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Nonspecific Interaction of *Escherichia coli* Pyrenyl RNA Polymerase Holoenzyme with Synthetic Polynucleotides As Monitored by Fluorescence Spectroscopy

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ABSTRACT: A derivative of RNA polymerase containing approximately 2 pyrene equiv per enzyme molecule has been used to study the interaction of RNA polymerase with poly[d(A-T)]·poly[d(A-T)] and poly[d-(G-C)]·poly[d(G-C)]. As monitored by fluorescence spectroscopy, pyrenyl RNA polymerase displays a unique set of conformational changes with each synthetic polynucleotide as a function of temperature. An increase in the fluorescence intensity was observed for both polynucleotides at 5 °C. A decrease was observed in the case of poly[d(A-T)]·poly[d(A-T)] at 25 and 37 °C, whereas no discernible perturbation was observed in the case of poly[d(G-C)]·poly[d(G-C)]. Different salt dependencies were observed for the interaction of pyrenyl RNA polymerase with these polynucleotides at 5 and 25 °C. Further characterization of these interactions as well as correlation of the observed fluorescence changes to the corresponding open and closed complexes was carried out with heparin. The interaction between pyrenyl RNA polymerase and poly[d-(A-T)]·poly[d(A-T)] at 25 °C was quantified by using two different methods. The graphical method of Schwarz and Watanabe [Schwarz, G., & Watanabe, F. (1983) *J. Mol. Biol. 163*, 467-484] yielded values of 115-228 for the apparent cooperativity parameter, 31-43 for the apparent lattice number, (0.9-1.4) × 10^7 M^{-1} for the observed association constant, and (4.8-7.8) × 10^4 M^{-1} for the apparent intrinsic association constant, whereas the curve-fitting method of McGhee and von Hippel [McGhee, J. D., & von Hippel, P. H. (1974) *J. Mol. Biol. 86*, 469-489] yielded values of 116-228, 35-37, (0.9-1.1) × 10^7 M^{-1}, and (5.0-7.6) × 10^4 M^{-1} for the corresponding constants. All values are in terms of base pairs for DNA concentration.

The process of transcription involves the intricate and specific interplay between RNA polymerase and the DNA template. The importance of DNA structure in this process has been well documented in terms of conserved sequences centered at −10 and −35 base pairs which define promoter sites (Hawley & McClure, 1983; von Hippel et al., 1984; McClure, 1985), the distance between the −10 and −35 base pair sequences required for optimal transcriptional activity (Brosius et al., 1985;

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Mulligan et al., 1985), the influence of DNA structure in the spacer separating the −10 and −35 regions on transcription (Auble & deHaseth, 1988), and also the effect of superhelical density on transcriptional efficiency (Wood & Lebowitz, 1984; Brahms et al., 1985; Aoyama & Takanami, 1988). Furthermore, alterations in the structure of DNA during transcription have also been demonstrated. As the closed complex isomerizes to an open one, between 10 and 15 base pairs are melted out (Saucier & Wang, 1972; Wang et al., 1977; Melnikova et al., 1978; Reisbig et al., 1979). This melting out process is accompanied by a decrease in the superhelical density of the DNA (Saucier & Wang, 1972). Except for the demonstration of a conformational change in RNA polymerase upon the formation of a specific complex with the A1 promoter of bacteriophage T7 in neutron small-angle scattering studies (Heumann et al., 1988), there is limited information about structural alterations in the enzyme that may accompany these changes in the DNA structure. In this study, fluorescently labeled RNA polymerase (pyrenyl RNA polymerase) has been used to monitor the structure of the enzyme as it interacts with synthetic polynucleotides under a variety of conditions. Furthermore, the perturbation in the fluorescence spectrum of pyrenyl RNA polymerase has been used to quantify these interactions.

Materials and Methods

The materials and experimental methods used for the preparation and characterization of the pyrenyl derivative of RNA polymerase have been described in the preceding paper (Johnson et al., 1991). All pyrenyl derivatives used in this study contained approximately 50% active enzyme molecules and were at least 90% saturated with σ subunit. The poly[d(A-T)][1] used in these studies had an $s_{20,w}$ of 19.9 S, and the poly[d(G-C)] had an $s_{20,w}$ of 6.4 S as reported by P-L Biochemicals. These values correspond to an average polymer length of 9700 base pairs for poly[d(A-T)] and 370 base pairs for poly[d(G-C)].

Spectroscopic Measurements. An IBM 9430 spectrophotometer was used to record all absorption spectra. The extinction coefficients used to determine the concentration of the various protein and nucleic acid solutions are listed in the preceding paper (Johnson et al., 1991). Unless otherwise stated, the concentration of polynucleotides is given in terms of phosphate groups. Fluorescence measurements were performed with a Spex Model 1681 spectrofluorometer which was interfaced to a Spex DM1B minicomputer. All samples had an absorbance of less than 0.05 to prevent inner filter effects. Spectra were generated by 90° transverse excitation of the sample in either a 10 × 2 mm or a 10 × 10 mm cuvette. Data were acquired in the ratio mode every 0.5 nm with an integration time of 2 s. The excitation and emission bandwidths were 1.8 and 4.5 nm, respectively. All spectra have been corrected for wavelength-dependent effects, and the background fluorescence from the sample in the absence of protein has been subtracted. There is a 3–5% estimated error in the measurement of the intensity of fluorescence. Unless otherwise stated in the text, the buffer used in the spectral studies contained 50 mM Tris-HCl (pH 8.0), 0.1 M KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT, and 10% glycerol. This buffer will be referred to in the text as buffer A plus 0.1 mM DTT.

