DNA Polymerase *β*. 5. Dissecting the Functional Roles of the Two Metal Ions with Cr(III)dTTP[1]

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DNA polymerases catalyze the synthesis of DNA with a fidelity ranging from 1000 to 100 000.[2] Although the kinetic properties of DNA polymerases have been investigated extensively,[3−5] the physical origin of this high fidelity is still poorly understood. The crystal structures of DNA polymerase *β* (Pol *β*) from rat brain show the presence of two Mg^{2+} ions in the active site[6a,b,7] (Figure 1). Functionally, it has been established that DNA polymerases require divalent metal ions for catalysis.[8,9] However, no kinetic evidence that *two* divalent metal ions are required has been obtained for DNA polymerases, nor have the roles of the two metal ions been dissected functionally. We here report the use of Cr(III)dNTP coupled with stopped-flow fluorescence to show the requirement of two metal ions, and to dissect their functional roles in the catalysis by Pol *β* from rat brain (overexpressed in *Escherichia coli*).[5] Pol *β* can serve as a good model for nucleotidyl transfer reactions because of its small size (39 kDa) and simplicity (without exonuclease or proof-reading activities).

Using a synthetic DNA primer/template containing the 2-aminopurine nucleotide analogue (2-AP) (Figure 2A), we recently reported two phases of fluorescence changes in the stopped-flow fluorescence assay of nucleotide incorporation catalyzed by Pol *β* (Figure 2B).[10] The fast phase was attributed to a conformational change step because its rate dependence on [dTTP] is hyperbolic. The rate of the slow phase corresponds to product formation. With a dideoxynucleotide-terminated primer, we have successfully dissected the slow fluorescence change and the chemical step, and attributed the slow phase to a conformational change that is the rate-limiting step in the reaction.[10]

Cr(III)·nucleotide complexes have been extensively used as analogues of Mg(II)·nucleotide complexes to solve enzymological problems because of their exchange-inert properties.[11−13] When the Pol *β*·DNA complex was mixed with *β*,*γ*-bidentate Cr(III)-

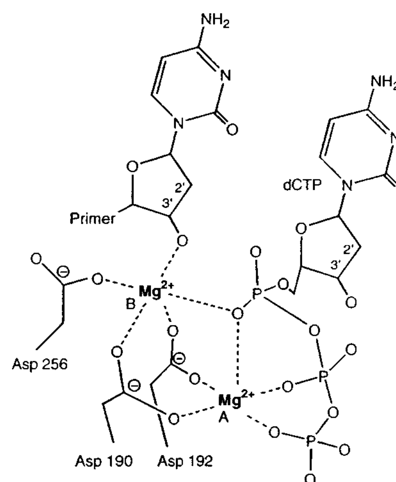
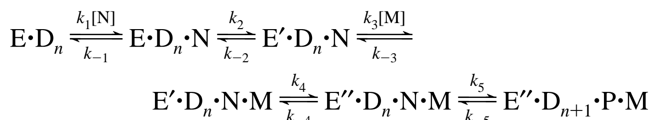


Figure 1. Schematic diagram of the active site of Pol *β* showing two metal ions, the nucleotide-binding ion (site A) and the catalytic ion (site B).[6]

dTTP[14] in the absence of Mg^{2+}, only the fast phase was observed (Figure 2C). However, the slow phase was restored upon addition of Mg^{2+} (Figure 2D). Under the latter condition the substrates were turned over, and the rate of the slow phase was comparable to the rate of product (21-mer) formation measured by the rapid chemical quench assay. In the absence of any metal ions (dTTP alone) neither fluorescence changes nor catalysis occurred. These results clearly indicate that the fast conformational change is induced by the binding of Cr(III)dTTP and the slow conformational change by the binding of Mg^{2+}. On the basis of the crystal structure shown in Figure 1, Cr^{3+} and Mg^{2+} should occupy the metal ion sites A and B, respectively.[6c]

The results described above, taken together with our pre-steady-state kinetic analyses reported previously, strongly support the mechanism we proposed for Pol *β*:[10]



$$E{\cdot}D_n \underset{k_{-1}}{\overset{k_1[N]}{\rightleftharpoons}} E{\cdot}D_n{\cdot}N \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E'{\cdot}D_n{\cdot}N \underset{k_{-3}}{\overset{k_3[M]}{\rightleftharpoons}} E'{\cdot}D_n{\cdot}N{\cdot}M \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E''{\cdot}D_n{\cdot}N{\cdot}M \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} E''{\cdot}D_{n+1}{\cdot}P{\cdot}M$$

where E, D, N, M, and P represent Pol *β*, DNA template/primer, MgdNTP, the second Mg^{2+} ion, and inorganic pyrophosphate, respectively. On the basis of this detailed mechanism, we interpret that the role of the first Mg^{2+} ion is to facilitate the dNTP to bind properly (step 1), which induces the first (fast) conformational change (step 2) associated with base-pairing. The first conformational change facilitates binding of the second Mg^{2+} ion (step 3), which in turn leads to the second (rate-limiting) conformational change (step 4) wherein the α-phosphate is closer to the 3′-OH of the primer in a geometry ready for the chemical step (step 5). This interpretation also supports the suggestion that the Mg^{2+} ion at site B is a catalytic metal ion.[6a,b]

Multiple conformational changes are most likely the strategy used by DNA polymerases to enhance their fidelity.[10] The base-pairing energy difference in the base-pairing step (associated with

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(1) This is paper 5 in the series DNA Polymerase *β*; for paper 4, see ref 10.
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(14) Cr(III)dTTP was prepared by mixing equal amounts of dTTP and $CrCl_3$ (aged or heated to replace Cl^- ligand with H_2O) in 5 mM MES buffer, pH 6.1, and incubating for 10 min.[12] HPLC analysis [Gruys, K. J.; Schuster, S. M. *Anal. Biochem.* **1982**, *125*, 66] indicated that the major product is *β*,*γ*-bidentate (mixture of four isomers). The mixed isomers were used directly since they interconvert to one another under the condition of the stopped-flow fluorescence experiments.

