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Effect of Different Divalent Cations on the Kinetics and Fidelity of RB69 DNA Polymerase

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Abstract

Although Mg^{2+} is the cation that functions as the cofactor for the nucleotidyl transfer reaction for almost all DNA polymerases, Mn^{2+} can also serve, but when it does, the degree of base discrimination exhibited by most DNA polymerases (pols) is diminished. Metal ions other than Mg^{2+} or Mn^{2+} can also act as cofactors depending on the specific DNA polymerase. Here, we tested the ability of several divalent metal ions to substitute for Mg^{2+} or Mn^{2+} with RB69 DNA polymerase (RB69pol), a model B-family pol. Our choice of metal ions was based on previous studies with other DNA pols. Co^{2+} , and to a lesser extent Ni^{2+} , were the only cations among those tested besides Mg^{2+} and Mn^{2+} that could serve as cofactors with RB69pol. The incorporation efficiency of correct dNMPs increased by 5-fold with Co^{2+} , relative to that of Mg^{2+} . The incorporation efficiencies of incorrect dNMPs increased by 2–17-fold with Co^{2+} , relative to that with Mg^{2+} depending on the incoming dNTP. Base selectivity was reduced even further with Mn^{2+} compared to that observed with Co^{2+} . Substitution of Mn^{2+} , Co^{2+} , or Ni^{2+} for Mg^{2+} reduced the exonuclease activity of RB69pol by 2-, 6-, and 33-fold, respectively, contributing to the frequency of misincorporation. In addition, Co^{2+} and Mn^{2+} were better able to extend a primer past a mismatch than Mg^{2+} . Finally, Co^{2+} and Mn^{2+} enhanced ground-state binding of both correct and incorrect dNTPs to RB69pol:dideoxy-terminated primer–template complexes.

Graphical Abstract

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Notes

The authors declare no competing financial interest.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b01350. Additional data showing the optimal divalent cation concentrations (Figure S1), equilibrium fluorescence titration results with increasing dTTP concentrations (or dCTP concentrations) (Figure S2), plots for incorporation of dATP opposite dT past DNA containing dA/dC mismatch (Figure S3), plots for incorporation of dATP opposite dT past DNA containing dA/dG mismatch (Figure S4), exonuclease activity of RB69pol using Ni^{2+} (Figure S5), and calculation of $K_{d,ca}$ in competition with Mg^{2+} (Figure S6) (PDF)

DNA polymerases play a vital role in replicating genomic DNA with remarkably high fidelity.¹ All DNA polymerases need Mg^{2+} or Mn^{2+} to catalyze both nucleotidyl transfer and intrinsic $3' \rightarrow 5'$ exonuclease activities. Although DNA polymerases employ the physiologically relevant Mg^{2+} , they can use other divalent metal ions such as Mn^{2+} even though Mn^{2+} reduces base selectivity.^{2–5} Two metal ions have been shown to be required for primer extension; one metal ion occupying the “A” site helps to lower the pK_a of the terminal $3'$ -OH group on the primer and coordinates both the α -phosphate of incoming dNTP and the $3'$ -OH of the primer strand, which facilitates its nucleophilic attack on the dNTP's α -phosphorus atom.⁶ The other metal ion occupying the “B” site coordinates the α -, β -, and γ -phosphate oxygens of the incoming dNTP and helps to neutralize the developing negative charge of the transition state in the nucleotidyl transfer reaction, assisting the departure of PP_i . The effect of metal ions on the fidelity of DNA replication has been studied for several pols.^{2,7–12} Some metal ions have been shown to be mutagens and carcinogens possibly due in part to the fact that DNA replication fidelity is reduced.^{2,7–11,13}

Pelletier et al.³ have conducted extensive structural studies of pol β ternary complexes with many different metal ions and showed that only Mg^{2+} , Mn^{2+} , Cd^{2+} , and Zn^{2+} catalyzed dNMP incorporation. Egli et al.⁵ reported that Mg^{2+} , Mn^{2+} , and Ca^{2+} are active for Dpo4-catalyzed polymerization but Co^{2+} , Ni^{2+} , Cu^{2+} , Zn^{2+} , Ba^{2+} , and Sr^{2+} are not. Pol β and Dpo4 are X- and Y-family pols and have different preferences for metal ion cofactors; however, there have not been extensive studies of the effect of divalent cations on the kinetics and fidelity of B-family pols. Thus, we decided to determine the metal ion preference for RB69pol, a high-fidelity model B-family DNA polymerase.^{14,15}

Here we report how different divalent cations influence base selectivity by determining the pre-steady-state kinetic parameters for primer extension with incoming dNTPs that are either complementary or unmatched with the templating base. We have also determined the effect of various divalent cations on the ground-state binding affinity of dTTP and dCTP against a templating dAP by recording their ability to quench 2AP fluorescence as a function of dNTP concentration. We have also looked at the influence of different divalent metal ions on their ability to allow RB69pol to extend primers bearing mismatched bases at their $3'$ -termini. In addition, we have investigated how these active divalent cations affect the exonuclease activity of RB69pol. Finally, we speculate about why Mg^{2+} , Mn^{2+} , Co^{2+} , and to a much lesser extent Ni^{2+} are the only metal ions among those tested that can activate the pol and exo activities of RB69pol and why Co^{2+} and Mn^{2+} reduce base selectivity.

MATERIALS AND METHODS

Chemicals

All chemicals were of the highest grade available and were used as purchased. dNTPs were from Roche (Burgess Hill, U.K.). T4 polynucleotide kinase was from New England Biolabs (Ipswich, MA), and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was from MP Biomedicals (Irvine, CA). The metal ion salts $[\text{MgCl}_2, \text{MnCl}_2, \text{CaCl}_2, \text{Zn}(\text{CH}_3\text{COO})_2, \text{CoCl}_2, \text{CdSO}_4, \text{CuSO}_4, \text{NiCl}_2, \text{SrCl}_2, \text{BeCl}_2, \text{BaCl}_2, \text{and CrKSO}_4]$ were from Fluka and were reported to be >99% pure.

DNA Substrates

Oligonucleotides were synthesized at the Keck Facility (Yale University), and primers labeled with fluorescein at the 5'-end were obtained from Integrated DNA Technologies (Coralville, IA). Oligonucleotides were gel purified using polyacrylamide gel electrophoresis (PAGE) [19:1 (w/v) acrylamide/bis(acrylamide) gels containing 8 M urea]. A fluorescein-labeled primer was annealed to unlabeled templates when they were heated to 95 °C and then slowly cooled to 25 °C.¹⁶ For studies using 2AP as the templating base, the primer was labeled with [γ -³²P]ATP using T4 polynucleotide kinase. The P/T sequences used in all assays are shown in Figure 1. To simplify the interpretation of data, a common primer sequence was annealed to different templates where the templating base was varied.

