APPLICATION OF CHEMICAL PROBES TO STUDY THE KINETIC MECHANISM OF DNA POLYMERASES

DISSERTATION

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Kinetic and structural basis of high-fidelity DNA replication by DNA polymerases has been the subject of extensive studies for several decades; however, it is still highly controversial. Presented here, a mechanistic analysis of DNA polymerases provides strong support to our hypothesis that chemistry is the rate-limiting step and, therefore, the main contributor to fidelity for DNA polymerase β (Polβ).

The substitution-inert Rh(III)dNTP was used as substrate analog in stopped-flow experiments to dissect two metal ions. Rh(III)dNTP binding to the polymerase-DNA binary complex results in the fast fluorescence change. At the same time, the slow fluorescence change occurs after mixing of Polβ-DNA-Rh(III)dNTP with Mg$^{2+}$. These results suggest that the subdomain-closing conformational change occurs before and the rate-limiting step occurs after binding of the catalytic Mg$^{2+}$. The results were further supported by a “sequential-mixing” stopped-flow experiment without use of analogs, which rules out the possibility that the discrepancy between experimental and computational results is due to the use of analogs.

pH dependence of the kinetic constants obtained in stopped-flow experiments demonstrates that pH selectively influences different steps in Polβ reaction pathway, which makes pH an additional variable in probing the polymerase mechanism.
Alteration of reaction buffer viscosity selectively perturbs conformational (rather than chemical) steps. At pH 7.0, increasing buffer viscosity selectively decreases the rate of the fast fluorescence transition, supporting our hypothesis that this phase reflects the subdomain-closing conformational change. By selectively increasing the rate of chemistry (high pH) and decreasing the rates of conformational steps (high viscosity) we created a situation in which the rapid quench rate of dNTP incorporation is faster than the rate of the second fluorescence change in stopped-flow. This dissection of the rates of chemistry and the slow fluorescence transition bolstered the hypothesis that the slow fluorescence change reflects the subdomain-reopening conformational step.

Application of the methodology used in our Polβ studies to Klenow Fragment (KF) revealed similarities in the kinetic mechanisms between the two DNA polymerases. KF resembles Polβ in that subdomain-closing is the only detectable conformational step before chemistry, but differs from Polβ in that subdomain-reopening in KF is considerably slower than phosphodiester bond formation.
Dedicated to my parents, Antonina and Mikhail
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CHAPTER 1

INTRODUCTION

1.1 DNA polymerase fidelity and the rate-limiting step in the course of single-nucleotide incorporation

Conservation of genetic information is of ultimate importance in all living organisms. DNA polymerases – enzymes that play a central role in DNA replication and repair – are required to be very accurate in order to maintain genome invariability. The measure of a DNA polymerase’s ability to accomplish accurate DNA synthesis is termed \textit{fidelity}.

Most DNA polymerases involved in DNA replication and repair are remarkable in their ability to effectively discriminate against incorrect nucleotides: incorrect nucleotide incorporation frequency is ranging from $10^{-3}$ to $10^{-6}$ \cite{1}. This ability is even more amazing since a DNA polymerase must choose a correct nucleotide from a pool of four structurally similar nucleotides as the identity of the correct nucleotide continuously changes during each round of nucleotide incorporation. The frequency of incorrect nucleotide incorporation is significantly lower than predicted from free energy differences between correct and incorrect base pairing in solution \cite{2}. 
The kinetic and structural basis of high-fidelity DNA replication by DNA polymerases has been the subject of extensive studies for several decades. Early kinetic studies of Klenow Fragment of *E. coli* DNA polymerase I (KF) (3-6) and bacteriophage T7 DNA polymerase (7, 8) concluded that DNA polymerases achieve dNTP selectivity by an induced-fit mechanism involving a rate-limiting conformational change (9). Subsequently structural studies showed a subdomain-closing conformational change upon dNTP binding to E·DNA binary complex, which led to the “dogma” that this “open-to-closed” protein structural transition is the rate-limiting step prior to chemistry and therefore the major determinant of fidelity (1). However, a large body of latter pre-steady state kinetic and structural studies suggest that this may not be universally true and it is likely that for some DNA polymerases the chemical step is the rate-limiting step and therefore the main contributor to fidelity (10).

The hypothesis of a rate-limiting conformational change in DNA polymerases has its ground on the observation of a small “thio-effect.” Phosphorothioate analogs have been commonly used as a mechanistic probe to determine whether the chemical step is rate-limiting (11, 12). It is rationalized that less electronegative sulfur is less effective than oxygen at stabilizing an associative transition state for phosphoryl transfer. The “thio-effect” is defined as the ratio of phosphoryl transfer rates for a phosphate substituted with a sulfur atom to that of an unsubstituted phosphate. When dNTP analogs, in which a nonbridging oxygen on the $\alpha$-phosphate is replaced with a sulfur atom (dNTP$\alpha$S), is used as a substrate analog in DNA polymerase studies, the ratio $k_{\text{pol, dNTP}}/k_{\text{pol, dNTP}}$ is smaller than predicted based on the ratio of rates of model reactions of
phosphothioates in solution. The observed small value of the “thio-effect” was interpreted as evidence that a step other than chemistry is rate-limiting (9). However, it is now commonly accepted that application of phosphothioates as mechanistic probes may be inappropriate for enzymatic phosphoryl transfer reactions (10, 13, 14).

Since our proposed kinetic mechanism contradicts the commonly accepted view in the field, the burden is on us to provide further evidence for our hypothesis. Even though there are a number of facts indicating that in the case of polymerase β (Pol β) the substrate-induced subdomain closing occurs at a rapid rate, it is still necessary to demonstrate that Pol β is not an exception from other DNA polymerases. In the present work, we provide more data to sustain our hypothesis by extensive mechanistic analysis of two polymerases: Pol β and KF.

1.2 DNA polymerase β background

Most of the DNA polymerase studies in our laboratory were performed with rat DNA polymerase β (Pol β) (15). It is a member of the X-family of nucleotidyl transferase proteins. Among various members of X-family there are three other mammalian DNA polymerases that have been identified: Pol λ, Pol μ, and Pol σ1 (16). Homologues of mammalian Pol β are found in yeast, plants, and viruses (17-19). Polymerase β is found to be essential for organism survival, thus Pol β knockout mice demonstrate lethality during an early embryonic stage (20).

Pol β lacks 3’→5’ exonuclease activity (a “proofreading” activity that is found in most replicative polymerases), but it demonstrates 5’-deoxyribose phosphatase (5’-dRP)
and AP lyase activities (15). Pol β plays a central role in the base excision repair (BER) pathway. In short, BER includes the following major steps: (1) Damaged DNA base is removed by one of lesion specific DNA glycosylases, generating an abasic DNA sites. (2) An AP-endonuclease creates a cut between the 5′-phosphate of the abasic deoxyribose and the 3′-hydroxyl of the previous base. (3) The resulting deoxyribose phosphate “flap” is then incised by the N-terminal lyase domain of Pol β. (4) The C-terminal polymerase domain of Pol β incorporates a dNTP to generate nicked DNA. (5) The nicked DNA is then sealed by DNA ligase.

Because of its small size (39-kDa) and a natural absence of exonuclease activity, Pol β is considered to be an ideal model for structural and mechanistic studies of DNA polymerases in general. Mammalian Pol β can be expressed in E. coli at high levels. It can be easily purified, and it is stable and active in a broad range of conditions. All these characteristics make Pol β a perfect enzyme for biochemical and biophysical studies.

1.3 Pol β structure and catalytic mechanism

More than fifty X-ray crystal structures have been solved for Pol β in its different liganded states: free enzyme(21), enzyme-dNTP binary complex (21), enzyme-DNA binary complexes (22, 23), enzyme-DNA-dNTP ternary complexes (23-25), pre-chemistry and post-chemistry intermediates (26), complexes with mismatches at 3′OH of primer (27, 28), complexes with damaged template (29). The crystal structures indicate that Pol β contains two distinct catalytic domains: an N-terminal 8-kDa lyase domain and
a C-terminal 31-kDa polymerase domain (Figure 1). The polymerase domain displays a canonical polymerase hand-like architecture consisting of thumb, palm, and fingers subdomains.

Figure 1. Structure of Pol β-DNA-dNTP ternary complex (15)

Pol β functional domains are named using the nomenclature of Steitz (30) and colored as follows:

lyase domain – gray

thumb subdomain (DNA binding) – purple

palm subdomain (catalytic) – gold

fingers subdomain (dNTP binding) - green
The thumb subdomain (along with the 8-kD domain) is extensively involved in DNA binding and displays a small backbone conformational change upon formation of E·DNA binary complex (31). The palm subdomain contains the conserved catalytic aspartate triad, which is intimately involved in coordination of two divalent metal ions assisting the nucleotidyl transferase reaction. The fingers subdomain is significantly involved in dNTP binding. Comparison of Pol β·DNA binary complex and Pol β·DNA·dNTP ternary complex structures indicates that the fingers subdomain changes conformation upon binding of correct dNTP (23, 31). The conformation of the enzyme in the binary complex is termed an “open” conformation, whereas the enzyme in ternary complex is in a “closed” conformation. DNA substrate also undergoes a subtle conformational change upon correct dNTP binding (32).

A number of local rearrangements accompany the global “open-to-closed” conformational change (Figure 2). Applying molecular dynamic computational methods, several amino acid residue rearrangements have been found to play a central role in the conformational closing of the fingers subdomain (33-35). In the catalytically active closed conformation, Asp 192 serves as one of the ligands required for binding of active site metal ions. In the inactive open conformation, this amino acid residue forms a salt bridge with Arg258. Closing of the fingers subdomain results in repositioning of Phe272 in such way that it disturbs the Asp192-Arg258 interaction. Arg258 undergoes a side chain rotation that results in new interactions with Glu295 and Tyr296. As a result of Arg258 repositioning, Asp192 becomes capable of efficient interaction with active-site metals. It has been proposed that Arg258 reorientation might be a slow pre-chemistry
conformational step which, in accordance with the induced-fit hypothesis, makes a major contribution in DNA polymerase fidelity.

Figure 2. Local rearrangements of active-site residues that accompany the “open-to-closed” conformational change of Pol β (15)
Based on crystal structures, the general chemical mechanism of Pol β and other DNA polymerases has been inferred (23, 36). As shown in Figure 3, polymerases require two magnesium ions to catalyze the phosphoryl transfer reaction. One metal ion binds the enzyme active site as a metal-dNTP complex (the nucleotide-binding ion, site A). This metal ion plays role in stabilizing the negative charge that builds up on the leaving group and assists in PPi dissociation. The presence of the Mg²⁺ ion tends to direct any H⁺ delivered during catalysis to the β-phosphate (with release of Mg²⁺ to the more negatively-charged terminal phosphate), which further enhances PPi as a leaving group (37). The other metal ion (the catalytic magnesium ion, site B) activates the primer's 3'-OH for in-line nucleophilic attack on the α-phosphate of the incoming nucleotid. The pentacoordinated transition state structure is stabilized by both metal ions. Details on how the active-site residues actually contribute to the various steps in the chemical process are not completely understood.
Figure 3. Schematic diagram of the Pol β active site showing the two magnesium ions required for catalysis (23)

The nucleotide-binding ion (site A), is considered to be associated with the incoming dNTP in the kinetic scheme. The catalytic ion (site B), binds the active site independently of the incoming nucleotide.
1.4 Proposed kinetic mechanism of Pol β

Pol β has been studied extensively by pre-steady state kinetic analyses in our group, as well as other research groups (38-42). In 1997, we first applied stopped-flow fluorescence assays to monitor changes in conformation associated with the catalytic cycle of Pol β (43). The observed biphasic change of fluorescence was initially interpreted as multiple conformational changes before the phosphodiester bond formation step. Following studies using a DNA substrate with dideoxy-terminated primer and Cr(III)dNTP exchange-inert complexes led to a new model suggesting that the faster phase of fluorescence transition is associated with a step preceding chemistry, while the slower phase of fluorescence transition reflects a step after chemistry (26). At the same time we showed that the crystal structure of the E·DNA·Cr(III)dNTP ternary complex (in the absence of catalytic metal ion) already exists in the closed form (26). These results, along with the observation that Cr(III)dNTP can induce only the faster phase of fluorescence transition in the absence of the catalytic metal ion, led us to the conclusion that the subdomain-closing conformational change of Pol β is already complete before the step which determines the rate of the slow fluorescence change.

The stopped-flow transient fluorescence results have also been substantiated under a variety of conditions (44). The reaction progress has been monitored using either of two different fluorescent probes: (1) Pol β’s sole tryptophan residue at position 325, which reports protein conformational transitions (Figure 4A), and (2) 2-aminopurine (2AP) on the templating DNA strand, which reports DNA structural changes (Figure 4B). Regardless of which of these probes is used, two fluorescence transitions are detected:
one with a rate identical to that of dNTP incorporation as determined by rapid-quench experiments under single turnover conditions, and the other with a rate significantly greater (Figure 4). Our current work addressed the structural bases of the two fluorescence transitions. Here we present new data that strongly sustain our hypothesis that the fast fluorescence transition is associated with the subdomain-closing conformational change and the slow fluorescence transition corresponds to the subdomain reopening (while its rate limited by the rate of the chemical step).

Based on the kinetic studies, we propose a minimal kinetic scheme of single-nucleotide incorporation catalyzed by Pol β (Figure 5). Here the nucleotide-induced “open-to-closed” conformational change (step 2) and “reopening” conformational change (step 6) are relatively fast steps, whereas chemistry (step 4) is the rate limiting-step.
Figure 4. Superimposition of rapid chemical quench and stopped-flow fluorescence assays

(A) Rapid chemical quench (open circles ○) and stopped-flow tryptophan fluorescence (blue •) assays for incorporation of dCTP into an 18/36AP DNA substrate. The rapid quench data points fit to a single exponential with $k_{RQ} = 0.430 \text{ s}^{-1}$. The stopped-flow data points fit to a double exponential with $k_{\text{fast}} = 64.4 \text{ s}^{-1}$ and $k_{\text{slow}} = 0.457 \text{ s}^{-1}$. (B) Similar assays for incorporation of dCTP into an 18/35AP DNA substrate. The fluorescence change from 2-AP instead of Trp was monitored.
Figure 5. Kinetic scheme of single-nucleotide incorporation

\[ E = \text{Pol } \beta \text{ in open finger conformation;} \]

\[ E' = \text{Pol } \beta \text{ in closed finger conformation;} \]

\[ D_n = \text{DNA;} \]

\[ N = \text{M·dNTP;} \]

\[ M = \text{catalytic metal ion;} \]

\[ P = \text{M·PP}_i. \]

In the text, the \textit{binary} complex refers to the \( E \cdot D_n \) state, while the \textit{ternary} complex refers to the \( E' \cdot D_n \cdot N \) state (after the conformational change).
CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Ultra-pure dNTP and G-25 microspin columns were purchased from GE Healthcare. Radiolabeled $[\gamma-^{32}\text{P}]\text{ATP}$ was purchased from MP Biomedicals. T4 polynucleotide kinase was obtained from New England BioLabs. Reverse phase C_{18} cartridges were obtained from the Waters Corp. Amicon protein concentrators were obtained from Millipore. Diethylaminoethyl (DEAE) and P-11 cellulose were purchased from Whatman. All other chemicals were obtained from Sigma-Aldrich.

