Proximity of the Nucleoside Monophosphate and Triphosphate Binding Sites on Deoxyribonucleic Acid Polymerase*

Thomas R. Krugh

ABSTRACT: *Escherichia coli* DNA polymerase exhibits both a deoxyribonucleoside triphosphate binding site and a second site that binds nucleotides that have both a 3′-hydroxyl group in the ribose configuration *and* a 5′-phosphate linkage. The specificity of the 3′-hydroxylribonucleotide binding site suggested that this site is related to the site that binds the primer terminus of a DNA chain. A paramagnetic analog of ATP was used to bind a spin-label substrate in the triphosphate binding site. Adenosine 5′-monophosphate was bound in the monophosphate binding site and the nuclear magnetic resonance relaxation rates of the C_2 proton were measured. The separation between the unpaired electron on the paramagnetic substrate and the C_2 proton of AMP, when both are bound to DNA polymerase, is 7.1 ± 0.6 Å. This shows that the two binding sites are adjacent and strongly supports the assertion that the 3′-hydroxylribonucleotide binding site is the site that binds the primer terminus of a deoxyribonucleic acid chain.

Deoxyribonucleic acid dependent DNA polymerase, isolated from *Escherichia coli*, is a multifunctional enzyme that plays a role in the reproduction and repair of DNA. The functions and physicochemical properties of the enzyme have been reviewed by Kornberg (1969). DNA polymerase catalyzes the formation of a phosphodiester bond between the α-phosphate of a deoxyribonucleoside 5′-triphosphate and the 3′-hydroxyl terminus of a DNA chain. In the presence of magnesium the enzyme has two nucleotide binding sites, one that binds deoxyribonucleoside triphosphates and a separate site that binds both deoxyribonucleoside mono- and diphosphates. The triphosphate site will bind a variety of nucleotide analogs (Englund *et al.*, 1969) but the triphosphate group appears to be essential. The mono- and diphosphate binding site will also bind a variety of analogs but there is a strict specificity for nucleotides with a 3′-hydroxyl group in the ribo configuration and a 5′-orthophosphate linkage (Huberman and Kornberg, 1970). The specificity of the monophosphate binding site suggests that this is the site that binds the primer terminus of a DNA chain.

If the monophosphate binding site is the site that binds the primer terminus of a DNA chain, then we expect the monophosphate site to be adjacent to the triphosphate binding site. We have measured the proximity of these two sites in an effort to determine the mechanism and the structure of the active center of DNA polymerase. This proximity was determined by binding a paramagnetic analog of ATP in the triphosphate binding site, and measuring the effect of the unpaired electron on the nuclear magnetic resonance relaxation rates of a proton of AMP,[1] bound in the monophosphate binding site. The separation between the unpaired electron on the ATP analog and the C_2 proton of AMP was calculated from the change in the relaxation rate of the C_2 proton due to the presence of the unpaired electron.

The change in the relaxation rate of water when a paramagnetic ion is bound to a macromolecule was first reported by Eisinger *et al.* (1961) for binding to DNA. Cohn and Leigh (1962) observed the effect for paramagnetic ions bound to proteins. Cohn and coworkers (*e.g.*, Cohn, 1963, and O'Sullivan and Cohn, 1966) have extended this technique to the study of enzyme mechanisms by replacing magnesium with manganese (a paramagnetic ion) as the metal ion activator. Mildvan and Weiner (1969) examined the interaction of a spin-labeled analog of NAD with alcohol dehydrogenase. From the line widths of the nuclear magnetic resonance signals they measured the transverse relaxation rates ($1/T_2$) of individual substrate protons and obtained information on the structural properties of ternary complexes. Mildvan and Cohn (1970) have provided an excellent review of the use of paramagnetic probes to elucidate enzyme mechanisms.