Enzymes

Wild-type *exo*⁺ and *exo*⁻ RB69pol were overexpressed in *Escherichia coli*, purified, and stored as previously described.¹⁷ Pol β was a generous gift from S. Wilson (National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC). An RW382 culture of an *E. coli* strain harboring the Dpo4 expression plasmid was provided by W. Yang (National Institutes of Health, Bethesda, MD). Protein expression was conducted as described previously.¹⁸ Cells were harvested and lysed using a microfluidizer followed by heat denaturation at 75 °C to remove the thermolabile *E. coli* proteins followed by ultracentrifugation at 45000 rpm for 1 h to remove the cell debris. The supernatant was dialyzed overnight against heparin A buffer that contained 150 mM NaCl, 50 mM Tris (pH 7.4), and 1 mM EDTA (pH 8.0), loaded onto a heparin column, and washed with buffer A. Dpo4 was eluted using heparin buffer B containing 800 mM NaCl, 50 mM Tris (pH 7.4), and 1 mM EDTA (pH 8.0). Fractions were analyzed via sodium dodecyl sulfate (SDS)-PAGE, and those containing Dpo4 as shown by SDS-PAGE were pooled and dialyzed overnight against protein storage buffer containing 20 mM Tris-HCl (pH 7.3), 100 μ M EDTA, and 5% (w/v) glycerol. The dialyzed protein was concentrated to 6 mg/mL, flash-frozen in liquid nitrogen, and stored in small aliquots at -80 °C.

Steady-State Kinetic Assays for the Metal Ion Dependence of RB69pol

Steady-state kinetic assays were used to screen selected metal ions for their ability to support the pol and *exo* activities of RB69pol. A typical assay mixture contained 66 mM Tris-HCl (pH 7.3), 200 nM 13/18-mer, 40 nM RB69pol *exo*⁻ enzyme, and 10 mM CoCl₂. The mixture was preincubated at 23 °C for 10 min and the reaction initiated by adding 500 μ M dTTP. Aliquots (10 μ L) were quenched with 0.5 M EDTA at 10, 20, 40, 60, and 120 s. The experiment was repeated using MnCl₂, CaCl₂, Zn(CH₃COO)₂, CdSO₄, CuSO₄, NiCl₂, SrCl₂, FeCl₂, BaCl₂, and CrKSO₄. To determine the optimal concentrations of Co²⁺, Mn²⁺, and Ni²⁺ (which were the only metal ions that worked), rates of primer extension were measured under steady-state conditions using different concentrations of these metal ions ranging from 2 to 20 mM.

Nucleotide Incorporation under Single-Turnover Conditions Catalyzed by *Exo*⁻ RB69pol

Rapid chemical quench assays were performed at 23 °C with an RQF3 rapid chemical quench instrument (Kintek Corp.). *Exo*⁻ RB69pol (1 μ M) was incubated with a 13/18-mer

P/T (80 nM) in assay buffer containing EDTA (0.1 mM) and 66 mM Tris-HCl (pH 7.3) and mixed with dTTP (10–500 μ M) containing MnCl_2 , CoCl_2 , or NiCl_2 (10 mM) in assay buffer (final concentrations after mixing). The reactions were quenched with 0.5 M EDTA (pH 8.0) at various times ranging from 3 ms to 1 s. For the incorporation of an incorrect nucleotide, the dNTP concentrations were varied from 50 μ M to 3 mM and reactions were quenched at times ranging from 500 ms to 180 min. Products were separated by PAGE [19:1 (w/v) acrylamide/bis-(acrylamide) gels containing 8 M urea], visualized using a FUJI scanner equipped with a 473 nm fluorescent laser signal, and quantified using MULTIGAUGE (Science Lab 2005) and GraphPad Prism. Assays involving 2AP as the templating base were conducted similarly except that the gels were visualized using a STORM imager (Molecular Imaging) and quantified using ImageQuant (GE Healthcare) and GraphPad Prism.

Exonuclease Assays with Exo^+ RB69pol Using Mn^{2+} , Co^{2+} , and Ni^{2+}

Rapid chemical quench assays were performed as mentioned above to determine the rate of excision (k_{exo}) in the presence of Mn^{2+} , Co^{2+} , and Ni^{2+} . RB69pol exo^+ (1 μ M) was incubated with 13/18-mer (80 nM) in reaction buffer containing EDTA (0.1 mM) and mixed with 10 mM Co^{2+} , Mn^{2+} , or Ni^{2+} , in assay buffer. The reactions were quenched with 0.5 M EDTA (pH 8.0) at times ranging from 4 ms to 5 s. The exonuclease reaction in the presence of Ni^{2+} was slow; hence, assays were conducted on the benchtop (10 s to 30 min).

Pre-Steady-State Kinetic Experiments for Insertion and Extension beyond a Mismatched P/T

Single-turnover experiments were conducted as described above except that the P/T terminus contained a dA/dC or dA/dG mismatch as shown in Figure 1. Exo^- RB69pol (1 μ M) was incubated with 13/18-mer (80 nM) in assay buffer containing EDTA (0.1 mM) and mixed with dATP (0.05–3 mM) containing Mg^{2+} (10 mM), Co^{2+} (10 mM), or Mn^{2+} (10 mM), in assay buffer. The reactions were quenched with 0.5 M EDTA (pH 8.0) at various times ranging from 0.5 s to 20 min. Six different dATP concentrations were employed for each k_{pol} and $K_{\text{d,app}}$ determination.

Equilibrium Fluorescence Titrations

The emission spectra of deoxy-primer/templates (dP/T) or dideoxy-primer/templates (ddP/T) containing 2AP were recorded at 23 °C with a Photon Technology International Alphascan scanning spectrofluorometer. The titration mixture contained 66 mM Tris-HCl (pH 7.3), 200 nM P/T (13/18-mer; dP/T or ddP/T) with 2AP as the templating base (Figure 1), 1 μ M exo^- RB69 pol, and either 10 mM MgCl_2 , 10 mM CoCl_2 , 10 mM MnCl_2 , or 2 mM CaCl_2 with varying dTTP and dCTP concentrations. A dP/T was used to conduct the titration in the presence of 2 mM Ca^{2+} because Ca^{2+} prevents primer extension and allows the dNTP to bind to the binary complex in the absence of chemistry.¹⁹ Samples were excited at 313 nm, and fluorescence emission spectra were recorded from 335 to 450 nm after 2 min to allow the reaction to come to equilibrium where no further change in fluorescence intensity was observed. The intensities for all samples were corrected by subtracting the intensity of the blank sample and protein that contained 66 mM Tris-HCl (pH 7.3) and the respective metal ion concentration mentioned above. Peak emission intensities at 365 nm were plotted as a function of dTTP concentration (or dCTP concentration) and fit to a

hyperbolic equation to obtain the ground-state equilibrium dissociation constant (K_{dg}) for incoming dTTP (or dCTP).

Measurement of $K_{d,app}$ for Ca^{2+} in Competition with Mg^{2+} for Primer Extension

Competition experiments were performed as described above. Briefly, a mixture containing exo^- RB69pol (1 μ M) and 13/18-mer (80 nM) in assay buffer containing EDTA (0.1 mM) was mixed with dTTP (1 mM) containing Mg^{2+} (10 mM) and Ca^{2+} (50 μ M), in assay buffer. The reactions were quenched with 0.5 M EDTA (pH 8.0) at intervals ranging from 4 to 100 ms. The experiment was repeated with varying Ca^{2+} concentrations (from 0.1 to 20 mM).

Nucleotide Incorporation Assays for Dpo4 and Pol β

Dpo4 (20 nM) was incubated with 13/18-mer (1 μ M) in reaction buffer containing EDTA (0.1 mM) and mixed with dTTP (1 mM) containing Co^{2+} (10 mM) in the standard assay buffer in the absence and presence of 2 mM DTT. Reactions were quenched with 0.5 M EDTA (pH 8.0) at intervals ranging from 1 to 15 min. Assays for pol β were conducted by incubating pol β (1 μ M) with 13/18-mer (80 nM) in reaction buffer containing EDTA (0.1 mM) and mixed with dTTP (1 mM) containing Co^{2+} (10 mM) in standard assay buffer. Reactions were quenched with 0.5 M EDTA at intervals ranging from 10 to 80 s.

