Burgess, R. R., & Jendrisak, J. J. (1975) *Biochemistry 14*, 4634.
Buttlaire, D., & Cohn, M. (1974) *J. Biol. Chem. 249*, 5741.
Buttlaire, D., Reed, M., & Himes, R. (1975) *J. Biol. Chem. 250*, 261.
Carr, H. Y., & Purcell, E. M. (1954) *Phys. Rev. 94*, 630.
Chamberlin, M., Kingston, R., Gilman, M., Wiggs, J., & de Vera, A. (1983) *Methods Enzymol. 101*, 540.
Chatterji, D., & Wu, F. Y.-H. (1982a) *Biochemistry 21*, 4651.
Chatterji, D., & Wu, F. Y.-H. (1982b) *Biochemistry 21*, 4657.
Chuknyisky, P. P. (1989) *J. Magn. Reson. 84*, 153.
Chuknyisky, P. P., Rifkind, J. M., & Eichhorn, G. L. (1986) *Biophys. J. 49*, 530a.
Chuknyisky, P. P., Rifkind, J. M., Tarien, E., & Eichhorn, G. L. (1987) *Biophys. J. 51*, 152a.
Cohn, M., Diefenbach, H., & Taylor, J. S. (1971) *J. Biol. Chem. 246*, 6037.
Eichhorn, G. L., Chuknyisky, P. P., Rifkind, J. M., & Tarien, E. (1988) *FASEB J. 2*, 4253.
Heumann, H., Stöckel, P., & May, R. (1982) *FEBS Lett. 148*, 91.
Knight, W. B., Dunaway-Mariano, D., Ransom, S. C., & Villafranca, J. J. (1984) *J. Biol. Chem. 259*, 2886.
Koren, R., & Mildvan, A. S. (1977) *Biochemistry 16*, 241.
Leigh, J. S., Jr. (1970) *J. Chem. Phys. 52*, 2608.
Levine, B. J., Orphanos, P. D., Fischmann, B. S., & Beychok, S. (1980) *Biochemistry 19*, 4808.
Lowe, P. A., Hager, D. A., & Burgess, R. R. (1979) *Biochemistry 18*, 1344.
Maggio, E. T., Kenyon, G. L., Mildvan, A. S., & Hegeman, G. D. (1975) *Biochemistry 14*, 1131.
Peacocke, A. R., Richards, R. E., & Sheard, B. (1969) *Mol. Phys. 16*, 177.
Redfield, A. G. (1957) *IBM J. Res. Dev. 1*, 19.
Reed, G., & Cohn, M. (1972) *J. Biol. Chem. 247*, 3073.
Shaner, S. L., Piatt, D. M., Wensley, C. G., Yu, H., Burgess, R. R., & Record, M. T., Jr. (1982) *Biochemistry 21*, 5539.
Slepneva, I. A., & Weiner, L. M. (1981) *FEBS Lett. 130*, 283.
Studier, W. F. (1969) *Virology 39*, 562.
Taylor, J. S., Leigh, J. S., & Cohn, M. (1969) *Proc. Natl. Acad. Sci. U.S.A. 64*, 219.
Villafranca, J. J., & Raushel, F. M. (1982) in *Advances in Inorganic Biochemistry* (Eichhorn, G. L., & Marzilli, L. G., Eds.) Vol. 4, p 289, Elsevier Biomedical, New York.
Villafranca, J. J., Balakrishnan, M. S., & Wedler, F. C. (1977) *Biochem. Biophys. Res. Commun. 75*, 464.
Yager, T. D., & von Hippel, P. H. (1988) in *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology* (Neidhardt, F. C., Ed.) p 1241, American Society of Microbiology, Washington, DC.

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

poly(dT) templates, and with either ATP or pUpU in *i*.

In order to understand the interaction geometry of the substrates it is obviously necessary to look at a variety of substrates and templates, since the geometry may be affected by this variation. We picked ATP substrates and poly(dAdT)·poly(dAdT) template as the first combination because with it we could utilize the greatest amount of previously accumulated data. Nevertheless we had to determine distances in the $i + 1$ site in the presence of poly(dAdT)·poly(dAdT) and to reevaluate some distances from previous data, taking into consideration the newly understood proximity of ATP in the *i* and $i + 1$ sites. The previously determined distances as well as those reported here were calculated from the paramagnetic effect of Mn(II) on the measured nuclear relaxation rates of hydrogen and phosphorus atoms in the ATP substrates. Fortunately, substitution can be accomplished separately in each subsite as a consequence of the different stabilities of the Mn complex in *i*, where it replaces Zn(II), and in $i + 1$, where it replaces Mg(II).

The ATP–poly(dAdT)·poly(dAdT) substrate–template combination does not lead to RNA synthesis, since complementary base interaction cannot occur except for the initial base. The system was selected partly for this reason, since active RNA synthesis will lead to a variety of substrates in the *i* site, and thus make it impossible to make NMR measurements on any one substrate. With this combination, substrate is placed into both the *i* and $i + 1$ sites (see below) but no bond formation occurs between the two substrates.

In this paper we shall present first the new results determined here. We shall then review the previously determined results and the implications our new data have upon them. Finally we shall consolidate all of these results with the metal–metal distances from the previous paper to construct a model for the interaction of the substrates on the enzyme.

Theoretical Basis for Distance Calculations: Metal–Substrate Distances. The metal–substrate distance calculations in this study are based upon the Solomon–Bloembergen description of nuclear relaxation in the presence of a paramagnetic species (Solomon, 1955; Solomon & Bloembergen, 1956; Bloembergen, 1957; Bernheim et al., 1959; Bloembergen & Morgan, 1961). The application of this approach to biological systems has been extensively reviewed (Mildvan, 1977; Mildvan & Gupta, 1978; Mildvan et al., 1980; Jardetzky & Roberts, 1981).

The effect of a paramagnetic enzyme-bound metal upon the longitudinal relaxation rate ($1/T_{1p}$) of a magnetic substrate nucleus exchanging into the enzyme complex from solution is a function of several parameters: the lifetime of the enzyme–substrate complex (τ_m), the relative stoichiometry of the magnetic nucleus and paramagnet in the complex (q)—in this instance [ATP]/[Mn] = 1, the correlation time, which modulates relaxation (τ_c), the distance from the unpaired electron to the magnetic nucleus in the complex (r), and the usually small outer-sphere contribution to the relaxation rate ($1/T_{os}$). These five parameters are related by eq 1, where f

$$1/(fT_{1p}) = q/(T_{1M} + \tau_m) + 1/(fT_{os}) \quad (1)$$

is the ratio of the concentration of the enzyme-bound paramagnetic metal (Mn) to the total substrate in solution and $1/T_{1M}$, the relaxation rate of the nucleus in the complex, is given by

$$1/T_{1M} = (C/r)^6 f(\tau_c) \quad (2)$$

In eq 2, C is a constant that depends upon the metal–nucleus interaction under investigation (Mildvan et al., 1980). For Mn(II) interacting with ^1H and ^{31}P the values are 812 and 601, respectively. The correlation function $f(\tau_c)$ depends upon the Larmor precessional frequencies of both the electron and the magnetic nucleus at a given magnetic field strength. Assuming that the outer-sphere contribution to relaxation is small and that the normalized relaxation time (fT_{1p}) is not exchange limited, then $fT_{os} \gg fT_{1p} \gg \tau_m$ and the distance r may be evaluated from eq 3.