To determine the proximity of the binding sites on DNA polymerase we have measured the longitudinal and transverse relaxation rates of the C^2 proton of enzyme-bound AMP, when it is interacting with the unpaired electron of bound Tempo-ATP (Figure 1). To ensure that the observed effect is a result of an interaction when both substrates are bound to the enzyme molecule, an excess of TTP was added to compete with Tempo-ATP for the triphosphate binding site. From equilibrium dialysis experiments A. Kornberg and D. L. Brutlag (private communication, 1970) found that Tempo-ATP competes with dATP for the triphosphate binding site. The binding constants of these substrates are about equal and thus the bulky paramagnetic group does not appear to distort the triphosphate binding site. The nuclear magnetic resonance experiments show that the monophosphate and triphosphate binding sites are adjacent and confirm the assertion that the 3′-hydroxylribonucleotide binding site is the site that binds the primer terminus of a DNA chain.

Theory

Paramagnetic Relaxation of Nuclear Spin Systems. The theory of the relaxation of nuclear spin systems by paramagnetic ions has been discussed elsewhere (*e.g.*, see Mildvan

* From the Department of Chemistry, Stanford University, Stanford, California 94305. *Received January 25, 1971.* Present address: Department of Chemistry, University of Rochester, Rochester, N. Y. 14627. This investigation was supported by a National Institutes of Health Fellowship (1 F02 GM39658-01).

[1] Abbreviations used are: AMP, adenosine 5′-monophosphate; NAD, nicotinamide-adenine dinucleotide; Tempo-ATP, *N*-6-(2,2,6,6-tetramethyl-4-aminopiperidine 1-oxyl)adenosine 5′-triphosphate; TTP, thymidine 5′-triphosphate.

and Cohn, 1970, and references therein), and will only be presented here in a form that is necessary to interpret the results of the present experiments. The magnitude of the magnetic moment of an electron is about 700 times that of a proton and therefore the proton–electron dipole–dipole interaction is usually the dominant relaxation mechanism for protons in the vicinity of unpaired electrons. The relaxation rates of a proton directly interacting with a paramagnetic ion are shown in eq 1 and 2. Equation 1 is from Reuben *et al.*

$$\frac{1}{T_{1M}} = \frac{2}{15}S(S+1)\frac{\gamma_I^2 g^2 \beta^2}{r^6}\left\{\frac{\tau_{c_2}}{1+(\omega_I-\omega_S)^2\tau_{c_2}^2} + \frac{3\tau_{c_1}}{1+\omega_I^2\tau_{c_1}^2} + \frac{6\tau_{c_2}}{1+(\omega_I+\omega_S)^2\tau_{c_2}^2}\right\} + \frac{2}{3}S(S+1)\left(\frac{A}{\hbar}\right)^2\left[\frac{\tau_{e_2}}{1+(\omega_I-\omega_S)^2\tau_{e_2}^2}\right] \quad (1)$$

$$\frac{1}{T_{2M}} = \frac{1}{15}S(S+1)\frac{\gamma_I^2 g^2 \beta^2}{r^6}\left\{4\tau_{c_1} + \frac{3\tau_{c_1}}{1+\omega_I^2\tau_{c_1}^2} + \frac{\tau_{c_2}}{1+(\omega_I-\omega_S)^2\tau_{c_2}^2} + \frac{6\tau_{c_2}}{1+\omega_S^2\tau_{c_2}^2} + \frac{6\tau_{c_2}}{1+(\omega_I+\omega_S)^2\tau_{c_2}^2}\right\} + \frac{1}{3}S(S+1)\left(\frac{A}{\hbar}\right)^2\left[\tau_{c_1} + \frac{\tau_{e_2}}{1+(\omega_I-\omega_S)^2\tau_{e_2}^2}\right] \quad (2)$$

(1970), and eq 2 is similar to Connick and Fiat (1966) with the scalar term from Abragam (1961). ω_I and ω_S are the Larmor precession frequencies of the proton and electron, respectively. The τ terms are correlation times and the other symbols have their usual meaning.

In the present experiments the unpaired electron and the proton of interest are on separate molecules and we have assumed that the relaxation due to scalar coupling of the spins is negligible (*i.e.*, ($A \cong 0$). The variables in each equation are the correlation function $f(\tau_c)$, the paramagnetic relaxation rates $1/T_{1M}$, and $1/T_{2M}$, and the distance r. The contributions to the relaxation rates, due to the unpaired electron, are determined from the experiments as described below. The value of the correlation function was determined from the ratio of $1/T_{2M}$ to $1/T_{1M}$.

