Magnetic Resonance and Kinetic Studies of the Role of the Divalent Cation Activator of RNA Polymerase from *Escherichia coli*†

Ruth Koren and Albert S. Mildvan*

ABSTRACT: The interactions of Mn^{2+}, substrates, and initiators with RNA polymerase have been studied by kinetic and magnetic resonance methods. As determined by electron paramagnetic resonance, Mn^{2+} binds to RNA polymerase at one tight binding site with a dissociation constant less than 10 μM and at 6 ± 1 weak binding sites with dissociation constants 100-fold greater. The binding of Mn^{2+} to RNA polymerase at both types of sites causes an order of magnitude enhancement of the paramagnetic effect of Mn^{2+} on the longitudinal relaxation rate of water protons, indicating the presence of residual water ligands on the enzyme-bound Mn^{2+}. A kinetic analysis of the Mn^{2+}-activated enzyme with poly(dT) as template indicates the substrate to be MnATP under steady-state conditions in the presence or absence of the initiator ApA. ATP and UTP interact with the tightly bound Mn^{2+} to form ternary complexes with ~50% greater enhancement factors. The dissociation constant of MnATP from the tight Mn^{2+} site as determined by longitudinal proton relaxation rate (PRR) titration (4.7 μM) is similar to the K_M of MnATP in the ApA-initiated RNA polymerase reaction (10 ± 3 μM) but not in the ATP-initiated reaction (160 ± 30 μM). Similarly, the dissociation constant of the substrate MnUTP from the tight Mn^{2+} site (90 μM) is in agreement with the K_M of MnUTP (101 ± 13 μM) when poly[d(A-T)]·poly[d(A-T)] is used as template, indicating the tight Mn^{2+} site to be the catalytic site for RNA chain elongation. Manganese adenylyl imidodiphosphate (MnAMP-PNP) has been found to be a substrate for RNA polymerase. It has the same affinity as MnATP for the tight site but, unlike the results obtained with MnATP, the enhancement is decreased by 43% in the enzyme-Mn-AMP-PNP complex. These results suggest that the enzyme-bound Mn^{2+} interacts with the leaving pyrophosphate group. The initiators ApA and ApU and the inhibitor rifamycin interact with the enzyme-Mn^{2+} complex producing small (15–20%) decreases in the enhancement. The dissociation constant of ApA estimated from PRR data ($\leq$1.5 μM) agrees with that determined kinetically (1.0 ± 0.5 μM) as the concentration of ApA required to produce half-maximal change in the K_M of MnATP. In the presence of the initiation specific reagents ApA, ApU, or rifamycin, the affinity of the enzyme-Mn complex for ATP or UTP shows little change. However, ATP and UTP no longer increase the enhancement factor of the tightly bound Mn^{2+} but decrease it by 30–55%, indicating a change in the environment of the Mn^{2+}-substrate complex on the enzyme when the initiation site is either occupied or blocked. Although the role of the six weak Mn^{2+} binding sites is not clear, the presence of a single tightly bound Mn^{2+} at the catalytic site for chain elongation which interacts with the substrate reinforces the number of active sites as one per molecule of holoenzyme and provides a paramagnetic reference point for further structural studies.

All RNA polymerases require a divalent cation, such as Mg^{2+} or Mn^{2+}, for activity (Burgess, 1971). Although many studies have been made of the complex catalytic properties of RNA polymerase and of the interaction of substrates, templates and other effectors with the enzyme from *Escherichia coli* (Burgess, 1971; Chamberlin, 1974), little is known about the site of action of the essential divalent cation. Thus it is not clear whether Mg^{2+} or Mn^{2+} is required simply to bind to the nucleoside triphosphate substrate, or to the initiation or elongation sites of the enzyme or to a combination of these sites. Magnetic resonance techniques with paramagnetic probes such as Mn^{2+} have proven useful in determining the role of metal ions in the mechanism of action of various enzymes (Mildvan and Cohn, 1970) including DNA polymerase (Slater et al., 1972; Sloan et al., 1975). The present work has been undertaken to determine the function of the divalent metal cofactor of *E. coli* RNA polymerase. To this end magnetic resonance techniques (EPR and PRR)[1] have been used to study the interaction of the paramagnetic activator Mn^{2+}, as well as substrates and initiators with RNA polymerase. The results, in conjunction with a steady-state kinetic analysis of the Mn-activated enzyme, have clarified the role of the divalent cation cofactor of RNA polymerase. A preliminary report of this work has been published (Koren et al., 1976b).

Experimental Procedures

Materials

Enzyme Preparation. E. coli RNA polymerase was prepared by two different methods. Preparation I was prepared by the earlier procedure of Burgess (1969a) using 10 lb of *E. coli* B purchased from Grain Processing Co. The preparation was 92% pure as judged by gel electrophoresis in the presence of sodium dodecyl sulfate. From such gels 17 ± 3% of the holoenzyme protein was estimated to be the σ subunit in accord with the predicted value of 19 ± 3% for one σ subunit per holoenzyme (Burgess, 1969b). Preparation II was prepared from 5 lb of the bacteria by Burgess' more recent procedure (Burgess

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[1] Abbreviations used: EPR, electron paramagnetic resonance; PRR, longitudinal proton relaxation rate; AMP-PNP, adenylyl imidodiphosphate; UV, ultraviolet; NMR, nuclear magnetic resonance.

and Jendrisak, 1975) using polymin P (Zillig et al., 1970) and a DNA-cellulose column. Preparation II was estimated to be 96% pure by sodium dodecyl sulfate gel electrophoresis. The σ subunit constituted 16 ± 3% of the holoenzyme protein. As with preparation I, this corresponds to approximately 1 mol of σ subunit per mol of holoenzyme. The presence of all other subunits in both preparations in the appropriate stoichiometry was also verified by gel electrophoresis in the presence of sodium dodecyl sulfate (Burgess, 1969a,b). Specific activities of the two preparations were comparable. Enzyme concentrations were determined by UV absorption, using $\epsilon_{280} = 0.65$ $(mg/ml)^{-1}$ assuming a molecular weight of 500 000 for the holoenzyme (Burgess, 1969b). Nucleoside triphosphates were purchased from Sigma, dissolved in water, and neutralized at 2 °C with KOH. The purity of all nucleotides was established by thin-layer chromatography. Concentrations were determined by UV absorption and in the case of ATP by enzymatic assay as well (Lamprecht and Trautschold, 1965). 5′-Adenylyl imidodiphosphate (AMP-PNP), purchased from P-L Biochemicals, was found to be 90% pure by thin-layer chromatography on polyethylenimine plates using 1 M LiCl as solvent. No ATP or ADP was detected by enzymatic assay (Lamprecht and Trautschold, 1965). [^{3}H]ATP and [^{3}H]UTP were purchased from New England Nuclear Corp. All reagents were the purest available commercially. All solutions were treated with Chelex 100 (Bio-Rad) before use to remove metal contaminants. Poly(dT) (average molecular weight 4×10^5), purchased from P-L Biochemicals (Lot No. 508-68), was dissolved in 50 mM Tris-Cl (pH 7.5) and the concentration was determined by UV absorbancy using $\epsilon_{264} = 22.4$ $(mg/ml)^{-1}$. Poly[d(A-T)]·poly[d(A-T)], free of guanosine and cytosine nucleotides, generously provided by Dr. S. S. Agarwal of this Institute, had been prepared by de novo synthesis using homogeneous *E. coli* DNA polymerase I as described by Radding and Kornberg (1962). The average molecular weight was $>4 \times 10^6$ as estimated by the sedimentation constant (>18 S) in neutral sucrose gradients. The concentration was determined by UV absorbancy in 0.1 M KCl, pH 7.0, using $\epsilon_{260} = 20.0$ $(mg/ml)^{-1}$. The dinucleoside monophosphates, ApA and ApU purchased from Sigma, were dissolved and neutralized as described above. Their concentrations were determined by UV absorption using $\epsilon_{257} = 2.78 \times 10^4$ M^{-1} for ApA (Ts'o et al., 1966) and 2.32×10^4 M^{-1} for ApU (P-L Biochemicals, 1975).