2.2 DNA polymerase $\beta$ purification

Recombinant rat Pol $\beta$ was purified as previously described from an overexpressing *E. coli* strain BL21(DE3) (pLysS, pET17-Pol $\beta$) (42). Briefly, the crude protein extract containing Pol $\beta$ was passed through DEAE-cellulose to remove nucleic acids. The pass-through fraction was loaded onto a P-11 ion-exchange column equilibrated with a buffer containing 0.3 M KCl in Buffer A (50 mM Tris HCl, 1 mM DTT, pH 8.0) and eluted with 0.3 M – 1M gradient of KCl (in Buffer A). Fractions containing Pol $\beta$ were combined and
the sample was concentrated to a volume of 2 mL using Amicon protein concentrators. The sample then was loaded onto a Superdex 75 Column (GE Healthcare) and eluted with buffer C (150 mM KCl, 2 mM DTT, and 100 mM Tris HCl, pH 8.0) at 1 mL/min in 1mL fractions. Peak fractions were combined, diluted with an equal volume of glycerol, aliquoted and then quickly frozen in liquid nitrogen. The purified enzyme was stored at -80 °C. The Pol β concentration was determined by using a 280 nm extinction coefficient of 21,200 M⁻¹ cm⁻¹. The enzyme was apparently homogenous on the basis of SDS/PAGE analysis developed using the silver staining method.

2.3 DNA substrates

The sequences of primer/template DNA substrates used in this study are shown in Figure 6. Custom synthesized oligomers were purchased from Integrated DNA Technologies (Coralville, IA). Each DNA oligomer was further purified by 18% (w/v) polyacrylamide/7M urea denaturing gels and extracted with 500 mM ammonium acetate and 1 mM EDTA. The extracted oligomers were subsequently desalted with a Sep-Pak C₁₈ cartridge and eluted using methanol: water (60:40) solvent. After removing the solvent with vacuum, the oligomers were resuspended in TE buffer (10 mM Tris HCl, 0.1 mM EDTA, pH 8.0) and the concentration was determined by UV using a calculated molar absorption coefficient (45). The oligomers were then stored at −20 °C.

DNA substrates used in the chemical quench experiments were 5’-end labeled using T4 polynucleotide kinase and [γ⁻³²P]ATP (4500 Ci/mol) according to the manufacturer’s protocol. The T4 polynucleotide kinase was inactivated by heating at 70 °C for 20 min.
The labeled primer was separated from unreacted ATP and Mg\(^{2+}\) using a G-25 microspin column with a small amount of Chelex 100 resin added to it. The appropriate unlabeled DNA-template was added and annealed by heating to 80 °C followed by gradual cooling to room temperature.

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**19/36AP**

\[5' - \text{GCC TCG CAG CCG TCC AAC C} \]
\[3' - \text{CGG AGC GTC GGC AGG TTG GT}^\text{A} \text{TCA GTG GAG TTA GGT} \]

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**19dd/36AP**

\[5' - \text{GCC TCG CAG CCG TCC AAC C}_{\text{dd}} \]
\[3' - \text{CGG AGC GTC GGC AGG TTG GT}^\text{A} \text{TCA GTG GAG TTA GGT} \]

---

**18/36AP**

\[5' - \text{GCC TCG CAG CCG TCCAAC} \]
\[3' - \text{CGG AGC GTC GGC AGG TTG GT}^\text{A} \text{TCA GTG GAG TTA GGT} \]

---

**18dd/36AP**

\[5' - \text{GCC TCG CAG CCG TCCAAC C}_{\text{dd}} \]
\[3' - \text{CGG AGC GTC GGC AGG TTG GT}^\text{A} \text{TCA GTG GAG TTA GGT} \]

---

**18/35AP**

\[5' - \text{GCC TCG CAG CCG TCC AAC} \]
\[3' - \text{CGG AGC GTC GGC AGG TTG GT}^\text{A} \text{TCA GTG GAG TTA GGT} \]

---

**18dd/35AP**

\[5' - \text{GCC TCG CAG CCG TCCAAC C}_{\text{dd}} \]
\[3' - \text{CGG AGC GTC GGC AGG TTG GT}^\text{A} \text{TCA GTG GAG TTA GGT} \]

---

Figure 6. Sequences of DNA substrates

\(^\text{A}\) represents 2-aminopurine. \(C_{\text{dd}}\) represents 2',3'-dideoxycytidine 5'-monophosphate.
2.4 Rapid chemical quench experiments

A rapid quench instrument (KinTek instrument Corp, State College, PA) was used for reaction times ranging from 2 ms to 100 s. Pol β and DNA were preincubated at least for 5 minutes before the start of the reaction. A typical reaction was initiated by rapid mixing of two solutions: solution A contained of 400 nM DNA and 1 µM enzyme, and solution B contained dNTP (concentration varied for different experiments). Typically, both solutions contained equal concentrations of MgCl₂ and 1 mM DTT in an appropriate reaction buffer. Reactions were rapidly quenched by mixing with EDTA (300 mM, final concentration) or HCl (1M, final concentration). In the case of an acid quench, the reaction products were neutralized with a solution containing 1 M Tris, 10 M NaOH, and 0.6 M EDTA. The reaction products were mixed with formamide (1:1) and run on 19 % (w/v) polyacrylamide/7 M urea denaturing gels. The disappearance of substrate and the formation of product were visualized with a STORM 840 PhosphorImager (Molecular Dynamics) and analyzed using ImageQuant 5.0 software (Molecular Dynamics).

2.5 Stopped-flow fluorescence assays

Experiments were performed on an Applied Photophysics SX 18MV Stopped-Flow apparatus. The excitation wavelengths were 285 nm and 312 nm for tryptophan and 2-aminopurine respectively, with a spectral bandpass of 4 nm. Emission was monitored using a 340 nm bandpass filter (Corion, 10 nm) for tryptophan and a 360 nm high pass filter (Corion) for 2-aminopurine. All reaction buffers were filtered with a 0.45 µm filter and degassed prior to use. Primer and template oligomers were mixed with a
1.1 : 1 ratio and annealed by heating to 80 °C and slowly cooling to room temperature. A typical experiment involved rapid mixing of two solutions: (A) 400 nM annealed DNA substrate and 1 μM polymerase, and (B) dNTP (varied concentrations). Typically, both solutions contained equal concentrations of MgCl₂ and 1 mM DTT in an appropriate reaction buffer. At least seven trials were performed and averaged. Profiles were typically collected using a logarithmic time scale (10 s) with 1000 data points.

2.6 Data analysis

Data obtained from rapid quench kinetic assays were analyzed by nonlinear regression using Sigma Plot software (Jandel Scientific) with equation 1:

\[
[DNA_{n+1}] = A(1 - e^{-k_{RQ}t})
\]

(equation 1)

where A and \(k_{RQ}\) represent the amplitude and the observed rate constant, respectively.

Stopped-flow data were analyzed using Applied Photophysics software. Equations 2 and 3 were used where appropriate:

\[
fluorescence = Ae^{-kt} + C
\]

(equation 2)

\[
fluorescence = A_1e^{-k_{fast}t} + A_2e^{-k_{slow}t} + C
\]

(equation 3)

where A is the amplitude of a fluorescence change, \(k\) is an observed rate constant and C is an offset constant.
CHAPTER 3

USE OF RHODIUM(III) NUCLEOTIDE COMPLEXES TO DISSECT
THE ROLES OF TWO METAL IONS IN THE POL β REACTION
MECHANISM

3.1 Exchange-inert metal-nucleotide complexes

DNA polymerase structures suggest a two-metal ion mechanism for dNTP incorporation (36, 46). These two magnesium ions are designated as the nucleotide-binding Mg$^{2+}$ ion (as a part of MgdNTP), and the catalytic Mg$^{2+}$ ion (Figure 3). In 1998 our research group used Cr(III)dTTP in stopped-flow fluorescence assays as a substitute for MgdTTP, which allowed us to demonstrate that the fast phase of the fluorescence change is induced by metal·dNTP binding in the absence of the catalytic metal ion (47). The goal of the present work is to take this previous study one step further and to perform kinetic analyses starting from the Pol β·DNA·MdNTP ternary complex. Since we were unable to accomplish this with Cr(III)dNTP, possibly because Cr(III)dNTP was not stable enough, we pursued the use of Rh(III)dNTP.

Unlike Mg$^{2+}$ nucleotide complexes, which are in rapid equilibrium in aqueous solution (with exchanges rates of 5000 s$^{-1}$) (48), exchange-inert nucleotide complexes
have ligand exchange rates measured in days under non-basic conditions (49). While our previous studies have demonstrated the usefulness of substitution-inert metal ion Cr(III) for mechanistic studies of DNA polymerases, Cr(III) is not the best metal ion for such studies, even though it has been the most widely used. Exchange-inert metal·nucleotide analogs have been synthesized and characterized for Cr(III), Co(III) (49), and Rh(III) (50). Of these, rhodium has been used least frequently in enzymatic studies (50-54), and has never been used in studies of polymerases. We chose Rh(III)dNTP for the present study of Pol β because, of the three metal·nucleotide complexes studied, Rh(III)NTP has been shown to be most structurally similar to MgNTP. For example, the Rh--O bond lengths are very close to Mg--O bond lengths (2.02 Å and 2.08 Å, respectively), as determined by X-ray structures with pyrophosphate (55, 56). At equilibrium, both MgNTP and Rh(III)NTP exist in an approximate 1:1 ratio of bidentate and tridentate isomers (50).

3.2 Synthesis of Rh(III)dNTP analogs

The synthesis and complete characterization of Rh(III)ATP has been described previously (50). The same procedure was followed to synthesize Rh(III)dCTP. In this complex Rh(III) coordinates the triphosphate moiety and is termed here as the phosphate complex, to be distinguished from the cytosine ring coordinated complex termed here as the “pre-complex,” which is also described in this work.

Briefly, 10 mM Rh(H_2O)_6(ClO_4)_3 was reacted with 10 mM dCTP at pH 3.0 at 80 °C. Rh(H_2O)_6(ClO_4)_3 was prepared from rhodium(III) chloride hydrate as described
by Ayres and Forrester (57). Product formation was monitored by $^{31}$P-NMR (Figure 7). It can be seen from the $^{31}$P-NMR spectrum that after 20 min of heating the reaction mixture contained bidentate and tridentate isomers of Rh(III)dCTP as well as non-reacted dCTP and products of dCTP degradation. The estimated yield of Rh(III)dCTP before purification was in the range of 30-40%.

Figure 7. $^{31}$P-NMR spectrum of dCTP reacted with Rh(ClO$_4$)$_3$($H_2$O)$_6$ at 80°C for 20 min

The product was purified as follows. The reaction mixture was loaded onto a 1.5 × 30 cm Dowex-1×200 (Cl$^-$ form) column and eluted with 10 mM HCl at 4 °C. Fractions containing Rh(III)dCTP were combined, pH was adjusted to 5.0 with K$_2$CO$_3$, and the sample was concentrated to a volume of 1 mL with high vacuum at 10 °C. The sample was then loaded onto a 15 mm × 160 cm G-10 gel filtration column and eluted with
2 mM MES buffer pH 5.0 at 4 °C. The appropriate fractions were collected and Rh(III)dCTP concentration was determined by UV using a molar absorption coefficient of 9100 M⁻¹ cm⁻¹ at 272 nm.

Using ion-exchange chromatography (Dowex-1, Cl⁻ form) we were able to isolate bi- and tridentate complexes of Rh(III)dCTP (Figure 8). We found that even though there was no observable dissociation of Rh(III)dCTP into free Rh(III) and dCTP, there was considerable inter-conversion between stereo and coordination isomers at the kinetic assay conditions. Rh(III)dCTP complexes showed ³¹P NMR properties similar to those described for Rh(III)ATP (50). Additional characterization was performed by ¹H NMR and ¹³C NMR. The ¹³C NMR spectrum of Rh(III)dCTP was found to be very similar to that of free dCTP, which ruled out the possibility of deaminated product. The purified product was used for rapid quench experiments.

The pre-complex of Rh(III)dCTP was synthesized from Rh(III) compounds containing 2-3 Cl⁻ ligands by heating of 10 mM Rh(III) chloride hydrate with 10 mM dCTP at 80 °C. Product formation was monitored by ¹H-NMR (Figure 9), which showed that the Rh(III) ion coordinates with the cytosine ring but not with the triphosphate moiety.
Figure 8. $^{31}$P NMR spectra of dCTP and purified Rh(III)dCTP complexes.

(A) dCTP; (B) bidentate Rh(III)dCTP; and (C) tridentate Rh(III)dCTP
3.3 Kinetic assay conditions and DNA substrates

In choosing enzyme assay conditions, we took into consideration that, similar to other exchange-inert nucleotide analogs, Rh(III)dCTP complexes become unstable at high pH (50), and Pol β loses activity at low pH. Consequently, a compromised pH 7.0 was used in these studies. The standard assay buffer consisted of 100 mM MOPS, 50 mM KCl, 10% (w/v) glycerol, 1 mM DTT at pH 7.0; and a typical assay was conducted at 25 °C.