$$r^6 = C^6 q f T_{1p} f(\tau_c) \quad (3)$$

The value of $1/T_{1p}$ may be obtained by subtracting the relaxation rate for a diamagnetic metalloenzyme from that found when a paramagnetic metal has displaced the diamagnetic one in the metalloenzyme; in our experiments Mn has displaced Mg in the $i + 1$ site. The correlation time τ_c, needed to determine the value of the correlation function $f(\tau_c)$, is most easily evaluated by examining the frequency dependence of $1/T_{1p}$. The correlation function, $f(\tau_c)$ is given by eq 4, where ω_I and ω_S are the nuclear and electron Larmor frequencies. Lastly, the determination of q by titration of the paramagnetic metalloenzyme with substrate while measuring any of a number of parameters, such as the relaxation rate of solvent water protons (Mildvan & Engle, 1972) or EPR intensity (Bean et al., 1977), or by equilibrium dialysis, will allow calculation of r. The evaluation of q for the present system was carried out by Bean et al. (1977), and confirmed by us (preceding paper), using EPR titration of the diamagnetic metalloenzyme with Mn(II).

$$f(\tau_c) = 3\tau_c/(1 + \omega_I^2\tau_c^2) + 7\tau_c/(1 + \omega_S^2\tau_c^2) \quad (4)$$

Experimental Procedures

The preceding paper should be consulted for discussion of materials, enzyme preparation, and substitution of Mn(II) for Zn(II).

Preparation of Enzyme Solutions for NMR Studies. NMR samples were prepared in a 50 mM Tris/200 mM KCl buffer, with D_2O substituted for the water solvent. The enzyme and poly(dAdT)·poly(dAdT) solutions were deuterated by repeated dilution with the buffer in D_2O and concentration by vacuum dialysis. Trace-metal impurities were removed from all solutions by treatment with Chelex 100. The concentrations were ATP, 10 mM; RNA polymerase, 50–100 μM; and Mn, 0–25 μM. In the solutions with template the concentration of admixed poly(dAdT)·poly(dAdT) was 50 base pairs per enzyme molecule.

NMR Measurements. Longitudinal relaxation times (T_1) of the proton nuclei of ATP were measured as a function of Mn concentration at 200 MHz on a Varian XL-200 by using a 180°–τ–90° inversion–recovery pulse sequence (Carr & Purcell, 1954). Phosphorus longitudinal relaxation times of ATP were obtained with the same pulse sequence on a Varian XL-200 spectrometer operating at 81 MHz.

Solvent proton relaxation rate experiments were conducted to ascertain if any significant amount of Mn(II) binds to the poly(dAdT)·poly(dAdT) template when the template is bound to the enzyme. The E-titration method (Dwek, 1975) was used to determine the binding constant of Mn to the template, the enzyme, and the enzyme–template complex. The apparent enhancement of the solvent water protons relaxation rates (ϵ^*)[1] was determined at constant Mn(II) concentration (20 μM) and variable concentrations of the enzyme (20–100 μM) or

[1] ϵ^* is defined as $(1/T_{1p}^*)/(1/T_{1p})$; the asterisk indicates the presence of the macromolecule.

Table I: Longitudinal Relaxation Rate of ATP with Mn(II) in $i + 1$ and No Template Present[a]

	$1/T_1$ (s^{-1})					
Mn_t (μM)	H_1	H_8	$H_{1'}$	P_α	P_β	P_γ
0	1.01	0.50	0.79	0.46	0.39	0.28
1				2.10	2.49	2.44
2				3.46	4.50	4.18
3				4.93	7.58	7.46
4				7.75	9.26	10.35
5	1.16	13.25	1.98			
10	2.06	31.35	3.17			
15	3.12	42.55	4.50			
20	4.08	59.88	5.62			
25	4.76	87.72	7.87			

[a] Results of a typical titration. For proton T_1 experiments, [RNA polymerase] = 50 μM; for ^{31}P T_1 experiments [RNA polymerase] = 100 μM.

enzyme–template complex (20–50 μM), with the template to enzyme ratio held constant at 50 base pairs per enzyme molecule. The data were analyzed by using eq 5, where $[E]_t$

$$1/\epsilon^* = 1/(n[E]_t)K_D/\epsilon_b + 1/\epsilon_b \quad (5)$$

is the total enzyme concentration, n the number of binding sites, K_D the dissociation constant, and ϵ_b the characteristic binary enhancement of the metal ion–enzyme complex.

Construction of Active-Site Model. The final three-dimensional model of two ATP substrate molecules bound at the active site of *Escherichia coli* RNA polymerase was generated by using the nonlinear least-squares algorithm IMSL ZXSSQ as implemented in Dock, a software program for the interactive display and manipulation of molecules (Stodola, 1988). Dock was developed under the direction of Helen Berman at the Fox Chase Cancer Research Center by Robert Stodola and Frank Manion of the Fox Chase Center and by William P. Wood, Jr., of Smith-Kline Beckman. A Digital Equipment Corp. Vax 11/780 was used as the host computer with an Evans and Sutherland PS-390 display subsystem.

Adjustable parameters in our model include the x, y, and z coordinates of the two ATP substrates and one metal ion (the other ion being fixed), rotation around the x, y, and z axes by each substrate, and rotation around the eight bonds in each substrate that can alter the substrate conformation.

Constraints in the model include the experimentally measured distance between the two metal ions and the metal–substrate distances in the i and $i + 1$ sites. With only these constraints it is not possible to arrive at a unique structure. However, including the geometric constraints required for an S_N2 mechanism, assuming that the bases must be able to hydrogen bond to contiguous bases on the template, and eliminating van der Waals overlap between nonbonded atoms allows us to generate what we believe is a unique structure. This was demonstrated by allowing the least-squares procedure to start from a variety of initial conformations, leading either to a structure consistent with the one presented here or to a structure that violates at least one of the distance constraints or one of the above assumptions.

Results

Metal–Substrate Distances in the Absence of Template. The effect of Mn(II) bound at the $i + 1$ site on the longitudinal relaxation rate of the protons and phosphorus atoms of ATP bound to *E. coli* RNA polymerase is shown in Table I. At the saturating concentrations used for these experiments the bulk of the ATP exists as the unbound species. Because there is fast exchange between free and enzyme-bound (at both the i and $i + 1$ subsites) substrate, the observed relaxation rates are the weighted averages of the relaxation rates in the different species present. The actual relaxation rate of enzyme-bound ATP with Mn(II) at the $i + 1$ site and no template was obtained from the observed relaxation rate by using eq 6 and known equilibrium constants (Koren &

$$1/(fT_{1p})_{ob} = 1/(fT_{1p})_{MnATP}[MnATP]/[Mn]_t + 1/(fT_{1p})_{i+1\ MnATP}[i + 1\ MnATP]/[Mn]_t \quad (6)$$

Mildvan, 1977), where $1/T_{1p}$ is the paramagnetic contribution to the relaxation rate of the species (MnATP is the binary complex, $i + 1$ MnATP is the complex containing Mn in the $i + 1$ site of the enzyme and ATP bound to that site). The value of $1/(fT_{1p})_{ob}$ was obtained from the slope of a plot of $1/T_1$ vs the total Mn concentration, $[Mn]_t$. The relaxation rate in the binary Mn–ATP complex, $1/(fT_{1p})_{MnATP}$, was obtained from a control experiment in which ATP was titrated with known amounts of Mn(II) in the absence of enzyme.

