The mechanism of action of T7 DNA polymerase

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The recent crystal structure determination of T7 DNA polymerase complexed to a deoxynucleoside triphosphate and primer–template DNA has provided the first glimpse of a replicative DNA polymerase in a catalytic complex. The structure complements many functional and structural studies of this and other DNA polymerases, allowing a detailed evaluation of proposals for the mechanism of nucleotidyl transfer and the exploration of the basis for the high fidelity of template-directed DNA synthesis.

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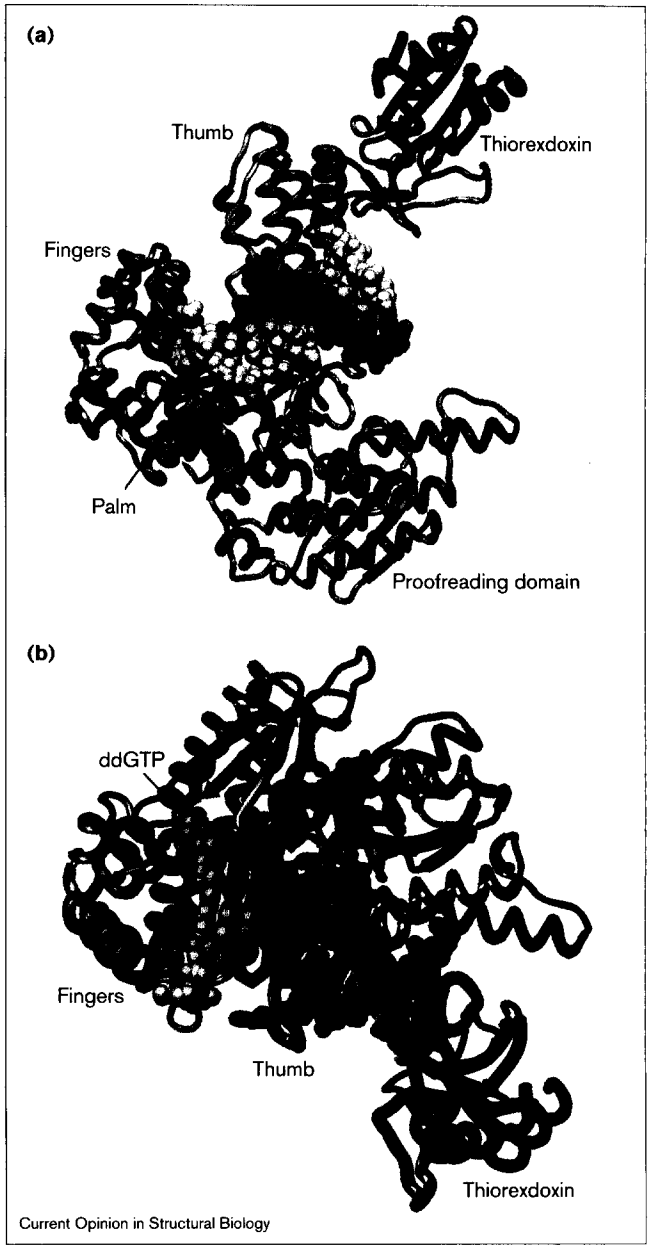
Abbreviations

ddNTP	2′,3′-dideoxynucleoside triphosphate
dNTP	2′-deoxynucleoside triphosphate
Pol I	polymerase I
rNTP	ribonucleotide triphosphate
RT	reverse transcriptase

Introduction

Recent crystal structure determinations of DNA polymerases complexed to DNA and/or nucleotide substrates suggest how functionally important residues and two metals within the polymerase active site contribute to the catalytic efficiency and fidelity of DNA synthesis. The first structure of a DNA polymerase, the Klenow fragment from *Escherichia coli* DNA polymerase I, revealed hand-like features, termed the fingers, palm and thumb subdomains, that define a DNA-binding groove (reviewed by Joyce and Steitz [1]). More recent structures of the related DNA polymerases from *Thermus aquaticus* (Taq DNA polymerase) [2–4], *Bacillus stearothermophilus* (Bst DNA polymerase) [5,6••] and bacteriophage T7 (T7 DNA polymerase) [7••] complexed to DNA and/or nucleotide substrates have shown how the fingers and thumb move in response to substrate binding. These substrate-induced movements suggest that DNA polymerases flicker between an open state, which allows the active site to sample nucleotides and the DNA primer–template to slide into position for the next catalytic cycle, and a closed state, which configures the active site for nucleotide incorporation. Several recent reviews have examined the structure and function of DNA polymerases and their interactions with other proteins at the replication fork [8–11]. Our review focuses on the nucleotidyl transfer reaction and relates it to the crystal structure of T7 DNA polymerase complexed to both nucleotide and DNA substrates