As shown in our previous work, the amplitude and direction of fluorescence change during the course of single nucleotide incorporation depends on the excited fluorophore, the DNA substrate, and the reaction conditions (44). This study used two fluorophore probes – Trp325 (the single tryptophan of Pol β) and 2-aminopurine – to monitor fluorescence changes associated with different enzyme-DNA conformations during the reaction pathway. For tryptophan fluorescence, it was found that the 18/36AP DNA substrate (Figure 6) provides a good signal-to-noise ratio (44). Also, as it has been shown previously, the best signal-to-noise ratio in 2-aminopurine fluorescence stopped-flow experiments can be obtained with the 2-AP modification in +1 position relative to the nascent base pair (44). Therefore 18/35AP DNA substrate (Figure 6) was chosen for our studies.
3.4 Characterization of Rh(III)dCTP complexes

The reaction products of six aqua coordinated rhodium and dCTP were analyzed by NMR spectroscopy. Corresponding spectra shown in Figure 7 demonstrate that the major products of the reaction are bi- and tridentate phosphate coordinated complexes. $^{13}$C and $^1$H-NMR (Figure 9) spectra indicate the presence of C6 and H6 splitting. Careful analysis of the proton splitting revealed the 9 doublets with the same coupling constant, which was likely a consequence of the presence of a mixture of different Rh(III)dCTP stereoisomers in the sample. Since there were no significant changes in the H6 and C6 chemical shifts, rhodium does not likely coordinate cytidine ring directly, but instead might interact with the ring through water molecules (outer sphere). This type of coordination is typical for Mg-nucleotide triphosphate complexes (58).
Purified product was used for rapid quench experiments, which revealed that the Rh(III)dCTP complex could be accepted as a substrate by Pol β, although the rate of Rh(III)dCTP incorporation was substantially slower than the rate of MgdCTP incorporation (Figure 10). This observation can be attributed to several possible factors. In using substitution-inert complexes, it is important to keep in mind that their
“inertness,” or stability, differs from one another and is also very sensitive to a number of factors including pH and the nature of other ligands in the metal coordination sphere. Furthermore, each complex, when formed, consists of a mixture of several different positional and stereo isomers (49). For the more stable complexes, each isomer can be isolated and tested individually as a substrate analog in an enzyme catalyzed reaction. This is how inert complexes have been used conventionally. Though not usually specified, the non-inert Mg(dNTP) complex consists of a similar set of isomers. When Mg(dNTP) binds to an enzyme in the ground state, the enzyme is likely to accept more than one isomer (or even all isomers). However, only one specific isomer can exist at the transition state. Based on early studies with adenylate kinase (59), it is believed that in proceeding from the ground state to the transition state, the enzyme is able to convert an incorrect isomer to the correct isomer. In the case of Cr(III)dNTP previously described in references (26, 47), a mixture of stereoisomers was used directly. It was assumed that stereochemically incorrect isomers were converted to the correct stereoisomer in the process of catalysis, though it cannot be ruled out that some of the isomers were excluded from binding or released after binding (and neither of these will alter the results, as long as all processes are fast). One single isomer was observed in the crystal structure of the intermediate Pol β-DNA-Cr(III)dNTP complex (26).

When Rh(III)dNTP (also a mixture of isomers) was used as a substrate analog for rapid quench experiments, the rate of incorporation was significantly slower than that of the natural Mg(dCTP) substrate (Figure 10). While this could suggest that Rh(III) is not a good analog of magnesium, the slow incorporation rate could also be observed because
Rh(III)dNTP isomers are more inert and the interconversion between isomers becomes rate-limiting. This problem was overcome by using a “pre-complex” of Rh(III)dCTP as described below.

Figure 10. Rapid quench assays of MgdCTP and Rh(III)dCTP incorporation into 18/36AP DNA substrate by Pol β

Red circles (●) – MgdCTP; violet triangles (▲) – Rh(III)dCTP. The data points fit to a single exponential equation with rate constants of 0.367 s⁻¹ for MgdCTP and 0.064 s⁻¹ for Rh(III)dCTP.
When chloride ions are present in the rhodium coordination sphere, the efficiency of phosphate complex formation decreases as the number of Cl atoms increases. There was no observable by $^{31}$P-NMR Rh(III)-phosphate coordination when Rh(III) chloride hydrate (3 Cl' in Rh(III) coordination sphere) was used as a reactant. However, changes in $^1$H-NMR spectrum were detected (Figure 9). First, there was a shifting of the H6 proton peak from 8.1 ppm to 7.2 – 7.4 ppm, which might be an indication of direct metal interaction with the cytidine ring. Second, a complex pattern of splitting at 8.0 – 8.1 ppm was observed, which could be attributed to formation of triphosphate - cytidine ring chelates. As determined by Yu Wang from our group, longer reaction time results in establishment of equilibrium between phosphate coordinated and cytidine base coordinated complex. The existence of such equilibrium can explain unsuccessful attempts to isolate the ring coordinated (also called here the “pre-complex”) and phosphate coordinated complexes.

In order to investigate the role of the triphosphate group in the stability of the “pre-complex,” we studied reactions of partially hydrated Rh(III) chloride with CMP and cytosine. Yu Wang demonstrated that the reaction with CMP resulted in predominant formation of nitrogen coordination products. This suggested that the presence of the $\beta$ and $\gamma$ phosphoryl group is required to shift the equilibrium toward the phosphate coordinated complex. On the other hand, high reactivity of cytosine with Rh(III) chloride suggested that the ring coordinated complex formation does not depend on the presence of the triphosphate moiety.
In the “pre-complex,” Rh(III) coordinates the cytosine ring possibly through a nitrogen ligand. This is a reasonable assumption since it has been shown that Rh(III) can coordinate the adenine ring rather than the phosphate moiety of AMP (the phosphate is likely an outer sphere ligand) (60). This hypothesis has been supported by the observation that UTP, which differs from CTP by the hybridization state of N3 and the oxygen at C4, is unable to form ring coordinated complex (shown by Yu Wang).

When the product of the reaction of Rh(III) chloride with dCTP was used in kinetic assays, it was found to be an efficient substrate for Pol β. Comparing Rh(III)dCTP complexes, obtained with the rhodium(III) six aqua compound and the chloride substituted compound, we found that the most striking difference between them is the presence of the ring coordinated complex. We believe that the “pre-complex” is more labile than the phosphate complex and that it is converted rapidly to the correct isomer of the phosphate complex after binding to the active site of Pol β. It must be mentioned that it is not completely proven that it is the ring coordinated complex that acts as a good substrate analog. It is also not clear why it would be a better analog than the phosphate complex. If the ring coordinated complex is indeed a good substrate analog, a polymerase must be able to bind this type of complex and then facilitate its isomerization to the correct triphosphate coordinated stereoisomer. On the other hand, we cannot exclude that Pol β binds phosphate coordinated Rh(III) complex (with possibly of outer-sphere coordination, so it is not detectable by $^{31}$P-NMR). Also, it is possible that presence of chloride in the coordination sphere of rhodium(III) favors the formation of the correct phosphate coordinated stereoisomer.
3.5 Use of Rh(III)dCTP to dissect roles of two metal ions

Rh(III)dCTP complexes were used as nucleotide analogs in stopped-flow experiments. First, Trp fluorescence was utilized to monitor changes in enzyme conformation during the reaction pathway. Figure 11 shows the Trp fluorescence change upon mixing preformed Pol β-DNA binary complex with Mg-dCTP (Figure 11A) or upon mixing the binary complex with Rh(III)dCTP in the absence of Mg$^{2+}$ (Figure 11C). In the first case, the observed fluorescence change has a biphasic character and can be fit to a double exponential equation (equation 3) with $k_{\text{fast}} = 64.4$ s$^{-1}$ and $k_{\text{slow}} = 0.457$ s$^{-1}$.

Whereas the fluorescence change induced by Rh(III)dCTP binding fit well to a single exponential equation (equation 2) with $k = 75.3$ s$^{-1}$. It should be noticed that the kinetic trace shown in Figure 11C resembles the kinetic trace observed in the case of dideoxy-terminated primer (Figure 11B). Moreover, remarkably similar kinetic traces have been previously observed in the case of Cr(III)dNTP, as described in reference (26). In order to rule out the possibility that the observed fluorescence decay was a result of fluorescence quenching by rhodium (III) chloride hydrate or dCTP, we performed a series of control stopped-flow experiments. The results of these experiments clearly demonstrate that neither dCTP nor RhCl$_3$·3H$_2$O alone can induce noticeable fluorescence changes (Figure 12).
Figure 11. Stopped-flow Trp fluorescence assays using MgdCTP and Rh(III)dCTP analogs

(A) Incorporation of MgdCTP into the Pol β·18/36AP binary complex; (B) MgdCTP binding to Pol β·18dd/36AP binary complex; (C) Rh(III)dCTP binding to Pol β·18/36AP binary complex
Figure 12. Negative controls (stopped-flow experiments)

Tryptophan fluorescence upon mixing of Pol β-DNA binary complex with (A) dCTP in the absence of Rh(III); (B) Rh(III) chloride hydrate in the absence of dCTP
We also used 2-aminopurine as a fluorescent probe to monitor Enzyme·DNA conformation dynamics. Figure 13A shows the fluorescence change associated with incorporation of dCTP in 18/35AP by Pol β. Again, the fluorescence change has biphasic character, and the slow phase was absent when chemistry was precluded by using dideoxy-terminated primer. As can be seen in Figure 13B, use of Rh(III)dCTP in the absence of Mg\(^{2+}\) resulted in the fast phase of fluorescence change only. Therefore, we were able to obtain identical results for two different fluorescent probes – tryptophan and 2-aminopurine. This suggests that the step responsible for the fast fluorescent transition involves global structural rearrangements of Pol β·DNA complex, and therefore this transition likely represents the subdomain-closing conformational change.

The results of these experiments reaffirm the results previously obtained with Cr(III)dNTP, in that metal·dNTP alone (in the absence of the second catalytic metal ion) can induce the subdomain-closing conformational change \((26, 47)\). Furthermore, the use of Rh(III)dNTP analogs and optimized conditions have allowed us to examine the reaction pathway starting from the Pol β·DNA·Rh(III)dNTP ternary complex. These studies yielded novel information presented in the next section.
Figure 13. Stopped-flow 2-AP fluorescence assays using Mg-dCTP and Rh(III)dCTP analogs

(A) Mg-dCTP incorporation into Pol β·18/35AP binary complex; (B) Rh(III)dCTP binding to Pol β·18/35AP binary complex
3.6 Use of Rh(III)dCTP to show than the slow step occurs after addition of catalytic Mg$^{2+}$ ion

We next used stopped-flow experiments to directly monitor the fluorescence change induced by binding of the second metal ion to the ternary complex. When Pol β was pre-incubated with DNA substrate and Rh(III)dCTP and the reaction was initiated by Mg$^{2+}$, the fast phase of tryptophan fluorescence disappeared, and only the slow phase was observed (Figure 14A). The fluorescence kinetics has a single exponential character and no indication of a lag-phase is observed. Similar results were also obtained in stopped-flow experiments by monitoring the change in 2-aminopurine fluorescence during the course of dCTP incorporation from ternary complex Pol β·18/35AP·Rh(III)dCTP (Figure 14C). The results shown in Figure 14 unequivocally demonstrate that the slow fluorescence change occurs upon addition of Mg$^{2+}$ to the E·DNA·MdNTP ternary complex (step 3, Figure 5).

When analogous experiments were repeated with dideoxy-terminated primers, no change in fluorescence was observed (Figure 14B). This observation suggests that the slow phase of fluorescence change is not caused directly by binding of the catalytic Mg$^{2+}$ ion (step 3 on the kinetic scheme (Figure 5)); instead, it occurs after addition of Mg$^{2+}$ to the Enzyme·DNA·MdNTP ternary complex (i.e., after step 3).
Figure 14. Stopped-flow fluorescence assay starting from the E·DNA·Rh(III)dCTP ternary complex

(A) Trp fluorescence change upon addition of Mg$^{2+}$ to Pol β·18/36AP·Rh(III)dCTP;
(B) Addition of Mg$^{2+}$ to Pol β·18dd/36AP·Rh(III)dCTP, Trp fluorescence;
(C) 2-AP fluorescence change upon addition of Mg$^{2+}$ to Pol β·18/35AP·Rh(III)dCTP
A tryptophan fluorescence

B tryptophan fluorescence

C 2-AP fluorescence
Finally, we addressed the issue that, based only on results of fluorescence stopped-flow assays, we could not completely rule out the possibility that a fluorescence silent slow step occurs before binding of the second magnesium ion. In order to sustain our conclusions, we decided to perform experiments with purified Rh(III)dCTP analogs in rapid quench assays. In these experiments, the rate of single-nucleotide incorporation from the ternary complex (Pol β·DNA·Rh(III)dCTP) was compared with the rate of Rh(III)dCTP incorporation into the binary complex (Pol β·DNA). If there existed a rate-limiting step before the formation of the closed ternary complex, the rate of incorporation starting from Pol β·DNA·Rh(III)dCTP complex would be faster than the rate of incorporation into the binary complex. In the first case, the ternary complex was formed by pre-incubation of Pol β, DNA and purified Rh(III)dCTP. Then the reaction was initiated by rapid mixing with 10 mM MgCl₂. In the second case, the reaction was initiated by rapid mixing of preformed binary Enzyme·DNA complex (in the presence of Mg²⁺) and Rh(III)dCTP. As can be seen in Figure 15, the rate constants of single-nucleotide incorporation were found to be identical in both reactions. These results strongly support our hypothesis that the formation of the closed ternary complex cannot be a rate-limiting step for single dNTP incorporation.
Figure 15. Rapid quench assays of single-nucleotide incorporation starting from the ternary or binary complex

Pink circles (●) – incorporation from Pol β·DNA·Rh(III)dCTP ternary complex upon addition of Mg$^{2+}$

Violet triangles (▲) – Rh(III)dNTP incorporation into Pol β·DNA binary complex

The rapid quench data points fit to a single exponential equation with rate constants of $0.065 \pm 0.003$ for the incorporation from the ternary complex and $0.064 \text{s}^{-1} \pm 0.004$ for the incorporation into the binary complex.
3.7 Conclusion

The substitution-inert Rh(III)dNTP was used to show for the first time that the slow fluorescence change occurs after mixing of Pol β·DNA·Rh(III)dNTP with Mg$^{2+}$. These results, along with crystal structures (26), suggest that the subdomain-closing conformational change occurs before binding of the catalytic Mg$^{2+}$ while the rate-limiting step occurs after binding of the catalytic Mg$^{2+}$. These results provide new evidence for the mechanism we have proposed previously, but do not support the results of recent computational studies (34, 35, 61). Two of the central points in these studies are that “the binding of the catalytic magnesium and the rearrangement of Arg258 may be coupled and represent a slow step in Pol β’s closing before chemistry,” and that “Pol β subdomain closing requires presence of both nucleotide-binding and catalytic metal ions.” Both points contradict our results since our data clearly indicate that the subdomain-closing conformational change occurs before binding of the catalytic Mg$^{2+}$, while the rate-limiting step occurs after binding of the catalytic Mg$^{2+}$.

