Metal Binding to DNA Polymerase I, Its Large Fragment, and Two 3',5'-Exonuclease Mutants of the Large Fragment*

(Received for publication, December 14, 1989)

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DNA polymerase I (Pol I) is an enzyme of DNA replication and repair containing three active sites, each requiring divalent metal ions such as Mg2+ or Mn2+ for activity. As determined by EPR and by 1/T1 measurements of water protons, whole Pol I binds Mn2+ at one tight site (KD = 2.5 μM) and ~20 weak sites (KD ~ 600 μM). All bound metal ions retain one or more water ligands as reflected in enhanced paramagnetic effects of Mn2+ on 1/T1 of water protons. The cloned large fragment of Pol I, which lacks the 5',3'-exonuclease domain, retains the tight metal binding site with little or no change in its affinity for Mn2+, but has lost ~12 weak sites (n = 8, KD = 1000 μM). The presence of stoichiometric TMP creates a second tight Mn2+ binding site or tightens a weak site 100-fold, dGTP together with TMP creates a third tight Mn2+ binding site or tightens a weak site 166-fold. The D424A (the Asp356 to Ala) 3',5'-exonuclease deficient double mutant of the large fragment of Pol I 3',5'-exonuclease-deficient double mutant has lost the tight metal binding site and four weak metal binding sites. The binding of dGTP to the polymerase active site of the D355A,E357A double mutant creates one tight Mn2+ binding site with a dissociation constant (KD ~ 3.6 μM), comparable with that found on the wild-type enzyme, which retains one fast exchanging water ligand. Mg2+ competes at this site with a KD of 100 μM. It is concluded that the single tightly bound Mn2+ on Pol I and a weakly bound Mn2+ which is tightened 100-fold by TMP are at the 3',5'-exonuclease active site and are essential for 3',5'-exonuclease activity, but not for polymerase activity. Additional weak Mn2+ binding sites are detected on the 3',5'-exonuclease domain, which may be activating, and on the polymerase domain, which may be inhibitory. The essential divalent metal activator of the polymerase reaction requires the presence of the DNA polymerase I (Pol I) from Escherichia coli is an enzyme of DNA replication and repair which catalyzes template-directed DNA synthesis, 3',5'-DNA "proofreading" hydrolysis and 5',3'-DNA hydrolysis on three separate domains of a single polypeptide chain of 103,000 daltons (Brutlag et al., 1969; Klenow and Overgaard-Hausen, 1970; Ollis et al., 1985; Freemont et al., 1986; Derbyshire et al., 1988). NMR studies of the conformations of substrates and templates bound at the polymerase site (Ferrin and Miltvan, 1986, 1986; Mullen and Miltvan, 1988) and kinetic studies of DNA polymerization (Kuchta et al., 1987, 1988) have provided insight into the reaction mechanism of high fidelity DNA synthesis by this enzyme. X-ray crystallographic studies of complexes containing DNA bound at the 3',5'-exonuclease site of the large (Klenow) fragment of Pol I have provided structural insight into the mechanism of the order of magnitude increase in fidelity produced by the 3',5'-exonuclease (Freemont et al., 1988).

Each enzymatic activity of Pol I requires divalent metal ions such as Mg2+ or Mn2+. Slater et al., (1972) established that Pol I has multiple classes of divalent metal ion binding sites. Employing x-ray crystallography and genetic modification, Ollis et al., (1986) and Derbyshire et al., (1988) detected two adjacent divalent cations at the 3',5'-exonuclease active site in an enzyme-dNMP complex. The 3',5'-exonuclease activity was profoundly decreased by the D424A single mutation of a ligand for one of these metals. This activity was also greatly decreased by the D355A,E357A double mutation of two ligands for the other metal, one of which, D355, is shared by both metals. The single mutation resulted in undetectable metal binding at one of the two sites, and the double mutation abolished metal binding at both sites. These mutations had no effect on polymerase activity (Derbyshire et al., 1988). The cloning and overproduction of these mutants (Derbyshire et al., 1988) and of the wild-type large fragment (Joyce and Grindley, 1983) have permitted us to determine quantitatively in solution the affinities as well as the active

** This work was supported by Grant NP645 from the American Cancer Society, National Institutes of Health Grant DK29616 (to A. S. M.), and National Institutes of Health Postdoctoral Fellowship GM12658-01 (to G. P. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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site locations and liganding environments of the bound divalent cations on DNA polymerase I.

