Magnetic Resonance and Kinetic Studies of Initiator–Substrate Distances on RNA Polymerase from *Escherichia coli*†

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ABSTRACT: α,β,γ-tridentate CrATP, a substitution inert paramagnetic complex of ATP, does not replace MgATP as a substrate in the elongation reaction catalyzed by RNA polymerase but does function as an RNA chain initiator as judged by three criteria. CrATP ($K_M = 97 \pm 6$ μM) stimulates the incorporation of labeled AMP into an RNA chain, is itself incorporated into the RNA chain unless blocked by an authentic initiator (ApA), and competes against the authentic initiators, [γ-^{32}P]ATP and ApA, with K_i values of 75 ± 21 μM. At higher levels, CrATP is a weak mixed-type inhibitor of chain elongation, with a parabolic competitive effect ($K_i = 0.5 \pm 0.1$ mM) and a linear uncompetitive effect ($K_i = 1.2 \pm 0.2$ mM). Similarly, β,γ-bidentate $Co^{3+}(NH_3)_4ATP$ is a potent initiator of RNA polymerase ($K_M = 103 \pm 10$ μM) which is incorporated into the RNA chain and a weak linear competitive inhibitor ($K_i = 7 \pm 2$ mM) of RNA chain elongation, but it is not a substrate for elongation. In the absence of template, the diamagnetic effects of the RNA polymerase–Mg^{2+} complex on the longitudinal ($1/T_1$) and transverse ($1/T_2$) relaxation rates of the H_6, H_5, and H_1' protons of the elongation substrate, CTP, at 100 and at 220 MHz yield rotational correlation times for bound CTP (1 ns $\leq \tau_r \leq$ 13 ns). These values are much shorter than τ_r calculated for the entire protein molecule (400 ns), indicating incomplete immobilization of the bound substrate, in accord with previous fluorescence polarization data with 1,N^6-etheno-ATP. The paramagnetic effects of CrATP at the initiation site on $1/T_1$ of the substrate CTP at the elongation site, together with the dipolar correlation time as determined by the frequency dependence of $1/T_1$ of water protons in the same complex, were used to calculate initiator to substrate distances on RNA polymerase of 8.6, 7.9, and 11.0 Å to the H-6, H-5, and H-1′ protons of CTP, respectively. Despite the absence of template, these distances define an average conformational angle at the glycosidic bond of bound CTP ($\chi = 90 \pm 15°$) appropriate for base pairing with a DNA template, since the same χ angle is found in double helical B-DNA. The absence of any detectable paramagnetic effects of CrATP on the EPR spectrum or on the dipolar correlation time of Mn^{2+} bound at the elongation site of RNA polymerase indicates a Cr^{3+} to Mn^{2+} distance greater than 10–11.5 Å. The conformations and arrangement of the bound initiator, substrate, and metal activator on RNA polymerase, based on the present and previous distance determinations [Bean B. L., Koren, R., and Mildvan, A. S. (1977), *Biochemistry 16,* 3322], permit stacking of the bases of the initiator and substrate and direct transfer of the nucleotidyl group of the substrate to the initiator.

The covalent reactions catalyzed by RNA polymerase from *E. coli* have been divided into two broad steps (Chamberlin, 1974):

$$\text{Pu·Nuc} + \text{N}_{\text{ppp}} \xrightarrow[M^{2+}]{\text{Enz, DNA}} \text{Pu·Nuc-p-N} + \text{PP}_i \quad (1)$$

$$\text{Pu·Nuc-p-N} + n\text{N}_{\text{ppp}} \xrightarrow[M^{2+}]{\text{Enz, DNA}} \text{Pu·Nuc-(pN)}_{n+1} + n\text{PP}_i \quad (2)$$

In the chain-initiation step (step 1) the enzyme catalyzes the transfer of the nucleotidyl group of the substrate, bound at the elongation site, to the initiator, a purine nucleotide bound at the initiation site, with the displacement of pyrophosphate. All subsequent nucleotidyl-transfer (elongation) reactions (step 2) occur at the elongation site, extending the RNA chain with a base sequence defined by the DNA template (Chamberlin, 1974). The initiation site is specific for the binding of the purine nucleotides (Wu and Goldthwait, 1969a,b). Further, the initiation step is probably the rate-limiting step for the overall reaction as shown by separate measurements of steps 1 and 2 (Rhodes and Chamberlin, 1974, 1975). Accordingly, the dissociation constants of various purine nucleotides from the initiation site agree well with their respective K_M values (Anthony et al., 1969; Wu and Goldthwait, 1969a,b; Koren and Mildvan, 1977). This rate-limiting initiation can be bypassed by the use of dinucleotides with enhanced affinities for the initiation site, such as ApU, UpA, and ApA (Downey and So, 1970; Downey et al., 1971; Niyogi and Stevens, 1965; Hoffman and Niyogi, 1973; Koren and Mildvan, 1977; Bean et al., 1977).

RNA polymerase also has an absolute requirement for a divalent metal cation such as Mg^{2+} or Mn^{2+} (Burgess, 1971). A single tight binding site for Mn^{2+} at the elongation site of the enzyme has been detected which satisfies the divalent cation requirement for activity (Koren and Mildvan, 1977). The paramagnetic effects of Mn^{2+} bound at this site on the relaxation rates of the initiator ApU and the substrate ATP in the absence of template have been used to calculate distances from the bound Mn^{2+} to the bound initiator (~10 Å) and to the bound substrate (~5 Å) (Bean et al., 1977). In order to further clarify the arrangement of the initiator and the substrate sites, the distance between them is required.

CrATP, a tridentate, substitution inert paramagnetic analogue of ATP, is an inhibitor of a large number of ATP-utilizing enzymes (Janson and Cleland, 1974) and is a substrate for hexokinase (Danenberg and Cleland, 1975). It has previ-

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ously been used as a paramagnetic probe to measure the intersubstrate distance on pyruvate kinase (Gupta et al., 1976). Similar methods have been used to obtain intersubstrate distances on dehydrogenases (Mildvan and Weiner, 1969; Mildvan et al., 1970), transcarboxylase (Fung et al., 1976), and on DNA polymerase I (Krugh et al., 1971). In this paper, CrATP is shown to occupy the initiation site of RNA polymerase and is used as a paramagnetic reference point to determine the distance between the initiation and elongation sites of RNA polymerase. A preliminary report of this work has been published (Stein and Mildvan, 1977).

Experimental Procedure

Materials

RNA polymerase was purified by the method of Burgess and Jendrisak (1975) from 5 lb of *E. coli* (K12, ¾ log phase, grown on enriched medium), purchased from the Grain Processing Co., Muscatine, Ill. Two such preparations of the enzyme were used which were estimated to be 95 and 92% pure by acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, staining with coomassie blue. The content of σ subunit was 100% in the first preparation and 60% in the second. However, the specific activity of the second preparation (400 units/mg) was 80% of the activity of the first preparation (500 units/mg) by the assay of Burgess (1969) and did not yield significantly different NMR results. Protein concentration was determined by UV absorption at 280 nm, using an extinction coefficient of 0.65 $(mg/mL)^{-1}$ (Richardson, 1966) and a molecular weight of 500 000 (Chamberlin, 1974).

The paramagnetic, α,β,γ-tridentate, substitution inert complex, CrATP, was synthesized according to the method of DePamphilis and Cleland (1973) and purified on a Bio-Rad Analytical Grade 50W-X_2 column, eluting with 0.1 M aniline. The CrATP was stored at liquid nitrogen temperature before use or at −70 °C between experiments. The diamagnetic, β,γ-bidentate substitution inert complex, $Co^{3+}(NH_3)_4ATP$, was synthesized by the method of Cornelius et al. (1977) and was stored as described for CrATP.