Data Analysis

The amount of product formed at each dNTP concentration at different times was fit by nonlinear regression to eq 1, and the observed rates of product formation at different dNTP concentrations were determined

$$[\text{product}] = A(1 - e^{-k_{\text{obs}}t}) \quad (1)$$

where A is the observed amplitude of product formation and k_{obs} is the observed rate constant at a particular dNTP concentration. To determine the kinetic parameters k_{pol} (the maximal rate of nucleotide incorporation) and $K_{d,app}$ (the concentration of dNTP at which the rate of nucleotide incorporation is half of k_{pol}), k_{obs} was plotted against the dNTP concentration, and data were fitted to eq 2.

$$k_{\text{obs}} = \frac{k_{\text{pol}}[\text{dNTP}]}{K_{d,app} + [\text{dNTP}]} \quad (2)$$

where k_{obs} is the observed rate at a given dNTP concentration. In cases in which saturation was not achieved with the incoming dNTP, data were fit to eq 3.

$$k_{\text{obs}} = \frac{k_{\text{on}}[\text{dNTP}]}{1 + [\text{dNTP}]/K_{\text{d, app}}} \quad (3)$$

where k_{on} is defined as the ratio of k_{pol} to the $K_{\text{d, app}}$ value for the incoming dNTP.

Rates of nucleotide excision were determined by plotting the amount of labeled primer from the P/T remaining as a function of time, and data were fit to the following equation

$$Y = \lambda e^{-kt} + C \quad (4)$$

where Y is the concentration of the DNA substrate remaining during the reaction, λ is the observed amplitude of the substrate remaining, and k is the observed rate constant.

Equilibrium fluorescence titration data were fit to the hyperbolic equation

$$f = f_0 + \frac{f_{\text{max}}[\text{dNTP}]}{K_{\text{d, g}} + [\text{dNTP}]} \quad (5)$$

where f is the observed fluorescence intensity, f_0 is the starting fluorescence, f_{max} is the maximal decrease in fluorescence intensity at a saturating dTTP concentration, and $K_{\text{d, g}}$ is the ground-state equilibrium dissociation constant for the incoming dNTP. Equilibrium fluorescence data involving tight dNTP binding relative to the enzyme concentration were fit to a quadratic equation.

$$\Delta F = F_0 + \frac{F_{\text{max}}[D_0 + S_0 + K_d - \sqrt{(D_0 + S_0 + K_d)^2 - 4D_0S_0}]}{2D_0} \quad (6)$$

where F is the fluorescence signal change, F_0 is the starting fluorescence, F_{max} is the maximal fluorescence signal change at saturating dNTP concentrations, D_0 is the total DNA concentration, S_0 is the total dNTP concentration, and $K_{\text{d, g}}$ is the ground-state equilibrium dissociation constant for the incoming dNTP.

Data for competition experiments using Ca^{2+} were fit to eq 1 to obtain the observed rates for product formation. The K_{d} for Ca^{2+} was obtained by fitting the data using eq 7.

$$k_{\text{obs}} = \frac{k_{\text{pol}}[\text{Mg}]/K_{\text{d, Mg}}}{1 + [\text{Mg}]/K_{\text{d, Mg}} + [\text{Ca}]/K_{\text{d, Ca}}} \quad (7)$$

where k_{obs} is the observed rate at a given Ca^{2+} concentration, $K_{\text{d,Mg}}$ is the K_{d} for Mg^{2+} , and $K_{\text{d,Ca}}$ is the K_{d} for Ca^{2+} when it is competing with Mg.

RESULTS

Effect of Divalent Metal Ions on the Kinetics of Primer Extension Catalyzed by RB69pol

Steady-state kinetic assays were conducted to determine if metal ions other than Mg^{2+} and Mn^{2+} were able to activate RB69 polymerase. We found that only Co^{2+} and to a lesser extent Ni^{2+} supported the pol activity of RB69pol. We also tried Fe^{2+} , Ca^{2+} , Zn^{2+} , Cd^{2+} , Sr^{2+} , Ba^{2+} , Cu^{2+} , and Cr^{3+} because these cations were tested with other pols;^{3,5} however, they did not support the pol activity of RB69pol. The rate of dTMP incorporation opposite dA was determined as a function of Co^{2+} , Mn^{2+} , and Ni^{2+} concentrations first under steady-state conditions to find the optimal concentration, which was ~10 mM for all three cations (Figure S1). We then used this concentration for pre-steady-state single-turnover experiments to investigate their effect on misincorporation and bypassing a mismatch at the primer terminus.

Single-Turnover Kinetics for Correct Nucleotide Incorporation by Exo⁻ RB69pol

Although Mg^{2+} is the physiologically relevant cation that activates RB69pol *in vivo*, DNA polymerases from human,²⁰ viral,²¹ and bacterial⁸ sources can also utilize Mn^{2+} , Co^{2+} , Ni^{2+} , and Cd^{2+} *in vitro*.³ Ca^{2+} has also been shown to activate Dpo4,⁵ but this is an exception because Ca^{2+} is inactive with every other DNA pol that has been tested. The apparent dissociation constant for dTTP ($K_{\text{d,app}}$) in the presence of Mg^{2+} , Mn^{2+} , Co^{2+} , and Ni^{2+} was determined under single-turnover conditions, where the RB69pol concentration was 12-fold greater than that of the primer-template (P/T). Under these conditions, ~95% of the DNA is bound to the enzyme. Product concentrations obtained at different dTTP concentrations were plotted against time and fitted to eq 1 to yield k_{obs} at each dTTP concentration. These rates were plotted against dTTP concentration to extract a k_{pol} of $>300 \text{ s}^{-1}$ and a $K_{\text{d,app}}$ of $56 \text{ }\mu\text{M}$ with Mg^{2+} . k_{pol} values for the incorporation of dTMP opposite dA using Co^{2+} and Mn^{2+} were also $>300 \text{ s}^{-1}$. Accurate k_{pol} values for Mg^{2+} , Co^{2+} , and Mn^{2+} could not be determined as the reaction proceeds faster than the 2 ms dead time of the Kintek instrument. However, the k_{pol} for incorporation of dTMP opposite dA in the presence of Ni^{2+} was much lower (0.6 s^{-1}). $K_{\text{d,app}}$ values for dTTP incorporation with these metal ions were between 16 and $61 \text{ }\mu\text{M}$ (Table 1). To directly compare the incorporation efficiencies in the presence of Mg^{2+} , Co^{2+} , and Mn^{2+} , single-turnover assays were conducted at $6 \text{ }^{\circ}\text{C}$ with 66 mM MES buffer (pH 6.3) to reduce the k_{obs} . The results summarized in Table 2 show that when Mg^{2+} was replaced with Co^{2+} or Mn^{2+} , the efficiency of incorporation of dTMP opposite dA increased by 5- or ~3-fold, respectively. We note that lowering the pH from 7.3 to 6.3 may influence the metal binding by altering the protonation states of acidic side chains that coordinate the metal ions.

To investigate the effect of Mg^{2+} , Co^{2+} , and Mn^{2+} on ground-state binding affinity ($K_{\text{d,g}}$) and the apparent equilibrium dissociation constant ($K_{\text{d,app}}$) of the incoming dNTPs (Scheme 1), we performed single-turnover kinetic assays using P/T containing 2AP as the templating base (DNA_P) and measured the $K_{\text{d,g}}$ values with correct and incorrect incoming nucleotides.

