An intramolecular FRET system monitors fingers subdomain opening in KlenTaq1

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Abstract

A major goal of polymerase research is to determine the mechanism through which a nucleotide complementary to a templating DNA base is selected and delivered to the polymerase active site. Structural evidence suggests a large open-to-closed conformational change affecting the fingers subdomain as being crucial to the process. We previously designed a FRET system capable of measuring the rate of fingers subdomain closure in the presence of correct nucleotide. However, this FRET system was limited in that it could not directly measure the rate of fingers subdomain opening by FRET after polymerization or in the absence of DNA. Here we report the development of a new system capable of measuring both fingers subdomain closure and reopening by FRET, and show that the rate of fingers subdomain opening is limited only by the rate of polymerization. We anticipate that this system will scale down to the single molecule level, allowing measurement of fingers subdomain movements in the presence of incorrect nucleotide and in the absence of DNA.

Keywords: polymerase; KlenTaq1; subdomain movement; FRET; stopped-flow; kinetics

DNA polymerases are responsible for template-directed replication of DNA. They do so by selecting a 2'-deoxyribonucleoside-5' triphosphate (dNTP) based on its complementarity to a templating base and incorporating it to the 3' end of a primer DNA strand. Other factors than Watson-Crick base-pairing contribute to discrimination between correct and incorrect dNTPs, and it has been shown that DNA polymerases actively participate in the process of nucleotide selection (for review, see Rothwell and Waksman 2005). As DNA polymerases are central to the overall fidelity of DNA synthesis, and thus to genome stability, there is great interest in understanding the mechanisms involved in nucleotide selection.

Structural and kinetic data have accumulated for many different types of DNA polymerase with different functions, rates of incorporation, and frequencies of misincorporation. Most share a similar basic structural organization and mode of nucleotide incorporation. A minimal model for the template-directed incorporation of nucleotide by DNA polymerases, as elucidated first for Klenow (Kuchta et al. 1987, 1988; Dahlberg and Benkovic 1991; Eger et al. 1991) and subsequently for several other polymerases (Patel et al. 1991; Wong et al. 1991; Capson et al. 1992; Kati et al. 1992; Washington et al. 2001), is shown in Figure 1A. According to this model, the two substrates bind to the polymerase (E) sequentially, with the p/t DNA substrate binding first to form an enzyme:primer/template (E:p/t) complex (step 1), followed by the binding of dNTP to give an E:p/t:dNTP complex (step 2). After nucleotide binding, a conformational change occurs in the protein (step 3 in Fig. 1A), producing the E':p/t:dNTP complex, required for subsequent formation of the phosphodiester bond (step 4 in Fig. 1A, leading to the

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Figure 1. Minimal scheme for incorporation of a nucleotide by KlenTaq1 and design of the FRET system. (A) Schematic diagram of the nucleotide incorporation cycle for KlenTaq1 DNA polymerase. For details, see text. (B) Positions of the fluorophores used to monitor fingers subdomain movement in KlenTaq1. The open binary (E:p/t) complex, strongly colored, is superposed over the closed ternary complex (E':p/t:ddCTP), weakly colored, both shown as a α backbone trace. For each, the palm subdomain is colored purple; the fingers, green; the thumb, blue; and the N-terminal vestigial proofreading domain, yellow. The nucleotide-binding O-helix is colored magenta in the E:p/t complex and red in the E':p/t:ddCTP complex. DNA from the closed ternary complex is shown in stick representation with the primer strand in yellow, the template strand in turquoise, and the incoming nucleotide in orange. The positions of the labels, residues 649 and 825 (the only two cysteines in KlenTaq1), are shown in red and purple spheres, respectively. The distances between residues in the open and closed forms are indicated. This panel was generated using the program PyMOL (Delano Scientific).

E':p₊₁/t:PP_i complex), and release of pyrophosphate (step 5 in Fig. 1A, leading to the E:p₊₁/t complex). Finally, the extended DNA strand is either released (distributive synthesis) or translocated to allow incorporation of the next nucleotide (processive synthesis). For most polymerases, step 3 is the rate-limiting step for polymerization and has a rate constant termed k_{pol} .

