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Role of the catalytic metal during polymerization by DNA polymerase lambda

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Abstract

The incorporation of dNMPs into DNA by polymerases involves a phosphoryl transfer reaction hypothesized to require two divalent metal ions. Here we investigate this hypothesis using as a model human DNA polymerase λ (Pol λ), an enzyme suggested to be activated *in vivo* by manganese. We report the crystal structures of four complexes of human Pol λ . In a 1.9 Å structure of Pol λ containing a 3' OH and the non-hydrolyzable analog dUpnpp, a non-catalytic Na⁺ ion occupies the site for metal A and the ribose of the primer-terminal nucleotide is found in a conformation that positions the acceptor 3' OH out of line with the α -phosphate and the bridging oxygen of the pyrophosphate leaving group. Soaking this crystal in MnCl₂ yielded a 2.0 Å structure with Mn²⁺ occupying the site for metal A. In the presence of Mn²⁺, the conformation of the ribose is C3' endo and the 3'-oxygen is in line with the leaving oxygen, at a distance from the phosphorus atom of the α -phosphate (3.69 Å) consistent with and supporting a catalytic mechanism involving two divalent metal ions. Finally, soaking with MnCl₂ converted a pre-catalytic Pol λ /Na⁺ complex with unreacted dCTP in the active site into a product complex via catalysis in the crystal. These data provide pre- and post-transition state information and outline in a single crystal the pathway for the phosphoryl transfer reaction carried out by DNA polymerases.

INTRODUCTION

DNA polymerases are crucial for a large number of DNA transactions [1–3]. They are essential for genome replication, they perform gap-filling synthesis in several DNA repair pathways, they perform translesion DNA synthesis and they contribute to somatic hypermutation of immunoglobulin genes. These roles all depend on the simple reaction that they catalyze, the incorporation of a nucleoside monophosphate onto the 3' terminus of a DNA chain and the release of pyrophosphate. This reaction consists of a nucleophilic substitution in which the 3' oxygen of the primer-terminal nucleotide donates an electron pair to form a phosphorus-oxygen bond with the phosphorus of the α -phosphate of the incoming deoxynucleoside triphosphate. Concomitantly, the bond between this phosphorus atom and the oxygen bridging the α and β -phosphates is broken, resulting in the release of pyrophosphate. By analogy to the mechanism proposed for a 3' exonuclease reaction [4], a mechanism for this reaction was proposed in 1993 [5] and remains current today. DNA polymerases were proposed to catalyze the reaction with the help of two divalent metal ions, which *in vivo* are generally believed to be Mg²⁺, but

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sometimes may possibly be Mn^{2+} [6]. One metal (A, or catalytic) is suggested to serve a catalytic role by lowering the pK_A of the 3'-OH at the primer terminus and/or by positioning it in a proper geometry for catalysis. The other metal (B, or dNTP-binding) coordinates the triphosphate moiety of the incoming dNTP, facilitating dNTP binding and subsequent release of pyrophosphate.

This two metal ion model is consistent with the stereochemistry of the polymerization reaction [7] and with a number of structural studies of DNA polymerases. Several groups have reported DNA polymerase crystal structures with metal ions present. However, in some cases only one metal is observed, in other cases the metals are not physiological (Ca^{2+}), and in still other cases the identity of the metal is uncertain. Moreover, the geometry at the active site is not usually consistent with an in line nucleophilic attack by the 3'-oxygen. In fact, the 3'-oxygen is often missing because a favorite method to trap pre-catalytic complexes of DNA polymerases is to employ a dideoxy-terminated primer (i.e., missing the 3'-OH). The exception is a recent study by Wilson and colleagues [8] describing the structure of a pre-catalytic complex of DNA polymerase β bound to gapped DNA, with a non-hydrolyzable nucleotide analog and two divalent metal ions bound at the active site. This structure (discussed further below), and the present study of DNA polymerase λ , strongly support the proposal that two divalent metal ions are required for catalysis.

