FOR THE RECORD

Bidentate and tridentate metal-ion coordination states within ternary complexes of RB69 DNA polymerase

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Abstract: Two divalent metal ions are required for primer-extension catalyzed by DNA polymerases. One metal ion brings the 3′-hydroxyl of the primer terminus and the α-phosphorus atom of incoming dNTP together for bond formation so that the catalytically relevant conformation of the triphosphate tail of the dNTP is in an α,β,γ-tridentate coordination complex with the second metal ion required for proper substrate alignment. A probable base selectivity mechanism derived from structural studies on Dpo4 suggests that the inability of mispaired dNTPs to form a substrate-aligned, tridentate coordination complex could effectively cause the mispaired dNTPs to be rejected before catalysis. Nevertheless, we found that mispaired dNTPs can actually form a properly aligned tridentate coordination complex. However, complementary dNTPs occasionally form misaligned complexes with mutant RB69 DNA polymerases (RB69pols) that are not in a tridentate coordination state. Here, we report finding a β,γ-bidentate coordination complex that contained the complementary dUpNpp opposite dA in the structure of a ternary complex formed by the wild type RB69pol at 1.88 Å resolution. Our observations suggest that several distinct metal-ion coordination states can exist at the ground state in the polymerase active site and that base selectivity is unlikely to be based on metal-ion coordination alone.

Keywords: nucleotidyl transfer; base selectivity; base discrimination; DNA polymerases; bidentate coordination state; tridentate coordination state; divalent metal-ion coordination; closed preinsertion ternary complex

Introduction

Replicative bacteriophage RB69 DNA polymerase (RB69pol) and many other replicative polymerases (pols) exhibit an extraordinary high degree of base selectivity during nucleotide incorporation, whereas lesion bypass pols such as Dpo4 exhibit reduced base selectivity.[1,2] To understand the structural basis for base selectivity of a pol, two closely related structures of replication ternary complexes

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of a pol need to be determined for pairwise comparison, one containing a complementary incoming dNTP and the other containing a noncomplementary dNTP. This could potentially reveal distinct geometric features attributable to specific base-pairing differences. A comparison of this type has been carried out with Dpo4 where it was found that the triphosphate tail of the complementary incoming dNTP adopts a "chair-like" conformation and forms a substrate-aligned, α,β,γ-tridentate coordination complex.[3] However, the triphosphate tail of noncomplementary dNTPs in Dpo4 complexes adopt a "goat tail-like" conformation that differs from the α,β,γ-tridentate coordination.[3] These complexes contained dT/dGTP or modified templating base adducts such as cyclobutane pyrimidine dimers or benzo[α]pyrene diol epoxides. The differences in substrate alignment between complementary and noncomplementary dNTPs appear to be consistent with the notion that mispaired dNTPs should somehow inhibit chemistry. Thus, Yang and coworkers proposed that a mismatched nascent base pair would lead to poor alignment of the metal ions, and as a consequence, inhibit nucleotide incorporation.[3] In pol β and the Bacillus polymerase fragment (Bst pol), noncomplementary incoming dNTPs failed to induce the fully closed conformation,[4,5] which reduces the rate of nucleotidyl transfer and supports an induced-fit hypothesis.[6,7] However, with RB69pol, noncomplementary incoming dNTPs can form a fully closed conformation but with only subtle differences in the polymerase active site.[8]

In the tridentate coordination state of pol's ternary complexes, one of the two divalent metal ions is responsible for bringing the α-phosphorus atom (Pα) of the incoming dNTP's triphosphate tail close enough to the 3′-hydroxyl group of the primer terminus (*pt*O3′) for nucleotidyl transfer, whereas the second metal ion simultaneously coordinates three oxygen atoms of the triphosphate tail and assists in the departure of pyrophosphate during catalysis.[9] These features are conserved in all pols of known structure, including Dpo4, pol β, Bst pol, and RB69pol.[9] When the triphosphate tail is not in the tridentate state, Pα of the incoming dNTPs is not optimally coordinated with the divalent metal ion in the B site so that any altered coordination states are not likely to be as catalytically relevant as the α,β,γ-tridentate state, or may be off the main reaction pathway.[3,9] However, if bidentate coordination states were on the kinetic pathway, interconversion between bidentate and tridentate states would be necessary before the incoming dNTP could proceed to the transition state for nucleotidyl transfer. For complementary incoming dNTPs, the tridentate state is likely to be more stable than other coordination states and there is a low-energy barrier for interconversion. Thus, the triphosphate tail of complementary incoming dNTPs prefers to adopt a more catalytically competent, α,β,γ-tridentate state. In contrast, for noncomplementary dNTPs, a bidentate state might be more stable. If conversion from the more stable bidentate to the catalytically relevant tridentate state has a higher energy barrier than the barrier for release of noncomplementary dNTPs, noncomplementary dNTPs will likely be rejected. Although these coordination differences have been proposed as a possible nucleotide selection mechanism,[3] direct evidence supporting it is lacking, in part because the ternary complexes of replicative pols containing noncomplementary incoming dNTPs are often too unstable to be captured and crystallized for structural studies.