Rifamycin SV, purchased from P-L Biochemicals, was dissolved in 1.7% ethanol in water, and its concentration was determined spectrophotometrically using $\epsilon_{314} = 32.2$ $(mg/ml)^{-1}$ (Stecher, 1968). Polymin P was generously provided by the BASF Wyandotte Corp., Parsippany, N.J.

Methods

Preparation of Enzyme Solutions. Prior to kinetic experiments, RNA polymerase was dialyzed overnight against 70 mM Tris-HCl (pH 7.5) and 0.2 M KCl (referred to as buffer A). For NMR and EPR experiments the enzyme was precipitated by adding solid $(NH_4)_2SO_4$ (0.5 g/ml) and centrifuged at 15 000 rpm for 20 min. The pellet was redissolved in buffer A, desalted by passage through a Sephadex G-25 column, and concentrated by vacuum dialysis against the same buffer to a final concentration of ~15 mg/ml. No loss of enzyme activity was observed during the course of the magnetic resonance experiments.

Magnetic Resonance Techniques. Two methods, EPR and the enhancement (ϵ) of the longitudinal proton relaxation rate (PRR) of water at 24.3 MHz, were used to study the interaction of Mn^{2+} with *E. coli* RNA polymerase, with poly(dT) and with various nucleotides and small ligands as previously described (Slater et al., 1972; Mildvan and Engle, 1972). The EPR method was used to determine the concentration of free Mn^{2+} in a mixture of free and bound Mn^{2+} (Cohn and Townsend, 1954). The PRR method was used independently to detect and characterize binary, ternary, and quaternary complexes. The observed enhancement (ϵ^*) is defined as the ratio of the paramagnetic contribution to $1/T_1$ of water protons in the presence of a Mn^{2+} complex to that in the presence of Mn^{2+} alone and consists of the weighted average of the enhancements due to all forms of Mn^{2+} (Mildvan and Cohn, 1970; Mildvan and Engle, 1972). The PRR data were analyzed as previously described (Mildvan and Engle, 1972) to yield enhancement factors of binary (ϵ_b), ternary (ϵ_T), and quaternary (ϵ_q) complexes. The dissociation constants relevant to a ternary enzyme-metal-ligand (E-M-L) interaction are defined as follows (Mildvan and Engle, 1972):

$$K_1 = [M][L]/[ML],\ K_2 = [E][ML]/[EML]$$

$$K_3 = [EM][L]/[EML],\ K_D = [E][M]/[EM]$$

$$K_A' = [EL][M]/[EML], \text{ and } K_S = [E][L]/[EL]$$

From their definitions it can be shown that

$$K_1K_2 = K_3K_D = K_A'K_S \quad (1)$$

For each system studied K_1 and K_D were directly determined by EPR titrations, while K_3 and K_S were determined by PRR titrations, permitting the calculation of K_2 and K_A'. The PRR data were analyzed by the computer program of Reed et al. (1970). Quaternary systems were analyzed in a similar manner, with the difference that E-S was substituted for E in the definitions of the dissociation constants, E-S denoting the enzyme in the presence of a saturating concentration of the first substrate, which was then titrated by a second substrate. Theoretical titration curves were calculated using those dissociation constants which minimized the percent standard deviation of the enhancements of the ternary or quaternary complex over the entire range of the titrations.

Kinetic Experiments. The assay used for the steady-state kinetic analysis of RNA polymerase measured the incorporation of [^{3}H]ATP into an acid-insoluble product during the initial period of incorporation, using poly(dT) as template. The reaction mixture, in a total volume of 0.05 ml, contained components in the following concentrations: 50 mM Tris-Cl (pH 7.5), 0.15 M KCl, 0.05 mg/ml poly(dT) (132 μM nucleotide phosphorus), and varying concentrations of [^{3}H]ATP (~20 cpm/pmol), ApA and Mn^{2+}. Each reaction mixture contained 2–4 pmol of RNA polymerase. The reaction mixture was incubated for 10 min at 24 °C and was stopped by adding 2 ml of 1 M $HClO_4$ containing 20 mM sodium pyrophosphate. The acid-insoluble radioactivity was measured as described by Loeb (1969). The kinetic data were analyzed by double-reciprocal plots using the integrated Michaelis-Menten equation (Dixon and Webb, 1964) when necessary since ATP incorporation occasionally exceeded 10% of the initial concentration. Kinetic constants were evaluated using a nonlinear least-squares program (Wilkinson, 1961) on a Wang desk computer.

To determine the K_M of MnUTP, poly[d(A-T)]·poly[d(A-T)] was used as template, Mn^{2+} was at a constant saturating concentration (1 mM), and [^{3}H]ATP and UTP concentrations were independently varied over the concentration range of 10 to 500 μM. Other components present were

TABLE I: Binary Complexes of Mn^{2+} with RNA Polymerase.[a]

Enzyme Preparation	Tight Sites			Weak Sites		
	n_1	K_D (μM)	ϵ_b	n_2	K_D (μM)	ϵ_b
I	1.0 ± 0.1	1.9 ± 0.2	6.0 ± 1.5	6 ± 1	200 ± 100	10.5 ± 1.5
II	1.2 ± 0.3	9.0 ± 1	11.8 ± 1.8	6.5 ± 1.5	900 ± 300	11.8 ± 1.8

[a] ϵ_b, the enhancement of the longitudinal PRR due to bound Mn^{2+}, was determined by $1/T_1$ studies of water and EPR as previously described (Mildvan and Engle, 1972; Slater et al., 1972).[2] n refers to the number of Mn^{2+}-binding sites and K_D is the dissociation constant of Mn^{2+}.