Rearranging eq 1, with the assumption that the hyperfine coupling between the unpaired electron and the proton is negligible, we get at 60 MHz

$$r(\text{Å}) = 544\left[T_{1M}\left(\frac{3\tau_{c_1}}{1+14.2\times10^{16}(\tau_{c_1})^2} + \frac{7\tau_{c_2}}{1+6.15\times10^{22}(\tau_{c_2})^2}\right)\right]^{1/6} \quad (3)$$

At 100 MHz we get eq 4. From high-resolution nuclear

$$r(\text{Å}) = 544\left[T_{1M}\left(\frac{3\tau_{c_1}}{1+39.4\times10^{16}(\tau_{c_1})^2} + \frac{7\tau_{c_2}}{1+16.9\times10^{22}(\tau_{c_2})^2}\right)\right]^{1/6} \quad (4)$$

magnetic resonance experiments we were unable to detect any effect of the nitroxide radical on the chemical shifts of the AMP protons. Therefore, from Swift and Connick (1962)

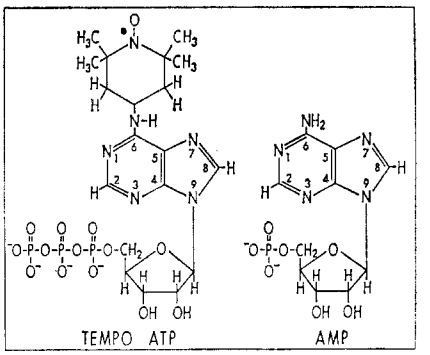

FIGURE 1: Structures of Tempo-ATP and AMP.

and Luz and Meiboom (1964) we may write

$$\frac{1}{T_{1p}} = \frac{pq}{T_{1M}+\tau_M} \quad (5)$$

$$\frac{1}{T_{2p}} = \frac{pq}{T_{2M}+\tau_M} \quad (6)$$

where $1/T_{1p}$ and $1/T_{2p}$ are the observed paramagnetic contributions to the relaxation rates, q is the number of substrate molecules which can simultaneously interact with the paramagnetic probe, p is the ratio of the substrate molecules which are being relaxed by the nitroxide, to the total concentration of substrate, and τ_M is the residence time of the substrate on the enzyme molecule. The most convenient way to determine the relative contributions of T_{1M} and τ_M is to measure the temperature dependence of $1/T_{1p}$. Experimentally, we calculate the paramagnetic contributions to the relaxation rates, $1/T_{1p}$ and $1/T_{2p}$, by measuring the relaxation times of the substrate when the paramagnetic ion is bound to the macromolecule ($T_{1\,\text{bound}}$ and $T_{2\,\text{bound}}$) and also free in solution ($T_{1\,\text{free}}$ and $T_{2\,\text{free}}$).

$$\frac{1}{T_{1p}} = \frac{1}{T_{1\,\text{bound}}} - \frac{1}{T_{1\,\text{free}}} \quad (7)$$

$$\frac{1}{T_{2p}} = \frac{1}{T_{2\,\text{bound}}} - \frac{1}{T_{2\,\text{free}}} \quad (8)$$

Experimental Section

The Tempo-ATP was a gift from M. R. Atkinson, D. L. Brutlag, and A. Kornberg. The samples of DNA polymerase were also gifts from A. Kornberg and D. L. Brutlag. The enzyme was dialyzed into a D_2O–phosphate buffer (5 mM) to reduce the size of the water signal in the nuclear magnetic resonance spectrum.

The measurements of relaxation times at 100 MHz were performed on a Varian HA-100 spectrometer which was modified as described below. The measurements at 60 MHz were performed on a Varian HR-60 spectrometer which was interconnected to components of the HA-100 to provide a field–frequency lock and frequency swept spectra.[2] The

[2] Details of the arrangement are available from L. Durham, M. Bramwell, and S. Seaver, Department of Chemistry, Stanford University, Stanford, Calif. 94305.

TABLE I: Effect of Tempo-ATP on the Relaxation Rate of the C_2 Proton of AMP.

