Single-molecule and ensemble fluorescence assays for a functionally important conformational change in T7 DNA polymerase

Guobin Luo1, Mina Wang2, William H. Konigsberg3, and X. Sunney Xie4

1Department of Chemistry and Chemical Biology, Harvard University, Cambridge, MA 02138; and 2Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT 06520

Edited by Robert J. Silbey, Massachusetts Institute of Technology, Cambridge, MA, and approved May 30, 2007 (received for review January 31, 2007)

We report fluorescence assays for a functionally important conformational change in bacteriophage T7 DNA polymerase (T7 pol) that use the environmental sensitivity of a Cy3 dye attached to a DNA substrate. An increase in fluorescence intensity of Cy3 is observed at the single-molecule level, reflecting a conformational change within the T7 pol ternary complex upon binding of a dNTP substrate. This fluorescence change is believed to reflect the closing of the T7 pol fingers domain, which is crucial for polymerase function. The rate of the conformational change induced by a complementary dNTP substrate was determined by both conventional stopped-flow and high-time-resolution continuous-flow fluorescence measurements at the ensemble-averaged level. The rate of this conformational change is much faster than that of DNA synthesis but is significantly reduced for noncomplementary dNTPs, as revealed by single-molecule measurements. The high level of selectivity of incoming dNTPs pertinent to this conformational change is a major contributor to replicative fidelity.

DNA polymerases (DNA pols) are essential components of replisomes that ensure faithful replication of cellular DNA (1). In addition to their role in replication, some DNA pols specialize in DNA repair (2). DNA pols also have important applications in molecular biology, such as in DNA sequencing (3) and in the PCR (4).

DNA pols catalyze the formation of a phosphodiester bond between the 3'-OH of a DNA primer strand and the α-phosphorous atom of an incoming dNTP. Continuous rounds of catalysis lead to a DNA duplex through extension of a nascent DNA primer having a sequence complementary to the template. The error rate during DNA replication can be as low as 10−10 to 10−15, which is crucial for polymerase function. The rate of the conformational change induced by a complementary dNTP substrate was determined by both conventional stopped-flow and high-time-resolution continuous-flow fluorescence measurements at the ensemble-averaged level. The rate of this conformational change is much faster than that of DNA synthesis but is significantly reduced for noncomplementary dNTPs, as revealed by single-molecule measurements. The high level of selectivity of incoming dNTPs pertinent to this conformational change is a major contributor to replicative fidelity.

It is of great interest to understand the molecular basis for the high fidelity exhibited by replicative DNA pols. Structural studies on T7 pol (13), HIV reverse transcriptase (14), human pol β (polβ) (15), Bacillus stearothermophilus pol I (16), and KlenTaq polymerase (17) have shown that binding of the complementary dNTP results in formation of a ternary complex in which the fingers subdomain of DNA pols has closed so that the incoming dNTP is encapsulated, with all possible hydrogen bonds and electrostatic interactions satisfied. As shown in Fig. 1B, T7 pol provides a good example of these features. Stopped-flow fluorescence measurements with 2-aminopurine (2AP), a probe for base stacking, have been used to detect conformation changes in polβ (18), T4 pol (19), and KF (20). The results suggested that the fingers domain may be in rapid equilibrium between the open and closed states before the putative rate-limiting step. Consistent with this observation, a recent stopped-flow study with fluorescence resonance energy transfer (FRET) on KlenTaq indicated that the motion of the fingers subdomain is also a fast step for this enzyme (21). In contrast, a kinetic study of a fluorescently labeled T7 pol showed that a conformational change, which is partially rate-limiting, could be responsible for base selectivity (22).

In light of the apparent discrepancies among different experimental approaches, additional assays probing the conformational change triggered by dNTP binding are necessary to fully understand the relationship between conformational dynamics and function of DNA pols. Here we report an assay to measure the T7 pol conformational change, based on the fluorescence intensity variation of a Cy3-labeled primer/template (P/T)-T7 pol complex due to environmental changes surrounding the dye (23). Upon the binding of a complementary dNTP, a fast conformational change is observed that is not rate-limiting for DNA synthesis. This change can be observed at both the single-molecule and ensemble-average levels. In general, real-time single-molecule studies (24–26) have the potential to identify transient intermediates without the need for synchronization, thus helping to elucidate enzymatic reaction mechanisms (27–29). Although spontaneous conformational fluctuations have been directly measured by single-molecule measurements (30, 31), less information is available about functionally important conformational changes at the single-molecule level. Here we use single-molecule techniques to directly

Author contributions: G.L. and X.S.X. designed research; G.L. and M.W. performed research; G.L., M.W., and W.H.K. contributed new reagents/analytic tools; G.L. and M.W. analyzed data; and G.L., W.H.K., and X.S.X. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Abbreviations: DNA pol, DNA polymerase; KF, the Klenow fragment of Escherichia coli polymerase I (KF) (11), the conformaional change (step 3) has been proposed to be rate-limiting for complementary dNTPs. This supposition has been supported by evidence from the elemental effect of αS-dNTP utilization, pulse–chase, and pulse–quench experiments (10, 12). Step 3 is important for base selection by most polymerases because, in addition to the initial dNTP binding step, it serves as a kinetic checkpoint to select against noncomplementary dNTPs (7). The selectivity arising from the difference in catalytic rates between complementary and noncomplementary substrates contributes a factor of 103 to 104 to replication fidelity (5).
observe stochastic events of switching between two conformational states of T7 pol. One particular advantage offered by single-molecule measurements is that rate constants of conformational changes for both the forward and reverse directions can be determined independently from a single time trace.

In this particular system, the fast forward conformational change cannot be resolved with single-molecule observations because of limited time resolution. To measure the fast kinetics, we have used a conventional stopped-flow apparatus and continuous flow in a microfluidic device with high time resolution. Michaelis–Menten kinetics for the conformational change upon binding of a complementary dNTP was observed and found not to be rate-limiting. We have assigned this conformational change to closing of the fingers domain in T7 pol.

We found that the rate constant of this conformational change is highly dependent on the complementarity of the dNTP substrate. Slow conformational changes induced by noncomplementary dNTP binding are difficult to observe at the ensemble level because they are rare events. However, infrequent conformational changes can easily be identified in a single-molecule time trace. Our results indicate that the rate constant of the conformational change is markedly reduced with noncomplementary bases. Therefore, this conformational change, presumably reflecting fingers domain closing, plays a crucial role in determining base selectivity and, thus, DNA replication fidelity.

**Results and Analysis**

**Single-Molecule Binding and Dissociations of T7 Pol-Cy3-P/T Complexes.** The single-molecule fluorescence experimental setup is shown in Fig. 2 [also see supporting information (SI) Appendix, Section 2 for details]. We first performed single-molecule experiments to observe the binding between T7 pol and immobilized Cy3-P/T. The fluorescence intensity of a single Cy3-P/T was stable. However, upon introduction of T7 pol in phosphate buffer, the fluorescence of Cy3-P/T fluctuated between two levels: a baseline level and a level with a 40% higher intensity (Fig. 3A). We attribute the increase in fluorescence intensity to the binding of T7 pol to the Cy3-P/T, which changes with the local environment of Cy3 (Fig. 3D, and see Discussion). This change is due to the reduced relaxation rate of Cy3 from its excited state, consistent with the higher fluorescence quantum yield of Cy3 in more viscous solvents.

