Numerous studies have been undertaken to establish the mechanism of deoxyribonucleoside triphosphate (dNTP) binding and template-directed incorporation by DNA polymerases. It has been established by kinetic experiments that a rate-limiting step, crucial for dNTP selection, occurs before chemical bond formation. Crystallographic studies indicated that this step may be due to a large open-to-closed conformational transition affecting the fingers subdomain. In previous studies we established a Fluorescence Resonance Energy Transfer (FRET) system to monitor the open to closed transition in the fingers subdomain of Klentaq1. By comparing the rates of the fingers subdomain closure to that of the rate-limiting step for Klentaq1, we showed that fingers subdomain motion was significantly faster than the rate-limiting step. We have now used this system to characterise DNA binding as well as complete a more extensive characterisation of incorporation of all four dNTPs. The data indicates that DNA binding occurs by a two step association, and that dissociation of the DNA is significantly slower in the case of the closed ternary complex (E':p/t:dNTP). The data for nucleotide incorporation indicates a step occurring before dNTP-binding which differs for all four nucleotides. As the only difference between the (E:p/t) complexes is the templating base, it would suggest an important role for the templating base in initial ground state selection.

DNA Polymerases constitute the central component of the DNA replication machinery. The basic role of a DNA polymerase is to catalyse the addition of a deoxynucleoside monophosphate (dNMP) to a primer strand based on its complementarity to a template base provided by a template strand. Various DNA polymerases have been discovered, specialised for different roles, and despite sharing a common catalytic mechanism for the nucleotidyl transfer reaction, vary considerably in their accuracy during template directed DNA synthesis. Most polymerases studied, irrelevant of their specificity, share a rate-limiting conformational change, which occurs before chemistry and is utilised during selection of a correct nucleotide.

A basic model for DNA polymerases was proposed based on early kinetic work on T4 DNA polymerase (1), T7 DNA polymerase (2,3), HIV-1 Reverse Transcriptase (HIV-1 RT) (4) and the Klenow fragment of DNA polymerase I from Escherichia coli (Klenow) (5-8). In this model, the first step is binding of the primer/template (p/t) substrate to the unliganded polymerase (E). For polymerases in which spectroscopic techniques have been applied to study p/t binding, a more complex interaction between the enzyme and the substrate has been observed, displaying either multiple binding modes or a sequential binding mode for HIV-1 RT and polymerase β (pol β), respectively (9-12). After p/t binding, there follows an initial loose binding of the dNTP substrate. This initial ground state binding of the dNTP is used by some polymerases such as HIV-1 RT and T7 DNA polymerase, which are both replicative enzymes, to discriminate against non-complementary dNTPs by a factor of 250 fold and 390 fold, respectively (2,4). For repair polymerases, the differences between correct and incorrect nucleotide binding are much smaller. Klenow only selects against incorrect nucleotide incorporation on average by a factor.
of 3.4, yeast polymerase η by a factor of 4, and polymerase β by a factor of 20 (13) (7,8,14,15). Binding of the correct nucleotide leads to a conformational change which converts a loose E:p/t:dNTP ternary complex into a tight activated E’:p/t:dNTP complex capable of undergoing chemistry. The rate of this conformational transition was named \( k_{pol} \). In general, this step is the point at which the strongest discrimination occurs. It is also the slowest, rate-limiting, step in the nucleotide incorporation cycle. After chemistry occurs, there then follows the release of the pyrophosphate product and either translocation or dissociation of the DNA product.

Structural studies of DNA polymerase as exemplified by Klentaq1, a family A repair enzyme, have solved many intermediates in the reaction pathway including the apo form of the polymerase, the E:p/t and E:dNTP binary complexes as well as the ternary complex, E’:p/t:dNTP which was formed using a “non-reactive” 3’ primer terminus with the next correct nucleotide bound to form a “trapped closed” ternary complex (16-23). The structural states reveal a limited conformational change occurring in the thumb subdomain upon p/t binding and a large conformational change occurring in the fingers subdomain upon binding of the correct nucleotide to the E:p/t complex. The change in conformation of the fingers subdomain results in the formation of the binding site for the nascent base pair. The original idea was that the open to closed transition seen structurally corresponds to the kinetically-defined “conformational change” required for selection.