The extent to which each step contributes to the overall selection against incorrect nucleotide depends on the function of the enzyme: Replicative polymerases preferentially bind correct nucleotide over incorrect nucleotide at step 2 by a factor of several hundred, 390-fold for T7 DNA polymerase 1, and 250-fold for HIV-1 reverse transcriptase (Wong et al. 1991; Kati et al. 1992), whereas repair enzymes show far lower discrimination: 3.4-fold for Klenow, fourfold for yeast polymerase η , and 20-fold

for polymerase β (Kuchta et al. 1987, 1988; Werneburg et al. 1996; Ahn et al. 1997; Washington et al. 2001). However, for almost all polymerases, the greatest contribution to nucleotide selection takes place at the rate-limiting step (with the rate k_{pol}). For T7 DNA polymerase 1, k_{pol} is 2000 times slower for incorrect nucleotide than for correct nucleotide, while for Klenow correct nucleotide is favored by a factor of 5000 (Kuchta et al. 1987, 1988; Patel et al. 1991; Wong et al. 1991).

KlenTaq1, an active truncated form of *Thermus aquaticus* DNA polymerase 1, is a member of the best structurally characterized family of DNA polymerases—family A. Family A polymerases have served as model systems for studying nucleotide selection by DNA polymerases. KlenTaq1 (Fig. 1B) consists of two domains: a catalytically inactive N-terminal domain, corresponding to the 3'–5' exonuclease domains in homologous polymerases, and a C-terminal polymerase domain. The polymerase domain is further subdivided into the palm, thumb, and fingers subdomains, named according to an analogy likening the overall structure to that of a right hand (Ollis et al. 1985). The palm subdomain contains the active site residues; the thumb subdomain is responsible for DNA binding, and the fingers subdomain contains the O-helix, the nucleotide-binding site.

Comparison of the crystal structures of the E:p/t complex of KlenTaq1 and a “trapped” ternary E':p/t:dNTP complex of the same enzyme suggested a large inward movement in the fingers subdomain as being responsible for the rate-limiting step 3 (Li et al. 1998b). Equivalent conformational changes between an open E:p/t complex and a closed E':p/t:dNTP complex have also been observed in other polymerases where structures of both the binary (E:p/t) and precatalytic ternary complexes (E':p/t:dNTP) are available (Pelletier et al. 1994; Doublié et al. 1998; Huang et al. 1998; Li et al. 1998b; Franklin et al. 2001; Ling et al. 2001, 2003; Temiakov et al. 2004; Berman et al. 2007). However, the assumption that this conformational change is rate-limiting was called into question by indirect evidence from various systems (Zhong et al. 1997; Vande Berg et al. 2001; Kim et al. 2003; Purohit et al. 2003; Shah et al. 2003). Direct evidence was provided when a fluorescence resonance energy transfer (FRET) system with an acceptor fluorophore attached to the outside of the fingers subdomain of KlenTaq1, and a donor fluorophore linked to a p/t substrate was developed (Rothwell et al. 2005). Using this system, it was possible to monitor directly closure of the fingers subdomain upon nucleotide binding, providing clear evidence that the fingers subdomain movement is fast and not rate-limiting. This FRET system was also used to measure the nucleotide concentration dependence of fingers subdomain closure, revealing a pre-equilibrium between two E:p/t conformations, only one of which is capable of binding nucleotide

(Rothwell and Waksman 2007). It was hypothesized that this equilibrium might be due to the movement of the templating base between a “preinsertion” state, where the base is flipped out of stacking arrangement with the previous template base, and an “intermediate preinsertion” state, where the templating base is in a conformation such that it can base-pair with an incoming nucleotide bound to the O-helix of the fingers subdomain in the open form (Temiakov et al. 2004; Rothwell and Waksman 2007). This model would explain the differences in the pre-equilibrium for the four different nucleotides, and the ability of polymerases to discriminate between correct versus incorrect nucleotide even before fingers subdomain closure.

There is significant interest in understanding the exact role of the fingers subdomain movement: Since dNTPs binds to the O-helix of the fingers subdomain in the open form (Beese et al. 1993; Li et al. 1998a) while the active site of the polymerase is only formed completely in the closed form, the fingers subdomain has a clear structural role in nucleotide selection and delivery. Whether the fingers subdomain closure is triggered by nucleotide binding, or whether the open and closed states are in rapid equilibrium regardless of the presence of nucleotide (or indeed DNA), has important implications for the mechanism of nucleotide selection, and thus for the overall fidelity of the polymerase. Unfortunately, ensemble level studies—limited to the study of the average behavior of a large number of molecules—are unable to distinguish between these two possibilities. Thus, thorough analysis of the movement of the fingers subdomain requires experimental techniques that measure the properties of individual protein molecules rather than averages, e.g., single molecule FRET.