To calculate internuclear distances from the data of Table I the correlation time, τ_c, which modulates nuclear relaxation, must be known. We used the relationship $1/\tau_c = 1/\tau_R + 1/\tau_s$, where τ_R is the rotational correlation time of the substrate nuclei and τ_s is the electron relaxation time. The value of τ_s employed was 2.48×10^{-9} s at 200 MHz, calculated from the known frequency dependence of water relaxation rates in the complex containing i ApU and $i + 1$ ATP (Bean, et al., 1977). The value of τ_R used was 1.9×10^{-9} s found for the protons of ATP in the same complex and evaluated from the diamagnetic T_1/T_2 ratio (Bean et al., 1977). This leads to a τ_c of 1.07×10^{-9} s and values of $f(\tau_c)$ from eq 4 of 1.14×10^{-9} s at 200 MHz and 2.5×10^{-9} s at 81 MHz. We assume that our substitution of ATP in the i site for ApU does not cause a substantial change in the τ_c of ATP at the $i + 1$ site. Because of the sixth power dependence of eq 3 even a 50% error in the value of τ_c would result in only a small error in the final distance. The values of $1/(fT_{1p})$ and the internuclear distances calculated from eq 3 using a value of $q = 1$ are given in Table II.

Table II: Effect of Mn(II) on Paramagnetic Relaxation Rates of ATP Alone and in $i + 1$ with No Template Present[a]

	relaxation rate ($s^{-1} \times 10^{-3}$)			
	$1/(fT_{1p})_{ob}$	$1/(fT_{1p})_{MnATP}$	$1/(fT_{1p})_{i+1MnATP}$	r^{*b} (Å)
H_2	1.81	1.60	9.98	5.8
H_8	29.4	25.8	171.0	3.5
$H_{1'}$	2.44	2.30	7.95	5.9
P_α	17.5	15.5	39.7	3.9
P_β	22.8	20.3	57.9	3.5
P_γ	25.2	21.2	78.5	3.5

[a] Correlation time, $\tau_c = 1.07 \times 10^{-9}$ s; correlation function, $f(\tau_c) = 1.14 \times 10^{-9}$ at 200 MHz, 2.5×10^{-9} at 81 MHz. [b] * represents a weighted average of distances to both the i and the $i + 1$ sites. See text.

Metal-Substrate Distances in the Presence of Template. We have also investigated the effect of a DNA template on metal binding to the enzyme and the relaxation rates of enzyme-bound ATP in the presence of a template. Analysis of water proton relaxation rates at 200 MHz using eq 5 yielded a value of 6.7×10^{-4} M for the dissociation constant of Mn(II)–poly(dAdT)·poly(dAdT) complex in 50 mM Tris/200 mM KCl (pH 7.9) buffer (data not shown). The dissociation constant K_D for Mn(II)–RNA polymerase has been determined by EPR both in the absence ($K_D = 1.9 \times 10^{-6}$ M) (Koren & Mildvan, 1977) and in the presence (Slepneva & Weiner, 1981) of poly(dA) ($K_D = 3.5 \times 10^{-6}$ M) or poly(dT) ($K_D = 2.0 \times 10^{-6}$ M) templates. Thus, Mn(II) binding to the enzyme is nearly 2 orders of magnitude stronger than to the

Table III: Longitudinal Relaxation Rate of ATP with Mn(II) in $i + 1$, in the Presence of Poly(dAdT)·Poly(dAdT)[a]

	$1/T_1$ (s^{-1})					
$[Mn]_t$ (μM)	H_2	H_8	$H_{1'}$	P_α	P_β	P_γ
0	0.93	1.27	1.14	0.30	0.35	0.22
1				1.80	1.61	2.08
2				3.98	4.74	3.79
3				5.68	6.94	7.94
4				7.52	10.94	9.17
5	2.40	1.36	12.58			
10	4.05	2.42	27.55			
15	5.13	3.15	38.76			
20	6.21	4.72	52.08			
25	7.52	5.56	66.67			

[a] Results of a typical titration. For proton T_1 experiments, [RNA polymerase] = 50 μM; for ^{31}P T_1 experiments, [RNA polymerase] = 75 μM.

Table IV: Paramagnetic Relaxation Rates of ATP with Mn in $i + 1$ in the Presence of Poly(dAdT)·Poly(dAdT) Template[a]

	relaxation rate ($s^{-1} \times 10^{-3}$)			r^{*}[b]
	$1/(fT_{1p})_{ob}$	$1/(fT_{1p})_{MnATP}$	$1/(fT_{1p})_{i+1MnATP(T)}$	(Å)
H_2	2.14	1.60	23.0	4.6
H_8	26.1	25.8	40.6	4.2
$H_{1'}$	2.54	2.30	11.9	4.5
P_α	18.3	15.5	62.1	3.9
P_β	22.9	20.3	71.4	3.6
P_γ	23.8	21.2	69.5	3.5

[a] Correlation time, $\tau_c = 1.07 \times 10^{-9}$ s; correlation function, $f(\tau_c) = 1.14 \times 10^{-9}$ at 200 MHz, 2.5×10^{-9} at 81 MHz. [b] * represents a weighted average of distances to both the i and the $i + 1$ sites. See text.

template and the presence of the template does not significantly affect the affinity of the metal ion toward the enzyme.

The effect of a poly(dAdT)·poly(dAdT) template on the longitudinal relaxation rate of the protons and phosphorus nuclei of ATP bound to *E. coli* RNA polymerase in the presence of $i + 1$ Mn is shown in Table III. The binding constants of Koren and Mildvan (1977) were used to calculate $1/(fT_{1p})$ values for the nuclei of ATP under these conditions, using eq 6, but with $i + 1$ MnATP replaced by $i + 1$ MnATP(T); i.e., the complex of enzyme with Mn in the $i + 1$ site, ATP and template. The data in the presence of template were analyzed in a manner identical with that employed in the absence of template. Because the enhancement ϵ^* of the solvent water proton relaxation rate at 200 MHz (data not shown) is the same in both the presence and the absence of a DNA template, τ_s is the same in both complexes. We assume that τ_R is likewise not significantly affected by the presence of a template. Thus the same value of τ_c was employed in both sets of calculations. The values of $1/(fT_{1p})$ and the internuclear distances for the $i + 1$ MnATP(T) complex calculated by using eq 3 are presented in Table IV.