Like Pol β , Pol λ is a family X polymerase that fills short gaps during DNA repair [9]. Pol λ and Pol β have similar properties, although Pol λ appears to display a larger flexibility with respect to the substrates on which it can polymerize [10]. This flexibility was first suggested by the observation that Pol λ generates deletion errors at an extremely high rate [11], and is now thought to be a feature of the enzyme that facilitates its role *in vivo*. The phenotypes of mice deficient in this polymerase indicate that Pol λ plays a role in the V(D)J process of antigen gene diversification [12], and several reports implicate Pol λ in Base Excision Repair [13–15] and the repair of double-strand breaks through the Non-Homologous DNA End-Joining pathway [16–18]. It is thus interesting to examine the details of the polymerization reaction as catalyzed by Pol λ in order to understand whether specific catalytic features can account for these special properties.

The structure of the catalytic core of human Pol λ has been extensively characterized [9,10, 19–22]. Although structurally similar to Pol β , Pol λ displays some unique structural differences. For instance, upon dNTP binding, Pol β undergoes a large scale conformational change in which its thumb subdomain rotates by about 25° degrees to assemble a catalytic conformation. By contrast, comparison of pre- and post-catalytic complexes of Pol λ suggests that no such subdomain motion takes place throughout the Pol λ catalytic cycle [21]. Instead, a catalytic conformation is achieved through the movement of the DNA template strand and the side chains of a few active site residues (see Fig. 2A in [21]). Moreover, several structures of Pol λ bound to misaligned and/or mismatched substrates illustrate the substrate flexibility of the enzyme and have identified some of the structural elements that are thought to facilitate this flexibility [10,22].

A number of structures of Pol λ bound to a normal DNA duplex have provided insights into the reaction mechanism. However, to date no pre-catalytic structure of Pol λ containing all the atoms involved in the reaction has been obtained, and the role, or even the presence, of the catalytic metal ion has been uncertain [21]. Here, we provide additional structures of Pol λ that include the essential catalytic atoms. These structures extend the characterization of the Pol λ reaction mechanism and strongly support the catalytic relevance of previous structures.

MATERIALS AND METHODS

Proteins and nucleotides

The human Pol λ catalytic core with a C543A mutation to eliminate intermolecular disulfide bond formation was expressed and purified as described [21]. Oligonucleotides Td (5'-CGGCAGTACTG), Pd (5'-CAGTAC) and DT (5'-GCCG), for the dUpnpp structures and Tc (5'-CGGCGGTACTG), Pc (5'-CAGTAC) and DT, for the dCTP structures were from Oligos Etc. dCTP was from GE Healthcare and dUpnpp was from Jena Bioscience.

Protein crystallization and structure determination

Crystals were grown essentially as described [21]. Briefly, crystals were formed using the hanging drop method by mixing 2 μ l of the protein solution containing DNA and the appropriate nucleotide (10 mM) with 2 μ l of reservoir solution containing 10–20% 2-propanol, 0.2 M sodium citrate and 0.1M sodium cacodylate pH 5.5. The protein solution contains 100 mM NaCl and 1 mM $MgCl_2$. The crystals that did not require soaking were transferred in five steps to a solution containing 19% 2-propanol (dCTP) or 21% (dUpnpp), 0.2 M sodium citrate, 0.1M sodium cacodylate pH 5.5, 100 mM NaCl, 1mM $MgCl_2$ and 25% (w/v) ethylene glycol. Data sets for crystals containing Mn^{2+} ions were made possible by soaking crystals for one hour in a solution containing 19% (dCTP soak) or 21 % (dUpnpp soak) 2-propanol, 0.1 M sodium cacodylate pH 5.5, 0.2 M sodium citrate and 200 mM $MnCl_2$. Subsequently, they were soaked three times for 45' into a solution containing 19% (dCTP soak) or 21 % (dUpnpp soak) 2-propanol, 0.1 M sodium cacodylate pH 5.5, 1 mM sodium pyrophosphate, 300 mM NaCl and 20 mM $MnCl_2$. After soaking, the crystals were transferred in five steps to a solution containing 0.1 M sodium cacodylate pH 5.5, 0.3 M NaCl, 20 mM $MnCl_2$, 1 mM sodium pyrophosphate, 19% (dCTP soak) or 21% (dUpnpp) 2-propanol and 25% (w/v) ethylene glycol. All crystals were frozen in liquid nitrogen and then mounted on a goniometer in a cold stream of nitrogen at $-178^\circ C$ for data collection. Data was collected on a Rigaku 007HF generator equipped with Varimax HF mirrors and a Saturn 92 detector. All data were processed using the HKL2000 software [23].