The suggestion that the rearrangement of Arg258 can be the rate-limiting step is not supported by our data, though it cannot be entirely ruled out. While there is almost certainly some movement of side chains induced by binding of the catalytic metal ion, unless this results in a detectable intermediate species, it cannot be considered as a distinct step since it cannot be observed experimentally. Thus, even if the binding of the catalytic metal ion induces a slow isomerization, which is immediately followed by nucleotidyl transfer, this isomerization would not be kinetically distinct from nucleotidyl transfer and therefore would have to be considered as a part of the chemical step. As
shown in Figure 5, we define the chemical step (step 4) as the step between the identifiable intermediate structure closest to the chemical transition state (Pol $\beta$-DNA·MgdNTP·Mg, which on the basis of crystal structures is essentially identical to Pol $\beta$-DNA·Cr(III)dNTP except for the orientation of some side chains of residues involved in the binding of the catalytic Mg$^{2+}$), and the identifiable intermediate structure immediately after the transition state (Pol $\beta$-DNA·MgPP$_1$·Mg), whose structure is not yet available but is likely to be nearly identical to the structure of Pol $\beta$-DNA·Cr(III)PCP) (23, 26). Like any step, the chemical step can be further dissected into multiple microscopic steps through the use of a variety of experimental or computational techniques on different time scales. It cannot be ruled out that the reorientation of a certain active site residue is the slowest microscopic step within the chemical step, if the chemical step is further dissected. However, this hypothesis is yet to be proven experimentally.
The second main point of the computational results, which strongly contradicts our experimental data, is that binding of both Mg\(^{2+}\) ions is required for subdomain closing. The authors of the computational study argue that such discrepancy can result from the use of Cr(III) rather than magnesium (61) in the crystal structure of the intermediate Pol β-DNA-Cr(III)dNTP ternary complex, which exists in the closed form in the absence of the catalytic metal ion(26). If this claim was true, then the stopped-flow results with MgdNTP (in the absence of free Mg\(^{2+}\)) would be very different from the results of exchange-inert metal-dNTP analogs. The next chapter presents evidence that MgdNTP acts very similarly to Rh(III)dNTP, which allows us to rule out the possibility that use of metal analogs causes the experimental result to be non-natural or erroneous.
CHAPTER 4

PROBING OF POL β KINETIC MECHANISM USING ONLY NATURAL SUBSTRATES AND CO-FACTORS

4.1 Rationale behind experimental design

While use of various analogs (e.g., dideoxy-terminated primers, dNTPαS, and substitution inert metal-nucleotide complexes) has made it possible to dissect the microscopic steps and to characterize the structures of intermediates involved in Pol β reaction pathway, it is conceivable that these analogs may cause unexpected or unknown perturbations to the reaction mechanism. Thus, whenever the experimental properties of Pol β are shown to differ from those of other polymerases, or differ from results of computational studies with Pol β, the use of analogs is often claimed to be the main cause of such discrepancy. One way to address this issue is to employ multiple analogs. If the results and interpretations for all of them are consistent, as described in the previous section, then the danger of misinterpretation due to an analog-introduced artifact can be minimized. However, the best approach to entirely eliminate the possibility of an analog-introduced artifact is to simply employ an experimental condition that utilizes only natural substrates but which achieves the same effect conferred by the analog.
Nucleotide triphosphates naturally exist as Mg$^{2+}$ complexes. The binding affinity of a nucleotide triphosphate toward a magnesium ion can vary depending on pH, temperature and ionic strength. For example, at pH, temperature and ionic strength close to our reaction conditions, ATP binds Mg$^{2+}$ with log$K_{eq} = 4.8$ (which corresponds to a $K_d$ equal to 16 µM) (58). It is feasible, by varying concentrations of dNTP and Mg$^{2+}$ (and possibly other magnesium chelators) to find conditions where the concentration of free Mg$^{2+}$ approaches zero, while maintaining the desired concentration of MgdNTP substrate. Here this is accomplished by modulating Mg$^{2+}$ concentrations to achieve the same effect conferred by Cr(III)dNTP and Rh(III)dNTP (namely, saturation of the MgdNTP binding site while maintaining essentially no free Mg$^{2+}$).

4.2 Experimental details

*Sequential flow/stopped-flow experiments.* Experiments were performed using Applied Photophysics SX 18MV Stopped-Flow apparatus that can be operated in sequential mixing mode as shown in Figure 16. Typically, Solution A contained 2 µM Pol β, 800 nM 19/36AP DNA substrate, and 1 mM EDTA in the reaction buffer (100 mM MOPS, 50 mM KCl, 1 mM DTT, 10 % glycerol, pH 7.0). Solution B contained 2 mM dATP, 1 mM EDTA, and a varied concentration of MgCl$_2$ in the reaction buffer. Solution C contained 22 mM MgCl$_2$ in the reaction buffer. First, solutions A and B were rapidly mixed and then, after a 150 ms delay, the reaction was initiated by rapid mixing with solution C.
The excitation wavelength was 312 nm with a spectral bandpass of 4 nm. The emission from 2-aminopurine was monitored using a 360 nm high pass filter (Corion). All reactions were performed at 25 °C. Typically, a minimum of ten trials were performed and averaged. Profiles were collected using a split time base (0.1 s/5 s) with 1000 data points.

Figure 16. Scheme of the stopped-flow apparatus in the sequential mixing mode
MgdATP binding experiments were performed using the standard operation mode of the stopped-flow instrument. In such experiments the solutions A and B were rapidly mixed and the data were collected using a split time base (0.1 s/5 s) with 1000 data points.

*MgdNTP concentration* was calculated using WEBMAXC STANDARD program (http://www.stanford.edu/~cpatton/webmaxcS.htm) using the following parameters: temperature 25 °C, pH 7.0, ionic strength 0.08. Table 5.3 summarizes the conditions used in MgdATP binding and sequential mixing experiments.

<table>
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<th>[Mg$^{2+}$]$_{free}$ µM</th>
<th>[MgdATP] µM</th>
<th>[MgEDTA] µM</th>
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<td>1.3</td>
<td>28.7</td>
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<td>627</td>
</tr>
<tr>
<td>0.90</td>
<td>11</td>
<td>122</td>
<td>767</td>
</tr>
<tr>
<td>1.2</td>
<td>31</td>
<td>270</td>
<td>899</td>
</tr>
</tbody>
</table>

Table 4.1. Calculated concentrations of Mg$^{2+}$ species in the presence of 1 mM dATP and 1 mM EDTA
4.3 Binding of MgdATP to binary Pol β-19/36AP complex

Taking into account that Pol β requires a relatively high concentration of free Mg$^{2+}$ for optimal activity, whereas it binds MgdNTP tightly ($K_{d\text{ app}}^{\text{Mg}} = 1.0$ mM and $K_{d\text{ app}}^{\text{dATP}} = 46 \mu$M (44)), it is possible to “dissect” the two metal ions merely by limiting the amount of Mg$^{2+}$ in the reaction buffer. As shown in Table 4.1, in a solution of 1 mM dATP, 1 mM EDTA, and 1.2 mM MgCl$_2$, the calculated concentration of free Mg$^{2+}$ is 31 µM. This is well below the $K_{d\text{ app}}^{\text{Mg}}$ and thus insufficient to support catalysis. At the same time calculated concentration of MgdATP is 270 µM, well above the $K_{d\text{ app}}^{\text{dATP}}$, and thus sufficient to saturate the MgdATP binding site.

Figure 17 shows results of 2-AP fluorescence stopped-flow assays in which preformed Pol β-DNA binary complex was rapidly mixed with varied concentration of MgdATP. As can be seen, under these conditions, the binding of MgdATP induced the fast phase of fluorescence change only. These results resemble the data obtained with Cr(III)dTTP and Rh(III)dCTP, supporting the validity of using these metal ion complexes in our studies.
Figure 17. Stopped-flow 2-AP fluorescence assay of MgATP binding to Pol β·19/36AP binary complex

The final calculated concentrations of MgATP are, according to Table 4.1 and from bottom to top: 1.3, 3.1, 9.8, 25, 67, 122 and 270 μM.
4.4 Pre-bound nucleotide incorporation upon addition of access Mg\textsuperscript{2+}

In order to reproduce results of single-nucleotide incorporation from the E·DNA·Rh(III)dNTP ternary complex without using analogs, we utilized the sequential mixing mode of the stopped-flow instrument. Specifically, Pol β·DNA binary complex was pre-mixed with MgdATP under conditions with limiting amount of magnesium to form Pol β·DNA·MgdATP complex; then, after 150 ms of delay, the reaction was initiated by addition of excess Mg\textsuperscript{2+}. In agreement with the Rh(III) results, the fast fluorescence transition was absent, presumably occurring during the 150 ms delay before addition of excess Mg\textsuperscript{2+}. Only the slow phase was observed, which fits well to a single exponential with $k = 1.09$ s\textsuperscript{-1} (Figure 18A). In a control experiment, when Pol β·DNA and dATP were pre-mixed in the absence of magnesium for 150 ms, subsequent mixing with an excess of Mg\textsuperscript{2+} resulted in biphasic fluorescence change (Figure 18B).
Figure 18. Three-syringe sequential mixing stopped-flow experiments for a nucleotide incorporation from ternary complex

(A) Addition of the excess Mg\textsuperscript{2+} to the Pol β·19/36AP·MgdATP ternary complex;

(B) Addition of the excess Mg\textsuperscript{2+} to the Pol β·19/36AP binary complex preincubated with dATP in the absence of Mg\textsuperscript{2+}. 
4.5 Conclusion

In conclusion, the above results suggest that the fast nucleotide-induced phase of the fluorescence change does not require catalytic Mg\(^{2+}\) binding, which confirms the results of experiments with exchange-inert metal·dNTP complexes. Therefore, the discrepancy between experimental and computational results cannot be simply due to the use of analogs in our studies.

Based on dynamics simulations, Yang et al proposed following sequence of events preceding the chemical step: (1) binding of catalytic Mg\(^{2+}\); (2) binding of MgdNTP; (3) subdomain closing (61). Our results suggest that binding of the two metal ions is separated by the conformational step. Therefore we propose the following: MgdNTP binding triggers the subdomain-closing conformational change, which results in (or is coupled with) reorganization of the active site, thus creating the site for catalytic Mg\(^{2+}\) binding. This proposed scheme contradicts the results of computational studies, however agrees with kinetic results obtained with yeast reverse transcriptase (Ty1 RT) (62). The latter study revealed cooperative binding of the two ions (specifically, nucleotide-binding metal ion binds first and facilitates the catalytic metal ion binding).

The closed conformation of active site has evolved to stabilize transition state of the chemical step. The catalytic Mg\(^{2+}\) ion is essential for such stabilization, or it is essential for stabilization of substrate in its near active conformation (NAC). So, it is quite reasonable to assume that the closed conformation of enzyme is thermodynamically more stable in the presence of the catalytic magnesium ion. However, it is important to point out that, even though the closed conformation is stabilized by catalytic Mg\(^{2+}\), it is not
induced by catalytic Mg$^{2+}$ binding. At the same time, departure of catalytic Mg$^{2+}$ after phosphodiester bond formation might destabilize the closed conformation and facilitate reopening. In connection to this, it is interesting to note that the catalytic Mg$^{2+}$ binding site (in closed enzyme conformation) is proposed to be easily accessible to solvent (61).
CHAPTER 5

pH DEPENDENCE OF THE STEPS IN POLYMERASE β

REACTION PATHWAY

5.1 Basis of pH effect on enzyme catalysis

An enzyme activity often depends on protonation/deprotonation of the ionizable groups in the enzyme active sites. Protonation/deprotonation can be important events in an enzyme’s catalytic circle. Thus a general acid (general base) catalyst must be in its protonated (deprotonated) state in order to facilitate the catalyzed reaction. The analysis of the pH dependence of kinetic constants could provide valuable information about an enzyme mechanism.

The basis of the effects of pH on enzyme-catalyzed reactions can be summarized as following:

(i) pH-dependence of an enzyme functional group ionization is governed by the Henderson-Hasselbalch equation:

\[ \text{pH} = pK_a + \log \frac{[A]}{[AH]} \]
(ii) Essential catalytic groups in the active-site are often only functional in one of their ionization states; therefore, the effectiveness of an enzyme depends on the concentration of such an active form.

\[
k = \frac{k^{HA}[H^+]}{K_a + [H^+]} + \frac{k^A K_a}{K_a + [H^+]} \quad \text{(equation 4)}
\]

Here, \( k \) is an observable kinetic characteristic, \( k^{HA} \) is the value of this kinetic attribute for the protonated state and \( k^A \) corresponds to the deprotonated state, \( K_a \) is the ionization constant.

For doubly ionizing system a kinetic parameter depends on \([H^+]\) as

\[
k = \frac{k^{H2A}[H^+]^2 + k^{HA} K_{a1}[H^+] + k^A K_{a1} K_{a2}}{K_{a1} K_{a2} + K_a [H^+] + [H^+]^2} \quad \text{(equation 5)}
\]

pH dependence studies have been performed for a number of enzymes involved in phospho-group transfer or hydrolysis (63-68). It has been shown that the polymerization rate of a number of polymerases depends on pH (69-72). However, a detailed analysis has not yet been performed for any nucleotidyl transferases. The objective of the studies presented here is to reveal steps in the kinetic pathway of Pol β that are affected by pH.
5.2 Experimental details

DNA substrates. Primer/template 19/36AP DNA substrate was used in the majority of experiments with two exceptions when 19dd/36AP and 18/35AP DNA substrates were used (Figure 6). All rapid chemical quench and 2AP fluorescence stopped-flow experiment were performed as described in Materials and Methods in Chapter 2.

Reaction buffers. A set of eight reaction buffers with pH varied from 6.2 to 8.8 were prepared according Table 5.1. pH was measured at 37 °C and ionic strength was adjusted with KCl to a value of 81mM. All reactions were performed in the presence of 1mM DTT and 10% glycerol.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>BisTris, mM</th>
<th>Tris, mM</th>
<th>KCl, mM</th>
<th>pH (measured at 37 °C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.2</td>
<td>70.0</td>
<td>0</td>
<td>39.6</td>
<td>6.13</td>
</tr>
<tr>
<td>6.6</td>
<td>60.0</td>
<td>10.0</td>
<td>49.8</td>
<td>6.53</td>
</tr>
<tr>
<td>7.0</td>
<td>42.5</td>
<td>27.5</td>
<td>50.0</td>
<td>6.93</td>
</tr>
<tr>
<td>7.4</td>
<td>27.5</td>
<td>42.5</td>
<td>50.0</td>
<td>7.37</td>
</tr>
<tr>
<td>7.6</td>
<td>20.0</td>
<td>50.0</td>
<td>51.5</td>
<td>7.54</td>
</tr>
<tr>
<td>8.0</td>
<td>0</td>
<td>70.0</td>
<td>56.9</td>
<td>7.93</td>
</tr>
<tr>
<td>8.4</td>
<td>0</td>
<td>70.0</td>
<td>68.9</td>
<td>8.33</td>
</tr>
<tr>
<td>8.8</td>
<td>0</td>
<td>70.0</td>
<td>75.6</td>
<td>8.72</td>
</tr>
</tbody>
</table>

Table 5.1. Composition of the buffer set used for pH studies
Concentration of MgdNTP. Dependence of observed rates on nucleotide substrate concentration was performed by varying the concentration of MgdATP, while keeping the concentration of free Mg\(^{2+}\). Calculation of [MgdATP] was performed using WEBMAXC STANDARD program (http://www.stanford.edu/~cpatton/webmaxcS.htm) with the following parameters: temperature 37 °C, pH varied according the experimental conditions, ionic strength 0.081.