Figure 1



Structure of the T7 DNA replication complex. **(a)** The T7 DNA polymerase structure (shown in purple), with primer (dark pink), template (yellow) and incoming nucleotide (cyan), provides the first glimpse of a processive DNA polymerase in a catalytic complex. The incoming nucleotide fits snugly in the polymerase active site, at the junction between the fingers and palm subdomains. *E. coli* thioredoxin (green) is bound to the tip of the thumb subdomain and is poised over the DNA exiting the polymerase. **(b)** A view of the T7 DNA polymerase active site, obtained by rotating the model in (a) by 90° about a horizontal axis. The incoming nucleotide (cyan) is tightly sandwiched between the O helix (dark purple) and the 3′-end of the primer. The thumb of the polymerase grips the DNA primer–template five to eight base pairs away from the polymerase active site.

(Figure 1), a state closely resembling a catalytic complex for nucleotide incorporation. Recent genetic and biochemical analyses of the active sites of T7 DNA polymerase and the related polymerases of the *E. coli* DNA polymerase I (Pol I) family have clarified the roles of the conserved residues that accelerate the rate of DNA synthesis, control nucleotide substrate selection [12,13•,14•] and/or influence the fidelity of DNA synthesis [15,16••,17•]. The congruence of this ensemble of polymerase structures with the functional data sheds new light on the mechanism of phosphoryl transfer that is catalyzed by Pol I-type DNA polymerases. Features of the active sites of both the nonhomologous, pol α-type polymerase from the *Salmonella* phage RB69 [18••] and the mammalian repair polymerase β [19,20••] are remarkably similar to those of the Pol I-type enzymes, suggesting that these diverse DNA polymerases employ closely related strategies for copying a DNA template.

Two metals in the polymerase active site

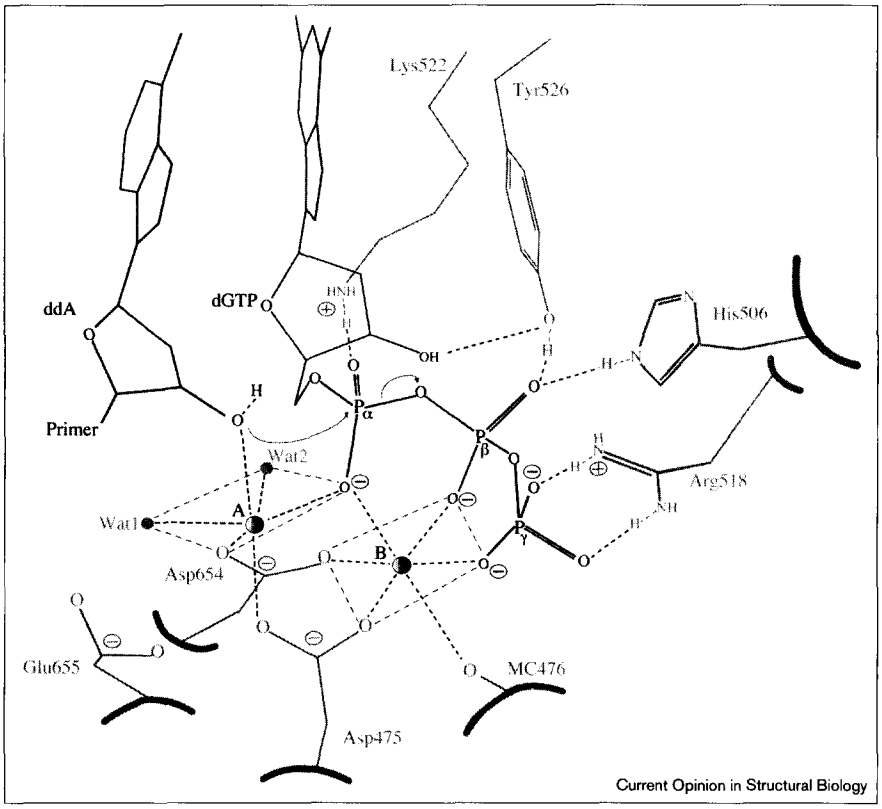
DNA polymerases catalyze the nucleophilic attack of a bound nucleoside 5′-triphosphate by the 3′-hydroxyl of a DNA primer, resulting in the incorporation of a nucleoside monophosphate into DNA and the release of pyrophosphate [21,22]. Like most other enzymes that catalyze phosphoryl transfer reactions [23–25], DNA polymerases are metal-dependent enzymes [1,21]. Pioneering biochemical and spectroscopic studies of *E. coli* Pol I suggested that one or more metals engage the β and γ phosphates of the incoming nucleoside triphosphate [26,27]. Steitz [22] proposed that two metals in the polymerase active site of *E. coli* Pol I provide catalytic assistance, a model based on stereochemical considerations and crystallographic studies of the stably bound metals in the 3′→5′ proofreading exonuclease site of Pol I [28,29]. However, the metals are not tightly affixed within the polymerase site in the absence of the nucleotide and the primer–template. Thus, in order to view the full catalytic complex, it was necessary to trap a polymerase engaging both DNA and nucleotide within a ternary complex [7••,19,20••].

