Role of Divalent Cations in the 3′,5′-Exonuclease Reaction of DNA Polymerase I[†]

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ABSTRACT: X-ray studies of the proofreading 3′,5′-exonuclease site of the large (Klenow) fragment of DNA polymerase I have detected a binuclear metal complex consisting of a pentacoordinate metal (site A) which shares a ligand, Asp-355, with an octahedral metal (site B) [Freemont, P. S., Friedman, J. M., Beese, L. S., Sanderson, M. R., & Steitz, T. A. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 8924–8928; Beese, L. S., & Steitz, T. A. (1991) *EMBO J. 10*, 25–33]. Kinetic studies of the activation of the 3′,5′-exonuclease reaction by Co^{2+}, Mn^{2+}, or Mg^{2+}, at low concentrations of DNA, reveal sigmoidal activation curves for the three metal ions with Hill coefficients of 2.3–2.4 and $K_{0.5}$ values of 16.6 μM, 4.2 μM, and 343 μM, respectively. The binding of Co^{2+} to the enzyme results in the appearance of an intense visible absorption spectrum of the metal ion with maxima at 633, 570, and 524 nm and extinction coefficients of 190, 194, and 150 M^{-1} cm^{-1}, respectively, suggesting the formation of a pentacoordinate Co^{2+} complex. Optical titration with Co^{2+} yields a sigmoidal titration curve which is best fit by assuming the cooperative binding of three Co^{2+} ions with a $K_{0.5}$ of 39.9 μM, comparable to the value of 16.6 μM obtained kinetically. Displacement of Co^{2+} by 1 equiv of Zn^{2+}, which binds tightly to the A site of the 3′,5′-exonuclease, shifts the optical spectrum to 524 nm and lowers the extinction coefficient to 30 M^{-1} cm^{-1}, indicative of octahedral coordination. EPR spectra of the enzyme-bound Co^{2+}, both in the absence or in the presence of Zn^{2+} to occlude site A, argue against tetrahedral Co^{2+} coordination and are consistent with pentacoordinate and octahedral complexation of the metal, as detected optically and by X-ray crystallography. It is suggested that three metal ions bind cooperatively to activate the exonuclease, one of which is pentacoordinate (site A) while the other two are octahedral. The binding of TMP reduces the ligand symmetry at the pentacoordinate site. One of the two octahedrally bound metal ions is probably at site B of the binuclear complex while the other binds nearby and, either directly or indirectly, facilitates the formation of the binuclear complex.

Escherichia coli DNA polymerase I is an enzyme of DNA replication and repair which has three distinct catalytic activities: DNA polymerase, a 3′,5′-exonuclease involved in proofreading, and a 5′,3′-exonuclease which functions in nick translation. Each activity has been shown to exist on separate domains of the complete enzyme, 928 residues in length. Pol I is proteolytically cleaved into two fragments. The large C-terminal Klenow fragment (residues 326–928) contains the polymerase and 3′,5′-exonuclease activities, while the small N-terminal fragment (residues 1–325) shows 5′,3′-exonuclease activity (Brutlag et al., 1969; Klenow & Overgard-Hansen, 1970; Kornberg, 1980). X-ray analysis of the large fragment of pol I (Ollis et al., 1985; Freemont et al., 1986; Derbyshire et al., 1988; Beese & Steitz, 1991) and kinetic (Kuchta et al., 1987, 1988; Catalano & Benkovic, 1989) and metal-binding studies (Mullen et al., 1990) of site-directed mutants of the cloned large fragment indicate that the 3′,5′-exonuclease activity is on the middle domain (residues 326–542) and that the polymerase activity is on the carboxy-terminal domain (residues 543–928) of pol I. Crystallographic studies, as well as kinetic studies of the large fragment with specifically cross-linked DNA, have further shown that the polymerase and 3′,5′-exonuclease sites are spatially separated by ~33 Å, permitting these sites to function independently.