When the concentration of T7 pol was reduced, the durations of the lower fluorescence intensity level were lengthened, but the duration of the higher intensity level remained constant. The distribution of the duration of lower and higher fluorescence intensity levels is related to polymerase binding and dissociation.
The presence of T7 pol and a complementary dCTP in which the dCTP association and dissociation rate constants (3.6 dd 50 nM of dCTP, with exponential fits (blue curves). C) Statistics of the lifetimes of the T7 pol binary and ternary states with levels. (With 10 nM T7 pol and 50 nM dCTP. The fluorescence intensity histogram, 12612 dideoxy-terminated primer (DNA synthesis and nucleotide excision, we used a nonextendable to focus on probing the conformational change in the absence of the complementary incoming dNTP on Cy3 fluorescence, we introduced the Cy3-P/T, the fluorescence intensity of the Cy3-P/T does not enhance can be tentatively identified and is highlighted in Fig. 4.Interestingly, when Sequenase (USB, Cleveland, OH), an exonuclease-deficient T7 pol with a 28-residue deletion (33), binds to the Cy3-P/T, the fluorescence intensity of the Cy3-P/T does not change significantly. We have tested the reactivity of Sequenase by using fluorescently labeled dCTP and Cy3-P/T and have confirmed that Sequenase still binds the DNA substrate. On the basis of these observations, the region of the T7 pol required for Cy3 fluorescence enhancement can be tentatively identified and is highlighted in Fig. 3C, with the estimated position of the Cy3 probe circled. Kinetics, and both fit single exponential functions (Fig. 3B). The association and dissociation rate constants (3.6 × 10^7 M^-1 s^-1 and 0.67 s^-1, respectively) are comparable to previously reported ensemble measurements (10).

Interestingly, when Sequenase (USB, Cleveland, OH), an exonuclease-deficient T7 pol with a 28-residue deletion (33), binds to the Cy3-P/T, the fluorescence intensity of the Cy3-P/T does not change significantly. We have tested the reactivity of Sequenase by using fluorescently labeled dCTP and Cy3-P/T and have confirmed that Sequenase still binds the DNA substrate. On the basis of these observations, the region of the T7 pol required for Cy3 fluorescence enhancement can be tentatively identified and is highlighted in Fig. 3C, with the estimated position of the Cy3 probe circled.

**Single-Molecule Observations of T7 Pol Conformational Changes Upon Complementary dNTP Binding.** To evaluate the effect of the complementary incoming dNTP on Cy3 fluorescence, we introduced Mg^{2+}, which is essential for both synthesis and exonuclease activity. To focus on probing the conformational change in the absence of DNA synthesis and nucleotide excision, we used a nonextendable dideoxy-terminated primer (ddP) and a phosphorothioate linkage between the penultimate and 3’ terminal nucleotide residue, which prevents exonuclease digestion (34) (see Materials and Methods). This primer was annealed to the same Cy3-labeled template.

Fig. 4A shows the intensity trajectories of the Cy3-ddP/T in the presence of T7 pol and a complementary dCTP in which the dCTP concentration was varied from 10 nM to 5 μM. After addition of dCTP, the intensity fluctuation pattern of Cy3 fluorescence in the complex changed significantly. Three different intensity levels of Cy3 fluorescence are seen in the intensity histogram (Fig. 4B). As mentioned above, the lowest level represents the free Cy3-ddP/T, the middle level corresponds to the T7 pol binary complex, and the highest level corresponds to the environment of Cy3 associated with the T7 pol ternary complex with dCTP. We also observed a similar fluorescence increase in Cy3-ddP/T:T7 pol complex upon ddCTP binding (see SI Appendix, Section 15).

Because the incoming dCTP is bound at the O helix (Fig. 1B) (13), some distance from the Cy3 probe, there is no direct interaction between Cy3 and the dCTP. We attribute the highest intensity level to a conformational state induced by dCTP binding. The reverse conformational change can be observed because the subsequent chemical step is blocked.

Individual binding and dissociation events can be clearly resolved in the single-molecule time traces at low concentrations of dCTP (~10–100 nM). The durations of T7 pol binding on DNA (the middle and the highest levels) are longer than those in the absence of dCTP, indicating that the binding of the complementary dCTP significantly stabilized the complex between T7 pol and DNA (see SI Appendix, Section 8 and Fig. S5).

The rate constants of the forward and backward conformational changes are easily extracted from the single-molecule time traces ([Fig. 4C]). The distribution of time intervals of the highest level is exponential, with a decay constant of ~0.9 s^-1, reflecting the rate constant of the reverse conformational change. This rate constant is independent of dCTP concentration. The distribution of middle intensity level durations is also exponential, with a decay rate constant proportional to dCTP concentration. At 50 nM dCTP, this pseudo-first-order rate constant is ~0.7 s^-1. At higher concentrations of dCTP (500 nM to 5 μM), the middle level cannot be observed because of the limited time resolution.

We distinguish two different situations, as follows:

**Scheme 1**

\[
\text{Pol-DNA} + \text{dCTP} \rightarrow \text{Pol-DNA}d\text{CTP}
\]

**Scheme 2**

\[
\text{Pol-DNA} + \text{dCTP} \rightarrow \text{Pol-DNA}d\text{CTP} \rightarrow \text{Pol}^*\text{-DNA}d\text{CTP}
\]

The intermediate intensity level represents the binary complex on the left sides of the equations, but it is not clear whether the highest intensity level represents a nascent ternary (Pol-DNA-dCTP) complex (Scheme 1) or an ensuing conformer of the ternary complex (Pol*-DNA-dCTP in Scheme 2). The rates of Cy3 fluorescence increases have different dCTP concentration dependencies for Scheme 1 and Scheme 2. The dependence is linear at low dCTP concentrations for both schemes; however, Scheme 2 would be expected to exhibit saturation behavior at high dCTP concentrations. Unfortunately, our single-molecule measurements do not provide sufficiently high time resolution at high dCTP concentrations to enable us to distinguish between the two schemes.

**Ensemble-Averaged Stopped-Flow and Continuous-Flow Experiments to Determine the Rate of the Conformational Change.** To achieve higher time resolution and to distinguish the two schemes above, we performed ensemble-averaged measurements to monitor rates of change in Cy3 fluorescence upon dCTP binding.

The traces in Fig. 5A show the temporal profiles of Cy3 fluorescence after the T7 pol-Cy3-ddP/T binary complex was mixed with increasing dCTP concentration by using a stopped-flow instrument. The fluorescence scans fit best to double exponentials. The fast phase, which is proportional to dCTP concentrations in the range of ~1–20 μM, accounts for the majority (~90% amplitude) of the intensity increase. This phase likely corresponds to the conformational change induced by dCTP binding as seen in the single-
molecule fluorescence trajectories (Fig. 4 A and B). The bimolecular rate constant obtained from stopped-flow experiments (2.2 × 10^7 M⁻¹s⁻¹, obtained at higher dCTP concentrations) is consistent with the rate obtained from single-molecule observations (1.4 × 10^7 M⁻¹s⁻¹, obtained with 50 nM dCTP). The minor increase in fluorescence intensity (~10% amplitude) of the slow phase (~1 s⁻¹) is independent of dCTP concentration and may reflect a shift in equilibrium, converting the remaining free Cy3-dddP/T to bind T7 pol because of the depletion of the T7 pol-Cy3-dddP/T binary complex as a consequence of dCTP binding. This minor phase is not considered in evaluating the rate constant.

At higher dCTP concentrations, the rate of the fluorescence change is so fast (>1,000 s⁻¹) that the stopped-flow instrument cannot capture it. We therefore constructed a continuous-flow device, using hydrodynamic focusing to achieve fast mixing at the microsecond time scale (35) (Fig. 5B; also see SI Appendix, Section 14 and Figs. S9 and S10 for details). The measured temporal profiles of Cy3-dddP/T-T7 pol fluorescence increase after mixing with dCTP are shown in Fig. 5C. The rate constants can be obtained from single exponential fits of the traces.

The dependence of the rates of the major fluorescence enhancement on dCTP concentrations from both measurements follows the Michaelis–Menten equation

\[ k_{\text{obs}} = \frac{k_{\text{max}} [\text{dCTP}]}{[\text{dCTP}] + K_d} \]

(Fig. 5D). The \( k_{\text{max}} \) and \( K_d \) for dCTP binding are 4,500 s⁻¹ and 200 μM, respectively. Had the fluorescence increase arisen solely from dCTP binding, the concentration dependence would be linear. The fact that we observed Michaelis–Menten kinetics provides direct proof that the fluorescence increase is related to a conformational change triggered by substrate binding (Scheme 2). This result rules out Scheme 1. Moreover, we reach the important conclusion that the observed conformational change is not rate-limiting in DNA synthesis because \( k_{\text{max}} \) is much greater than the nucleotidyl transfer rate (~300 s⁻¹) (10). We note that other conformational changes could occur after this conformational change and might limit the rate for the nucleotidyl transfer reaction (22).