However, a large body of evidence now indicates that the kinetically-defined conformational change does not correspond to the motion of the fingers subdomain for most polymerases. For polymerase β (pol β), a mutation of Asp 276, a residue that makes contact with the incoming nucleotide only in the closed conformation, results in an increase in free energy of dNTP ground state binding (24) suggesting that the rate-limiting conformational change is not the open to closed structural transition, but instead is triggered in the closed polymerase conformation (24). Similar conclusions were drawn from inspection of the rate-limiting kinetics of wild-type pol β and a pol β variant mutated at Tyr265, a residue that does not make any contact with the DNA or the dNTP (25,26). Fluorescence studies on pol β also indicated that motions in the fingers subdomain appear to be fast but the extent to which it is not rate-limiting was not addressed (27). The conclusions that the rate-limiting conformational change actually occurs in the closed conformation would appear to also apply to the repair enzymes, such as Klentaq1 and Klenow. It was shown using the Klenow fragment and 2-aminopurine (2-AP) as a reporter that a 2-AP at the templating position (the first unpaired template base) undergoes a sizable and very rapid decrease in fluorescence associated with dNTP-binding (28). If the motion of the templating base is coupled to the open-to-closed transition affecting the fingers subdomain, then the open-to-closed conformational transition affecting the fingers subdomain must also be at least as fast. In contrast, for T7 DNA polymerase, recent fluorescence studies support a rate-limiting conformational change affecting the fingers subdomain (29). However, despite the positioning of a fluorophore in the fingers subdomain it is unclear whether this probe is monitoring the overall movement of the fingers subdomain or more subtle changes occurring in this subdomain. Finally, we developed a FRET system on Klentaq1 in which the motion of the fingers subdomain was directly monitored upon nucleotide binding and incorporation: this study indicates that the fingers subdomain motion is fast and not rate limiting (23).

We have now used this FRET system to characterise DNA-binding as well as complete a more extensive characterisation of binding and incorporation of all four dNTPs. The data indicates that DNA-binding occurs by a two step association, and that dissociation of the DNA is significantly slower in the case of the closed ternary complex, E’:p/t:dNTP. The data for nucleotide incorporation indicates a step occurring before nucleotide-binding and fingers subdomain closure. The dependence of this step on nucleotide concentration differs for each dNTPs. As the only difference between the E:p/t complexes is the templating base, it would suggest an important role for the templating base in initial dNTP ground state binding.

**Experimental procedures**

**Purification and labelling of Klentaq1 and KT**

Rothwell and Waksman (2007)
Klentaq1 was purified according to Korolev et al. (1995) (16). The mutation Val 649 to Cys (KT_{V649C}) was introduced using the Stratagene Quick change kit and the sequence confirmed by DNA sequencing. Purification of the mutant protein was performed as for wild-type Klentaq1. The concentration of protein was calculated using a molar extinction coefficient of 69,622 cm$^{-1}$ M$^{-1}$ at 280 nm. Cysteine 649 was labelled with the acceptor fluorophore Alexa$^{594}$-C$_2$ maleimide (Molecular Probes, Leiden, Netherlands) according to reference (23) to form Alexa$^{594}$-labelled Klentaq1 (KT$_{V649C}^{A594}$). Labelling efficiencies were typically 90% or greater.

Nucleotides

100 mM solutions of deoxynucleoside triphosphates (dNTP), and dideoxynucleoside triphosphates (ddNTP) were purchased from GE Healthcare (Amersham, UK).

Oligonucleotides

The Alexa 488-labelled DNA primer strand was purchased from IBA (Göttingen, Germany). The donor Alexa 488 probe was coupled using an NHS activated Alexa 488 dye from Molecular Probes (Leiden, Netherlands) on a commercially available 5-C6-Amino-2'-deoxythymididine. The primer used, p$^{A488}\_G$, was a 40-mer (sequence 5'–CAGGCCCACTGGGTCACTCCAGGCGCTGAGCAGGCTACGGTAGGCTGCGACG–3') in which the donor labelled base is indicated in bold. The template oligonucleotides used in this study have the following sequences (bold indicates the base-pairing region). The templates are called t_A, t_C, t_G, t_T, with the letters in capital corresponding to the next dNMP to be incorporated.

$t_A$ (5'–TGGTTAATCTCTGCGTACGTAGGCTGCGACGTGCCGACTGACCAGGCGCTGAGCAGGCTACGGTAGGCTGCGACG–3')$

t_C (5'–TGGTTAATCTCTTGGATACGTAGGCTGCGACGTGCCGACTGACCAGGCGCTGAGCAGGCTACGGTAGGCTGCGACG–3')$

t_G (5'–TGGTTAATCTCTTAGGATACGTAGGCTGCGACGTGCCGACTGACCAGGCGCTGAGCAGGCTACGGTAGGCTGCGACG–3')$

t_T (5'–TGGTTAATCTCTGCTTAGTACGTAGGCTGCGACGTGCCGACTGACCAGGCGCTGAGCAGGCTACGGTAGGCTGCGACG–3')$

The hybridization of primer and template was performed according to Rothwell et al. (2005) (23). Stock solution of 100 µM were made in 50 mM Tris pH 7 and 20 mM NaCl.

Steady state titration measurements

Steady state titration experiments to determine the affinity of the E:p/t interaction were recorded using a Fluormax-3 (Jobin Yvon Horiba) at 20°C. A solution of p$^{A488}\_G$ at a concentration of 100 nM in 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 2 mM MgCl$_2$ was made and the fluorescent signal of the donor was monitored over time until a stable signal was observed. Following addition of increasing amount of KT$_{V649C}^{A594}$ (see Figure 1A), the sample was mixed and was allowed to equilibrate until a stable signal was observed. Alexa 488 was excited at 496 nm and the fluorescence emission recorded at 520 nm with slits set at 4 nm and 1 nm for excitation and emission, respectively. Data was normalised to 1 using Origin 7.0 (OriginLab Corporation) and the signal of the donor-labelled p$^{A488}\_G$ as a reference. The FRET system was used here because it yielded better data quality than when the unlabelled enzyme was used. It was also used in order to remain consistent with the pre-steady state measurements (see below).