Soln	[AMP] (M)	Tempo-ATP (μM)	DNA Polymerase (μM)	$1/T_1$ (sec^{-1})	$1/T_{1p}$ (sec^{-1})
1	0.119	0	0	0.300	0
2	0.115	85	0	0.353	0.053[a]
3	0.115	105	0	0.373	0.073[a]
4	0.038	0	52.3	0.243	
5	0.036	127	0	0.360	0.117[b]

[a] Solution 1 used as reference. [b] Solution 4 used as reference. This is not an ideal comparison since solution 4 has DNA polymerase.

relaxation times, T_1 and T_2, were determined by the $T_{1\rho}$ method (Sykes, 1969) except that the method was modified to provide for frequency pulsing instead of field pulsing. This modification allows for the use of the field-frequency lock section of the HA-100 and provides the field stabilization required to perform time-averaging measurements. The frequency pulsing was performed by replacing the sweep oscillator of the lock box with a stable voltage controlled oscillator. A simple RC circuit was designed to allow the operator to pulse the spectrometer off resonance and then sweep back to the center of resonance. In addition, a timing device was constructed that would activate both a Varian C-1024 time averager and the pulsing circuit so that the relaxation measurements of weak signals could be time averaged. A detailed description of the apparatus is available from the author. Sykes and Wright (1970) have recently reported similar modifications.

Variable-temperature measurements were performed with Varian Models V6040 and V4540 temperature regulators. The actual probe temperature was determined from the chemical shifts of methanol, placed in the probe before each sample. All samples were placed in the same μ cell (Kontes Glass Co., about 50-μl capacity) to minimize the effects of magnetic field inhomogeneity.

Paramagnetic impurities may influence the results, so care was taken to eliminate undesirable paramagnetic ions. The D_2O (Bio-Rad) was distilled and Na_2AMP (Sigma Chemical Co., St. Louis, Mo., lot 89B-7360) was treated with Chelex-100 (Bio-Rad). In addition, the enzyme solutions were made 5 mM in Mg^{2+} to facilitate the binding of the substrates and minimize the binding of paramagnetic metal ions. The $MgCl_2$ (Baker Analyzed Reagent) was dissolved in deionized water and the TTP (P-L Biochemicals Lot 5809) was dissolved in distilled D_2O. The concentrations of the AMP, Tempo-ATP, and DNA polymerase solutions were determined by ultraviolet absorption.

Results

The electron paramagnetic resonance spectrum of Tempo-ATP bound to DNA polymerase (A. Kornberg and D. L. Brutlag, unpublished results) showed that the molecule was "strongly immobilized," *i.e.*, the tumbling rate of the radical is $<10^7$ sec^{-1} (Hamilton and McConnell, 1968). Therefore the bound Tempo-ATP has approximately the same tumbling rate as the large enzyme molecule. Preliminary measurements also showed that the water relaxation rate was enhanced when the Tempo-ATP was bound to DNA polymerase, compared to an equivalent concentration of the radical ion in buffer solution.[3] The effect of free Tempo-ATP on the relaxation rate of the C_2 proton of AMP was measured and the results are listed in Table I. At these low concentrations of Tempo-ATP we note that $1/T_{1p}$ is linearly dependent upon the concentration of Tempo-ATP. These experiments are intended only to give an estimation of the magnitude of the paramagnetic effect of free Tempo-ATP at the concentration used in the experiments with DNA polymerase. The effect of Tempo-ATP on sodium acetate was measured and when [Tempo-ATP] = 1×10^{-4} M we found that $1/T_{1p}$ = 0.02–0.03 sec^{-1}. The larger values of the paramagnetic relaxation rate for the Tempo-ATP, AMP solutions are probably due to base stacking of the nucleotides (*e.g.*, see Schweizer *et al.*, 1965). Base stacking will also affect the relaxation rate of AMP protons, as may be inferred from the data in Table I. The AMP concentration of solution 4 is about one-third the concentration of solution 1 and the relaxation rate has decreased even though DNA polymerase is present. This indicates that the relaxation rate of the C_2 proton is concentration dependent. We are presently studying this effect. It also shows that the AMP and DNA polymerase solutions do not contain paramagnetic impurities which bind to DNA polymerase near the AMP binding site. In a separate control experiment we found that the relaxation rate of acetate protons increased 0.02 sec^{-1} when Tempo-ATP was bound to DNA Polymerase.