While the polymerase active site interacts with a template–primer duplex, elongating the latter, the 3′,5′-exonuclease site catalyzes the hydrolysis of single-stranded DNA, yielding 5′-deoxynucleotides as products. For the 3′,5′-exonuclease to function in proofreading therefore requires the duplex DNA to melt by four or five base pairs to generate a single strand which reaches the exonuclease site, permitting the hydrolytic excision of a 5′-dNMP from the primer terminus (Cowart et al., 1989). The product, dNMP, inhibits the 3′,5′-exonuclease reaction (Que et al., 1978) and, as shown by X-ray, occupies the same site on the enzyme as the terminus of single-stranded DNA, thus locating the 3′,5′-exonuclease active site (Ollis et al., 1985; Freemont et al., 1988).

The proofreading 3′,5′-exonuclease requires divalent cations such as Mg^{2+}, Mn^{2+}, or Zn^{2+} for activity (Lehman & Richardson, 1964; Kornberg, 1980). X-ray studies in the presence of dNMP have shown the presence of two divalent cations at the exonuclease site designated sites A and B. The metal at site A is pentacoordinate, utilizing Asp-355, Glu-357, Asp-501, a water molecule, and a phosphate oxygen of dNMP as monodentate ligands. The weaker binding metal at site B, which is octahedral, is detected in the crystal only in the presence of dNMP (Freemont et al., 1988; Beese & Steitz, 1991), and in solution its binding affinity is increased 100-fold by the nucleotide TMP (Mullen et al., 1990). Metal B shares with metal A a phosphate oxygen of dNMP, as well as the other carboxylate oxygen of Asp-355 as ligands, forming a binuclear complex. In addition, metal B also coordinates another phosphate oxygen of dNMP as well as three water ligands to complete the octahedral complex. One of these waters is hydrogen bonded to Asp-424, providing an explanation for the loss of metal binding at site B in the D424A mutant, as detected both in the crystal (Derbyshire et al., 1988;

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Beese & Steitz, 1991) and in solution (Mullen et al., 1990).

Several lines of evidence suggest that metal ions must be present at both sites A and B for exonuclease activity. Thus, a comparison of the dissociation constant of Mg^{2+} from site A (38 μM) with the K_M for Mg^{2+} in the 3′,5′-exonuclease reaction (1.7–3.0 mM) indicates that one or more weak sites must be occupied for catalysis to occur but does not establish the role of the tight site (Lehman & Richardson, 1964; Slater et al., 1972; Derbyshire et al., 1991). The D424A mutation which blocks metal binding at site B (Derbyshire et al., 1988) and weakens metal binding at site A by an order of magnitude (Mullen et al., 1990) decreases exonuclease activity by 10^5-fold. Similarly, the double mutation D355A + E357A, which abolishes metal binding at both sites A and B as detected crystallographically (Derbyshire et al., 1988) and in solution (Mullen et al., 1990), also profoundly inactivates the exonuclease. While these studies indicate an important role of site B in catalysis, and possibly also of site A of the binuclear complex, Mullen et al. (1990) detected the binding of four additional Mn^{2+} ions to the 3′,5′-exonuclease domain, one of which was near enough to Mn^{2+} at sites A or B to induce dipolar relaxation. To further investigate the catalytic role, interactions, and coordination geometries of divalent cations at the active site of the 3′,5′-exonuclease in solution, the interaction of Co^{2+} with the enzyme was studied by kinetics, visible spectroscopy, and EPR spectroscopy. The results indicate that three Co^{2+} ions bind cooperatively to activate the 3′,5′-exonuclease. One of these sites appears to be pentacoordinate, and the others are octahedral. The cooperativity of the interaction detected by both kinetic and binding studies establishes the importance of the binuclear site, as well as a third site, in activating the 3′,5′-exonuclease reaction.

