^{-1}$). This must mean that an $E{\cdot}D_{ddT}{\cdot}$ddGTP open complex or an $E{\cdot}D_{ddT}{\cdot}$6-HEX-ddGTP open

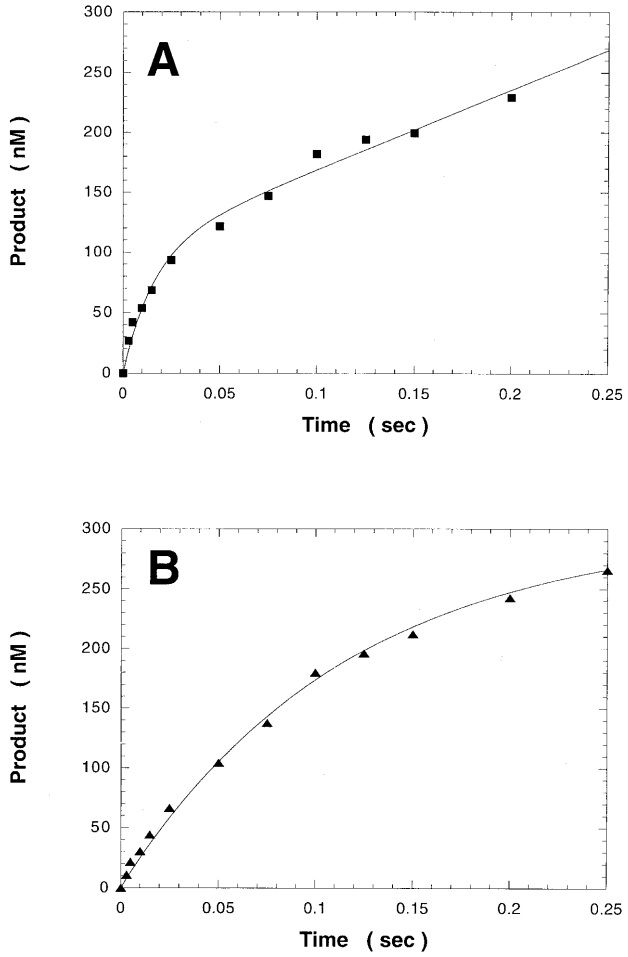

Figure 3. Pre-steady-state burst kinetics for dye-labeled terminator incorporation by AmpliTaq FS. A pre-incubated solution of AmpliTaq FS polymerase (100 nM) plus 5′-dye-labeled duplex DNA (500 nM) was mixed with Mg^{2+} plus dye-labeled ddTTP (160 µM) as indicated in a rapid chemical quench flow apparatus (all concentrations are given as final concentrations following mixing in the instrument). (**A**) The plot shows the time course incorporation pattern for TAMRA-ddTTP (■). The data were fitted to a burst kinetic equation [$k_{obs} = A(1 - \exp^{-rt}) + k_{ss}t$], where k_{obs} is the observed rate of the reaction, A is the burst amplitude in nM which is proportional to the Enz·DNA concentration, r is the burst rate, t is the time in seconds and k_{ss} is the steady-state turnover rate. The burst amplitude was 100 ± 10 nM with a burst rate of 60 ± 12 s^{-1} and a steady-state rate of 7 ± 1 s^{-1}. (**B**) The plot shows the time course incorporation for FAM-ddTTP (▲). All reaction conditions were the same as those described above. The plot shows a fit to a single exponential curve with a burst amplitude of 300 ± 8 nM and an initial rate of 8 ± 1 s^{-1}.