**The Effect of Mg²⁺.** We found that Mg²⁺ is required for the observed conformational change. Removal of Mg²⁺ from the solution by EDTA completely abolished the fluorescence increase of Cy3 upon dCTP binding. Single-molecule measurements of dCTP binding were also performed in the presence of 20 and 100 μM of Mg²⁺ (see SI Appendix, Section 5 and Fig. S2). At lower Mg²⁺ concentrations, we found a significantly lower rate for the forward transition at equivalent dCTP concentrations. The rate of the reverse transition was independent of Mg²⁺ concentration. We characterized the effect of Mg²⁺ on the affinity of dCTP by titrating fluorescence intensity with dCTP at different Mg²⁺ concentrations (see SI Appendix, Sections 4, 10, and 11 and Fig. S7A) and then determined that the binding constant for Mg²⁺ was ~5 mM (see SI Appendix and Fig. S7B).

Even at very low Mg²⁺ concentration (~3 μM) (see SI Appendix, Section 6 and Fig. S3), dCTP binding still induced a conformational change in the complex between T7 pol and the extendable Cy3-P/T without resulting in dCMP incorporation (SI Appendix, Section 9 and Fig. S6). We used a FRET quencher, Dabsyl-labeled dTTP, to test whether base incorporation occurs at this low concentration of Mg²⁺ (SI Appendix, Section 9), but no incorporation was found in this time period. Therefore, under these conditions, a conformational change can occur when only one Mg²⁺ is present in the catalytic center (B site), but nucleotidyl transfer will not occur. This result indicates that the two essential Mg²⁺ ions play different roles in the catalytic center and is consistent with previous reports (36, 37).

**Single-Molecule Measurements with Noncomplementary dNTP.** Can a noncomplementary dNTP induce a conformational change? The fluorescence increase upon noncomplementary dNTP binding is difficult to detect at the ensemble level because the equilibrium strongly favors reversal of the conformational change. When a high concentration (0.25 mM) of the noncomplementary dTTP or dGTP was added, together with the T7 pol, to the immobilized Cy3-P/T, we observed enhancement in the single-molecule fluorescence time traces, but the frequency was much lower than with the complementary dNTP (Fig. 6). The rate constants for the forward transitions were estimated to be 0.02 and 0.2 s⁻¹ for dTTP and dGTP, respectively, ~10^5 times lower than for dCTP. However, the reverse transition (2 s⁻¹ for dTTP and 1.7 s⁻¹ for dGTP) was only slightly faster than that observed with the complementary dCTP, indicating that the conformation of the transition state more closely resembles the final, rather than the initial, state (see Discussion).

The observation of ~10⁴-fold discrimination against noncomplementary dNTPs in the forward conformational change upon dNTP
binding indicates that such conformational change is crucial for base selectivity and is a major factor contributing to the high fidelity of DNA replication.

**Discussion**

**Cy3 as a Sensitive Probe for the Functionally Important Conformational Change.** We selected Cy3 as a probe because of its high fluorescence quantum yield and its environment-dependent fluorescence. The photophysical properties of this dye have been studied extensively (38). The torsional motion of double bonds (Fig. 3D) in its excited state can bring Cy3 back to its electronic ground state without photon emission. Constraints of the torsional motion hinder the nonradiative decay pathway, giving a higher fluorescence quantum yield. In fact, the fluorescence quantum yield of Cy3 has strong solvent viscosity dependence. This property makes Cy3 a valuable probe for detecting subtle conformational alterations within an intact protein or protein complex.

The fluorescence enhancement of Cy3-P/T upon T7 pol binding is due to the steric hindrance of the excited-state torsional motion, as indicated in Fig. 3C. In the T7 pol complex, Cy3 may reside within a “cavity” formed by the DNA major groove and the polymerase thumb, finger, and exonuclease domains. Residues in this cavity can hinder the internal torsion of Cy3 and enhance its fluorescence. We noted that the fluorescence intensity of Cy3 barely increases upon Sequenase binding, possibly because of the 28-aa deletion and the noted that the fluorescence intensity of Cy3 barely increases upon Sequenase binding, possibly because of the 28-aa deletion and the subsequent disruption of this proposed cavity in Sequenase.

From analysis of available kinetic and structural information, we propose that the rise in Cy3 fluorescence reflects a conformational change in the polymerase ternary complex subsequent to dNTP binding. Additional evidence in support of this proposal is as follows. (i) With a noncomplementary dNTP, the events of fluorescence increase occurred much less frequently than with the complementary dNTP. (ii) In the absence of Mg2+, there was no fluorescence increase upon complementary dNTP binding. (iii) If substrate binding alone was able to induce a change in Cy3 fluorescence, then we would not have observed Michaelis–Menten behavior when the complementary dNTP was added to the T7 pol-DNA binary complex.

**Fluorescence Change Reflects Fingers Domain Closing.** We have assigned the Cy3 fluorescence changes to conformational changes in the T7 pol ternary complex, observed by x-ray crystallography after dNTP binding, is the open-to-closed transition in the fingers domain. The incoming dNTP initially binds to the fingers subdomain in the open state by means of interactions between the triphosphate tail and the conserved, positively charged side chains in the O helix (38).

A key relating the observed fluorescence change to the actual structural change in T7 pol is the Mg2+ dependence. It is known that Mg2+ is crucial for the open-to-closed transition because it stabilizes the closed complex (13), forming a coordination complex with the dNTP triphosphate tail as well as with the highly conserved Asp and Glu residues in the palm domain. Formation of the Mg2+ coordination complex in the active site also requires closing of the fingers to bring the dNTP triphosphate tail and the conserved Asp and Glu residues close enough for them to ligate the Mg2+. The dNTP-induced fluorescence increase in the T7 pol complex was observed only in the presence of Mg2+, indicating that the fluorescence change relies on the formation of the Mg2+ coordination complex. Because of the geometric constraints of the Mg2+ ligands, such a fluorescence change can only occur when the fingers domain of T7 pol is closed. This connection is further reinforced by the observation that the conformational change can also be induced by ddCTP, which produced an equivalent conformational state as shown by x-ray crystallography (13).

Clossing of the fingers subdomain (Fig. 1B), by itself, does not appear to have a major effect on the environment of Cy3, according to the x-ray structure. Instead, the conformational change in the DNA template has a more significant effect on the environment of Cy3. In the open form of the DNA pol complex, the DNA templating base is flipped out of its helical conformation (38). Accompanying the closing of fingers, the templating base rotates into the active site and assumes a position as if it were actually part of a duplex. This movement of the templating base repositions the Cy3 probe so that it senses a different environment, giving rise to the observed increase in fluorescence intensity. Additional evidence for this explanation also comes from previous reports. In particular, kinetic studies have shown that the incoming dNTP is likely to form H bonds with the templating base while T7 pol is still in the open state (38). Likewise, a recent crystal structure of the T7 RNA polymerase, which has close structural homology to T7 DNA pol, shows that such interaction occurs in the open form of the T7 RNA pol complex (39).

**Closing of T7 Pol Fingers Domain Is a Fast Step Triggered by dNTP Binding.** It has not been resolved in the literature whether reversible open-to-closed conformational changes occur before binding of dNTP substrates (21). Our single-molecule experiment can address this question. Because no increase of fluorescence intensity of the T7 pol-Cy3-P/T complex was found in the single-molecule fluorescence time traces in the absence of dNTP, there is no evidence for the existence of dynamic equilibrium between open and closed states before dNTP binding. Therefore, binding of an incoming dNTP is required to induce fingers closing.

Our observations show that the fingers closing is a rapid step in the T7 pol nucleotidyl transfer reaction and is not rate-limiting, which is in agreement with the kinetic results reported for polβ (20), KF (20), and Klentaq1 (21). We note that fast conformational change upon binding of the complementary base is necessary for high replication efficiency. If the conformational change upon binding of complementary dNTP were to be rate-limiting, the overall DNA replication rate would be significantly reduced because of competitive inhibition by noncomplementary dNTPs and NTPs.