Experimental Procedures

Materials

Preparation of Enzyme. The Klenow fragment was purified to homogeneity from the *E. coli* strain CJ155 and stored as previously described (Joyce & Grindley, 1983; Mullen et al., 1990). Enyzme purity was judged by the observation of a single band in SDS–PAGE and by a polymerase specific activity of 9000 ± 500 units/mg and a 3′,5′-exonuclease specific activity of 900 ± 50 units/mg. The enzyme was further purified free of divalent cations by elution from a Sephadex G-25 column at 4 °C which had been thoroughly washed with 20 mM EDTA/16 mM K^+–TES, pH 7.5/32 mM KCl and equilibrated with 16 mM K^+–TES, pH 7.5/32 mM KCl. Elution was with the same buffer and salt, in the absence of EDTA. TES rather than Tris buffer was used since the former shows little or no tendency to complex with Co^{2+} (Serpersu et al., 1988). The protein concentration was spectrophotometrically determined using $A^{1\%}_{280} = 9.3$ and the molecular weight of 68 000 for the Klenow fragment.

$[^3H](dT)_{300}$. For use as substrate in the 3′,5′-exonuclease studies, $[^3H](dT)_{300}$ was prepared and purified as described by Kelly et al. (1970). The final $[^3H](dT)_{300}$ stock solution (0.23 mM in nucleotide residues) had a specific radioactivity of 3.04 cpm/nmol and was diluted appropriately for the kinetic studies. All compounds were of the highest purity commercially available. To remove trace metals, all buffer and salt solutions, except those containing divalent cations, were passed through the K^+ form of Chelex-100 (Bio-Rad) before use.

Methods

Enzyme Assays. The polymerase and 3′,5′-exonuclease activities were assayed by measuring the rate of incorporation of $[^3H]$d(ATP) to poly(dA-dT) template–primer and the rate of release of 5′-mononucleotide from $[^3H](dT)_{300}$, respectively (Setlow, 1974). In the standard assays, 1 unit of activity is defined as 10 nmol of total nucleotide residues polymerized or released in 30 min.

Kinetic Studies. Kinetic analyses of metal activation of the 3′,5′-exonuclease reaction were carried out with Co^{2+}, Mg^{2+}, and Mn^{2+} chlorides (0.25–200 μM) in 16 mM K^+–TES buffer, pH 7.5, containing 32 mM KCl at 24 ± 1 °C. In addition, each reaction mixture (200 μL) contained $[^3H](dT)_{300}$ (0.575–1.15 μM). After preincubation for 5 min, the reactions were started by adding enzyme at a final concentration of 64.5 nM. The reactions were stopped after 2 min by applying 50 μL of each reaction mixture to Whatman DE81 ion-exchange filter paper which had been processed as described by Setlow (1974). The kinetic data were analyzed by Lineweaver–Burk double-reciprocal plots and by Hill plots.

Co^{2+} Binding Studies. The binding of Co^{2+} to the 3′,5′-exonuclease was monitored spectrophotometrically using a Perkin Elmer λ-9 spectrophotometer at 24 ± 1 °C. In a typical experiment, the concentrated enzyme (133 μM in 16 mM K^+–TES buffer, pH 7.5, containing 32 mM KCl) was titrated by the stepwise addition of an appropriate aliquot of $CoCl_2$ solution, recording the spectrum from 300 to 800 nm after each addition. Titration curves were constructed by plotting the absorption at 633 nm against the total Co^{2+} concentration, $[Co]_T$, and fitted by computing the concentrations of free and bound Co^{2+} from eqs 1–3. In eqs 1–3, the subscripts f and

$$(K_{0.5})^n = \frac{[E][Co]_f^n}{[E\text{–}Co_n]} \quad (1)$$

$$[E]_T = [E]_f + [E\text{–}Co_n] \quad (2)$$

$$[Co]_T = [Co]_f + n[E\text{–}Co_n] \quad (3)$$

T refer to free and total species, n is the Hill coefficient, and $K_{0.5}$ is the nth root of the product of the consecutive dissociation constants $K_1K_2 \cdots K_n$ for each of the n individually bound Co^{2+} ions, which is equal to the concentration of free Co^{2+} at half-maximal change in absorbance. For $n = 3$, which provided the best fit to the data, eq 4 derived from eqs 1–3 was

$$27x^4 - 27([E]_T + [Co]_T)x^3 + 9([Co]_T^2 + 3[E]_T[Co]_T)x^2 - ([Co]_T^3 + 9[Co]_T^2[E]_T + K_{0.5}^3)x + [E]_T[Co]_T^3 = 0 \quad (4)$$

used to calculate the concentration of bound Co^{2+} (x), where $x = 3[E\text{–}Co_3]$, using the program MACSYMA. For $n = 2$, eq 5 was used.