TABLE II: Dissociation Constant and Enhancement Factors of Binary Complexes of Mn^{2+} with Ribonucleotide Derivatives.[a]

Complex	K_1 (μM)	ϵ_a
MnApA	103 000 ± 8 000	
MnApU[b]	225 000 ± 50 000	2.9 ± 0.5
MnATP	13.2 ± 1.5	1.2 ± 0.2
MnAMP-PNP	10.4 ± 1.9	1.2 ± 0.2
MnUTP	10.9 ± 0.6	1.0 ± 0.1
MnCTP	20.3 ± 1.0	1.3 ± 0.2

[a] Dissociation constants (K_1) were determined by EPR at 24 ± 1 °C and enhancement factors (ϵ_a) were determined by PRR and EPR at 22 ± 2 °C in the presence of 50 mM Tris-Cl (pH 7.5)–150 mM KCl. [b] Determined by Dr. Bruce Bean.

RNA polymerase (preparation II, 4 pmol per assay), 50 mM Tris-Cl, pH 7.5, and 0.15 M KCl (T = 24 °C). The same template and conditions were used to show that AMP-PNP was a substrate, with the following exceptions: RNA polymerase (preparation I, 7.3 pmol per assay), 200 μM [^{3}H]UTP (specific activity 5.1 cpm/pmol), 0.9 mM $MnCl_2$, and either 660 μM AMP-PNP or 520 μM ATP. The reactions were stopped and the data were analyzed as described above.

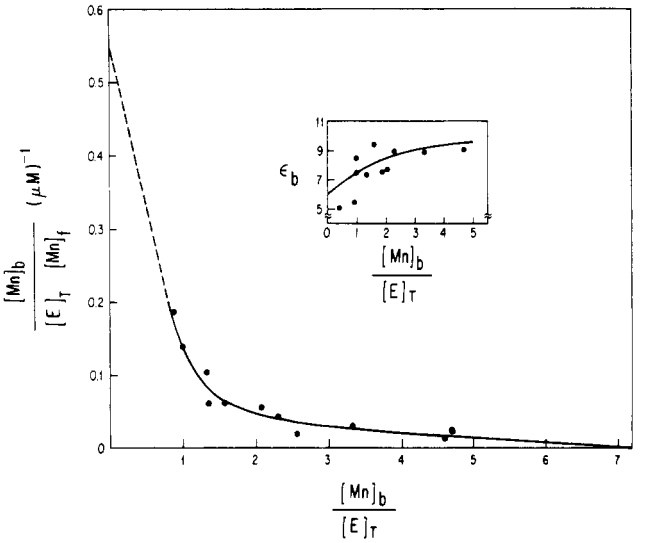

FIGURE 1: Scatchard plot of the binding of Mn^{2+} to RNA polymerase. Solutions contained: RNA polymerase (22.4 μM or 11.2 mg/ml, preparation I), $MnCl_2$ (20–500 μM), 50 mM Tris-Cl buffer (pH 7.5), and 150 mM KCl. T = 24 °C. Inset: Plot of ϵ_b, the enhancement of the PRR due to bound Mn^{2+}, as a function of site occupancy in the same solutions. $[Mn]_f$ indicates free Mn^{2+} and $[Mn]_b$ indicates enzyme-bound Mn^{2+}.

Results

Properties of Binary Enzyme–Mn^{2+} Complexes. The binding of Mn^{2+} to *E. coli* RNA polymerase was studied by titration of the enzyme with Mn^{2+} measuring the free Mn^{2+} by EPR. The tight binding of Mn^{2+} at a single site on the enzyme was indicated qualitatively by the absence of detectable free Mn^{2+} until the total Mn^{2+} concentration was approximately equal to the total enzyme concentration. At Mn^{2+} concentrations exceeding the enzyme concentration, free Mn^{2+} was detected, and the titration data could be analyzed quantitatively. The data from such a titration, shown in the form of a Scatchard plot (Figure 1), have been fit with an average deviation of 11.3% by a calculated curve which assumes the existence of 1.0 ± 0.1 tight Mn^{2+} binding sites with a dissociation constant K_D = 1.9 ± 0.2 μM and 6 ± 1 weak binding sites with a K_D = 200 ± 100 μM (Table I). Values of n and K_D beyond these limits produced much poorer fits to the data. Parallel PRR measurements of the paramagnetic effects of Mn^{2+} on $1/T_1$ of water protons (Figure 1, inset) for the same points yield enhancement factors (ϵ_b), ranging from 5 to 9. Analysis of the ϵ_b values as a function of occupancy yields an ϵ_b of 6.0 ± 1.5 for the tight site and an average ϵ_b of 10.5 ± 1.5 for the weak sites (Table I). The second enzyme preparation showed similar stoichiometry in the binding of Mn^{2+} at 1.2 ± 0.3 tight sites and 6.5 ± 1.5 weak sites, but the dissociation constants for Mn^{2+} were fivefold greater, K_1 = 9.0 ± 1 μM and K_2 = 900 ± 300 μM, respectively (Table I). Moreover, for this preparation ϵ_b was independent of occupancy and had a higher average value of 11.8 ± 1.8 at both classes of sites.[2]

Binary Complexes between Mn^{2+}, Ribonucleotides, and Derivatives. Prior to studying the ternary enzyme–Mn–ligand complexes, EPR studies of the binding of Mn^{2+} to ATP, AMP-PNP, UTP, CTP, ApA, and ApU were carried out under appropriate conditions (Table II). Parallel PRR titrations of the same systems were done to obtain enhancement factors (ϵ_a) of the binary Mn–ligand complexes. The results are summarized in Table II.

Ternary Complexes between Mn^{2+}, Enzyme, and Initiation Specific Reagents. To determine the role of the tight Mn^{2+} binding site on RNA polymerase, 1:1 mixtures of enzyme (20–26 μM, preparation I) and Mn^{2+} were titrated with ApA, an initiator of RNA polymerization (Downey and So, 1970; Downey et al., 1971), measuring $1/T_1$ of water protons. The data (Figure 2) indicate that ApA at concentrations comparable to that of the enzyme–Mn^{2+} complex produces a small

[2] These observations were made at 25–30 μM RNA polymerase. At higher enzyme concentrations (80 μM), slightly higher values of ϵ_b (16 ± 2) were detected at the tight Mn^{2+} site. The reason for the fivefold greater dissociation constant and twofold greater ϵ_b value for Mn^{2+} interacting with preparation II is not known. One possible explanation for both of these differences is that preparation II, prepared by the procedure of Burgess and Jendrisak (1975), donates one less ligand to the tightly bound Mn^{2+} than does preparation I prepared by the earlier procedure of Burgess (1969a). For consistency, comparisons of kinetic and binding data were made only with the same preparation.

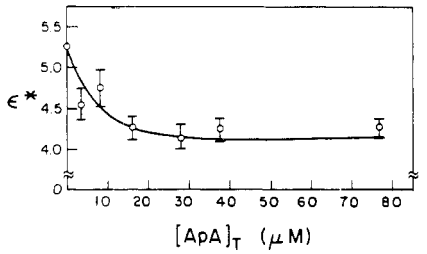

FIGURE 2: PRR titration of the 1:1 RNA polymerase-Mn^{2+} complex with ApA. A solution containing RNA polymerase (23.9 μM, preparation I) and $MnCl_2$ (16.9 μM) was titrated with ApA by measuring the enhancement of the PRR. The titration was done in the presence of 50 mM Tris-Cl (pH 7.5) and 150 mM KCl at 21 °C. The experimental points are the average of two identical titrations, and the error bars indicate the observed range of ϵ^*.