We have engineered several Nascent Basepair-binding Pocket (NBP) mutants of RB69pol that have enabled us to capture ternary complexes containing nearly all noncomplementary incoming dNTPs in crystals and to determine their structures.[8,10] With these NBP mutants, we found that the triphosphate tail of both correct and incorrect incoming dNTPs adopt the same tridentate coordination state,[8] suggesting that the coordination differences for nucleotide selection developed for Dpo4 is not likely to be applicable to these NBP mutant RB69pols. For example, we found that the triphosphate tail of dUpNpp (a nonhydrolyzable analog of dUTP), when opposite dA, is in a β,γ-bidentate coordination state with Mg^{2+} in ternary complexes with an RB69pol triple mutant (tm) (L561A, S565G, and Y567A).[9] In that structure, *pt*O3′ forms a hydrogen bond with the α-phosphate of dUpNpp.[9] Only one Mg^{2+} is bound near the B metal ion site.[9] This β,γ-bidentate complex was consistently observed in duplicate structure determinations under the same experimental conditions. When Mg^{2+} was replaced with Mn^{2+}, only the tridentate coordination state was seen (pdb accession 3SI6[9]). With the quadruple mutant (qm) (L415A, L561A, S565G, and Y567A), we once again observed the tridentate Mg^{2+} coordination state for dUpCpp (another nonhydrolyzable analog of dUTP, pdb accession 3SPY[9]) when paired opposite dA. These observations suggest that the relative stability between tridentate and bidentate coordination states has been shifted in these complexes. It is not likely that amino acid substitutions in these NBP mutants are responsible for the stability differences since both tridentate and bidentate states have been found with each of these NBP mutants. Here, we report a β,γ-bidentate coordination state in the crystal structure of the wild type (wt) RB69pol ternary complex containing a complementary incoming dUpNpp determined at 1.88-Å resolution and provide evidence that the bidentate state does not result from amino acid substitutions in the NBP mutants of RB69pol.

Table I. *Crystallographic Statistics for Data Collection and Structure Refinement*

Space group	$P2_12_12_1$
Unit cell dimensions [a, b, c (Å)]	75.24, 120.20, 130.21
Resolution range (Å)[a]	50.0–1.88 (1.96–1.88)
Number of reflections	
Unique	96,145
Redundancy	2.1 (2.1)
Completeness (%)	99.8 (99.5)
R_{merge} (%)[b]	7.7 (87.1)
I/σ	16.0 (1.2)
Refinement Statistics	
# of protein residues	903
# of Water molecules	552
# of Ca^{2+} ions	6
# of template nucleotides	18
# of primer nucleotides	13
# of dNpNpp molecules	1
# of reflections	91,050
R_{o2p}[c]	2.64
R_{work} (%)[d]	18.8 (28.3)
R_{free} (%)[e]	21.9 (29.7)
r.m.s.d.[f]	
Bond length (Å)	0.008
Bond angle (°)	1.151
PDB access code	3UIQ

[a] The highest resolution shell statistics are in parenthesis.
[b] $R_{merge} = <\Sigma_{hkl}\ \Sigma_j\ |\boldsymbol{I}_j(hkl) - <\boldsymbol{I}(hkl)>|>/<\boldsymbol{I}(hkl)>$, merging statistics for all symmetry-mates.
[c] The observation-to-parameter ratio, where the number of parameter is four times the number of atoms in the final model.
[d] $R_{work} = \Sigma_{hkl}\ |F_{obs}(hkl) - F_{calc}(hkl)|\ /\Sigma_{hkl}\ |F_{obs}(hkl)$, crystallographic R-factor.
[e] R_{free}, Cross-validation R-factor for ~5% of the total unique reflections that have been randomly selected.
[f] r.m.s.d.: root-mean-square deviation from ideal values.