Table 5.2 shows an example of a set of experimental conditions used for nucleotide dependence analysis at pH 8.33.

<table>
<thead>
<tr>
<th>[dATP](_{tot}), µM</th>
<th>[Mg(^{2+})](_{tot}), mM</th>
<th>[MgdATP], µM</th>
<th>[Mg(^{2+})](_{free}), mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00</td>
<td>3.50</td>
<td>0.99</td>
<td>3.50</td>
</tr>
<tr>
<td>2.50</td>
<td>3.50</td>
<td>2.47</td>
<td>3.50</td>
</tr>
<tr>
<td>5.00</td>
<td>3.51</td>
<td>4.94</td>
<td>3.50</td>
</tr>
<tr>
<td>10.0</td>
<td>3.51</td>
<td>9.87</td>
<td>3.50</td>
</tr>
<tr>
<td>25.0</td>
<td>3.53</td>
<td>24.7</td>
<td>3.50</td>
</tr>
<tr>
<td>50.0</td>
<td>3.55</td>
<td>49.4</td>
<td>3.50</td>
</tr>
<tr>
<td>100</td>
<td>3.60</td>
<td>98.8</td>
<td>3.50</td>
</tr>
<tr>
<td>200</td>
<td>3.70</td>
<td>198</td>
<td>3.50</td>
</tr>
<tr>
<td>400</td>
<td>3.90</td>
<td>395</td>
<td>3.50</td>
</tr>
<tr>
<td>750</td>
<td>4.24</td>
<td>741</td>
<td>3.50</td>
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<tr>
<td>1250</td>
<td>4.73</td>
<td>1230</td>
<td>3.50</td>
</tr>
<tr>
<td>1750</td>
<td>5.23</td>
<td>1730</td>
<td>3.50</td>
</tr>
</tbody>
</table>

Table 5.2. Concentrations of MgdATP used for [dNTP] dependence analysis at pH 8.33
Concentration of free Mg$^{2+}$. Dependence of observed rates on free Mg$^{2+}$ (catalytic metal ion) concentration was performed by varying the concentration of [Mg$^{2+}$]$_{\text{free}}$ while adjusting the total concentration of Mg$^{2+}$ to minimize variation of [MgdATP]. The concentrations were calculated using WEBMAXC STANDARD program (http://www.stanford.edu/~cpatton/webmaxcS.htm) with the following parameters: temperature 37 °C, pH varied according the experimental conditions, ionic strength 0.081.

Table 5.3 shows an example of a set of experimental conditions used for catalytic Mg$^{2+}$ dependence analysis at pH 8.33.

<table>
<thead>
<tr>
<th>[Mg$^{2+}$]$_{\text{tot}}$, mM</th>
<th>[dATP]$_{\text{tot}}$, µM</th>
<th>[Mg$^{2+}$]$_{\text{free}}$, mM</th>
<th>[MgdATP], µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.50</td>
<td>500</td>
<td>0.129</td>
<td>371</td>
</tr>
<tr>
<td>0.70</td>
<td>500</td>
<td>0.271</td>
<td>429</td>
</tr>
<tr>
<td>0.90</td>
<td>500</td>
<td>0.446</td>
<td>454</td>
</tr>
<tr>
<td>0.75</td>
<td>500</td>
<td>0.777</td>
<td>473</td>
</tr>
<tr>
<td>1.00</td>
<td>500</td>
<td>1.51</td>
<td>486</td>
</tr>
<tr>
<td>1.25</td>
<td>500</td>
<td>2.51</td>
<td>491</td>
</tr>
<tr>
<td>1.50</td>
<td>500</td>
<td>4.51</td>
<td>495</td>
</tr>
<tr>
<td>2.00</td>
<td>500</td>
<td>6.50</td>
<td>495</td>
</tr>
<tr>
<td>3.00</td>
<td>500</td>
<td>9.50</td>
<td>496</td>
</tr>
<tr>
<td>4.00</td>
<td>500</td>
<td>12.5</td>
<td>498</td>
</tr>
<tr>
<td>5.00</td>
<td>500</td>
<td>15.5</td>
<td>499</td>
</tr>
</tbody>
</table>

Table 5.3. Concentrations of free Mg$^{2+}$ used for [Mg$^{2+}$] dependence analysis at pH 8.33
5.3 Analysis of dependence of observed rates on dNTP concentration

We used 2-aminopurine fluorescence stopped-flow to analyze the rates of fast and slow fluorescence transitions at varied pH ranging from 6.2 to 8.8. An example of the stopped-flow kinetic traces collected at pH 7.54 is shown in Figure 19. In general, the fluorescence change fits well to a double exponentials equation (equation 3), however at higher dNTP concentration and higher pH a triple exponential equation provides the best fit. In the latter case, we were not able to assign the slowest rate to any step in the Pol β reaction pathway, and its nature remains to be clarified. Here it is important to mention that this phase has a relatively small amplitude (less than 10% of the signal) and it is significantly slower than the rate of single-nucleotide incorporation.

Typically, the amplitude of fluorescence change and rates of fast and slow phases increase as the concentration of MgGTP increases. If it is assumed that the closed E′·Dₙ·N complex has higher fluorescence intensity comparing to binary E·Dₙ and E·Dₙ₊₁ complexes (as well as open E·Dₙ·N and E·Dₙ₊₁·P) (73), then a larger amplitude indicates a larger concentration of the closed complex (E′·Dₙ·N). This observation is entirely consistent with the Pol β reaction scheme.
Figure 19. Nucleotide concentration dependent stopped-flow 2-AP fluorescence assays performed at pH 7.54
dATP concentration varied from 1.00 µM to 1.50 mM (final concentrations).
The rate of fast phase shows a hyperbolic dependence on dNTP concentration. For each pH analyzed, the saturations curves were constructed by plotting the observed rate vs. [dATP], which were subsequently fit to a single rectangular hyperbola with a non-zero intercept (equation 7). A representative example of such analysis is shown in Figure 20. According Pol β kinetic mechanism (Figure 5), fitting to equation 7 reveals values of the thermodynamic constant $K_{d}^{dNTP}$, microscopic constant $k_2$ (conformational closing step) and an apparent constant $k_{open}$, which reflects the rate of disappearance of closed E′:D·N complex, and is essentially a combination of the microscopic constants $k_{-2}$ and $k_4$.

\[
k_{fast} = \frac{k_2[N]}{K_d^N + [N]} + \frac{k_{-2}K_d^M}{K_d^M + [M]} + \frac{k_4[M]}{K_d^M + [M]} \quad \text{(equation 6)}
\]

\[
k_{fast} = \frac{k[N]}{K_d^N + [N]} + k_{open} \quad \text{(equation 7)}
\]

In order to focus on the fast conformational step exclusively, we performed similar stopped-flow experiments using dideoxy-terminated 19dd/36AP DNA. Data analysis using hyperbolic equation 7, as well as a global fitting to the Pol β kinetic scheme (Pro-Kineticist II, Applied Photophysics Ltd) results in values of $K_d^{dNTP}$, $k_2$, and $k_{-2}$ which are in a reasonable agreement with the corresponding constants obtained using the 19/36AP DNA substrate.
Figure 20. dATP concentration dependence of the rate of the fast fluorescence change (pH 7.54)

Figure 21. dATP concentration dependence of the rate of the slow fluorescence change (pH 7.54)
The observed rate of the slow phase showed a hyperbolic dependence on dNTP concentration as well (Figure 21). However, the saturation curves fit well to a simple rectangular hyperbola (equation 8), which is in agreement with the kinetic scheme that suggests the following expression for the kinetic parameters \( k_{pol} \) and \( K_{d,app} \).

\[
k_{slow} = \frac{k_{pol} [N]}{[N] + K_{d,app}} \quad \text{(equation 8)}
\]

\[
k_{pol} = \frac{k_2 k_4}{k_2 + k_4 + \frac{k_{-2} K_d^M}{K_d^M + [M]}}
\]

\[
K_{d,app} = K_d^N \frac{k_4 + \frac{k_{-2} K_d^M}{K_d^M + [M]}}{k_2 + k_4 + \frac{k_{-2} K_d^M}{K_d^M + [M]}}
\]
The kinetic and thermodynamic parameters obtained from the analysis of nucleotide concentration dependence of the fluorescence transients at different pH are summarized in Table 5.5 (Section 5.7).

To insure that the rate of slow phase matches the rate of chemistry, we performed rapid chemical quench assays at varied pH. This analysis was done using two different DNA substrates: 19/36AP (dATP as incoming nucleotide) and 18/35AP (dCTP as incoming nucleotide). The experiments were conducted at 37 °C as well as at 25 °C. For all sets of conditions the rate of single-nucleotide incorporation (as determined by the rapid quench) were found to be very similar to the rate of the slow phase of fluorescence (as determined in the stopped-flow). Figure 22 and Table 5.4 show such similarities in rates observed for dCTP incorporation into 18/35AP DNA substrate at 25 °C and varied pH.
Figure 22. pH dependence of the observed rate constants of single-nucleotide incorporation $k_{RQ}$ and the slow phase of fluorescence change $k_{slow}$.

<table>
<thead>
<tr>
<th>pH</th>
<th>$k_{RQ}$, s$^{-1}$ (determined in rapid quench)</th>
<th>$k_{slow}$, s$^{-1}$ (determined in stopped-flow)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.63</td>
<td>0.182</td>
<td>0.178</td>
</tr>
<tr>
<td>6.94</td>
<td>0.569</td>
<td>0.573</td>
</tr>
<tr>
<td>7.40</td>
<td>1.59</td>
<td>1.62</td>
</tr>
<tr>
<td>7.57</td>
<td>2.71</td>
<td>2.80</td>
</tr>
<tr>
<td>7.99</td>
<td>5.95</td>
<td>6.36</td>
</tr>
<tr>
<td>8.40</td>
<td>16.7</td>
<td>13.5</td>
</tr>
<tr>
<td>8.79</td>
<td>20.5</td>
<td>21.3</td>
</tr>
</tbody>
</table>

Table 5.4. Observed rate constants of single-nucleotide incorporation ($k_{RQ}$) and slow phase of fluorescence change ($k_{slow}$) at different pH.
5.4 Analysis of dependence of observed rates on the catalytic magnesium ion concentration

To study [Mg$^{2+}$] effect on kinetics of single-nucleotide incorporation, we performed a series of 2-aminopurine fluorescence stopped-flow experiments at varied pH. In each experiment we varied the concentration of free Mg$^{2+}$ while trying to maintain the saturated concentration of MgdNTP. Figure 23 demonstrates a representative series of such experiments that were performed at pH 7.54. In most cases, traces fit well to a double exponential equation (equation 3). However, at higher pH the kinetic traces showed triple exponential time dependence. As mentioned above, the slowest rate obtained from such a triple exponential fit was significantly slower than the rate of single nucleotide incorporation and could not be reasonably assigned to any step in Pol $\beta$ reaction pathway.

At all pH studied, the amplitude of fluorescence change had its maximum at low [Mg$^{2+}$] and decreased to some degree with increasing [Mg$^{2+}$]. This observation is entirely consistent with Pol $\beta$ kinetic scheme (Figure 5): the formation of closed ternary complex – a species with more intensive fluorescence signal – does not require presence of the catalytic metal ion. On the other hand, saturation of the enzyme active site with catalytic Mg$^{2+}$ results in faster chemistry, and therefore reduces accumulation of the closed complex. Similarly, the fluorescence change amplitude decreases as the rate of single-nucleotide incorporation increases at high pH.
Figure 23. Magnesium concentration dependent stopped-flow 2-AP fluorescence assays performed at pH 7.54.

Free Mg$^{2+}$ concentration varied from 130 µM to 15.5 mM (final concentrations)
The rate of the fast phase does not have a simple dependence on free Mg\(^{2+}\) concentration. According to the kinetic scheme (Figure 5), if microscopic rate constant \(k_2\) is larger than \(k_4\) (chemical step), then the fast rate should decrease upon increasing [Mg\(^{2+}\)] (equation 6). *Vice versa*, if chemistry is faster than “subdomain opening” \((k_2 < k_4)\), then the rate of the fast fluorescence change should increase with increasing [Mg\(^{2+}\)] (43). Therefore one could expect that at low pH (when the rate of dNTP incorporation is slowed), the fast fluorescence rate would decrease as [Mg\(^{2+}\)] increases. One could also expect that at high pH, it would increase with increasing concentrations of catalytic Mg\(^{2+}\).

In reality, at all pH the dependence of the fast phase rate constant on [Mg\(^{2+}\)] has very similar character: first, there is an increase in rate with increasing [Mg\(^{2+}\)], and then the rate steadily decreases (Figure 24). A possible explanation for this unexpected behavior is that Mg\(^{2+}\) has an inhibitory effect (see more discussion below). The small increase in rate at the first points in the Mg\(^{2+}\) titration can be attributed to the fact that the concentration of MgdNTP complex might be non-saturated under reaction conditions of low [Mg\(^{2+}\)]. It is interesting to note that the maximum value of the fast fluorescent phase does not have a pronounced pH-dependence (Figure 25).
Figure 24. Mg$^{2+}$ concentration dependence of the rate of the fast fluorescence change

Figure 25. Absence of pronounced pH effect on the maximum observed rate constant of
the fast fluorescence transition
According to the proposed Pol β kinetic mechanism (Figure 5), the rate of the slow phase of fluorescence change should follow a hyperbolic dependence on magnesium ion concentration. Indeed, the analysis of the slow phase dependence reveals that in pH range from 6.2 to 7.4 it fits to a simple rectangular hyperbolic equation. A representative example of such analysis is shown in Figure 26. However, at higher pH the Mg\(^{2+}\) becomes inhibitory before it reaches saturating concentration. Nevertheless, applying Eadie-Hofstee linearization technique, we were able to obtain \(K_{d\text{app}}^{\text{Mg}}\) and \(k_{\text{pol}}\) parameters for all pH analyzed. An example of \([\text{Mg}^{2+}]_{\text{free}}\) dependence of the slow rate and Eadie-Hofstee linearization (\(k_{\text{slow}}\) vs. \(k_{\text{slow}}/[\text{Mg}^{2+}]_{\text{free}}\)) is shown in Figure 27. The kinetic parameters that have been obtained from the analysis of magnesium concentration dependence are summarized in Table 5.5 (Section 5.7)
Figure 26. Free Mg$^{2+}$ concentration dependence of the rate of slow fluorescence change at pH 7.0 (The inset shows Eadie-Hofstee plot.)