Pre-steady-state kinetic assays using incorporation of dTMP opposite 2AP showed trends in k_{pol} and $K_{\text{d,app}}$ values similar to those for incorporation of dTMP opposite dA at 23 °C. The k_{pol} values were $>300 \text{ s}^{-1}$ in the presence of Mg^{2+} , Co^{2+} , and Mn^{2+} , while the $K_{\text{d,app}}$ values were 167, 50, and $20 \mu\text{M}$, respectively. Similarly, assays were repeated using incorporation of dCMP opposite dAP. Figure 2 shows the plots for incorporation of dCMP opposite dAP in the presence of 10 mM Mn^{2+} at 23 °C. Assays involving incorporation of dTMP opposite dAP were repeated at 6 °C using 66 mM MES buffer (pH 6.3) to directly compare the k_{pol} values obtained with the three metal ions. Among the three cations, the highest incorporation efficiency was obtained in the presence of Co^{2+} ($4 \mu\text{M}^{-1} \text{ s}^{-1}$) followed by Mn^{2+} ($2 \mu\text{M}^{-1} \text{ s}^{-1}$) and Mg^{2+} ($0.6 \mu\text{M}^{-1} \text{ s}^{-1}$). Incorporation efficiencies with DNA containing 2AP as the templating base (DNA_P) were in general ~5-fold lower than that of DNA containing dA as the templating base with all three metal ions (Table 2).

Single-Turnover Kinetics for Incorporation of the Incorrect Nucleotide by Exo⁻ RB69pol

To study the effect of Co^{2+} , Mn^{2+} , and Ni^{2+} on base selectivity, single-turnover experiments were conducted with incorrect incoming dNTPs. We tested purine:purine, purine:pyrimidine, and pyrimidine:pyrimidine mismatches. In general, Mg^{2+} and Co^{2+} showed the greatest base selectivity with all the mispairs (Table 1). While the $K_{\text{d,app}}$ values with Mg^{2+} , Co^{2+} , and Ni^{2+} were in a similar range, most of the reduction in base selectivity with Mn^{2+} resulted from a dramatic increase in k_{pol} values with incorrect incoming dNTPs. Figure 3 shows the plots for incorporation of dAMP opposite dC in the presence of 10 mM Co^{2+} at 23 °C. We note that we have compared the $k_{\text{pol}}/K_{\text{d,app}}$ values for correct nucleotide incorporation obtained at 6 °C with the $k_{\text{pol}}/K_{\text{d,app}}$ values for incorrect nucleotide incorporations obtained at 23 °C because the actual k_{pol} values at 23 °C for correct nucleotide incorporations were $>300 \text{ s}^{-1}$ with all three metal ions, so a direct comparison of $k_{\text{pol}}/K_{\text{d,app}}$ values could not be made.

Effect of Metal Ions on Ground-State Binding of dNTP to the Pol–P/T Binary Complex with Mg^{2+} , Mn^{2+} , Co^{2+} , and Ca^{2+}

To examine whether the different metal ions affect the ground-state equilibrium dissociation constant ($K_{\text{d,g}}$) for dTTP binding (opposite 2AP) to a ddP/T–RB69pol binary complex, equilibrium fluorescence titrations were conducted in the presence of Mg^{2+} , Co^{2+} , Mn^{2+} , and Ca^{2+} (Table 3). The $K_{\text{d,g}}$ for binding of dTTP opposite template 2AP in the presence of Ca^{2+} using a deoxy-terminated (dP/T) was 170 nM, with a 26% reduction in the amplitude, while the $K_{\text{d,g}}$ using a ddP/T was 440 nM and resulted in a 75% reduction in amplitude, indicating that the presence of an OH group at the 3'-terminus of the primer increased the affinity of dTTP for the binary complex by ~2.5-fold. Similar decreases in amplitude (72, 72, and 65%) were observed with Mg^{2+} , Co^{2+} , and Mn^{2+} , respectively. $K_{\text{d,g}}$ values obtained in the presence of Mg^{2+} , Co^{2+} , and Mn^{2+} were 34, 6, and $0.6 \mu\text{M}$, respectively. Figure 4 and Figure S2A show equilibrium fluorescence titration results for the RB69pol–ddP/T complex with increasing dTTP concentrations in the presence of 10 mM Mg^{2+} and 10 mM Co^{2+} , respectively.

To test whether similar discrimination would be observed for an incorrect incoming dNTP in the presence of these metal ions, we determined the $K_{\text{d,g}}$ values for dCTP binding opposite

2AP in the presence of Mg^{2+} , Mn^{2+} , and Ca^{2+} by measuring the change in 2AP fluorescence observed as a function of dCTP concentration. The $K_{d,g}$ values obtained for dCTP binding in the presence of Mg^{2+} , Mn^{2+} , and Ca^{2+} were 1400, 34, and 14 μM , respectively, demonstrating that Mn^{2+} and Ca^{2+} provide 40- and 100-fold tighter ground-state binding of the incorrect dNTP substrate (dCTP), respectively, compared to that of Mg^{2+} . Figure S2B shows equilibrium fluorescence titration results for the RB69pol-ddP/T complex with increasing dCTP concentrations in the presence of 10 mM Mn^{2+} .

Extension beyond a Mismatched P/T with Mg^{2+} , Mn^{2+} , and Co^{2+}

We determined how effectively RB69 pol could bury a mismatch in the presence of Mg^{2+} , Mn^{2+} , and Co^{2+} as this could result in mistakes being preserved during DNA replication if not removed by *exo* activity or base excision repair. Our choice of DNA containing a dA/dC mismatch (DNA_{ACMM}) and a dA/dG mismatch (DNA_{AGMM}) was based on the fact that the dA/dC purine-pyrimidine pair has a nascent wobble mispair with the correct size but with distorted geometry while the dA/dG represents a purine-purine mispair, which also has distorted geometry but does not form hydrogen bonds between the bases. With Mg^{2+} , the $K_{d,\text{app}}$ was 800 μM and the k_{pol} was 1 s^{-1} for extension beyond the dA/dC mismatch, indicating that the rate of incorporation of a correct dNMP was very slow compared to the rate of extension past a matched base pair at the primer terminus ($>300 \text{ s}^{-1}$). With Mn^{2+} , the $K_{d,\text{app}}$ was 650 μM , similar to that obtained in the presence of Mg^{2+} , but the k_{pol} was 17-fold higher, demonstrating that the correct dNMP was more readily incorporated beyond the dA/dC mismatch. Figure S3A–D shows the plots for incorporation of dAMP opposite dT in the presence of 10 mM Mg^{2+} and 10 mM Mn^{2+} at 23 °C. Correct dNMPs can also be incorporated past the dA/dC mismatch in the presence of Co^{2+} , but the $K_{d,\text{app}}$ for dAMP insertion was much higher than those with Mg^{2+} and Mn^{2+} , resulting in an only 1.8-fold increase in dNMP incorporation efficiency compared to that with Mg^{2+} . The kinetic parameters for extension past a dA/dC mismatch are summarized in Table 4.