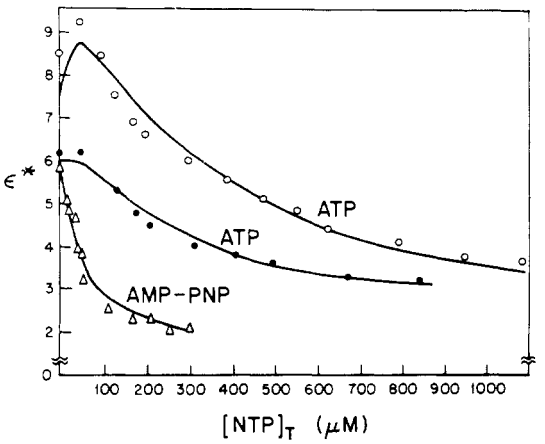

FIGURE 3: PRR titration of the RNA polymerase-Mn^{2+} complex with ATP and AMP-PNP. Solutions containing RNA polymerase (26.0 μM, preparation I, ○) and $MnCl_2$ (18.9 μM) or RNA polymerase (21.3 μM, preparation I, ●) and $MnCl_2$ (29.3 μM) were titrated with ATP. A solution containing RNA polymerase (21.3 μM, preparation I) and $MnCl_2$ (29.3 μM) was titrated with AMP-PNP. The titrations were performed at 21 ± 2 °C in the presence of 50 mM Tris-Cl (pH 7.5) and 150 mM KCl. The theoretical curves were computed by assuming the dissociation constants and enhancement factors given in Tables II and III.

but significant decrease in the observed enhancement factor to a value approaching 4.0. Because of the low affinity of ApA for Mn^{2+} and the low enhancement factor of the binary Mn-ApA complex (Table II), the titration curve of Figure 2 indicates the formation of a tight ternary enzyme-Mn-ApA complex rather than the simple removal of Mn^{2+} from the enzyme by ApA. Two titrations of the Mn^{2+} complex of preparation I and two titrations of the Mn^{2+} complex of preparation II with ApA gave results similar to those shown in Figure 2. Because of the small change in ϵ^*, the data were unsuitable for computer analysis. Graphical estimates of the dissociation constant (K_3) and the fractional decrease in enhancement (ϵ_T/ϵ_b) are given in Table III. Similar behavior was observed in titrations of the Mn^{2+} complex of preparation II with ApU, and the results of the graphical analysis are given in Table III.

These data indicate that dinucleoside monophosphates produce only small changes in the environment of the tightly bound Mn^{2+}. High levels of rifamycin, an inhibitor of initiation, also produced small (12–18%) decreases in ϵ^* of the enzyme-Mn^{2+} complex, arguing against direct interaction. Thus the addition of a large excess of rifamycin (1.9 mM) to a solution containing 26 μM enzyme (preparation I) and 25.1 μM $MnCl_2$ decreased ϵ^* from 5.6 to 4.6. Similarly, the addition

TABLE III: Dissociation Constants and Enhancement Factors of Ternary and Higher Complexes of RNA Polymerase, Mn^{2+}, and Ligands.[a]

Complex	K_2 (μM)	K_3 (μM)	K_D (μM)	K_S (μM)	ϵ_T/ϵ_b or ϵ_q/ϵ_T	PSTD (%)
Preparation I						
E-Mn-ApA		≤1.5[b]	2.0		0.80[b]	
E-Mn-AMP-PNP	6.3	25	2.1	33	0.56	11.0
E-Mn-ATP[c]	4.1	30	1.9	50	1.70	7.2
E-Mn-ATP[d]	5.3	35	1.9	73	1.20	7.5
Preparation II						
E-Mn-ApU		≤1.5[b]	9.0		0.85[b]	
E-Mn-ATP	18.4	27	9.0	74	1.63	6.4
E-Mn-UTP	90.3	140	8.0	85	1.53	4.6
(E-ApU)-Mn-ATP	9.3	30	4.1	70	0.45	6.9
(E-ApA)-Mn-UTP	44.6	77	5.5	70	0.50	11.3
(E-Rif)-Mn-ATP	16.3	27	7.5	72	0.70	6.5

[a] The dissociation constants are defined as follows: K_2 = [E]·[MS]/[EMS]; K_3 = [EM][S]/[EMS]; K_D = [E][M]/[EM]; K_S = [E][S]/[ES]. ϵ_b, ϵ_T, and ϵ_q are the enhancements of the PRR in the binary, ternary, and quaternary complexes, respectively. PSTD, which measures the quality of the fit of the theoretical titration curve to the data, is the percent standard deviation about ϵ_T or ϵ_q of all of the experimental points. [b] Determined graphically as described by Mildvan and Cohn (1966). [c] Enzyme concentration was 26 μM (Figure 3). [d] Enzyme concentration was 21 μM (Figure 3).

of 380 μM rifamycin to a solution containing 68 μM enzyme (preparation II) and 55 μM $MnCl_2$ decreased ϵ^* from 13.7 to 12.0. These results suggest that the tightly bound Mn^{2+} is not at the initiation site, as will be documented below.

Ternary Complexes of Enzyme, Mn^{2+}, and Ribonucleoside Triphosphate Substrates. Titration of 1:1 enzyme-Mn^{2+} complex with ATP at two levels of enzyme (preparation I, Figure 3) produced smaller decreases in the observed enhancement than could be explained by the simple removal of Mn^{2+} from the enzyme by ATP. However, the data could be fit theoretically by assuming the formation of a single ternary complex with an enhancement factor ~1.5-fold greater than that of the binary enzyme-Mn^{2+} complex (Table III) which also explains the ascending limb of the titration curve, at the highest enzyme concentration used (Figure 3). At higher levels, ATP, which has a high affinity for Mn^{2+} (Table II), removes Mn^{2+} from the enzyme accounting for a decrease in ϵ^*. Accordingly the ascending limb is less apparent at the lower level of enzyme (Figure 3). The data were analyzed by computer and Table III summarizes the parameters used to generate the theoretical curves of Figure 3.[3] Thus, unlike ApA, which decreases the enhancement of the enzyme-bound Mn^{2+} in its ternary complex, ATP increases the enhancement. Similar results were obtained for preparation II (Table III). An increase in ϵ^* is not, however, a general property of substrates as indicated by a titration with AMP-PNP (Figure 3) which decreases the enhancement factor by 43% (Table III). AMP-PNP was shown to be a substrate as follows. Using pol-

[3] The binding of ATP at an additional weak site, which is known to occur (Wu and Goldthwait, 1969a,b), did not significantly affect ϵ^* at the tight site since the data could be fit most simply without considering this interaction.