In the crystal structure of T7 DNA polymerase, the fingers, palm and thumb grip the primer–template such that the 3′-end of the primer strand is positioned next to the nucleotide-binding site, which is located at the confluence of the fingers and thumb. The incipient base pair formed between the nucleotide and the template base fits snugly into a groove between the fingers and the 3′-end of the primer (Figure 1b). Two metals in the polymerase active site, which are magnesium ions in the crystals, were identified by X-ray diffraction experiments [7••] (Figure 2). Metal A is adjacent to the primer 3′-end and metal B engages the triphosphate moiety of the nucleotide, positions that approximate those proposed for the two metal ion mechanism of DNA synthesis [22,28]. In a 1.85 Å resolution model of the T7 complex (S Doublié, T Ellenberger, unpublished data), the metal-binding sites are located 3.8 Å apart, with an average distance of 2.2 Å between the metals and their oxygen ligands, as expected for the octahedral coordination of magnesium by oxygen [30]. Metal B is coordinated with nearly perfect octahedral geometry by the pro-R_p oxygens of the α and β phosphates, a nonesterified oxygen of the γ phosphate and the carboxylate oxygens of the strictly conserved residues Asp475 and Asp654. A main-chain carbonyl oxygen from Ala476 completes the octahedral coordination of metal B (Figure 2). The interaction of metal B with the pro-R_p oxygens of the α and β phosphates provides an explanation for the inhibitory effects of phosphorothioate substitutions at these positions. A nucleotide with sulfur replacing the pro-R_p oxygen of the β phosphate is not incorporated into DNA by the Klenow fragment unless the thiophilic metal manganese is present [27]. On the other hand, the analogous substitution of sulfur for the pro-R_p oxygen of the α phosphate prevents nucleotide incorporation in the presence of either magnesium or manganese, which is suggestive of some steric interference by the larger sulfur atom. Metal A is held in the active site of T7 DNA polymerase by the pro-R_p oxygen of the α phosphate and by the two aspartic acid residues that also ligate metal B. Two water molecules also contribute to the octahedral coordination of metal A (Figure 2). The primer 3′-hydroxyl, which would complete the octahedral coordination of metal A, is missing from the chain-terminating 2′,3′-dideoxynucleotide triphosphate (ddNTP) that was incorporated by the polymerase during the crystallization experiment. The weaker electron density and higher thermal parameter of metal A (34.1 $Å^2$), in comparison to the fully ligated metal B (25.5 $Å^2$), are suggestive of disorder or incomplete occupancy of the metal A site in the absence of the primer 3′-hydroxyl. A 3′-hydroxyl group modeled on the primer is within 3.0 Å of the α phosphorus and these atoms define an angle of 162° with the α–β bridging oxygen of the pyrophosphate leaving group (Figure 2). This geometry is consistent with an in-line attack on the α phosphorus of the nucleotide by the 3′-hydroxyl of the primer.

In a remarkable example of convergent evolution, similar metal coordination geometry was revealed in recent crystal structures of two unrelated polymerases complexed to a nucleotide and DNA. Two metals are bound by three aspartic acid residues in the active sites of human DNA polymerase β [20••] and HIV-1 reverse transcriptase (RT) (H Huang, R Chopra, G Verdine, S Harrison, personal communication). In both of these enzymes, as in T7 DNA polymerase, one metal contacts nonbridging oxygens of all three phosphates of the bound nucleotide and the other metal is located between the α phosphate and the primer 3′-end. The conserved metal-binding geometry within the active sites of these three highly divergent polymerases suggests a universal strategy for the metal-assisted polymerization of nucleotides.