**EXPERIMENTAL PROCEDURES**

**Materials**

**Preparation of Enzymes**—Whole Pol I was prepared from _E. coli_ by the method of Jovin et al. (1969) as modified previously by Slater et al. (1979). Additionally, gel filtration was carried out on Sephadex G-100 to remove degraded fragments. In this gel filtration step, 10 mg of purified Pol I was loaded onto a 1 × 100-cm column and eluted using upward flow with 100 mM Tris-HCl, pH 7.5, containing 1 mM dithiothreitol.

Purification of the large fragment of Pol I from the _E. coli_ strain CJ155 was performed as described previously (Joyce and Grindley, 1983). The D424A and D355A,E357A enzymes were similarly purified from the appropriate genetically engineered mutants of _E. coli_ (Derbyshire et al., 1986). The enzymes were stored in either 50 mM K+ phosphate containing 0.5 mM dithiothreitol or 10 mM K₂ Pipes containing 1 mM dithiothreitol, pH 7.0, with 50% v/v glycerol at −20 °C or at an 85% ammonium sulfate solution at 0 °C. All enzymes were further purified free from divalent metal ions by elution from a G-25 Sephadex column at 4 °C, using 10 mM Tris-HCl, pH 7.5, and 32 mM KCl as elution buffer. In the absence of enzyme, using a Varian E-4 EPR spectrometer equipped with a variable frequency probe. The coordination number for fast exchanging waters coordinated to Mn²⁺ at 24 °C were determined by measuring the electron paramagnetic resonance signal intensity due to free Mn²⁺ in a mixture of free and bound Mn²⁺. A Scatchard plot of Mn²⁺ binding to whole Pol I and to its large fragment was studied by the EPR method which measures free Mn²⁺ in a mixture of free and bound Mn²⁺. A Scatchard plot of Mn²⁺ binding to whole Pol I based on EPR data (Fig. 1) may be fit most simply by assuming that the complete enzyme binds a single Mn²⁺ ion tightly (Kₒ = 2.5 ± 1.1 μM) and 20 ± 5 Mn²⁺ ions more weakly (Kₒ = 600 ± 300 μM) (Table I).

**RESULTS AND DISCUSSION**

*Mn²⁺ Binding to Whole Pol I and the Large Fragment of Pol I—Mn²⁺ binding to whole Pol I and to its large fragment was studied by the EPR method which measures free Mn²⁺ in a mixture of free and bound Mn²⁺. A Scatchard plot of Mn²⁺ binding to whole Pol I based on EPR data (Fig. 1) may be fit most simply by assuming that the complete enzyme binds a single Mn²⁺ ion tightly (Kₒ = 2.5 ± 1.1 μM) and 20 ± 5 Mn²⁺ ions more weakly (Kₒ = 600 ± 300 μM) (Table I). The presence of Mn²⁺-binding sites of intermediate affinity as found in whole Pol I, with little or no change in the presence of enzyme (Joyce and Grindley, 1983) have yielded the large fragment of Pol I (Mₘ = 68,000), which corresponds to the large proteolytic fragment (Klenow fragment) of the enzyme (Brutlag et al., 1969; Klenov and Overgaard-Hansen, 1970). The large fragment of Pol I retains the tight Mn²⁺ binding site, as found in whole Pol I, with little or no change.

The slightly reduced stoichiometry for Mn²⁺ binding at the tight site may be due to partial occupancy of this site by trace amounts of Zn²⁺ which is known to bind tightly at this site (Ferrin et al., 1983; Oliss et al., 1986).
in the affinity of this site for Mn$^{2+}$ (Table I) as determined by Scatchard analysis of both EPR and PRR data (Fig. 2, A and B). Therefore, the tight Mn$^{2+}$ binding site is not located on the 5',3'-exonuclease domain in Pol I. Differences between whole Pol I and the large fragment are found in a significantly reduced number of weak divalent cation binding sites on the large fragment ($n = 8.0 \pm 1.0$) (Table I), suggesting that they exist on the 5',3'-exonuclease domain.