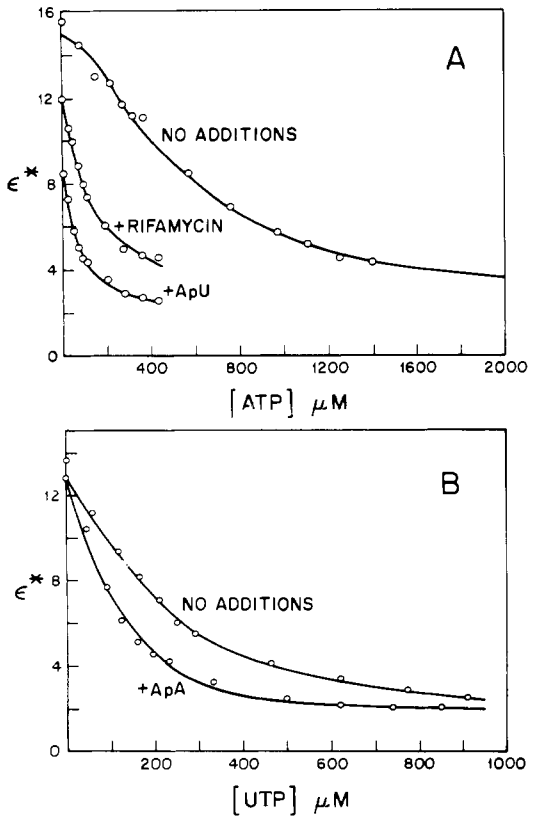

FIGURE 4: Titration of the RNA polymerase-Mn^{2+} complex with ATP and UTP. (A) ATP titrations were carried out as follows. No additions: solutions contained RNA polymerase (110 μM, preparation II) and $MnCl_2$ (89.0 μM); + rifamycin: solutions contained RNA polymerase (67.9 μM, preparation II), $MnCl_2$ (54.8 μM), and rifamycin (379 μM); + ApU: solutions contained RNA polymerase (33.8 μM, preparation II), $MnCl_2$ (27.9 μM), and ApU (262 μM). (B) UTP titrations were carried out as follows. No additions: solutions contained RNA polymerase (88.2 μM, preparation II) and $MnCl_2$ (71.2 μM); + ApA: solutions contained RNA polymerase (65.3 μM, preparation II), $MnCl_2$ (52.7 μM), and ApA (198 μM). Conditions were otherwise as described in Figure 3. The theoretical curves were computed using the dissociation constants and enhancement factors given in Tables II and III.

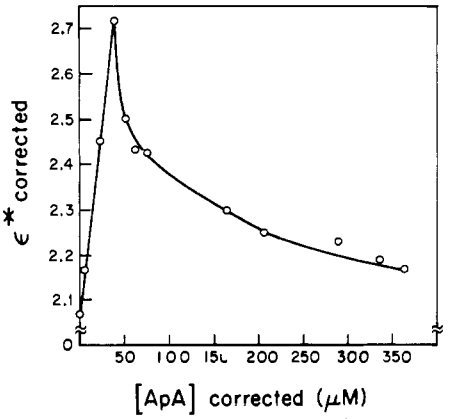

FIGURE 5: PRR titration of the weakly bound Mn^{2+} on RNA polymerase with ApA. A solution containing RNA polymerase (22.8 μM, preparation I) and $MnCl_2$ (207 μM) was titrated with ApA. The observed enhancement (ϵ*) and ApA concentrations were corrected by assuming that the interaction of Mn^{2+} and ApA at the tight site (Figure 2) is unaltered by occupancy of the weak sites by Mn^{2+} or ApA.

y[d(A-T)]·poly[d(A-T)] as a template, under conditions otherwise as described in Methods, the presence of AMP-PNP (660 μM) resulted in the incorporation of 1.40 nmol of [^{3}H] UTP into RNA while ATP (520 μM) resulted in the incorporation of 3.13 nmol. A similar observation has previously been made with the β,γ-methylene analogue of ATP, AMP-PCP (Simon et al., 1965). Hence a change in the nature of the leaving group of the substrate from pyrophosphate to imidodiphosphate has greatly altered the enhancement of the ternary complex suggesting that the leaving group interacts with the enzyme-bound Mn^{2+}. As with ATP, titration of the 1:1 enzyme-Mn complex (88 μM, preparation II) with UTP (Figure 4) revealed the existence of a ternary complex, the parameters of which are summarized in Table III. In all cases the titrations with ribonucleoside triphosphates could not be fit by assuming the simple removal of Mn^{2+} from the enzyme by the substrate but required the formation of ternary complexes. The affinity of the enzyme-Mn^{2+} complex for UTP as measured by K_3 (Table III) is lower than that for ATP, but the enhancement is similarly increased by ~50% in the ternary complex.

Deviations from hyperbolic behavior, due to the predominant formation of ternary enzyme-Mn^{2+}-nucleotide complexes at the beginning of the titration, were seen with ATP (Figures 3 and 4) but not with UTP (Figure 4). This is because of the lower affinity of UTP for the enzyme-Mn^{2+} complex than for free Mn^{2+} (Tables II and III).

Quaternary Complexes of Enzyme, Mn^{2+}, Ribonucleoside Triphosphates, and Initiation Specific Reagents. Titrations of the 1:1 enzyme-Mn^{2+} complex (preparation II) with ribonucleoside triphosphates in the presence of saturating concentrations of ApA, ApU, or rifamycin were performed and the results are presented in Figure 4. Again, the titration curves could not be fit theoretically by assuming the simple removal of Mn^{2+} from the enzyme by the substrates but required the assumption of quaternary complexes. The parameters of these quaternary complexes are summarized in Table III. It can be concluded from the dissociation constants obtained by computer fitting (Table III) that the dinucleoside monophosphates ApA and ApU do not interact competitively with ATP or UTP. Rather ApU and ApA tighten the binding of Mn^{2+} to the enzyme. The most striking feature of the quaternary complexes is that in contrast to the ternary complexes with ATP and UTP, in which $\epsilon_T > \epsilon_b$, the enhancement is decreased by ATP and UTP in the presence of the initiation specific reagents ApA, ApU, or rifamycin (Table III). Hence these reagents change the environment of the Mn-nucleoside triphosphate complex on the enzyme.

Titration of the Weak Mn^{2+} Binding Sites. A solution containing 25 μM RNA polymerase (preparation I) and an eightfold excess of $MnCl_2$ (200 μM), in which the tight binding site was fully occupied and the weak binding sites were initially 37% occupied, was titrated with ApA. The data were corrected to yield a titration curve for the weak Mn^{2+} sites (Figure 5) by making the simplest assumption that the small effect of ApA at the tight site is unaltered by the presence of Mn^{2+} at the weak sites. A biphasic titration curve of the response of Mn^{2+} at the weak sites is obtained. The results indicate that ApA, at a range of concentrations that decreases ϵ* at the tight site, increases the enhancement due to Mn^{2+} at the weak sites by 31%. At higher levels of ApA, ϵ* at the weak Mn^{2+} sites also decreases by 20% indicating the formation of higher complexes of ApA. Although the effects of ApA on ϵ* are small (Figure 5), they are well beyond our experimental error of ±5%. Because of the low magnitude of the effects and the complexity of this system, no theoretical fitting of the data was done.