The currently available FRET system described by Rothwell et al. (2005) for studying fingers subdomain movement in KlenTaq1, with one fluorophore attached to the substrate DNA and the other to the protein, is limited in that it cannot directly monitor structural changes succeeding fingers subdomain closure. KlenTaq1 also has a relatively low affinity for DNA (mid nM) and a high dissociation rate for this interaction. Due to the fact that single molecule experiments are typically performed at pM concentrations, a DNA- and protein-labeled FRET system in which the protein concentrations are far below the K_D of the interaction is unsuitable. An additional disadvantage is that changes in the apo-form of the enzyme or in the E:dNTP form of the enzyme cannot be detected.

Here we report the development of a FRET system for monitoring KlenTaq1 fingers subdomain movement in which both fluorophores are attached to the protein. This allows measurement both of fingers subdomain closure and of fingers subdomain opening through the entire incorporation cycle, with no lower limit to protein con-

centration. Ensemble level measurements on this system show the rates of dNTP incorporation and fingers subdomain closure to be comparable to those obtained using the singly labeled protein–singly labeled DNA system previously developed. In addition, we are now able to record and measure directly the fingers subdomain opening after dNTP incorporation, and we show that this motion is limited only by k_{pol} . This system has the potential to be used at the single molecule level to resolve the kinetics of fingers subdomain closure and opening.

Materials and Methods

Protein expression and labeling

Protein was expressed and purified as previously described (Korolev et al. 1995). Mutations were introduced using a Stratagene QuikChange Kit. Protein concentration was determined spectrophotometrically using an extinction coefficient at 280 nm of $69,622 \text{ M}^{-1} \text{ cm}^{-1}$.

Labeling of the $\text{KT}^{\text{V649C, E825C}}$ double mutant was carried out at room temperature with a protein concentration of $100 \mu\text{M}$ in a labeling buffer consisting of 150 mM Tris-HCl (pH 7.5), 200 mM NaCl, and 1 mM EDTA. Protein was first labeled for 2 h in $110 \mu\text{M}$ Alexa 647 C_2 -maleimide (Invitrogen). This yielded a mixture of singly labeled, doubly labeled, and unlabeled proteins. The reaction was quenched by the addition of 2-mercaptoethanol to a final concentration of 5 mM , and excess free dye was removed by spin filtration (Millipore, 50-kDa cutoff). The singly labeled species was separated from the unlabeled and doubly labeled species by anion exchange chromatography using a 6-mL Resource-Q column (GE Healthcare) in a buffer consisting of 20 mM Tris (pH 7.5) and 1 mM EDTA, and eluted over a gradient of 0 – 500 mM NaCl. The singly labeled protein was collected and the concentration determined before it was further labeled for 2 h with a 1.5 times molar excess of Alexa 488 C_5 -maleimide (Invitrogen). The double-labeled, donor- and acceptor-containing protein was purified using a Resource-Q column as described above. The degree of labeling was determined as per the manufacturer's instructions and was typically between 85% and 105% for both Alexa 488 and Alexa 647.

The single mutants KT^{V649C} and KT^{E825C} were each labeled with a 1.5-fold molar excess of either Alexa 488 C_5 -maleimide or Alexa 647 C_2 -maleimide, and the unlabeled and singly labeled proteins were separated as described above. The extent of labeling was as for the doubly labeled protein.

Nucleotides

We purchased 100 mM solutions of dNTP and ddNTP from GE Healthcare.

Primer/template

DNA oligonucleotides for primer (p), primer labeled with Alexa Fluor 488 at the sixth base from the 3' end ($\text{p}^{\text{A488.6}}$), template with dCTP as the next correct nucleotide (t_C), and template with dGTP as the next correct nucleotide (t_G) were ordered from IBA. The sequences are as follows, with

base-pairing regions on the templates highlighted in bold. The labeled base in $p^{A488.6}$ is shown in *italics*.

p: 5'-CAG CGC CAC TGG GTC AGT CCG AGC CGT CGC
AGC CTA CCG T-3',
 $p^{A488.6}$: 5'-CAG CGC CAC TGG GTC AGT CCG AGC CGT
CGC AGC CTA CCG T-3'
t_C: 5'- TGG TTA ATC TCT CTA GAC **GGT AGG CTG CGA**
CGG CTC GGA CTG ACC CAG TGG CGC TG -3'
t_G: 5'- TGG TTA ATC TCT TAG CAC **GGT AGG CTG CGA**
CGG CTC GGA CTG ACC CAG TGG CGC TG -3'

Primer/templates were prepared by mixing at a ratio of 0.9:1 primer:template in a buffer consisting of 50 mM Tris-HCl (pH 7.0) and 20 mM NaCl, heating to 95°C for 5 min, and then cooling slowly to room temperature over several hours to give $p^{A488.6}$ /t_C, p/t_C, and p/t_G.