Pre-steady state kinetic measurements

All stopped flow experiments were performed at 20 °C in 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 2 mM MgCl$_2$. Data were recorded using a Hi-Tech scientific DX-2 stopped flow system with excitation of Alexa 488 at 493 nm. Donor and acceptor fluorescence were separated using the filters XF3084 (Glen Spectra; bandpass range 510-570 nm) for Alexa488 and XF3028 (Glen Spectra; bandpass range 615-650 nm) for Alexa 594. Slits were set at 10 nm for both excitation and emission. The voltage on both photo multipliers varied depending on the conditions and optimized for signal to noise. Each trace shown represents the average of at least 3 "shots".

To monitor formation of the E:p/t complex a solution of 20 nM p$^{A488}\_G$ was mixed rapidly with a varying concentration of KT$_{V649C}^{A594}$ (100 – 1200 nM) and the signal produced from the decrease in the donor fluorescence of the labelled DNA was recorded. Although a signal decrease is also observed by mixing labelled DNA with the unlabelled wild-
type enzyme, larger concentrations of the p_{A488.6}/t_G are needed, typically 100 nM, to produce a recordable signal. Thus, the labelled enzyme was used for all experiments.

For p/t dissociation experiments from the binary E:p/t or ternary E':p/t:dNTP complex, the p/t was terminated as previously described (23) to produce a complex of 1 µM KT^{V649C[A594]}:500 nM p_{A488.6}/t_GTE (where t_GTE indicates a t_G primer terminated by ddGTP with the next nucleotide to be incorporated being dCTP). For DNA dissociation experiment from the binary E:p/t complex, the 1 µM KT^{V649C[A594]}:500 nM p_{A488.6}/t_GTE complex was loaded into one sample chamber with the second sample chamber containing 0.2 mg/ml Heparin or 20 µM unlabelled p/t (final concentration). For p/t dissociation experiments from the ternary E':p/t:dNTP complex, the E':p/t:dNTP was first produced from the KT^{V649C[A594]}:p_{A488.6}/t_GTE complex by adding dCTP to a final concentration of 250 µM. The resulting closed ternary complex was mixed rapidly with 0.2 mg/ml Heparin to monitor dissociation kinetics.

For nucleotide incorporation experiments, preincubated KT^{V649C[A594]} (1 µM final concentration) and p_{A488.6}/t_G (500 nM final concentration), where X is the next nucleotide to be incorporated, were loaded into one sample chamber with the second sample chamber containing varying concentrations of the corresponding dNTP to be incorporated.

In all stopped flow experiments except for the DNA dissociation experiments a "blank" run was performed in which reaction buffer was mixed rapidly with either the labelled p_{A488.6}/t_G alone for DNA binding experiments or the KT^{V649C[A594]}:p_{A488.6}/t_G (where X is the next nucleotide to be incorporated) complex for dNTP incorporation experiments. This was subtracted from each individual data set using Origin 7.0. Data were fitted using Grafit 5.0 (Erithacus Software Limited) between 0.02 s to 1.5 s according to the models described in the text.

Results

We previously designed a FRET system to monitor the fingers subdomain motion of Klentaq1 upon nucleotide incorporation (23). The system consists of a primer/template (p/t) labelled 6 bases away from the 3' terminus of the primer with the donor fluorophore Alexa 488 called p_{A488.6}/t_G (where X is the next nucleotide to be incorporated). In combination with Klentaq1 labelled with Alexa 594 at an introduced cysteine at position 649 in the fingers subdomain, called KT^{V649C[A594]} a measurable change in FRET occurs. This is due to the fingers subdomain closure on nucleotide binding which results in the acceptor fluorophore in the fingers subdomain moving closer to the donor fluorophore on the p/t DNA. We have now extended the use of this system to monitor the association of the p/t to Klentaq1 and perform a more complete characterisation, involving all 4 dNTPs, of the fingers subdomain motion upon nucleotide incorporation.

**Primer/template DNA-binding to Klentaq1**

To investigate the affinity and binding of KT^{V649C[A594]} to p/t, both steady state and pre-steady state association experiments were performed.

For the steady state titration experiments, a fixed concentration of donor labelled p_{A488.6}/t_G of 100 nM was used and increasing amounts of KT^{V649C[A594]} was added (Figure 1A). The data was fitted with the equation below where F, F_{max} and F_{min} are the recorded, maximum, and minimum fluorescence signals, respectively. E_{0} and S_{0} are the initial concentrations of KT^{V649C[A594]} and p_{A488.6}/t_G, respectively, and K_D is the dissociation constant:

\[
F = F_{\text{min}} - \left[\frac{(F_{\text{max}} - F_{\text{min}})\left(E_{0} + S_{0} + K_{D}\right) - \left(\sqrt{(E_{0} + S_{0} + K_{D})^{2} + 4E_{0}S_{0}} - (E_{0} + S_{0})\right)}{2E_{0}}\right] \]

Fitting of the data gave a K_D value of 86.5±8.8 nM, which is slightly lower than the published value of 100 nM (31).