A similar titration of the 1:8 enzyme-Mn^{2+} mixture (preparation I) with ATP was performed and a titration curve for the weak sites was calculated as described above. This curve

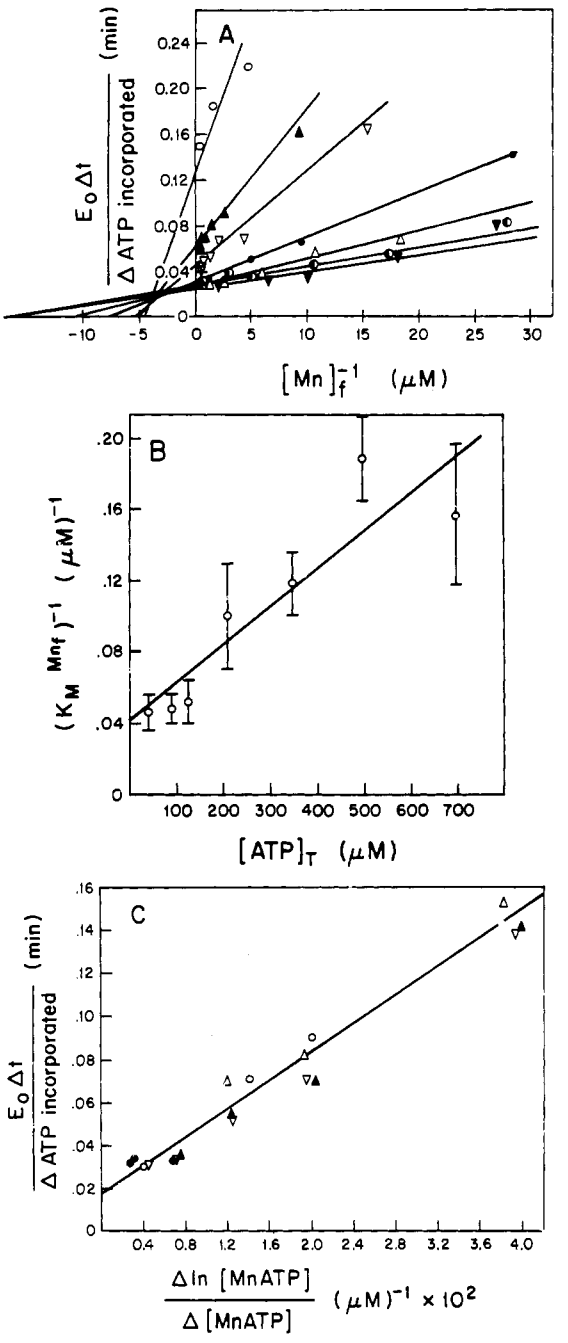

FIGURE 6: Steady-state kinetics of RNA polymerase, activated by Mn^{2+} using poly(dT) as template in the absence of ApA. (A) Double-reciprocal plots of the rate of incorporation of [^{3}H]ATP into poly(rA) as a function of the concentration of free Mn^{2+} at the following total ATP concentrations: (○) 43.5 μM; (▲) 87.0 μM; (▽) 131 μM; (●) 218 μM; (△) 350 μM; (◐) 491 μM; (▼) 702 μM. (B) Extrapolation to zero concentration of ATP in a plot of the K_m of free Mn^{2+} as a function of total ATP concentration calculated from the data of Figure 6A. (C) Treatment of the data in Figure 6A by the integrated Michaelis-Menten equation in terms of MnATP concentration at the following total concentrations of $MnCl_2$: (○) 109 μM; (△) 156 μM; (▲) 215 μM; (▽) 312 μM; (●) 391 μM. Other components present in a total volume of 0.05 ml were poly(dT) (0.05 mg/ml), RNA polymerase (2 pmoles, preparation I), 50 mM Tris-Cl, pH 7.5, and 150 mM KCl, T = 24 °C. Other experimental details are given in Methods.

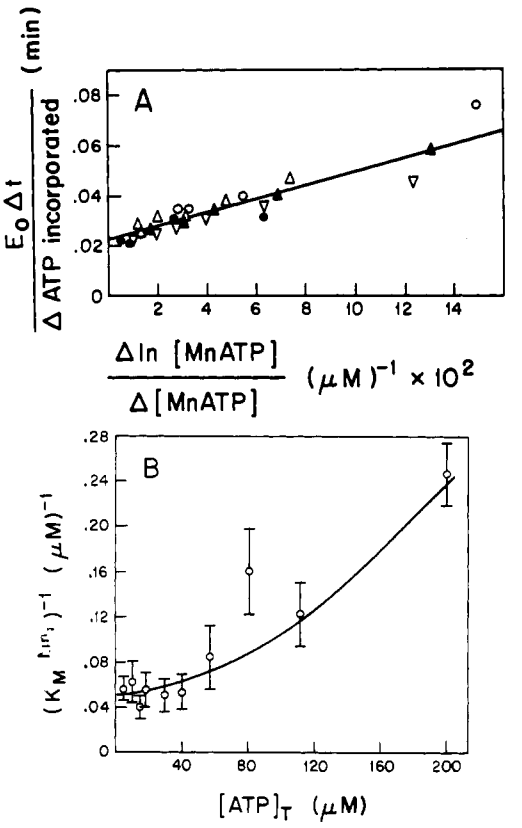

FIGURE 7: Steady-state kinetics of RNA polymerase activated by Mn^{2+} using poly(dT) as template in the presence of ApA. (A) The rate of incorporation of [^{3}H]ATP into poly(rA) in the presence of ApA (42.5 μM) is analyzed by the integrated Michaelis-Menten equation in terms of MnATP concentration at the following total concentrations of $MnCl_2$: (○) 45.8 μM; (△) 62.9 μM; (▲) 98.8 μM; (▽) 228 μM; (●) 333 μM. (B) Extrapolation to zero concentration of ATP in a plot of the K_M of free Mn^{2+} as a function of total ATP concentration. Conditions were identical with those described in Figure 6 except that the amount of enzyme was either 2 or 4 pmol (preparation I) in each 0.05-ml assay.

revealed only a monotonic decrease in ϵ^*, approaching the enhancement of the binary MnATP complex. Such behavior could be explained by removal of Mn^{2+} from the weak sites by ATP, although the existence of a ternary E-MnATP complex at the weak sites, with a dissociation constant ≥150 μM for the MnATP from the enzyme and an $\epsilon_T \sim \epsilon_a \sim 1.2$, could not be ruled out.

The presence of rifamycin (3.6 mM) had no detectable effect on either the enhancement or the extent of binding of Mn^{2+} to the weak sites of RNA polymerase as determined by PRR and EPR respectively in a solution containing 15.7 μM enzyme (preparation I) and 245 μM $MnCl_2$. Under these conditions the tight Mn^{2+} site was fully occupied and 1.8 of the six weak binding sites were occupied. These results indicate that neither substrates nor rifamycin interact strongly with Mn^{2+} at the weak sites.