We note that other conformational changes could occur after the one that we observed. These ensuing conformational changes might be rate-limiting for the nucleotidyl transfer reaction. In fact, Tsai and Johnson (22) observed a much slower conformational change by a fluorescence label at a different location. A possible change could consist of a small adjustment of amino acid side chains that ligate Mg2+ in the A metal site, as suggested in ref. 40.

**Conformational Change Plays a Major Role in Determining DNA Replication Fidelity.** Because the nucleotidyl transfer step is precluded in our experiment, we have been able to determine the rate
of the conformational change. We observed that binding of non-complementary dNTPs can also give rise to an increase of Cy3 fluorescence, with amplitude similar to that for complementary dNTP. However, a much lower rate of fluorescence intensity transitions was obtained with noncomplementary vs. complementary dNTPs. For noncomplementary bases, the rate of the conformational change is comparable to the overall rate of incorporation and could be the rate-limiting step (41).

The catalytic efficiency $k_{cat}/K_m$ for complementary vs. non-complementary dNTP substrates determines the extent of base discrimination by DNA pols (5, 22). The rate of fingers closing for complementary dNTP substrates is $10^4$ to $10^5$ times higher than for noncomplementary dNTPs. Therefore, the conformational change we observed serves as a major kinetic checkpoint to discriminate against noncomplementary dNTP incorporation.

Fig. 7 shows an energy diagram for the dNTP-binding step and the open-to-closed transition of the T7 pol complex for complementary and noncomplementary dNTPs. The variation in stability of closed forms with different dNTP substrates affects the rate of fingers closing significantly but has a minimal effect on the rate of fingers opening. This implies that the transition state for the fingers closing has a structure that more closely resembles the closed, rather than open, form. Perturbations affecting the closed form should affect the transition state in a similar way. As a result, the more stable the T7 pol closed complex is, the lower the barrier (and affect the transition state in a similar way. As a result, the more stable the T7 pol closed complex is, the lower the barrier (and higher the rate) for the open-to-closed transition. Therefore, it is the molecular interactions between the T7 pol-DNA binary complex and the incoming dNTP that give rise to the enhanced replication fidelity, in addition to the fidelity provided by the Watson–Crick base pairing (42–45).

In summary, the photophysical properties of Cy3 have allowed us to observe the conformational change in the T7 pol ternary complex before phosphodiester bond formation. Structural information, together with Cy3 fluorescence enhancement mechanisms and Mg$^{2+}$ dependence of conformational change, suggest that the transition we observed is assigned to closing of the fingers subdomain. Based on the ensemble-averaged observations, this conformational change is a very rapid, non-rate-limiting step. The conformational change makes a major contribution to fidelity because it allows efficient discrimination against the incorporation of non-complementary bases.

**Materials and Methods**

**Materials.** All reagents were the highest grade obtainable and were used without further purification. See *SI Appendix, Section 1* for details.

**P/T Preparation.** If not otherwise specified, the following oligonucleotides were used as the primer and template strands of the P/T primer, 5′-GCCCTCAGCGCAGCGCACTTCG-3′ (P); template, 3′-BiotinTEG-CGGAGCTGCGAGTTGTTGAGTGGCCC-5′ (Cy3-T). The duplex Cy3-P/T was prepared by mixing the Cy3-T and P oligos at a 1:1.5 ratio in 50 mM phosphate buffer, pH 7.5.

We made observations by using 40 mM Tris buffer, pH 7.5, containing 10 mM MgCl₂ in experiments in which the dNTP substrate was present. A thiophosphoryl primer with the same sequence as primer P, 5′-GCCCTCAGCGCGCACTTCG-3′ (ddP), where * is the phosphorothioate linkage between the deoxynucleoside residues and ddA is the dideoxynucleoside residue (see *SI Appendix, Section 1*), was prepared and annealed to the Cy3-T. In experiments involving the ddP primer, 20 µM ddATP was added to the reaction mixture to ensure that the primer always had a ddA residue at its 3′ terminus, in case it is cut by the exonuclease activity of T7 pol.

**Microscope Sample Preparation and Single-Molecule Imaging.** Biotin-functionalized glass coverslips and flow cells were prepared as described in ref. 46. See *SI Appendix, Section 2* for details of sample preparation and single-molecule fluorescence imaging.

**Ensemble-Averaged Fluorescence Measurements.** For stopped-flow fluorescence measurements, see *SI Appendix, Section 13*. For continuous-flow measurements using hydrodynamic focusing (35), see *SI Appendix, Section 14*.

We thank Drs. Antoine M. van Oijen, Paul Blainey, Hong Zhang, and Jeremy Agresti and Mr. Brian English for their suggestions and help. This work was supported by the Department of Energy, Office of Science, Office of Basic Energy Science, Chemical Sciences (X.S.-X.) and by U.S. Public Health Service Grant GM063276-01 (to W.H.K.).

SI Appendix

1. Sources of reagents and preparation of the DNA substrate for observation in solution with magnesium ions

T7 pol, T4 DNA polymerase and KF were purchased from Amersham biosciences (Piscataway, NJ). Sequenase was from USB Corporation (Cleveland, OH). DNA oligomers were custom synthesized by IDT Inc. (Coralville, IA) or MWG-biotech inc. (High Point, NC) followed by additional HPLC or PAGE purification. dNTPs were purchased from Invitrogen (Carlsbad, CA). 2’,3’-Dideoxynucleoside triphosphates (ddNTPs) were purchased from Trilink biotechnology (San Diego, CA). Functionalized PEG-NHS and biotin-PEG-NHS were purchased from Nektar Therapeutics (San Carlos, CA).

T7 DNA polymerase requires magnesium ions for its synthesis and editing functions. In order to ensure that the observation of polymerase-dNTP interactions occur at the same template/primer junction, the editing functions that excise nucleotide residues from the primer strand need to be suppressed. For this purpose a modified primer (IDT) with the sequence was used:

Primer: 5’-GCCTCGCAGCCGTCCAACCAACT*C -3’ (thioP)

where * denotes the phosphorothioate modification that was introduced to prevent the exonuclease digestion of the primer. The phosphorothioate bond from chemical DNA synthesis has two configurations $R_p$ and $S_p$ for the chiral phosphorus atom. The $S_p$ configuration of the phosphorothioate cannot be digested by the exonuclease activity of polymerases(1). To remove the exonuclease susceptible $R_p$ portion of the primer, 10 μL 100 μM thioP primer was incubated with ~4 μM T7 pol for 1 hr in the presence of 10 mM Mg$^{2+}$. After incubation, the solution was heated to 90 °C for ~30 min to inactivate the T7 pol. The remaining oligonucleotide was resistant to exonuclease digestion by T7 pol. The DNA duplex Cy3-thioP/T was formed by mixing the Cy3-T oligo and the
selected thioP with a 1/1.5 ratio in phosphate buffer. To form the same sequence as the Cy3T/P duplex, a ddA base was incorporated at the 3’ primer terminus by adding into a 10 μl solution of 4 μM Cy3-thioP/T duplex with 10 mM MgCl₂, 1 μl of 10 mM ddATP and 1 μL of 1 μM Sequenase solution and incubated for 10 min. The Cy3-ddP/T duplex prepared in this way has the same sequence as the Cy3-P/T, but the ddP primer terminus lacks the 3’-OH group. Thus the primer in the duplex is not extendable. To maintain the ddA base at the 3’ end of the primer, 20 μM ddATP was added to the solution during single molecule observations or for fluorescence titration.

2. Microscope sample preparation and single molecule imaging:

Biotin-functionized glass coverslips were prepared by the following procedure. After thorough cleaning with ethanol and 1 M sodium hydroxide, the No.1 microscope glass slips (VWR Inc., West Chester, PA) were amine-functionalized by treatment with 2% 3-aminopropyl-triethoxysilane in dry acetone for 2 minutes. After rinsing with deionized water, the slips were incubated for 4 hours in a solution of amine-reactive poly(ethylene glycol) (100 mg/ml mPEG-SPA (MW = 5,000) and 1 mg/ml biotin-PEG-CO₂NHS (MW = 3,400) in 100 mM pH 8.3 NaHCO₃).