Figure 27. Free Mg$^{2+}$ concentration dependence of the rate of slow fluorescence change at pH 8.72 (The inset shows Eadie-Hofstee plot.)
5.5 Mg\textsuperscript{2+} inhibition

The actual nature of the inhibitory Mg\textsuperscript{2+} effect on Pol β catalysis cannot be understood completely based on our current knowledge. However, we can exclude some possibilities. First, the ionic strength effect can be ruled out. Even though addition of extra KCl slows down rates of both fluorescence phases, the ionic strength must be nearly doubled (from 80 to 150 mM) to produce the same effect as the addition of 10mM MgCl\textsubscript{2}. Second, the assumption that the binding of one extra Mg\textsuperscript{2+} ion by Pol β results in enzyme inhibition is not likely to be correct. Analysis of the Mg\textsuperscript{2+} titration data using a model for this type of substrate inhibition (equation 9) demonstrates that the experimental data does not agree with the model.

\[
    k_{\text{slow}} = \frac{k_{\text{pol}} [Mg]}{K'_d + [Mg] + \frac{[Mg]^2}{K_i}} \quad (\text{equation 9})
\]

It appears that Mg\textsuperscript{2+} inhibition is a general phenomenon. In almost all plots of enzyme activity as a function of magnesium ion cofactor concentration, the initial increase in activity with increasing [Mg\textsuperscript{2+}] is followed by a steady decrease in activity (48). Such Mg\textsuperscript{2+} inhibition phenomenon can be possibly explained as a form of competition for the substrate binding between the metal ion and enzyme. It is likely that DNA polymerases respond to high Mg\textsuperscript{2+} concentration in a similar to other enzymes manner. Therefore one of the hypotheses that can reasonably explain the inhibitory effect of Mg\textsuperscript{2+} is a depletion of available MgdNTP substrate at high Mg\textsuperscript{2+} concentration due to
Mg$_2$ dNTP formation (MgdNTP + Mg ⇌ Mg$_2$ dNTP, log$K_{eq} = 1.7$ or $K_d = 20$ mM (74)).

An alternative possibility is suggested by results of kinetic assays performed at pH 9.2. At this pH, 10mM Mg$^{2+}$ has a large inhibitory effect, such that the rate of the slow phase obtained in stopped-flow is reduced from 32.84 s$^{-1}$ (at optimal Mg$^{2+}$ concentration) to 7.37 s$^{-1}$. Interestingly enough, the rate obtained in the rapid quench experiment reduces only to approximately 18 s$^{-1}$. This observation suggests that Mg$^{2+}$ influences the rate of the post-chemistry conformational change (observed in stopped-flow experiments) to a greater extent than the rate of chemistry (measured in rapid quench experiments).

Therefore, it is possible that Mg$^{2+}$ dissociation is required for the post-chemistry conformational step. However, the results of experiments performed at pH 9.2 might be questionable because Pol β becomes rather unstable at this pH.

5.6 pH profiles of kinetic constants

The pH-effect on the values of kinetic and thermodynamic constants was analyzed over pH range of 6.2 – 8.8. The following kinetic and thermodynamic parameters were determined (Table 5.5):

$K_d^{dNTP}$ – a dissociation constant corresponded to ground binding of dNTP by Pol β·DNA binary complex $E:D_n + N \Leftrightarrow E:D_n:N$

$K_{d_{app}}^{dNTP}$ – a kinetic constant that reflects the equilibrium concentration of $E':D_n:N:M$ species.

$k_2$ – a microscopic rate constant of the subdomain-closing conformation step
\( k_{\text{open}} \) – a kinetic constant that reflects the rate of disappearance of the closed E’·D·N complex

\( K_{d\text{ app}}^{\text{Mg}} \) – a dissociation constant corresponded to the catalytic metal binding to Pol β·DNA·dNTP closed ternary complex E’·D·N + M ⇌ E’·D·N·M

\( k_{\text{pol}} \) – a maximum value of \( k_{\text{slow}} \) obtained from dependence of the rate of the slow fluorescence transition on free Mg\(^{2+}\) concentration (equal to a maximum value of the rate constant of single nucleotide incorporation under standard experimental conditions)

\( k_4 \) – an estimated microscopic rate constant of the chemical step.

<table>
<thead>
<tr>
<th>pH</th>
<th>( K_d \text{ dNTP, \mu M} )</th>
<th>( K_{d\text{ app}} \text{ dNTP, \mu M} )</th>
<th>( k_2, \text{s}^{-1} )</th>
<th>( k_{\text{open}}, \text{s}^{-1} )</th>
<th>( K_{d\text{ app}} \text{ Mg, mM} )</th>
<th>( k_{\text{pol}}, \text{s}^{-1} )</th>
<th>( k_4, \text{s}^{-1} ) estimated</th>
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<td>0.304</td>
<td>34.3</td>
<td>64.5</td>
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</table>

Table 5.5. Kinetic and thermodynamic parameters obtained at different pH
As shown in Figure 28, the rate of the conformational step does not have a distinct pH-dependence in the range from 6.2 to 8.8. There are several potentially ionisable amino acid residues that are involved in key events during the course of conformational closing. For example, Tyr 271 moves upon subdomain closing, breaking a bond with the template and forming new bond with the primer 3’-end base. Another example is the repositioning of Arg258 mentioned earlier: Arg258 forms a salt bridge with Asp292 in the open enzyme conformation, but it makes a new interaction with Tyr296 in the closed conformation (33). However, the absence of the pH effect on the rate of the first fluorescent transition suggests that the protonation/deprotonation state of these amino acid residues does not change within the analyzed pH range.

Figure 28. pH profile of the microscopic rate constant $k_2$ (red circles, ●) and kinetic constant $k_{\text{open}}$ (blue triangles, ▲).
It is interesting to notice that $K_d^{dNTP}$ decreases more than ten fold (from 166 µM to 6.86 µM) when the pH changes from 7.6 to 8.8 (see Table 5.5 and Figure 29). This suggests that one deprotonation is required for dNTP ground state binding. It is not very likely that this ionization corresponds to the substrate deprotonation. (The pKa value of MgATP is around 4.8 (58)). Analysis of the crystal structure of binary Pol β-MgdNTP complex (21) indicates that dNTP could bind initially in the active site by making interactions with Ser180, Arg183, Cly189, Arg149, Asp190. It is not quite clear whether the deprotonation of any of these residues is responsible for the observed pH dependence of $K_d^{dNTP}$. There is a possibility that apparent decreased $K_d^{dNTP}$ is just a consequence of

![Figure 29. pH profile of dNTP dissociation constant $K_d^{dNTP}$](image)
Mg$^{2+}$ inhibition. Even though [dNTP] dependence experiments were performed at the optimal [Mg$^{2+}$] for each pH, this Mg$^{2+}$ concentration might be already inhibitory by substantially reduce the concentration of the available Mg:dNTP. This would result in apparent lowering of $K_{d}^{\text{dNTP}}$.

The rate constant of the slow phase increases from 1.08 s$^{-1}$ at pH 6.2 to 34.3 s$^{-1}$ at pH 8.8 (Figure 30). The profile fits well to a single deprotonation model (equation 4) and results in $\text{pKa}_{\text{app}} = 7.74$. This suggests that at least one deprotonation of the substrates-enzyme quaternary E·D·N·M complex is required for the catalysis. At the same time the $K_{d}^{\text{app}}_{\text{Mg}}$ decreases from 18.7 mM at pH 6.2 to 0.304 mM at pH 8.8 (Figure 31). Analysis of the pH dependence using a single deprotonation equation 4 provides $\text{pKa}_{\text{app}}$ value close to 5.8. Nevertheless, since this value is out of the experimentally analyzed pH range, it should be considered only as a rough approximation.
Figure 30. pH profile of the kinetic rate constant $k_{pol}$

Figure 31. pH profile of Mg$^{2+}$ dissociation constant $K_{d_{app}}^{Mg}$
As shown in Figure 32, $k_{pol}/K_{d\text{ app}}^{\text{Mg}}$ increases rapidly as pH increases, also suggesting a deprotonation event that happens prior to catalytic Mg$^{2+}$ binding. The $k_{pol}/K_{d\text{ app}}^{\text{Mg}}$ pH profile can not fit satisfactory to either single or double ionization models (equations 4 and 5). However, linearization of the pH dependence by plotting the logarithm of $k_{pol}/K_{d\text{ app}}^{\text{Mg}}$ vs. pH reveals a slope equal to 1.8, which suggests that two deprotonation events might take place. The lack of success in data fitting to a double ionization model could be due to the fact that one ionization occurs at a pH below the analyzed range. Therefore, it was impossible to deconvolute pKa for individual residues.

Figure 32. Effect of pH on $k_{pol}/K_{d\text{ app}}^{\text{Mg}}$
5.7 Conclusion

The rate of single-nucleotide incorporation is greatly influenced by pH. The data presented here provides a detailed characterization of pH dependence of the steps in the Pol β reaction pathway. It was found that the conformational closing is not affected by pH ($k_2$ is pH independent), whereas chemistry appeared to be very sensitive to pH change. The pH profile of $k_{pol}$ shows that the rate of single-nucleotide incorporation decreases by a factor of 10 per pH unit. The pH dependence curve shows a point of inflexion at a pH 7.74, implying that the catalysis depends on a functional group that has a pKa close to 7.7.

It has been proposed that Pol β facilitates the nucleotidyl transfer reaction by general base catalysis with a base accepting proton from 3’-OH group of the primer concomitant with attack on the α-phosphate of the incoming nucleotide triphosphate. Our results suggest that a group with a pKa of 7.7 could be such a general base. On the basis of Pol β crystal structures (Figure 33) and computational studies, it was proposed that Asp 256 plays the role of the general base (24, 33). New high-resolution structure of Pol β pre-catalytic complex (75) revealed that the attacking nucleophile and Oδ2 of Asp256 are indeed in close proximity (2.8 Å). Therefore these two atoms could form a hydrogen bond, and Asp256 could effectively abstract proton from 3’-OH. However, Asp256 is involved in binding of catalytic Mg$^{2+}$, and such interaction with a metal ion would decrease the ability of Asp256 to be a proton acceptor (30) (or Asp256 in its protonated state would have decreased affinity to catalytic Mg$^{2+}$).
Figure 33. Details of Pol β active site organization (15)
The crystal structure of Pol β·DNA·dUPNPP complex (75) indicates that O2 of α-phosphate and 3’ OH are also in hydrogen bonding distance (3.13 Å). Earlier, quantum chemical calculations of the nucleotidyl transfer reaction catalyzed by Pol β suggests that O2 of the incoming nucleotide α-phosphate may play the role of a general base, accepting the proton from the 3’-hydroxyl (76). It is proposed that this proton is redirected toward the β-phosphate group, which creates an additional stabilization of negative charge that is localized on the departing pyrophosphate. A similar role of a substrate as a general base has been suggested for GTP hydrolysis by Ras p21 (77).

Another possibility is that the pKa = 7.7 corresponds to ionization of a water molecule bound to the catalytic Mg$^{2+}$ (Figure 33), which implies specific base catalysis (similar to the proposed mechanism of 3’-5’ exonuclease of KF) (78). This Mg-coordinated water is only 2.81 Å apart from the 3’- OH, which make it a reasonable proton acceptor (75). Alternatively, the pH dependence of $k_{pol}$ may be related to the pKa value of the 3’-OH hydroxyl itself. In any case, the environment of the protein would have to perturb the pKa of possible ionizable groups by several pH units. (For example, the pKa of free side chain of aspartyl residue is 3.86; and pKa of Mg(H$_2$O)$_6^{2+}$ is 11.42). Taking into account that the active site carries several negatively charged groups, it is reasonable to assume that a pKa of Asp or α-phosphate might be significantly higher. In structures where several charged groups reside in close proximity, the side-chain pKa values may be drastically perturbed. Indeed, short distance between two electronegative atoms would require a large energetic penalty, which could be abated by placing a proton between the negatively charged atoms (79). An illustration for such perturbation of pKa
values can be demonstrated by low molecular weight dicarboxylic acid models. The first and second pKa values of maleic acid (cys isomer) are perturbed to lower and higher values respectively relative to those of fumaric acid (trans isomer), where intramolecular hydrogen bonding is not possible (Figure 34) (79). In the case of Pol β active site, Pα oxygen could share proton with a non-bridging oxygen of β-phosphate (3.0 Å), a γ-phosphate oxygen (3.1 Å) or Asp192 (3.1 Å). A pKa of Asp256 could be also easily perturbed because of potential proton shearing with Asp190 (3.0 Å) or Asp192 (3.1 Å). Therefore both the α-phosphate and Asp256 may be reasonable candidates to play the role of a general base accepting the proton from the 3’-OH (24, 76).

Figure 34. Perturbation of pKa values in model dicarboxylic acid systems
(a) maleic acid; (b) fumaric acid (79)
It is important to point out that, even though the protonation state of the group with pKa\textsuperscript{app} = 7.7 greatly influences the rate of chemistry, this functional group does not necessarily need to act as a general base. It is obvious that protonation of \textit{any} magnesium-binding ligands in the Pol \( \beta \) active site would significantly change bond lengths between the metal and ligands. Therefore, the active site geometry might be less optimal for catalysis if one of the groups is protonated.

The pH profile of \( k_{\text{pol}}/K_{\text{d,app}}^{\text{Mg}} \) revealed that at least two deprotonation events are required for effective transition state stabilization. One ionizable functional group appears to have a pKa near 7.7, which probably corresponds to the same deprotonation event that was observed for the pH dependence of \( k_{\text{pol}} \). The second ionizable functional group has an estimated pKa value of 5.8. Since this value is outside of the pH range analyzed, it was impossible to deconvolute pKa’s for individual residues from the \( k_{\text{pol}}/K_{\text{d,app}}^{\text{Mg}} \) pH profile.

The second ionization event could correspond to a residue in closed ternary complex that needs to be deprotonated for the effective binding of the catalytic metal ion. One of the possible candidates might be Asp256. This residue provides its carboxylic oxygen for catalytic Mg\textsuperscript{2+}, but not for the nucleotide binding metal ion. Therefore Mg\textsuperscript{2+}NTP binding and the conformational closing should not depend on the protonation state of Asp256. If efficient binding of the catalytic metal ion indeed requires deprotonation of Asp256, then it is unlikely that Asp256 can act as a general base by accepting a proton from the 3’-OH of a DNA substrate.
Deprotonation of Asp190 or Asp191 may not be entirely excluded either. It is interesting to note that the $K_d^{dNTP}$ substantially decreases upon increasing pH. Even though values of $K_d^{dNTP}$ obtained from dNTP concentration dependence of the fast fluorescence phase are somehow doubtful because of the Mg$^{2+}$ inhibitory effect at high pH, the observed trend in $K_d^{dNTP}$ values might support the assumption that Asp190 or Asp191 ionization is required for the binding of both active-site metal ions.