The effect of the ATP at the i site on these distances calculated in the $i + 1$ site will be considered below.

Discussion

Geometry in the i + 1 Site. A comparison of the distances determined in the absence and presence of poly(dAdT)·poly(dAdT) template in Tables II and IV, respectively, reveals no detectable change in the distances from $i + 1$ Mn to the phosphates in $i + 1$ ATP. Some small changes are observed in the distances to the nucleoside portion of the molecule, so that the template appears to affect the substrate selectively in a way that does not involve the phosphates.

Distances from Mn(II) to H_2, H_8, and $H_{1'}$ of ATP in the $i + 1$ site were previously determined by Bean et al. (1977) in the absence of template. In these experiments the i site was occupied by ApU so that the confusion with ATP in the i site was avoided. These results are compared with ours in the top three lines of Table V. Clearly, our results in the absence of template, and ATP in the i site, are virtually identical with their results in the absence of template, and ApU in the i site. Apparently the change from ATP to ApU in the i site does not affect the metal to ATP distances in the $i + 1$ site. These distances are also similar to those determined in the binary Mn(II)–ATP complex, with no enzyme present (Sloan & Mildvan, 1976). These data are included in Table V for comparison. In the bottom lines of Table V the distances that we have determined from $i + 1$ Mn(II) to the triphosphate region of $i + 1$ ATP are compared with some similar studies previously carried out by Slepneva and Weiner (1981). They had measured distances from $i + 1$ Mn(II) to the phosphates with no template present, as well as in the presence of either poly(dA) or poly(dT) template. In the comparison with our results in Table V we have listed their data for poly(dA) template, because we considered that template most similar to ours in that it was not complementary for the substrates.

Geometry in the i Site. Distances from the Mn in the i site to i ATP have been previously measured by Chatterji et al. (1984) in the presence and absence of $(dAdT)_{60}$ template. In fact, the conditions of the present experiments were selected partly to make it possible to utilize the data obtained in that study. Their distances are reproduced in Table VI, columns a and b. [Similar distances were obtained when Co(II), instead of Mn(II), substituted for Zn(II) in the i site, and we therefore include distances to $H_{1'}$, although these were measured only with Co(II).] It was concluded that the template made a difference in the i site distances. We believe that this conclusion is correct, although the differences between the data in columns a and b in Table VI should not be attributed entirely to the template affect, as will be discussed below.

Construction of a Model for Substrate Interaction at the Active Site. The purpose of this and the preceding paper has been to bring together a sufficient number of parameters to make possible the construction of a working model for the interaction of the two substrates at the active site of the enzyme. For the conformation at the i site we use the results of Chatterji and Wu (1984, Table 6b) for ATP in the presence of an alternating dA-dT template (they used the 60 mer). For

Table V: Distances (Å) from $i + 1$ Mn to $i + 1$ ATP Substrate

	without template[a]		with template[a]		
atom	previous study	present study	previous study	present study	comparison with Mn–ATP[e]
H_2	5.6 ± 0.9[b]	5.8 ± 0.6*		4.6 ± 0.6*	6.4 ± 0.5
H_8	4.0 ± 0.4[b]	3.5 ± 0.4*		4.2 ± 0.5*	4.5 ± 0.5
$H_{1'}$	5.7 ± 0.7[b]	5.9 ± 0.6*		4.5 ± 0.6*	6.2 ± 0.6
P_α	4.0 ± 0.1*[c]	3.9 ± 0.4*	4.0 ± 0.2*[c,d]	3.9 ± 0.7*	3.2 ± 0.3
P_β	3.7 ± 0.1*[c]	3.5 ± 0.4*	3.8 ± 0.2*[c,d]	3.6 ± 0.4*	3.1 ± 0.3
P_γ	3.8 ± 0.1*[c]	3.5 ± 0.4*	3.8 ± 0.1*[c,d]	3.5 ± 0.4*	3.1 ± 0.3

[a] * represents a weighted average of distances to both the i and the $i + 1$ sites. See text. [b] Distance determined with ApU in the i site (Bean et al., 1977). [c] From Slepneva and Weiner (1981). [d] Distance determined with poly(dA) template. [e] From Sloan and Mildvan (1976).

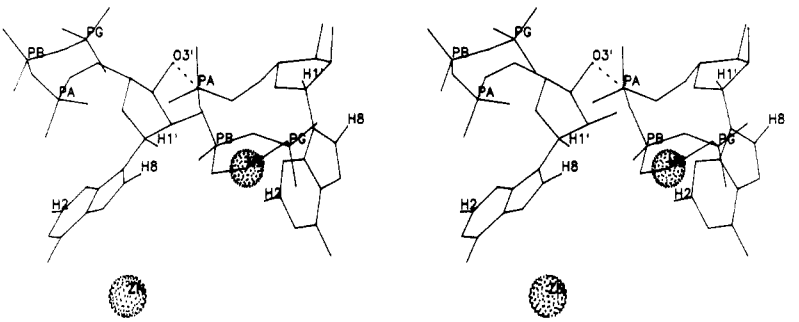

FIGURE 1: Stereo model of geometrical relationship between two ATP molecules on the *i* and *i* + 1 sites of *E. coli* RNA polymerase, based on NMR measurements presented in this paper, or performed by Bean et al. (1977), Slepneva and Weiner (1984), and Chatterjee et al. (1984), and EPR measurements in the preceding paper. Parameters used for and determined from the model as described in the Experimental Section are listed in Table VII. The positions of the metal ions are labeled according to the metals usually present, although both were substituted by Mn(II) to obtain the data. The labeled atoms are those from which distances to the metals had been determined; PA, PB, and PG represent P_α, P_β and P_γ, respectively. $O_{3'}$ has also been labeled because of its importance in bond formation. To avoid confusion, heteroatoms are not identified and hydrogen atoms to which distances were not calculated are not represented. The dotted line schematically represents the internucleotide bond to be formed. The *i* site is on the left, the *i* + 1 site on the right. We consider this model the best representation of the geometry obtained with two ATP substrates and alternating A-T template. See text for assumptions implicit in the structure.

Table VI: Distances (Å) from *i* Mn to *i* ATP[a]

	(a) without template[b]	(b) with $(dAdT)_{60}$ template[c,d]
H_2	4.8 ± 0.5	6.5 ± 0.5*
H_8	4.3 ± 0.4	4.4 ± 0.4*
$H_{1'}$	6.8 ± 0.8[e]	6.0 ± 1.2*[e]
P_α	11.2 ± 1.0	7.5 ± 0.8*
P_β	13.9 ± 1.2	9.4 ± 1.0*
P_γ	12.7 ± 1.5	9.8 ± 1.0*

[a] From Chatterjee et al., 1984. [b] Without Mg(II); ATP in *i* site only. [c] With Mg(II); ATP in both *i* and *i*+1. [d] * represents a weighted average of distances to both the *i* and *i* + 1 sites. See text. [e] Distance determined with Co(II) instead of Mn(II).