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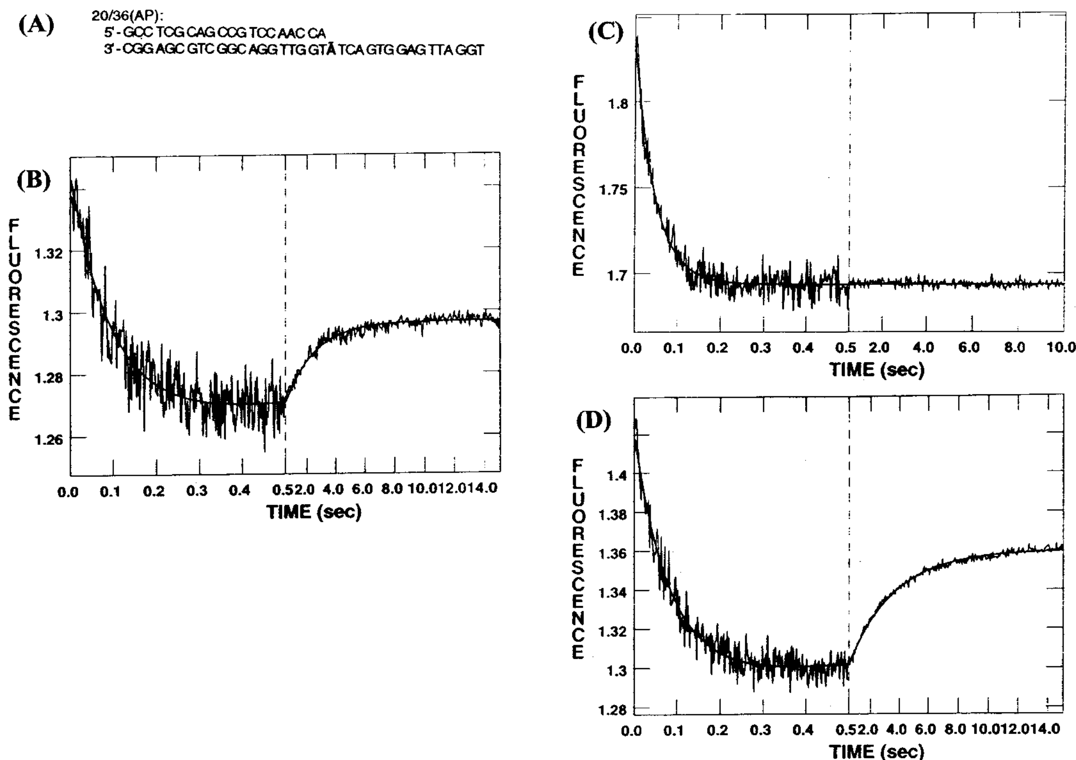

Figure 2. (A) Sequences of 20/36(AP) DNA substrates. **Ã** represents 2-aminopurine. (B–D) Stopped-flow fluorescence assays with MgdTTP (B), Cr(III)dTTP in the absence of Mg^{2+} (C), and Cr(III)dTTP in the presence of Mg^{2+} (D). In (B), syringe 1 contained 0.6 μM Pol β and 0.6 μM 20/36(AP) in buffer I (50 mM MOPS, 50 mM KCl, 10% glycerol, pH 7.0), and syringe 2 contained 200 μM dTTP and 2.2 mM Mg^{2+} in buffer II (5 mM MES, 50 mM KCl, 10% glycerol, pH 6.1). The reaction was initiated by mixing 80 μL solutions from each syringe at 20 °C, and the fluorescence signal was monitored during the reaction. Multiple experiments were collected and averaged (15–19 runs) to improve the signal-to-noise ratio. Two time windows were used to accurately measure the rates of both the fast and the slow phases. The data were fit to a double exponential to yield k_{obs} for both phases (10.0 ± 0.4 s^{-1}, 0.48 ± 0.04 s^{-1}). The condition for (C) was the same as that for (B), except that syringe 2 contained 200 μM Cr(III)dTTP instead of dTTP and Mg^{2+}, and the data were fit to a single exponential to give $k_{obs} = 21.0 \pm 0.5$ s^{-1}. The condition for (D) was the same as that for (C) except that 2 mM Mg^{2+} was added to syringe 1, and the data were fit to a double exponential to give $k_{obs} = 12.0 \pm 0.3$ and 0.32 ± 0.01 s^{-1}.

the fast conformational change) provides a selectivity in K_d in the range of 5–150 corresponding to 1–3 kcal/mol. The rate-limiting conformational change has been proposed to be the other (major) source of fidelity.[4,10] Since our results clearly demonstrate that one metal ion is essential for each of the two conformational change steps, we conclude that the two metal ions are important for the fidelity of Pol β. This can explain why the fidelity of DNA polymerases is sensitive to metal ions, and some metal ions are potential mutagens.[7,15]

In conclusion, we have successfully dissected the two conformational changes and the functional roles of the two metal ions in the mechanism of Pol β. The results have greatly enhanced our understanding of the catalytic mechanism and the origin of fidelity for Pol β in particular and DNA polymerases in general. In addition, the experimental approach used in this study can be used to further probe the mechanism of other polymerases.

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