To immobilize Cy3-P/T duplex, a streptavidin coating was introduced by flowing in 0.2 mg/ml streptavidin (Sigma) and incubated for 10 minutes to provide binding sites for the biotin-modified DNA duplex. After flushing out the excess streptavidin with 3ml phosphate buffer, 10 pM of biotinated Cy3-P/T duplex was introduced and incubated for 10 minutes. Finally the flow cell was flushed with 2 ml of phosphate buffer.

An inverted fluorescence microscope (Olympus IX70, Olympus America Inc., Melville, NY) was used for imaging of the fluorescent DNA duplex attached to the surface of the coverslip. The 532 nm laser (λ-Pro, Shanghai, China) was reflected by a dichroic mirror (Z532, Chroma, Rockingham, VA) and focused onto the edge of the back focal plane of a
microscope objective (60X PlanApo; N.A.=1.45; Olympus) to create a total internal
reflection excitation at the interface of glass and water at a power density of ~200W/cm².
The fluorescence of individual molecules was collected by the same objective and, after
passing 3 filters (dichroic mirror Z532, emission filter HQ575/60 and long pass filter
550LP; Chroma, Brattleboro, VT), focused onto a CCD camera (Cascade 512B, Roper
Scientific, Trenton, NJ) by the microscope tube lens. Fluorescence movies were recorded
at a speed of 5 frames per second. To prevent photobleaching of the fluorescent probe,
buffer solutions containing 0.1mg/ml glucose oxidase (Sigma), 0.025mg/ml catalase
(Roche), 0.4% (w/v) glucose and 1% beta-mecaptoethanol were used. T7 pol and dNTPs
were introduced through the tubing with an automated syringe pump (Harvard Apparatus,
PhD 2000, Holliston, MA). Fluorescence intensity time trajectories of individual
molecules were extracted from the CCD movie by integrating 2x2 pixels at the
fluorescence spot using a Matlab script. The long-term intensity drifts due to objective
focus drifts were corrected by dividing a polynomial function that fits the gradual
intensity changes in the trajectory. Typically about 50% of the Cy3 labeled DNA
molecules were active. We used more than 30 molecules to construct a decay histogram
for one experimental condition.

3. Intensity trajectories of various DNA polymerases upon incubation with Cy3-P/T

The binding of DNA polymerases other than T7 pol was also tested. Due to different
environments of the Cy3, the binding of various DNA pols have intensity enhancement
that are characteristic of the individual DNA pol. Figure S1A shows the fluorescence
intensity trajectories of the Cy3-P/T DNA duplex when flowing in T4 pol, KF and
sequenase. Binding of the T4 pol and KF leads to enhancement of the Cy3 fluorescence
intensity with different amplitudes. However, binding of Sequenase does not change the
fluorescence intensity of the Cy3-P/T duplex.

Under the condition where the Cy3 probe is closer to the primer-template junction, we
observed similar Cy3 fluorescence enhancement upon binding of T7 pol and Sequenase to the DNA substrate. Figure S1B shows typical fluorescence intensity trajectories of an immobilized Cy3 labeled DNA duplex, when flowing in 500 nM dNTPs along with T7 pol or Sequenase in Tris buffer with Mg$^{2+}$. When the 5’ overhang of the DNA template was filled by primer extension, binding of Sequenase enhanced the Cy3 fluorescence with an amplitude comparable to that of T7 pol.

Figure S1 A) Intensity trajectories of the Cy3-P/T DNA duplex in the presence of different DNA polymerases. The binding of KF induced a small but discernable increase of the fluorescence intensity of the Cy3 probe. T4 pol gives a remarkably distinct intensity enhancement. The binding of Sequence cannot be observed from the fluorescence intensity trajectory. B) Intensity trajectories of individual Cy3 labeled DNA duplex with sequence:
Primer 5’-GCCTCGCAGCCGTCCAACCACTCC -3’ (TP)

Template 3’-BiotinTEG-CGGAGCGTCGGCAGGTGGTTGAGGACCTTCTC-Cy3-5’ (LCy3T).

Reaction mixture of 500nM dNTPs with T7 pol or Sequenase was flowed in at about 20 second in the trajectory. Different from T7 pol, binding of Sequenase did not result in Cy3 fluorescence changes right after its introduction into the reaction mixture. With the completion of primer extension, the Cy3 moved closer to the polymerase active center; binding of T7 pol and Sequenase yielded similar fluorescence enhancement.

4. Relationship between $K_d$, $K_m$ and $K_{d,app}$ of dCTP

We noticed that a very low [dCTP] (on the order of 100 nM, much lower than ground state binding constant, which is about 10 μM, of correct dNTP) could drive the binding to favor the ternary complex. The biased equilibrium of the conformational change after dNTP binding can explain this.

The apparent binding constant $K_{d,app}$ of dCTP from our measurement, defined by the equilibrium constant for the equilibrium between the DNApol/DNA binary complex and all forms of the DNApol/DNA/dNTP ternary complex, is different from its $K_d$ and $K_m$.

Consider the following reaction mechanism to clarify their relationship,

$$
\begin{align*}
dCTP + Pol \cdot DNA & \xrightleftharpoons[k_1]{k_{-1}} Pol \cdot DNA \cdot dCTP \\
& \xrightleftharpoons[k_f]{k_b} Pol^* \cdot DNA \cdot dCTP
\end{align*}
$$

where an additional conformational change occurs after dCTP binding. $K_d = \frac{k_{-1}}{k_1}$, is the binding constant of the initial binding equilibrium. $K_m$ is defined as $K_m = \frac{k_{-1} + k_f}{k_1}$.

Calculation of $K_{d,app}$ needs to take into account all the forms of the ternary complex,

$$
K_{d,app} = \frac{[dCTP][Pol \cdot DNA]}{[Pol \cdot DNA \cdot dCTP] + [Pol^* \cdot DNA \cdot dCTP]}.
$$
With the relationship \[ [Pol^* \cdot DNA \cdot dCTP] = \frac{k_f}{k_b} [Pol \cdot DNA \cdot dCTP] \] and
\[ [Pol \cdot DNA \cdot dCTP] = \frac{k_{i1}}{k_{-1}} [Pol \cdot DNA][dCTP], \]
we have \[ K_{d,app} = \frac{k_{-1}}{k_{i1}(1 + k_f / k_b)} = K_d \frac{1}{1 + k_f / k_b}. \] In the case of T7 pol, \( k_f \) is much larger than \( k_b \). Finally the relationship \[ K_{d,app} \approx \frac{k_b}{k_f} K_d \approx \frac{k_b}{k_f} K_m \] explains why we obtained a much lower \( K_{d,app} \) of dCTP than the \( K_d \) obtained from previous kinetic measurements.

5. Intensity trajectories of T7 pol: Cy3-ddP/T using different concentrations of dCTP with 20 and 100 \( \mu \text{M} \) \( \text{Mg}^{2+} \)

Fluorescence intensity trajectories of the Cy3T/thioPddA duplex with T7 pol and with different concentrations of dCTP in the presence of 20 and 100 \( \mu \text{M} \) \( \text{Mg}^{2+} \) were also recorded. Figure S2A and B are examples of the fluorescence intensity trajectories obtained. The forward and reverse transitions can also be observed at these \( \text{Mg}^{2+} \) concentrations within the dCTP concentration range used. We observed a higher \( K_{d,app} \), compared to values at high (10 mM, where \( K_{d,app} \sim 50 \text{ nM} \)) \( \text{Mg}^{2+} \) concentrations. These observations agree well with the fluorescence intensity titration result.
Figure S2 Fluorescence intensity trajectories of the Cy3 probe upon polymerase binding to the P/T in the presence of different concentrations of dCTP in 20 and 100 μM Mg$^{2+}$.  A) With 20 μM magnesium ion, the switching between binary and ternary complexes can be best observed at ~500 nM dCTP.  B) With 100 μM Mg$^{2+}$, the switching can be best observed at ~200 nM dCTP.