Table VII: Distances and Angles Used for or Determined from Figure 2 Structure

	distances (Å)					
	from metal in *i* site			from metal in *i* + 1 site		
	to *i* ATP		to *i* + 1 ATP	to *i* + 1 ATP		to *i* ATP
	input[a]	computed best fit	computed	input[b]	computed best fit	computed
H_2	6.5	6.3	7.3	4.6	4.6	9.6
H_8	4.4	4.4	10.6	4.2	4.2	4.2
$H_{1'}$	6.0	6.2	10.4	4.5	4.6	5.3
P_α	7.5	7.5	8.2	3.9	3.8	9.2
P_β	9.4	9.4	7.6	3.6	3.6	10.9
P_γ	9.8	9.8	9.1	3.5	3.4	10.5

	other distances (Å)	
	input	computed
i Mn – *i* + 1 Mn[c]	6.7	6.6
i $O_{3'}$ – *i* + 1 P_α	2.5	2.8
i + 1 Mn – *i* + 1 O_β		2.6
i + 1 Mn – *i* + 1 O_γ		2.3

	angles (deg)	
	input	computed
i O_3 – *i* + 1 P_α–*i* + 1 $O_{\alpha\beta}$	180	161
i $C_{3'}$ – *i* $O_{3'}$ – *i* + 1 P_α	120	106
i $C_{2'}$ – *i* $C_{3'}$ – *i* + 1 P_α	109	99
i + 1 O_β–Mg–O_α	90	83
i + 1 P_β–O_β–Mg	130	121
i + 1 P_γ–O_γ–Mg	130	126

[a] From Table VI(b). [b] From Table IV. [c] From preceding paper.

the conformation at the *i* + 1 site we use the data from Table IV. We therefore have conformations for ATP at both *i* and *i* + 1 sites. We shall consider below what effects the ATP on each site has on the distance determination at the other, but at this point we use the conformational studies for the construction of a tentative model.

Of great importance for the construction of the model are points of contact or distances between the two substrates. A contact is achieved through the approach of the 3′-hydroxyl group in the *i* site to the α-phosphate group in the *i* + 1 site. This approach is required for the formation of the internucleotide bond. We would expect this approach to occur on the side of the phosphate opposite to that at which the pyrophosphate cleavage would occur in the usual S_N2 mechanism. In fact, Yee et al. (1979) have shown that the attack of the 3′-hydroxyl group of the *i* ATP upon the *i* + 1 P_α atom and the release of pyrophosphate occurs with inversion of configuration at the phosphorus atom. We therefore place the 3′-oxygen of the *i* ATP, P_α of the *i* + 1 ATP, and the oxygen of the leaving pyrophosphate bound to P_α, all in a linear (180°) arrangement. We fix the distance between the two reacting atomic centers at 2.5 Å, and not to exceed the limits of 2.2 and 3.0 Å, a range between the sum of their van der Waals radii of 3.3 Å and a P–O bond length of 1.8 Å.

A very important parameter for the model construction is the distance between the metals in the *i* and *i* + 1 sites determined in the preceding paper by substitution of Mn(II) for Zn(II) and Mg(II).

The distances between the *i* + 1 Mn and the triphosphate group of *i* + 1 ATP indicate that this group is within bonding distance of the metal. Our distances would be compatible with metal binding either to oxygens on all three phosphorus atoms or to oxygens of only the β- and γ-phosphates. Yee et al. (1979) have obtained evidence for coordination to β- and γ-oxygens only, and we have therefore placed these two oxygens in positions suitable for coordination to the metal.

Finally, we have considered the fact that the *i* + 1 α-phosphate must be in position to bond to the 3′-OH group, *but not* to the 2′-OH group in *i*. We have therefore included a parameter (the *i* $C_{2'}$, $C_{3'}$, *i* + 1 P_α angle) to prevent the 2′-OH group from competition with the 3′-group for bond formation.

From all these parameters, which include distances determined by NMR and EPR experiments, as well as deductions from known characteristics of RNA polymerase, a model for the geometric configuration at the active site of the enzyme is shown in Figure 1. This model incorporates many known features about substrate and enzyme–metal–substrate interaction with ATP substrates and with alternating A-T template. Its construction does involve a variety of assumptions, and we consider it a working model, to be verified or modified by further studies with different substrates and templates. Table

VII contains a list of parameters that were used as input in the construction of the model, along with the numbers generated for best fit to all of the parameters. The table also contains distances that were measured on the model from i Mn to $i + 1$ ATP, and from $i + 1$ Mn to i ATP, as well as the Mn(II)–oxygen coordination distances in the $i + 1$ site.

Effect of a Second ATP on the Determination of Distances from Metal to ATP in both the i and i + 1 Sites. In the calculation of distances from the metal in each subsite to the ATP nuclei in the same subsite we have ignored the presence of a second ATP in the other subsite. Since the ATP in each subsite is in rapid exchange with the ATP in solution, however, the value of $1/(fT_{1p})$ calculated from eq 1 is actually the weighted average of the relaxation rates for the substrate nuclei in both subsites. Because the relaxation rate has a nonlinear dependence upon the distance between the nucleus undergoing relaxation and the paramagnetic metal, the distances calculated from averaged relaxation rates do not represent linear averages of the distances to each substrate (Jardetzky & Roberts, 1981). Assuming identical spectral density functions and correlation times for the two subsites, the relationship between the distance calculated by using eq 3 and the two true distances from the paramagnetic ion to the substrate nuclei, r_i and r_{i+1}, is given in eq 7. The distances calculated by

$$r^* = \frac{r_i r_{i+1}}{[\frac{1}{2} r_i^6 + \frac{1}{2}(r_{i+1})^6]^{1/6}} \quad (7)$$

eq 3 when ATP occupies both the i and $i + 1$ sites have been labeled with an asterisk in all the tables, to indicate the fact that they are technically "average" distances. We now consider to what extent r^* is influenced by the "other" ATP. The cross-distances shown in Table VII will be helpful in this analysis.

Let us first take up the effect on distance measurements in the $i + 1$ site. We can start with the following generalizations: When an i ATP atom is closer than the corresponding $i + 1$ ATP atom to the $i + 1$ Mn, the actual distance from Mn to ATP in the $i + 1$ site is greater than r^*. We see from Table VII that this situation does not occur. When however an i ATP atom is farther away than the corresponding atom on $i + 1$ ATP from the $i + 1$ Mn, the actual distance to the $i + 1$ ATP is smaller, and in this case the *maximum* difference, from eq 7, is ~12%, within the range of uncertainty of the experiments. A comparison in Table VII of the measured distances r^* (which we have used as a measure of the $i + 1$ Mn to $i + 1$ ATP distances) with the cross-distance taken from Figure 1 shows the following: 1. The distances to the two H_8 atoms are the same, and therefore r^* is appropriately used for the $i + 1$ distance. 2. The distances to the $H_{1'}$ atoms are similar, and therefore, r^* is very nearly correct for the $i + 1$ distance. 3. There are large differences between the distances to the H_2 and to the three phosphates, so that the actual distances are clearly smaller than those calculated, but even a maximum decrease of 12% will not make much difference in these small numbers, as may be observed from the fact that the r^* values place the metal within bonding distance of the phosphate groups, and that therefore no very significant decrease is possible. For these reasons we believe that the use of r^* values is appropriate for the distances within the $i + 1$ site.