$$4x^3 - 4([E]_T + [Co]_T)x^2 + ([Co]_T^2 + 4[E]_T[Co]_T + K_{0.5}^2)x - [E]_T[Co]_T^2 = 0 \quad (5)$$

At the end of the Co^{2+} titrations, 1 equiv of $ZnCl_2$ was added, to selectively displace Co^{2+} from the A site by Zn^{2+}, which binds tightly at this site (Beese & Steitz, 1991; Ferrin et al., 1983).

EPR Spectra of Enzyme–Co^{2+} Complexes. The EPR samples (200 μL) were prepared by adding appropriate amounts of $CoCl_2$, $ZnCl_2$, and d(TMP) to the concentrated enzyme solution (151 μM) in a 3-mm (i.d.) quartz EPR tube (Wilmad, 708 PSQ). Each sample was quickly frozen in isopentane, cooled by a liquid nitrogen cold finger, and then stored in liquid nitrogen. EPR spectra were obtained with an IBM model ER-200D-SRC spectrometer operating at X-band and using a 100-KHz modulation frequency. The temperature was maintained at ~8 K by liquid helium using an Air Products Model LTD-3-110 liquid-transfer Heli-Tran cryogenic unit equipped with an APD-E temperature controller. The microwave power was 8 mW, the sweep time was 200 s, the time constant was 0.5 s, the modulation amplitude was 8

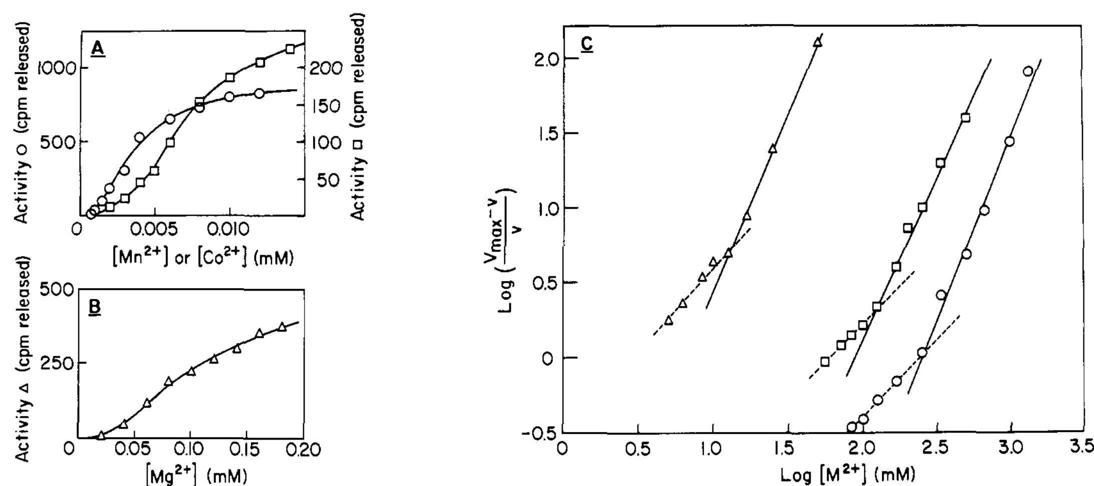

FIGURE 1: Activation of 3',5'-exonuclease by Co^{2+}, Mn^{2+}, and Mg^{2+}. (A) Activity of 3',5'-exonuclease as a function of the concentration of $CoCl_2$ (□) or $MnCl_2$ (○). Components present were 64.5 nM enzyme, 0.575 μM $[^3H](dT)_{300}$, 16 mM K^+–TES buffer, pH 7.5, and 32 mM KCl. T = 24 °C. (B) Activity of 3',5'-exonuclease as a function of the concentration of $MgCl_2$ (△). Components and conditions were as in (A) except that enzyme was 64.5 nM and $[^3H](dT)_{300}$ was 1.15 μM. (C) Hill plots of data from (A) and (B) for Co^{2+} (□), Mn^{2+} (○), and Mg^{2+} (△).