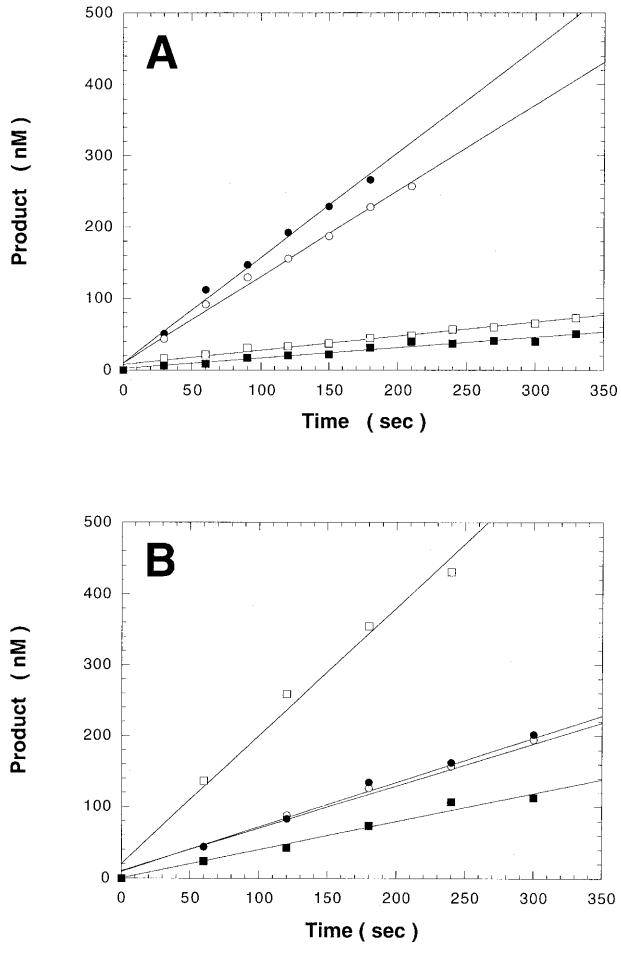

Figure 4. Measurement of the dissociation rate for the Enz·DNA·Nuc complex. (**A**) A preincubated solution of AmpliTaq FS (1 nM) plus 5′-(FAM)-25/36AC primer/template was mixed with 2.4 mM $MgCl_2$ plus 400 µM ddTTP, either alone or in the presence of 400 µM ddCTP (next incorrect nucleotide) or 400 µM ddGTP (the next correct nucleotide) or 400 µM HEX(II)·ddGTP (the next correct, fluorescein-labeled nucleotide). The steady-state rates of incorporation were: 1.5 ± 0.06 s^{-1} for dissociation of the Enz·DNA_{ddT} binary complex (ddTTP alone; ●) and 1.2 ± 0.04 s^{-1} in the presence of an additional nucleotide that cannot form a Watson–Crick base pair in the next template position (ddTTP + ddCTP; ○). The dissociation rate of the ternary complex, Enz·DNA_{ddT}·ddGTP, was slower or 0.15 ± 0.01 s^{-1} suggesting the formation of a tighter binding complex when the next correct nucleotide was also present in the reaction mixture (ddTTP + ddGTP; ■). The presence of a fluorescein dye attached via the propargylamine linker arm did not affect the ability of the polymerase to form a more slowly dissociating ternary complex (ddTTP + 6-HEX·ddGTP, 0.20 ± 0.1 s^{-1}; □). (**B**) The experiment was conducted in the same manner as the one described above except that the primer/template was 5′-(FAM)-25/36AG as shown in Table 1 so that the next correct nucleotide was a pyrimidine instead of a purine. The steady-state rates of incorporation were: 0.62 ± 0.01 s^{-1} (ddTTP-alone; ●) and 0.59 ± 0.02 s^{-1} with the next incorrect terminator also present in the reaction mixture (ddTTP + ddGTP; ○). The steady-state turnover rate when the next correct nucleotide was present was reduced to 0.39 ± 0.01 s^{-1} (ddTTP + ddCTP; ■). The dissociation rate of the ternary complex was about 3-fold faster than 'ddTTP alone' when the next correct nucleotide had an attached fluorescein dye or 1.8 ± 0.1 s^{-1} (ddTTP + TET·ddCTP; □).

complex could undergo a conformational change to form the more slowly dissociating closed structures. Such behavior is predicted from the results shown in Table 3. The selectivity assay value measured for ddGTP versus 6-HEX·ddGTP was, in fact, *unity* which predicted that this particular dye/linker arm combination on ddGTP should be 'transparent' to AmpliTaq FS. Other fluoresceins also attached to purines by a propargylamino linker arm behaved in a similar manner to 6-HEX-ddGTP in this assay (data not shown).

The steady-state turnover behavior for fluorescein dyes attached to pyrimidines was quite different. As shown in Figure 4B, AmpliTaq FS incorporated unlabeled ddTTP alone with a steady-state rate of 0.62 ± 0.01 s^{-1} and this rate was unaffected by the presence of ddGTP which for this template (Table 1) cannot form a correct base pair in the next template position (0.59 ± 0.02 s^{-1}).