Molecular replacement and refinement

Phases were calculated using molecular replacement from PDB entries 1XSP or 1XSN as a starting models. The programs O [24] and Coot [25] were used for model building and the models were refined with CNS [26]. In all cases the density was of sufficient quality to build most side chains and all backbone atoms for all but a few N-terminal residues. The quality of models was assessed with Molprobity [27] and all were found to have good stereochemistry (see Table I). For the dCTP-soak structure, two alternate conformations were necessary to model the density in the region surrounding the active site. These conformations were first modeled to fit the density and then lightly restrained to correspond to the pre- and post-catalytic conformations deduced from other structures. Figures were made using Molscript [28], Povscript+ [29] and POV-Ray (www.povray.org).

RESULTS

Two metal ions in the Pol λ active site

Previous structures of Pol λ contained only one of the two proposed divalent metal ions in the active site [21]. While the dNTP-binding metal was present, no density could be observed that would account for a second metal in the catalytic metal binding site. It was hypothesized that the absence of the catalytic metal was either due to the absence of the 3'-oxygen that would complete the coordination sphere or to the fact that the catalytic metal is only transiently bound during the transition state. Alternatively, the Pol λ polymerization mechanism could employ

only one divalent metal to coordinate the incoming triphosphate. To investigate the nature of metal usage during the Pol λ catalytic cycle, we crystallized Pol λ in complex with a non-hydrolyzable nucleotide analog, dUpnpp, and solved the structure to 1.90 Å resolution. As expected, this structure overlays well with the previous pre-catalytic and post-catalytic ternary complexes (PDB 1XSN and 1XSP; rmsd of 0.459 Å for 324 C- α atoms and rmsd of 0.586 Å for 322 C- α atoms, respectively). Interestingly, density was observed for an ion at the catalytic metal binding site (see Fig. 1). Because both ions possess the same number of electrons, it is not possible to distinguish between Na⁺ and Mg²⁺ on the basis of electron density. The observed density could thus correspond to either ion or to a mixture of both (i.e., each present in a subset of the molecules in the crystal). However, the coordination distances are slightly larger than expected for Mg²⁺ (2.1–2.5 Å), and the coordination sphere lacks the octahedral geometry usually observed for Mg²⁺. For these reasons, we assigned this metal as a Na⁺ ion. Interestingly, the 3'-oxygen of the primer terminal nucleotide was not in a conformation compatible with catalysis. This is unlike what would be expected from the overlay of previous structures ([21]; see Fig. 1). The 3'-oxygen is located 4.66 Å away from the phosphorus of the α -phosphate and it is not in line with this atom and the leaving oxygen.