We next investigated DNA-binding using stopped flow to determine the pre-steady state binding parameters. Figure 1B shows raw data obtained from the association kinetics of p_{A488.6}/t_G to KT^{V649C[A594]}. The donor labelled p/t was kept at a fixed concentration (20 nM end concentration) and mixed rapidly with increasing amounts of KT^{V649C[A594]} and the change in donor fluorescence was recorded. From the data, two exponential phases are apparent. The first exponential phase is protein concentration dependent. The rate constants (k_{obs}) for this phase obtained at various protein concentrations were plotted against protein concentration (Figure 1C) and the data were fitted using the linear equation:

\[
k_{\text{obs}} = k_{1}[E] + k_{-1} \]

where k_{1} and k_{-1} represent the forward and reverse rate constants for the first phase binding step. The rate constants k_{1} and k_{-1} are derived
from the slope and the intercept with the y-axis of the plot of $k_{obs}$ versus enzyme concentration, respectively (Figure 1C). For this step of p/t binding, values for $k_1$ of $5.3 \pm 0.2 \times 10^8$ M$^{-1}$ s$^{-1}$ and $k_2$ of $16.5 \pm 7.5$ s$^{-1}$ were determined. The $k_j$ value is inaccurate due to uncertainty about the intercept at the y-axis. Nevertheless, $K_P$ determined from $k_{pj} / k_j$ was approximately 31 nM, in the same order of magnitude as that obtained from steady state measurements of 86.5 nM. The second phase is not well resolved over the concentration range measured but appears to show no dependence on $K_{T_E}$ and has an average value of 16 s$^{-1}$. This step may represent an additional conformational change occurring after p/t binding, or may represent a partitioning into a second binding mode.

Association experiments were also performed with the labelled p/t and wild-type Klentaq 1 (data not shown). As indicated in the experimental procedure section, measurements had to be carried out at higher concentrations of 100 nM p$^{A488.6}$/t$_G$. However, very similar results (not shown) indicate that the two phases observed are not due directly to FRET, rather the acceptor fluorophore on the labelled p$^{A488.6}$/t$_G$ is amplifying the signal obtained from the reduction in the donor fluorescence on the labelled p/t.

In order to determine a more accurate value for the dissociation rate, a complex of 1 µM p$^{A488.6}$/t$_T$G was mixed with 0.2 mg/ml Heparin and the change in donor and acceptor fluorescence was recorded as the donor labelled p/t is replaced with Heparin (Figure 2; note that performing the same experiment with a non-terminated primer/template yields very similar results). It can be seen that the dissociation reaction is biphasic and can be described as the sum of two components with rate constants of 38.2 s$^{-1}$ and 3.2 s$^{-1}$. A possible explanation of this behaviour is that the p/t is distributed between two states when bound to the enzyme. Taken together with the association data a two step binding model can be proposed in which the two E:p/t complexes are interchangeable. We assign the slower rate as $k_2$ and the faster rate as $k_j$. Using the value of 38.2 s$^{-1}$ as $k_j$, together with the $k_1$ of $5.3 \times 10^8$ M$^{-1}$ s$^{-1}$ obtained from the E:p/t association data results in a $K_P$ value of 71.8 nM, which is in better agreement with the steady state value of 86.5 nM.

The dissociation of the p/t from the ternary complex (E':p/t:dCTP) was also investigated (Figure 3). The ternary complex was made as described in the Experimental Procedures section to produce a complex of 1 µM p$^{A488.6}$/t$_G$, 500 nM p$^{A488.6}$/t$_T$G, 250 nM dCTP. In this complex the substrate was terminated using ddGTP (which lack the 3’-OH group required for chemistry), and the next nucleotide to be incorporated, dCTP, is present. The ternary E’:p/t:dCTP complex produced was mixed rapidly with 0.2 mg/ml heparin and the increase in donor and decrease in acceptor signal monitored. In contrast to the binary complex the data is best fit by a triple exponential model. The fastest and first phase (55.9 s$^{-1}$) is comparable to $k_2$ in the scheme above and thus may represent dissociation from an open complex. However, the relative amount of this complex is now only 22 % as compared to 52 % seen for the binary complex (as judged by the amplitude changes). The next two phases, which contribute to the overall slowing of the dissociation from the ternary complex, as compared to the binary complex, have a rate of 0.86 s$^{-1}$ and 0.19 s$^{-1}$. The species represented by a rate of 0.86 s$^{-1}$ has the highest population contributing over 50% of the total signal while the species with a dissociation rate of 0.19 s$^{-1}$ contribute approximately 21% of the total signal. Because rate of dissociation is low for both species, they likely represent direct dissociation from the closed ternary complex.  