While the EPR method measures free Mn$^{2+}$, the PRR method detects enzyme-bound Mn$^{2+}$ by its enhanced paramagnetic effects on 1/T, of water protons. The large enhancement factor ($t_b = 11.0 - 7.8$) (Table I), suggesting that they exist on the 5',3'-exonuclease domain.

**Effect of TMP and dGTP on the Mn$^{2+}$ Binding Properties of the Large Fragment of Pol I**—In the presence of a stoichiometric amount of TMP, which binds at the exonuclease site (Derbyshire et al., 1988), the large fragment of Pol I binds two Mn$^{2+}$ ions tightly ($K_D = 9.7 \pm 2 \mu M$) and 7.0 \pm 1.0 weakly ($K_D = 1200 \pm 600 \mu M$) (Fig. 2, C and D, Table I). Hence the presence of TMP has created an additional tight Mn$^{2+}$-binding site on the enzyme probably by tightening one of the weak exchanging water ligands remain coordinated to Mn$^{2+}$, i.e. the bound metals are not buried in the protein.

**TABLE I**

| Enzyme | Nucleotide | Method | Tight sites | Weak sites |
|--------|------------|--------|-------------|------------|
|        |            |        | $n^a$ | $K_D$ | $s^b$ | $n^a$ | $K_D$ | $s^b$ |
| UNA polymerase I | EPR | | 0.90 \pm 0.10 | 2.2 \pm 1.1 | 20 \pm 5 | 600 \pm 300 |
| Large fragment | EPR | | 0.63 \pm 0.15 | 6.8 \pm 3.0 | 8.0 \pm 1.0 | 1000 \pm 500 |
| Large fragment | PRR | | 0.64 \pm 0.24 | 2.7 \pm 1.2 | 13.2 \pm 1.4 | 8.0 \pm 1.0 | 1200 \pm 400 |
| Large fragment | TMP | EPR | 1.86 \pm 0.15 | 10.0 \pm 2.0 | 7.0 \pm 1.0 | 1200 \pm 600 |
| Large fragment | TMP | PRR | 1.75 \pm 0.15 | 9.4 \pm 2.0 | 11.0 to 7.8 | 7.0 \pm 1.0 | 2600 \pm 2200 |
| Large fragment | TMP, dGTP | PRR | 2.80 \pm 0.33 | 7.8 \pm 2.0 | 7.0 \pm 1.5 | >600 |
| Large fragment | TMP, dGTP | EPR | 2.90 \pm 0.32 | 6.7 \pm 2.0 | 10.6 to 8.8 | 7.0 \pm 1.0 | 8.8 \pm 0.7 |
| D424A | EPR | | 1.0 \pm 0.2 | 67 \pm 25 | 7.0 \pm 1.0 | 3500 \pm 1500 |
| D424A | PRR | | 1.0 \pm 0.2 | 69 \pm 25 | 14.0 \pm 0.7 | 7.0 \pm 1.5 | 3500 \pm 1500 |
| D424A | TMP | EPR | 0.7 \pm 0.1 | 72 \pm 20 | 7.0 \pm 1.0 | 5400 \pm 3400 |
| D424A | TMP | PRR | 0.7 \pm 0.1 | 61 \pm 20 | 15.8 \pm 1.5 | 7.0 \pm 3.3 | 7000 \pm 2300 |
| D355A,E357A | dGTP | EPR | 1.0 \pm 0.2 | 3.6 \pm 1.8 | 4.0 \pm 1.0 | >650 |
| D355A,E357A | dGTP | PRR | 1.0 \pm 0.2 | 3.6 \pm 1.8 | 4.0 \pm 1.0 | >650 |

* Stoichiometric.
  * PRR enhancement factor due to bound Mn$^{2+}$ in binary, ternary, or higher complexes. Error is expressed as two standard errors of the mean.
  * A systematic decrease in the average enhancement of the tight sites was observed with increasing occupancy.