Mn²⁺ as a metal activator

Previous reports with Pol β suggest that Na⁺ ions could out compete the Mg²⁺ ion for the catalytic metal binding site [8]. When the above Pol λ crystals were soaked in a solution containing a higher MgCl₂ concentration, no difference was observed in the structure as a result of the soak (not shown). However, it has been suggested [6] that Pol λ might be activated by Mn²⁺ rather than Mg²⁺ *in vivo*. Indeed, Mn²⁺ is generally believed to bind to DNA polymerase-DNA complexes more tightly than Mg²⁺ [30,31], and Mn²⁺ activation can result in higher catalytic efficiencies [6,32]. Thus, we soaked the Pol λ -dUpnpp crystals in a solution containing Mn²⁺ ions (see Materials and Methods). The resulting crystal diffracted to 2.00 Å. After solving the structure, we could clearly observe density corresponding to a metal ion in the catalytic metal binding site (see Fig. 2) whose coordination sphere and coordination distances were consistent with Mn²⁺. Binding of this Mn²⁺ resulted in a change in the conformation of the ribose of the primer-terminal nucleotide from C2' endo to C3' endo, such that the 3'-oxygen was located in line with the leaving oxygen and at a distance from the phosphorus atom of the α -phosphate (3.69 Å) consistent with catalysis. From a crystallographic point of view, Mn²⁺ ions have the advantage that, when present in the crystal lattice, they act as anomalous scatterers. Thus, the presence of Mn²⁺ at the site for metal A could be unambiguously assessed (see Fig. 2). No other Mn²⁺ binding site could be identified in the structure (data not shown). Interestingly, no trace of Mn²⁺ was observed in the metal B binding site, indicating that, at least while the dNTP is bound to the enzyme, the dNTP-bound Mg²⁺ ion does not exchange with the metal in solution, or does so extremely slowly.

Na⁺ ions can inhibit catalysis

The structural analysis described above suggests that an excess of Na⁺ ions can inhibit catalysis by competing for binding with the catalytic metal. This is consistent with kinetic observations suggesting that high NaCl concentrations can inhibit the reaction [33], although an alternative explanation is that a solvent with a higher ionic strength can decrease binding of the protein to DNA. To test whether Na⁺ can indeed compete with the metal activator and inhibit the reaction, we set up crystallization trials of Pol λ in complex with a single-nucleotide gap and correct incoming dCTP in the presence of a high concentration of Na⁺ ions. The crystals obtained diffracted to 2.1 Å. Consistent with Na⁺ inhibition of the polymerization reaction, the incoming dCTP was unreacted and the ribose and 3'-oxygen were in conformations similar to those seen in the dUpnpp structure before soaking (see Figure 3A). In addition, the distance from the 3'-oxygen to the phosphorus of the α -phosphate was also similar to that observed before (4.85 Å), confirming that this conformation is catalytically inactive. Overall, this structure is

remarkably similar to a Pol λ pre-catalytic structure lacking the 3'-oxygen (rmsd of 0.419 Å for 322 C- α atoms; PDB 1XSN).

Catalysis in the crystal upon soaking with Mn²⁺ ions

Soaking of the dUpnpp crystals with Mn²⁺ ions was sufficient to stimulate binding of the catalytic metal and induce a conformational change of the ribose of the primer terminus toward an active conformation. Thus, soaking the dCTP/Na⁺ crystals with the same solution should stimulate catalysis. To test this, we performed a similar soak and solved the resulting structure to 2.1 Å resolution. A direct comparison of the data sets obtained before and after soaking indicates that the only differences between the two data sets are consistent with incorporation of dCTP into DNA within the crystal (Fig. 3B). However, the density suggested that the reaction did not proceed to completion, and a conformation corresponding to both the reacted and unreacted states of the incoming dCTP could be fit to the density. Interestingly, an anomalous density map indicates that Mn²⁺ is present in both metal binding sites (Fig. 3B), indicating that, once pyrophosphate is released, the pyrophosphate-Mg²⁺ complex now readily exchanges with the Mn²⁺-pyrophosphate present in the soaking solution.

The Pol λ catalytic cycle

Since Pol λ does not undergo a large scale protein conformational change, pre- and post-catalytic structures define the polymerization reaction [21]. Before this report, no pre-catalytic complex of Pol λ existed with all atoms involved in the reaction present. Our complex with dUpnpp fills this void and provides a clear depiction of the polymerization reaction (Fig. 4). With the catalytic metal present, the conformation of the active site is identical before and after catalysis, except for a small shift in the position of the phosphorus of the α -phosphate and the breaking and making of a new phosphorus-oxygen bond. The 3'-oxygen is located 5.28 Å away from the bridging oxygen (the nitrogen atom in dUpnpp) and 3.69 Å from the phosphorus of the α -phosphate, and it is clearly in line with the phosphorus of the α -phosphate and the bridging oxygen (see Fig. 4). As expected [7,21], the polymerization reaction results in an inversion in the stereochemical configuration of the phosphorus of the α -phosphate, strongly supporting an in line displacement mechanism.