In conclusion, the pre-steady state kinetic pH dependence studies of Pol β demonstrate that pH selectively influences different steps in Pol β reaction pathway, which makes pH an additional variable in probing Pol β mechanism. Importantly, stopped-flow results for Pol β suggest that the relative rates of microscopic steps can significantly vary as a function of assay conditions. For example, fingers subdomain closure is faster than chemistry by a factor of 20 at pH 6.2 and by less than a factor of 4 at pH 8.0, while the rates of both steps approach equality at pH near to 8.8. This fact can be important both during kinetic analyses of individual enzymes and also when attempting to make comparisons between different enzymes.
CHAPTER 6

USE OF REACTION BUFFER VISCOSITY AS A PROBE OF
CONFORMATIONAL STEPS

6.1 Application of viscogens in Pol β pre-steady state kinetic analysis

Solvent viscosity as a probe of steps involving enzyme conformational changes has been successfully used in steady-state experiments (80-84). If an enzyme reaction pathway includes a step involving spatial movements of the enzyme’s segments, then by using an assay buffer with altered viscosity this step can be selectively perturbed (84, 85). Here we applied variable buffer viscosity in single-turnover stopped-flow fluorescence studies of DNA polymerase β.

DNA substrates. We used two fluorophore probes – tryptophan and 2-AP – to monitor fluorescence change associated with different Enzyme-DNA conformations during the reaction pathway. As it has been mentioned previously (44), the 18/36AP DNA substrate provides the best signal-to-noise ratio in tryptophan fluorescence assays. The best signal-to-noise ratio in the 2-AP fluorescence stopped-flow experiments can be obtained with 2-AP modification in +1 position relative to the nascent base pair (44). Therefore 19/36AP and 18/35AP DNA substrates (Figure 6) were chosen for this work.
Reaction conditions. Assays were performed in one of the buffers described in Chapter 5 (Table 5.1) at 37 °C or in a buffer containing 50 mM KCl and 100 mM Mops, pH 7.0 at 25 °C. All reactions were performed in the presence of 1 mM DTT and a varied concentration of glycerol or sucrose. All rapid chemical quench and fluorescence stopped-flow experiments were performed as described in Materials and Methods (Chapter 2). If not specified otherwise, a typical reaction mixture would consist of 500 nM Pol β, 200 nM DNA, 400 µM dNTP, and 10 mM MgCl₂ (all final concentrations).

6.2 The fast fluorescence phase is slowed down by high reaction buffer viscosity

First, we used sucrose, a common viscogen, to probe the observed biphasic change of tryptophan fluorescence. These experiments were conducted under the conditions that were used in Rh(III)dNTP assays (pH 7.0 at 25°C, see Chapter 3 for details). As shown in Figure 35, the rate of the fast fluorescence transition decreased noticeably (from 61.6 s⁻¹ to 42.2 s⁻¹) as the sucrose concentration increased (from 0 % to 30 %). At the same time, the slow fluorescence transition did not slow down at all (0.591 s⁻¹ and 0.644 s⁻¹ in the buffer containing 0 % or 30 % sucrose, respectively).
Figure 35. Effect of reaction buffer viscosity in the stopped-flow tryptophan fluorescence assay

A double exponential fit resulted in parameters: standard reaction buffer (red ●) $k_{\text{fast}} = 61.6 \text{ s}^{-1}$ and $k_{\text{slow}} = 0.591 \text{ s}^{-1}$; 30% sucrose (cyan ○) $k_{\text{fast}} = 42.2 \text{ s}^{-1}$ and $k_{\text{slow}} = 0.644 \text{ s}^{-1}$.

Secondly, a 2-AP fluorescent probe was used to monitor changes in Enzyme-DNA conformation during the reaction pathway. As can be seen in Figure 36 and Table 6.1, the rate of the fast fluorescence transition decreased significantly with increasing sucrose percentages in the reaction buffer, while the slow transition rate remained almost unperturbed upon buffer viscosity variation.
In order to make sure that the observed effect is caused by the change of buffer viscosity rather than by an interaction with sucrose, we performed analogous stopped-flow experiments with varied concentrations of another viscogen - glycerol. As can be seen in Figure 37 and Table 6.1, glycerol has a similar effect on the rates of the fast fluorescence transitions. These results suggest that the fast fluorescence transition corresponds to a conformational change step and this step involves considerable physical movement of the Pol β·DNA complex. It is not very likely that a step, which involves only subtle active site rearrangements, could be perturbed by the higher viscosity. Therefore, our results support the hypothesis that the fast fluorescence transition corresponds to the subdomain-closing conformational change, and that the slow fluorescence transition is likely related to the rate of the chemistry step.
Figure 36. Effect of buffer viscosity in stopped-flow 2-AP fluorescence assay at pH 7.0

Bars show % change of fast fluorescence transition (pink) and slow fluorescence transition (cyan) vs. % (w/v) sucrose in the reaction buffer.

<table>
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<tr>
<th></th>
<th>No viscogen</th>
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<th>20% sucrose</th>
<th>30% sucrose</th>
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<td>$k_{slow}, s^{-1}$</td>
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<table>
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<td>0.892</td>
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Table 6.1. Effect of buffer viscosity on the rates of the fast and slow phases of 2-AP fluorescence change in stopped-flow assays

Assays were performed at pH 7.0.
Figure 37. Effect of the presence of sucrose or glycerol on the rate of the fast phase in stopped-flow 2-AP fluorescence assays

Red triangles ▲: $k_{fast}$ at varied concentration of sucrose

Blue circles ●: $k_{fast}$ at varied concentration of glycerol

The above results have found additional support from experiments with the substrate analogs dNTP\(\alpha\)S (performed by a former member of our group Soojin Lee) (86). The use of dCTP\(\alpha\)S in fluorescence stopped-flow assays showed that the fast phase rate is similar in cases of both natural substrate and thio-analog ($k_{fast} = 13.9 \text{ s}^{-1}$ for dNTP and $k_{fast} = 16.3 \text{ s}^{-1}$ for dNTP\(\alpha\)S). In contrast, the rate of the slow fluorescence transition was significantly slower in the reaction with dNTP\(\alpha\)S ($k_{slow} = 0.72 \text{ s}^{-1}$ for dNTP and $k_{slow} = 0.10 \text{ s}^{-1}$ for dNTP\(\alpha\)S). It is more likely that the chemical step would be perturbed by
the dNTPαS substrate analog, while a conformational change step would be unaffected. The data obtained in this work further support our interpretation that the second (slow) fluorescence transition reflects the rate of the chemistry step because the slow phase is perturbed differentially by dNTPαS.

The application of dNTPαS and reaction buffer with altered viscosity as mechanistic probes in the stopped-flow experiments allowed us to confirm the assignment of the slow and fast fluorescence transitions as the events reflecting the chemical and conformational change steps respectively. Thus, the stopped-flow experimental data indicate that the rate of the fast fluorescence transition can be affected by the presence of viscogens, whereas the slow transition remains insensitive to such agents. Complementarily, the application of the thio-substituted nucleotide analogs results in a decrease in the rate of the slow phase, while leaving the fast phase unaffected. Since viscosity is generally believed to perturb physical steps (conformational change), and dNTPαS is expected to selectively affect the chemical step, we can safely assign the slow fluorescence change to an event that has the rate of chemistry.

It is important to point out that the slow fluorescence transition is not likely caused by the phosphodiester bond formation directly. This is evident from the fact that this transition is observed in both 2-AP and tryptophan fluorescence assays, and that the tryptophan residue (Trp325) of Pol β is remote from the site of the chemical reaction. Instead, the fluorescence change is probably caused by another conformational change occurring after chemistry. Because the rate of the fluorescence change is limited by
chemistry, we suggest that the rate of the corresponding conformational change is faster than the chemical step under the employed experimental conditions.

### 6.3 Viscosity effect on the fluorescence changes at higher pH

The slow phase, which is not affected by the reaction buffer viscosity at pH 7.0, becomes sensitive to increased glycerol concentrations at pH 8.4 (Figure 38). This observation suggests that the rate-limiting step might change at higher pH, such that at high pH a conformational step controls the rate of the slow phase to greater extent than the chemistry does. Using a variety of reaction conditions (pH, temperature, various dNTP, and DNA substrate), we have observed that the slow fluorescence transition always matches (within experimental error) the rate of single-nucleotide incorporation determined in rapid chemical quench experiments (Figure 22 in Chapter 5). Therefore, the sensitivity of the slow phase to the increased buffer viscosity could be indicative that Pol β does have a pre-chemistry conformational change that becomes rate-limiting at high pH. Alternatively, the slow phase could originate from a slow post-chemistry conformational change. In order to clarify the origin of the viscosity effect on the fluorescence changes at higher pH, we performed rapid quench assays at pH 8.4 in reaction buffer containing 10% glycerol or 35% glycerol, as described in the section below.
Figure 38. Effect of reaction buffer viscosity in stopped-flow 2-AP fluorescence assays at pH 7.0 and pH 8.4

Bars show % change of fast fluorescence transition (pink) and slow fluorescence transition (cyan) vs. % (w/v) glycerol in the reaction buffer. (A) Assays were performed at pH 7.0. (B) Assays were performed at pH 8.4.
6.4 Viscosity effect on the rate of single-nucleotide incorporation: dissection of the chemical and slow conformational steps

Here we compare the results of the 2-AP fluorescence stopped-flow and rapid quench assays performed at pH 8.4 in reaction buffer containing either 10 % glycerol or 35 % glycerol (Figure 39 and Table 6.1): it can be seen that the rapid quench rate of dNTP incorporation is not affected by viscosity. The rate constant $k_{RQ}$ determined under the conditions of increased viscosity (35 % glycerol) equals 24.0 s$^{-1}$, which agrees within the experimental error with the values of 26.9 s$^{-1}$ and 24.3 s$^{-1}$ for the rapid quench and stopped-flow assays determined under standard conditions (10 % glycerol). In contrast, the rate constant of the slow phase determined under the conditions of increased viscosity in stopped-flow equals 7.89 s$^{-1}$, which is 3-fold slower than $k_{RQ}$.

To exclude the possibility of inefficient quenching by EDTA under the altered reaction conditions, we performed the rapid quench experiments with two alternative quenchers: formamide and hydrochloric acid. The acid quench resulted in very similar rate constants, whereas formamide appears to be a poor quencher.
Figure 39. Effect of reaction buffer viscosity in rapid acid quench assays.

Assays were performed at pH 8.4 in the reaction buffer containing 10% glycerol (red ●) or 35% glycerol (blue ●).

<table>
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<td>22.41</td>
<td>26.9</td>
<td>24.0</td>
</tr>
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</table>

Table 6.2. Rates of the slow fluorescence transition (obtained in stopped-flow experiments) and rates of single-nucleotide incorporation (obtained in rapid quench experiments). Assays were performed at pH 8.4.
Comparison of the rapid quench and stopped-flow data indicates that the slow fluorescence change originates from a conformational step following chemistry. Therefore by increasing the rate of chemistry (increasing of pH to 8.4) and selectively slowing down the rate of conformational changes (increasing the reaction buffer viscosity by addition 35% glycerol), we have dissected the two steps by creating a situation in which the rapid chemical quench rate of dNTP incorporation is faster than the rate of the second fluorescence change.

Although the slow phase of the fluorescence change could reflect any step after chemistry (subdomain reopening, PPi release, or DNA repositioning), we hypothesize that it corresponds to the subdomain-reopening conformational change because:

(i) Use of different fluorophores and DNA substrates reveal that the direction of the slow fluorescence change is always opposite to the direction of the fast fluorescence change corresponding to the subdomain-closing step (44).

(ii) The rate of the slow phase demonstrates a similar sensitivity to reaction buffer viscosity as does the rate of the fast subdomain-closing conformational change.

(iii) It is not very likely that DNA translocation causes the slow fluorescence phase, because the slow phase is still present when a gapped DNA substrate is used. Since the product of single-nucleotide incorporation into a gapped DNA substrate is a nicked DNA, the next nucleotide incorporation should not occur and therefore DNA translocation should not occur either. Even if DNA repositioning causes the fluorescence change, it
is likely that the DNA translocation event is coupled with the subdomain-
reopening conformational change (as suggested in recent structural studies
of T7 RNA Polymerase (87)).

6.5 Relaxation of the viscosity effect by decreasing magnesium concentration

Assuming that the viscosity effect at pH 8.4 is due to the selective increase of the
rate of the chemical step at high pH, one can hypothesize that keeping the same pH (8.4)
but selectively slowing down the rate of chemistry should relax the sensitivity of the slow
phase to the buffer viscosity. In Chapter 5, we demonstrated that the slow phase observed
in stopped-flow has a hyperbolic dependence on free Mg\textsuperscript{2+} concentration. If it is the rate
of a post-chemistry conformational step that is directly affected by Mg\textsuperscript{2+} concentration,
then the viscosity effect should be preserved under non-saturated Mg\textsuperscript{2+} concentration. On
the other hand, if reduced concentration of free Mg\textsuperscript{2+} selectively slows down the chemical
step (which is predicted by the proposed kinetic scheme for single nucleotide
incorporation by Pol β in Figure 5), then the second phase of fluorescence change should
become less sensitive to viscosity as free Mg\textsuperscript{2+} is decreased.