Similar results were observed with the dA/dG mismatch in the presence of Mg^{2+} , Mn^{2+} , and Co^{2+} , except that the $K_{d,\text{app}}$ values for incorporation of dAMP opposite a templating dT were within the same range (300–500 μM) (Table 4). The k_{pol} values for extension past a dA/dG mispair were 40–380-fold lower than those obtained with a dA/dC mispair. The $k_{\text{pol}}/K_{d,\text{app}}$ values in the presence of Co^{2+} and Mn^{2+} were 10- and 40-fold higher than those obtained with Mg^{2+} , respectively, suggesting that these metal ions with RB69pol allow it to more easily extend a primer past a mismatch compared to Mg^{2+} and hence would be expected to increase the frequency of mistakes during DNA replication. With Mg^{2+} , Mn^{2+} , and Co^{2+} , the efficiency of bypassing a dA/dG mismatch is approximately 1–2 orders of magnitude lower than that of extending past a dA/dC mismatch. Most of this decrease in efficiency results from a dramatic decrease in k_{pol} values with Mg^{2+} , Co^{2+} , and Mn^{2+} , as summarized in Table 4. Figure S4 shows the plots for incorporation of dAMP opposite dT in the presence of 10 mM Co^{2+} at 23 °C.

Relative Exonuclease Activity for *Exo*⁺ RB69pol with Mg^{2+} , Mn^{2+} , Co^{2+} , and Ni^{2+}

To test the effect of different metal ions on the exonuclease activity of RB69 pol, we determined the rate of base excision in the presence of Mg^{2+} , Co^{2+} , Mn^{2+} , and Ni^{2+} (Table

5). The excision rate decreased by 2- and ~6-fold when Mn^{2+} and Co^{2+} , respectively, were substituted for Mg^{2+} . Ni^{2+} on the other hand diminished the exonuclease activity by a factor of 33-fold as compared to that with Mg^{2+} . Figure S5 shows the plots for DNA degradation as a function of time in the presence of 10 mM Ni^{2+} at 23 °C.

Ca^{2+} Competes with Mg^{2+} for Metal Ion Binding Site(s)

Wang et al.⁶ have reported the crystal structure of a preinsertion ternary complex of RB69pol containing the 3'-hydroxyl group at the terminus of an extendable primer and a nonhydrolyzable dNTP analogue (dUpXpp; X = N or C) in the presence of Ca^{2+} and Mg^{2+} . Because both Mg^{2+} and Ca^{2+} can bind to the RB69pol active site, it was expected that Ca^{2+} could compete with Mg^{2+} for the metal ion binding site. Competitive inhibition assays were conducted in the presence of 10 mM Mg^{2+} for the incorporation of dTMP opposite dA in the presence of increasing Ca^{2+} concentrations. We determined that the Ca^{2+} concentration required to obtain the half-maximal incorporation rate under single-turnover conditions was $630 \pm 72 \mu\text{M}$, showing that Ca^{2+} competes with Mg^{2+} for the metal ion binding site (Figure S6).

Dpo4 and Pol β Can Be Activated by Co^{2+}

Dpo4 is the only DNA polymerase reported to utilize Ca^{2+} , but its incorporation efficiency was much lower than that with Mg^{2+} .⁵ Pol β and Dpo4 are the only known exceptions to DNA polymerases that are unable to use Co^{2+} for catalysis.^{3,5} Nevertheless, we decided to ask whether these pols could also employ Co^{2+} as a cofactor. We showed that Co^{2+} was competent in the nucleotidyl transfer reaction for both pols under our conditions (Figure 5A, C). When the assay with Dpo4 was repeated under conditions used by Egli et al. (where 2 mM DTT was included in their assays), no primer extension was observed (Figure 5B). Moreover, assays conducted in the absence of any divalent cation served as a negative control. No dNMP incorporation was seen even after incubation for 60 min. The differences between our results with pol β and those obtained by Pelletier et al. are addressed in the Discussion. Hence, apart from Mg^{2+} and Mn^{2+} , Co^{2+} can effectively replace Mg^{2+} for DNA polymerases from different families.

DISCUSSION

The effect of divalent metal ions on the replication fidelity of various DNA pols has been studied previously, but the rates of incorporation of correct and incorrect nucleotides have not been determined under single-turnover conditions in the presence of different metal ions except for Mn^{2+} .²² Co^{2+} , Mn^{2+} , and Ni^{2+} have been characterized as “mutagenic”, because they reduce the fidelity of DNA synthesis.^{2,7–11,13} Attempts have been made to address the reasons why these metal ions act as mutagens, but most of these studies have focused on the mutagenic behavior of Mn^{2+} .^{10,11} For example, substitution of Mn^{2+} for Mg^{2+} caused an 11–34-fold increase in the rate of misincorporation opposite an abasic site by T4 DNA pol.²² Mn^{2+} also promoted translesion DNA synthesis by herpes simplex virus type I.²³ In addition, Pelletier et al. have observed primer extension with pol β using blunt-ended DNA in the presence of Mn^{2+} rather than Mg^{2+} .³

Divalent metal ions can alter the fidelity of DNA replication at various points along the reaction pathway by (1) affecting the ground-state binding affinity of correct and incorrect dNTPs for DNA pol/P/T binary complexes,²⁴ (2) promoting misincorporation during primer extension,² (3) influencing exonuclease activity,²³ and (4) altering the efficiency of extension beyond a mismatch at the P/T terminus.²³ Aside from Mg^{2+} , only Mn^{2+} , Co^{2+} , and Ni^{2+} can activate RB69pol. When Mg^{2+} was replaced with Co^{2+} , the k_{pol} s for the correct incoming dTTP versus dA or dAP were greater than 300 s^{-1} , whereas with incorrect incoming dNTPs, the k_{pol} s were 3–10 times higher with Co^{2+} than with Mg^{2+} . The $K_{\text{d,app}}$ values for the incorrect incoming dNTP were generally higher with Co^{2+} than with Mg^{2+} , except for dATP versus dC and dTTP versus dC. Because saturation was not achieved with incoming dNTPs for dGTP versus dA and dGTP versus dT, we determined only the $k_{\text{pol}}/K_{\text{d}}$ values. In general, it appears that the level of base discrimination is reduced with Co^{2+} versus Mg^{2+} but not nearly as much as when Mn^{2+} replaced Mg^{2+} , where the k_{pol} values for the incorporation of incorrect dNMPs were as much as 500 times higher than those found with Mg^{2+} . Surprisingly, a decrease in base selectivity was not observed when Ni^{2+} was used in place of Mg^{2+} , although the only mismatch tested was dATP versus dC. Lower $K_{\text{d,app}}$ values were observed for most of the mispairs when Mn^{2+} was substituted for Mg^{2+} or Co^{2+} , and the largest differences were observed when dAMP was incorporated versus dC ($307 \mu\text{M}$ for Mn^{2+} , $1870 \mu\text{M}$ for Co^{2+} , and $1800 \mu\text{M}$ for Mg^{2+}). The exception was when dGMP was inserted opposite dT ($>2000 \mu\text{M}$ vs $725 \mu\text{M}$ for Mg^{2+}); however, the $k_{\text{pol}}/K_{\text{d,app}}$ for this pair was $1.1 \times 10^{-2} \mu\text{M}^{-1} \text{ s}^{-1}$ for Mn^{2+} and only $5.6 \times 10^{-5} \mu\text{M}^{-1} \text{ s}^{-1}$ for Mg^{2+} , which was why the level of base discrimination for dGTPs versus dT was so dramatically reduced with Mn^{2+} . It is also worth noting that the efficiency of correct insertions (dTTP vs dA or dAP) when assayed at 6°C instead of 23°C was greatest for Co^{2+} compared to those with Mn^{2+} and Mg^{2+} (Table 2).