DNA synthesis by *E. coli* Pol I and related enzymes results in the inversion of stereochemistry at the α phosphorus of the nucleotide [27,31,32], the signature of an in-line attack

Figure 2



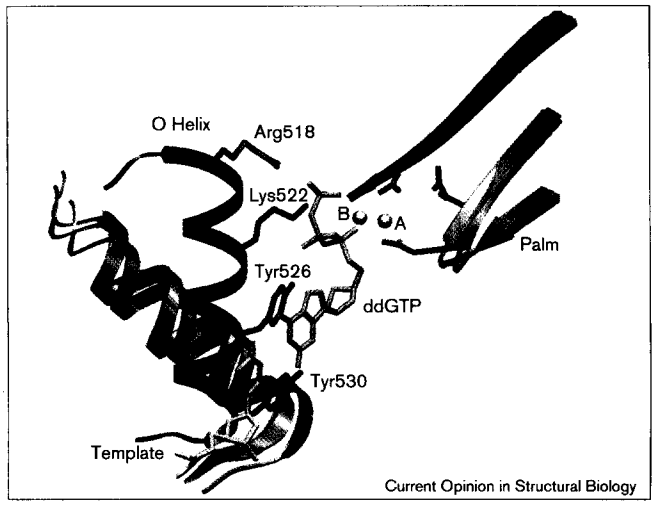
Schematic of the proposed mechanism of nucleotidyl transfer by T7 DNA polymerase. The modeled 3′-hydroxyl at the primer is shown to participate in an in-line nucleophilic attack on the α phosphorus of the incoming nucleotide, followed by the release of pyrophosphate. The two magnesium ions (labeled A and B) in the active site are ligated by two strictly conserved aspartates, Asp475 and Asp654 (Asp705 and Asp882, respectively, in *E. coli* DNA polymerase I). Both metals ligate the α phosphate of the incoming nucleotide and metal B also ligates the β and γ phosphates. Two water molecules, labeled Wat1 and Wat2, complete the octahedral coordination of metal A. MC476 refers to a contact by the mainchain carbonyl oxygen of residue Ala476.

on the α phosphorus by the primer 3′-oxygen, with the displacement of pyrophosphate. The polymerase selectively stabilizes the charge and stereochemistry of the transition state, which are determined by the extent of bonding of the α phosphoryl group to the attacking oxygen and to the pyrophosphate leaving group (for reviews, see [33–35]). Evidence from physical organic chemistry suggests that the transition states of nonenzymatic phosphoryl transfer reactions can have characteristics varying from extreme associative (pentacoordinate, oxyphosphorane-like) to dissociative (tricoordinate, metaphosphate-like) reaction pathways [36,37]. Nonenzymatic reactions of bisubstituted phosphoryl compounds, analogous to the α phosphoryl group of a nucleoside triphosphate, react via a transition state of intermediate character, similar to the transition state of a classical substitution nucleophilic biomolecular (S_N2) reaction. The extent to which enzymes can alter the structure of the transition state during phosphoryl transfer is unknown.

What does the structure of the T7 polymerase active site suggest about the catalytic mechanism of DNA synthesis? The metals bound in the active site of the polymerase assist catalysis by juxtaposing the reactive primer hydroxyl with the nucleotide α phosphate, lowering the entropic cost of nucleophilic addition. Two strictly conserved aspartic acids located on the floor of the active site (Asp475 and Asp654) form a bridge between the metals that holds them in the required orientation. Moreover, the interaction of metal B with the nonesterified oxygens of the α, β and γ phosphates aligns the scissile bond with respect to the attacking primer 3′-end, steering nucleophilic attack. There is no general base within the active site that could deprotonate the primer hydroxyl; however, the lowered pK_a of the metal-bound hydroxyl favors the formation of the 3′-hydroxide nucleophile. Although metal coordination is expected to decrease the nucleophilicity of the oxyanion, the metal serves to position the nucleophile and increases its concentration in the active site at physiological pH. The interaction of the positively charged metal with the 3′-oxygen of the primer shifts the equilibrium for deprotonation of the neutral hydroxyl in favor of the negatively charged hydroxide. The metals and Lys522 from conserved sequence motif B [38] contact both nonbridging oxygens of the α phosphate (Figure 2) and, thus, can counteract the negative charge of the α phosphorus that develops during bond formation with the primer 3′-oxygen.