DISCUSSION

The Pol λ catalytic pathway was previously defined by an overlay between a pre-catalytic complex lacking the 3'-oxygen and a post-catalytic complex containing pyrophosphate. We now report a pre-catalytic structure of Pol λ that contains all the atoms needed for catalysis. The new structural data confirm the catalytic pathway predicted by earlier structures and stress the important role of the catalytic metal in inducing the ribose of the primer-terminal nucleotide to achieve an active conformation. The present structures are consistent with the depiction of the reaction mechanism described in Batra et al. [8] for Pol β , an enzyme of the same family. In fact, an overlay of the Pol λ and Pol β complexes with dUpnpp (Fig. 5) suggests that the active site conformations of Pol β and Pol λ are virtually indistinguishable. This is remarkable, given the difference in the properties of Pol λ and Pol β and the fact that, unlike Pol β , Pol λ does not appear to undergo a large scale protein conformational change before and after catalysis [21]. Besides strongly reinforcing the proposed catalytic pathway, the similarity between the Pol λ and Pol β active sites indicates that even though the conformational changes that assemble the active site might not be conserved, catalysis by these two Family X polymerases is very similar.

The ability of Pol λ crystals to conduct catalysis upon soaking with the metal activator validates the catalytic relevance of the present structures and the proposed model for catalysis. The soaked crystals simultaneously capture pre- and post-catalytic information, revealing the in-

line displacement mechanism. The fact that catalysis can take place despite a limited soaking time and the low temperature at which the crystals are kept strongly argues in favor of a model where the crucial step for catalysis is defining proper active site geometry. In addition, it confirms that catalysis for Pol λ does not require a large scale protein conformational change.

The present study clearly demonstrates that polymerization by Pol λ requires two metal ions and, together with previous data, suggests that the catalytic metal may only be bound transiently. Moreover, it presents the clearest depiction to date of the polymerization reaction by Pol λ , clearly defining all the atoms involved in catalysis in the pre- and post-catalytic states. Our results confirm that Mn^{2+} can act as a metal activator *in vitro*. Even though the identity of the metal activator *in vivo* is still uncertain, this is consistent with previous reports indicating that polymerases can bind Mn^{2+} with higher affinity than Mg^{2+} . In this respect, the results are consistent with the suggestion by others that Mn^{2+} may activate Pol λ *in vivo* [6]. However, it is also possible that the preference for Mn^{2+} might be due in this case to the existence of an imperceptible distortion in the active site due to the presence of dUpnp (34).

This study completes the view of the polymerization reaction catalyzed by Pol λ with a correct substrate, and it provides a starting point to investigate catalytic differences for polymerization using other substrates, e.g., those encountered during BER or NHEJ, or those that would result in base substitutions, additions or deletions. The present structures also provide information needed to develop simulations to probe the nature of the transition state and further enhance our understanding of the polymerization reaction.

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Figure 1. Structure of Pol λ in complex with dUpnpp

Stereo view of the Pol λ active site. A Mg^{2+} ion occupies the metal B binding site (green), but a non-catalytic Na^{+} ion is present in the metal A binding site (light blue). As a result, the 3'-OH (black arrow) is located far from the phosphorus of the α -phosphate (see yellow dotted line) and is not in line with this atom and the bridging oxygen (nitrogen in dUpnpp) of the pyrophosphate leaving group. A simulated annealing Fobs-Fcalc omit map is shown (blue) contoured at 4σ . The three catalytic aspartates are shown in gray.

Figure 2. Soaking with Mn²⁺ induces a catalytic conformation

Stereo view of the Pol λ active site. A Mn²⁺ ion (magenta) now occupies the metal A binding site. The 3'-OH is now at a catalytically relevant distance (see yellow dotted line) from the phosphorus of the α-phosphate and both of these atoms are in line with the bridging oxygen (nitrogen in the analog) of the pyrophosphate leaving group. A simulated annealing F_{obs}-F_{calc} omit map contoured at 4σ is shown in blue and an anomalous difference density map contoured at 5σ is shown in magenta. The three catalytic aspartates are shown in gray.