Relaxation Measurements of the Ternary Complex AMP, DNA Polymerase, Tempo-ATP. Three series of experiments were performed to determine the proximity of the nucleotide binding sites. The data from these experiments are listed in Table II. These data show that binding Tempo-ATP to DNA polymerase increased the relaxation rate of the C_2 proton of AMP. To determine how much of this change in the relaxation rate is due to the bound Tempo-ATP interacting with the bound AMP we added TTP to the enzyme solution to compete with Tempo-ATP for the triphosphate binding site. A 90- to 140-fold excess of TTP was used and electron paramagnetic resonance spectra of the final enzyme solutions showed that the Tempo-ATP was no longer "strongly immobilized" but did show some "weak immobilization" which may be due to the viscosity of the solution or to weak electrostatic binding of the Tempo-ATP to the enzyme molecule. Since the TTP and Tempo-ATP have about the same binding constant, the large excess of TTP will mean that only a very small fraction of the Tempo-ATP will remain bound to the enzyme in the triphosphate binding site.

With the equations given previously, we may calculate the separation between the unpaired electron on Tempo-ATP and the C-2 proton of AMP, when both are bound to DNA polymerase. First, however, a few comments on the data are necessary. The activity of the enzyme was checked at the completion of each series of experiments. The enzyme from solution 3 showed 70% of normal activity, solution 5 had 100% activity, and solution 7 showed only 10% of normal activity. The recovered activity appears to be correlated with the duration and temperature of the experiments. Solutions 4 and 5 had the largest concentration of AMP and time-averaging techniques were not required. These experiments lasted about

[3] Dr. Ray Freeman is gratefully acknowledged for performing these preliminary experiments at Varian Associates, Palo Alto, Calif.

TABLE II: Nuclear Magnetic Resonance Relaxation Measurements of the C2 Proton of AMP.

Soln	[AMP] (M)	DNA Polymerase (μM)	[Tempo-ATP] (μM)	[TTP] (mM)	$1/T_1$ (sec^{-1})	$1/T_{1p}$[a] (sec^{-1})	$1/T_{1\rho}$ (sec^{-1})	Temp (°C)	Frequency (MHz)
1	0.038	52.3	0	0	0.243		0.89	35	100
2	0.036	50.4	126	0	0.472	0.099	3.51	35	100
3	0.035	48.5	122	11	0.373		1.32	35	100
4a	0.137	43.1	83	0	0.410	0.033	1.45	35	100
4b	0.137	43.1	83	0	0.467	~0		27	100
5a	0.132	41.5	80	11	0.377		1.02	35	100
5b	0.132	41.5	80	11	0.470			27	100
6a	0.096	45.4	133	0	0.505	0.132	1.82	35	60
6b	0.096	45.4	133	0	0.407	0.022		35	100
6c	0.096	45.4	133	0	0.357			40	100
7a	0.092	43.6	128	15	0.373		0.52	35	60
7b	0.092	43.6	128	15	0.385			35	100

[a] The concentration of Mg^{2+} is 5 mM and the enzyme solutions are in a D_2O–5 mM phosphate buffer (uncorrected pH 7.4). The experimental uncertainty is usually 0.01–0.015 sec^{-1}. Solutions 1, 2, 3, 6, and 7 were time averaged and only one value of $1/T_1$ was measured. For solutions 6 and 7 the data showed more scatter than usual and the uncertainty is probably ± 0.02 sec^{-1}.

TABLE III: Separation of the Nucleotide Binding Sites.

Enzyme Soln	$1/T_{1p}$ (sec^{-1})	T_{1M}[a] (sec)	$(1/T_{1\rho})_p/(1/T_{1p})$[b]	τ_c (sec)	r (Å)[c]	Enzyme[d] Act. (%)
1, 2, 3	0.099	7.9×10^{-3} ($\pm 2 \times 10^{-3}$)	22	9×10^{-9}	7.4	70
4a, 5a	0.033	4.4×10^{-3} ($\pm 3 \times 10^{-3}$)	13	7×10^{-9}	7.0	100
6a, 7a	0.132	2.1×10^{-3} ($\pm 1 \times 10^{-3}$)	10	9.6×10^{-9}	6.9	10
		Average value $r = 7.1 \pm 0.6$ Å (estimated error)				

[a] The uncertainties in T_{1M} are estimates. [b] From line-width measurements these values are 20, 4, and 9, respectively. [c] Separation between unpaired electron of Tempo-ATP and C-2 proton of AMP. [d] Tested at the completion of the experiments.