Nucleotide dependence of fingers subdomain closure

Figure 4A shows typical data obtained for single dCTP incorporation into the p$^{A488.6}$/t$_C$ complex for 3 different dCTP concentrations. Only the first 1.5 s of the trace are shown which show the increase in FRET (decrease in donor fluorescence and increase in acceptor fluorescence) due to fingers subdomain closure. The second component of the signal (not shown) consists in an increase in donor fluorescence and, although a corresponding decrease in acceptor fluorescence is observed, is not due to FRET (see reference (23) for details). The data shown in Figure 4A was fitted with a single exponential and slope and the change in FRET occurring during fingers subdomain closure in response to differing concentrations of dNTPs was determined. The

$$
E + p/t \stackrel{k_{pj}}{\rightleftharpoons} E:p/t_1 \stackrel{k_{1j}}{\rightarrow} E:p/t_2 \quad (3)
$$

The dissociation of the p/t from the ternary complex (E’:p/t:dCTP) was also investigated (Figure 3). The ternary complex was made as described in the Experimental Procedures section to produce a complex of 1 µM p$^{A488.6}$/t$_G$, 500 nM p$^{A488.6}$/t$_T$G, 250 nM dCTP. In this complex the substrate was terminated using ddGTP (which lack the 3’-OH group required for chemistry), and the next nucleotide to be incorporated, dCTP, is present. The ternary E’:p/t:dCTP complex produced was mixed rapidly with 0.2 mg/ml heparin and the increase in donor and decrease in acceptor signal monitored. In contrast to the binary complex the data is best fit by a triple exponential model. The fastest and first phase (55.9 s$^{-1}$) is comparable to $k_2$ in the scheme above and thus may represent dissociation from an open complex. However, the relative amount of this complex is now only 22 % as compared to 52 % seen for the binary complex (as judged by the amplitude changes). The next two phases, which contribute to the overall slowing of the dissociation from the ternary complex, as compared to the binary complex, have a rate of 0.86 s$^{-1}$ and 0.19 s$^{-1}$. The species represented by a rate of 0.86 s$^{-1}$ has the highest population contributing over 50% of the total signal while the species with a dissociation rate of 0.19 s$^{-1}$ contribute approximately 21% of the total signal. Because rate of dissociation is low for both species, they likely represent direct dissociation from the closed ternary complex.  

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rate constants derived from the single exponential fit (k_{obs}) were then plotted as a function of dNTP concentration (Figure 4B). In Figure 4B, each point is the average of at least 3 measurements with the error bars being generated from the standard error on averaging for each concentration. For dATP, dTTP and dCTP, due to a low amplitude of the FRET increase phase at low concentrations, it was only possible to reliably record down to 50 µM.

What can be observed immediately is that the rate of fingers subdomain closure decreases as a function of increasing dNTP concentrations. Such a trend in the data has been previously interpreted as an indication that the rate of a binding reaction is limited by a preequilibrium step occurring beforehand where the first step (the preequilibrium step) is slow compared to the second step (the binding step) (30). Thus, we propose here that the rate observed for fingers subdomain closure is limited by a step occurring beforehand. For such a case, the model proposed by Fersht (30) and adapted here is the following:

\[ E:{p/t} \Leftrightarrow (E:{p/t})^* + dNTP \leftrightarrow [(E:{p/t})^* : dNTP]_{closed} \]

(4)

Here, we omitted to show the (E:{p/t})^* : dNTP species because it immediately transitions to the [(E:{p/t})^* : dNTP]_{closed} form. Derivation by Fersht leads to (30):

\[ k_{obs} = k_{3} + k_{j3} \left( \frac{K_{D4}}{[dNTP] + K_{D4}} \right) \]

(5)

This model envisages two E:{p/t} complexes, E:{p/t} or (E:{p/t})^*, in equilibrium, only one of which is competent for dNTP binding.

It is interesting that for the different nucleotides the rates of \( k_{3} \) and \( k_{j3} \) vary. For both dATP and dTTP the forward and reverse rates are similar having values of 1.49 s\(^{-1}\) and 1.58 s\(^{-1}\) for \( k_{3} \) and 8.68 s\(^{-1}\) and 7.72 s\(^{-1}\) for \( k_{j3} \), respectively. However for dCTP incorporation, the forward and reverse rates are more similar, \( k_{3} \) being 4.07 s\(^{-1}\) and \( k_{j3} \) being 7.40 s\(^{-1}\), while for dGTP incorporation the forward and reverse rates are almost identical (\( k_{3} = 2.21 \) s\(^{-1}\) and \( k_{j3} = 1.56 \) s\(^{-1}\)). The rates would indicate that in the case of dATP and dTTP, the enzyme is predominantly in the E:{p/t} state; for dCTP less so, and dGTP would be predominantly in the (E:{p/t})^* state. Although the \( K_{D4} \) (see equation 5) values are inaccurate due to the lack of information at lower concentrations for dATP, dCTP, dTTP, there seems to be almost equivalent \( K_{D} \) values for the purine based nucleotides dATP and dGTP of 173 µM and 175 µM respectively, and for the pyrimidine based nucleotides dCTP and dTTP of 236 µM and 215 µM respectively. These values are higher than previously determined by quench flow measurements and probably do not represent the \( K_{D} \) for “ground state” binding recorded by this technique (31).