Steady-State Kinetic Analysis of Mn^{2+}-Activated RNA Polymerase. To clarify the role of the various binary, ternary, and quaternary complexes of RNA polymerase, a kinetic study was made of the Mn^{2+} activated reaction (Figure 6A) using poly(dT) as template and preparation I of the enzyme. Under these conditions, ATP serves as both initiator and substrate for chain elongation and the former process is believed to be rate limiting (Chamberlin, 1974; Rhodes and Chamberlin, 1974, 1975). The Michaelis constant of free Mn^{2+} increases with decreasing ATP concentration, approaching a value of 22 ± 4 μM in the limit of zero ATP (Figures 6A and 6B). This value does not agree with either of the dissociation constants of the Mn-enzyme complex (preparation I, Table I) but is similar to the dissociation constant of the binary Mn-ATP complex (13.2 ± 1.5 μM, Table II) suggesting that MnATP is the form of the substrate (or initiator) which binds to the

TABLE IV: Test of MnATP as Substrate for RNA Polymerase.

$[ApA]_T$ (μM)	$[ATP]_T$ (μM)	$[Mn]_T$ (μM)	$K_{M[Mn]_T}$ (μM)	$K_{M[ATP]_T}$ (μM)	$V_M/E_{0[Mn]_T\to\infty}$ (min^{-1})	$V_M/E_{0[ATP]_T\to\infty}$ (min^{-1})
0	90	Variable	86 ± 16		19 ± 2	
	Variable	109		65 ± 13		21 ± 4
	218	Variable	188 ± 60		44 ± 4	
	Variable	215		212 ± 40		64 ± 18
8.0	28.9	Variable	46 ± 18		23 ± 4	
	Variable	33.2		31 ± 10		17 ± 2

enzyme under steady-state conditions rather than free Mn^{2+} or free ATP (Dixon and Webb, 1964). This point is confirmed by plotting all of the kinetic data in terms of the concentration of the Mn-ATP complex (Figure 6C). All of the points fall on a single straight line, yielding a K_M for MnATP of 160 μM. The overall fit is not significantly improved by drawing individual lines through each set of points belonging to a given Mn^{2+} concentration. A third kinetic test which indicates that MnATP is the substrate (or initiator) is provided by comparing the K_M of total Mn^{2+} at a fixed concentration of ATP with the K_M of total ATP at the same fixed concentration of Mn^{2+} (Table IV). As shown by Dixon and Webb (1964), under these conditions, the respective K_M values of total Mn^{2+} and total ATP (as well as the V_{max} values) should agree for a mechanism in which the metal ligand complex is the substrate. Such behavior is observed, within the experimental error of the kinetic method (Table IV). Hence, by these three kinetic tests, the simplest assumption consistent with the kinetic data is that MnATP is the substrate (or initiator) with a K_M of 160 ± 30 μM. This value agrees with those previously found using Mg^{2+} in the ATP-initiated reaction (Anthony et l., 1969) and with a dissociation constant found by fluorescence (Wu and Goldthwait, 1969a) and by equilibrium dialysis (Wu and Goldthwait, 1969b) for a rifamycin-sensitive ATP binding site but does not agree with K_2, the dissociation constant of Mn-ATP from the tight Mn^{2+} binding site (Table III). By this additional criterion the tight Mn^{2+} site is probably not the initiator site of RNA polymerase.

Steady-State Kinetic Analysis in the Presence of ApA. The dinucleoside monophosphate ApA has previously been shown to act as an initiator of RNA chains with *E. coli* RNA polymerase (Downey and So, 1970; Downey et al., 1971). Hence the kinetics of chain elongation by ATP in the presence of a poly(dT) template may be examined more directly by using ApA as initiator. At saturating levels of ApA (42.5 μM), MnATP, rather than free Mn^{2+} or free ATP, is the substrate, by the same three criteria discussed above (Figure 7, Table IV). The K_M of MnATP (10 ± 3 μM) under these conditions is similar to the dissociation constant (K_2) of MnATP from the tight Mn^{2+} site (4.9 ± 0.5 μM, Table III), suggesting that the tight Mn^{2+} site is catalytically active in chain elongation.

In order to determine the dissociation constant of the enzyme-ApA complex by a kinetic method, reaction rates were measured at two constant levels of Mn^{2+} (41 and 340 μM) and at varying concentrations of ATP and ApA and were analyzed in terms of the substrate MnATP (Figure 8). The maximal velocity, extrapolated to infinite levels of MnATP, was independent of the concentration of ApA. However, the K_M of MnATP decreased with increasing ApA levels from a value of 150 ± 30 μM in the absence of ApA to a value of 10 ± 3 μM at ApA concentrations greater than 10 μM (Figure 8). These K_M values agree with those previously determined for MgATP

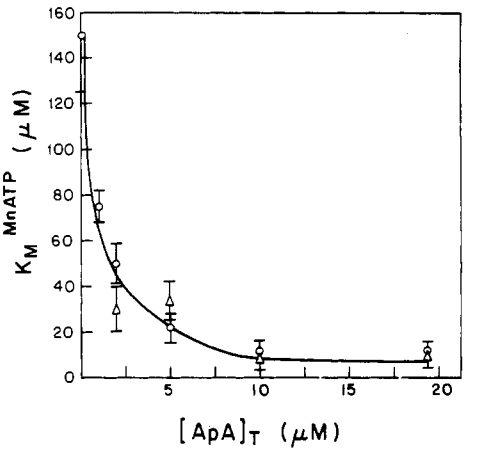

FIGURE 8: Effect of ApA on the K_M of MnATP. Kinetic experiments were carried out with enzyme preparation I at two different concentrations of $MnCl_2$: (○) 340 μM; (△) 41.0 μM. Assay conditions were otherwise identical with those of Figure 6.

in the initiation and elongation steps, respectively, under somewhat different conditions (Anthony et al., 1969; Rhodes and Chamberlin, 1974, 1975). The kinetically determined dissociation constant of ApA from RNA polymerase determined from the concentration of ApA required for half-maximal effect on the K_M of MnATP (Figure 8) is 1.0 ± 0.5 μM. This value is in reasonable agreement with the K_3 value of ApA determined by PRR (Table III) indicating that the binding of ApA detected by PRR is functional in initiation. Table V compares the dissociation constants determined by kinetics with those obtained by magnetic resonance studies.