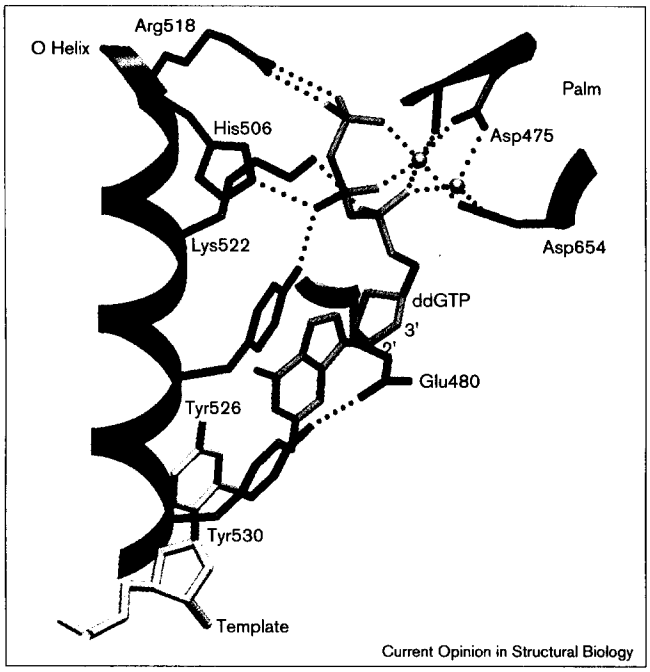
In the structures of T7 polymerase [7••] and polymerase β [20••], there are no direct contacts to the nucleotide's α–β bridging oxygen, which becomes the hydroxide of the pyrophosphate leaving group. The metals are more than 3.4 Å from the bridging oxygen, too far for direct ligation of this oxygen. It seems unlikely that metal B would forsake the octahedral coordination arrangement seen in the crystal structure (Figure 2) in order to bond with the oxygen of the pyrophosphate leaving group [10,22]. Instead, the

Figure 3



Open and closed conformations of the fingers subdomain. The structures of four Pol I family polymerases were superimposed by aligning their highly conserved palm subdomains. The unliganded Bst polymerase is shown in gold [5], the Klenow fragment editing complex is in pink [45], Taq polymerase complexed with DNA is in green [4] and the T7 DNA polymerase complex with DNA primer–template and incoming nucleotide is shown in purple [7••]. In the absence of DNA and/or nucleotide, the fingers subdomains of these polymerases (represented here by the O helix) are rotated away from the polymerase active site. In the T7 complex, the fingers adopt a substrate-induced closed conformation that brings conserved residues from the fingers subdomain into contact with the incoming nucleotide (shown in cyan).

Figure 4



Close-up of the nucleotide-binding site of T7 DNA polymerase (residue numbers for *E. coli* DNA polymerase I are given in parentheses). The incoming nucleotide (ddGTP, cyan) is ligated by four conserved residues from the fingers subdomain – His506, Arg518, Lys522 and Tyr526 (His734, Arg754, Lys 758 and Phe762) – and by the two metals in the active site (silver spheres). The ribose moiety stacks against the aliphatic part of Glu480 (Glu710), which acts as a 'steric gate' for the discrimination against ribonucleotides. Tyr530 (Tyr766) does not stack against the template base, as seen in the Taq and Bst DNA polymerase–DNA complexes [4,6••]. Instead, it is partially buried at the junction of the fingers and palm domains, where it donates a hydrogen bond to Glu480.

sidechain of Lys522 could easily move to neutralize the negatively charged pyrophosphate leaving group. This interaction of the homologous lysine of the Klenow fragment (Lys758) was previously inferred from a crystal structure of pyrophosphate bound to the Klenow fragment polymerase active site [39]. In addition to positioning the nucleotide for incorporation, the interactions of His506 and metal B with the nonbridging oxygens of the β phosphate also could also indirectly stabilize the charged pyrophosphate product. The phosphates of the bound nucleotide are almost completely enveloped by the polymerase and are inaccessible to solvent; more than 91% of the surface of the triphosphate group is buried. This secluded environment strengthens the electrostatic interactions between the charged transition state and the active site metals and amino acids. The redistribution of charge that accompanies the conversion of a nucleoside triphosphate into DNA and pyrophosphate might be the signal for the polymerase to release its grip and prepare for the next round of nucleotide binding and incorporation.