6. Determination of magnesium ion concentrations in phosphate buffer

Calmagite was used to quantify the free Mg$^{2+}$ in the 50 mM pH7.5 phosphate buffer that we used.  Shown in Fig. S3A, are a series of absorption spectra of 14 μM calmagite in 250mM pH10 borate buffer with different amounts of added magnesium ion.  The absorption spectrum of calmagite in 250 mM pH10 borate solution in the presence of 500 μM EDTA was also measured as a reference for zero magnesium ion concentration.  A calibration curve was made by plotting the ratio of the absorbance at 541 and 613 nm from the spectra versus the added magnesium ion concentration (Fig. S3B).  By comparing the $A_{541}/A_{613}$ ratio of the absorption spectrum with the calibration curve, we determined that the magnesium ion concentration in 50 mM phosphate buffer was about 3 μM.
Figure S3 Measurement the magnesium ion concentration in 50 mM phosphate buffer pH7.5.  A) Absorption spectra of 14 μM calmagite in different solutions as indicated in the figure.  B) Calibration curve for the determination of the magnesium ion concentration in the 50mM phosphate buffer. In the plot, the x-axis is the added amount of magnesium ion in the pH10 borate buffer. A second order polynomial fit was used to model the relationship between the absorbance ratio and the magnesium ion concentration. The blue dashed line shows the absorption ratio of calmagite in the phosphate buffer. As there are still tiny amounts of magnesium ions in the borate buffer, absorption spectrum of calmagite in the solution containing 500μM EDTA was used as a reference for the absorption ratio without magnesium (red dashed line).

7. Intensity trajectories of duplex Cy3-P/T with T7 pol and dCTP in 50 mM phosphate buffer containing 3 μM Mg$^{2+}$. 
We noticed that the binding of dCTP still induced similar conformational changes in the polymerase complex as observed in the fluorescence trajectories of Cy3-P/T in the phosphate buffer without EDTA. Fig. S4 shows examples of the fluorescence trajectories obtained with the Cy3-P/T duplex in a solution of 5nM T7 pol and different concentrations of dCTP. Repetitive binding events with the identical single DNA duplex can be observed. The $K_{d,\text{app}}$ of dCTP under these conditions is much greater than at high Mg$^{2+}$ concentration. In the dCTP concentration range of 1~10 μM, conformational changes of the polymerase can be observed from the fluorescence intensity trajectories. In the phosphate buffer, the non-complementary substrate, dGTP, at a concentration of 250 μM did not change the binding pattern of the trajectory (data not shown).
8. dCTP concentration dependence of the polymerase:DNA dissociation rate

We observed that the binding of the dCTP substrate to the polymerase/DNA complex changed the dissociation rate of the polymerase from the DNA. This observation can be explained by the following scheme,

\[
\begin{align*}
\text{dCTP} + \text{Pol} \cdot \text{DNA} & \rightleftharpoons \text{Pol} \cdot \text{DNA} \cdot \text{dCTP} \\
\downarrow & \quad \downarrow \\
k_{\text{diss,1}} & \quad k_{\text{diss,2}}
\end{align*}
\]

in which the polymerase dissociation from DNA can occur from both the binary and ternary complex. The dCTP concentration affects the equilibrium between the binary and ternary complexes with an apparent binding constant \( K_{d,\text{app}} \). The binding and dissociation of dCTP from T7 pol complexes is faster than the polymerase dissociation rate from its DNA complexes. The average dissociation rate then is determined by the population of the polymerase in binary and ternary complexes \( p_{\text{bin}} \) and \( p_{\text{ter}} \),

\[
k_{\text{diss}} = k_{\text{diss,1}} p_{\text{bin}} + k_{\text{diss,2}} p_{\text{ter}}.
\]

With the relation \( p_{\text{bin}} = \frac{K_{d,\text{app}}}{K_{d,\text{app}} + [\text{dCTP}]} \) and \( p_{\text{ter}} = \frac{[\text{dCTP}]}{K_{d,\text{app}} + [\text{dCTP}]} \),

the dCTP concentration dependent average dissociate rate of the polymerase is

\[
k_{\text{diss}} = k_{\text{diss,1}} \frac{K_{d,\text{app}}}{K_{d,\text{app}} + [\text{dCTP}]} + k_{\text{diss,2}} \frac{[\text{dCTP}]}{K_{d,\text{app}} + [\text{dCTP}]}.
\]
The amplitude of the change in $k_{diss}$ at different dCTP concentrations depends on the difference of $k_{diss,1}$ and $k_{diss,2}$. The change of $k_{diss}$ of DNA pol from Cy3-ddP/T upon dCTP binding with 10mM Mg$^{2+}$ is noticeable. In phosphate buffer the $k_{diss}$ changes by over 10 fold with the addition of dCTP. The reduced dissociation rate can be rationalized in terms of the structure of the T7 pol ternary complex, where the fingers domain is in the closed position, restricting the motion of the P/T. Shown in Fig. S5 is the [dCTP] dependence of $k_{diss}$ in phosphate buffer. Also shown is the curve used to fit the model presented above. The $K_{d,app}$ of dCTP is 6 μM under these conditions.

![Graph showing dCTP concentration dependence of the polymerase dissociation rate](image)

Figure S5 dCTP concentration dependence of the polymerase dissociation rate obtained from the statistics of the binding durations at different dCTP concentrations. A fit

$$k_{diss} = k_{diss,1} \frac{K_{d,app}}{K_{d,app} + c} + k_{diss,2} \frac{c}{K_{d,app} + c}$$

was used to model the data. An apparent dCTP binding constant $K_{d,app}$ of ~6 μM was obtained.

9. Incorporation test using phosphate buffer

Fluorescence resonance energy transfer (FRET) between Cy3 and Dabcyl was used to
test whether the polymerase reaction can occur at very low [Mg$^{2+}$]. A dNTP labeled with a fluorescence quencher, Dabcyl-dUTP, was synthesized by the reaction between Dabcyl-NHS (Sigma) and 5-aminoallyl-dUTP (Trilink Biotechnology). The reaction was carried out in 200 μL DMF at room temperature for 12 hr with addition of 1 nmol 5-amino-allyl-dUTP, 2 mg Dabcyl-NHS and 1 ml of triethylamine (Sigma, as a catalyst). After the reaction, the DMF was removed under vacuum. The Dabcyl-dUTP was purified from reaction mixtures with reverse phase HPLC. This substrate can be easily incorporated into the primer with Sequenase.

The incorporation assay was carried out with a 14nM duplex consisting of

Primer 5′-GCCTCGCAGCCGTCCAACCAACTCC -3′ (TP)
Template 3′-BiotinTEG-CGGAGCGTCGGCAGGTTGGTTGAGGACCTTCTC-Cy3-5′ (LCy3T)

, 400nM Dabcyl-dUTP and ~0.7 nM sequenase in a 1.5 mL buffer (50 mM Tris buffer with 10 mM MgCl$_2$ or 50mM phosphate buffer). The incorporation of Dabcyl-dUMP was followed by the change of the Cy3 fluorescence, as indicated in Fig. S6 when the experiment was carried out with 10 mM MgCl$_2$. In 50 mM phosphate buffer, the change of fluorescence due to Dabcyl-dUMP incorporation was not detectable.
Figure S6 Fluorescence intensity of the Cy3 probe in the LCy3T/TP DNA duplex in the presence of 400 nM Dabcyl-dUTP and 1.4 nM sequenase in different reaction buffers. Clearly, at a magnesium concentration of 3 μM, the incorporation reaction catalyzed by Sequenase is negligible during this period.

10. Determination of $K_{d,app}$ using fluorescence intensity titration

To explore the effect of $[\text{Mg}^{2+}]$ on the rate of conformational change, the change of fluorescence intensity of the T7 pol: Cy3-ddP/T complex upon addition of dCTP, at the ensemble-averaged level, were used to determine the $K_{d,app}$ of dCTP at different $[\text{Mg}^{2+}]$. In the fluorescence intensity titrations, different $[\text{Mg}^{2+}]$ were employed and measurements were made using a spectrofluorometer (Fluorolog III, Jobin Yvon Inc.). Solutions of 5nM DNA duplex (Cy3-ddP/T), 13nM T7 pol, 20 μM ddATP, and various $[\text{Mg}^{2+}]$ in 50mM pH7.5 phosphate buffer were titrated with increasing amounts of dCTP. The fluorescence intensities of the Cy3 probe (excited at 532nm and emission at 563nm) were recorded at each [dCTP].