The third Mn$^{2+}$-binding site created by the presence of dGTP is clearly on enzyme-bound dGTP rather than on free dGTP since its enhancement factor, $c_t = 10.5 \pm 2.7$, greatly exceeds the value of 2.2 found for binary Mn$^{2+}$-dNTP complex (Slater et al., 1972). The $c_0$ value for E-dGTP-Mn$^{2+}$ was determined from the average enhancement factor for the three tight sites (9.7 \pm 0.9) by factoring out the average enhancement factors of the other two tightly bound Mn$^{2+}$ ions independently measured in the E-Mn$^{2+}$-TMP complex. This value for $c_0$ will be confirmed in a later section by genetic deletion of the other two tight sites.

**Mn$^{2+}$ Binding to the D424A 3',5'-Exonuclease-deficient Mutant of the Large Fragment of Pol I**—With TMP bound at the 3',5'-exonuclease site, two metals were detected at this active site by a combination of genetic and x-ray crystallographic studies (Ollis et al., 1985; Steitz et al., 1987; Derbyshire et al., 1988) (Fig. 3). One of these metals (metal A) was coordinated by the 5'-phosphate of TMP and the carboxylates of Asp$^{355}$, Glu$^{357}$, and Asp$^{501}$, whereas the other metal (metal B) was also coordinated by the carboxylate of Asp$^{355}$ and formed a second-sphere complex with the carboxylate of Asp$^{350}$ and the phosphate of TMP (Fig. 3). In the absence of TMP, metal A remained bound to the wild-type crystalline enzyme, but metal B was not detected in the x-ray structure, suggesting that metal B was bound more weakly than metal A. The exonuclease-deficient mutant D424A also retained metal A but not metal B under the conditions of the crystallographic experiment, i.e., in the presence of TMP and 3 m (NH$_4$)$_2$SO$_4$.

We have studied Mn$^{2+}$ binding to the D424A mutant in the absence of nucleotides or metal-liganding salts (Fig. 4, A and B). Scatchard analysis of the binding data and PRR enhancements reveal the loss of approximately one weak site in comparison with the native large fragment and an order of magnitude weakening of the tight metal binding site (Table I).
The enhancement factor, $e_b$, for the tight Mn$^{2+}$ site on the D424A mutant (14.0 ± 0.7) agreed with that of the wild-type enzyme within experimental error (13.2 ± 1.4) (Table I), suggesting that the tightly bound Mn$^{2+}$ had neither gained nor lost water ligands in the mutant. Hence the weaker binding of Mn$^{2+}$ at this site in the D424A mutant could have resulted from loss of the electrostatic effect of the nearby Asp$^{424}$.

Unlike the wild-type large fragment, the D424A mutant showed no tightening of a weak metal-binding site in the presence of stoichiometric amounts of TMP (Fig. 4, C and D). Stated another way, in the presence of TMP, the D424A mutation caused the loss of one of the two tight Mn$^{2+}$-binding sites, and a weakening of the other one on the large fragment of Pol I (Table I).

The average enhancement factor of the residual weakly bound Mn$^{2+}$ in the binary complex of the D424A mutant ($e_b = 9.7 ± 2.1$) was greater than that obtained with the wild-type enzyme ($e_b = 5.7 ± 0.6$). This increase in the average $e_b$ value cannot be explained solely by the loss of a site with low
enhancement since the $c_b$ value of the lost site would have to be negative, a physical impossibility. Hence the observed increase in the average $c_b$ for weakly bound Mn$^{2+}$ remaining in the D424A mutant must result at least in part from the loss of dipolar interaction with Mn$^{2+}$ at site B which no longer exists (Fig. 3). Such dipolar interaction, which decreases $c_b$ due to a shorter electron spin relaxation time of Mn$^{2+}$ (Leigh, 1970; Gupta, 1977; Mildvan and Gupta, 1978) provides independent evidence for the loss of a weak metal binding site and requires that one or more of the weakly bound Mn$^{2+}$ ions in the wild-type enzyme is near site B, the weak site which was lost in the D424A mutant.