6 hr at 35° and the enzyme was 100% active at the end of the experiments. Solutions 1, 2, and 3 had the lowest AMP concentration and the experiments lasted 22 hr at 35°. The enzyme retained 70% activity and thus these enzyme solutions appear to be relatively stable at 35°. The experiments with solutions 6 and 7 required 17 hr but for 2 hr the solution was at 40°. This enzyme solution retained only 10% activity and suggests that 35° is about the highest temperature that the enzyme will remain stable for the time required to complete the experiments. Solutions 6 and 7 were included in the data because the critical experiment needed for the determination of the proximity of the binding sites (*i.e.*, solution 6a) was performed before the solution was raised to 40°.

Proximity of the Nucleotide Binding Sites. As mentioned earlier, the temperature dependence of $1/T_{1p}$ and $1/T_{2p}$ is needed to determine the relative values of T_{1M} and T_{2M} compared to τ_M in eq 5 and 6. The data from solutions 4 and 5 suggest that the relaxation effect decreases with decreasing temperature which indicates that τ_M is not negligible at 27°. However, $1/T_{1p}$ at 35° (0.033 sec^{-1}) is not much larger than experimental uncertainties, and as a result, the variable-temperature studies are inconclusive. The relative values of T_{1M} and τ_M will be discussed again later.

To calculate the separation between the unpaired electron on Tempo-ATP and the C_2 proton of AMP we need both the value of T_{1M} and the value of the correlation function $f(\tau_c)$. T_{1M} may be calculated from eq 5, if the values of p, q, and τ_M are known. Binding studies indicate that DNA polymerase has a single binding site for 3′-hydroxyl ribonucleotides (Huberman and Kornberg, 1970) and thus q equals one. The value of p is the ratio of $[\text{Tempo-ATP}]_{bound}$ to $[\text{AMP}]_{total}$ since the enzyme is saturated with AMP. The concentration of bound Tempo-ATP is calculated from its binding constant. K_D is $\sim 1 \times 10^{-5}$ at 5° (D. L. Brutlag, private communication) and for the present calculations we have assumed that K_D is 8×10^{-5} at 35°. In addition, we have assumed that $T_{1M} \gg \tau_M$, *i.e.*, the observed paramagnetic relaxation effect is not limited by the lifetime of the AMP molecules bound to the DNA polymerase. This assumption will be justified in the next section. The value of the correlation function was determined from the ratio $(1/T_{1\rho})_p/1/T_{1p}$. This is equivalent to $1/T_{2p}/1/T_{1p}$ if the following assumptions are valid: (1) $T_{1\rho} = T_2$ and (2) $T_{1M}, T_{2M} \gg \tau_M$. At low levels of the irradiating field $T_{1\rho}$ approaches T_2 (Sykes, 1969). Since the observed $T_{1\rho}$ is much shorter than T_1, it is clear that the level of H_1 used is at least approaching the limit where $T_{1\rho} = T_2$. Values of $1/T_{2p}$

were also calculated from changes in the line width of the nuclear magnetic resonance signals and agreed, within experimental error, with the measurements of $1/T_{1\rho}$. We will show that $T_{1M} \gg \tau_M$ in the next section, but we must assume that $T_{2M} \gg \tau_M$. The consequences of this assumption will also be discussed.

In the present series of experiments we have bound Tempo-ATP to DNA polymerase and measured the relaxation rates of the C_2 proton of AMP. The observed value is an average of both bound and free AMP and thus we must consider the interaction of bound Tempo-ATP with free AMP. In a separate experiment we found that the relaxation rate of acetate protons increased $\sim$0.02 sec^{-1} when Tempo-ATP was bound to DNA polymerase. When the Tempo-ATP is "chased off" the DNA polymerase the additional free Tempo-ATP will contribute to the relaxation rate of the AMP protons. Using the data in Tables I and II and the binding constant of Tempo-ATP we calculate that the additional free Tempo-ATP will increase the observed relaxation rate $\sim$0.02 sec^{-1}. These two corrections to T_{1p} are self-canceling and appear to have about the same magnitude. Using the equations given earlier, we have calculated the separation for each of the experiments listed in Table II. These values are given in Table III and we see that $r = 7.1 \pm 0.6$ Å. For the values of r listed in Table III the author neglected the above corrections since they are self-canceling and are no larger than the experimental uncertainty of 0.02 sec^{-1}.