Table I: Kinetic Parameters of 3',5'-Exonuclease with Various Metals[a]

metal	Hill coefficient low [metal]	Hill coefficient high [metal]	$K_{0.5}$[b] (μM)	V_{max} ($\times 10^2$ s^{-1})
Co^{2+}	2.3	1.2	16.6	2.46
Mn^{2+}	2.4	1.1	4.2	5.44
Mg^{2+}	2.4	1.2	343	4.90

[a] pH 7.5, 32 mM KCl/16 mM K^+–TES; temperature = 24 ± 1 °C. [b] Concentration of divalent cation which gave half-maximal velocity at $[^3H](dT)_{300}$ concentrations of 0.575 μM for Co^{2+} and Mn^{2+} and 1.15 μM for Mg^{2+}. The errors in the kinetic parameters are ±10%.

G, and the frequency was 9.42 GHz. Each spectrum was the summation of four scans.

Results and Discussion

Metal Activation of 3',5'-Exonuclease. Since the 3',5'-exonuclease of pol I is known to require divalent cations such as Mg^{2+} or Mn^{2+} for activity, it is reasonable to expect Co^{2+} to activate as well. Activation by Co^{2+} would be of interest because of the chromophoric and paramagnetic properties of this ion which make it a valuable probe of coordination geometry. Such activation by Co^{2+} was indeed observed. At low DNA concentration, to avoid substrate inhibition, a plot of exonuclease activity as a function of Co^{2+} concentration (Figure 1A) reveals a sigmoidal curve indicating positive cooperativity in the interaction of Co^{2+} with the enzyme. A Hill plot of the data (Figure 1C) is biphasic, yielding Hill coefficients of 2.3 at low Co^{2+} concentrations (≤8 μM) decreasing to 1.2 at higher levels of Co^{2+} as saturation is approached (Table I). The apparent dissociation constant $K_{0.5}$ is 16.6 μM, and the maximum specific activity, expressed as a turnover number, is 0.025 s^{-1} (Table I). Cooperativity either in metal activation of the 3',5'-exonuclease reaction or in metal binding to this enzyme has not previously been reported. However, such cooperativity is reasonable, on the basis of the presence of a binuclear metal complex at the exonuclease active site and the detection of other nearby metal binding sites in Mn^{2+} binding studies. Hence, the effects of Mg^{2+} and Mn^{2+} on the activity of the 3',5'-exonuclease were reinvestigated in detail, obtaining numerous points along the activation curve (Figure 1). As with Co^{2+}, positive cooperativity was also detected with Mg^{2+} and with Mn^{2+}, both with Hill coefficients of 2.4 at low metal concentrations, which decreased to 1.2 and 1.1 as saturation was approached (Table I).

Since Hill coefficients give a lower limit to the number of interacting sites, the Hill coefficients of 2.3–2.4 suggest that, initially, at least three divalent cations bind cooperatively to activate the 3',5'-exonuclease. The cooperative binding of three metal ions to the enzyme could occur if the binding of the first metal ion induces a ligand rearrangement, or other conformational change, which facilitates the subsequent binding of two additional metal ions. The $K_{0.5}$ values (Table I) indicate that Mn^{2+} binds most tightly and that Mg^{2+} binds least tightly to activate the enzyme, while Co^{2+} binds with intermediate affinity.