Open and closed conformations

A slow conformational change in the polymerase sets the tempo for nucleotide incorporation during the catalytic cycle [40–44]. For T7 DNA polymerase, the rate-limiting step in the catalytic cycle occurs after the nucleoside triphosphate binds and before it is incorporated into the DNA [44]. The crystal structure of T7 polymerase suggests that this decisive step is the closure of the fingers subdomain around the DNA and nucleotide. In the catalytic complex, the fingers rotate inwards by about 40° towards the active site, compared to their open position in the unliganded polymerase (S Doublié, T Ellenberger, unpublished data). A relaxed, more open conformation of the fingers is also seen in structures of other Pol I-type polymerases lacking nucleotide and/or DNA substrates in their active site (Figure 3) [2–5,45]. A similar, substrate-induced change between the open and closed forms of a polymerase has been documented in a series of crystal structures of mammalian DNA polymerase β [19,20••,46]. The C-terminal subdomain of polymerase β, which corresponds to the fingers subdomain of other polymerases, rotates inward towards the active site only when the correct nucleotide is paired with the DNA template [20••]. In the polymerase β–DNA complex lacking a nucleotide, the protein is in an open conformation that would permit the nucleotide-binding site to sample candidate substrates. A recent crystal structure of Taq DNA polymerase complexed to both DNA and a nucleotide also shows large-scale movement of the

fingers relative to their position in structures of the unliganded polymerase (G Waksman, personal communication).

The closed conformation of T7 DNA polymerase complexed to DNA and a nucleotide brings conserved, functionally important residues of the fingers subdomain into contact with the nucleotide substrate, configuring the active site for the phosphoryl transfer reaction (Figure 3). The base and deoxyribose sugar of the nucleotide are sandwiched between the 3′-end of the primer and the conserved Tyr526 residue of the O helix within the fingers subdomain [7••]. The triphosphate group is contacted extensively by the two metals described above and by the conserved residues His506, Arg518, Lys522 and Tyr526, also from the fingers subdomain (Figure 4). Alanine substitutions at any of the homologous positions in the Klenow fragment increase the Michaelis constant for the deoxynucleoside triphosphate (dNTP) substrate of the polymerase ($K_{m(dNTP)}$) and/or decrease the rate of synthesis [47–49]. Another conserved tyrosine, located at the C-terminal end of the O helix, is important for polymerase activity; its replacement in the Klenow fragment (Tyr766) with alanine or serine diminishes both the rate of DNA synthesis [48] and the fidelity of nucleotide insertion [15,17•,50]. A conservative substitution of this tyrosine with phenylalanine, however, has little effect on DNA synthesis [50,51], suggesting that a bulky aromatic sidechain is required here for the normal functioning of the polymerase. In the T7 DNA polymerase complex, the analogous O helix residue Tyr530 does not contact the base pair in the active site (Figure 4). Instead, its sidechain is partially buried at the junction of the fingers and palm subdomains, where the tyrosine could influence the movement of the fingers and indirectly affect the fidelity of DNA synthesis. A different orientation of this tyrosine is seen in the crystal structures of Bst DNA polymerase [6••] and Taq DNA polymerase [4] complexed to DNA. In these polymerase–DNA complexes, the tyrosine stacks against the template base, indicating that the fingers have opened in order to allow this interaction. The situation captured by these crystal structures is likely to represent another step in the polymerization cycle. This conformationally flexible tyrosine sidechain [52•] might chaperone each template base into the active site during DNA translocation, prior to the closure of the fingers.