**Discussion**

One major goal of this report was to establish the nature of p/t DNA binding to Klentaq1. Both DNA-association and DNA-dissociation binding experiments show that Klentaq1 can exist in two E:{p/t} states, E:{p/t\(_{1}\)} and E:{p/t\(_{2}\)} in scheme (3), both of which have similar \( K_{D} \) values and can interchange rapidly. The binding of a p/t DNA is structurally characterized by two events: i- the outwards motion of the thumb subdomain and ii- the ordering of the H\(_{1}\)H\(_{2}\) loop in the thumb subdomain and its wrapping around the p/t DNA to form a quasi cylinder surrounding the DNA (17,20). Thus, one could envisage step 1 of scheme (3) to correspond to the binding of the p/t DNA to the partially-formed p/t-binding surface of the apo form. This initial binding event would then be followed by a reorganization of the thumb subdomain leading to the structuring of the H\(_{1}\)H\(_{2}\) loop, step 2 of scheme 3. A major objection to this scenario is that the ordering of the H\(_{1}\)H\(_{2}\) loop would likely result in tighter binding for step 2, which is not what is observed.

A perhaps better explanation for the observation of two E:{p/t} complexes observed from the DNA-association and -dissociation experiments may be found in the following remarkable feature of the E:{p/t} complex structure: the stacking of the first paired template base onto the aromatic ring of Y671 (Figure 5A). This interaction provides a means to register the pairing of the first unpaired base of the template) swings in its stacking arrangement and, as shown in the ternary E:{p/t\(_{d}\)}dNTP complex structure, the templating base of the template strand (i.e. the first unpaired base of the template) swings in its place, positioning itself to form a Watson-Crick base pair with the correct incoming nucleotide (Figure 5B). If one assumes that the fingers domain in the E:{p/t} complex (i.e. in the absence of an incoming nucleotide) constantly oscillates...
between an open and closed form, then the DNA might be released from the stacking arrangement with Y671 and thus might be free to move within the confines of the p/t-binding site of the enzyme. Indeed, molecular dynamics simulations suggest that the DNA-binding site may form an electrostatic tube within the quasicylinder that the p/t-binding site forms and within which no preferential interaction with any particular residue occur leaving the DNA free to slide within the site. Thus, the E:p/t₁ and E:p/t₂ states may correspond to two binding modes within the p/t-binding site, these two modes only differing in relation to Y671 and the conformational transition affecting the fingers subdomain. Two p/t-binding modes have also been observed in HIV1-RT and a similar interpretation was suggested (9,12,32). For HIV1-RT, the two E:p/t species differ by only a 5 Å shift in the position of the nucleic acid. These two states were described as “educt” and “product” states in the polymerization reaction cycle whereby the “educt” state is a complex in which the nucleic acid is positioned to allow nucleotide incorporation and the product state corresponds to a state formed immediately after nucleotide incorporation, but before RT translates to the next nucleotide.

The second major goal of this report was to investigate how the fingers subdomain closure is linked to dNTP selection. The dependence of fingers subdomain closure on nucleotide concentration was thus studied for all nucleotides. The major finding is the observation of a step occurring before the dNTP-induced fingers subdomain closure takes place. The nature of the pre-equilibrium taking place prior to nucleotide-binding and/or fingers subdomain closure is hard to determine based on the present results. It is unlikely that this pre-equilibrium is due to the reverse transition of the fingers subdomain into an open conformation from a closed conformation, as this would be indicated by a large change in fluorescence of the donor and acceptor probes as the fingers subdomain first opens and then closes due to dNTP binding.

However, a plausible interpretation of this unexpected result may be derived from the observation that the dependence of fingers subdomain closure on nucleotide concentration is different for each nucleotide. As the only major difference between all four E:p/t complexes is the templating base of the p/t DNA, this would suggest that the first event in dNTP incorporation for Klentaq1 is an initial rearrangement of the templating base occurring before dNTP binding and/or fingers subdomain closure. Indeed, from the comparison of the E:p/t open complex with the E':p/t:ddNTP closed complex (Figure 5, A and B, respectively), one can infer that the templating base moves in and out of stacking arrangement with the first paired template base as Y671 moves out and in of the same stacking arrangement, the motions of the templating base and of Y671 seemingly concerted. Thus, one could envisage the templating base participating in an equilibrium between two conformations, which would have unique features dependent on the nature of the templating base. Temiakov et al. (2004) describe these two states of the templating base in T7 RNA polymerase as “the pre-insertion state” corresponding to the “out” conformation described in Figure 5A and “the insertion state” corresponding to the “in” conformation described in Figure 5B (33) and we shall adopt this terminology in the discussion below.