When the next correct unlabeled terminator (which for this template is ddCTP) was also present in the reaction mixture, the dissociation rate of the ternary complex was reduced to 0.39 ± 0.01 s^{-1} as expected from the behavior exhibited by unlabeled purines described above. However, when the next correct base was a fluorescein-labeled *pyrimidine*, 5-TET-ddCTP, the rate of dissociation of the ternary complex was not reduced but rather apparently accelerated by 3-fold to 1.8 ± 0.1 s^{-1}. Other fluorescein–pyrimidine combinations showed the same trend (data not shown). It should be noted that this dye effect only occurred when the fluorescein dye was attached to the terminator. Free dye had no effect on the dissociation rates (data not shown).

The dissociation rate for the ddTTP + 5-TET-ddCTP case in Figure 4B appeared to be accelerated because the 'ddTTP alone' steady-state turnover rate was artificially reduced due to the particular template sequence necessary for this experiment. Since the next template position following the A was a G (as shown for the first primer/template pair listed in Table 1), it was possible for ddTTP to form a ddTTP:dG mismatched base pair in the n + 1 template position. Therefore, the reaction in which ddTTP was present alone behaved as if another 'correct' nucleotide was also present and its turnover rate was slower than would have been expected. In this way, *Taq* Pol I behaves like *E.coli* Pol I which shows misincorporation rates that are saturable at concentrations of incorrect dNTPs within an order of magnitude of the correct bases (23). Apparently, the geometry of a G:T mismatch is close enough to that of a normal G:C base pair (24) to allow *Taq* DNA polymerase to undergo a conformational change. However, this mismatch does not allow the formation of as tight a complex as a true G:C base pair because the 'ddTTP alone' turnover rate as shown in Figure 4B was not reduced as much as the rate for 'ddTTP + ddCTP' (or ddTTP plus the next correct base). The fact that the 'ddTTP + 5-TET-ddCTP' steady-state rate (1.8 ± 0.1 s^{-1}) in Figure 4B was equivalent to the 'ddTTP alone' turnover rate shown in Figure 4A (1.5 ± 0.1 s^{-1}) further supports this explanation since a G:C base pair would compete out a G:T wobble pair. This indicates that the true rate for 'ddTTP alone' in Figure 4B would be expected to be ~1.8 s^{-1}.

While these results cannot rule out an additional 'fluorescein dye effect' on the chemistry step in the reaction, they do indicate significant interference with the ability of the polymerase to undergo a conformational change. More detailed kinetic/mechanism studies will be required to investigate any possible interference during the group transfer reaction.

CONCLUSION

Previous studies have shown that F667 versions of *Taq* Pol I exhibit a strong bias against 2′,3′-ddNTP terminators (12). Results presented in this paper show that this bias was unaffected by a representative rhodamine dye but exacerbated by several different fluoresceins. 6-TAMRA-ddTTP showed equivalent nucleotide rate and binding constants to unlabeled ddTTP for KlenTaq suggesting that this may be the reason why rhodamine terminators were developed first for *Taq* Pol I DNA sequencing methods. A representative fluorescein terminator, 6-FAM-ddTTP, on the other hand, showed a 40-fold lower rate constant but no change for the binding constant indicating that fluorescein dyes make dye-labeled terminator incorporation worse by affecting a step following ground-state nucleotide binding. Additional kinetic measurements using an F667Y version of *Taq* Pol (AmpliTaq FS) indicated that fluorescein dyes interfere with the ability of this enzyme to form a closed ternary complex following initial nucleotide binding but only when these dyes are attached to pyrimidines. The nature of this interaction is unknown. However, since it is specific for fluorescein dye structures and the larger the dye, the more the bias against its incorporation, this interaction must have both chemical and steric components. Detailed molecular modeling and/or crystallography studies of an active, conformationally closed, ternary complex that includes a fluorescein-labeled pyrimidine terminator may show where and how these dyes contact the enzyme. By minimizing or eliminating these interactions, it should be possible to develop fluorescein terminator chemistries for modified versions of *Taq* DNA polymerase that do not discriminate between deoxy- and dideoxynucleotides.

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