It is convenient to determine the apparent binding constant of dCTP using the fluorescence intensity titration. Consider the equilibrium as follows,
Since the polymerase is in excess relative to the DNA duplex, the free polymerase concentration can be treated as constant. There are four different states of the Cy3 probes, (i) in the free DNA duplex; (ii) in the polymerase/DNA binary complex; (iii) in the ternary complex before conformational change; and (iv) in the ternary complex after the conformational change. The total fluorescence intensity is the sum of the intensities of Cy3 probe in different states. Therefore we have

\[ I = p_{dna} I_{dna} + p_{pol-dna} I_{pol-dna} + p_{pol-dna-dCTP} I_{pol-dna-dCTP} + p_{pol*-dna-dCTP} I_{pol*-dna-dCTP} \]

where \( p \) refers to the population and \( I \) refers to the intensity. We can simplify this expression by combining the two ternary complex terms into one, as the equilibrium between them is not controlled by the dCTP concentration. In this case the equilibrium constant of the binary complex and ternary complex becomes \( K_{d,app} \).

\[ I = p_{dna} I_{dna} + p_{pol-dna} I_{pol-dna} + p'_{pol-dna-dCTP} I'_{pol-dna-dCTP} \]

With the equilibrium relations we have

\[ p_{dna} = \frac{K_{d,pol} K_{d,app}}{K_{d,pol} K_{d,app} + [Pol] K_{d,app} + [Pol][dCTP]} \]

\[ p_{pol-dna} = \frac{[Pol] K_{d,app}}{K_{d,pol} K_{d,app} + [Pol] K_{d,app} + [Pol][dCTP]} \]

and
\[ p_{\text{pol-dna-dCTP}}' = \frac{[\text{Pol}][\text{dCTP}]}{K_{d,\text{pol}}K_{d,\text{app}} + [\text{Pol}][\text{dCTP}] + \text{app} + [\text{Pol}][\text{dCTP}]} \]

The total fluorescence intensity is

\[ I = \frac{(I_{\text{dna}}K_{d,\text{pol}} + I_{\text{pol-dna}}[\text{Pol}])K_{d,\text{app}} + I_{\text{pol-dna-dCTP}}'}{K_{d,\text{pol}} + [\text{Pol}][\text{dCTP}] + \text{app} + [\text{Pol}][\text{dCTP}]} \]

In our titration experiments, [Pol] is about 10 nM and the \( K_{d,\text{pol}} \) is about 20 nM. With these parameters fixed, we can determine the \( K_{d,\text{app}} \) with the fluorescence titration.

Figure S7A shows an example of the fluorescence intensity as a function of [dCTP] in the presence of 100 \( \mu \text{M} \) MgCl\(_2\). The \( K_{d,\text{app}} \) for dCTP binding decreases with increasing \([\text{Mg}^{2+}\)]\) as shown in Fig. S7B. This result is in good agreement with the single-molecule observations, namely that Mg\(^{2+}\) facilitates the forward conformational change but has little effect on its reversal. The effect of [Mg\(^{2+}\)] on dCTP binding at the ensemble averaged level saturates in the mM range.

![Figure S7](image.png)

**Figure S7** A) An example of fluorescence intensity titration to determine the apparent binding constant \( K_{d,\text{app}} \) of dCTP to the T7 pol/DNA binary complex in the presence of 100 \( \mu \text{M} \) Mg\(^{2+}\). A fit \( I = \frac{(I_{\text{dna}}K_{d,\text{pol}} + I_{\text{pol-dna}}[\text{Pol}])K_{d,\text{app}} + I_{\text{pol-dna-dCTP}}'}{K_{d,\text{pol}} + [\text{Pol}][\text{dCTP}] + \text{app} + [\text{Pol}][\text{dCTP}]} \) is used to model the data and a \( K_{d,\text{app}} \) was
determined to be 0.30 μM. B) [Mg$^{2+}$] dependence of the $K_{d,app}$ of dCTP binding. At each [Mg$^{2+}$], the $K_{d,app}$ of dCTP was determined by fluorescence intensity titrations. The relation

$$K_{d,app} \approx K_m \frac{1 + [Mg^{2+}] / K_{d,Mg}}{k_{f1} / k_{b1} + (k_{f2} / k_{b2})[Mg^{2+}] / K_{d,Mg}}$$

was used to model the Mg$^{2+}$ dependence of the $K_{d,app}$. The binding affinity of magnesium $K_{d,Mg}$ = 5 mM was obtained from the curve fitting.

11. The $K_{d,app}$ for dCTP binding depends on [Mg$^{2+}$]

At very low [Mg$^{2+}$] these conformational changes still occur in the complex between T7 pol and DNA duplex Cy3-P/T with the extendable primer P, even in the absence of chemistry. The $K_d$ of Mg$^{2+}$ in the B site is much lower than the $K_d$ for Mg$^{2+}$ in the A site. In this case the catalytic A site (see in Fig. 1B in the main text) must be empty, otherwise phosphoryl transfer would occur. This is consistent with the results of the Tsai group on pol β, namely that one metal ion (in the B site) is required to trigger the conformational change(2, 3).

The dependence of the $K_{d,app}$ of dCTP binding on [Mg$^{2+}$] can be modeled using a scheme where the polymerase would have different rates of conformational change with different extents of Mg$^{2+}$ occupancy. As the binding of the Mg$^{2+}$ at the B site is relatively tight ($K_d=10 \text{ } \mu M$), in the Mg$^{2+}$ concentration range of 20 μM to 10 mM, the first magnesium-binding site B has been saturated. Therefore the [Mg$^{2+}$] dependence of dCTP binding for T7 pol is due to the second magnesium ion binding to the catalytic site (A site). Such dependence of the rate on the second Mg$^{2+}$ occupancy (in the A site) is consistent with the assignment of the observed conformational change to polymerase fingers closing, as the second Mg$^{2+}$ will further stabilize the closed conformation.

Our observations can be explained with the parallel kinetic scheme,
We assume the magnesium ion doesn’t affect the initial binding equilibrium of the dCTP to the polymerase open complex. From the derivation of the relation of $K_{d,app}$ and $K_d$, the apparent binding constant of dCTP can be calculated by

$$K_{d,app} \approx K_d \frac{[Pol \cdot DNA \cdot dCTP(Mg)] + [Pol \cdot DNA \cdot dCTP(Mg_2)]}{[Pol^* \cdot DNA \cdot dCTP(Mg)] + [Pol^* \cdot DNA \cdot dCTP(Mg_2)]}$$

With the relations of $[Pol^* \cdot DNA \cdot dCTP(Mg)] = \frac{k_{f1}}{k_{b1}}[Pol \cdot DNA \cdot dCTP(Mg)]$, $[Pol^* \cdot DNA \cdot dCTP(Mg_2)] = \frac{k_{f2}}{k_{b2}}[Pol \cdot DNA \cdot dCTP(Mg_2)]$, and $[Pol \cdot DNA \cdot dCTP(Mg_2)] = \frac{[Mg^{2+}][Pol \cdot DNA \cdot dCTP(Mg)]}{K_{d,Mg}}$, we have the expression of the apparent binding constant $K_{d,app} \approx K_d \frac{1 + [Mg^{2+}] / K_{d,Mg}}{k_{f1} / k_{b1} + (k_{f2} / k_{b2})[Mg^{2+}] / K_{d,Mg}}$. A $K_d$ of 5mM for $Mg^{2+}$ occupancy was obtained from curve fitting (Fig. S7B). This value is similar to that reported for the $K_d$ of $Mg^{2+}$ binding to the (catalytic) A site(3).

However, an alternative sequential kinetics scheme shown below can also explain our data.
Here $\text{Pol}^*$ and $\text{Pol}^{**}$ denote the closed form of T7 pol with one or two Mg$^{2+}$ coordination complexes formed.

