



Recent progress in dissecting molecular recognition by DNA polymerases with non-native substrates

Kaitlin M Pugliese^{1,3} and Gregory A Weiss^{1,2}

DNA polymerases must discriminate the correct Watson–Crick base pair-forming deoxynucleoside triphosphate (dNTP) substrate from three other dNTPs and additional triphosphates found in the cell. The rarity of misincorporations *in vivo*, then, belies the high tolerance for dNTP analogs observed *in vitro*. Advances over the last 10 years in single-molecule fluorescence and electronic detection of dNTP analog incorporation enable exploration of the mechanism and limits to base discrimination by DNA polymerases. Such studies reveal transient motions of DNA polymerase during substrate recognition and mutagenesis in the context of erroneous dNTP incorporation that can lead to evolution and genetic disease. Further improvements in time resolution and noise reduction of single-molecule studies will uncover deeper mechanistic understanding of this critical, first step in evolution.

Addresses

¹ Department of Chemistry, University of California, Irvine, CA 92697, United States

² Department of Molecular Biology and Biochemistry, 4122 Natural Sciences 1 (mailing: 1102 Natural Sciences 2), University of California, Irvine, CA 92697-2025, United States

Corresponding author: Weiss, Gregory A (gweiss@uci.edu)

³ Present address: Illumina, 5200 Illumina Way, San Diego, CA 92122, United States.

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Introduction

DNA polymerases have long served as models for successful and highly discriminating molecular recognition. The ability to select the correct deoxynucleoside triphosphate (dNTP) substrate from an assortment of closely related molecules is a key mechanistic step in the enzyme's catalysis of DNA polymerization. Advances over the last 5–10 years allow observation of nucleotide incorporation in real-time at ultrafast time scales via observation of enzyme motions during catalysis [1–4,5[•],6[•],7]. Incorporation of dNTP analogs in combination with these recently developed techniques provide

new insights into mechanisms of molecular recognition essential for the function of DNA polymerase [5[•],8,9].

Non-cognate substrates for DNA polymerization, including ribonucleoside triphosphates (rNTPs) and dideoxynucleoside triphosphates (ddNTPs), represent the molecular recognition challenge faced by DNA polymerase. The rNTPs, for example, are present at >10-fold higher concentrations than dNTPs in the cell [10], yet are rejected as substrates by DNA polymerase. Experiments with such substrates have generated insights into the enzyme's preferences and formed the basis for sequencing technologies (e.g., Sanger sequencing using ddNTPs) [8,9,11–13]. Base-modified dNTP analogs can probe molecular recognition of both the incoming dNTP substrate and an appropriately substituted template base [5[•],14–17,18[•],19[•]]. This review will survey the key biophysical techniques that have employed dNTP base and ribose analogs and the insights gained from these studies. Additionally, the limitations associated with current techniques and potential areas in which nucleotide analogs could be employed will be examined. The discussion will focus on studies of A-family polymerases, including the large fragments of DNA polymerase I from *Escherichia coli* (KF), *Thermus aquaticus* (KlenTaq), and *Bacillus stearothermophilus* (BF).

Crystallography provides insight with static visualization

High resolution crystal structures provide static images of polymerases at different stages of nucleotide incorporation. Historically, important crystallographic studies 'trapped' DNA polymerase in the 'closed' conformation—the result of a large reorientation of the fingers subdomain necessary for catalysis [20]. Comparison of the 'open' and 'closed' crystal structures revealed additional intra-protein and protein-primer/template (protein-P/T) interactions. New insights gained from crystal structures, including those in the presence of nucleotide analogs, can guide fluorescent and other experimental techniques to dissect nucleotide incorporation. For example, the recent discovery by time-resolved X-ray crystallography of a third Mg²⁺ cation necessary for catalysis revises our understanding of the 'closed' structure of DNA polymerase [21].

DNA polymerase crystal structures from the past several years present a more thorough picture of enzyme nucleotide selection through co-crystallization with non-cognate substrates (Table 1). For example, rNTPs result

Table 1

Crystal structures of DNA pol I with non-native nucleotide substrates.