Steady-state fluorescence measurements

All fluorescence scans were carried out at 20°C using a Fluoromax-3 (Jobin-Yvon Horiba). Samples were excited at 493 nm, and emission scans were measured, with the excitation and emission slits set to 4 nm. Spectra were measured for 1 μ M protein (for details, see text) in activity assay buffer, after addition of 900 nM p/t_G, after termination with 250 μ M ddGTP, and after addition of 250 μ M of the next correct (dCTP) or incorrect nucleotide (dATP). After each addition, a multigroup time trace was performed, and the fluorescent signal of both fluorophores, as well as the FRET signal, were monitored until the signal stabilized before an emission scan was measured. For the termination reactions, the temperature was raised to 30°C due to the fact that ddGTP incorporation is very slow at 20°C, before being lowered to 20°C to perform the emission scan.

Quench flow nucleotide incorporation assay

We pre-equilibrated 2 μ M doubly labeled protein and 1 μ M fluorescently labeled $p^{A488.6}$ /t_C in activity assay buffer (20 mM Tris-HCl at pH 7.5, 50 mM NaCl, 2 mM $MgCl_2$). The E:p/t complex was rapidly mixed with an equal volume of 500 μ M dCTP in activity assay buffer at 20°C and quenched after varying lengths of time between 0.5 and 60 sec with 0.6% trifluoroacetic acid, using a rapid quench apparatus (Kintek). Samples were mixed at a 2:1 ratio with denaturing gel loading buffer (0.1% orange G, 0.1% bromophenol blue, 10 mM EDTA in formamide) and run on an 8% polyacrylamide, 7 M urea sequencing gel to separate the extended (p_{+1}) from the unextended (p) primer. Gels were scanned and imaged using a FLA3000 fluorescence scanner (Fujifilm) with excitation at 496 nm and emission recorded above 520 nm. Background was subtracted from each band, and then the extent of product formation was determined as the intensity of the p_{+1} band divided by the sum of the intensities of the p and p_{+1} bands. Product formation as a function of time was fitted to a single exponential model using pro Fit (Quansoft) to determine the rate of single nucleotide incorporation.

Pre-steady-state stopped flow measurements

We preincubated 2 μ M labeled protein and 1.8 μ M p/t_C in activity assay buffer, then rapidly mixed with varying concentrations of dCTP in activity assay buffer using a stopped

flow apparatus (High Tech Scientific). A “blank” run in which the labeled E:p/t complex was mixed against buffer was also performed (0 μ M dCTP). The samples were excited at 493 nm, and the donor and acceptor signals were separated using filters XF3084 (Glen Spectra, bandpass range 510–570 nm) for the donor and RG665 (Schott; 665-nm longpass filter) for the acceptor. Slits were set to 10 nm for both excitation and emission, and the photomultipliers were adjusted to give a 70% photomultiplier response for both signals. The fluorescence of the donor and acceptor signals were monitored over the course of 30 sec, the background of 0 μ M dCTP was subtracted from each trace, and then the signals were fitted as described below using pro Fit (Quansoft). Each trace represents the average of at least three injections.

Results and Discussion

Using the crystal structures of the closed and open forms of Klentaq1 (Li et al. 1998b), a series of cysteine double mutants were rationally designed such that the distance between the two cysteines (d) is different in the open and closed forms but falls within the range $0.5 F_0 < d < 1.5 F_0$ in both cases, where F_0 is the Förster radius of the fluorophore pair Alexa 488–Alexa 647 (56 Å as determined by the manufacturer). One of these double mutants, $KT^{V649C, E825C}$, i.e., Klentaq1 with residues 649 and 825 mutated to cysteines (see Fig. 1B), was labeled with Alexa Fluor 647 C₂-maleimide as an acceptor fluorophore, giving a mixture of unlabeled $KT^{V649C, E825C}$, $KT^{V649C, E825C}$ singly labeled on either the V649C position ($KT^{V649C[A647], E825C}$) or the E825C position ($KT^{V649C, E825C[A647]}$), and $KT^{V649C, E825C}$ labeled on both positions ($KT^{V649C[A647], E825C[A647]}$). The unlabeled, singly labeled, and doubly labeled forms of Klentaq1 were separated by ion exchange chromatography (Fig. 2), and the singly labeled forms were further reacted with Alexa Fluor 488 C₅-maleimide as a donor fluorophore (Fig. 2) and repurified. The resulting double-labeled protein consists of both $KT^{V649C[A488], E825C[A647]}$ and $KT^{V649C[A647], E825C[A488]}$, which we refer to as $KT^{V649C, E825C[A488 - A647]}$.