Results and Discussion

The structure of the preinsertion ternary complex of wt RB69pol containing complementary dUpNpp has been refined at 1.88-Å resolution with R-factor and free R-factor of 19.0% and 22.0%, respectively (Table I). There are two Ca^{2+} bound near the A and B sites, interacting with the triphosphate tail of dUpNpp (Fig. 1). Each Ca^{2+} has about seven ligands with average coordination bond length of 2.50 ± 0.30 Å. Ca^{2+} bound near the B site is in a bidentate coordination complex with the β and γ phosphate groups of dUpNpp. Ca^{2+} bound near the A site interacts with the α-phosphate, but not with *pt*O3′. The CaA-CaB, CaA-*pt*O3′, *pt*O3′-Pα, and CaB-O2α distances are 3.33, 3.80, 3.74, and 4.10 Å, respectively. To convert the β,γ-bidentate to the α,β,γ-tridentate state (3SPY[9]), a number of torsion angles of dUpNpp have to be changed simultaneously according to the observed torsion angle differences between the two conformations, which ranges from 30° to 50° (Table II). How these changes take place cooperatively within the ternary complex depends on the free energy field within the pol active site, including contributions from the two divalent metal ions. These changes must also be accompanied by torsion rotations of protein side chains within the polymerase active site when the ground state proceeds to the transition state.[11–13]

The β,γ-bidentate complex was observed after cocrystallization of all the components rather than one component being replaced by soaking. This suggests that β,γ-bidentate coordination state is equal to or more stable than the α,β,γ-tridentate state under conditions where we have stalled nucleotidyl transfer, i.e., with Ca^{2+} and the use of the nonhydrolyzable dUpNpp analogs. The β,γ-bidentate complex observed previously, when we used the soaking-replacement methods,[9] might have been due to a kinetically trapped complex because the lattice interactions may have locked RB69pol in the closed conformation that would have restricted any large torsion motions during the replacement of Mg^{2+} for Ca^{2+}. Our current and previous results[9,14] suggest that the free energy landscape within the pol active site of wt RB69pol is not a simple uniform funnel. Multiple local minima may exist, particularly within chemically stalled ternary complexes, which might trap incoming dNTPs in a catalytically incompetent conformational state.

We have previously shown that RB69pol can form a closed ternary complex in the absence of Mg^{2+} in the A site.[15] If the β,γ-bidentate coordination state with only one bound metal ion represents a kinetic intermediate, the binding of Mg^{2+} to the A site, as a distinctive step, could be related to the bidentate-to-tridentate conversion within the ternary complex. Whether this conversion requires a transient reopening of the ternary complex remains to be determined but may now be addressable using computer simulation methods because high-resolution crystal structures for the start and end points are now available. All experimental methods designed to capture unstable intermediates have their own limitations because they may have altered the energy profile for the reaction. Based on our findings, we are cautious about interpreting results when using nucleotide analogs, a dideoxy-terminated primer, or catalytically inert divalent metal ions to stall nucleotidyl transfer, because they may have altered the free energy difference between the tridentate and bidentate states.[9] The most direct method to address the structural basis for base selectivity would be time-resolved crystallography after restarting a transiently stalled reaction in a crystal in synchrony.

The bidentate state observed in this study may have been underreported because the differences between bidentate and tridentate coordination states might be too small to be accurately resolved at medium resolutions. Having seen the bidentate state at 1.88-Å resolution, we have reexamined several of our unpublished lower-resolution structures and found a variety of bidentate coordination