Poly(dT) ($s_{20,w}$ = 7.14) and poly[d(AT)]·poly[d(AT)] ($s_{20,w}$ = 5.49) were purchased from P-L Biochemicals. They were dissolved in 50 mM K^+-Pipes[1] buffer, pH 6.7, containing 150 mM KCl. Concentrations were estimated by UV absorbance using extinction coefficients of 22.4 $(mg/mL)^{-1}$ at 264 nm for poly(dT) and 20.0 $(mg/mL)^{-1}$ at 260 nm for poly[d(AT)]·poly[d(AT)]. Nucleoside triphosphates were purchased from Sigma, dissolved in water, and adjusted to the desired pH with KOH at 2 °C. Concentrations were determined by UV absorption. Tritiated ATP and UTP were obtained from New England Nuclear Corp. The dinucleoside monophosphates, ApA and ApU, were purchased from Sigma, dissolved and neutralized as described above. Their concentrations were determined by UV absorption using extinction coefficients at 257 nm of 2.78×10^4 M^{-1} for ApA (Tso et al., 1966) and 2.32×10^4 M^{-1} for ApU (P-L Biochemicals, 1975). All solutions used in NMR experiments were passed through Bio-Rad Chelex 100 before use to remove trace metal ion contaminants.

Methods

Kinetic Experiments. Assays testing CrATP as a substrate for elongation measured the incorporation of [^{3}H]UMP into an acid-insoluble product, with poly[d(AT)]·poly[d(AT)] as template. The reaction mixture, in 0.05 mL, contained 50 mM K^+-Pipes (pH 7.0), 150 mM KCl, 1 mM $MnCl_2$, 60 μM ApU, 0.05 mg/mL template, 465 μM [^{3}H]UTP, 150 μM ATP, or CrATP when present and 5 pmol of enzyme. Experiments testing $Co^{3+}(NH_3)_4ATP$ as a substrate for elongation utilized poly(dT) (0.05 mg/mL) as template, ApA (58 μM) as initiator, $MgCl_2$ (10 mM) as activator, and $Co^{3+}(NH_3)_4$-[^{3}H]ATP (1.97 mM) or [^{3}H]ATP (50 μM) as substrates under conditions otherwise similar to those used for CrATP. Initiation by CrATP or $Co^{3+}(NH_3)_4ATP$ was detected by measurements of the incorporation of Cr[^{3}H]ATP or of $Co^{3+}(NH_3)_4$[^{3}H]ATP into the acid-insoluble product using [α-^{32}P]ATP as substrate and poly(dT) as template. Other kinetic experiments testing for initiation by CrATP or $Co^{3+}(NH_3)_4ATP$ measured the stimulation of incorporation of [^{3}H]AMP into the acid-insoluble product. Interaction of CrATP at the initiator site was also studied by its competition against authentic initiators, i.e., against the incorporation of [γ-^{32}P]ATP into the acid-insoluble product and against the ApA-induced stimulation of [^{3}H]AMP incorporation into the RNA product. Interaction of CrATP and $Co^{3+}(NH_3)_4ATP$ with the elongation site of the enzyme was studied by competition against the substrate [^{3}H]ATP at saturating levels (50 and 250 μM) of the initiator ApA. In all of these cases, poly(dT) (0.05 mg/mL) was used as the template. These reaction mixtures also contained, in a volume of 0.05 mL, 50 mM K^+-Pipes (pH 6.7), 150 mM KCl, 5 mM $MgCl_2$, and variable amounts of ATP, ApA, CrATP and/or $Co^{3+}(NH_3)_4ATP$. These mixtures were equilibrated at 24 °C after which the reaction was initiated by adding 0.5 to 31 pmol of enzyme, which had been dialyzed against the same buffer. The solution was incubated at 24 °C for 10 or 20 min and was stopped by the addition of 2 mL of 1 M $HClO_4$ containing 20 mM sodium pyrophosphate. The acid-insoluble radioactivity was measured as described by Loeb (1969). Kinetic constants were evaluated using a nonlinear least-squares program (Wilkinson, 1961) on a Wang desk computer.

CrATP and ATP are initiators of RNA polymerase, both with V_{max} values 25% of the V_{max} found with initiation by ApA under our conditions. Hence, the kinetic data measuring the inhibition of ApA initiation by CrATP required corrections for initiation by CrATP and ATP, particularly at high levels of CrATP. The amount of ATP incorporated into the product RNA, by ApA initiation only (v_{ApA}/v_{total}), was calculated using eq 3 (Segel, 1975), which takes into account the V_{max} and K_M values of each individual initiator (as indicated by the subscripts) measured in separate experiments.

$$\frac{v_{ApA}}{v_{total}} = \frac{\left(\frac{[ApA]}{K_{ApA}}\right)V_{ApA}}{\left(\frac{[ApA]}{K_{ApA}}\right)V_{ApA} + \left(\frac{[CrATP]}{K_{CrATP}}\right)V_{CrATP} + \left(\frac{[ATP]}{K_{ATP}}\right)V_{ATP}} \quad (3)$$

Preparation of Enzyme for NMR Measurements. The enzyme, which had been stored at −70 °C at concentrations of 5–15 mg/mL in 10 mM Tris-HCl (pH 7.9), containing 50% (v/v) glycerol, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 100 mM NaCl (Burgess and Jendrisak, 1975), was diluted fivefold with cold 50 mM K^+-Pipes buffer (pH 6.7) containing 150 mM KCl, precipitated by adding 0.5 g/mL of solid $(NH_4)_2SO_4$, and centrifuged at 15 000 rpm for 20 min. The pellet was

[1] Abbreviations used: NMR, nuclear magnetic resonance; EPR, electron paramagnetic resonance; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Me_4Si, tetramethylsilane.

redissolved in 50 mM K^+-Pipes buffer (pH 6.7) containing 150 mM KCl, and the $(NH_4)_2SO_4$ was removed by passage through a Sephadex G-25 column, equilibrated with the same buffer. The eluted protein was concentrated by vacuum dialysis. For proton NMR experiments, water was replaced with D_2O by sequentially diluting with deuterated buffer and reconcentrating by vacuum dialysis five times or by repeated dialysis against deuterated buffer.

Magnetic Resonance Measurements. Proton magnetic resonance spectra were obtained at 100 MHz using a Varian XL-100 FT NMR spectrometer and at 220 MHz using a Varian HR-220 FT system. Residual water signals at 100 MHz were eliminated by point irradiation using the proton decoupler. The longitudinal relaxation rates ($1/T_1$) of the H-6, H-5, and H-1′ protons of CTP as previously assigned (Tso et al., 1969) were determined by the pulsed Fourier transform 90°-homogeneity spoil-τ-90° method of McDonald and Leigh (1973). The transverse relaxation rates ($1/T_2$) were determined by the Carr-Purcell pulsed method at 100 MHz using a 90°-τ-180°-τ pulse sequence and by NMR line broadening at both 100 and 220 MHz (Mildvan and Engle, 1973).