Discussion

The agreement among the three experiments is encouraging. The value of r depends on the sixth root of the product of T_{1M} and τ_c and thus rather large errors in these values will not drastically affect the calculation of r. The sixth-root dependence also means that the magnitude of the paramagnetic effect rapidly diminishes as r increases and in the present experiments the maximum separation that would have an observable effect is $\sim$9 Å. The T_1 of the C_8 proton was measured in solutions 1 and 2 (1.55 and 1.5 sec). These values agree, within experimental error, and indicate that $r \gtrsim 9$ Å for the C_8 proton. This result will be verified in future experiments.

Comments on τ_c. The value of τ_c was determined from the ratio $(1/T_{1\rho})_p/1/T_{1p}$ and we found that the values for each of the experiments agreed fairly well. To calculate τ_c we assumed that $T_{1M}, T_{2M} \gg \tau_M$. To examine this assumption we divide eq 5 by eq 6 and obtain

$$1/T_{2p}/1/T_{1p} = \frac{T_{1M} + \tau_M}{T_{2M} + \tau_M} \qquad (9)$$

and we note from Table III that the ratio on the left-hand side is between 10 and 22. We therefore conclude that $T_{1M} \gtrsim 9\ \tau_M$ and the assumption that $T_{1M} \gg \tau_M$ is justified. We are unable to make the same statement for T_{2M} and by rearranging the above expression we can show that $T_{2M} \lesssim 1/10\ T_{1M}$. At 60 MHz this means that $T_{2M} \lesssim 2.1 \times 10^{-4}$ sec, a value that may be approaching τ_M or, in fact, the observed paramagnetic effect, $1/T_{2p}$, may be limited by the exchange rate (*i.e.*, $T_{2M} \lesssim \tau_M$). τ_M varies over a wide range for a variety of enzymes and substrates and must be determined experimentally (Mildvan and Cohn, 1970). If the T_2 relaxation is limited by the exchange rate, then the correlation time will be longer than the values reported in Table III. If we assume that a reasonable upper limit for τ_c is 1.5×10^{-8} sec, then the average value of r would be 6.5 Å. The agreement between τ_c obtained at 100 MHz (9×10^{-9} sec for solutions 1, 2, and 3) and 60 MHz (9.6×10^{-9} sec for solutions 6 and 7) suggests that $9 \times 10^{-9} < \tau_c < 1 \times 10^{-8}$ sec.

Interpretation of the Results. The 3′-hydroxylribonucleotide binding site on DNA polymerase is believed to be the binding site of the primer terminus of a growing chain of DNA. The triphosphate binding site is unquestionably the site at which the polymerization reaction takes place and if the monophosphate binding site is the primer terminus site, the two binding sites must be adjacent. These experiments showed that when Tempo-ATP and AMP are both bound to DNA polymerase the separation between the unpaired electron on Tempo-ATP and the C_2 proton of AMP is 7.1 ± 0.6 Å. This is about the separation expected from models of a DNA chain if the Tempo-ATP is placed in the position of the next nucleotide residue. This is not positive proof that the monophosphate binding site is the binding site of the primer terminus, but when added to the evidence put forth by Huberman and Kornberg (1970) it offers convincing arguments that the monophosphate binding site is the primer terminus binding site.

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Aspects of Chromosomal Structure I. Circular Dichroism Studies*

Thomas Wagner† and Thomas C. Spelsberg‡

ABSTRACT: Circular dichroism spectroscopy was used to examine the chromosomal components of intact nuclei, broken nuclei, isolated chromatin, and residual chromatin complexes from which specific chromosomal proteins had been removed. The DNA component of intact nuclei displayed a positive ellipticity band at 264 nm characterizing a nonconservative DNA spectrum. Upon lysis of the nuclei the conservative DNA spectrum characteristic of DNA in aqueous solution was seen to reappear. The DNA region of the chromatin circular dichroism spectrum was little different from that of broken nuclei.