Substrate Kinetics of MnUTP and MnATP. To determine the K_M of MnUTP, poly[d)a-T)]·poly[d(A-T)] was used as template and the substrates ATP and UTP were mutually varied at a fixed high level of Mn^{2+} (1 mM), as described in Methods using enzyme preparation II. Under these conditions initiation was brought about both by MnATP and by the template itself (Nath and Hurwitz, 1974; Springgate and Loeb, 1975). Double-reciprocal plots of the kinetic data showed parallel line "ping-pong" behavior when either MnATP or MnUTP were the variable substrates, as previously found in the Mg^{2+}-activated reaction (Rhodes and Chamberlin, 1974). The intercept replots were linear yielding K_M values of 68 ± 10 μM for MnATP and 101 ± 13 μM for MnUTP (Table V). The K_M of MnATP under these conditions is intermediate between that characteristic of MnATP as an initiator (160 ± 30 μM) and as a substrate for chain elongation (10 ± 3 μM). The K_M of MnUTP, which cannot initiate, reflects only its function as a substrate for chain elongation. Its value is in agreement with the dissociation constant of MnUTP from the tight Mn^{2+} site (K_2 = 90 μM) as determined by PRR titration

TABLE V: Comparison of Kinetic Parameters of Mn^{2+}-Activated RNA Polymerase with Dissociation Constants.[a]

Enzyme Preparation	Complex	Kinetic Parameter		Dissociation Constant, K_1, K_2, or K_3 (μM)
		K_M or K_A (μM)	Conditions	
I	E(MnATP)	10 ± 3	+ ApA	
		160 ± 30	No ApA	4.7 ± 0.6
	EMn(ApA)	1.0 ± 0.5	K_A (ApA) from Figure 8	≤1.5
	MnATP	22 ± 4	K_M of $[Mn]_f$ as ATP → 0	13.2 ± 1.5
II	E(MnATP)	68 ± 10	Poly[d(A-T)]·poly[d(A-T)] template [MnUTP] → ∞	18.4
	E(MnUTP)	101 ± 13	Poly[d(A-T)]·poly[d(A-T)] template [MnATP] → ∞	90.3

[a] Kinetic parameters and dissociation constants refer to component in parentheses dissociating from the complex.

(Tables III and V), indicating a rapid equilibrium ping-pong kinetic scheme.

Discussion

Mn^{2+}-binding studies to *E. coli* RNA polymerase, as previously found for DNA polymerase I (Slater et al., 1972), reveal more than one class of metal binding sites. A single high affinity site and six low affinity sites are found on RNA polymerase (Table I). The high enhancement factors indicate the presence of residual water ligands on the enzyme-bound Mn^{2+}.

Four lines of evidence indicate that the tight Mn^{2+}-binding site functions at the active site for RNA chain elongation. The first is the agreement of the K_M of MnATP, the substrate for chain elongation in the ApA initiated reaction, with K_2, the dissociation constant of MnATP from the tight Mn^{2+} site. No such agreement is found with the K_M of MnATP when MnATP functions as an initiator. Similarly, the K_M of MnUTP as a substrate for chain elongation agrees with its K_2 from the tight Mn^{2+}-binding site (Table V). Since K_M values need not be true dissociation constants, such agreement between K_M values and dissociation constants suggests a rapid equilibrium kinetic scheme for Mn^{2+}-activated RNA polymerase. Second are the relatively small effects of ApA, ApU, and of rifamycin on the enhancement at the tight Mn^{2+} site, as compared with the larger effects of ATP and UTP. Third, the initiation specific compounds ApA, ApU, and rifamycin do not prevent the interaction of ATP or UTP with the tightly bound Mn^{2+}, indicating that the tight Mn^{2+} site is not the initiator binding site. Fourth, in other studies of $1/T_1$ of the protons of ATP and the protons and phosphorus of ApU (Koren et al., 1976a) we have found the tightly bound Mn^{2+} to be significantly closer to the H_1' proton of bound ATP (4.9 ± 1.0 Å) than to the protons or phosphorus of bound ApU (9.0–10.5 Å).

Although less effective as a substrate than Mn-ATP, the Mn^{2+} complex of AMP-PNP has a very similar affinity for the tight Mn^{2+} site of the enzyme as measured by K_2 (Table III). The enhancement factor for Mn^{2+} at the tight site, however, is significantly different in the ternary complexes of ATP and AMP-PNP, indicating that a difference in the leaving pyrophosphate group alters the environment of the enzyme-bound Mn^{2+}. This finding is consistent with an enzyme–Mn–ATP bridge complex involving the leaving pyrophosphate group of ATP. In the mechanistically related enzyme, DNA polymerase I, direct coordination of the γ-phosphoryl group of dTTP to the enzyme bound Mn^{2+} has been established by ^{31}P NMR (Sloan et al., 1975).

The stoichiometry of a single elongation site per holoenzyme molecule, as determined by the present Mn^{2+} binding and kinetic studies (Tables I and V), is consistent with the stoichiometry found in previous ATP and UTP binding studies (Wu and Goldthwait, 1969a,b) but differs from the finding of two tightly bound Zn^{2+} ions in this metalloenzyme (Scrutton et al., 1971). The latter observation suggests a structural rather than a catalytic role for one or both of the Zn^{2+} ions. In this connection RNA polymerase B from yeast has been found to contain only one Zn^{2+} ion per mole (Lattke and Weser, 1976).

Although the initiation-specific compounds ApA, ApU, and rifamycin do not appear to interact directly with the tightly bound Mn^{2+}, two observations suggest that these compounds influence the environment of Mn^{2+} at the catalytic site. First, these compounds produce a small but significant decrease in ϵ^* (Table III). Second, ApA and ApU alter the response of the bound Mn^{2+} to ATP and UTP. Thus, in the absence of ApA, ApU, or rifamycin, ATP and UTP increase the enhancement of the tightly bound Mn^{2+}. However, when the initiation site is occupied by ApA or ApU or is inhibited by rifamycin, the triphosphate substrates decrease the enhancement (Table III), suggesting that initiators adjust the conformation of the catalytic site for chain elongation. All of these observations are consistent with proximity of the initiation and elongation sites as is required by the chemical nature of the product.

As with DNA polymerase I (Slater et al., 1972), Mn^{2+} substitutes for Mg^{2+} with RNA polymerase yielding slightly greater activity (Springgate and Loeb, 1975). Since Mg^{2+} is not paramagnetic, the present binding studies could not have been done with Mg^{2+}. However, the similarity of enzymatic rates indicates similar mechanistic roles for Mn^{2+} and Mg^{2+}.

The role of the 6 ± 1 weak Mn^{2+}-binding sites on RNA polymerase is not clear. The moderate affinity and high ϵ_b values (Table I) suggest chelate structures and thereby argue against the adventitious binding of Mn^{2+} at these sites. Moreover, levels of free Mn^{2+} required to cause 50% inhibition of RNA polymerase (~6 mM) are significantly greater than the dissociation constants of Mn^{2+} from these sites (Table I), indicating that they are not inhibitory sites. The average enhancement factor at these weak Mn^{2+} sites increases when ApA binds to RNA polymerase with an affinity appropriate to cause initiation, suggesting that one or more of these weak sites may be at or near the initiation site. Although no direct evidence for the interaction of rifamycin or of MnATP at these weak sites has been obtained, the possible binding of MnATP at these sites with a dissociation constant as high as its K_M as an initiator (150 μM) has not been ruled out. Binding sites for Mn^{2+} with intermediate affinity at which substrates can interact have previously been found on DNA polymerase I (Slater et al., 1972). Alternatively or additionally the weak Mn^{2+} binding sites on RNA polymerase may represent the template binding site as is suggested by the observation that

high levels of ApA decrease the enhancement at these sites (Figure 5). At high concentrations ApA, binding at multiple sites, may function as an analogue of the template.

The finding of a single tight Mn^{2+}-binding site at or near the catalytic site for chain elongation, which interacts with ATP or UTP, reinforces the number of active sites as one per molecule of holoenzyme and provides a paramagnetic reference point for mapping the conformation of enzyme-bound initiators and substrates.

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