The V_{max} values obtained with Co^{2+}, Mn^{2+}, and Mg^{2+} (0.025–0.054 s^{-1}, Table I) are comparable and very low as previously noted by others (Kuchta et al., 1988; Derbyshire et al., 1991). While these V_{max} values are more than 3 orders of magnitude slower than that of staphylococcal nuclease, a Ca^{2+}-activated DNase, measured under similar conditions, they are indistinguishable from V_{max} of the E43S mutant of staphylococcal nuclease in which the general base, Glu-43, has been mutated to Ser (0.035 s^{-1}; Serpersu et al., 1989). It is of interest that no obvious general base was detected crystallographically at the 3',5'-exonuclease site (Beese & Steitz, 1991), and no evidence for general base catalysis was obtained by kinetic studies as a function of pH (Derbyshire et al., 1991). Comparably low V_{max} values are also obtained in the endonuclease reaction catalyzed by a ribozyme (0.011 s^{-1}; Herschlag & Cech, 1990) which also lacks a general base.

Spectrophotometric Studies of Co^{2+} Binding. The binding of Co^{2+} to the Klenow fragment causes a marked intensification of the visible absorption spectrum of the metal ion resulting in absorption maxima at 633 nm (ϵ = 190 M^{-1} cm^{-1}), 570 nm (ϵ = 194 M^{-1} cm^{-1}), and 524 nm (ϵ = 150 M^{-1} cm^{-1}) (Figure 2, solid curve). The addition of 1 equiv of TMP per enzyme had no effect on the spectrum. A spectrophotometric titration of the enzyme with Co^{2+} in the presence of 1 equiv of TMP, monitoring the absorbance at 633 nm, reveals a sigmoidal increase in optical density as a function of Co^{2+} concentration (Figure 3). Identical behavior was observed in the absence of TMP (not shown). The solid curve which provides the best fit to the data was calculated by assuming that three Co^{2+} ions bind to the enzyme with complete cooperativity, i.e., with a Hill coefficient of 3 and with a $K_{0.5}$ = 39.9 μM, and that the E–$(Co^{2+})_3$ complex is the only species

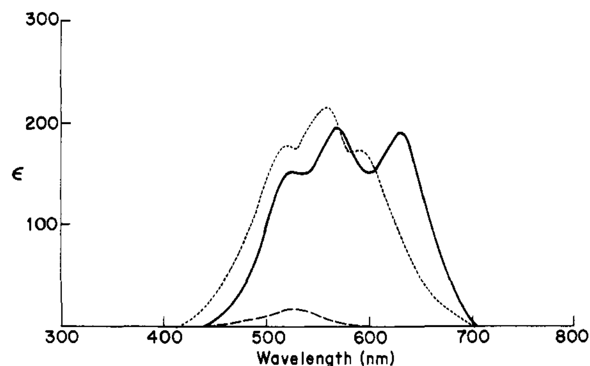

FIGURE 2: Visible spectra of Co^{2+}–3′,5′-exonuclease complexes. The solid curve is the spectrum of a solution containing enzyme (133 μM), $CoCl_2$ (798 μM), and TMP (133 μM) in the presence of 16 mM K^+–TES buffer, pH 7.5, and 32 mM KCl at 24 °C, corrected by subtraction of the dashed curve. The dashed curve is the spectrum obtained by adding 1 equiv of $ZnCl_2$ (133 μM) to the above solution. For comparison, the spectrum of the pentacoordinate carbonic anhydrase–Co^{2+}–benzoate complex (dotted line) is shown, which was obtained in 0.1 M phosphate buffer, pH 7.5 (Bertini et al., 1977). The extinction coefficients (ε) are expressed per site, assuming that the enzyme binds three Co^{2+} ions.

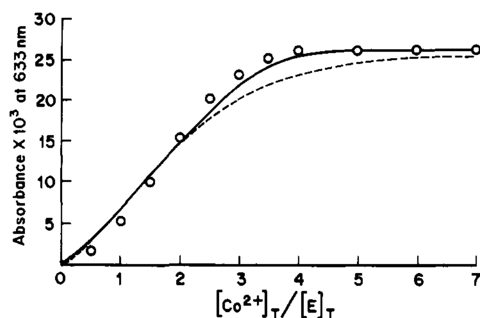

FIGURE 3: Spectrophotometric titration of 3′,5′-exonuclease with Co^{2+}, in the presence of TMP, monitoring the absorbance at 633 nm. Components present were 133 μM enzyme, 133 μM TMP, 16 mM K^+–TES, pH 7.5, and 32 mM KCl. T = 24 °C. The solid curve, which best fits the data, was calculated assuming a Hill coefficient of 3.0 and a $K_{0.5}$ of 39.9 μM. The dashed curve is the optimum fit obtained with a Hill coefficient of 2.0 and a $K_{0.5}$ of 106.4 μM.