In order to confirm the activity of doubly labeled $KT^{V649C, E825C[A488-A647]}$, and to measure the rate of incorporation of dCTP (k_{obs}), quench flow experiments were carried out. $KT^{V649C, E825C[A488-A647]}$ was preincubated with a limiting amount of fluorescently labeled primer/template ($p^{A488.6}$ /t_C), mixed rapidly with dCTP at 20°C, and quenched with trifluoroacetic acid after increasing periods of time. Primer extension as a function of reaction time was determined by the relative fluorescence intensities of the p and p_{+1} bands on a DNA sequencing gel and fitted to a single exponential model using pro Fit (Quansoft), giving a value for k_{obs} of $0.058 \pm 0.004 \text{ sec}^{-1}$ at 250 μ M dCTP. This value is the same within experimental error as the value of $0.064 \pm 0.004 \text{ sec}^{-1}$ previously determined by Rothwell et al. (2005) for the singly labeled $KT^{V649C[A594]}$ protein at the

Figure 2. Dual labeling of Klentaq1. Resource-Q elution profiles of (A) unlabelled $\text{KT}^{\text{V649C, E825C}}$, (B) $\text{KT}^{\text{V649C, E825C}}$ labeled with Alexa 647 at a 1:1 dye:protein ratio, and (C) the singly labeled fraction from B further labeled with Alexa 488 at a 2.5:1 dye:protein ratio. Absorbance at 280 nm is shown as a full line; at 488 nm, a dot-dashed line; and at 647 nm, a dashed line. The concentration gradient of NaCl, ranging from 0–500 mM, is shown as a dotted line.

same temperature and the same dCTP concentration, confirming that $\text{KT}^{\text{V649C, E825C[A488-A647]}}$ is active and that the rate of nucleotide incorporation is not significantly altered by the presence of a second fluorophore at position 825.

Next, steady-state fluorescence measurements were performed on $\text{KT}^{\text{V649C, E825C[A488-A647]}}$, as well as the singly labeled variants $\text{KT}^{\text{V649C[A488]}}$, $\text{KT}^{\text{V649C[A647]}}$, $\text{KT}^{\text{E825C[A488]}}$, and $\text{KT}^{\text{E825C[A647]}}$, to determine whether this system was suitable for monitoring fingers subdomain movement (Fig. 2). Fingers subdomain closure should result in an appreciable increase in FRET as the two fluorophores on the protein come into closer proximity (see Fig. 1). Fluorescence emission scans after excitation of the donor fluorophore at 493 nm were performed on (1) the enzyme alone (E); (2) after incubation of E with primer/template (p/t_G); (3) after termination of the primer by the addition of dideoxyguanosine triphosphate (ddGTP), which is incorporated onto the growing end of the primer strand to form $\text{E:p}_{+\text{ddGMP/t}}$ and prevents further elongation as it lacks the 3' hydroxyl group required for catalysis; and (4) after addition of the next correct nucleotide (dCTP) or incorrect nucleotide (dATP).

In Figure 3, A and B, the results for the doubly labeled $\text{KT}^{\text{V649C, E825C[A488-A647]}}$ under various conditions are shown, with free enzyme (E) in black. The donor only labeled $\text{KT}^{\text{V649C[A488]}}$ trace is shown as a black dotted line, and all fluorescence values are normalized to the

fluorescence of the $\text{KT}^{\text{V649C[A488]}}$ control. Addition of the p/t complex (red) and termination with ddGTP (green) leads to no significant change in the fluorescence signal. Addition of the next correct nucleotide (dCTP; blue in Fig. 3A) leads to an increase in FRET as seen by a decrease in the donor signal and an increase in the acceptor signal, whereas addition of an incorrect nucleotide (dATP) leaves the FRET signal unchanged (Fig. 3B). The increase in FRET observed when the correct nucleotide is provided indicates that the two fluorophores are moving closer together upon binding of the correct nucleotide, and would represent the transition from an “open” E:p/t form of Klentaq1 to a “closed,” nucleotide-bound (E':p/t:dNTP) ternary complex as seen crystallographically. In contrast, there is no signal change for the incorrect nucleotide (dATP). Thus stable closure of the fingers subdomain is only observable upon addition of a correct nucleotide to a terminated substrate. Figure 3, C and D, shows the equivalent traces for the two singly labeled donor-only controls, $\text{KT}^{\text{V649C[A488]}}$ and $\text{KT}^{\text{E825C[A488]}}$, respectively. Both exhibit substantially higher donor fluorescence than the donor-acceptor labeled protein, as expected in the absence of FRET. Although a slight change in donor fluorescence is