The rotational correlation time τ_r for CTP bound to the enzyme was determined by the frequency dependence of the diamagnetic effects of the enzyme on the longitudinal relaxation rates of the protons of CTP using eq 4 (Fung et al., 1976), where $1/T_{1d}$ is the difference in $1/T_1$ of a CTP proton in the presence and absence of the enzyme.

$$\frac{(1/T_{1d})_{100}}{(1/T_{1d})_{220}} = \frac{\left(\frac{5 + 8\omega_I^2\tau_r^2}{1 + 5\omega_I^2\tau_r^2 + 4\omega_I^4\tau_r^4}\right)_{100}}{\left(\frac{5 + 8\omega_I^2\tau_r^2}{1 + 5\omega_I^2\tau_r^2 + 4\omega_I^4\tau_r^4}\right)_{220}} \quad (4)$$

An upper limit to τ_r was also determined by eq 5 (Solomon, 1955), in which the transverse relaxation time (T_2) was determined by the Carr-Purcell pulsed method (Mildvan and Engle, 1973).

$$\frac{T_{1d}}{T_{2d}} = \frac{2\omega_I^4\tau_r^4 + 13\omega_I^2\tau_r^2 + 4}{8\omega_I^2\tau_r^2 + 5} \quad (5)$$

The paramagnetic contribution to the relaxation rates of these protons was determined by adding CrATP to a sample containing the substrate, CTP, $MgCl_2$, and enzyme, after the diamagnetic relaxation rates in the absence of CrATP had been determined. The paramagnetic effects on CTP due to free CrATP were determined by experiments lacking enzyme and in the presence of enzyme by adding excess ApA to saturate the initiation site displacing enzyme-bound CrATP. Both methods gave indistinguishable results. The data from the enzyme experiments were analyzed using eq 6:

$$\frac{1}{T_{1p}} = \frac{[\text{CrATP}]_{\text{bound}}}{[\text{CTP}]_{\text{total}}}(1/fT_{1p})_{\text{Enz}} + \frac{[\text{CrATP}]_{\text{free}}}{[\text{CTP}]_{\text{total}}}(1/fT_{1p})_{\text{free}} \quad (6)$$

(Fung et al., 1974), in which $1/T_{1p}$ is the difference in $1/T_1$ in the presence and absence of CrATP, and f is a normalization factor equal to $[\text{CrATP}]_{\text{bound}}/[\text{CTP}]$ or $[\text{CrATP}]_{\text{free}}/[\text{CTP}]$ as described by Luz and Meiboom (1964). The concentrations of bound and free CrATP were calculated from the dissociation constant of the RNA polymerase-CrATP complex obtained from three independent types of kinetic measurements. $(1/fT_{1p})_{\text{free}}$ is the normalized paramagnetic effect of free CrATP, and $(1/fT_{1p})_{\text{Enz}}$, the unknown to be determined, is the paramagnetic effect of enzyme-bound CrATP on the relaxation rates of the protons of CTP. The temperature for all relaxation measurements was maintained at 24 ± 1 °C by cooled nitrogen gas flow. The distances (r) from the Cr of enzyme-bound CrATP to the protons of enzyme-bound CTP were calculated using the dipolar term of the Solomon-Bloembergen equation (Solomon, 1955; Solomon and Bloembergen, 1956; Mildvan and Engle, 1973).

$$r\ (\text{Å}) = C[T_{1M}f(\tau_c)]^{1/6} \quad (7)$$

The parameter C is a collection of constants equal to 705 for the Cr(III)-proton interactions (Gupta et al., 1976); T_{1m} is the relaxation time of a nucleus of enzyme-bound CTP and is equal to $(fT_{1p})_{\text{Enz}}$ in the limit of fast exchange (Mildvan and Engle, 1973; Sloan and Mildvan, 1974). The correlation function $f(\tau_c)$ is given by

$$f(\tau_c) = \frac{3\tau_c}{1 + \omega_I^2\tau_c^2} + \frac{7\tau_c}{1 + \omega_s^2\tau_c^2} \quad (8)$$

where τ_c is the correlation time for dipolar interaction, ω_I is the nuclear precession frequency, and ω_s is the electron precession frequency.

The value of τ_c was obtained by measuring the paramagnetic effects of enzyme-bound CrATP under identical conditions on the longitudinal relaxation rates of water protons at 3, 8, 15, and 24.3 MHz on an NMR Specialties PS 60W spectrometer modified in this laboratory and at 100 MHz on the XL-100 as modified for measuring T_1 directly from the free-induction decay.[2] The T_1 measurements of water protons at all frequencies were made by the 180°-τ-90° null-point method (Mildvan and Engle, 1973).

The samples contained CTP (9.8 mM), $MgCl_2$ (11.2 mM), K^+-Pipes buffer (pH 6.7) (50 mM), KCl (150 mM), 35 to 70 μM RNA polymerase, when present, and 140 μM CrATP when present. The relaxation rates of the protons of water were corrected for the effects of free CrATP, as measured separately.

The paramagnetic effects of enzyme-bound CrATP on $1/T_1$ of water protons were fit to eq 7-9 by a computer search procedure as previously described (Maggio et al., 1975; Gupta, 1976; Bean et al., 1977).

$$\frac{1}{\tau_c} = B\left[\frac{\tau_v}{1 + \omega_s^2\tau_v^2} + \frac{4\tau_v}{1 + 4\omega_s^2\tau_v^2}\right] \quad (9)$$

In eq 9, B is a constant which depends on the symmetry of the ligand field at Cr^{3+}, and τ_v is the time constant for distortions of this symmetry (Bloembergen and Morgan, 1961).

In addition, a lower limit to τ_c was estimated by measuring the line width of the EPR spectrum of enzyme-bound CrATP, using a Varian E-4 EPR spectrometer together with a Nicolet 1074 computer for the accumulation of multiple scans. The samples contained 132 μM CrATP and 66.6 μM RNA polymerase and were otherwise identical to those used for the water proton relaxation measurements. A sample containing only buffer, salts, and CTP was used for baseline corrections. Due to the low concentration of CrATP, 256 sweeps were accumulated with a scan time of 2 min each. A 4000-G scan range centered at 3400 G was used to observe the Cr^{3+} resonance (Gupta et al., 1976). Other instrument settings were microwave power, 100 mW; modulation amplitude 40 G, and time constant, 1.0 s. The temperature was maintained at 24 ± 1 °C by cooled nitrogen gas flow. Since 75% of the CrATP was free in solution under these conditions as calculated from its dis-

[2] We are grateful to Mr. Sol Altstein for making this modification.

sociation constant, 75% of the baseline-corrected spectrum of free CrATP was subtracted by computer from the baseline-corrected spectrum containing enzyme. This correction had no apparent effect on the resultant line width of the CrATP signal, indicating that binding to RNA polymerase did not alter the line width of CrATP. The peak to peak line width ($\Delta\nu$) of the derivative EPR spectrum (in gauss) was used to determine a lower limit to τ_c using eq 10.

$$\tau_c \geq \frac{1}{(3\pi)(2.8 \times 10^6)(\Delta\nu)} \quad (10)$$

The effect of enzyme-bound CrATP on the intensity of the EPR spectrum of enzyme-bound Mn^{2+} was studied in order to estimate the distance between the two metals (Leigh, 1970; Villafranca, 1977). From a measure of the decrease in amplitude of the signal due to enzyme-bound Mn^{2+} in the presence of paramagnetic CrATP as compared to diamagnetic $Co^{3+}(NH_3)_4ATP$, and from the line width of this signal, a value of the interaction coefficient, C, can be obtained (see Table I from Leigh, 1973). This is used to measure the distance (r) by the following equation:

$$C = \frac{(g)_{Mn}[g^2(S)(S+1)]_{Cr}\beta^3\tau_{Cr}}{\hbar r^6} \quad (11)$$

In eq 11, g, β, S and $\hbar$ have their usual meanings (Mildvan and Engle, 1973) and τ_{Cr} is the longitudinal electron-spin relaxation time of enzyme-bound CrATP, which, in the present case, is the same as τ_c in eq 7–9. Under the experimental conditions, 64% of the Mn^{2+} present was free. Hence, the enzyme-bound Mn^{2+} spectrum was obtained by subtracting the spectrum of the free Mn^{2+} by computer. The EPR spectrometer settings were: microwave power, 100 mW; frequency, 9.15 GHz; modulation amplitude, 40 G; time constant, 1.0 s; scan time, 2 min with 32 scans accumulated; frequency range, 2000 G centered at 3200 G.

Results

Inactivity of CrATP and $Co^{3+}(NH_3)_4ATP$ as Substrates of RNA Polymerase. The activity of CrATP as a substrate for elongation was tested using poly[d(A-T)]·poly[d(A-T)] as the template and initiating with saturating amounts of ApU (60 μM) (Koren and Mildvan, 1977). The incorporation of [^{3}H]UMP into an acid-insoluble product in a 10-min assay was measured. In the absence of an adenine-containing substrate, chain elongation was not detected. Addition of ATP (152 μM) to the assay mixture induced significant UMP incorporation (1527 pmol), whereas CrATP (150 μM) did not increase the measured incorporation of UMP ($\leq$7 pmol) despite the presence of a divalent cation activator. This demonstrates that CrATP cannot function as a substrate for elongation. The inert β,γ-bidentate complex $Co^{3+}(NH_3)_4ATP$ was also shown to be inactive as a substrate for elongation, using poly(dT) as template, a saturating level of the initiator ApA (58 μM), and 2.0 mM $Co^{3+}(NH_3)_4$[^{3}H]ATP as the monitor of incorporation. After a standard 10-min assay, less than 0.02% of the ^{3}H label was incorporated at 24 °C and pH 6.7. Further 10 min assays after preincubating all components except the enzyme for increasing time intervals from 10 min to 2 h indicated that the slight incorporation observed was probably due to a slow breakdown of $Co^{3+}(NH_3)_4$[^{3}H]ATP making free [^{3}H]ATP available.

Tests of CrATP and $Co^{3+}(NH_3)_4ATP$ as Initiators of RNA Polymerase. A number of tests were conducted to determine if CrATP is an initiator of RNA polymerase. Initiation by CrATP was initially suggested by its ability to activate the incorporation of [^{3}H]AMP into RNA with poly(dT) as template. In order to minimize initiation by ATP, which is required for chain elongation, the concentration of this substrate was kept at 25 μM. This is well below the K_M of ATP in the overall reaction (150 ± 20 μM) determined under identical conditions which measures the affinity of ATP for the initiation site (Wu and Goldthwait, 1969; Koren and Mildvan, 1977). CrATP caused a 6.6-fold increase in incorporation of [^{3}H]AMP into product, over that observed in samples which contained only ATP, suggesting that CrATP is an initiator of RNA polymerase. The amount of [^{3}H]AMP incorporated due to initiation by ATP in the absence of CrATP was subtracted from all points to obtain an initial estimate of initiation by CrATP. The initial K_M value of CrATP (87 ± 5 μM) obtained from a linear double-reciprocal plot of this data was used as a starting point for iterations to estimate the amount of poly(A) formation initiated by CrATP only, based on the K_M, V_{max}, and concentrations of the two competing initiators CrATP and ATP. The K_M for ATP was measured as 150 ± 20 μM as mentioned above while the apparent V_{max} was approximately equal to the V_{max} for CrATP initiation under the conditions of these experiments. After three iterations, a constant value of 97 ± 6 μM was obtained for the K_M of CrATP as an initiator, by a linear least-squares fit. Similarly, $Co(NH_3)_4ATP$ also produced a 3.3-fold activation of RNA polymerase with an iterated K_M of 103 ± 10 μM, suggesting that this stable metal complex of ATP is also an initiator with a similar affinity for the initiation site.

Direct evidence that CrATP is an initiator requires the demonstration that it is incorporated into the RNA product. In order to determine this, we synthesized Cr[^{3}H]ATP and tested its incorporation into an acid-insoluble product using poly(dT) as template (Table I). In the absence of ATP, about 0.1% of the labeled CrATP was incorporated into the product in 30 min. This incorporation was probably due to contamination of the Cr[^{3}H]ATP by 0.1% free [^{3}H]ATP. After allowing this background incorporation to run to completion in the absence of substrate, unlabeled ATP was added to start the reaction. Additional CrATP was then incorporated to the extent of 9 ± 2 times the value for the control reaction (Table I). Since it has been shown that CrATP is not a substrate for elongation, this incorporation must have been due to the incorporation of CrATP at the 5′-end of the product RNA. Consistent with this view, the presence of the initiator ApA (400 μm) in these experiments inhibited the incorporation of Cr[^{3}H]ATP by 61 ± 2% (Table I). In these experiments, CrATP also increased the incorporation of the substrate for elongation, as monitored by [α-^{32}P]AMP labeling of the acid-insoluble product, by a factor of 1.65 ± 0.20 over that found with ATP alone in accord with the previous activation experiments.

In a similar manner, more direct evidence for initiation by $Co^{3+}(NH_3)_4$[^{3}H]ATP was obtained (Table I). Without preincubation, the presence of ATP resulted in a 4.9 ± 0.9-fold increase in the incorporation of $Co^{3+}(NH_3)_4$[^{3}H]ATP into RNA as compared with control reactions without ATP. Moreover, in these experiments, incorporation of the substrate for elongation, [α-^{32}P]ATP was also stimulated 3.6- and 5.8-fold by 202 and 404 μM $Co^{3+}(NH_4)_4ATP$, respectively. As shown in the double-reciprocal plot of Figure 1, CrATP inhibits initiation by ApA, an extremely efficient initiator with poly(dT) as template (Koren et al., 1977). In order to calculate the rate of formation of RNA initiated only by ApA, corrections had to be made for initiation by CrATP and ATP. This was done as described under Methods using eq 3. The K_M for CrATP used in this correction was 97 μM as determined separately. The K_M for ApA determined in similar experiments

TABLE I: Incorporation of Cr[^{3}H]ATP[a] or $Co^{3+}(NH_3)_4$[^{3}H]ATP[b] into the RNA Product.

Preincub (min)	Cr[^{3}H]ATP concn (μM)	[α-^{32}P]ATP concn (μM)	ApA concn (μM)	pmol of Cr[^{3}H]ATP incorp	pmol of [^{32}P]AMP incorp
30	51.6	0	0	0.6	
30	51.6	24	0	6.6	651
30	51.6	24	400	2.8	850
Preincub (min)	**$Co^{3+}(NH_3)_4$[^{3}H]ATP concn (μM)**	**[α-^{32}P]ATP concn (μM)**		**pmol of $Co^{3+}(NH_3)_4$ATP incorp**	**pmol of [^{32}P]AMP incorp**
0	202	0		1.0	
0	202	24		4.9	256
0	404	0		1.9	
0	404	24		12.9	415

[a] The reaction mixtures in 0.05 mL contained: 0.05 mg/mL poly(dT), 5 mM $MgCl_2$, 50 mM K^+-Pipes (pH 6.7), 150 mM KCl, 31.3 pmol of RNA polymerase, and the designated amounts of ApA, [α-^{32}P]ATP (9.3 cpm/pmol), and [^{3}H]CrATP (464 cpm/pmol). The reaction mixtures minus ATP were preincubated at 24 °C as indicated. The reaction was initiated by adding ATP, where indicated (otherwise an equal volume of H_2O), and stopped after 20 min as described under Methods. [b] $Co^{3+}(NH_3)_4$[^{3}H]ATP (46.2 cpm/pmol) was used in place of Cr[^{3}H]ATP, and the reactions were initiated by adding 6 pmol of enzyme and stopped after 10 min. The specific activity of the [α-^{32}P]ATP was 10.0 cpm/pmol. Otherwise, all conditions were the same as above.

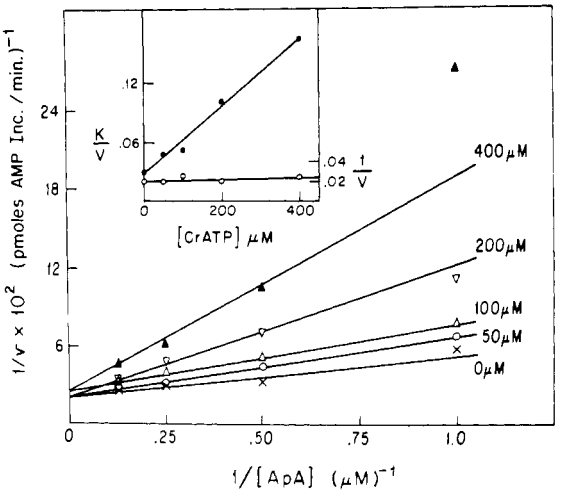

FIGURE 1: Double-reciprocal plot of the inhibition by CrATP of ApA initiation of RNA polymerase. The conditions and assay mixtures were as given in the text, except the concentrations of ApA and CrATP were varied as shown. The insert shows secondary plots of the slope (K/V) and intercept ($1/V$) against CrATP concentration.

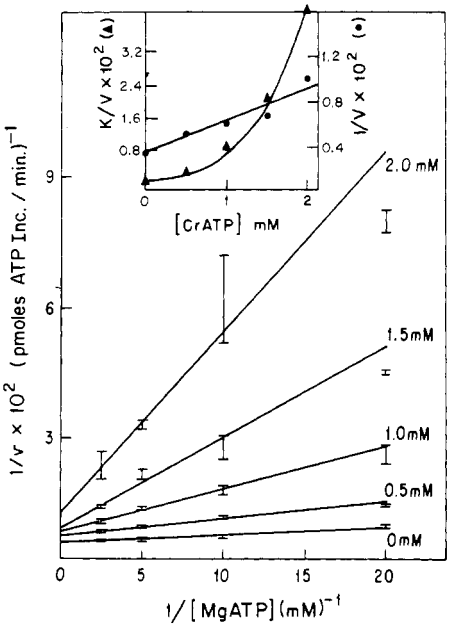

FIGURE 2: Double-reciprocal plot of the inhibition by CrATP of AMP incorporation into RNA at a saturating level of the initiator ApA. Assay conditions and components were as given in the text, except that ApA was at 250 μM, the concentrations of [^{3}H]ATP and CrATP were varied as shown. The insert shows secondary plots of the slope and intercept against CrATP concentration.

without added CrATP was 1.9 μM, while the K_M for ATP was 150 ± 20 μM as mentioned previously. The corrections for initiation by ATP were very small, but larger corrections were necessary for CrATP initiation at high levels of this initiator. A double-reciprocal plot of the corrected data (Figure 1) indicates that CrATP is a linear competitive inhibitor with respect to ApA with a K_i of 90 ± 15 μM.

That CrATP inhibits initiation by ATP is shown by the decreased incorporation of [γ-^{32}P]ATP into RNA which can occur only at the 5′ end. A plot of the reciprocal of the velocity of [γ-^{32}P]ATP incorporation against CrATP concentration was linear, yielding half-maximal inhibition at 120 ± 20 μM CrATP as determined by a least-squares analysis. Correcting for the presence of ATP which was present at a level (150 μM) equal to its K_M value, the K_i of CrATP as a competitive inhibitor of initiation by ATP is calculated as 60 ± 15 μM. In the same experiment, no inhibition by CrATP of the rate of RNA synthesis was observed, as measured by the rate of incorporation of [^{3}H]AMP into the acid-insoluble product. Thus, initiation by CrATP is established and its average dissociation constant from the initiator site is 86 ± 25 μM as determined by three independent methods, the K_M of CrATP as an initiator (97 ± 6 μM), and its competitive K_i against two other initiators (75 ± 21 μM). Similarly, initiation by $Co^{3+}(NH_3)_4$ATP is established and a comparable dissociation constant of 103 ± 10 μM is estimated from its K_M as an initiator.

Inhibition of RNA Chain Elongation by CrATP and $Co^{3+}(NH_3)_4$ATP. Although unable to act as a substrate for elongation, CrATP weakly inhibits chain elongation at higher levels in a complex manner. Figure 2 shows the effect of varying levels of CrATP on the rate of incorporation of [^{3}H]AMP into an RNA chain, using poly(dT) as template and initiating with saturating amounts of ApA to prevent any effect of CrATP at the initiation site. The results indicate mixed in-

TABLE II: Diamagnetic Effects of RNA Polymerase on the Relaxation Rates of the Protons of CTP at 100 and 220 MHz.[a]

	100 MHz					220 MHz				τ_r ($s \times 10^9$)	
Proton	$1/T_1$ (CTP) (s^{-1})	$1/T_1$ (Enz + CTP) (s^{-1})	$1/T_{1d}$ (s^{-1})	$1/fT_{1D}$ (s^{-1})	$1/fT_{2D}$ (s^{-1})	$1/T_1$ (CTP) (s^{-1})	$1/T_1$ (Enz + CTP) (s^{-1})	$1/T_{1D}$ (s^{-1})	$1/fT_{1D}$ (s^{-1})	from $(T_{1D})_{220}/(T_{1D})_{100}$	from $(T_{1D})_{100}/(T_{2D})_{100}$
H-6	1.36 ± 0.33	2.56 ± 0.42	1.2 ± 0.5	140 ± 60	480 ± 90	1.45 ± 0.15	1.45 ± 0.15	≤0.21	≤25	≥1.3	<5.8
H-5	0.90 ± 0.10	2.22 ± 0.29	1.3 ± 0.3	160 ± 40		0.82 ± 0.15	0.89 ± 0.16	≤0.22	≤26	≥4.8	
H-1′	0.57 ± 0.02	1.23 ± 0.10	0.66 ± 0.10	79 ± 12	1090 ± 350	0.55 ± 0.03	0.80 ± 0.04	0.25 ± 0.05	30 ± 6	0.9	<12.8

[a] Components present were CTP (10.0 mM), RNA polymerase, when present (78–84 μM), $MgCl_2$ (11.0 mM), K^+-Pipes buffer (pH 6.7) in H_2O (50 mM), KCl (105 mM) in a D_2O solvent ($T = 24 \pm 1$ °C). f is here defined as [RNA polymerase]/[CTP], since the enzyme is saturated with 10.0 mM CTP, the dissociation constant of which is 230 μM, as determined by equilibrium dialysis (Wu and Goldthwait, 1969b).

hibition by CrATP consisting of a parabolic competitive (slope) effect with an apparent K_i of 0.5 ± 0.1 mM and a linear uncompetitive (intercept) effect with a K_i of approximately 1.2 ± 0.2 mM. Changing the level of ApA from 50 to 250 μM had no effect on these results, indicating that the binding of CrATP at the initiation site has been eliminated in these experiments. Thus, in addition to the initiation-site binding of CrATP, other weaker sites exist at or near the elongation site of the enzyme. In contrast, $Co^{3+}(NH_3)_4ATP$ appears to be a simple linear competitive inhibitor of elongation with much weaker binding ($K_i = 7 \pm 2$ mM).

Diamagnetic Effects of RNA Polymerase on the Relaxation Rates of the Protons of CTP. The proton NMR spectrum of CTP at 100 and 220 MHz contains three easily resolvable resonances which have previously been assigned (Tso et al., 1966). Doublets ($J = 7.6$ Hz) at 8.08- and at 6.30-ppm downfield from $(CH_3)_4Si$ have been assigned to the cytidine H_6 and H_5 protons, respectively. A doublet ($J = 2.6$ Hz) at 6.16 ppm has been assigned to the ribose H_1' proton. The presence of RNA polymerase caused marked increases in the longitudinal ($1/T_1$) and transverse ($1/T_2$) relaxation rates of these protons (Table II). At 220 MHz, much smaller effects of the enzyme on $1/T_1$ of the protons of CTP were noted. The large frequency dependence of $1/T_{1D}$ and the absence of nonexponential relaxation behavior argue against a significant contribution of spin diffusion (Kalk and Berendsen, 1976) to the relaxation rates of CTP under our conditions. Rotational correlation times ranging from 0.9 to >4.8 ns for enzyme-bound CTP were estimated from the $(1/T_{1p})_{100}/(1/T_{1p})_{220}$ ratio using eq 4 (Table II). Although pulsed T_2 values were determined only at 100 MHz, the broadening of the proton resonances of CTP induced by the enzyme at 100 MHz increased by a factor of 3 ± 1 at 220 MHz, indicating a predominant chemical-shift contribution to $1/T_{2D}$. Hence, only upper-limit values to τ_r of <5.8 to <12.8 ns could be estimated from the T_{1D}/T_{2D} ratios at 100 MHz using eq 5 (Table III). As calculated from Stokes law (Abragam, 1961; Cohn and Edsall, 1943) a spherical molecule with the molecular weight of RNA polymerase (500 000) would have a rotational correlation time of ~400 ns or even greater due to dimerization (Chamberlin, 1974). Hence, the results of Table II suggest that the bound CTP molecule is only partially immobilized by the enzyme. These findings are consistent with the incomplete polarization of the fluorescence of 1,N^6-etheno-ATP when bound to RNA polymerase (Mildvan et al., 1978) and provide independent evidence for some local mobility of the enzyme-bound substrate.

Paramagnetic Effects of Enzyme-Bound CrATP on the Longitudinal Relaxation Rates of CTP. The paramagnetic properties of Cr^{3+} and the relatively strong binding of CrATP to the initiation site, as well as the observations that pyrimidine nucleotides bind solely to the elongation site (Wu and Goldthwait, 1969a,b), make CrATP a suitable probe for distance measurements from the initiation site to the elongation site of RNA polymerase. Initially, the diamagnetic relaxation rates of the protons of CTP were measured in the presence of RNA polymerase. CrATP was added and the effect on the proton relaxation rates measured (Table III). At the low levels of CrATP (≤100 μM) and at the saturating level of CTP (10.0 mM) used in these experiments, the occupancy by CrATP of the elongation site ($K_i = 0.5$ mM) and of ancillary sites ($K_i = 1.2$ mM) may be assumed to be negligible. To establish this point, a saturating amount of ApA was added to displace CrATP from the initiation site, allowing a measurement of the paramagnetic effect of free CrATP in the samples containing enzyme (Table III). These effects were indistinguishable from those measured by adding CrATP to CTP in solutions lacking enzyme but which were otherwise identical. Adding ApA to the CrATP–MgCTP system in the absence of enzyme had a negligible effect on the relaxation rates of the CTP protons. This control indicates that the decreases in the relaxation rates induced by ApA in the presence of the enzyme were due to the displacement of CrATP from the initiation site. Under our experimental conditions, RNA polymerase was observed to enhance the paramagnetic effects of CrATP on $1/T_{1p}$ of the protons of CTP by factors ranging from 1.6 ± 0.3 to 1.9 ± 0.1 (Table III), establishing the formation of a quaternary enzyme–CrATP–MgCTP complex. Using eq 6 (see Methods), the paramagnetic effects of enzyme-bound CrATP on the longitudinal relaxation rates of the protons of enzyme-bound CTP ($1/fT_{1p}$) were calculated (Table III).

Calculation of Distances from CrATP to CTP on RNA Polymerase. The use of eq 7 to calculate distances from a paramagnetic center to an enzyme-bound ligand from the $1/fT_{1p}$ values of the ligand requires that the $1/fT_{1p}$ values not be limited by the rate of chemical exchange of the ligand out of the paramagnetic environment on the enzyme. If $1/fT_{1p}$ is exchange limited, only upper-limit distances can be calculated (Mildvan and Engle, 1973). Fast exchange for CTP from the paramagnetic environment ($>10^5$ s^{-1}) is expected, since ATP, a substrate which binds to the elongation site an order of magnitude more tightly than CTP or UTP (Wu and Goldthwait, 1969b; Koren and Mildvan, 1977), exchanges at a rate ($>2 \times 10^5$ s^{-1}; Bean et al., 1977) much greater than the $1/fT_{1p}$ values of CTP (Table IV). Also, the diamagnetic effect of RNA polymerase on $1/fT_{2D}$ of the H_1' proton of CTP (Table

TABLE III: Paramagnetic Effects of the RNA Polymerase-CrATP Complex on the Longitudinal Relaxation Rates of the Protons of CTP at 100 MHz.[a]

Proton	[Enzyme] (μM)	[CrATP] (μM)	$1/T_1$ (s^{-1})	$1/T_{1p}$ (s^{-1})	$(1/fT_{1p})(Enz)$[b] (s^{-1})
H-6	77.8	0	1.79 ± 0.16		
	77.8	50	2.94 ± 0.25	1.14 ± 0.29	401 ± 31
	77.8	100	3.33 ± 0.16	1.54 ± 0.22	225 ± 32
	77.8	100[c]	2.99 ± 0.11	1.20 ± 0.19	
	54.0	0	1.35 ± 0.06		
	54.0	50	2.30 ± 0.12	0.95 ± 0.13	386 ± 97
	54.0	50[c]	1.89 ± 0.15	0.54 ± 0.14	
					Av 337 ± 96
H-5	77.8	0	1.35 ± 0.14		
	77.8	50	3.08 ± 0.13	1.73 ± 0.19	753 ± 29
	77.8	100	3.13 ± 0.12	1.78 ± 0.18	357 ± 39
	77.8	100[c]	2.27 ± 0.11	0.92 ± 0.18	
	54.0	0	1.11 ± 0.10		
	54.0	50	2.70 ± 0.24	1.59 ± 0.24	653 ± 154
	54.0	50[c]	2.00 ± 0.21	0.89 ± 0.21	
					Av 588 ± 197
H-1′	77.8	0	1.54 ± 0.11		
	77.8	50	1.75 ± 0.12	0.21 ± 0.16	36 ± 26
	77.8	100	2.22 ± 0.12	0.68 ± 0.16	114 ± 322
	77.8	100[c]	2.00 ± 0.11	0.46 ± 0.15	
	54.0	50	1.47 ± 0.14	0.18 ± 0.13	90 ± 56
	54.0	50[c]	1.29 ± 0.10	0 ± 0.10	
					Av 80 ± 39

[a] In addition to the indicated amount of enzyme and CrATP, all solutions contained in D_2O the components listed in Table II. [b] Calculated from eq 6 under Methods, using 86 ± 25 μM as the K_d for CrATP from the initiation site of RNA polymerase. f is here defined as [E-CrATP]/[CTP]. [c] 100 μM ApA was used to displace CrATP from the enzyme. [d] A negligible effect of free CrATP was found under these conditions.

TABLE IV: Paramagnetic Effects of the RNA Polymerase-CrATP Complex on the Longitudinal Relaxation Rates of Water Protons at Five Frequencies.[a]

[RNA pol] (μM)	$(1/T_{1p})_{Enz}$[b] 3 MHz (s^{-1})	8 MHz (s^{-1})	15 MHz (s^{-1})	24.3 MHz (s^{-1})	100 MHz (s^{-1})	$B \times 10^{-21}$ s^{-2}	$\tau_\nu \times 10^{13}$ s	$\tau_c \times 10^{10}$[c] s
70	0.329	0.271	0.266	0.286				
						1.0 ± 0.5	6.3 ± 2.0	4 ± 2
35	0.204	0.196	0.205	0.213	0.240			

[a] Components present are given under Methods. [b] $(1/T_{1p})_{Enz}$ is the contribution of enzyme-bound CrATP to the relaxation rate of water protons where the CrATP is 26 and 14% enzyme bound in the presence of 70 and 35 μM RNA polymerase, respectively. [c] Calculated at 100 MHz.

TABLE V: Calculation of Distances from CrATP to the Protons of CTP on RNA Polymerase.[a]

Proton	$1/fT_{1p}$ (s^{-1})	$f(\tau_c)$ ($s \times 10^9$ [b])	r (Å)
H-6	337 ± 96	1.1 ± 0.5	8.6 ± 0.9
H-5	588 ± 197	1.1 ± 0.5	7.9 ± 0.9
H-1′	80 ± 39	1.1 ± 0.5	11.0 ± 1.6

[a] The conditions are given in Table III. [b] Calculated at 100 MHz.

II) sets a lower limit to the exchange rate of CTP which is also significantly greater than the $1/fT_{1p}$ values of the protons of CTP (Table III).

Moreover, the $1/fT_{2D}$ values of the protons of CTP on RNA polymerase are not exchange limited, since the line broadenings increase with magnetic field between 100 and 220 MHz, by factors of 3 ± 1 as discussed in the preceding section.

In order to calculate the Cr^{3+}-CTP distances, the correlation time for the dipolar effect of enzyme-bound CrATP on the protons of CTP is required. This parameter was determined by measuring the effects of enzyme-bound CrATP on the longitudinal relaxation rates of water protons at five frequencies ranging from 3 to 100 MHz. The best value of τ_c, as determined by a computer fit of the data to eq 7-9, is (4.0 ± 2.0) × 10^{-10} s (Table IV), a value typical of the electron-spin relaxation time of Cr^{3+} complexes (Gupta et al., 1976). Consistent with this, a lower limit to τ_c of 0.74 × 10^{-10} s was estimated independently from the line width of the EPR spectrum of enzyme-bound CrATP. The best fit τ_c value corresponds to an $f(\tau_c)$ value of 1.13 ± 0.54 × 10^{-9} s^{-1} at 100 MHz which was then used in eq 7 to calculate the distances from enzyme-bound CrATP to the various protons of enzyme-bound CTP (Table V).

Since distances both to the cytidine ring and to the sugar were determined, the dihedral angle (χ) at the glycosidic bond was thereby defined. Model-building studies using the distances and errors in Table V yielded a glycosidic conformational angle for CTP bound at the elongation site of RNA

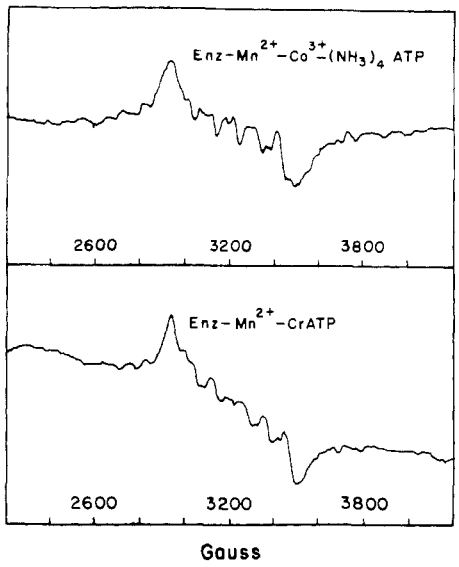

FIGURE 3: EPR spectra of RNA polymerase bound Mn^{2+} with either $Co(NH_3)_4ATP$ (upper) or CrATP (lower) bound at the initiation site. These spectra were obtained by subtracting the spectrum of free Mn^{2+}, $Co^{3+}(NH_3)_4ATP$, or CrATP (32 scans), buffer, and salts from the spectrum of the complete system (32 scans). Components present in the complete system were RNA polymerase (44.5 μM) and $MnCl_2$ (48 μM, 36% of which was bound as measured by EPR). $Co(NH_3)_4ATP$ or CrATP (0.5 mM), buffer, and salts were as given in the text. The instrumental settings were given under Methods.

polymerase ($\chi = 90 \pm 15°$) indistinguishable from that previously found for ATP bound at the same site (Bean et al., 1977).[3]

Limiting Distance from CrATP to the Divalent Cation Binding Site of RNA Polymerase. Previous studies from this laboratory (Koren and Mildvan, 1977; Bean et al., 1977) showed the presence of a single tight Mn^{2+} binding site at the elongation site of RNA polymerase, the occupancy of which was necessary for catalysis. In principle the distance between CrATP bound at the initiation site and Mn^{2+} bound at the elongation site may be calculated from the paramagnetic effects of CrATP on the electron-spin relaxation time of the bound Mn^{2+}. Two methods based on this principle have been described, the effect of CrATP on the transverse electron-spin relaxation time as measured by the amplitude of the EPR spectrum of bound Mn^{2+} (Villafranca et al., 1977; Leigh, 1970), and the effect of CrATP on the longitudinal electron-spin relaxation time as measured by the enhancement of the water relaxation rate due to enzyme-bound Mn^{2+} (Gupta, 1977). Both of these methods were used.

With the EPR method, under our conditions, only 36% of the Mn^{2+} was bound to the enzyme. Hence, the EPR spectrum of enzyme-bound Mn^{2+} was obscured by the more intense signal of free Mn^{2+}. Mathematical subtraction by computer of the spectrum due to the calculated amount of free Mn^{2+} was used to obtain the broadened spectrum characteristic of enzyme-bound Mn^{2+} (Figure 3).

Further subtraction of the free Mn^{2+} signal yielded an apparent increase in intensity due to the inversion of the spectrum of free Mn^{2+}. The derivative peaks in the EPR spectrum of the bound Mn^{2+} so generated showed the same amplitude, within experimental error (±10%), in the presence of the paramagnetic CrATP or the diamagnetic $Co^{3+}(NH_3)_4ATP$ complex (Figure 3). Indistinguishable results were obtained when the correction for free Mn^{2+} was determined experimentally by equilibrium dialysis. Our error level yields a maximal paramagnetic effect of CrATP on Mn^{2+} of $\leq 10\%$ from which a minimal Cr^{3+} to Mn^{2+} distance on the enzyme of ≥ 11.5 Å is estimated using eq 11.

Similarly, with the proton relaxation method, CrATP and $Co^{3+}(NH_3)_4ATP$ had indistinguishable effects on the enhancement of the longitudinal water proton relaxation rate due to the Mn^{2+} bound in the enzyme–initiator–MnCTP complex ($\epsilon_t = 18.5 \pm 0.6$) within the experimental error. From the latter, a lower limit Cr^{3+} to Mn^{2+} distance ≥ 10.0 Å was calculated as previously described (Gupta, 1977). These findings indicate a large distance (≥ 10–11.5 Å) between CrATP and Mn^{2+} on RNA polymerase.

Discussion

The stable metal complexes of ATP are inactive as substrates of RNA polymerase or DNA polymerase[4] presumably because the metal cannot coordinate to the protein to form an enzyme–metal–substrate bridge complex which is, apparently, a requirement of the RNA polymerase reaction (Koren and Mildvan, 1977; Bean et al., 1977) and of the DNA polymerase reaction (Sloan et al., 1975).

Both the α,β,γ-tridentate CrATP and the β,γ-bidentate $Co^{3+}(NH_3)_4ATP$ complexes can, however, function as initiators of RNA polymerase. This is not surprising, since the initiation site is relatively insensitive to changes in the polyphosphate structure (Chamberlin, 1974). In fact, AMP and ADP can function as initiators in the pyrophosphate-exchange reaction of the enzyme (Krakow and Fronk, 1969), and MnATP can initiate RNA synthesis (Koren and Mildvan, 1977). In addition, dinucleotides which do not possess any 5′-terminal phosphate groups are extremely efficient initiators (Downey and So, 1970; Niyogi and Stevens, 1965; Bean et al., 1977). The large diamagnetic effects of RNA polymerase on the relaxation rates of the substrate for elongation, CTP, may be explained by a partial immobilization of the bound substrate and are consistent with the partial polarization of fluorescence of the substrate analogue 1,N^6-etheno-ATP by the enzyme (Mildvan et al., 1977). The incomplete immobilization of the substrates by RNA polymerase has been correlated with a greater error rate in template copying by this enzyme, as compared with DNA polymerase I (Mildvan et al., 1977), although the possibility of further immobilization in the presence of a template has not been studied.

Since the Cr^{3+} ion is paramagnetic, the binding of CrATP at the initiation site of the enzyme provides a second paramagnetic reference point on RNA polymerase. Previously, Mn^{2+} bound tightly at the elongation site of the enzyme (Koren and Mildvan, 1977) had been used to determine the distances to a dinucleotide initiator, ApU, and to a substrate for elongation, ATP, bound to the enzyme (Bean et al., 1977). Seven distances from the bound Mn^{2+} to the carbon-bound protons and phosphorus of the initiator ApU were between 9.0 and 10.5 Å, while three distances to the substrate protons were much smaller ranging from 4.0 to 5.7 Å. These previous measurements would predict a wide range of possible distances between the initiator and the substrate varying from 3.3 to 16.2 Å.

[3] A χ angle of 270 ± 15° would also fit the present data on CTP and the previous data with ATP (Bean et al., 1977), but the resulting syn conformation for the bound substrates is highly unlikely, since it would be unsuitable for base pairing with a template.

[4] $Co^{3+}(NH_3)_4$-dATP is not a substrate of DNA polymerase I with poly[d(AT)]·poly[d(AT)] as template (P. J. Stein and T. M. Li, unpublished observations).

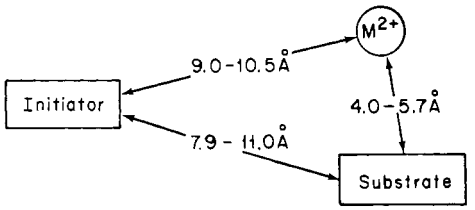

FIGURE 4: Spatial arrangement of the functional sites on RNA polymerase based on the present and previous NMR measurements (Bean et al., 1977).

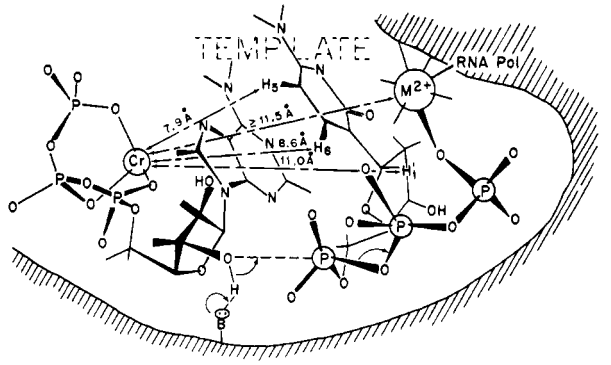

FIGURE 5: Arrangement of the initiator, CrATP, the elongation substrate, CTP, and the divalent cation activator, M^{2+}, on RNA polymerase consistent with the indicated distances and with previously determined distances (Bean et al., 1977). The arrangement permits stacking of the bases of the initiator and substrate and direct interaction between the 3′-OH group of the initiator and the α-phosphorus of the substrate.

The present studies clarify the distances between the initiator CrATP and the substrate CTP to be from 7.9 to 11.0 Å (Table V) and provide independent evidence with a pyrimidine substrate that the conformational angle of the substrate bound at the elongation site is 90° at the glycosidic bond, i.e., is equal to the glycosidic χ angle of double-helical B-DNA.[3] As pointed out previously, this conformation for the substrate seems appropriate for base pairing with a DNA template (Bean et al., 1977). Figure 4 shows a schematic arrangement of the functional sites on RNA polymerase in accord with the present and previous studies. Since the present EPR experiments indicate the Cr^{3+}–Mn^{2+} separation to be 11.5 Å or greater, it is likely that the nucleotide bases of both the initiator and the substrate are located between the catalytic divalent cation and the Cr^{3+} probe attached to the initiator. A structural model consistent with the present data, as well as with the previous studies of the conformations of the bound initiator and substrate (Bean et al., 1977), is shown in Figure 5.

Although the arrangement of the initiator and substrate in Figure 5 does not provide a unique fit to the data and is subject to possible alteration in the presence of a template, the model easily accommodates the binding of the initiator and substrate to the enzyme in a manner which permits the stacking of their respective bases. It also permits a nucleophilic attack by the 3′-hydroxyl oxygen of the initiator on the α-phosphorus atom of the substrate, as is necessary for the initiation reaction (eq 1). For these reasons, as well as the χ angle of 90° of the bound substrates despite the absence of the template and the similar dissociation constant of the enzyme–substrate complex found both in the absence and in the presence of the template (Koren and Mildvan, 1977), no large changes in the arrangement or conformations of the initiator and substrate in the presence of the template are anticipated. Nevertheless, further NMR studies in the presence of templates, which are much more difficult to carry out, will be necessary to establish the structural model and mechanism of Figure 5.

Acknowledgments

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Characterization of Phosphatidylthreonine in Polyoma Virus Transformed Fibroblasts†

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ABSTRACT: A threonine phospholipid in polyoma virus-transformed hamster embryo fibroblasts has been characterized as phosphatidylthreonine. The identification has been made by chemical and enzymatic hydrolysis. Acid hydrolysis of the phospholipid produces free threonine. Mild alcoholysis produces a water-soluble derivative having the properties of glycerophosphorylthreonine. Hydrolysis with phospholipase C produces phosphorylthreonine which on prolonged acid hydrolysis yields threonine. Phosphatidylthreonine in the cell is more accessible to reaction with fluorodinitrobenzene than is phosphatidylserine. Phosphatidylthreonine also has been found as a major aminophospholipid in two other polyoma-transformed hamster cell lines and in the BHK-21/C13 line including the PVT-3 and TS-3 lines, the latter derived from BHK cells. Only a trace amount of phosphatidylthreonine occurs in normal liver, kidney and spleen of the adult mouse, in normal liver and kidney of the adult hamster, in whole mouse and hamster embryos, and in mouse 3T3 cells and SV_{40}-transformed 3T3 cells.

Threonine has been found in egg yolk lipids (Rhodes & Lea, 1957). Phosphatidylthreonine has been reported to occur in tuna muscle (Igaraski et al., 1958). The complete characterization of phosphatidylthreonine was not carried out in these previous studies.

In a recent paper we reported the marked elevation of a threonine phospholipid in polyoma transformed hamster embryo fibroblasts (Mark-Malchoff et al., 1977). This lipid was provisionally identified as phosphatidylthreonine. We now have characterized this lipid as phosphatidylthreonine by chemical and enzymatic hydrolysis.

Experimental Procedure

Polyoma virus transformed hamster cells including the HFT-91[1] cells (HTC-3049-91TC, Hare, 1967) and PVT-3 cells (HTC-3049-3, Hare, 1964) and hamster embryo cells (HEF) were grown in tissue culture as described previously (Mark-Malchoff et al., 1977). TS-3 cells were obtained from Dr. Walter Eckhart of the Salk Institute and have been maintained at 32 °C. The BHK-21/C13 line of baby hamster kidney was obtained from the American Type Culture Association. Mouse 3T3 (Swiss) and SV-40 transformed 3T3 cells were originally obtained from Dr. Howard Green. These cell

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[1] Abbreviations used: HFT, polyoma virus transformed hamster embryo fibroblasts; HEF, normal hamster embryo fibroblasts; FDNB, 1-fluoro-2,4-dinitrobenzene; TNBS, 2,4,6-trinitrobenzenesulfonate; TNP, trinitrophenyl; DNP, dinitrophenyl; TLC, thin-layer chromatography; PS, phosphatidylserine; PT, phosphatidylthreonine; PE, phosphatidylethanolamine; ET, ethanolamine.

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