detectable spectroscopically at 633 nm. The data could not be fit satisfactorily by assuming two cooperatively binding Co^{2+} ions (Figure 3). While the $K_{0.5}$ value obtained spectroscopically is 2.4-fold greater than that obtained kinetically, the kinetic studies were carried out in the presence of DNA which likely raised the affinity of the enzyme for Co^{2+}.

As noted above, the presence of 1 equiv of TMP per enzyme did not significantly alter the visible spectrum or the sigmoidal titration curve. However, the addition of 1 equiv of Zn^{2+} per enzyme in the presence of a 7-fold excess of Co^{2+} profoundly decreased the intensity of the visible spectrum to an extinction coefficient of 30 M^{-1} cm^{-1} and shifted the absorption maximum to 524 nm (Figure 4, dashed curve). Since Zn^{2+} is known to have a high affinity for the A site, it is clear that the original absorption spectrum was dominated by Co^{2+} bound at site A, and the weak absorption observed in the presence of Zn^{2+} is due to Co^{2+} bound at the two other sites, one of which is probably site B.

Coordination Geometry Based on Visible Spectra. The profound differences in the visible spectra of Co^{2+} bound at site A and at the other two sites indicate differences in coordination geometry at these sites. Ligand field theory predicts that optical transitions of four coordinate or tetrahedral Co^{2+} complexes usually give rise to intense absorptions (ε > 300) in the higher wavelength region of 625 ± 50 nm, while tran-

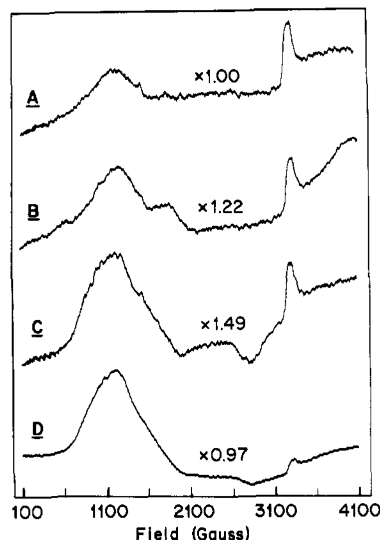

FIGURE 4: EPR spectra of Co^{2+}–3′,5′-exonuclease complexes at 8 K. Components present: (A) 151 μM enzyme, 151 μM $CoCl_2$; (B) as in (A) with 302 μM TMP; (C) as in (B) with 151 μM $ZnCl_2$; (D) as in (C) except that $CoCl_2$ was 755 μM. Other components present were 16 mM K^+–TES, pH 7.5, and 32 mM KCl. EPR conditions are given under Methods. The numbers on the spectra give the relative gains.

sitions of six coordinate or octahedral Co^{2+} complexes have very weak absorption (ε < 30) at lower wavelengths (525 ± 50 nm). Pentacoordinate Co^{2+} complexes show intermediate features: moderate absorption (50 < ε < 250) with several maxima between 525 and 625 nm (Horrocks et al., 1980; Bertini & Luchinat, 1984). On the basis of these criteria, the optical spectra of Co^{2+}–3′,5′-exonuclease complexes obtained in the absence and presence of Zn^{2+} suggest site A to be pentacoordinate and site B and the third site to be hexacoordinate. A spectrum very similar to that of site A of Co^{2+}–3′,5′-exonuclease has been obtained for the bovine carbonic anhydrase–Co^{2+}–benzoate ternary complex (Figure 4, dotted curve), which has been assigned a pentacoordinate geometry (Bertini et al., 1977). The small differences in the absorption positions and extinction coefficients may result from the different liganding atoms in the two cases: three nitrogens and two oxygens in the carbonic anhydrase–Co^{2+}–benzoate complex and five oxygens in the 3′,5′-exonuclease–Co^{2+} complex.