Figure 3. Fluorescence spectra of Klentaq1 under different substrate conditions. In each case, the black trace is 1 μM Klentaq1; the red trace, 1 μM Klentaq1 + 900 μM p/t; the green trace, 1 μM Klentaq1 + 900 μM p/t + 250 μM ddGTP; and the blue trace, 1 μM Klentaq1 + 900 μM p/t + 250 μM ddGTP with either 250 μM dCTP (A) or 250 μM dATP (B). Fluorescence of the $\text{KT}^{\text{V649C[A488]}}$ control is shown as a black dotted line. (A) Doubly labeled Klentaq1, with the added dNTP being the correct nucleotide (dCTP). (B) Doubly labeled Klentaq1, with the added dNTP being an incorrect next nucleotide (dATP). (C) Singly labeled $\text{KT}^{\text{V649C[A488]}}$, with the added dNTP being the correct next nucleotide (dCTP). Fluorescence of $\text{KT}^{\text{V649C[A647]}}$ is shown in pink. (D) Singly labeled $\text{KT}^{\text{E825C[A488]}}$ (black trace), with the added dNTP being the correct next nucleotide (dCTP). Fluorescence of $\text{KT}^{\text{E825C[A647]}}$ is shown in pink.

observable upon binding of the next correct nucleotide to the $\text{KT}^{\text{V649C[A488]}}\cdot\text{p}_{\text{+ddGMP/t}}$ complex (blue in Fig. 3C), this signal is small and leads to an increase in donor fluorescence, whereas for the $\text{KT}^{\text{V649C, E825C[A488-A647]}}$ system a large reduction in donor fluorescence is observed with a corresponding increase in acceptor fluorescence. Excitation of the acceptor-only labeled controls ($\text{KT}^{\text{V649C[A647]}}$ and $\text{KT}^{\text{E825C[A647]}}$; pink in Fig. 3C,D, respectively) at 493 nm gives a negligible fluorescent emission signal (<1% of the total acceptor fluorescence in the doubly labeled form). Thus we attribute the decrease in donor fluorescence and the increase in acceptor fluorescence observed in $\text{KT}^{\text{V649C, E825C[A488-A647]}}$ upon addition of the correct nucleotide to a distance change between the two fluorophores on fingers subdomain closure. Our $\text{KT}^{\text{V649C, E825C[A488-A647]}}$ FRET system is therefore capable of measuring movement of the fingers subdomain during the nucleotide incorporation cycle.

In order to measure the rates of fingers subdomain closure and opening during nucleotide incorporation, pre-steady-state stopped-flow measurements were carried out at 20°C. $\text{KT}^{\text{V649C, E825C[A488-A647]}}$ was preincubated with p/t_C and then rapidly mixed with varying concentrations of dCTP, and the fluorescence of the donor and acceptor fluorophores was monitored. Control runs with the four singly labeled protein controls, $\text{KT}^{\text{V649C[A488]}}$, $\text{KT}^{\text{E825C[A488]}}$, $\text{KT}^{\text{V649C[A647]}}$, and $\text{KT}^{\text{E825C[A647]}}$, were performed in the same way at 500 μM and 0 μM dCTP.

At all nucleotide concentrations, using $\text{KT}^{\text{V649C, E825C[A488-A647]}}$, a rapid FRET increase phase can be observed, where the donor signal decreases and the acceptor signal increases, followed by a slow relaxation in which FRET decreases. For the two donor-only labeled controls, there is no donor signal change upon addition of nucleotide (Fig. 4) and negligible acceptor signal (data not shown). For the acceptor-only labeled controls, there is no measurable signal within the voltage range of the photomultiplier (data not shown). Thus we confirm that the anti-correlated changes in donor and acceptor fluorescence observed when mixing $\text{KT}^{\text{V649C, E825C[A488-A647]}}\cdot\text{p/t}$ with dCTP are due to FRET. As the FRET signal monitors fingers subdomain motion, the first fast FRET increase phase represents fingers subdomain closure while the slow FRET decrease phase can be interpreted as fingers subdomain opening. Because the FRET increase and decrease phases occur on very different timescales, such that the FRET increase phase is complete before the subsequent FRET decrease phase has appreciably started, the two phases were fitted separately. Note that, for both phases, the anti-correlated changes in donor and acceptor fluorescence are in good agreement, with fitting yielding similar rates.