These observations are taken as evidence of a unique geometry for nuclear DNA due to specific DNA packaging in the nucleus. A specific change in DNA geometry within isolated chromatin indicated by circular dichroism spectroscopy is observed concomitant with the removal of a major portion of histone IV (f2a1) from chromatin, suggesting a DNA conformation determining role for this histone. The observed conformational change in the DNA component of chromatin shows a direct correlation with the RNA template activity of the chromatin. On the basis of this evidence histone IV is suggested to act as a general repressor of RNA synthesis through its conformational effect on the DNA template. The results of this study indicate that the conformation of DNA in the chromosomes of eucaryote cells is determined by both the nuclear environment of the chromosomal material and the specific interactions between the DNA and chromosomal protein components of the chromosome.

The structural study of eucaryote chromosomes represents a particularly difficult problem because of the large number of protein and nucleic acid molecules which constitute these genetic structures. The complexity of the problem is magnified by the structural interrelationships between these macromolecular components and the structural effects of the native milieu of chromosomal material. Because of the primary genetic role played by the DNA component of the chromosome, the particular geometry of DNA in the active chromosome is of central importance in studies of chromosomal structure. Studies of the optical rotatory properties of isolated complexes of nucleohistone or chromatin (Oriel, 1966; Tuan and Bonner, 1969; Permogorov *et al.*, 1970; Simpson and Sober, 1970; Shih and Fasman, 1970; Wilhelm *et al.*, 1970; Henson and Walker, 1970) suggest a change in conformation of the DNA due to interaction with chromosomal proteins. Conformational changes in DNA due to interaction with specific histone proteins (Fasman *et al.*, 1970; Shih and Fasman, 1970) and models for histones such as poly-L-lysine (Cohen and Kidson, 1968; Shapiro *et al.*, 1969) are indicated by changes in the rotatory properties of the DNA. Conversely, X-ray diffraction studies have concluded that the DNA configuration found in nucleohistones remains essentially the same as DNA in aqueous media (Wilkins *et al.*, 1959; Zubay and Wilkins, 1962, 1964; Pardon *et al.*, 1967). Although these combined studies provide valuable information regarding the effects of chromosomal protein on DNA structure in isolated or reconstituted DNA complexes, none attempt to take into account the role played by the nuclear packaging of chromosomes. The unusually high density of DNA in the interphase nucleus (Mirsky and Osawa, 1961) suggests that the nucleoprotein material of nuclei is closely packed in some unique manner. It would not seem unusual that this packaging could alter the protein–DNA interactions within the chromosome and affect the geometry of chromosomal DNA.

In the present study we wish to investigate the combined effects of chromosomal packaging within the nucleus and chromosomal protein interactions on the geometry of chromosomal DNA. Circular dichroism spectroscopy has been utilized to examine DNA geometry within the interphase nucleus, within isolated chromatin, and within residual chromatin complexes from which certain chromosomal proteins have been removed. The DNA template activity of the isolated chromatin and subchromatin fractions utilized in this structural study are presented.

Experimental Section

Materials. Rat thymus (male Sprague–Dawley) nuclei were prepared from fresh rat thymus glands by the method of Blobel and Potter (1966). The resulting nuclear preparation was washed into 0.01 M Tris buffer (pH 7.0) containing 0.0033 M $CaCl_2$ for circular dichroism spectroscopy.

Rat thymus chromatin was prepared from rat thymus (male Sprague–Dawley) glands frozen immediately after sacrificing the animals. As needed, these glands were thawed and the nuclei isolated and purified by the method of Blobel and Potter (1966). The isolation of chromatin from these purified nuclei has been described and is based on previous studies of

* From the Department of Chemistry, Ohio University, Athens, Ohio 45701 (T. W.), and from the Department of Obstetrics and Gynecology, Vanderbilt University School of Medicine, Nashville, Tennessee 37203 (T. C. S.). *Received December 7, 1970.*

† To whom inquiries should be directed.

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