These results, obtained in solution, are consistent with the x-ray studies of the crystalline enzyme and provide additional quantitative information. Thus, we conclude that two Mn$^{2+}$ ions bind at the 3',5'-exonuclease site on the native enzyme in the absence of dNMP. One Mn$^{2+}$ ion binds tightly and one binds weakly. The presence of TMP raises the affinity of the wild-type enzyme for the weak Mn$^{2+}$-binding site. The D424A mutation abolishes Mn$^{2+}$ binding at the weak site and weakens Mn$^{2+}$ binding at the tight site, likely due to proximity of the two sites. The tight Mn$^{2+}$-binding site is probably site A, and the weak Mn$^{2+}$-binding site is probably site B detected in the crystal structure (Fig. 3). Our ability to detect metal occupancy at site B even in the absence of dNMP may well be due to the lower ionic strength of the present Mn$^{2+}$-binding experiments.

Metal Binding to the D355A,E357A 3',5'-Exonuclease-deficient Mutant of the Large Fragment—Further evidence that the tight Mn$^{2+}$-binding site, which is weakened by the D424A mutation, is indeed at the 3',5'-exonuclease site was obtained by solution studies of Mn$^{2+}$ binding to the D355A,E357A double mutant. EPR studies of solutions containing the double mutant (58-190 µM) and MnCl$_2$ (11 µM to 4.6 mM) did not detect tight binding of Mn$^{2+}$ to the enzyme, but only weak binding sites. Since EPR detects free Mn$^{2+}$, and since most of the Mn$^{2+}$ was free in these studies, the EPR data were not suitable for analysis to determine the $n$ and $K_b$ values of the residual weak sites. The PRR data, which detects the enhanced effect of bound Mn$^{2+}$, was more sensitive and could be analyzed by a Scatchard plot (Fig. 5) to yield both $n$ and $K_b$ values (Table I).

In agreement with the EPR observations, the analysis of the PRR data revealed that the tight Mn$^{2+}$-binding site was lost in the double mutant. In addition, 4 ± 1 weak sites were also lost in the double mutant, in comparison with the wild-type large fragment (Table I). Hence the mutated residues Asp355 and Glu357 contribute significantly to the binding of Mn$^{2+}$ at the tight site, presumably by direct coordination, and at 4 ± 1 weak sites either directly or indirectly. One of the weak sites which is lost in the D355A,E357A double mutant is probably site B detected in the crystal structure, since one

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**FIG. 3.** The 3',5'-exonuclease active site as determined by x-ray crystallography (Ollis et al., 1986; Steitz et al., 1987; Joyce and Steitz, 1987). The two divalent metal-binding sites are labeled A at the tight site and B at the weak site. The figure is modified from Steitz et al. (1987) and reproduced with permission.

**FIG. 4.** Scatchard plot of Mn$^{2+}$ binding to the D424A mutant of the large fragment of Pol I (A and B) and the D424A mutant-TMP complex (C and D) as determined by EPR (A and C) and PRR (B and D) measurements. Conditions are described in Fig. 1. Enzyme concentrations of 165, 217, and 239 pM (A and B) and 42 µM (C and D) were used. The concentration of TMP was 42 µM (C and D). Parameters used to calculate the theoretical curves are given in Table I.
of the mutated residues, Asp355, is also a ligand for Mn$^{2+}$ bound at site B (Fig. 3). These studies in solution thus confirm the theoretical curve are given in Table I.

In the double mutant, the average enhancement factor $\varepsilon_b$ for the residual weakly bound Mn$^{2+}$ ($\varepsilon_b = 15.7 \pm 1.6$) was significantly greater than the corresponding $\varepsilon_b$ value of the wild-type enzyme (5.7 $\pm$ 0.6) (Table I). As in the case of the D424A mutant, this increase in $\varepsilon_b$ cannot be due solely to the selective loss of Mn$^{2+}$ sites with low $\varepsilon_b$, since these $\varepsilon_b$ values would have to be negative, but requires an increase in $\varepsilon_b$ of one or more of the weakly bound Mn$^{2+}$ ions remaining in the D355A,E357A mutant. As discussed for the D424A mutant where a similar effect was observed (Table I), such an increase in $\varepsilon_b$ probably results from the loss of dipolar interactions, which were present in the wild-type enzyme, between weakly bound Mn$^{2+}$ ions remaining in the double mutant and Mn$^{2+}$ bound at sites B, A, or at one or more of the other lost sites.