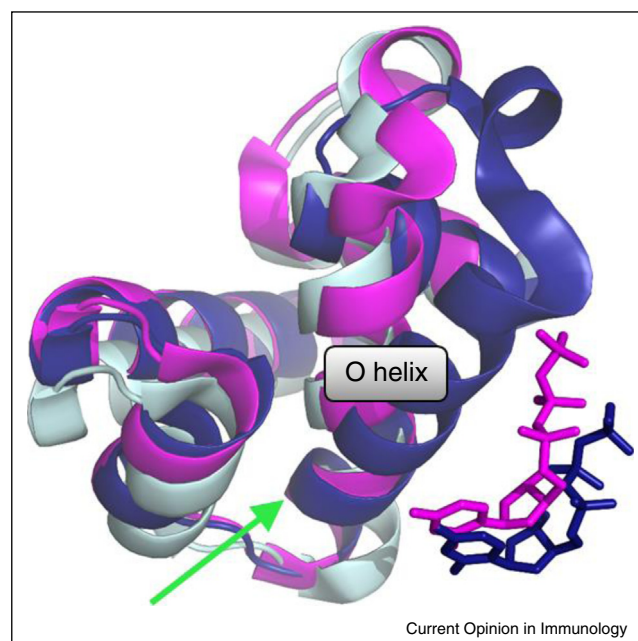
| Organism | Template base | Analog substrate | PDB ID | Resolution (Å) | Reference |
|------------------------------|---------------|-----------------------|--------|----------------|-----------|
| <i>B. stearothermophilus</i> | dG | ddCTP | 4DQP | 1.74 | [12] |
| <i>B. stearothermophilus</i> | dG | rCTP | 4DS5 | 1.68 | [12] |
| <i>B. stearothermophilus</i> | dG | ddTTP | 3HP6 | 1.81 | [13] |
| <i>B. stearothermophilus</i> | dG | dTTP | 3HPO | 1.75 | [13] |
| <i>B. stearothermophilus</i> | dA | dCTP (tautomer) | 3PX0 | 1.73 | [22] |
| <i>B. stearothermophilus</i> | dA | ddTTP | 3PV8 | 1.52 | [22] |
| <i>B. stearothermophilus</i> | dA | ddCTP (wobble) | 3PX4 | 1.58 | [22] |
| <i>B. stearothermophilus</i> | dA | ddCTP (tautomer) | 3PX6 | 1.59 | [22] |
| <i>B. stearothermophilus</i> | dC | ddGTP | 3TI0 | 1.62 | [22] |
| <i>B. stearothermophilus</i> | dT | ddATP | 3THV | 1.61 | [22] |
| <i>T. aquaticus</i> | dG | dT ^{spin} TP | 3OJU | 2.0 | [14,17] |
| <i>T. aquaticus</i> | dG | dT ^{dend} TP | 3OJS | 1.9 | [14,17] |
| <i>T. aquaticus</i> | dNaM | d5SICSTP | 3SV3 | 2.1 | [15,16] |
| <i>T. aquaticus</i> | dA | dU ^{ene} TP | 5E41 | 1.8 | [19**] |
| <i>T. aquaticus</i> | dT | dA ^{ene} TP | 5SZT | 1.8 | [19**] |
| <i>T. aquaticus</i> | dA | dU ^{kyr} TP | 4DFK | 1.65 | [19**,23] |
| <i>T. aquaticus</i> | dT | dA ^{kyr} TP | 4DF4 | 2.2 | [19**,23] |
| <i>T. aquaticus</i> | dG | dC*TP | 4DFM | 1.89 | [23] |
| <i>T. aquaticus</i> | dA | dT*TP | 4DFJ | 1.9 | [23] |
| <i>T. aquaticus</i> | dC | dG*TP | 4DFP | 2.0 | [23] |
| <i>T. aquaticus</i> | dT | dA*TP | 4DF8 | 2.0 | [23] |

in non-productive conformations of the O helix in the fingers subdomain [12]. One of the most interesting discoveries identified an 'ajar' BF conformation in the presence of a mismatched dNTP; the position of the active site's O helix is intermediate between the 'open' and 'closed' states via a kink at residue G711 (Figure 1) [13]. Complexes with rare tautomers of incoming dNTPs [22], bulky dNTP analogs [14], and unnatural base pairs [15,16] also resulted in an 'ajar' conformation. Taken together, these results indicate that various structures of polymerases can accommodate binding, but not catalysis, of some non-cognate substrates, and the resultant ajar conformations represent frozen states along the pathway for nucleotide discrimination.