Coordination Geometry Based on EPR Spectroscopy. EPR spectroscopy at low temperature (4–20 K) provides an independent approach to the coordination geometry of high-spin Co^{2+} complexes (Kennedy et al., 1972; Banci et al., 1982). While EPR spectra of low symmetry Co^{2+} complexes generally give three g values which are related to the ligand geometry, in practice, the g values alone often fail to distinguish between tetrahedral, pentacoordinate, and octahedral Co^{2+} complexes. However, hyperfine coupling of the unpaired electrons with the ^{59}Co nucleus is usually larger in five- and six-coordinate complexes than in tetrahedral complexes, resulting in well-defined splittings or broader resonances at low field for five- and six-coordinate complexes than for tetrahedral species (Kennedy et al., 1972; Fung et al., 1974; Bencini et al., 1981).

The EPR spectra of Co^{2+}–3′,5′-exonuclease complexes in the absence (Figure 4A) and presence of TMP (Figure 4B–D) and in the absence (Figure 4A,B) and presence of Zn^{2+} (Figure 4C,D) are, with the possible exception of Figure 4C, quite featureless. However, in all cases, a broad low-field resonance is found, suggesting hyperfine coupling. Increasing the concentration of Co^{2+} to a 5-fold excess over the enzyme (Figure

4D) increases the intensity of the EPR spectrum obscuring any hyperfine splitting due to the occupancy of additional sites, but the low-field resonance remains broad. Hence, from the above arguments, tetrahedral coordination is unlikely for the three cooperatively bound Co^{2+} ions, in accord with the optical spectra.

The addition of TMP to the enzyme–Co^{2+} complex (Figure 4B) results in the appearance of a new transition at 1859 G (g = 3.62), indicating a decrease in the symmetry of the Co^{2+} complex. In the presence of Zn^{2+} (Figure 4C), which displaces Co^{2+} from site A, this transition is not detected at g = 3.62 but as a shoulder at 1512 G (g = 4.45) on the major transition at 1218 G (g = 5.53), suggesting that TMP interacts more strongly with metal A than with metal B to decrease the ligand symmetry.

Conclusions

Kinetic studies with Co^{2+}, Mg^{2+}, and Mn^{2+} and direct binding studies with Co^{2+} indicate that three metal ions bind cooperatively to activate the 3′,5′-exonuclease reaction of pol I. Optical and EPR spectra of the Co^{2+} complex suggest a pentacoordinate geometry at one site and an octahedral geometry at the other two sites. The Zn^{2+} ion selectively displaces Co^{2+} from the former site. The EPR spectra suggest stronger interaction of TMP with the pentacoordinate metal, decreasing the symmetry of the complex. These geometric findings in solution are consistent with X-ray studies of the enzyme–TMP complex in the presence of Zn^{2+} and Mg^{2+}, which revealed a binuclear complex with a pentacoordinate Zn^{2+} at site A and an octahedral Mg^{2+} at site B (Beese & Steitz, 1991), and with NMR studies which detected the presence of a third divalent cation near the binuclear complex (Mullen et al., 1990). Beese and Steitz (1991) have proposed specific roles in the exonuclease mechanism for the two metals of the binuclear complex. Metal A is proposed to activate the attacking water, and metal B promotes the departure of the leaving 3′-oxygen, while both cations activate the phosphodiester group. The role of the third metal ion, which binds cooperatively with the binuclear system, may be indirect, adjusting the protein structure and thereby facilitating the formation of the binuclear complex. Alternatively, since it may be near sites A and B, the third metal ion may play a more direct role in catalysis but, due to low occupancy in the crystal, may have escaped detection.

Acknowledgments

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Registry No. Co, 7440-48-4; Mn, 7439-96-5; Mg, 7439-95-4; DNA polymerase, 9012-90-2; 3′,5′-exonuclease, 79393-91-2.

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