Metal Binding at the Polymerase Active Site—Using the D355A,E357A double mutant which lacks the tight metal-binding site, we have investigated Mn$^{2+}$ binding at the polymerase active site in the presence of the substrate, dGTP. A Scatchard analysis of the EPR data for Mn$^{2+}$ binding to the enzyme-dGTP complex indicates the appearance of one tight metal binding site ($K_D = 3.6$ $\mu$M) in addition to the 4 $\pm$ 1 weak metal binding sites which were detected in the absence of the nucleotide (Fig. 6A, Table I). Because of the high concentrations of enzyme and dGTP required for $>$90% complexation, the levels of free Mn$^{2+}$ were too low for detection until saturating levels of Mn$^{2+}$ were approached, resulting in a larger error ($\pm$ 50%) in the $K_D$ of Mn$^{2+}$ at the tight site. In a competition experiment monitored by PRR, Mn$^{2+}$ displaced Mn$^{2+}$ from the tight polymerase site created by dGTP with a $K_D$ of 100 $\pm$ 20 $\mu$M (Fig. 6B).

The $\varepsilon_b$ value for the tightly bound Mn$^{2+}$ (10.8 $\pm$ 0.9) is much greater than that found for a binary Mn$^{2+}$-dNTP complex ($\varepsilon_b = 2.2$ $\pm$ 0.1) (Slater et al., 1972) establishing the existence of a ternary enzyme-dGTP-Mn$^{2+}$ complex in which the binding of dGTP to the enzyme has created a tight Mn$^{2+}$-binding site. This increase in the enhancement factor is a result of the increased correlation time of Mn$^{2+}$ due to binding to the enzyme-dGTP complex and is in quantitative agreement with the average value (10.5 $\pm$ 2.7) calculated above for the wild-type enzyme. The dissociation constant of Mn$^{2+}$ from the ternary complex ($K_D = 3.6$ $\mu$M) is 2.9-fold tighter than the $K_D$ value of a binary Mn$^{2+}$-dNTP complex under similar conditions (Slater et al., 1972), indicating that the enzyme has raised the affinity of bound dGTP for Mn$^{2+}$, possibly by contributing a ligand to the metal. Since dGTP would be expected to donate probably two (Burgers and Eckstein, 1979) and, at most, three ligands to octahedral Mn$^{2+}$ (Cohn and Hughes, 1962; Sternlicht et al., 1965; Sloan et al., 1975; Sloan and Milván, 1976), three or four water ligands would remain coordinated to Mn$^{2+}$ if the enzyme did not contribute any additional ligands. To examine this point, the number of residual fast exchanging water ligands on the tightly bound

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**Table II**

| [Mn$^2+$] | 1/(T$\rho_i$) (X 10$^{-6}$ s$^{-1}$) |
|-----------|-------------------------------|
| $\mu$M    | frequency (MHz)               |
| 15.0      | 24.3                          | 30.0 | 40.0 | 50.0 | 59.8 |
| 54.1      | 6.42                          | 6.35 | 5.66 | 4.46 | 3.40 | 2.69 |
| 72.1      | 6.50                          | 6.37 | 5.65 | 4.46 | 3.44 | 2.74 |

*The normalized longitudinal relaxation rates of water protons (1/T$\rho_i$), where $f = [Mn^{2+}]/[H_2O]$, as a function of precession frequencies of protons ($\omega$) and unpaired electrons ($\omega_e$) were analyzed according to the following equations (Mildvan and Gupta, 1978; Mildvan et al., 1980): $1/T\rho^{-1} = gC/\rho f(\tau_s) = 3\tau_s/(1 + \omega^2\tau_s^2) + 7\tau_s/(1 + \omega^2\tau_s^2) + 1\tau_s^{-1} = 1/\tau_S = [C/(1 + \omega^2\tau_s^2) + 4\tau_s/(1 + \omega^2\tau_s^2)],$ where $q$ is the number of fast-exchanging water ligands, $C$ is a product of physical constants, equal to 812 $\mu$A/s$^{-1}$ for Mn$^{2+}$-proton interactions, $\tau_s$ is the metal nuclear distance, $\tau_p$ is the dipolar correlation time, $\tau_s$ is the longitudinal electron spin-relaxation time, B is the field splitting parameter, and $r_i$ is a time constant for motion of the water ligands which modulates B. The dipolar correlation times $\tau_s$ are given for 24.3 MHz, and the $q$ value is averaged over all frequencies.