Nucleotide analogs used in many biotechnological applications contain various functional groups, including fluorescent tags and sites for bioconjugation, on the C5 position of pyrimidines or the C7 position of 7-deazapurines (exemplary structures can be found in [18**]). Recent crystal structures further illustrate the flexibility of the KlenTaq active site to accept different modified nucleotides, suggesting specific residues required to stabilize the unnatural conformation [17]. The authors also identified alkene-modified nucleotides with more efficient incorporation than their native counterparts as supported by crystal structures of catalytically competent complexes. Why the polymerase preferentially incorporates alkene-modified nucleotides over alkyne-modified nucleotides remains unclear, however [19**]. Using inspiration from the accommodating polymerase active site, a more comprehensive study with BF observed aromatic bases of dNTPs with strong affinity for an active-site arginine, which interacts with the dNTP via a π -cation

interaction [24**]. A recent review further expands on these polymerase crystal structures solved in the presence of modified nucleotides [18**]. Despite the great deal of

Figure 1



An 'ajar' conformation of *Bst* polymerase (magenta, PDB ID: 3HT3) was identified in the pathway from 'open' (light blue, PDB ID: 1L3T) to 'closed' (dark blue, PDB ID: 1LV5) for a mismatched incoming dNTP. The motion of the O helix, responsible for molecular recognition of the dNTP, is especially notable for a 'kinking' effect along the conformational pathway. The position of this kink along the O helix at residue G711 is indicated by the green arrow.

knowledge gained from crystal structure determination in the presence of nucleotide analogs, these static images by themselves do not provide adequate information about the kinetics and dynamic motions of specific and non-specific dNTP and dNTP analog incorporation events.

From static to dynamic: molecular dynamics simulations

Molecular dynamics (MD) simulations model protein motions using the crystal structures of DNA polymerase as starting points. The technique can uncover key aspects of enzymatic catalysis not observable by conventional experimental techniques. For example, the driving forces behind conformational transitions associated with catalysis, including nucleotide selection, can be identified. Simulated fluctuations of BF in the absence of substrate model the 'open' to 'ajar' transition as requiring less than 20 ns. This BF simulation suggested that the 'ajar' intermediate acts as an energetic barrier to opening, and promotes correct dNTP incorporation [25]. A recent crystal structure of an 'open' ternary BF complex allowed simulation of the events in active site assembly, which appear to include desolvation of the incoming dNTP [26*]. As described, computer simulations have theorized persistently elusive rapid motions. However, simulations over short time scales require days to weeks of computational analysis. In any case, more advanced experimental techniques are required to support such data.

Molecular dynamics via single-molecule fluorescence experiments

Conformational fluctuations occur over a broad range of time-scales in equilibrium, and cannot be detected through ensemble-averaged experiments due to averaging from the large number of non-synchronous molecules in the population [27]. To address this limitation, single-molecule biophysical methods to measure DNA polymerase reactions can answer unresolved questions about nucleotide selectivity and DNA replication fidelity [28]. Fluorescent techniques, developed largely over the last two decades, allow observations of previously inaccessible transient intermediates during correct or mutagenic incorporation by DNA polymerases.

The low quantum yields of fluorescent base analogs often limit their value in single-molecule studies, which can require sensitive probes of the local environment. In a newly developed assay, however, single-molecule fluorescence with 2-aminopurine and pyrrolo-C-containing templates were characterized for the first time in DNA and RNA, respectively [29]. A new thymidine analog, *N,N*-dimethylaniline-2'-deoxythymidine, has sensitive fluorescent properties that could be adapted to single-molecule experiments [30**]. Characterization of fluorescent analogs in single-molecule experiments sets the stage for their application to polymerase dynamics during nucleotide incorporation. However, environmentally sensitive

fluorophores alone do not always clarify which structural changes are associated with the fluorescent fluctuations.

Förster resonance energy transfer (FRET)-based assays permit distance measurements by defined placement of two fluorophores that undergo energy transfer only when in close proximity to one another. Single-molecule FRET (smFRET) is especially powerful, as it measures conformational motions along reaction pathways at millisecond timescales [31]. For these experiments, crystallographic information can suggest the placement or choice of fluorophores after defining the interaction to be explored. Non-nucleotide fluorophore pairs provide a direct readout of conformational transitions during incorporation of dNTP analog substrates by DNA polymerase. Alternatively, fluorescent base analogs have potential as acceptor fluorophores on the P/T [32] or as the incoming dNTP substrate.