With respect to ground-state binding affinities for dTTP versus dAP as measured by 2AP fluorescence quenching, Mn^{2+} –RB69pol complexes had the lowest dissociation constant (K_{dg}), almost equal to that of complexes containing Ca^{2+} followed by Co^{2+} and then Mg^{2+} . For an incorrect dNTP (dCTP vs dAP), the K_{dg} differences were much greater between Mg^{2+} and Mn^{2+} (1400 and 34) (Table 3), consistent with the enhanced ability of dCMP to be inserted opposite 2AP by Mn^{2+} compared to Mg^{2+} (Table 1). This might be due to the ability of Mn^{2+} , in contrast to Mg^{2+} , to accommodate nucleotide residues other than the four normal bases in the nucleotide binding pocket for reasons discussed below.

As far as the ability of the various divalent cations to promote the nucleophilicity of the primer's 3'-OH is concerned, their effect on reducing the $\text{p}K_{\text{a}}$ of bound water is about the same for Mg^{2+} and Mn^{2+} but considerably lower for Co^{2+} (Table 6). If this was the main effect responsible for differences in k_{pol} , then Co^{2+} and Ni^{2+} would be expected to be more effective than Mg^{2+} and Mn^{2+} , which was not the case. Apart from activating the 3'-hydroxyl group, the ionic radius of metal ion A plays a crucial role in determining the proximal distance between the 3'-hydroxyl group and α -phosphorus atom of the incoming dNTP as the transition state is approached. The ionic radius of Mg^{2+} is $0.86 \text{ \AA}$, while those of Mn^{2+} , Co^{2+} , and Ni^{2+} are 0.81 , 0.89 , and $0.83 \text{ \AA}$, respectively, values very close to that of Mg^{2+} , allowing all these metal ions to bring the 3'-hydroxyl group and α -phosphate atom of the incoming dNTP close enough for reaction (Table 6). In addition, the coordination

geometry of metal ions A and B plays a crucial role in the nucleotidyl transfer reaction. Wang et al.⁶ have reported the crystal structure of a preinsertion ternary complex of RB69pol containing the 3'-hydroxyl group at the terminus of an extendable primer and a nonhydrolyzable dUTP analogue (dU_pN_{pp}) in the presence of Ca²⁺, Mg²⁺, and Mn²⁺. Mg²⁺ in the B metal ion site exhibits a nearly perfect octahedral geometry, and Mg²⁺ in the A site shows a distorted octahedral geometry. Similar to Mg²⁺, Mn²⁺ in the B metal ion site exhibits a nearly perfect octahedral geometry and Mn²⁺ in the A metal site also exhibits a distorted octahedral geometry caused by the displacement of the 3'-hydroxyl group at the 3'-terminus of the primer. Also, both Mn²⁺ and Mg²⁺ (in the B site) form an α,β,γ -tridentate complex with the triphosphate tail of dU_pN_{pp}.⁶ In addition to Mg²⁺ and Mn²⁺, Co²⁺ and Ni²⁺ are also able to form octahedral complexes,²⁵ and even though crystal structures of RB69pol with these metal ions are not currently available, it is likely that Co²⁺ binds in a fashion similar to that of Mg²⁺ in the A and B sites. The observation that only Co²⁺, Mn²⁺, and Ni²⁺ are able to activate RB69pol is consistent with (1) the similar ionic radii of these metal ions compared to that of Mg²⁺, (2) the formation of octahedral complexes as found with Mg²⁺,²⁵ and (3) their ability to lower the pK_a of the 3'-hydroxyl group of the primer.

It has been reported that Ca²⁺ does not act as a metal ion cofactor for RB69pol.⁶ Ca²⁺ is not able to effectively lower the pK_a of the 3'-hydroxyl group of the primer (12.8) compared to Mg²⁺ (11.4), and the ionic radius of Ca²⁺ (1.1 Å) is significantly larger than that of Mg²⁺ (0.86 Å). Thus, Ca²⁺ is ineffective in polarizing the hydroxyl group for nucleophilic attack. Also, crystal structures of RB69pol ternary complexes show that Ca²⁺ is displaced toward the 3'-hydroxyl group of the primer terminus.⁶ As a result, Ca²⁺ is unable to bring the 3'-hydroxyl group close enough to the α -phosphorus atom of the incoming dNTP for nucleotidyl transfer.

The mutagenic behavior of Mn²⁺ has been rationalized on the basis of the fact that Mg²⁺ is considered a harder metal ion than Mn²⁺, implying that Mn²⁺ is somewhat more polarizable than Mg²⁺.²⁶ When Mg²⁺ or Mn²⁺ is in a hexahydrated complex (Mn[H₂O]₆²⁺ or Mg[H₂O]₆²⁺, respectively) and the inner sphere coordination number is changed from 6 to 5 to 4, there is a greater energy penalty with Mg²⁺ than with Mn²⁺, indicating less rigid coordination requirements for Mn²⁺ complexes that would allow more freedom for mismatched dNTPs to be accessible to the nucleotide binding pocket.²⁶ Pelletier et al.³ have suggested that because transition metal ions bind more tightly to carboxylate groups and the triphosphate moiety of dNTPs than Mg²⁺ does, this difference could account for the decrease in base selectivity in the presence of Mn²⁺.

Primer Extension past dA/dC and dA/dG Mismatches

Extension beyond a mismatch at a P/T terminus can contribute to the number of errors in the nascent DNA.²⁷ When we investigated the influence of three of the four active metal ions (Mg²⁺, Mn²⁺, and Co²⁺) on the efficiency of extending a primer beyond a mismatch at the primer terminus, it was clear that Mn²⁺ was much more effective than Mg²⁺ mainly because of the higher *k*_{pol} values (Table 4) observed with Mn²⁺, Co²⁺ was also able to serve as an activating cation in this reaction with *k*_{pol} values for Pur/Pyr mismatches at the primer

terminus that were similar to those observed with Mn^{2+} , but the $K_{d,\text{app}}$ with Co^{2+} was greater than 2 mM in contrast to those with Mg^{2+} and Mn^{2+} . Without a crystal structure for this ternary complex, we are unable to provide a structural rationale for this result. On the other hand, when there was a Pur/Pur mismatch that had to be extended by RB69pol with Co^{2+} , the $K_{d,\text{app}}$ values were in the same range as that found with Mg^{2+} and Mn^{2+} . In this situation, the k_{pol} for primer extension was 10 times higher than the k_{pol} observed with Mg^{2+} .

Effect of the Active Metal Ions on the Exonuclease Activity of RB69pol

As shown in Table 5, Mg^{2+} was the most active cation for promoting base excision, but the difference among Mg^{2+} , Mn^{2+} , and Co^{2+} was not that great. Ni^{2+} supported the exo activity, but it was much less effective than the other metal ions. In contrast to our results, the 3′–5′ exonuclease activity of T4 DNA polymerase was reported to be unaffected by substitution of Mn^{2+} for Mg^{2+} .²³ Also, the rates of base excision with Mg^{2+} (2 mM), Mn^{2+} (4 mM), and Co^{2+} (4 mM) were nearly identical for *E. coli* DNA pol I.⁸ Clearly, divalent cations affect the exonuclease activity of pols, but the differences are minor compared to their influence on misincorporation.