The importance of geometry versus hydrogen bonds

DNA polymerases synthesize DNA with an error frequency of 10^{-3} to 10^{-5} per nucleotide incorporated, without exonucleolytic proofreading [53]. The mechanism of this high selectivity of template-directed DNA synthesis has remained elusive despite intensive study. If a polymerase discriminated against misincorporation only on the basis of the relative hydrogen-bonding energies of correctly matched versus mismatched base pairs, it would make a mistake about every 5 to 150 nucleotides [53,54]. The vastly lower error rates of most DNA polymerases suggest that their active sites exaggerate the energetic penalty for mispairing with the template over that measured for mismatches in duplex DNA [54–58]. The importance of the shape of the polymerase active site in the 'geometric selection' of correct nucleotides has been recognized for some time [53,59,60]. A Polymerase might block the binding of incorrect nucleotides or it could fail to adopt a catalytically productive conformation when the nucleotide and template are mismatched. This is a type of induced-fit mechanism, in which nonideal substrates impede the rate-limiting conformational change that converts the inactive (open) polymerase into the active (closed) form [20••,44,54,61,62]. In addition to these steric restrictions, the immersion of the nucleotide in the nonpolar surroundings of the active site will enhance the favorable energy of Watson–Crick hydrogen bonds and disfavor irregular base pairings that leave hydrogen-bonding groups unsatisfied [63]. In the T7 DNA polymerase complex, a sharp kink in the DNA template exposes the template base that is to be paired with the incoming nucleotide. More than 90% of the surface of the base pair is buried from solvent (Figure 1b) and the polymerase makes extensive contacts with the bases in the active site using nonpolar, van der Waals' interactions. These steric and electrostatic constraints of the polymerase active site neatly and selectively specify the shape and hydrogen-bonding arrangement of normal Watson–Crick base pairs. Is hydrogen bonding important? The Klenow fragment polymerase selectively incorporates nucleosides with nonpolar bases that have little or no hydrogen-bonding capacity [16••]. The nonpolar thymine analog difluorotoluene (compound F) is selectively incorporated opposite adenine in the template [16••], or opposite the adenine analog 4-methylbenzimidazole (compound Z) [64••]. Likewise, the nucleoside triphosphate of Z is selectively incorporated opposite thymine or compound F. The Klenow fragment utilizes the nonpolar nucleosides much less efficiently than their natural counterparts, however, presumably reflecting the cost of forgoing Watson–Crick hydrogen bonding. Even so, much of the selectivity of DNA polymerization is maintained with little hydrogen bonding between nucleotide and DNA template, emphasizing the importance of substrate shape in polymerase fidelity.

Selection for deoxyribonucleoside triphosphates

Most DNA polymerases strongly reject nucleotides with modifications of the 2′-deoxyribose sugar, such as ribonucleotide triphosphates (rNTPs) or ddNTPs. Although rNTPs are 10-fold more abundant than dNTPs in the cell [21], they are effectively excluded from the genome by replicative DNA polymerases. In the T7 polymerase complex, the C2′-position of the nucleotide sugar is wedged between the aromatic ring of Tyr526 and the aliphatic carbons of the Glu480 sidechain (Figure 4). This small hydrophobic pocket in the nucleotide-binding site does not accommodate the 2′-hydroxyl of rNTPs. The analogous glutamic acid of the Klenow fragment (Glu710) acts as a 'steric gate' that excludes rNTPs [14•].

Substituting an alanine for Glu710 opens the gate and reduces the $K_{m(rNTP)}$. Chain-terminating ddNTPs lacking a 3′-hydroxyl are also incorporated poorly by most DNA polymerases, with one exception being T7 DNA polymerase. A single residue in T7 polymerase, Tyr526 in the O helix, accounts for its leniency towards ddNTPs [12]. Substitution of the phenylalanine at the corresponding positions in Taq DNA polymerase and the Klenow fragment with a tyrosine makes these enzymes tolerate ddNTPs as well [12,13•]. Conversely, phenylalanine substitution in the T7 enzyme boosts its discrimination against ddNTPs several 1000-fold. The structure of T7 DNA polymerase complexed to DNA and a normal substrate dGTP (S Doublié, T Ellenberger, unpublished data) shows that the 3′-hydroxyl of the incoming nucleotide and the hydroxyl of Tyr526 are both in a position to donate a hydrogen bond to the pro-S_p oxygen of the β phosphate (Figure 2). One or both of these interactions with the β phosphate are apparently required for efficient polymerase activity. Enzymes lacking the tyrosine hydroxyl require a nucleotide with a 3′-hydroxyl for efficient incorporation.