We now consider the effect of the $i + 1$ ATP upon distance measurements in the i site—a somewhat more complicated matter.

We have already noted the differences in the measured distances (Chatterji & Wu, 1984) from i Mn to i ATP in the presence and absence of template, shown in Table VI. We now take into account the fact that the two sets of distances in Table VI were determined under conditions that differed not only in the presence or absence of template. The experiments without template contained no Mg(II) and those with template contained enough Mg(II) to occupy the metal position in the $i + 1$ site. In the absence of an added divalent cation there is only one ATP binding site, the i site; but in the presence of such a cation, there is an additional strong ATP binding site (Wu & Goldthwaite, 1969), which we assume to be the $i + 1$ site.

Thus, the experiments lacking both template and Mg bind a single ATP in the i site only, whereas those experiments that include both template and Mg bind two ATP substrate molecules, one each in the i and $i + 1$ sites.

One might ask whether the $i + 1$ site might be unoccupied, even in the presence of Mg(II), in the presence of a template that does not promote RNA synthesis with the substrates, e.g., poly(dAdT)·poly(dAdT) and ATP. However, Slepneva and Weiner (1981) have shown by equilibrium dialysis that the addition of pUpU to an enzyme–Mn(II)–ATP–poly(dA) complex results in the substitution of pUpU for half the ATP in the original complex. Apparently the pUpU had replaced i ATP but not $i + 1$ ATP. Thus, in the presence of Mg(II) the enzyme places ATP into both the i and $i + 1$ sites.

The differences between the two sets of data in Table VI, columns a and b, can therefore be attributed to a combination of effects due to the $i + 1$ ATP as well as the template, both of which are present in (b) but not in (a). It should not be assumed a priori that the template is solely responsible for the differences, since the $i + 1$ ATP must be taken into account.

In fact, we considered the possibility that the $i + 1$ ATP was solely responsible for the differences. We took the distances in Table VIa as the distances from i Mn to ATP in the i site, since these data were obtained without ATP in $i + 1$. We then took the values from Table VIb to be the weighted average of the distances from i Mn to i and $i + 1$ ATP, according to eq 7, and then used that equation to calculate distances from i Mn to $i + 1$ ATP. An attempt to construct a model for the interaction between the two ATP molecules utilizing these distances proved incompatible with some of the other requirements of the model that will be indicated below. We conclude that the differences between the two data sets cannot be explained solely by the presence of the $i + 1$ ATP and, therefore, must be due in part to the presence of the template.

We now ask what effect the presence of the $i + 1$ ATP could have had on the measurement of the distances in the i site. We note that the distances from i Mn to the β- and γ-phosphorus atoms in $i + 1$ are smaller than the corresponding distances measured in our model to the corresponding phosphorus atoms in i (Table VII). The significance of this result is that the distances to these phosphorus atoms in i are actually larger than measured. Therefore the triphosphate group is further extended away from the i nucleoside than the calculations had indicated. The position of the i triphosphate, unlike that of the $i + 1$ triphosphate, has little consequence for the RNA synthesis mechanism; the triphosphate does not participate in bond formation. We are therefore not concerned with the effect on the triphosphate. The effect on the i nucleoside moiety is quite important, however, and we note that the distances from i Mn to points on $i + 1$ ATP (Table VII) are all greater than those to points on i ATP. Therefore the i nucleoside distances from i Mn are smaller than calculated, particularly for H_8 and $H_{1'}$, again with a limit of 12%.

For these reasons we believe that the presence of the two substrates does not affect the distance calculations in each subsite to an extent that would change the structure of our model. The structure shown in Figure 1 is therefore not greatly influenced by this circumstance.

A comparison of the distances calculated from the NMR results and the best-fit distances that the computer generated for the construction of the model (Table VII) reveals that there is little difference; the distance measured in the i and $i + 1$ sites are therefore compatible. In order to place these distances into the model, it was necessary to adjust the angle from the i O_3, through P_α to the $i + 1$ oxygen on the other side of P_α from 180 to 161°, an angle that is nevertheless still reasonable for the S_N2 mechanism for bond formation.

Comparison of the Active-Site Structure with Other Studies on the Two Substrates. The present study is the first attempt to make use of the presence of bound metals in *both* the i and $i + 1$ sites to understand the stereochemistry involved in the formation of the internucleotide bond in RNA synthesis. As we have already noted, previous studies had utilized one or the other of these metals and thus were able to characterize the i or the $i + 1$ subsites separately. There have been some previous attempts to determine the relative positions of the two substrates, albeit without reference to both metals, and we should like to examine the results of these studies in light of the structure shown in Figure 1.

Stein and Mildvan (1978) had established the proximity of the subsites by placing a Cr(III) complex of ATP into the i site and CTP into the $i + 1$ site. Cr(III)–ATP was an excellent probe for determining the proximity of substrates, since Cr(III) remains firmly attached to its ligands and is displaced with difficulty. Distances were calculated from the Cr(III) attached to the triphosphate of ATP in the i site to $i + 1$ Mn (11.5 Å), to $i + 1$ $H_{1'}$ (10.9 Å), and to the base (~8 Å). These distances cannot be accommodated by our model, as might be anticipated, for this system differs greatly from ours in that the Cr(III) does not displace the intrinsic Zn(II), which remains attached to the enzyme that now contains both Zn(II) and Cr(III) in the i site. Binding to Cr(III) would be expected to change the conformation and position of ATP in the i site, so that the geometry of interaction with the $i + 1$ ATP cannot be determined in this way—even though the experiment was very successful in determining proximity.

Distances between $i + 1$ Mn and ApU in the i site were determined (Bean et al., 1977) in the absence of any substrate in $i + 1$, and with no template. All of the distances ranged between 9 and 10.5 Å. These distances do not fit the Figure 1 structure; in fact, they are incompatible with the 3′-OH in i coming close enough to the α-phosphate in $i + 1$ to make bond formation possible. We do not attempt to explain this incompatibility at the moment. Perhaps a substrate is required in $i + 1$, or a template is required to produce a bond-formation mode in the enzyme. Perhaps it matters to the structure of the enzyme complex whether ApU or ATP is in the i site. Perhaps ApU is a not quite proper substrate since it does not have a triphosphate on the 5′-terminus. These are all interesting questions that future experiments should answer.

In a third study of substrate relationships the Zn(II) in i was displaced by Co(II), and UpA and UTP were placed in the i and $i + 1$ sites, respectively, with each substrate bound to a fluorescent reporter moiety, 1-aminonaphthalene-5-sulfuric acid (AmNS). Distances were measured from the two reporters in i and $i + 1$ to the i Co(II); the distances were 17.4 and 17.5 Å and were not significantly different in the presence and absence of template (Wu & Tyagi, 1987). These distances are much larger than those determined by any other technique in the RNA polymerase active site, because the size of the reporter molecule is added to that of the substrate. It is therefore difficult to relate these distances to other distances measured on the active site, and therefore to the Figure 1 model. The study supports considerable flexibility within the RNA polymerase active site, since fluorescently labeled nucleotides apparently are accommodated within the cavity.