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**Fig. 5.** Scatchard plot of Mn$^{2+}$ binding to D355A,E357A mutant of the large fragment of Pol I as determined by PRR measurements. Conditions are described in Fig. 1. Enzyme concentrations of 58-190 $\mu$M were used. Parameters used to calculate the theoretical curve are given in Table I.

**Fig. 6.** Metal binding to the D355A,E357A mutant of the large fragment of Pol I in the presence of dGTP. The enzyme and dGTP concentrations were 86 $\mu$M. Other conditions are described in Fig. 2. A, Scatchard plot of Mn$^{2+}$ binding to the enzyme-dGTP complex determined by PRR data. B, Mn$^{2+}$ displacement of Mn$^{2+}$ from the enzyme-Mn$^{2+}$-dGTP complex, at a total Mn$^{2+}$ concentration of 72 $\mu$M. Theoretical curve was calculated assuming simple competition between Mn$^{2+}$ ($K_D = 0.6$ $\mu$M) and Mn$^{2+}$ ($K_D = 100 \pm 20$ $\mu$M).


Mn$^{2+}$ was determined by analysis of the frequency dependence of the PRR of water (Table II). The results indicate that in the ternary enzyme-dGTP-Mn$^{2+}$ complex, only one rapidly exchanging water ligand remains coordinated to Mn$^{2+}$. Hence two or three water ligands of Mn$^{2+}$ have either been replaced by ligands from the enzyme or alternatively have been occluded by the enzyme such that they exchange with solvent at a rate slower than 10$^6$s$^{-1}$.

CONCLUSIONS

From the Mn$^{2+}$ binding data for Pol I, its large fragment, and the 3',5'-exonuclease-deficient mutants of the large fragment, we can infer the distribution of Mn$^{2+}$-binding sites among the three domains of Pol I and some of the properties of these sites. Based on affinity for the enzyme, two types of sites are detected, tight sites with dissociation constants in the micromolar range and weak sites with dissociation constants in the millimolar range. The locations of these sites are summarized in Fig. 7. Approximately 12 weak Mn$^{2+}$-binding sites reside on the 5',3'-exonuclease domain, since ~12 fewer weak sites are detected on the whole Pol I. One or more of these sites may be essential for the 5',3'-exonuclease reaction, since this reaction is known to require a divalent cation (Klett et al., 1968) and this requirement is typically satisfied by 7 mM Mg$^{2+}$ (Lehman and Richardson, 1964; Deutscher and Kornberg, 1969).

The 3',5'-exonuclease domain binds one Mn$^{2+}$ tightly using the carboxylate ligands Asp$^{355}$ and Glu$^{357}$, since mutation of these residues abolishes the tight binding of Mn$^{2+}$ (Table I) as well as 3',5'-exonuclease activity (Derbyshire et al., 1988). This site corresponds to site A in the crystallographic model of the exonuclease active site (Fig. 3). The previous view that the tight Mn$^{2+}$-binding site on Pol I detected in the absence of a substrate is at the polymerase site (Slater et al., 1972) is ruled out. This tight site, now known to be site A, binds Mg$^{2+}$ with a dissociation constant of 38 ± 9 μM (Slater et al., 1972) and has a high affinity for Zn$^{2+}$ (Ferrin et al., 1983; Ollis et al., 1985). The occupancy of this site alone by Mg$^{2+}$ is insufficient to activate the 3',5'-exonuclease reaction since the apparent $K_M$ for Mg$^{2+}$ is approximately 1.7 mM, and the optimum Mg$^{2+}$ is between 7 and 20 mM with whole Pol I (Lehman and Richardson, 1964). A more recent estimate of the $K_M$ for Mg$^{2+}$ with the cloned large fragment is approximately 3 mM.2 An adjacent Mn$^{2+}$ on the 3',5'-exonuclease domain is weakly bound at or near Asp$^{357}$ in the absence of dNTP and tightly in the presence of dNTP, since mutation of this residue removes the tight site created by dNTP, induces an order of magnitude lowering of the affinity for the tightly bound Mn$^{2+}$ (Table I), and abolishes 3',5'-exonuclease activity (Derbyshire et al., 1988). This site, which binds Mn$^{2+}$ 100-fold more tightly in the presence of dNTP (Table I), is most likely site B in the crystallographic model (Fig. 3). At least four additional weak Mn$^{2+}$-binding sites are located on the exonuclease domain, since three in addition to site B are lost in the D355A,E357A double mutant, and at least one remaining Mn$^{2+}$ is near enough to Mn$^{2+}$ that was bound at site B or A to undergo dipolar relaxation. Occupancy of site B and possibly one or more of the other weak metal binding sites is necessary to explain the high $K_M$ for Mg$^{2+}$ in the 3',5'-exonuclease reaction.