For the FRET increase phase, according to the pre-equilibrium model of Rothwell and Waksman (2007), the nucleotide binding step can be subdivided into two

Figure 4. Representative stopped flow traces of the labeled KlenTaq1: primer/template complex after addition of varying concentrations of correct nucleotide. The donor signals are shown in green (500 μM dCTP), turquoise (250 μM dCTP), and blue (50 μM dCTP), and the acceptor signals are in red (500 μM dCTP), orange (250 μM dCTP), and yellow (50 μM dCTP). The background signal of KlenTaq1 mixed with 0 μM dCTP has been subtracted from each trace, and each represents the average of at least three measurements. The black dashed lines represent lines of best fit for each data set (for details, see text). The pink and gray dotted lines are the donor traces for the $\text{KT}^{\text{V649C[A488]}}$ and $\text{KT}^{\text{E825C[A488]}}$ controls, respectively. (A) The first 0.5 sec, with single exponential fits to the fingers closure phase. The fitted rates for the data shown are 10.5 sec^{-1} , 15.7 sec^{-1} , and 22.2 sec^{-1} for the donor at 500 μM , 250 μM , and 50 μM , respectively, and 12.1 sec^{-1} , 14.8 sec^{-1} , and 23.8 sec^{-1} for the acceptor at the same concentrations. (B) The entire 30 sec, with the fingers opening phase (3–30 sec) fitted to a single exponential. The fitted rates for the data shown are 0.216 sec^{-1} , 0.229 sec^{-1} , and 0.133 sec^{-1} for the donor at 500 μM , 250 μM , and 50 μM , respectively, and 0.218 sec^{-1} , 0.217 sec^{-1} , and 0.125 sec^{-1} for the acceptor at the same concentrations.

kinetically distinct components, with a pre-equilibrium between two forms of the E:p/t complex, only one of which is capable of binding nucleotide. The observed rate of fingers subdomain closure following nucleotide binding (k_{app}) is given by Equation 1 (where notation by Rothwell and Waksman [2007] is used):

$$\text{E:p/t} \xrightleftharpoons[k_{-3}]{k_3} (\text{E:p/t})^* + \text{dNTP} \xrightleftharpoons[K_{\text{D4}}]{K_{\text{D4}}} [(\text{E:p/t})^*:\text{dNTP}]_{(\text{closed})}$$

$$k_{\text{app}} = k_3 + \frac{k_{-3}K_{\text{D4}}}{[\text{dNTP}] + K_{\text{D4}}} \quad (1)$$

When the first step is slow relative to the second step, this model accounts for the observed inverse relationship between nucleotide concentration and rate of nucleotide binding observed by Rothwell and Waksman (2007). Fingers subdomain closure data (between 0 and 0.5 sec) were fitted to a single exponential model using pro Fit (Quansoft), with a rate k_{app} as in Equation 1. Plotting k_{app} against [dCTP] (Fig. 5A) gives the same relationship as previously observed by Rothwell and Waksman (2007) supporting the pre-equilibrium model of nucleotide binding. Fitting to Equation 1 gives the values: $k_3 = 8.68 \pm 1.61 \text{ sec}^{-1}$, $k_{-3} = 20.67 \pm 1.65 \text{ sec}^{-1}$, and K_{D4} of $97.3 \pm 38.5 \mu\text{M}$, in fair agreement with the previously published values for dCTP of $k_3 = 4.07 \text{ sec}^{-1}$, $k_{-3} = 7.40 \text{ sec}^{-1}$, and $K_{\text{D4}} = 236 \mu\text{M}$ (Rothwell and Waksman 2007).

Figure 5. Secondary plots of stopped flow data, with the rates of the fast and slow FRET phases as a function of nucleotide concentration. Both donor and acceptor data were used, and data represent an average of three separate experiments. The data were fitted according to the models described in text with lines of best fit to the equations as described in the text, generated using pro Fit (Quansoft). (A) The rate of the fast FRET increase phase (k_{app}) as a function of nucleotide concentration. (B) The rate of the slow fingers opening phase (k_{obs}) as a function of nucleotide concentration.