Assumptions Implicit in the Structure. Before discussing the implications of the structure shown in Figure 1, we consider it useful to examine the assumptions implicit in that structure.

First there are the assumptions contained in the NMR and EPR methods; these include simplifications of equations made on the basis that one parameter is so much smaller than another that it can be ignored. These assumptions are generally quite good; for example, outer-sphere contribution to the relaxation rate should generally be very small. Nevertheless, the equations lose precision as a consequence of these simplifications. The importance of these assumptions is related to the accuracy of the results that can be obtained by these methods; errors are of the order of 10%. Within such error limits the simplifications are generally valid. And the errors do not prevent the construction of the model of Figure 1; the geometry shown in this model would not differ much by 10% changes in various distances.

Next we make the assumption that the 3′-hydroxyl group in the i site is poised to form a bond with the α-phosphate group in the $i + 1$ site, and we arbitrarily assign a value of 2.5 Å within a range from 2.2 to 3.0 Å for the distance from the i oxygen to the $i + 1$ P_α. We consider it almost an axiom that a major purpose of the enzyme is to provide conditions favorable for bond formation and that, therefore, the functional groups between which bond formation is to occur are expected to be positioned close to each other. The selected range is a compromise between the sum of the van der Waals radii and the final distance after bond formation has occurred, but here again the general structure shown in Figure 1 would be only slightly affected by changing this distance from one extreme to the other. The basic assumption remains, however, that the two groups are brought close together.

We have considered the assumptions involved in the substitution of Mn(II) for Zn(II) and Mg(II) in the preceding paper.

We are also concerned with the fact that some enzyme molecules in any preparation (in our laboratory and elsewhere) may be inactive, according to the Chamberlin et al. assay. This matter has puzzled us to the extent that we have a project underway to determine the reason for this apparent inactivity. Until more is learned about the apparently "inactive" molecules, and since most of the molecules are active, we assume that our results are not significantly altered by the "inactive" molecules. We are reassured in this assumption by the fact that the same metal–metal distances are obtained with enzyme preparations of different activity, according to the Chamberlin et al. assay.

Finally, we must be aware of the major assumption that it is possible to utilize substrates and templates that are not complementary and do not lead to RNA synthesis and still assume that the enzyme brings these entities close together in a manner similar to what occurs under conditions of RNA synthesis. Again, we rely on the probability that the structure of the two subsites is designed in such a way as to place the two substrates into an appropriate geometric relationship. We already have evidence that this relationship is subject to some change that depends on the template–substrate

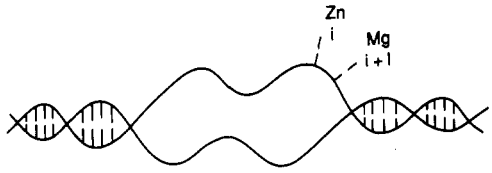

FIGURE 2: Schematic of base pairing in the *i* and *i* + 1 sites of *E. coli* RNA polymerase, to demonstrate the presence of these sites in the unwound portion of the template. Our results do not pinpoint the exact position of the sites, but the figure reflects the close proximity of the unwinding and catalytic activities of the enzyme (Shi et al., 1988).

combination—we discuss this matter below—yet we assume that in a fundamental way the *i* and *i* + 1 sites remain intact. These matters will be tested in future experiments. For the present we consider Figure 1 as the best representation of much that is known on the structure at the active site of the enzyme, under the conditions selected for these studies. It is the only model that can be constructed from the combination of constraints provided by the experimental results, and the assumptions that have been listed here and in the section on model construction. In that sense the model is unique. Taken in conjunction with different assumptions of course, the experimental results could lead to somewhat different models. For example, if it is not assumed that the ATP bases should be in a position to H bond to DNA bases, a different structure could ensue. However, it must be remembered that the experiments determine the distance between the metals as well as between metals and points on the substrate. The error range in the experiments could change the model very little. In the following sections we consider the significance of this model in terms of what it tells us about the relationships between critical structural elements during bond formation in RNA synthesis. We believe that the major significance of the model has yet to be determined, since it rests on the changes that can take place in the active-site structure, as will be discussed below.

Structural Features of the Active-Site Model and Implications for Function. Figure 1 shows only the two metals and the two substrates associated with the active site of the RNA polymerase. The position of the DNA template can be imagined in the stereo presentation as between the viewer and the adenines of the ATPs, in position to hydrogen bond in the proper locations for Watson–Crick base pairing. The position of the protein is uncertain. Presumably some of the outer contours of the substrates fit into a protein crevice. We think of the protein as being in part below the substrate molecules in the stereo presentation, but enveloping the Zn and Mg atoms to which it is bound. In such a structure the protein would wrap around the β- and γ-phosphates in *i* + 1, in line with its participation in the cleavage of the triphosphate group leading to the removal of pyrophosphate in the *i* + 1 subsite.

The Bases Are Not Parallel. One of the characteristics of the B form of DNA is that the nucleotide bases are parallel; in fact, base stacking is believed responsible for a substantial part of the stability of the double helix. Inspection of Figure 1 reveals that the bases in *i* and *i* + 1 are not parallel. When one takes into account the fact that the unwound region of DNA is involved (Figure 2), it becomes clear that the bases should not be expected to be parallel.

Metal Binding to Substrates. There have been previous suggestions that the metal in the *i* site binds the adenine base on ATP at N_7 (Chatterji & Wu, 1982) or through π bonding to the heterocyclic ring (Chatterji et al., 1984). We believe that Figure 2 shows that the distance of *i* Mn(II) to adenine in *i* is too large to permit either type of binding to take place. Evidence for the lack of binding of the metal in *i* and its failure to act directly in the "catalytic" process has been provided by the work of Giedroc and Coleman (1986). We do not know what role is played by the metal in that site; it could of course be a structural role on the enzyme.

On the other hand, the metal in *i* + 1 is close enough to the triphosphate in *i* + 1 to form bonds, as has been previously pointed out. The metal probably is catalytically active and could be involved in the cleavage of pyrophosphate from the ATP molecule.

Is the Enzyme Flexible? It was shown in the preceding paper that the distance between the metals in the *i* and *i* + 1 sites—at least when these metals are Mn(II)—changes from its value on the enzyme alone as substrate and template are added consecutively to the enzyme. Although we must be careful in drawing conclusions from distance changes of relatively low magnitude and high error, we believe that the change from 5.2 to 6.7 Å produced by the addition of both substrate and template is a real change and therefore reflects a change in the structure of the enzyme, on which the metals are located.

The change due to template appears to involve both the *i* and *i* + 1 subsites. Results obtained in the *i* site (Chatterji et al., 1984) indicate that the presence of template changes the *i* ATP conformation, even though some of the differences previously attributed to the template are due to the presence of *i* + 1 ATP. Figure 1 shows that the *i* Mn (substituting Zn) is between the *i* and *i* + 1 substrate.