Comparison of Preferences of Metal Ions for Different DNA Polymerases

All DNA polymerases known to date utilize Mg^{2+} as a cofactor. Most pols can also use Mn^{2+} and Co^{2+} , albeit with reduced fidelity.^{8–10,13} There are two known exceptions, namely, human pol β and Dpo4,⁵ which cannot be activated by Co^{2+} . However, under our experimental conditions, both of these pols allowed primer extension with Co^{2+} . This apparent discrepancy might be explained by the different assay conditions. Pelletier et al.³ used blunt-ended DNA for their crystal soaking experiments with pol β complexed with DNA where the templating base is absent,³ while we used a P/T containing dA as the templating base. With Dpo4, Egli et al.⁵ used 2 mM DTT in their extension assays; however, DTT is a strong reducing agent (standard electrode potential of -0.33 V)²⁵ and is capable of reducing Co^{2+} because the standard reduction potential for Co^{2+} is -0.28 V . This could explain the absence of extension products observed by these authors as opposed to our conditions where DTT was absent and primer extension was observed (Figure 5C).

Studies of pol β have shown that Zn^{2+} and Cd^{2+} were active³ while RB69pol was not able to use either one of these metal ions for catalysis. Ni^{2+} can support catalysis for all DNA polymerases, albeit with greatly reduced activity except for human pol α ,²⁸ human pol β ,³ and Dpo4.⁵ Moreover, Dpo4 is the only polymerase that can utilize Ca^{2+} as a cofactor, although Ca^{2+} is much less active than Mg^{2+} .⁵ Clearly, DNA polymerases from different families have different metal ion cofactor preferences in addition to Mg^{2+} , which is universally active. This suggests that size, charge, coordination geometry, and the ability to lower the $\text{p}K_{\text{a}}$ of the primer's 3′-OH group are not the only determinants of metal ion specificity. Our results extend the information obtained from previous studies that have been conducted to elucidate the role of different metal ions in the replication fidelity of different DNA polymerases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

2AP	2-aminopurine
RB69pol	bacteriophage RB69 DNA polymerase
dNTP	deoxynucleoside triphosphate
Dpo4	DNA polymerase IV from <i>Sulfolobus solfataricus</i>
exo⁻	DNA polymerase lacking the exonuclease proofreading activity
exo⁺	DNA polymerase exhibiting both the polymerase and exonuclease activities
k_{obs}	observed rate constant
k_{pol}	maximal rate of dNMP incorporation
K_{d,app}	apparent equilibrium dissociation constant for dNTP that supports the half-maximal rate of dNMP incorporation
K_{d,g}	ground-state equilibrium dissociation constant for an incoming dNTP from a DNA pol–DNA–dNTP ternary complex
MES	2-(<i>N</i> -morpholino)-ethanesulfonic acid
pol	polymerase
wt	wild-type RB69pol

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DNA _{13T}	5'- GC GGA CTG CTT AC-3' 3'-GCG CCT GAC GAA TG T CCT-5'
DNA _{13A}	5'- GC GGA CTG CTT AC-3' 3'-GCG CCT GAC GAA TG A CCT-5'
DNA _{13G}	5'- GC GGA CTG CTT AC-3' 3'-GCG CCT GAC GAA TG G CCT-5'
DNA _{13C}	5'- GC GGA CTG CTT AC-3' 3'-GCG CCT GAC GAA TG C CCT-5'
DNA _P	5'- GC GGA CTG CTT AC-3' 3'-GCG CCT GAC GAA TG P CCT-5'
DNA _{Pdd}	5'- GC GGA CTG CTT AC _{dd} -3' 3'-GCG CCT GAC GAA TG P CCT-5'
DNA _{ACMM}	5'- GC GGA CTG CTT AA-3' 3'-GCG CCT GAC GAA T C T CCT-5'
DNA _{AGMM}	5'- GC GGA CTG CTT AA-3' 3'-GCG CCT GAC GAA TG T CCT-5'

Figure 1.

Primer/template sequences used for the pre-steady-state kinetic assays and equilibrium fluorescence titrations. The templating base is shown in bold. P represents 2-aminopurine as the templating base; C represents the dideoxy-terminated cytosine.

Figure 2.

Concentration dependence of the rate of incorporation of dCTP opposite 2AP as the templating base. RB69pol (1 μ M) was preincubated with DNA_p (80 nM) in reaction buffer and was mixed with increasing concentrations of dCTP (25, 50, 100, 250, 500, and 1000 μ M) containing 10 mM Mn²⁺. Reactions were quenched with 0.5 M EDTA (pH 8.0) at various times ranging from 3 to 100 ms. All data were obtained at 23 °C. (A) Gel images showing incorporation of dCTP opposite dAP at various dCTP concentrations. (B) Plots of the amount of extended DNA product obtained as a function of time at various dCTP concentrations. Points are experimental, while curves are based on a fit of the data to eq 1. (C) Single-exponential rates plotted as a function of dCTP concentration and fitted to eq 2 to obtain a k_{pol} of 122 ± 5 s⁻¹ and a $K_{d,app}$ of 160 ± 22 μ M.

Figure 3.

Concentration dependence of the rate of incorporation of dATP opposite dC as the templating base. RB69pol (1 μ M) was preincubated with DNA_C (80 nM) in reaction buffer and was mixed with increasing concentrations of dATP (0.25, 0.50, 1, 1.75, and 3 mM) containing 10 mM Co²⁺. Reactions were quenched with 0.5 M EDTA (pH 8.0) at various times ranging from 0.5 to 60 s. All data were obtained at 23 °C. (A) Gel images showing incorporation of dAMP opposite dC at various dATP concentrations. (B) Plots of the amount of extended DNA product obtained as a function of time at various dATP concentrations. Points are experimental, while curves are based on a fit of the data to eq 1. (C) Single-exponential rates plotted as a function of dATP concentration and fitted to eq 2 to obtain a k_{pol} of 0.23 ± 0.02 s⁻¹ and a $K_{d,app}$ of 1.87 ± 0.52 mM.

Figure 4.

Equilibrium fluorescence titration of the RB69pol-ddP/T complex with increasing dTTP concentrations. (A) Fluorescence emission spectra of the RB69pol-ddP/T-dTTP complex are shown as solid lines in the presence of 10 mM MgCl₂. The concentration of DNA_{Pdd} was 200 nM, and that of RB69pol was 1 μM. The dTTP concentrations were 0, 0.02, 0.04, 0.1, 0.25, 0.5, 1, 2, 5, 10, 20, 50, 100, 200, 500, 1000, and 2000 μM (from top to bottom, respectively). The solid black line at the bottom is the fluorescence spectrum of DNA_{Pdd} alone. (B) Fluorescence intensities at 365 nm fitted to a hyperbolic equation. Titration of dTTP vs 2AP in the presence of 10 mM MgCl₂ gives a $K_{d,g}$ of 34 ± 1 μM. F represents the change in fluorescence in the direction of quenching, and F increases with an increase in dTTP concentration.

Figure 5.

Co^{2+} can be utilized as a cofactor for pol β and Dpo4. Pol β ($1 \mu\text{M}$) was incubated with 80 nM DNA_{13A} and 1 mM dTTP in buffer containing 10 mM Co^{2+} , and aliquots were withdrawn at the noted times. Dpo4 (20 nM) was incubated with $1 \mu\text{M}$ DNA_{13A} and 1 mM dTTP in buffer containing 10 mM Co^{2+} , and aliquots were withdrawn at the noted times. (A) Gel images of the products of DNA_{13A} (fluorescein-labeled 5'-end primer) extension using pol β at various times. (B) Phosphorimages of the products of DNA_{13A} extension using Dpo4 in the presence of 2 mM DTT at various times. (C) Phosphorimages of the products of DNA_{13A} extension using Dpo4 in the absence of 2 mM DTT at various times.