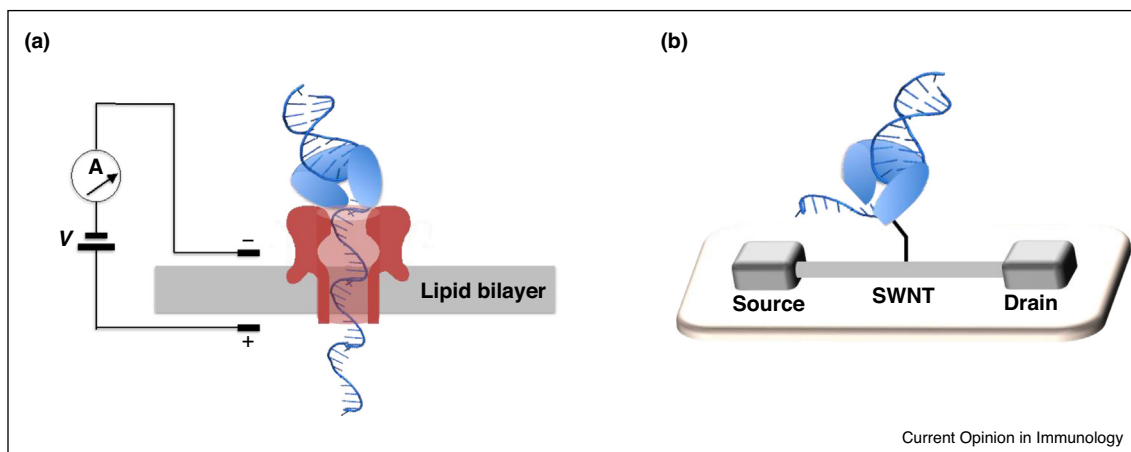
Unique intermediate FRET signatures, suggested to be fidelity checkpoints, occur between the 'open' and 'closed' states of KF in the presence of mismatched dNTPs [33,34*,35]; these signatures are likely representative of the 'ajar' crystal structures previously observed for BF [13]. Complementary nucleotides also produce an intermediate FRET signature that is short-lived and barely detectable, indicating the transient presence of this state along the catalytically relevant reaction pathway [33]. Observation of this intermediate step, previously unobserved for correct nucleotides, underscores the importance of this sensitive single-molecule technique.

In recent lifetime-resolved smFRET measurements, fluorogenic and pro-fluorogenic substrates indicated local changes in the microenvironment of intermediate or final reaction products [36,37]. This use of fluorogenic substrates as FRET acceptors could be coupled with a recently developed smFRET sensor that avoids use of a functionalized polymerase [38*]. While such a system would certainly be appreciated in the investigation of phosphoryl transfer and pyrophosphate release by DNA polymerase, careful design of substrates will be required for successful catalysis. Overall, smFRET can uncover important aspects of nucleotide incorporation, but its limited time resolution can conceal rapid subdomain dynamics during this mechanism.

Current and potential single-molecule sequencing techniques as mechanistic tools

Polymerase-driven single-molecule technologies with potential for DNA sequencing can double as formidable tools for understanding the enzyme's fidelity mechanism (Figure 2). By taking advantage of the enzyme's biophysical characteristics during the substrate recognition stage of polymerase catalysis, some sequencing approaches serve both basic research and applied methodology. One

Figure 2



Single-molecule electronic techniques to measure DNA polymerase activity in the presence of nucleotide analogs. **(a)** Nanopore sequencing is a commercialized technology that reads DNA nucleobases via an ionic current blockage unique to each base. The development of this technique also enabled measurements of distinct DNA polymerase-P/T complexes in the presence of mismatched nucleotides or other incorrect substrates. **(b)** Single-walled carbon nanotubes (SWNTs) functionalized with polymerase molecules provide a read-out of enzyme motion. This type of measurement is possible due to charged amino acids on the polymerase surface that change the SWNT conductivity. Observations of novel signals for nucleotide analogs compared to native, complementary nucleotides during base incorporation support the potential of this technique for DNA sequencing.

current example is a strategy used with nanopore devices, which are comprised of a thin membrane containing a biological or solid-state nanopore through which captured ssDNA can cause changes in ionic current [39,40]. Another DNA polymerase-monitoring technology relies on voltage-gating of single-walled carbon nanotube field-effect transistors (SWCNT-FETs). These devices, developed in our lab, provide a readout of the charged amino acids on the enzyme's surface that move within 1 nm of the nanotube during catalysis [4].