The template has no effect on the Mn–phosphate distances in *i* + 1, but it appears to decrease the distances to base and ribose, particularly to H_2 (5.8 to 4.6 Å) and $H_{1'}$ (5.9 to 4.5 Å). On the other hand, the distance to H_8 seems to increase, indicating that perhaps H_8 moves in a direction opposite that of H_2 and $H_{1'}$. Such movement would indicate a change in the tilt of the adenine base upon addition of template. These changes in the active-site structure are confirmed by the change in the Mn EPR spectrum shown in the preceding paper.

If we assume that changes in substrate interaction geometry reflect changes in the enzyme, evidence therefore exists that the active-site structure of the enzyme can undergo changes upon the addition of template. Other evidence indicates that different substrates also produce conformational transitions, e.g., ApU in the *i* site (Bean et al., 1977) is further from *i* + 1 Mn than ATP in the same site. There seems thus to be some flexibility in the enzyme active site.

Since one of the major functions of the enzyme must be to create the most favorable conditions for the formation of a bond between the substrates, we wonder whether this apparent flexibility exists to help to differentiate between "correct" and "incorrect" substrates. Perhaps the optimal conditions for bond formation are provided when the correct complementary base is introduced into the *i* + 1 site, and perhaps a less than optimal conformation is produced when the wrong nucleotide is placed into that site. Future experiments with a variety of templates and substrates should tell us whether this hypothesis is correct.

Figure 1 and changes in the structures represented in it during the course of RNA synthesis, as well as under different conditions for that synthesis, should provide an extremely useful handle for probing many aspects of the mechanism and regulation of RNA synthesis at the point of bond formation. It is expected that these studies will be complementary to other studies on RNA synthesis and regulation at points other than the site of internucleotide bond formation.

Added in Proof

An electron microscopy study has led to a model of the

enzyme contour (Darst et al., 1989). This model shows a cleft that is presumed to be at the active site. If so, it would contain the i and $i + 1$ subsites and bind to the substrates shown in our Figure 1.

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REFERENCES

Bean, B. L., Koren, R., & Mildvan, A. S. (1977) *Biochemistry 16*, 3322.
•Bernheim, R. A., Brown, T. H., Gutowsky, H. S., & Woessner, D. E. (1959) *J. Chem. Phys. 30*, 950.
Bloembergen, N. (1957) *J. Chem. Phys. 27*, 572.
Bloembergen, N., & Morgan, L. O. (1961) *J. Chem. Phys. 34*, 842.
Carr, H. Y., & Purcell, E. M. (1954) *Phys. Rev. 94*, 630.
Chatterji, D., & Wu, F. Y.-H. (1982) *Biochemistry 21*, 4657.
Chatterji, D., Wu, C.-W., & Wu, F. Y.-H. (1984) *J. Biol. Chem. 259*, 284.
Darst, S. A., Kubalek, E. W., & Kornberg, R. D. (1989) *Nature 340*, 730.
Dwek, R. A. (1975) in *Nuclear Magnetic Resonance in Biochemistry*, Chapter 11, Oxford University Press, London.
Eckstein, F., Armstrong, V., & Sternbach, H. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2987.
Giedroc, D., & Coleman, J. (1986) *Biochemistry 25*, 4969.
Koren, R., & Mildvan, A. S. (1977) *Biochemistry 16*, 241.
Jardetzky, O., & Roberts, G. C. (1981) in *NMR in Molecular Biology*, Academic Press, New York.
Mildvan, A. S. (1977) *Acc. Chem. Res. 10*, 246.
Mildvan, A. S., & Engle, J. L. (1972) *Methods Enzymol. 26C*, 654.
Mildvan, A. S., & Gupta, R. K. (1978) *Methods Enzymol. 49G*, 322.
Mildvan, A. S., Granot, J., Smith, G. M., & Liebman, M. N. (1980) in *Advances in Inorganic Biochemistry 2* (Darnall, D. W., & Wilkins, R. G., Eds.) Vol. 2, p 211, Elsevier Biomedical, New York.
Shi, Y., Gamper, H., Van Houten, B., & Hearst, J. E. (1988) *J. Mol. Biol. 199*, 277.
Slepneva, I. A., & Weiner, L. M. (1981) *FEBS Lett. 130*, 283.
Sloan, D. L., & Mildvan, A. S. (1976) *J. Biol. Chem. 251*, 2412.
Solomon, I. (1955) *Phys. Rev. 99*, 559.
Solomon, I., & Bloembergen, N. (1956) *J. Chem. Phys. 25*, 261.
Stein, P., & Mildvan, A. (1978) *Biochemistry 17*, 2675.
Stodola, R. (1988) in *DOCK: A Program for the Interactive Display of Molecules*, Version 5E 01, Feb 1988.
Wu, C.-W., & Goldthwait, D. A. (1969) *Biochemistry 8*, 4450.
Wu, F. Y.-H., & Tyagi, S. C. (1987) *J. Biol. Chem. 262*, 13147.
Yager, T. D., & von Hippel, P. H. (1988) in *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology* (Neidhardt, F. C., Ed.) p 1241, American Society of Microbiology, Washington, DC.
Yee, D., Armstrong, V., & Eckstein, F. (1979) *Biochemistry 18*, 4116.

Spin-Labeled Oxazolopyridocarbazole as a Probe for Studying Nonintercalating DNA Groove Binding Ligands

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ABSTRACT: A spin-label (P-OPC) composed of the nitroxide-containing ring proxyl linked at the C1 position of the intercalating fluorescent chromophore oxazolopyridocarbazole (OPC) has been synthesized. The spin-labeled OPC was found to interact with DNA and polynucleotides according to an external minor groove binding mode with association constant values K_{app} ranging from 10^5 to 10^6 M^{-1}. External binding was obvious from the inability of P-OPC to increase the length of sonicated DNA upon binding, the low unwinding angle (9.6°) of circular PM2 DNA, and the low energy transfer from DNA bases to bound chromophore. Binding of P-OPC to DNA or polynucleotide results in a strong immobilization of the proxyl moiety, resulting in the appearance of an asymmetric and broad ESR spectrum with a maximal hyperfine splitting of 56.5 G. In the equilibrium conditions, the occurrence of superimposed ESR spectra related to the P-OPC fraction undergoing rapid motion and to the P-OPC fraction immobilized allows the estimation of the concentrations of free and DNA-bound spin-label. The external mode of binding to DNA as well as the characteristics of the ESR spectra make P-OPC suitable for the determination of DNA binding parameters of nonintercalating ligands using competition experiments. The measurement of the binding constants of distamycin A to poly[d(A-T)] and poly[d(G-C)] is taken as an example.

The great majority of antitumor and antiviral agents as well as a number of antibiotics exert their pharmacological activities by interfering with DNA. Attention has been recently focused on agents that reversibly bind to DNA in the helical grooves or along the surface of the phosphate backbone of the helix [see Zimmer and Wähnert (1986) for review]. This class of

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