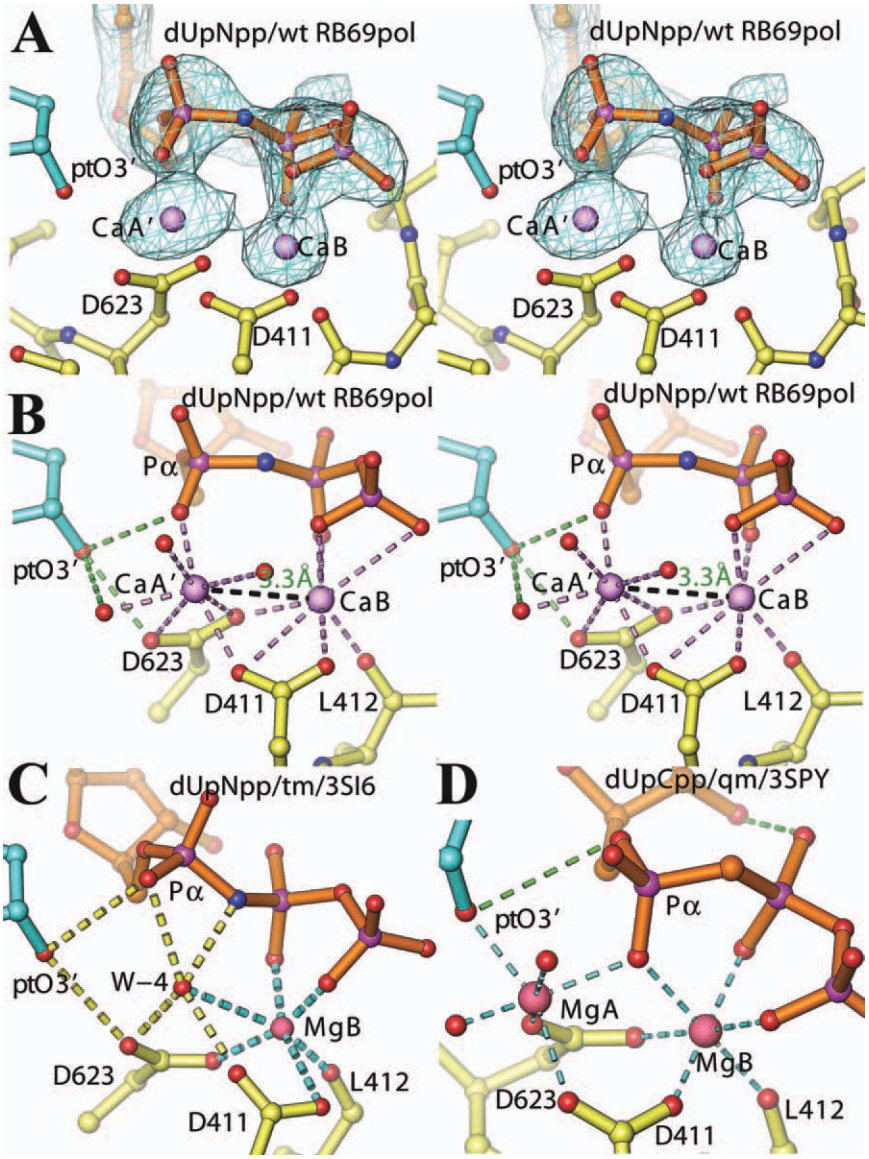

Figure 1. The structure of a β,γ-bidentate coordination complex formed by wt RB69pol. A: Stereodiagram of an omit Fo-Fc electron density map contoured at 5.2 σ at 1.88 Å resolution unambiguously shows that there are two Ca^{2+} (CaA′ and CaB) bound to the triphosphate tail of dUpNpp. At lower contoured levels, all coordination water molecules can be seen (not shown). B: Stereodiagram of the bidentate coordination complex. Each Ca^{2+} has 7 ligands. CaA′ does not bind to *pt*O3′, which could form three hydrogen bonds with D623, an ordered water molecule and/or with O2α of dUpNpp. C: A previous structure (3SI6) of the β,γ-bidentate coordination complex formed by tm RB69pol with only one Mg^{2+} bound. D: A previous structure (3SPY) of the classic tridentate coordination complex formed by qm RB69pol.

states with dNTPs that have been stalled in the ternary complex containing Ca^{2+}. Because the bidentate conformational states with complementary incoming dNTPs have been observed at much low frequency than the tridentate state, their possible role in the reaction pathway is not apparent. For the record, this study combined with those reported previously[9] has shown that bidentate coordination states with complementary incoming dNTPs within the closed ternary complexes are likely to be a common feature with all pols. Computer simulations must take into account this unusual observation when addressing nucleotide selection mechanisms.

Materials and Methods

Portions of the experimental details for this study have been described in a recent publication on structural studies of replication ternary complexes formed by wt, tm, and qm RB69pols containing dUpXpp opposite a complementary templating base.[9] In brief, the ternary complex was crystallized at pH 6.5 using poly (ethylene glycol) 350 monomethyl ether in the presence of 150 m*M* $CaCl_2$. X-ray diffraction data

Table II. *Torsion Angles and Their Differences Between a Tridendate and Bidentate Coordination States of dUpXpp in Degrees*[a]

Torsion angle definition	Bidentate coordination	Tridendate coordination	Differences
C3′-C4′-C5′-O5′	64.4	51.2	−13.2
C4′-C5′-O5′-Pα	−162.2	−151.7	+10.5
C5′-O5′-Pα-N3α	67.9	99.2	+31.3
O5′-Pα-X3α-Pβ	−21.4	−65.7	−44.3
Pα-X3α-Pβ-O3β	−183.4	−133.3	+50.1
X3α-Pβ-O3β-Pγ	63.0	56.8	−6.2
Pα-O3β-Pγ-O1γ	−13.5	18.8	+32.3

[a] Difference in the torsion angles of glycosidic bond between the Ca2+/dUpNpp wt and the Mg2+/dUpCpp qm RB69pol ternary complexes (3SPY) is −6.7° for a reference of possible error bar in the structure determination, where X3α is NH and CH2, respectively.

were collected at 110 K at beamline 24ID-E, the Northeast Collaborative Access Team (NECAT), Advanced Photon Source (APS), Argonne National Laboratory (ANL), Argonne, IL. Data were processed using HKL2000 (Table I).[16] The structure was determined using the automated molecular replacement method phaser and refined with Refmac.[17,18] We have unambiguously identified all bound Ca^{2+} in the structure based on outstanding features in the Fo-Fc difference densities and the number of coordination ligands often greater than 6. Refined coordinates and X-ray diffraction data are available in PDB under the accession number of 3UIQ (Table I).

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