The remaining 3 ± 1 weak Mn$^{2+}$-binding sites are probably on the polymerase domain or, less likely, on the 3',5'-exonuclease domain, remote from the exonuclease active site. Although the polymerase domain does not bind Mn$^{2+}$ tightly in the absence of a substrate, the binding of dNTP induces the tight binding of Mn$^{2+}$ at the polymerase active site, indicating that the Mn$^{2+}$ is coordinated by the enzyme-bound substrate. A ligand to Mn$^{2+}$ may also be donated by the enzyme which would explain the higher affinity for Mn$^{2+}$ and the single fast exchanging water ligand remaining on the metal. A comparison of the dissociation constant for Mn$^{2+}$ at the site induced by dGTP ($K_D$ = 3.6-7.8 μM, Table I) with the kinetically determined Michaelis constant for free Mn$^{2+}$ in the polymerization reaction ($K_4$ = 9.8 ± 2.8 μM, Slater et al., 1972)

2 V. Derbyshire, N. D. F. Grindley, and C. M. Joyce, personal communication.

FIG. 7. Schematic three-dimensional structure of Pol I modified from Ollis et al. (1985) depicting tight (T) and weak (W) divalent metal ion binding sites detected in the present studies. The structure and location of the 5',3'-exonuclease domain is unknown and is represented by a dashed circle. The weak site adjacent to the tight site in the 3',5'-exonuclease (middle) domain is tightened 100-fold in the presence of TMP. The conformation of the base and sugar of the bound dNTP substrate is based on intramolecular nuclear Overhauser effect studies (Ferrin and Mildvan, 1985, 1986). The location of the dNTP substrate is based on intermolecular nuclear Overhauser effect studies (Ferrin and Mildvan, 1985, 1986), photoaffinity labeling (Joyce et al., 1985), and on the binding of substrates to a peptide fragment of Pol I consisting of residues 728-777 (Mullen et al., 1989). The location of the triphosphate moiety is speculative, and the βγ coordination of the metal is based on kinetic studies (Burgers and Eckstein, 1979) and NMR measurements (Sloan et al., 1975).
indicates this to be the active site for DNA polymerization. At higher levels, Mn$^{2+}$ inhibits polymerization with a $K_i$ of 600 ± 300 μM (Slater et al., 1972) consistent with the occupancy of one or more of the weak binding sites (Table I). In vivo, the relevant activator is Mg$^{2+}$ which binds ligands more weakly than Mn$^{2+}$ does and is spectrophotometrically inert. By competition with Mn$^{2+}$, the $K_D$ of Mg$^{2+}$ from the polymerase-dGTP complex found here (100 ± 2 μM) is comparable with the dissociation constants of binary Mg$^{2+}$-dNTP complexes (Mildvan and Cohn, 1966), indicating little additional contribution to affinity by the enzyme at the polymerase active site. Like Mn$^{2+}$, at higher levels, Mg$^{2+}$ also inhibits polymerization ($K_i$ = 3 mM, Travaglini et al., 1975) probably due to the occupancy of one or more of the weak metal binding sites. The modest (≤ 3-fold) increase in the affinity of dGTP for divalent cations induced by binding of the nucleotide to the enzyme may be due to a change in metal-nucleotide coordination from tridentate α,β,γ in the binary metal-dGTP complex, to bidentate β,γ in the ternary complex, consistent with activation of the leaving pyrophosphate group by the activating metal (Fig. 7).

Acknowledgments—We are grateful to Catherine M. Joyce and to Victoria Derbyshire for generously providing us with the mutant enzymes and the E. coli strains for the wild type and double mutant enzymes and for their helpful comments.

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