The second phase, the fingers subdomain opening phase, occurs on a much longer timescale than does fingers subdomain closure, suggesting that fingers subdomain opening is either slow or is limited by a rate-limiting step preceding opening (Fig. 4B). The data at each nucleotide concentration between 3 and 30 sec were fitted using a single exponential model with a rate k_{obs} . This single exponential model provides a reasonable fit to the data (Fig. 4B). At concentrations of dCTP above 500 μ M, a slight reduction in the k_{obs} was seen (data not shown), indicating an inhibition of this process at high dNTP concentrations.

The secondary data k_{obs} versus [dNTP] were then plotted (Fig. 5B) and fitted to a model adapted from the model for k_{pol} of Patel et al. (1991) as in Equation 2 below:

$$E : p/t + dNTP \xrightleftharpoons{K_D} E' : p/t : dNTP \xrightleftharpoons{k_1} E : p_{+1}/t + PP_i$$

$$k_{obs} = \frac{k_1 [dNTP]}{[dNTP] + K_D} \quad (2)$$

Here, k_1 is the rate-limiting step for fingers opening after nucleotide binding. Fitting of the data yields a $K_D = 65.0 \pm 11.5$ μ M and $k_1 = 0.232 \pm 0.016$ sec^{-1} . The rate of opening, k_{obs} , at 250 μ M dCTP is 0.197 sec^{-1} . Although this value is over three times greater than the measured rate of dNTP incorporation (k_{pol}) at the same dNTP concentration, we believe that the FRET-decrease phase reflects k_{pol} for the following reasons: First, the difference between the observed rates of nucleotide incorporation and fingers opening is very small compared with that between the rate of fingers subdomain closure and k_{pol} (~ 100 fold in Rothwell et al. 2005), and second, the FRET-decrease phase after closure may reflect a succession of steps that include fingers subdomain opening

followed by a potential re-equilibrium phase between structural isoforms in the DNA-bound enzyme. We, thus, would like to suggest that the rate of fingers subdomain opening after polymerization is limited mostly by k_{pol} . Although these measurements were performed at 20°C, the K_D value observed here is in good agreement with that measured previously at 60°C for dCTP incorporation (33).

Previously, a FRET system has successfully been used to monitor the movement of the fingers subdomain of KlenTaq1 during nucleotide binding; however, it was limited by the requirement for fluorescently labeled DNA and its inability to monitor fingers subdomain opening directly via FRET (Rothwell et al. 2005). Here, we report the design of a FRET system with both fluorophores attached to the protein and demonstrate its ability to monitor fingers subdomain movement both upon nucleotide binding and after catalysis of phosphodiester bond formation. This system supports previous observations of fingers subdomain behavior during nucleotide binding using a different fluorophore pair, and eliminates potential problems caused by movement of the DNA substrate within the active site. We show that the rate of fingers subdomain opening is similar to k_{pol} , indicating either that fingers subdomain opening occurs after the rate-limiting step or that fingers subdomain opening is slow.

From this study and others, it is clear that the enzyme oscillates between an open and closed form of the fingers subdomain, a conformational change likely to be important in the delivery of nucleotides to the active site. Although ensemble FRET studies are useful in monitoring conformational transitions where all individual molecules converge conformationally toward a relatively stable end state (like the closed ternary complex of KlenTaq1), they do not enable the observation of rapidly transient conformations in a mixture of unsynchronized conformational states as is likely the case for the fingers subdomain of KlenTaq1 when presented with an incorrect nucleotide or in the apo form. For this, single molecular FRET is the technique of choice. The previously described $KT^{V649C[A594]}.p^{A488.6/t}$ system (Rothwell et al. 2005) was very useful for determining the rate of the open to closed transition of the fingers subdomain in KlenTaq1. However, the relatively low affinity of KlenTaq1 for its p/t substrate (mid nM) and the fast dissociation of the p/t substrate make this system unsuitable for the harsh requirements of low concentration (typically pM) of single molecule spectroscopy. The dual labeled KlenTaq1 system presented here ($KT^{V649C, E825C[A488-A647]}$) has the advantage over the previous system in that there is no requirement for labeled DNA. A system with unlabeled p/t substrate is ideal for single molecule FRET studies, as it overcomes the requirement for DNA to be at a similar concentration to enzyme, which in turn determines the minimum working protein concentration; thus, we anticipate

that the FRET system described here will be used to further our understanding of the role of the fingers subdomain in nucleotide selection by DNA polymerases at a single molecule level.

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