While the pre-insertion versus insertion states equilibrium of the templating base may account for the observed base-specific behaviour, the observed dependence on nucleotide concentration can only be explained if one of these states is involved in nucleotide ground state binding. This implies that the templating base might participate in nucleotide binding before the fingers subdomain has closed. One could envisage the incoming nucleotide binding directly in the active site as is shown in Figure 5C and the templating base in its insertion state interacting with the incoming nucleotide through Watson-Crick pairing as shown in Figure 6A. In the configuration shown in Figure 5C, which was observed crystallographically by Li et al. (1998) (17), the incoming nucleotide is in the active site i.e. bound to the catalytic residues D610 and D785 with only one Mg ion involved; however, the fingers subdomain is not clamped onto the nucleotide as it is in the open conformation and Y671 locates opposite the base of the incoming nucleotide. To account for the dNTP concentration dependence for fingers subdomain closure described here, the Y671 would need to move in and out of stacking arrangement with the first paired template base in the open state of the fingers subdomain to allow the templating base to move in and out of the insertion state site as shown in Figure 6A.

A second model is suggested by Temiakov et al. (2004) who have described a
state of T7 RNA polymerase, where the templating base would locate between the pre-insertion and insertion state sites (Figure 6B). In that third state, termed “intermediate pre-insertion state”, the templating base would be close enough to the incoming nucleotide bound to the nucleotide-binding site (the O-helix in the case of Klentaq1) in the open fingers subdomain to interact with the incoming nucleotide though Watson-Crick base pairing. Thus, the pre-equilibrium which we observed here, as it must be templating base- and dNTP concentration-dependent, would consist of an equilibrium between a “pre-insertion state” and an “intermediate pre-insertion state”, the nature of which would differ depending on the identity of the templating base.

Whether the pre-equilibrium revealed here reflects the “pre-insertion” versus “insertion” states equilibrium or the “pre-insertion” versus “intermediate pre-insertion” states equilibrium of the templating base and Y671, both are sufficient to explain the observed nucleotide concentration dependence of fingers subdomain closure. For both models there would be little difference in the ground state binding affinity of each of the dNTPs, rather closing of the fingers subdomain would amplify what little differences there are between Watson-Crick and non Watson-Crick base pairs and provide a closed reaction site in which nucleotide incorporation could occur.

Replicative DNA polymerases, such as T7 DNA polymerase, appear to be capable of strong discrimination in the ground state. It would be interesting to perform similar experiments on T7 DNA polymerase to determine how this greater level of discrimination is seen.

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References
Figure legends

Figure 1. Binding of the acceptor labelled KT\textsuperscript{V649C[A594]} to donor labelled p\textsuperscript{A488.6}/t\textsubscript{G}. A) Steady state titration of the acceptor labelled KT\textsuperscript{V649C[A594]} against 100 nM p\textsuperscript{A488.6}/t\textsubscript{G}. Excitation was at 493 nM and emission was recorded at 520 nm. The data was fitted as described in the text and gave a value of 86.5±8.8 nM. B) Pre-steady state kinetic measurement of p/t binding. A typical stopped flow result is shown in which a 20 nM sample of p\textsuperscript{A488.6}/t\textsubscript{G} was mixed rapidly with 500nM KT\textsuperscript{V649C[A594]}. Excitation was at 493 nm and the donor fluorescence was collected using band pass filter between 510-570 nm. The inset in Figure 1B shows the first 50 ms of the binding. The data was fitted with a single exponential (dot), single exponential with slope (dash) and a double exponential (solid) with the best fit being a double exponential. C) Dependence of the pseudo-first order rate constant on KT\textsuperscript{V649C[A594]} concentration. A constant concentration of 20 nM p\textsuperscript{A488.6}/t\textsubscript{G} was mixed with increasing amount of KT\textsuperscript{V649C[A594]}. k\textsubscript{i} and k\textsubscript{f} were determined by the slope of the linear fit and the intercept of the line with the y-axis and yielded values of 5.3x10\textsuperscript{3}±0.2 x10\textsuperscript{8} M\textsuperscript{-1} s\textsuperscript{-1} and 16.5±7.5 s\textsuperscript{-1}, respectively.

Figure 2. Kinetics of dissociation of the donor-labelled p\textsuperscript{A488.6}/t\textsubscript{G\textsubscript{TE}} from the acceptor-labelled KT\textsuperscript{V649C[A594]}. A complex of 1 \mu M KT\textsuperscript{V649C[A594]}:500 nM p\textsuperscript{A488.6}/ t\textsubscript{G\textsubscript{TE}} was mixed rapidly with 0.2 mg/ml Heparin. The terminated substrate was used in order to provide a direct comparison between the dissociation of the binary (E:p/t) and the ternary (E':p:t:dCTP) complexes, although the non terminated p/t gives essentially the same results. Excitation was at 493 nm. The donor fluorescence (green) was collected using band pass filter between 510-570 nm and the acceptor fluorescence (red) using a band pass filter between 615-650 nm. Two time scales were recorded and superimposed on the graph. The data was fitted with a double exponential (dash) for both donor and acceptor and gave values of 38.70±0.33 s\textsuperscript{-1} and 37.70±0.35 s\textsuperscript{-1} for the first phase and 3.09±0.01 s\textsuperscript{-1} and 3.42±0.01 s\textsuperscript{-1} for the second phase for the donor and acceptor, respectively. The relative amplitude of the first phase as compared to the total amplitude is approximately 52% for both donor and acceptor.