Figure 3. Metal-activated catalysis

A. Pre-catalytic complex of Pol λ with dCTP. Under these crystallization conditions, Na⁺ occupies the metal A binding site (light blue), resulting in a non-catalytic conformation. As a result, the substrate (yellow) is unreacted. A simulated annealing Fobs-Fcalc omit map contoured at 4σ is shown in blue. **B.** The crystals shown in (A) were soaked in the presence of Mn²⁺ atoms. To clearly observe the effects of the soak, a Fobs(pre)-Fobs(post) difference density map was calculated. This map only expresses the differences between the two datasets (pre- and post-soak). It is contoured at 5σ (light blue) and at -5σ (yellow). Thus, yellow indicates electron density appearing upon soaking the crystal, while light blue indicates electron density that disappeared in response to the soak. Superimposed to the Fobs(pre)-Fobs(post) difference density map is an anomalous difference density map contoured at 8σ (red). This map indicates the position of the Mn²⁺ atoms after the soak.

Figure 4. The inline displacement reaction

An overlay of the Pol λ precatalytic complex with dUpnpp and a post-catalytic complex (PDB 1XSP) reveals the details of the inline displacement reaction. The polymerization reaction results in an inversion in the stereochemical configuration of the phosphorus atom of the α-phosphate group. The line of transfer is shown as a yellow dotted line.

Figure 5. Overlay of the Pol β and Pol λ complexes with dUpnpp

The superimposition is centered on the active site (19 C α -atoms, Pol λ residues 413 to 431, rmsd 0.251Å). The active site of Pol λ is shown and the equivalent atoms from the Pol β structure are shown transparent in the same colour.

Table I

Summary of crystallographic data

Data Set	dUpnpp	dUpnpp (soak)	dCTP	dCTP (soak)
PDB ID				
Unit cell dimensions (a x b x c)	55.08 x 61.56 x	56.09 x 61.41 x	55.89 x 62.85 x	56.09 x 63.48 x
Space group	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁
Number of observations	177784	179719	97319	100103
Unique reflections	37287	33210	29762	26491
R _{sym} (%) (last shell) ^a	8.8 (58.7)	5.8 (41.3)	11 (31.6)	5.9 (44.4)
I/σI (last shell)	15.0 (2.3)	16.6 (1.7)	10.3 (2.4)	20.8 (2.3)
Completeness (%) (last shell)	97.6 (94.3)	98.9 (94.0)	99.5 (96.4)	88.2 (72.6)
Refinement Statistics				
Resolution (Å)	1.9	2.0	2.1	2.1
R _{cryst} (%) ^b	21.2	21.9	20.9	25.7
R _{free} (%) ^c	23.2	24.4	24.6	28.9
Number of complexes in asymmetric unit	1	1	1	1
Mean B value (Å)	40.6	38.3	41.6	47.8
RMS deviation from ideal values				
Bond length (Å)	0.005	0.005	0.005	0.017
Bond angle (°)	1.1	1.1	1.1	1.2
Dihedral angle (°)	22.0	21.8	21.9	22.3
Improper angle (°)	0.8	0.8	0.9	2.0
Ramachandran statistics[27]				
Residues in:				
Favored regions	97.2%	95.9%	98.1%	92.3%
Disallowed regions	0%	0%	0%	0.31%

^aR_{sym} = $\Sigma(|I_i - \langle I \rangle|) / \Sigma(I_i)$, where I_i is the intensity of the ith observation and $\langle I \rangle$ is the mean intensity of the reflection.

^bR_{cryst} = $\Sigma||F_o| - |F_c|| / \Sigma|F_o|$, calculated from working dataset.

^cR_{free} is calculated from 5% of data randomly chosen in each case not to be included in refinement.