Scheme 1. Minimal Kinetic Scheme Depicting the Ground-State Binding Affinity ($K_{d,g}$) and Apparent Binding Affinity ($K_{d,app}$) for an Incoming dNTP^a

^aEDN represents the open conformation of the ternary complex, whereas FDN represents the closed conformation.

Summary of Pre-Steady-State Kinetic Parameters for Incorporation of dNMPs by Wild-Type RB69pol Using Different Metal Ions with 66 mM Tris-HCl (pH 7.3) at 23°C

Table 1

metal ion	dNTP	template	k_{pol} (s^{-1})	$K_{d,app}$ (μM)	$k_{pol}/K_{d,app}$ ($\mu M^{-1} s^{-1}$)
Mg^{2+}	dTTP	dA	$>300^a$	56	>5
	dTTP	2AP	$>300^a$	167	>1.8
	dCTP	2AP			3.5×10^{-3b}
	dGTP	dA	0.003	784	3.8×10^{-6}
	dATP	dC	0.08	1800	4.4×10^{-5}
	dTTP	dC	0.09	1700	5.3×10^{-5}
	dGTP	dT	0.041	725	5.6×10^{-5}
Co^{2+}	dTTP	dA	$>300^a$	30	>10
	dTTP	2AP	$>300^a$	50	>6
	dCTP	2AP			5.0×10^{-2b}
	dGTP	dA			3.0×10^{-4b}
	dATP	dC	0.2	1870	1.4×10^{-4}
	dTTP	dC	1.2	1300	9.2×10^{-4}
	dGTP	dT			6.8×10^{-5b}
Mn^{2+}	dTTP	dA	$>300^a$	16	>19
	dTTP	2AP	$>300^a$	20	>15
	dCTP	2AP	122	160	7.2×10^{-1}
	dGTP	dA	0.23	509	4.5×10^{-4}
	dATP	dC	3	307	9.7×10^{-3}
	dTTP	dC	23	1090	2.0×10^{-2}
	dGTP	dT			1.1×10^{-2b}
Ni^{2+}	dTTP	dA	0.6	61	9.8×10^{-3}
	dATP	dC	0.0003	243	1.2×10^{-6}

^a Actual k_{pol} values could not be determined as the reaction proceeds faster than the dead time of the instrument (2 ms).

Represents the ratio of K_d to K_d to K_d as saturation is not achieved with the incoming NTP. Standard deviations were within 10–20 and 30% for K_d and K_d values, respectively.

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Table 2

Summary of Pre-Steady-State Kinetic Parameters for Incorporation of dTMP by RB69pol Using Different Metal Ions with 66 mM MES (pH 6.3) at 6 °C^a

metal ion	dNTP	templating base	k_{pol} (s ⁻¹)	$K_{\text{d,app}}$ (μM)	$k_{\text{pol}}/K_{\text{d,app}}$ (μM ⁻¹ s ⁻¹)
Mg ²⁺	dTTP	dA	88	22	4
	dTTP	2AP	38	62	0.61
Co ²⁺	dTTP	dA	297	15	20
	dTTP	2AP	139	36	4
Mn ²⁺	dTTP	dA	88	8	11
	dTTP	2AP	60	31	2

^aStandard deviations were within 10–20 and ~30% for k_{pol} and $K_{\text{d,app}}$ values, respectively.

Table 3

Equilibrium Fluorescence Titration Data for RB69pol Using Mg^{2+} , Co^{2+} , Mn^{2+} , and Ca^{2+} with 2AP as the Templating Base^a

metal ion	P/T	dNTP	$K_{d,g}$ (μM)	quench amplitude (%)
Mg^{2+}	ddP/T	dTTP	34	68
Mg^{2+}	ddP/T	dCTP	1400	72
Co^{2+}	ddP/T	dTTP	6.0	72
Co^{2+}	ddP/T	dCTP	ND ^b	
Mn^{2+}	ddP/T	dTTP	0.6	65
Mn^{2+}	ddP/T	dCTP	34	63
Ca^{2+}	dP/T	dTTP	0.17	26
Ca^{2+}	ddP/T	dTTP	0.44	75
Ca^{2+}	ddP/T	dCTP	14	46

^aThe concentrations of Mg^{2+} , Co^{2+} , Mn^{2+} , and Ca^{2+} used in the titrations were 10, 10, 10, and 2 mM, respectively. Additional details are provided in the text. Standard deviations were within 5–30% for $K_{d,g}$.

^bNot determined.

Table 4

Pre-Steady-State Kinetic Parameters for Extension beyond dA/dC, and dA/dG Mismatches for RB69pol Using Mg^{2+} , Co^{2+} , and Mn^{2+} , Where dAMP Is Being Inserted opposite a Templating dT

metal ion	P/T mismatch	k_{pol} (s^{-1})	$K_{\text{d,app}}$ (μM)	$k_{\text{pol}}/K_{\text{d,app}}$ ($\mu\text{M}^{-1} \text{s}^{-1}$)
Mg^{2+}	dA/dC	1	800	1.2×10^{-3}
Mg^{2+}	dA/dG	0.006	350	1.7×10^{-5}
Co^{2+}	dA/dC			2.2×10^{-3a}
Co^{2+}	dA/dG	0.05	310	1.7×10^{-4}
Mn^{2+}	dA/dC	17	650	2.6×10^{-2}
Mn^{2+}	dA/dG	0.37	500	7.4×10^{-4}

^aThis value represents the ratio of the k_{pol} to the $K_{\text{d,app}}$ value as saturation is not achieved with the incoming dNTP. Standard deviations were within 10–20 and ~30% for k_{pol} and $K_{\text{d,app}}$ values, respectively. dAMP was used as the incoming nucleotide opposite dT. Additional details are provided in Materials and Methods.

Table 5

Summary of k_{exo} Values for Wild-Type RB69pol Using Different Metal Ions with a Complementary P/T (DNA_{13A})^a

metal ion	k_{exo} (s ⁻¹)	x -fold decrease ^b
Mg ²⁺	6.6	1.0
Co ²⁺	1.0	6.6
Mn ²⁺	3.0	2.0
Ni ²⁺	0.2	33

^aStandard deviations were within 10–20% for k_{exo} .

^bDefined as $(k_{\text{exo}})_{\text{Mg}^{2+}}/(k_{\text{exo}})_{\text{other metal ion}}$.

Table 6

Ionic Radii, Coordination Geometries, and pK_a Values of Water Molecules Coordinated to Mg^{2+} , Co^{2+} , Mn^{2+} , Ni^{2+} , and Ca^{2+} ²⁵

	Mg^{2+}	Co^{2+}	Mn^{2+}	Ni^{2+}	Ca^{2+}
ionic radius (Å)	0.86	0.89	0.81	0.83	1.1
coordination geometry ^a	Oct	Oct	Oct	Oct	Oct
	Td	Td	Td	Td	PBP
	Sq		Sq	Sq	HBP
	TBP		TBP		
pK_a of the water molecule	11.4	10.0	11.5	10.6	12.8

^aTd represents tetrahedral, Sq square planar, TBP trigonal bipyramidal, Oct octahedral, PBP pentagonal bipyramidal, and HBP hexagonal bipyramidal.