We tested the viscosity effect at \([\text{Mg}\textsuperscript{2+}]_{\text{free}} = 0.27 \text{ mM}\) and pH 8.4 and found that the
slow phase rate was not affected by an increase in viscosity up to 30% glycerol (Figure
40). However, at higher glycerol concentrations the viscosity effect became pronounced
again. Therefore, decreasing the magnesium concentration results in relaxation of the
viscosity effect. These results are very similar to the viscosity effect at pH 7.0, where
chemistry is also selectively slowed down.
Figure 40. Viscosity effect on the slow phase under different reaction conditions

Blue (●): pH 8.4 and [Mg$^{2+}$]$_{free}$ = 9.6 mM
Cyan (●): pH 8.4 and [Mg$^{2+}$]$_{free}$ = 0.27 mM
Red (●): pH 7.0 and [Mg$^{2+}$]$_{free}$ = 9.6 mM

6.6 Conclusion

The application of viscogens to probe the fast and slow phases in fluorescent stopped-flow assays, along with the complementary results of experiments with dNTPαS nucleotide analogs, strongly support our assignment of the fast phase of the fluorescence change to the global conformational rearrangement required to form the closed Pol β ternary complex. Comparison of the Pol β pre- and post-chemistry (i.e. prior to the pyrophosphate release) ternary complexes reveals negligible structural changes, (26)
suggesting that phosphodiester bond formation itself is not responsible for the slow fluorescence transition. We, therefore, hypothesize that the slow fluorescence transition corresponds to a conformational change occurring after chemistry. Note, that this post-chemistry conformational change would be insensitive to altered viscosity as long as a preceding step remained rate-limiting. This would explain why the slow fluorescence transition was unaffected by increased viscosity at pH 7.0. To confirm that the event responsible for the slow fluorescence transition is not the chemical step itself, we attempted to differentially perturb the rate of chemistry and the rate of the slow fluorescence transition. By changing the buffer pH (from 7.0 to 8.4) we increased the rate of Pol β-catalyzed single dNTP incorporation. By concurrently increasing the buffer viscosity (from 10% to 35% glycerol), we selectively slowed down conformational steps, creating a situation in which the rate of dNTP incorporation (determined by rapid quench) is faster than the rate of the second fluorescence change in stopped-flow. This dissection of the rate of chemistry and the rate of the slow fluorescence change is consistent with our hypothesis that the slow fluorescence change reflects a conformational step after chemistry (most likely the subdomain reopening).
CHAPTER 7

KINETIC ANALYSIS OF KLENOW FRAGMENT REACTION PATHWAY

7.1 Klenow Fragment of *E. coli* DNA Polymerase I: Background

DNA polymerase I of *E. coli*, discovered in 1956 by Arthur Kornberg, is the first identified DNA polymerase (88). It was also the first DNA polymerase with a solved crystal structure (89) and the first DNA polymerase for which a detailed kinetic mechanism was established (6).

DNA polymerase I is a high fidelity enzyme that makes on average only one error per 10⁵-10⁶ nucleotides. It belongs to the Family A of DNA polymerases and is an essential enzyme which plays two major roles in *E. coli*: the processing of Okazaki fragments and DNA repair. According to its biological roles, DNA polymerase I demonstrates three enzymatic activities: 3'→5' exonuclease, 5'→3' exonuclease (editing), and polymerase. A large C-terminal fragment of *E. coli* DNA polymerase I – Klenow Fragment (KF) – has polymerase and 5’-3’ exonuclease editing activities, located on two distinct structural domains. Site-directed mutagenesis of key residues in the 5’→3’ exonuclease active site resulted in KF(exo-) variants with abolished exonuclease
activity, however, their DNA polymerase activity was not affected. Such exonuclease-deficient KF has been commonly used as a model DNA polymerase.

Based on small magnitudes of the “thio-effect” for correct nucleotide incorporation, pioneering kinetic studies of KF(exo-) concluded that a step other than chemistry must be rate-limiting (5). At the same time, the results of pulse-chase experiments suggested existence of a conformationally closed ternary complex (E’·DNA·dNTP) (3). As such, these studies led to the hypothesis stating that DNA polymerases control their fidelity by an induced-fit mechanism that implies a rate-limiting conformational change (1, 14). This paradigm has been subsequently utilized in a number of kinetic studies of several DNA polymerases (as an example see references (42, 70, 90). As a consequence, it is commonly believed that the majority of DNA polymerases utilize an induced-fit mechanism of fidelity, and although Pol β indeed lacks a rate-limiting step prior chemistry, it is likely to be an exception. If Pol β is an exception, there must be critical differences in the results of kinetic assays comparing Pol β and KF. Here we applied the methodology used in our Pol β studies to make a direct kinetic comparison of Polβ and KF mechanisms.

7.2 Experimental details

Purification of KF(exo-). Klenow fragment D355A/E357A mutant with abolished 3´→5´ exonuclease activity was purified from overexpressing E. coli strain CJ375 according the procedure described by Catherine Joyce (91). Briefly, protein expression was induced by increasing temperature to 42 °C and after 2 hours the cells were
harvested. After cell lysis (in the presence of lysozyme), sonication, and centrifugation, the crude protein extract was fractioned by addition of ammonium sulfate. The KF containing fraction was dialyzed against Buffer A (50 mM Tris HCl, pH 7.5, 1 mM DTT), loaded onto a Mono Q HR column (GE Healthcare) and eluted with 0 M – 0.5 M gradient of NaCl (in Buffer A). Fractions containing KF were pooled, dialyzed against 1.7 M (NH₄)₂SO₄ in Buffer A, loaded onto a phenyl-Superose column, and eluted with 1.7 M – 0 M gradient of (NH₄)₂SO₄ (in Buffer A). Fractions containing KF were combined, and the sample was concentrated to a volume of 0.5 mL using Amicon protein concentrators. The sample was then applied onto a Superose 12 HR gel filtration column (GE Healthcare) and eluted with a buffer containing 100 mM Tris HCl, pH 7.5, and 2 mM DTT. The peak fractions were combined, diluted with an equal volume of glycerol, aliquoted, and rapidly frozen in liquid nitrogen. The purified enzyme was stored at -80 °C. The KF concentration was determined by using a 280 nm extinction coefficient of 55,300 M⁻¹ cm⁻¹. The enzyme was apparently homogenous based on SDS/PAGE analysis developed using the silver staining method.

**DNA substrates and kinetic assay conditions.** The DNA substrates previously used for Pol β studies (Figure 6) were employed in KF assays as well. Most reactions were performed at 20 °C in the reaction buffer containing 100 mM Tris HCl, pH 7.5, 1 mM DTT. A typical reaction mixture consisted of 200 nM DNA, 500 nM KF, 30 µM dNTP, and 10 mM MgCl₂ (all final concentrations). Rapid quench and stopped-flow 2-AP fluorescence assays were performed as described in Material and Methods (Chapter 2).
7.3 Stopped-flow kinetic analysis of KF.

Figure 41 shows fluorescence change associated with the incorporation of dATP in 19/36AP DNA substrate by KF. The fluorescence change has a biphasic character which fits well to a double exponential equation with parameters $k_{\text{fast}} = 260$ s$^{-1}$ and $k_{\text{slow}} = 3.1$ s$^{-1}$. Similarly to Pol β results, when a dideoxy-terminated primer was used with KF, only the fast phase of fluorescence change was observed.

Figure 41. Stopped-flow 2-AP fluorescence assay of incorporation of dATP into 19/36AP DNA substrate by KF

Inset shows fluorescence change induced upon dATP binding to KF·19dd/36AP abortive binary complex.
When dCTP incorporation into 18/36AP DNA substrate was examined, both phases of the fluorescence changes were faster (the first fluorescence transition was barely detectable) and the directions of fluorescence change were inverted (Figure 42). Single exponential fit of the fluorescence growth resulted in $k_{\text{slow}} = 21.9 \text{ s}^{-1}$ and $k_{\text{fast}}$ was estimated to be larger than 300 \text{ s}^{-1}. The slow phase was not observed when a dideoxy-terminated 18dd/36AP was used.

Figure 42. Stopped-flow 2-AP fluorescence assay of incorporation of dCTP into 18/36AP DNA substrate by KF

Inset shows dCTP binding to KF·18dd/36AP abortive binary complex.

The different appearance of the fluorescent traces is not surprising since it has been previously shown for Pol β that the direction and amplitude of fluorescence change is
very sensitive to the reaction conditions (DNA, dNTP, and Mg$^{2+}$)(44). More unexpected was the fact that the rates observed in the stopped-flow assays did not match the value of $k_{pol}$ determined in earlier studies of KF(exo-) (92). Since such data discrepancy might be due to the use of different (2-AP modified) DNA substrates in our studies, we decided to test the rate of single nucleotide incorporation in a rapid quench experiment using our DNA substrates.

7.4 Rapid quench analysis of single-nucleotide incorporation catalyzed by KF

Figure 43 shows rapid quench kinetics of single dNTP incorporation into different DNA substrates. In each case, the time course of the formation of extended primer (product) was fit to a single exponential equation. For all DNA substrates, the observed rate constant of product formation, $k_{RQ}$, was significantly smaller than the values of $k_{fast}$ and significantly larger than the values of $k_{slow}$ observed from stopped-flow experiments under the same conditions (Table 7.1). Such differences in the rate constants cannot be simply due to inefficient quenching with EDTA, because an acid quench gave very similar rates for dATP incorporation into the 19/36AP DNA substrate.
Figure 43. Rapid quench assay of single-nucleotide incorporation by KF
(A) dATP incorporation into 19/36AP DNA (B) dCTP incorporation into 18/36AP DNA.
<table>
<thead>
<tr>
<th></th>
<th>Rapid quench, $k_{RQ}, s^{-1}$</th>
<th>Stopped-flow</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>$k_{fast}, s^{-1}$</td>
</tr>
<tr>
<td>19/36AP + dATP</td>
<td>95.8</td>
<td>260</td>
</tr>
<tr>
<td>18/36AP + dCTP</td>
<td>104</td>
<td>&gt;300</td>
</tr>
<tr>
<td>20/36AP + dTTP</td>
<td>11.7</td>
<td>N/D</td>
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Table 7.1. Rate constants obtained from KF pre-steady state kinetic assays

$k_{RQ}$ is the rate of single-nucleotide incorporation obtained in rapid quench experiments,

$k_{fast}$ and $k_{slow}$ are rates of the fast and slow fluorescence transitions obtained in stopped-flow experiments.

These observations resemble the results obtained for Pol β under high pH and high viscosity conditions. Perhaps, the most reasonable interpretation of these results is that, by analogy with Pol β, the fast and slow fluorescence changes correspond to a fast subdomain-closing before chemistry and a slow subdomain-reopening after chemistry, respectively.

### 7.5 Two-nucleotide incorporation

To further verify that the slow fluorescence change for KF represents a step in the KF reaction pathway, we performed a rapid quench experiment with 18/36AP DNA substrate in the presence of both dCTP and dATP (Figure 44A). The formation of 20mer (the product of two nucleotide incorporation) was slower than the product formation in the course of single nucleotide incorporation. At the same time, the rate of the second
nucleotide incorporation matched the rate of the slow fluorescence change in the experiment with 18/36AP DNA (Table 7.2). Similarly, sequential incorporation of dATP and dTTP into 19/36AP DNA revealed that the rate of 21mer formation is limited by a step with the rate similar to the rate of the slow fluorescence change in 19/36AP stopped-flow assay (Figure 44B and Table 7.2). These results indicate that the slow phase of fluorescence change corresponds to a physical step in KF reaction pathway, which limits the rate of the second but not the first dNTP incorporation. This constitutes unequivocal evidence that the slowest step occurs after chemistry.

<table>
<thead>
<tr>
<th>Stopped-flow, $k_{\text{slow}}$</th>
<th>Rapid quench</th>
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<tbody>
<tr>
<td>18/36AP</td>
<td>$k_{18\rightarrow19}$</td>
</tr>
<tr>
<td>22 s$^{-1}$</td>
<td>105 s$^{-1}$</td>
</tr>
<tr>
<td>19/36AP</td>
<td>$k_{19\rightarrow20}$</td>
</tr>
<tr>
<td>3.1 s$^{-1}$</td>
<td>96 s$^{-1}$</td>
</tr>
</tbody>
</table>

Table 7.2. Rates of the first and second nucleotide incorporation by KF
Figure 44. Rapid quench time course for the two-nucleotide extended primer formation (A) dCTP and dATP incorporation into 18/36AP DNA, (B) dATP and dTTP incorporation into 19/36AP DNA.
7.6 Application of Rh(III)dNTP analogs in KF stopped-flow experiments

We utilized exchange-inert Rh(III)dCTP complexes to probe the contribution of nucleotide-binding and catalytic metal ions in the events associated with the fast and slow phases of a stopped-flow fluorescence change. Figure 45A shows the fluorescence change associated with incorporation of dCTP into 18/35AP DNA substrate. The kinetic trace has typical biphasic character, and only the fast phase is present when the DNA substrate is substituted with its dideoxy-terminated analog, 18dd/35AP. Rapid mixing of Rh(III)dCTP with KF·18/35AP binary complex induces fast fluorescence transition only. At the same time, addition of Mg\(^{2+}\) to the preformed KF·18/35AP·Rh(III)dCTP ternary complex results in fast phase disappearance. However, a noticeable lag phase precedes the slow fluorescence decay. The presence of a lag phase is entirely consistent with the suggestion that the chemical step (initiated upon addition of catalytic Mg\(^{2+}\)) occurs before the conformational step associated with the slow fluorescence transition.

The application of Rh(III)dCTP as a mechanistic probe of KF kinetics confirmed the similarity of Pol \(\beta\) and KF mechanisms. Therefore, it is reasonable to propose that the binding of Mg\(dNTP\) is necessary and sufficient to induce the fingers subdomain-closing conformational change. The slow fluorescence change occurs only after the binding of the second metal ion to the KF·DNA·MdNTP ternary complex.
Figure 45. The application of Rh(III)dCTP in stopped-flow 2-aminopurine fluorescence assays of KF.

(A) dCTP incorporation into 18/35AP DNA substrate (inset shows assay with dideoxy-terminated 18dd/35AP substrate analog) (B) Rh(III)dCTP binding to KF·18/35AP binary complex (C) Mg$^{2+}$ induced nucleotide incorporation from KF·18/35AP·Rh(III)dCTP ternary complex.
7.7 Conclusion

Since our hypothesis that chemistry is the rate-limiting step for Pol β differs from earlier conclusions that the fingers subdomain-closing conformational change is rate-limiting for the majority of DNA polymerases, it is important to consider whether Pol β represents an exception from this rule, as suggested recently (14). The results presented here suggest that in similarity to Pol β, the fast fluorescence change in KF stopped-flow assays represents subdomain closing before chemistry, while the slow change corresponds to subdomain reopening after chemistry. It is important to note that the latter conclusion is consistent with the findings of Dahlberg and Benkovic, who previously described a slow post-chemistry conformational change in KF (3). KF resembles Pol β in that the fast subdomain-closing is the only detectable conformational change before chemistry. However, Pol β and KF differ in that the latter has a rate-limiting step after phosphodiester bond formation, most likely the subdomain reopening, although PPi release or DNA translocation cannot be totally ruled out.

Recently, kinetic similarity with Pol β was observed in studies of Klentaq1 (93). Direct monitoring of the fingers subdomain motions using fluorescence resonance energy transfer (FRET) indicates that Klentaq1 subdomain closing is substantially faster than the rate of single-nucleotide incorporation. Additionally, recent computer simulations of the free energy landscape for correct and mismatched nucleotides incorporation by T4 polymerase also indicate that the chemical step (namely, P–O dissociation) is rate-limiting (13).
Collectively, our results and the results of other research groups indicate that chemistry is likely the rate-limiting step for some DNA polymerases through the step of dNTP incorporation. Even though it would be hasty to conclude that all DNA polymerases have a common rate-limiting step, the statement that Pol β is an exception to the rule is no longer valid.


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