Recent nanopore sequencing systems measure motion of motor proteins with approximately 7–8 times more spatial sensitivity than FRET [41,42]. A-family polymerases captured atop a nanopore in an applied electric field allow detection of polymerase-dependent DNA extension with single-base resolution [1]. The rate of polymerization by the DNA polymerase controls ssDNA translocation through the pore [2,3,43–45]. One notable study using nanopores analyzed rNTP-bound versus dNTP-bound ternary complexes and demonstrated distinct intermediate complexes with increasing stability. This study established the first step of complementary base recognition, followed by a deoxyribose recognition state, then fingers-closing, and finally an active site rearrangement [9]. These nanopore-based experiments have also recognized enzyme-P/T binary complex and enzyme-P/T-dNTP ternary complex discrimination [43], incorrect dNTP binding [2], and pre-equilibrium kinetics [46]. Nanopore devices based on $\phi 29$ DNA polymerase have facilitated understanding of deoxyribose discrimination [8], substrate binding, and dynamics of translocation [7,47].

Other strategies could be adapted to observe polymerases within a nanopore and further analyze catalysis. Advances in the nanopore system itself, including the optimization of applied potential and salt concentration, increase the system's sensitivity for identifying differences in nucleotide structure [48]. In one strategy, nanopores with internally bound proteins [49] could evaluate a single enzyme over long time scales by measuring small changes in the protein's conformation. In a second strategy, translocation of a protein-P/T complex through a nanopore could sequentially evaluate various single-molecules and permit determination of an activity distribution from single-molecule populations [50,51].

A new single-molecule electronic technique for examining DNA polymerase was initially developed in our lab to detect conformational transitions during catalysis by various enzymes, including KF [4]. Although the exact nature of the observed conformations for KF is unclear, distinct states have been observed for dNTP analogs [5**]. These unique states implicate active site residues in a fidelity-checking mechanism, and are likely affected by changes in the analogs' electronic distribution. This result provides a novel opportunity — perhaps 'electronic' analogs can be optimized with electronic-based experiments in the same way that fluorescent base analogs are with fluorescent-based techniques. Despite a recent report that touted the short probe-able distance from electronic biosensors as a limitation [52], this distance-limited sensitivity can in fact be an advantage to reduce noise from more distant and extraneous protein motion. Recently, the dNTP-induced fingers-closing transition was observed

with a commercial biosensor that also revealed previously unidentified tight binding states for Taq and KF, and could presumably be expanded to measure analog incorporation [6*]. The location of attachment is significant for the thermal stability of immobilized enzymes [53] and rapid, stable immobilization in a reproducible, specific orientation is required for consistent, sensitive detection [54].

Concluding remarks

Nucleotide analogs that are easily accepted by polymerases provide a strategy to probe the enzyme's response to closely related substrates and explore the mechanistic underpinnings of mutagenesis. The enzymes described here exhibit broad tolerance for dNTP analogs, which make their tremendous fidelity all the more remarkable. The alternative and intermediate conformations trapped by such analogs clarify how the enzymes test each potential substrate's fit before catalysis.

Continued development of techniques to study DNA polymerase offers both new DNA sequencing approaches and insight into this machine at the heart of cell division and life. Experimental limitations inherent to the study of the rapid, non-rate-limiting steps of the DNA polymerase reaction, however, prevent complete structural characterization of its mechanistic intermediates. Even the millisecond timescale of state-of-the-art fluorescent experimental techniques can be insufficient for observing subdomain motions during nucleotide incorporation. Techniques with improved time resolution, elongated time scales, and minimal non-native modifications are required to further elucidate the dynamic processes of DNA polymerase catalysis. Improvement in single-molecule biophysical techniques will continue to clarify the precise nature of nucleotide selection through experiments with non-native substrates.

Conflict of interest

Kaitlin Pugliese is currently an employee at Illumina.

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