From the derivation of the relation of $K_{d,app}$ and $K_d$, the apparent binding constant of dCTP $K_{d,app}$ can be calculated by

$$K_{d,app} \approx K_d \frac{[\text{Pol} \cdot \text{DNA} \cdot \text{dCTP}]}{[\text{Pol}^* \cdot \text{DNA} \cdot \text{dCTP}] + [\text{Pol}^{**} \cdot \text{DNA} \cdot \text{dCTP}(\text{Mg})]}.$$

Finally we have $K_{d,app} \approx K_d \frac{1 + [\text{Mg}^{2+}] / K_{d,Mg}}{k_{f1} / k_{b1} + k_{f1} / k_{b1} \cdot (1 + k_{f1} / k_{b1}) \cdot [\text{Mg}^{2+}] / K_{d,Mg}}$. This expression is equivalent to that from the previous kinetic scheme. Therefore, the same value of $K_{d,Mg}$ can still be obtained from the $[\text{Mg}^{2+}]$ dependence of the $K_{d,app}$ of dCTP.

The major difference between these two kinetic schemes is whether the two Mg$^{2+}$ complexes can be formed in one step. With the current data, we cannot discriminate between them.

12. Experimental result using a different duplex and the complimentary dTTP

The DNA duplex with sequence

**Primer:** 5’-GCCTCGCAGCCGTCCAACCAACTCC -3’ (TP)

**Template:** 3’-BiotinTEG-CGGAGCGTCGCCAGTTGGTGAGGACCTTCTC-Cy3 5’ (LC y3T)

was also tested for binding of T7 pol and dTTP. Figure S8A shows examples of the
fluorescence trajectories in the solution of 5nM T7 pol and different concentrations of dTTP in the phosphate buffer. Repeated binding events at the same single DNA duplex can be observed. Similar to the situation when using the duplex Cy3-P/T, longer binding durations and a second intensity level appeared with the addition of dTTP. The dTTP concentration dependence of the polymerase dissociation rate is shown in figure S8B.

Figure S8 Experimental result with T7 pol and the DNA duplex LCy3T/TP duplex and dTTP. A) Fluorescence intensity trajectory of the Cy3 probe upon polymerase binding to DNA in the presence of different dTTP concentrations in 50 mM phosphate buffer (with ~3 μM magnesium ion). Elongated polymerase binding duration at high dTTP concentration, and transition between polymerase binary and ternary complexes can be observed from these trajectories. B) dTTP concentration dependence of the polymerase dissociation rate. A fit $k_{\text{diss}} = k_{\text{diss, app}} \frac{K_{d, app}}{K_{d, app} + [dTTP]} + k_{\text{diss, 2}} \frac{[dCTP]}{K_{d, app} + [dTTP]}$ is used to model the data. The apparent binding constant of dTTP in this case is ~14 μM.
13. Stopped-flow fluorescence measurements

Stopped-flow fluorescence measurements were carried out with an Applied Photophysics SX.18MV stopped-flow spectrofluorometer at 22°C. The instrument triggered a rapid mixing of solutions from two different syringes. One syringe contained 50 nM Cy3-ddP/T duplex, 200 nM T7 pol and 10 μM ddATP in 40 mM pH 7.5 Tris buffer with 10mM MgCl$_2$. The other syringe was filled with different concentrations of dCTP in 40 mM Tris buffer pH 7.5 containing 10mM MgCl$_2$. After excitation at 546 nM with a mercury lamp, the fluorescence of the Cy3-ddP/T was detected by a PMT after passing through a 570BP filter and recorded as a function of time. The fluorescence signal was collected for 1s. Two to four traces were averaged for each set of conditions. The dead time of the instrument is 2 ms.

14. Continuous-flow fluorescence measurements using a hydrodynamic focusing device

Since the conformational change of T7 pol: Cy3-T/P: dCTP ternary complex is too fast to be resolved with our stopped-flow instrument, we constructed a microfluidic continuous-flow device based on hydrodynamics focusing according to the report by Knight et al.(4) to make the kinetic measurements. Soft lithography technique was used for the fabrication of this device. Fig. S9 shows the setup for continuous-flow fluorescence measurements.
Fig S9 Setup for the continuous-flow fluorescence measurement using hydrodynamics focusing to measure the fast conformational change of the T7 pol ternary complex upon dCTP binding. Hydrodynamic focusing can be realized by applying appropriate pressures to the central and side stream inlets of the microfluidic device. The depths of the microfluidic channels are 20 μm. The width of the central inlet channel, two side channels and focused channel are 10, 40, 40 μm, respectively. To illuminate a large enough area, an Olympus 10x NA 0.25 objective was used to image the flow, the final magnification of the system is 15x. Under these conditions one pixel corresponds to 1.067 μm. The inset in the lower right corner is a typical fluorescence image of the focused stream.

Fast mixing is realized by focusing the central stream into a very narrow range, so that solute in the side streams can diffuse into the central stream within a short time. The width of the focused stream can be controlled by the pressure ratio of the central and side channel inlets. A typical pressure of 6.0 psi for the side and 5.8 psi for the central stream was used, leading to a volume flow rate of 2.5 μl/min, corresponding to a line speed of ~75 μm/ms at the center of the channel. The width of the stream in this condition is ~300
nm, measured by the Cy3 fluorescence intensity ratio between the focused area and the pre-focusing area. The estimated mixing time at this stream width is about 50 μs for the dCTP substrate.

In our experiment the central stream is a pH 7.5 50mM Tris Buffer with 10mM Mg$^{2+}$, 100nM Cy3-P/ddT and 1 μM T7 DNA polymerase, and the side stream is a pH 7.5 50mM Tris Buffer with 10mM Mg$^{2+}$ and different concentrations of dCTP. The kinetic information can be obtained by measurement of the fluorescent intensity profile at different positions of the focused stream, translated into different time points after mixing. 500 frames of the fluorescent images were collected, and averaged to obtain and averaged fluorescence image of the focused stream.

The fluorescence intensities at different time were obtained by fitting the line profile in the image across the stream with a Gaussian function. To correct for the uneven illumination of the sample, the intensity profile is normalized by dividing the intensity trace of a control image, which was obtained when a buffer solution without dCTP was supplied in the side channels.

Fig. S10 shows the normalized fluorescence time traces of the Cy3-ddP/T: T7 pol complex when mixing with different concentrations of dCTP.
Fig. S10 Normalized fluorescence intensity trace of Cy3-ddP/T: T7 pol complex after mixing with different concentrations of dCTP solution. For 10 and 50 μM dCTP, the line speed of the central stream was 75 μm·ms$^{-1}$. For dCTP concentrations of 100, 200, 500 μM, a higher line speed of the central stream at 150 μm·ms$^{-1}$ was used to increase the time resolution. The relative amplitude of the intensity change is about
25%, matching the expected value. The rate constants for the fluorescence increase were obtained by fitting the traces with a single exponential rise function. At 10 μM dCTP, the rate constant obtained by this method (220 s\(^{-1}\)) is consistent with the value obtained from stopped-flow fluorescence measurements.

15. Observation of the conformational change induced by ddCTP binding

The structure of the closed form of T7 pol replication complex was obtained using 3’-dideoxy terminated primer and a dideoxy nucleotide triphosphate ddNTP as the incoming substrate. We also made observation using the Cy3-ddP/T: T7 pol complex and the ddCTP to better connect the fluorescence observation and the previous structural information. A similar fluorescence increase of the Cy3 probe can also be observed upon ddCTP binding, as shown in Fig. S11. The affinity of ddCTP to the T7 pol complex is found to be only slightly lower than that of dCTP. With this observation, we can make a direct connection between this Cy3 fluorescence intensity increase to the fingers closing found by X-ray crystallography.
Fig. S11 Fluorescence intensity trajectories of the Cy3-ddP/T upon polymerase binding in the presence of different concentrations of ddCTP. Similar fluorescence intensity enhancement after ddCTP binding to that shown in Fig. 4A in the main text can be observed. The apparent binding constant of ddCTP is on the range of sub-micromolar concentration.

References:

