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Structural Studies on the Active Site of *Escherichia coli* RNA Polymerase. 1. Interaction of Metals on the *i* and *i* + 1 Sites

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ABSTRACT: The two substrates between which an internucleotide bond is formed in RNA synthesis occupy two subsites, *i* and *i* + 1, on the active site of *Escherichia coli* RNA polymerase, and each subsite is associated with a metal ion. These ions are therefore useful as probes of substrate interaction during RNA synthesis. We have studied interactions between the metals by EPR spectroscopy. The Zn(II) in the *i* site and the Mg(II) in the *i* + 1 site were substituted separately or jointly by Mn(II). The proximity of the metals was established by EPR monitoring of the titration at 5.5 K of the enzyme containing Mn(II) in *i* with Mn(II) going into the *i* + 1 site, and the 1:1 ratio of the metals in the two sites was confirmed in this way. The distance between the two metals was determined by EPR titration at room temperature of both the enzyme containing Zn(II) in *i* and Mn(II) in *i* with Mn(II) going into the *i* + 1 site, making use of the fact that EPR spectra are affected by dipolar interactions between the metals. The distances calculated in the presence of enzyme alone, in the presence of enzyme and two ATP substrates, and when poly(dAdT)·poly(dAdT) was added to the latter system ranged from 5.2 to 6.7 Å.

The regulation of RNA synthesis is one of the most important processes in molecular biology, and its mechanism involves a variety of components, many of which are concerned with initiation and termination. We shall concern ourselves with the possibility that regulation can occur at the point of bond formation and have begun a series of investigations to determine whether the geometry of interaction at that point can change in a way that can have regulatory significance.

The synthesis of RNA is mediated by the enzyme RNA polymerase, which in *Escherichia coli* has a molecular mass of $\sim^1/_2$ million daltons, and consists of five subunits (two α, β, and β' for core enzyme, plus σ factor in holoenzyme). This enzyme is involved in a variety of functions required in the regulation of RNA synthesis, but its central function is to bring together two substrates for the purpose of forming an internucleotide bond between them. In this central process of RNA synthesis the oxygen atom at the 3′ terminus of the growing RNA chain reacts with the α-phosphorus of a nucleoside triphosphate (NTP) to form a phosphodiester bond, with the concomitant loss of pyrophosphate.

Clearly, a major function of the enzyme must be to bring together the two substrates in a geometrical relationship that is optimal for the formation of the bond. It is possible that the enzyme permits this optimal geometry to be attained only when the substrates are the "correct" substrates for transcription, and that the enzyme can edit transcription in this way; we consider this possibility a hypothesis to be tested. At any rate, the determination of the geometrical relationship between the substrates and the possible changes in this relationship during RNA synthesis is likely to lead to many insights into the mechanism of RNA synthesis, and the goal of this and subsequent papers is to understand this relationship.

The part of the enzyme on which the two substrates come into contact can be considered the active site. On that site the enzyme brings together not only the two substrates, but also the DNA template, a portion of whose sequence is recognized by base pairing with each of the two substrates. Since the active site involves *two* substrates, it can be considered as consisting of two subsites, one for each substrate. Each of these subsites contains a metal ion bound to it.

One subsite we call the *i* site, after Yager and von Hippel (1988); it has been given other names, such as the initiation site (Anthony et al., 1969), since it is here that the first nucleoside triphosphate (NTP) hydrogen bonds to the initial DNA base to be copied. During subsequent stages of transcription this site binds the nucleoside at the 3′-terminus of the growing RNA chain. At the *i* site a Zn(II) is strongly bound to the protein (Chatterji & Wu, 1982a); we are not concerned here with the second zinc bound elsewhere to the protein. The second subsite we call the $i + 1$ site (Yager & von Hippel, 1988); it has been called the elongation site, since it is here that the second and all succeeding NTPs hydrogen bond to successive DNA bases. This site contains Mg(II), which is much less strongly bound than the Zn(II) on the *i* site; in fact, the enzyme is isolated in an inactive form without a metal on the $i + 1$ site, and a divalent cation must be added to produce the active enzyme in vitro. The difference in the strength of attachment of the two metals to the enzyme is very important in this study, since it makes possible the substitution of Mn(II) separately for each of the naturally occurring metals associated with the two subsites. This paramagnetic metal has already been used as a structural probe in previous investigations, in which distances from the metal in $i + 1$ to substrate atoms have been obtained by Bean et al. (1977) and Slepneva and Weiner (1981) and from the metal in the *i* site to points on the substrate by Chatterji and Wu (1982b). It occurred to us that the interrelationships between the substrates can be determined from some of these previously determined metal–substrate distances, augmented by some others not previously determined and, most importantly, by measuring the distance between the two metal atoms in the two subsites.

Obviously these measurements can be made with a variety of templates and substrates. For a complete mechanistic picture of the bond-forming reactions of RNA synthesis, a variety of substrate–template combinations must be examined. For the initial experiments, we chose to work with a poly(dAdT)·poly(dAdT) template and an ATP substrate in both *i* and $i + 1$ sites. This particular template–substrate combination was selected to provide distances complementary to those measured in prior studies. The poly(dAdT)·poly(dAdT) template–ATP substrate system is incapable of template-directed RNA synthesis. We assume that the template and substrate are therefore poised for RNA synthesis without actual bond formation taking place. We consider it an advantage to have both *i* and $i + 1$ sites occupied by a triphosphate, just as they shall be for the initial step of RNA synthesis. Later studies will show whether changes in template and substrates lead to changes in the orientations that we observe.

The present study is not concerned with the nature of the subunit or subunits to which the metals and the substrates are bound.

In this paper we report the studies on the determination of metal–metal distances on the enzyme. The measurements are made on enzyme without substrate or template, as well as in the presence of substrate alone, and finally in the presence of both template and substrate. Preliminary results of these studies have been reported previously (Chuknyisky et al., 1986, 1987; Eichhorn et al., 1988). The following paper considers metal–substrate distances and presents a model for substrate–substrate interaction.

Theory for Determination of Metal–Metal Distances

The distance between the Mn substituted for Zn in the *i* site and the Mn in the $i + 1$ site of RNA polymerase may be found essentially by the technique developed by Leigh (1970). This method analyzes the interaction between two dissimilar paramagnetic centers bound to a rigid lattice where an observed spin is influenced by the presence of a nearby spin undergoing rapid spin–lattice relaxation. This approach has been applied to determine the distance between a free radical and a paramagnetic metal ion (Leigh, 1970; Cohn et al., 1971; Taylor et al., 1969), between different metal ions such as Mn(II) and Cr(III) (Villafranca et al., 1977; Balakrishnan & Villafranca, 1978) and between two Mn(II) ions (Villafranca & Roushel, 1982; Knight et al., 1984).

The major change in the EPR spectrum of the observed spin in the presence of the perturbing spin is an "apparent" diminution without a noticeable broadening or appearance of new lines in the observed EPR signal. The line width of this signal is given by

$$\delta H = C'(1 - 3\cos^2\theta_R)^2 + \delta H_0 \qquad (1)$$

where the constant C' is defined as

$$C' \equiv (g\beta\tau_c\mu^2)/(r^6\hbar) \qquad (2)$$

δH_0 is the line width in the absence of a perturbing spin, θ_R is the angle between the applied magnetic field and the vector joining the two spins, and r is the interspin distance. τ_c is the correlation time, which modulates relaxation, μ is the magnetic moment of the spin producing the relaxing field, g is the electron g factor, and β is the Bohr magneton. For a spin bound to a rigid lattice, τ_c is identical with T_{1e}, the electron spin–lattice relaxation time. Equation 1 indicates that when $\theta_R \approx 54°$, and therefore, $1-3\cos^2\theta_R \approx 0$, the line width of the observed signal δH is approximately equal to that of the unperturbed signal, δH_0; however, the apparent intensity of the signal will be diminished because only that fraction of the randomly oriented spin pairs bound to the enzyme which have an interspin vector oriented in the range of $\theta_R \approx 54°$ with the magnetic field will be detected. For the other spin pairs the dipolar relaxation will result in a large broadening of the signal ($\sim$2000 G), which eliminates the possibility that the contributions of those spins will be observed. From the relative amplitude of the resultant EPR signal (the ratio of signal heights of the derivative line shapes of perturbed and unperturbed signals) the parameter C' characterizing the strength of the dipolar relaxation may be determined from the relationship of relative amplitude as a function of $C'/\delta H_0$ calculated and presented graphically by Leigh (1970). If τ_c is

known or may be evaluated, then the distance between the interacting paramagnetic centers may be calculated by eq 2.

The Leigh technique is based upon equations describing the effect of fluctuating magnetic fields on the relaxation times of resonance lines (Redfield, 1957). The Redfield equations are strictly applicable only when the following conditions are satisfied:

$$\tau_c < T_{2e} \tag{3}$$

and

$$\tau_c\omega_s > 1 \tag{4}$$

where τ_c is equal to T_{1e}, the electron spin relaxation time of the perturbing spin, and ω_s is the electron spin Larmor precession frequency. T_{2e} is the spin–spin relaxation time of the perturbed spin, which may be calculated from the line width of the observed signal:

$$1/T_{2e} = \sqrt{3}\pi g\delta H/(7.144 \times 10^{-7}) \tag{5}$$

T_{1e} may be derived from NMR measurements of the frequency dependence of water proton relaxation rates. The correlation time τ_c for water molecules on the enzyme–paramagnetic metal ion complexes is expressed by the equation

$$1/\tau_c = 1/T_{1e} + 1/\tau_R + 1/\tau_M \tag{6}$$

where τ_R is the rotational correlation time of the complex and τ_M is the lifetime of the ligands in the first coordination sphere of the metal ion (Peacocke et al., 1969). For water ligands on proteins it has been found that τ_R is long enough and could be ignored in eq 6 (Abragam, 1961). Maggio et al. (1975) showed that $1/\tau_M \ll 1/T_{1e}$ for most of the macromolecular complexes of Mn(II). Therefore, the electron spin–lattice relaxation time T_{1e} of the metal ion is a good approximation for the correlation time of water molecules in the complex, i.e., $\tau_c = T_{1e}$, and the frequency dependence technique described below can be used for the determination of T_{1e}.

The paramagnetic effect, $1/T_{1p}$, of Mn(II) in the *i* site on the water proton longitudinal relaxation rates was calculated by using

$$1/T_{1p} = 1/T_1(\mathrm{Mn}) - 1/T_1(\mathrm{Zn}) \tag{7}$$

where T_1(Mn) is the water proton relaxation time in an enzyme with Mn substituted for Zn in the *i* site of RNA polymerase, and T_1(Zn) is the relaxation time for the native RNA polymerase with Zn in the same site. The frequency dependence of $T_{1p}(\nu)$ is given by

$$\frac{1/T_{1p}(\nu_1)}{1/T_{1p}(\nu_2)} = \frac{f[\tau_c(\nu_1)]}{f[\tau_c(\nu_2)]} \tag{8}$$

where $f[\tau_c(\nu)]$ is the correlation function given by eq 9 at the indicated proton NMR frequency. ω_I and ω_s are the nuclear and electron Larmor frequencies, respectively.

$$f[\tau_c(\nu)] = 3\tau_c/(1 + \omega_1^2\tau_c^2) + 7\tau_c/(1 + \omega_s^2\tau_c^2) \tag{9}$$

Bloembergen and Morgan (1961) have shown that T_{1e} disperses as a function of frequency according to

$$1/T_{1e} = B[\tau_\nu/(1 + \omega_s^2\tau_\nu^2) + 4\tau_\nu/(1 + 4\omega_s^2\tau_\nu^2)] \tag{10}$$

where B is a constant related to the zero-field splitting of Mn(II), and τ_ν is a time constant for distortion of the complex.

The values of B and τ_ν were varied in an iterative computer search (Chuknyisky, 1989) to find the values of T_{1e} that yield the best fit between the experimentally determined left side of eq 8 and the calculated right side for NMR frequencies of 20, 80, and 200 MHz. Once B and τ_ν were determined, T_{1e} values for different metalloenzyme complexes were calculated for 15 MHz, which corresponds to the Larmor precession frequency of 9.8 GHz in the EPR experiments.

Experimental Procedures

Materials. Frozen cells of *E. coli* K12 were obtained from grain Processing Co., Muscatine, IL. Lysozyme and calf thymus DNA were purchased from Cooper Biomedic, and T7 DNA was prepared essentially as described by Studier (1969). Poly(dAdT)·poly(dAdT) was obtained from P. L. Biochemicals and was purified by dialysis against 10 mM Tris, 100 mM KCl, 0.1 mM dithiothreitol, and 5% glycerol. ATP was purchased from Sigma, D_2O (99.8%) from Norell, and [^{3}H]CTP from ICN. Ultra-Pure Tris-HCl was obtained from Bethesda Research Laboratories. Metal standards for atomic absorption spectrophotometry were from J. T. Baker. The cellulose powder CF11 was obtained from Whatman and Ultragel AcA22 from LKB. Chelex-100, Bio-Rex 70, and Bio-Phore gels were purchased from Bio-Rad. All other reagents were of the highest grade obtainable commercially and were used without additional purification.

Enzyme Preparation. E. coli RNA polymerase was prepared by the method of Burgess and Jendrisak (1975) with the modifications of Lowe et al. (1979). The AcA22 column was used as suggested by Levine et al. (1980). The purity of the preparation was checked by acrylamide gel electrophoresis and found to be better than 95%. The concentration of the enzyme was determined from the extinction coefficient (ϵ_{280} = 3.25 × 10^2 mM^{-1} cm^{-1}), assuming a molecular mass of 500 000 daltons. The catalytic activity of the enzyme was assayed as described by Burgess (1969) and Chamberlin et al. (1983). When assayed by the latter method, the activity is presumably obtained as a percentage of enzyme molecules that are active in the initiation process. Fractional activity measurements of our RNA polymerase preparations, using T7 DNA, have ranged from 50 to 80% when the enzyme was added directly to DNA. If the enzyme was first diluted approximately 10-fold, much lower activities (in the 30–40% range) were obtained. We believe that the higher activities are the more nearly correct, but in view of the fact that the measured activity seems to depend on the dilution process, we do not make much of the absolute values for the activity, but use the assay mainly to determine the relative activities of, e.g., zinc- and manganese-substituted enzymes.

Substitution of Mn(II) for Zn(II) in RNA Polymerase. Selective substitution of Mn(II) for the Zn(II) located in the *i* site of RNA polymerase was carried out by a modification of the method developed by Chatterji and Wu (1982a). Before metal ion substitution the σ subunit was removed from the core enzyme by the method of Lowe et al. (1979). The purity of the core RNA polymerase was tested by acrylamide gel electrophoresis and found to be greater than 95%. The core enzyme at 5–10 mg/mL concentration was denatured at 23 °C for 2.5 h by dialysis against 50 mM Tris-HCl (pH 8) buffer containing 7.0 M urea, 0.1 mM EDTA, 10 mM dithiothreitol, 10–100 μM Mn(II), and 5% glycerol. The core enzyme was then reconstituted by dialysis for 100 min at 23 °C against reconstitution buffer [50 mM Tris-HCl (pH 8), 20 mM β-mercaptoethanol, 200 mM KCl, 10 mM $MgCl_2$, 1.0 mM EDTA, 10–100 μM Mn(II), and 20% glycerol]. The previously separated σ subunit was then added to the partly reconstituted core enzyme and the dialysis continued overnight at 4 °C. The removal of excess or loosely bound Mn(II) was accomplished by dialysis against a third buffer [10 mM Tris-HCl (pH 8), 10 mM EDTA, 0.1 mM dithiothreitol, 100 mM KCl, and 5% glycerol] at 4 °C, for 8–12 h with a change of buffer every 2–3 h. EDTA was removed from the enzyme solution by additional dialysis against the third buffer without

EDTA at 4 °C for 9 h with renewing of the buffer every 2–3 h.

The unmodified method of Chatterji and Wu (1982a) gives ~65% Mn substitution, as shown in the original paper and confirmed by us. Ni(II), Cu(II), or Co(II) substitution in the *i* site led to over 90% substitution. Our modification led to nearly 100% substitution of Mn for Zn, as was required for the quantitative studies in this work. Substitution of Mn for Zn was 98.5% for the titration without substrate and template (Figure 4A) and 99.8% for the titration in the presence of substrate (Figure 4B) and substrate and template (Figure 4C). The average percentage from five successful substitutions is 96.7%, with maximum deviation of 11% from the average. The activity of the enzyme with Mn substituted for Zn in the *i* site was the same as the activity of the enzyme reconstituted with Zn, as has been reported previously (Chatterji & Wu, 1982a).

Preparation of Enzyme Solutions for Magnetic Resonance Studies. Samples for magnetic resonance studies were generally in 10 mM Tris-HCl, 100 mM KCl, 0.1 mM dithiothreitol, 5% glycerol (pH 8) buffer. These conditions are applicable to the experiments shown in all the figures (1–5), except that for convenience the titrations of the Zn–enzymes shown in Figure 4 were done with 500 mM NaCl. RNA polymerase is subject to dimerization under some ionic strength conditions (Shaner et al., 1982; Heumann et al., 1982), and we wanted to make sure that our data are not subject to change with varying conditions of salt concentration. We therefore carried out both titrations shown in Figure 4A in the presence of 250 mM KCl, as well as the 100 and 500 mM salt generally used in the figure, and found that the data obtained in 250 mM salt were identical with those at the other salt concentrations. Thus, our results are not affected by ionic strength. If any dimers are present in any of the solutions, they do not affect our studies on the enzyme active site.

In the titration procedures involving substrate or template Mn was added to the enzyme solutions and incubated for 10 min prior to the addition of ATP or poly(dAdT)·poly(dAdT) previously equilibrated to pH 8 in the same buffer. The ratio of template to enzyme was 50–100 base pairs per enzyme molecule. The concentrations of ATP and poly(dAdT)·poly(dAdT) were determined from their UV absorbances at 260 nm ($\epsilon = 1.5 \times 10^4$ and 6.6×10^3 M^{-1} cm^{-1}, respectively). Trace-metal impurities were removed from all solutions by treatment with Chelex 100.

NMR Measurements. The longitudinal relaxation rates of the solvent water protons were determined at 20, 80, and 200 MHz by using a 180°–τ–90° pulse sequence (Carr & Purcell, 1954). An IBM Minispec PC-20 NMR instrument with a built-in program for the inverse recovery method was used for 20 MHz. Data acquisition at 80 MHz was performed on a Bruker IBM NMR spectrometer, and T_1 was calculated on an IBM PC computer. For these measurements a coaxial cell filled with D_2O was inserted in the samples placed in 5-mm tubes. The measurements at 200 MHz were accomplished with a Varian XL-200 FT NMR spectrometer.

EPR Measurements. EPR spectra (X-band) at cryogenic temperatures were recorded on a Jeolco JES spectrometer. Temperatures in the range 5–80 K were maintained to ±1 K by using an Air Products digital temperature controller to regulate the liquid helium or liquid nitrogen evaporation flow from a Dewar. Spectra at 23 °C (296 K) were obtained with an IBM Instruments ER 200 D-SRC spectrometer using 50-μL Kimble microcapillary pipets of 1.0-mm inside diameter with the ends capped by Critoseal. For titration experiments, samples of varying Mn concentration were placed in different microcapillary pipets. Reproducibility of the EPR spectra and of the atomic absorption metal determination was checked at all points of the titration curves. Samples with the same metal concentration showed less than 3% deviation in amplitude of the EPR signal, measured for either a single transition or for the average of the six lines in the hyperfine splitting.

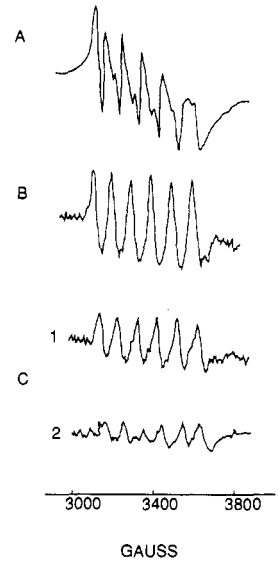

FIGURE 1: Low-temperature (5.5 K) EPR spectra of RNA polymerase–Mn(II) complexes. The enzyme concentration is 72 μM. (A) Mn(II) substituted for Mg(II) in *i* + 1 site, Zn(II) in *i* site, gain 200. (B) Mn(II) substituted for Zn(II) in *i* site, no metal in *i* + 1 site, gain 2000. (C) Mn(II) in both *i* and *i* + 1 sites, gain 2000. The ratios (*i* + 1) Mn(II) to enzyme for spectra C1 and C2 are 0.58 and 0.87, respectively.

Metal Detection. The concentration of Mn(II) and Zn(II) in enzyme-containing solutions and in control buffers was determined by using a Perkin-Elmer 5000 atomic absorption spectrophotometer with automatic burner control. The wavelengths used for determination of Mn and Zn were 279.5 and 213.9 nm with slits of 0.2 and 0.7 nm, respectively. The enzyme solutions were diluted to a concentration of 2–6 μM prior to use.

Results and Discussion

Low-Temperature EPR Spectra of Mn(II) in the i and i + 1 Sites. The distance between the two metals in *i* and *i* + 1 was determined by EPR techniques, in which Mn(II) was substituted for Zn(II) in *i* as well as for Mg(II) in *i* + 1. Before going to the distance measurements themselves, we consider the EPR spectra of Mn in the separate sites under low-temperature (5.5 K) conditions that maximize the EPR intensity. Fortunately, it is possible to replace the two metals separately. To place Mn(II) into the *i* + 1 site it is merely necessary to add Mn(II) to the intact enzyme; because Mn(II) binds much more strongly to that site than anywhere else on the intact enzyme (except to the *i* site, but that site is inaccessible in the intact enzyme), virtually all the Mn added up to 1 mol per enzyme binds to the *i* + 1 site (Koren & Mildvan, 1977). Mn(II) could be placed into the *i* site by a modification (described above) of the procedure devised by Chatterjee and Wu (1982a).

The EPR spectra of Mn(II) in *i* + 1 and *i* at 5.5 K are shown in Figure 1, spectra A and B, respectively. The EPR spectrum of the *i* + 1 site is very similar to that of $Mn(H_2O)_6^{2+}$ frozen in the presence of Sephadex to prevent aggregation (Reed & Cohn, 1972). The fine structure in this powder spectrum is attributed to the small zero-field splitting, which makes it possible to detect the formally forbidden transitions

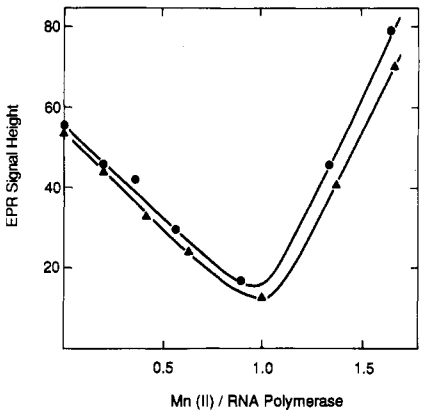

FIGURE 2: Titration of 80 μM RNA polymerase containing Mn(II) in *i* site with Mn(II) at low temperature (5.5 K), (●) without template and (▲) with template. The enzyme concentration is 80 μM. The ratio of poly(dAdT)·poly(dAdT) template to enzyme is 50–100 base pairs per enzyme molecule.

in which the nuclear spin quantum numbers change ($\Delta M_I = \pm 1$) (usual selection rules dictate $\Delta M_I = 0$ for allowed transitions).

The EPS spectrum of Mn in the *i* site at 5.5 K appears to be more isotropic than that for Mn in the *i* + 1 site. This difference can perhaps be attributed to the greater line width of Mn in *i* than in *i* + 1. Thus, the peak to trough difference for the lowest field transition is 29 G for *i* and 16 G for *i* + 1. With such a line width it is impossible to detect any fine structure. This broad line width at such low temperature is presumably due to the short electronic relaxation time (T_{1e}) for this complex and can be attributed to the strong coordination of Mn to this intrinsic metal binding site. In most other Mn–protein complexes previously studied by EPR the Mn can be readily removed by metal-complexing agents. The short relaxation time and broader signals also explain the order of magnitude lower signal intensity for Mn in the *i* site than for Mn in the *i* + 1 site (spectra A and B in Figure 1 were obtained at a gain of 200 and 2000, respectively). Neither signal is as intense as that of free hydrated Mn(II), which is 4 times as strong as that of Mn(II) in *i* + 1.

When Mn(II) was added to the *i* + 1 site of the enzyme that already contained Mn(II) in *i* (in a ratio of 0.58 *i* + 1 Mn to 1.0 *i* Mn and 0.87 *i* + 1 Mn to 1.0 *i* Mn), the intensity of the EPR spectra was greatly diminished, as shown in Figure 1, spectra C1 and C2, respectively. The decrease in intensity of the EPR spectra by the addition of Mn(II) to *i* + 1, where the EPR spectrum should be more intense than for Mn(II) in *i*, demonstrates the interaction of the two Mn(II) atoms and, therefore, their proximity.

This interaction is shown more quantitatively by the titration of the enzyme containing Mn(II) in *i* with Mn(II) in Figure 2. The initial EPR signal of *i* Mn is diminished until an equimolar quantity of Mn has been added to *i* + 1; the addition of excess Mn leads to enhancement of the EPR signal, because the excess goes to points on or off the enzyme that are not close to the Mn in *i*. The line shapes of the spectra containing excess Mn are similar to those of free Mn(II) in frozen solution. This titration demonstrates very clearly that there is one strong binding site of Mn(II) on the enzyme, as previously shown by Bean et al., (1977) and Slepneva and Weiner (1981), and also confirms that virtually all the Mn(II) added to intact enzyme goes into the *i* + 1 site. The presence of poly(dAdT)·poly(dAdT) template produces a slight decrease in the intensity at all points in the titration curve.

These studies at 5.5 K are useful in establishing some differences in the EPR behavior of Mn in the two sites and in determining the proximity of these sites. They are, however, not useful for distance calculation for several reasons; particularly the correlation time of *i* Mn is comparatively long to meet the condition of the Leigh theory that τ_c, or T_{1e}, of the perturbing spin must be less than T_{2e} of the perturbed spin. Conditions at room temperature were more favorable for the measurement of this distance.

Table I: Spin–Lattice Relaxation Time for Mn(II) in *i* and *i* + 1

frequency (MHz)	T_{1e} (s) Mn(II) in *i* ($\times 10^{10}$)[a]	T_{1e} (s) Mn(II) in *i* + 1 ($\times 10^{9}$)[b]
8		1.6
15	2.67	1.7
20	2.68	
24.3		1.9
40		2.5
80	2.73	
100		6.4
200	3.01	

[a] No metal ion in *i* + 1. [b] From Bean et al. (1977), with Zn in *i*.

The Observed Metal Site and the Perturbing Metal Site. Before any measurements of distance between the metals could be carried out, it was first necessary to determine whether the conditions existed for the use of the Leigh method. Application of this method requires, first of all, the assumption that dipolar relaxation effects represent the dominant interaction, and that exchange does not constitute a significant part of the interaction. It is characteristic of the dipolar relaxation mechanism that the signal intensity of the EPR spectrum is decreased without any change in line shape or splitting of peaks, whereas exchange phenomena are accompanied by such changes in the spectrum. The spectra at 5.5 K (Figure 1) are in line with a dominant dipolar relaxation interaction. Spectra C1 and C2 are diminished versions of the unperturbed Mn spectra that do not reveal any indication of splitting. It is of course possible that splitting could occur with a magnitude that cannot be observed in Figure 1, and we cannot therefore rule out exchange as a component of the interaction. But if exchange occurs, it must be a minor component, and we can assume that dipolar relaxation interaction constitutes the major effect of Mn atoms upon each other.

The spectra obtained at 5.5 K provided the first hint that one site did in fact perturb the other. The Mn signal at the *i* site was only $^1/_{10}$ as strong as that at the *i* + 1 site. When the enzyme was heated to liquid nitrogen temperature (77 K) and room temperature, 23 °C (296 K), no signal could be detected from *i* Mn at all, while the signal from *i* + 1 Mn was readily observed. These phenomena indicate that the correlation time, τ_c, of *i* Mn is shorter than τ_c for *i* + 1 Mn. The spin–lattice relaxation time, T_{1e}, which is taken to be equivalent to τ_c (see above), was determined for Mn on both sites at 23 °C (296 K) by NMR measurements of the frequency dependence of the paramagnetic effect of the Mn on the water proton relaxation rates (Table I). The table shows that T_{1e} for *i* Mn is $^1/_{10}$ that of T_{1e} for *i* + 1 Mn.

Measurement of T_{1e} for *i* Mn and T_{2e} for *i* + 1 Mn demonstrates that *i* Mn meets the conditions of perturbing metal and *i* + 1 Mn meets those for the observed (to be perturbed) paramagnet, according to eqs 3 and 4. Table II shows that T_{1e} of *i* Mn is always considerably lower than T_{2e} of *i* + 1 Mn, and that $T_{1e}\omega_s$ is always >1. Under the conditions the observed paramagnet is the *i* + 1 Mn.

Room-Temperature EPR and Measurement of the Distance between Mn in i and Mn in i + 1. At room temperature Mn in the *i* site becomes EPR-invisible, despite the fact that the

Table II: Leigh Method Relationships

	T_{1e} (i Mn) × 10^{10} (s)	T_{2e} (i + 1 Mn) × 10^{10} (s)	$T_{1e}\omega_s$
enzyme	2.7 ± 0.5	18.0 ± 0.6	17 ± 3
enzyme + substrate[a]	9.8 ± 1.9	15.0 ± 0.6	59 ± 12
enzyme + substrate[a] + template[b]	8.5 ± 1.7	20.9 ± 0.9	51 ± 10

[a] ATP in i and i + 1. [b] Poly(dAdT)·poly(dAdT).

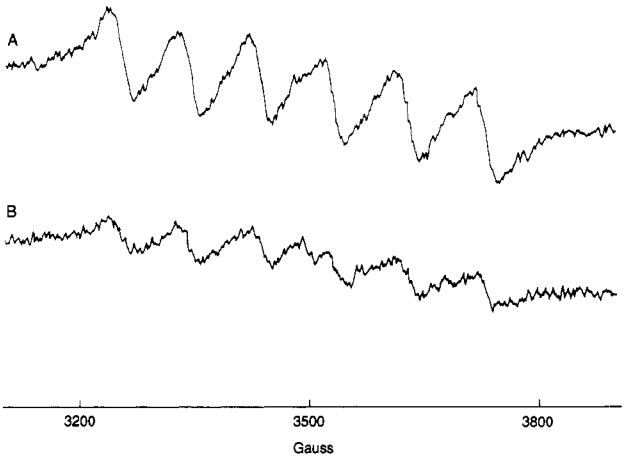

FIGURE 3: Effect of Mn(II) in i site of RNA polymerase on EPR spectrum of Mn(II) in i + 1 site, at room temperature, 23 °C (296 K). Concentration of the enzyme is 175 μM. (A) Mn(II) in i + 1 site alone, with a ratio of Mn(II) to enzyme of 1.15; (B) Mn(II) in both i and i + 1 sites, with a ratio of (i + 1)Mn(II) to enzyme of 1.25. Mn(II) in i site is EPR-silent at room temperature.

concentration of enzyme and Mn was 175 mM. The room temperature, 23 °C (296 K) EPR spectrum of Mn in i + 1 is shown in Figure 3A; 1.15 mol of Mn had been added per mole of enzyme. At this temperature the spectrum is quite isotropic compared to the spectrum at 5.5 K. The spectrum of enzyme containing Mn in both i and i + 1 sites is shown in Figure 3B; in this case, 1.25 mol of Mn has been added per mole of enzyme (and, therefore, per mole of Mn in the i site). In spite of the fact that more Mn has been added to the enzyme with Mn in the i site (Figure 3B) than to the enzyme with Zn in the i site (Figure 3A), the intensity of the signal is much lower with Mn in the i site, obviously because of the effect of the i Mn on the EPR spectrum of the i + 1 Mn.

This effect of i Mn on the EPR of i + 1 Mn was then used to measure the distance between the two Mn atoms. The details of the procedure have been described above, but the critical result required for the calculation (in addition to T_{1e}, vide supra) is the magnitude of the perturbation of the EPR intensity of i + 1 Mn by i Mn. The change of EPR signal height is best determined by titration, which would have been easily carried out if the i Mn were perturbed by the i + 1 Mn, since the amount of Mn at the i + 1 site can be readily changed by incremental addition of Mn. The Mn concentration at the i site remains constant throughout an experiment, once the zinc has been replaced by Mn.

Since, however, the perturbed Mn is in i + 1 and the perturbing Mn in i, we carried out two sets of titrations of the enzyme with Mn going into i + 1, as shown in Figure 4. One titration was of the enzyme with Zn in i and the other was of the enzyme with Mn in i. In both sets of titrations an increase of the slope occurs after 1 mol of Mn has been added to the enzyme. In the curves with i Mn, the addition of a given increment of Mn produces a considerably lower EPR intensity than in the curves with i Zn; the difference between the i Mn and i Zn curves is due to the perturbing action of i Mn in the

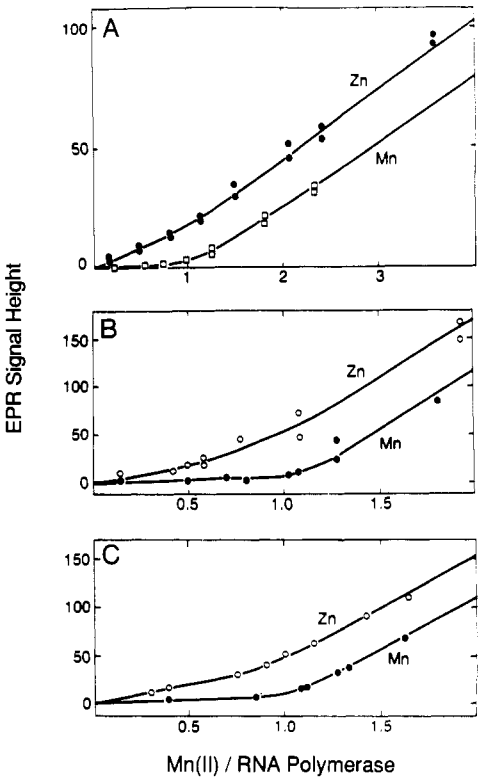

FIGURE 4: Titration of RNA polymerase containing Zn or Mn in i site with Mn going into i + 1 site at 23 °C (296 K). (A) 175 μM enzyme, no added substrate or template; (B) 80 μM enzyme, 320 μM ATP; (C) 80 μM enzyme, 320 μM ATP, and 50–100 base pairs of poly(dAdT)·poly(dAdT) per enzyme molecule.

Table III: Analysis of EPR Data

	relative amplitude	$C' \times 10^{-2}$	r (Å)
enzyme alone	0.15 ± 0.02	6.6 ± 3.0	5.2 ± 0.6
enzyme + ATP substrates	0.17 ± 0.02	11.2 ± 5.2	6.0 ± 0.7
enzyme + ATP substrates + template	0.18 ± 0.03	5.0 ± 3.0	6.7 ± 0.7

former but not in the latter. Hence this difference provides the data required for the determination of the distances.

The distances were measured under three sets of conditions: in the presence of enzyme alone, in the presence of enzyme and ATP substrate, and in the presence of enzyme, substrate, and poly(dAdT)·poly(dAdT) template. The distances were 5.2, 6.0 and 6.7 Å, respectively (Table III).

Since the error of these EPR-determined distances is of the order of 10–11%, we do not know how significant the differences measured under the three conditions are; we believe that the difference between the value for enzyme alone and for enzyme + substrates + template is significant. If the differerences in the distances are meaningful as alterations of the active-site conformation, these changes might be expected to be accompanied by changes in the EPR spectrum, which is sensitive to conformational alterations that would change the environment of the Mn. The EPR of the Mn in i + 1 does in fact change as substrate and then template are added (Figure 5).

The addition of ATP to Mn in the i + 1 site (Figure 5A) produces a spectrum with a prominent first peak of the sextet, typical of the EPR spectrum of the Mn–ATP complex in solution, indicating that i + 1 Mn is bound to ATP. Of course, the Mn is bound to the enzyme as well, as indicated, for example, by the interaction with Mn in the i site (Figure 3). Thus, the change in spectrum can be attributed to the dis-

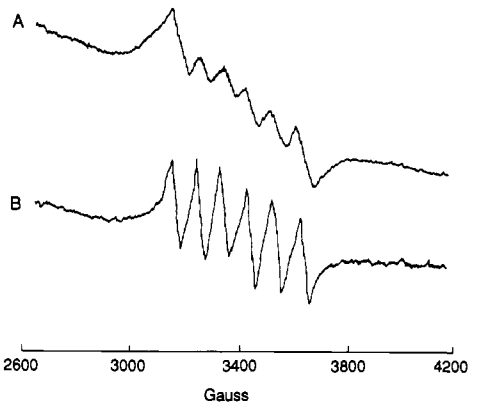

FIGURE 5: EPS spectra of Mn(II) in $i + 1$ site of RNA polymerase at room temperature, 23 °C (296 K). The ratio of Mn(II) to enzyme is 0.7 (A) 80 μM enzyme plus 320 μM ATP. (B) 80 μM enzyme plus 320 μM ATP and 50–100 base pairs of poly(dAdT)·poly(dAdT) per enzyme molecule.

placement of H_2O ligands by the ATP. An analogous similarity between the spectrum of Mn–ATP and the ternary enzyme complex has been reported in other cases where the substrate binds together with the divalent metal ion (Buttlaire & Cohn, 1974; Buttlaire et al., 1975). The addition of template to the enzyme–ATP substrate complex brings about a reversion to the type of spectrum obtained in the absence of ATP (Figure 5B), possibly indicating a loosening of the binding between Mn and ATP. At any rate, it is clear that the template affects the EPR spectrum. Taken together with the change in the metal–metal distance, we can conclude that a structural change occurs in the enzyme when template is added.

Comments and Conclusions

The presence of metal ions in a biological system, particularly in an enzyme, can frequently be used as a probe of structure. The presence of zinc and magnesium in close proximity to each other in *E. coli* RNA polymerase makes these metals extremely useful for determining the relationships between the i and $i + 1$ subsites of the enzyme active site. Since both of these metals are associated with the enzyme active site, where the internucleotide bond is formed, it is no surprise that they are in close proximity to each other; in fact, a different result would not have been easy to explain.

It is a time-honored practice in enzyme mechanism studies to substitute a spectroscopically active metal ion for an inactive one in a metalloenzyme, and to assume that the newly incorporated metal functions in the same way as the metal that has been displaced. Yet the assumption is always open to question. In this particular instance, manganese replaces zinc and magnesium, although it is obviously quite different in its electronic characteristics from either of these displaced metals. We know in fact that manganese in the $i + 1$ site can foster synthetic processes that do not occur with magnesium in the site. Nevertheless, these metals occupy the positions of the metals they replaced and produce an enyzme that is as active in support of template-directed internucleotide bond formation as the enzyme containing the displaced metals. It seems therefore most reasonable to expect that the incorporated metals participate in enzyme function in the same manner as the displaced metals, and that the mechanism is not substantially changed. But we should not forget the fact that the enzyme has indeed been altered.

The accuracy of the distance measurements is not high, in line with other such measurements made in solution, yet such measurements are important to determine structure in solution. Although the differences in the distances in enzyme with and without substrate and template are not great, they are in line with changes in the EPR spectrum at the $i + 1$ site.

The EPR titration curves in Figures 2 and 4 probably represent the most accurate evaluation to date of the 1:1 stoichiometry between the metal bound to the $i + 1$ site and enzyme (and metal in the i site). This stoichiometry is of critical importance in this and many other studies. One might ask in this connection whether this stoichiometry could be an artifact in view of the apparent presence of "inactive" molecules in RNA polymerase, as discussed above. One type of molecule could bind two Mn by displacing not only the i Zn, but also both intrinsic Zn atoms, and the other type might bind none, thus leading to an average of one. Such a scheme is readily refuted by the fact that it would require variability in the amount of Zn displaced by Mn as a function of enzyme activity. Actually the Zn to Mn stoichiometry is always 1:1, regardless of the activity. We believe that this finding settles the matter. The clearcut 1:1 stoichiometry shown by analysis as well as in the EPR titration data is clearly in accord with Mn replacing one Zn, and not the other, in all of the enzyme molecules.

The EPR spectra themselves contain some information about the nature of the Mn(II) complexes. The much lower intensity of the spectrum of i Mn, reflected in the lower T_{1e} value, is in line with the much stronger binding of the intrinsic metal in the i site, as compared to the metal in the $i + 1$ site. The latter site loses its metal during preparation procedures; routinely, metal must be added to restore activity.

The EPR characteristics of the $i + 1$ Mn support a structure of the Mn enzyme complex, in which the Mn is bound to enzyme and water molecules, and in which the water molecules are displaced at least in part by ATP. The addition of template appears to loosen the ATP attachment to the Mn, perhaps a consequence of interaction with the template (although this particular template has not been designed for hydrogen bonding to ATP).

In the following paper the results presented here will be combined with studies on metal–substrate distances to provide a basis for the structure proposed for the interaction of the substrates at the active site.

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Structural Studies on the Active Site of *Escherichia coli* RNA Polymerase. 2. Geometrical Relationship of the Interacting Substrates

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ABSTRACT: Since a major function of RNA polymerase must be to bring together substrates in the optimal configuration for internucleotide bond formation, studies have been undertaken to understand the geometrical relationship of the two substrates. A model has been constructed for the geometry of interaction of two ATP molecules poised on the active site of the *Escherichia coli* enzyme for the formation of the first bond in RNA synthesis. The model is based primarily on the distance, measured by EPR, between the two metals in the *i* and *i* + 1 subsites, as well as distances, measured by NMR, from each metal to points on the substrate in the same subsite, in the presence of a poly(dAdT)·poly(dAdT) template. Both the Zn(II) in the *i* site and the Mg(II) in *i* + 1 are displaced by Mn(II). The nucleotide bases are not parallel to each other, in line with the reaction of the ATP molecules with DNA within the transcription bubble. The metal in the *i* site appears too far removed from substrate to participate in catalysis, but the metal in *i* + 1 is in position to bind to the β- and γ-phosphate groups and probably is involved in cleavage of the triphosphate, as has been previously suggested.

RNA polymerase brings together two substrates for internucleotide bond formation at the active site of the enzyme. The two substrates are located on two subsites called *i* and *i* + 1 (Yager & von Hippel, 1988), and each of these subsites in turn is bound to a metal ion, Zn(II) in *i* and Mg(II) in *i* + 1. In the preceding paper we measured the distance between these metals, when each was substituted by Mn(II), in the presence and absence of ATP substrates and a poly(dAdT)·poly(dAdT) template. We found the metal–metal distance to vary from 5.2 to 6.7 Å.

These measurements represent the beginning of an effort to understand the geometrical relationship of the two substrates between which bond formation occurs, since we believe that this relationship contains many clues to the mechanism of RNA synthesis. The interaction geometry can be deduced from the metal–metal distance in conjunction with distances from these metals to the substrates.

Some of the metal–substrate distances have been previously determined. In the *i* site, Chatterji and Wu (1984) measured the distances to ATP in the presence and absence of poly(dAdT)·poly(dAdT) template. In the *i* + 1 site, Bean et al. (1977) determined distances to protons in ATP, with ApU in *i*, and with no template; and Slepneva and Weiner (1981) found the distances to ATP phosphate with poly(dA) and