Figure 3. Kinetics of dissociation of p\textsuperscript{A488.6}/t\textsubscript{G\textsubscript{TE}} from the ternary complex. A complex of 1 \mu M KT\textsuperscript{V649C[A594]}:500 nM p\textsuperscript{A488.6}/ t\textsubscript{G\textsubscript{TE}}:250 \mu M dCTP was mixed rapidly with 0.2 mg/ml Heparin. Excitation was at 493 nm and the donor fluorescence (green) was collected using band pass filter between 510-570 nm and the acceptor fluorescence (red) using a band pass filter between 615-650 nm. The data was fitted with a double exponential (dot), and a triple exponential (dash) for both donor and acceptor with the triple exponential model providing the best fit. Fitting of the data gave values of 69.40±0.33 s\textsuperscript{-1} and 42.40±0.35 s\textsuperscript{-1} for the first phase, 0.72±0.01 s\textsuperscript{-1} and 1.01±0.01 s\textsuperscript{-1} for the second phase, and 0.13±0.01 s\textsuperscript{-1} and 0.26±0.01 s\textsuperscript{-1} for the third phase for the donor and acceptor, respectively. The relative amplitude of the phases is 22% for phase 1 for both donor and acceptor, 62% for donor and 52% for acceptor for phase 2 and, 16% and 26% for donor and acceptor, respectively, for phase 3. The inset shows a comparison between dissociation of the binary complex (lighter shades of green and red for donor and acceptor, respectively) and the ternary complex (green and red for donor and acceptor, respectively).

Figure 4. Dependence of fingers subdomain closure on dNTP concentration. A) Raw data obtained for single nucleotide incorporation by KlenTaq. A complex of 1 \mu M KT\textsuperscript{V649C[A594]}:500 nM p\textsuperscript{A488.6}/t\textsubscript{C} was mixed rapidly with increasing amounts of dCTP. Excitation was at 493 nm and the donor fluorescence was collected using band pass filter between 510-570 nm and the acceptor fluorescence using a band pass filter between 615-650 nm. 3 concentrations of nucleotides are shown. Red is 1000 \mu M, green is 500 \mu M, and blue is 250 \mu M. The rich colours show the donor signal and the faded colours show the acceptor signal. The data was fitted between 0.02-1.5 s with a single exponential with slope. B) Secondary data obtained from the rate obtained from the FRET-increase phase for dATP (◊), dCTP (□), dGTP (△), dTTP (○). The data was fitted using equation 5 and gave values of k\textsubscript{i}...
and $k_3$ of $1.49\pm0.28$ s$^{-1}$ and $8.68\pm0.34$ s$^{-1}$ and $K_{D4}$ of $172.9\pm31.6$ µM; $4.07\pm0.37$ s$^{-1}$ and $7.40\pm0.44$ s$^{-1}$ and $K_{D4}$ of $235.7\pm66.9$ µM; $2.21\pm0.13$ s$^{-1}$ and $1.56\pm0.12$ s$^{-1}$ and $K_{D4}$ of $175.2\pm60.1$ µM; and $1.58\pm0.38$ s$^{-1}$ and $7.72\pm0.35$ s$^{-1}$ and $K_{D4}$ of $215.9\pm51.1$ µM for dATP, dCTP, dGTP, and dTTP, respectively.

**Figure 5.** Crystal structures of the E:p/t (A), E':p/t:ddNTP ternary closed (B), and E:p/t:ddNTP ternary open (C) complexes. In each A, B, and C, the panel on the left is a schematic diagram of the stereo-view panel of the structure shown at right. In the stereo-view panels at right, the fingers, palm, and thumb subdomains are shown in C-α trace representation, color-coded in green, magenta, and dark blue, respectively. The O-helix is shown in red. The p/t DNA is shown in stick representation, color-coded in yellow and cyan for the template and primer strands, respectively. The first templating base is shown in dark blue. The incoming nucleotide and the Mg ions bound to it are shown in ball-and-stick representation, color-coded in orange. Y671 is shown in ball-and-stick representation with carbon, oxygen, and nitrogen color-coded in light gray, red, and blue, respectively. In the panels at left, the color-coding is the same as in the corresponding panels at right. The first paired template base is indicated as are the various sites mentioned in the text. In A, a representation of a nucleotide is shown in tuned orange color: it has been added bound to the O-helix as is observed in the E:dNTP structures of Li et al. (1998) (17).

**Figure 6.** Models for templating base- and dNTP concentration- dependence of fingers subdomain closure. In both panels, definition and color-coding of the various players are as in Figure 5. In each case, the equilibrium is with the pre-insertion state and thus, only the state of the templating base corresponding to its dNTP interacting state is shown. **A.** Binding of the dNTP to the templating base in its insertion state. **B.** Binding of the dNTP to the templating base in its intermediate pre-insertion state.
Figure 1
Rothwell and Waksman (2007)
Figure 2

Rothwell and Waksman (2007)
Figure 3

Rothwell and Waksman (2007)
Figure 5

Rothwell and Waksman (2007)
A pre-equilibrium before nucleotide binding limits fingers subdomain closure by KLENTAQ1
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