Minor-groove interactions and fidelity check

DNA polymerases occasionally make mistakes that are subject to editing by the 3′→5′ proofreading exonuclease activity of many polymerases. The rapid rate of DNA synthesis catalyzed by T7 polymerase (300 nucleotides s^{-1}) slows dramatically (to 0.01 s^{-1}) following the misincorporation of a nucleotide. The exonucleolytic reaction (2.3 s^{-1}) is then favored and the DNA shifts from the polymerase site to the 3′→5′ exonuclease site without dissociating [44,54]. The mechanism of DNA transfer between the polymerase and exonuclease sites is not known, but mutations that alter the structure of the exonuclease site shift the balance in favor of DNA binding to the polymerase site and against binding to the exonuclease site [65•,66]. How does the polymerase detect a misincorporated base and initiate the transfer of DNA to the exonuclease site? In crystal structures of T7 DNA polymerase, Taq DNA polymerase, DNA polymerase β, Bst DNA polymerase and HIV-1 RT complexed to DNA, the DNA primer–template near the polymerase active site has an A-form structure with a widened minor groove (reviewed by Brautigam and Steitz [10]). These enzymes make sequence-independent contacts to the widened minor groove of the DNA immediately downstream of the polymerase active site. A sheared base pair, caused by a primer–template mismatch, would compromise these interactions with the floor of the polymerase, perhaps triggering the movement of the DNA to the 3′→5′ exonuclease site. In the T7 polymerase complex, the strictly conserved residues Arg429 and Gln615 contact the minor groove of the base pair at the 3′-end of the primer. Alanine substitutions at these positions in the Klenow fragment decrease its affinity for primer–template DNA and reduce the k_{cat} of DNA synthesis [47,67]. Arg429 of T7 polymerase also contacts O4′ of the incoming nucleotide. The truncation of this sidechain in the corresponding alanine mutant of the Klenow fragment decreases the fidelity of nucleotide insertion and reduces the barrier for the extension of a mismatch [17•], consistent with the perturbation of the nucleotide-binding site. A total of four base pairs at the 3′-end of the template are contacted in the minor groove by the T7 polymerase and mismatches at any of these positions cause the polymerase to stall [54].

Processivity

Replicative DNA polymerases incorporate thousands of nucleotides during each encounter with a DNA template. Many of these replication enzymes are attached to a ring-shaped sliding clamp that encircles the DNA and tethers the polymerase onto the template [11]. In contrast, T7 DNA polymerase has adopted the host protein *E. coli* thioredoxin as its processivity factor [68]. Thioredoxin binds tightly to the polymerase [69] and stimulates the rate and processivity of DNA synthesis [68,70]. This interaction with thioredoxin orders a proteolytically sensitive loop, located at the tip of the thumb subdomain, between α helices H and H1. It is most likely this thioredoxin-binding loop plays a direct role in processivity. Although it does not contact DNA in the crystal structure (Figure 1), we hypothesize that this subdomain can swing towards the DNA exiting the polymerase. The addition of thioredoxin to the polymerase protects seven additional DNA base pairs from exonuclease digestion (S Tabor, CC Richardson, personal communication). Mutations affecting three lysines in the thioredoxin-binding loop (Lys300, Lys302 and Lys304) prevent phage growth, decrease the affinity of the polymerase for DNA and lower its processivity [71]. The addition of the thioredoxin-binding domain to the Klenow fragment confers a thioredoxin-dependent increase in its processivity [72•], suggesting that most of the processivity function is located within this compact domain. The thioredoxin-binding domain is not large enough to function as a *bona fide* sliding clamp that encircles the DNA. Instead, it might slide along the DNA, acting as an electrostatic tether that prevents the dissociation of the polymerase–DNA complex. Similar models have been proposed for UL42, the processivity factor of the herpes simplex virus DNA polymerase [73].

Conclusions

Crystal structures of the nonhomologous and structurally different enzymes T7 DNA polymerase, DNA polymerase β and HIV-1 RT have revealed similar, substrate-induced changes in the conformation of these proteins. Upon binding to DNA and nucleotide substrates, all three polymerases adopt a closed conformation that surrounds the nucleotide–template base pair and configures the polymerase active site for DNA synthesis. This nascent base pair lies in a narrow hydrophobic pocket that accommodates an undistorted Watson–Crick base pair and selects against mismatches. Conserved residues and two metals in the active sites of these polymerases align the incoming nucleotide for an in-line attack by the 3′-end of the primer and stabilize the charged transition state of nucleotidyl transfer. The enzyme must then open in order to allow the next template base and

its complementary nucleotide access to the polymerase active site for the next catalytic cycle.

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Residues in the active site of a Klenow fragment polymerase were examined for their effects on the efficiency of ddNTP incorporation, relative to normal dNTP substrates. Of the many mutant polymerases examined, only residue substitutions involving Phe762 selectively relieved discrimination against ddNTPs, primarily by increasing their rate of incorporation.

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