[1] Abbreviations: poly[d(A-T)], poly[d(A-T)]·poly[d(A-T)]; poly[d(G-C)], poly[d(G-C)]·poly[d(G-C)]; RPase, RNA polymerase; P-RPase, pyrenyl RNA polymerase; CD, circular dichroism.

Determination of Binding Parameters. The binding of pyrenyl RNA polymerase holoenzyme to synthetic polynucleotides was monitored by fluorescence spectroscopy. Two methods were used to determine the binding parameters: the graphical method of Schwarz and Watanabe (1983) and the least-squares fitting procedure of McGhee and von Hippel (1974). Binding parameters obtained with these methods for finite lattices are dependent on chain length (Epstein, 1978). Thus, the parameters obtained are apparent values.

In the method of Schwarz and Watanabe (1983), the maximum perturbation in the fluorescence (Z) was determined for the fluorescently labeled protein in the presence of poly[d(A-T)]. The fluorescence of a solution containing a large molar excess of polynucleotide over protein was measured as a function of dilution with buffer. Under these experimental conditions, the percent of bound protein was varied while the ratio of polynucleotide concentration (C_p) to protein concentration (C_A^0) remained constant. A plot is constructed of fluorescence per total protein concentration (F/C_A^0) versus $1/C_A^0$ (e.g., Figure 4). The complexes formed between the protein and DNA dissociate as the sample is diluted, and the fluorescence approaches f_A, which is the fluorescence per unbound protein molecule. The amount of protein bound to the DNA approaches C_A^0 as $1/C_A^0$ approaches zero. Thus, extrapolation of the linear region of the line to zero leads to f_a which is the fluorescence per protein molecule bound to DNA. If it is assumed that the perturbation in the fluorescence is the same for DNA-bound protein in contact with zero, one, or two contiguous protein molecules, then the maximum perturbation possible upon binding of DNA is

$$Z = 1 - (f_a/f_A) \quad (1)$$

After Z has been determined, then the lattice number (n), observed association constant (K_{obs}), cooperativity parameter (q), and intrinsic association constant (K_{int}) can be determined. A solution containing a known concentration of polynucleotide is titrated with fluorescently labeled protein. A plot of fluorescence versus the concentration of protein deviates from a straight line (e.g., Figure 5). The observed fluorescence in this case is a linear combination of the fluorescence due to unbound (f_A) and bound (f_a) protein. The final linear increase in fluorescence at high protein concentrations indicates that saturation of nearly all of the available DNA binding sites has occurred. Extrapolation of this line to the abscissa leads to the value of C_A^0 at the intercept which can then be used to calculate the number of deoxyribonucleotide bases per bound protein by

$$n = ZC_p/C_A^0 \quad (2)$$

The number of deoxyribonucleotide base pairs per bound protein molecule (n') can be determined by using this value. The fraction of occupied DNA binding sites (θ) is defined from the graph in Figure 5 as $\theta = y/y_\infty$. The horizontal distance between the two parallel lines is defined as y_∞. For each data point obtained in the titration of poly[d(A-T)] by pyrenyl RNA polymerase, a distance y is also defined. It is the horizontal distance between the data point and the line representing the variation in the fluorescence as a function of protein concentration in the absence of polynucleotide. Because the observed fluorescence is a linear combination of the fluorescence due to unbound (f_A) and bound (f_a) protein, the fraction of sites occupied at any given concentration of protein can be obtained in this manner. The concentration of free protein is calculated from

$$C_A = C_A^0 - \theta C_p/n \quad (3)$$

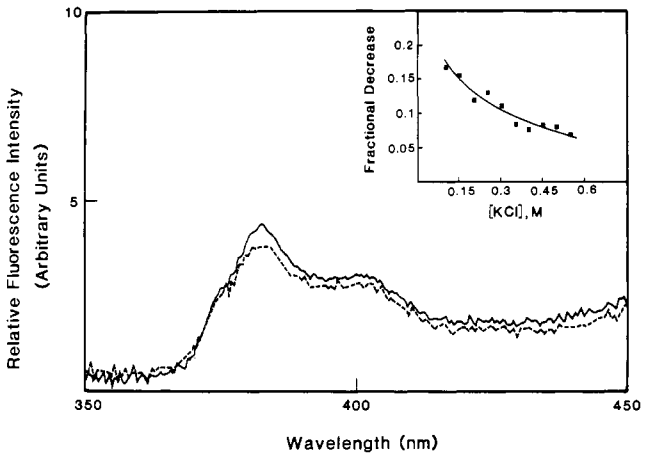

FIGURE 1: Effect of poly[d(A-T)] on the fluorescence spectrum of pyrenyl RNA polymerase at 25 °C. The fluorescence spectrum of pyrenyl RNA polymerase in buffer A plus 0.1 mM DTT is given by the solid line. The dashed line is the spectrum in the presence of poly[d(A-T)]. This spectrum has been corrected for dilution. The excitation wavelength was 330 nm. The inset shows the dependence of the perturbation as a function of salt concentration. A solution containing poly[d(A-T)] in a complex with pyrenyl RNA polymerase in buffer A + 0.1 mM DTT was titrated with a stock solution of 2 M KCl. The change in the fluorescence was monitored at 383 nm. All values have been corrected for dilution.

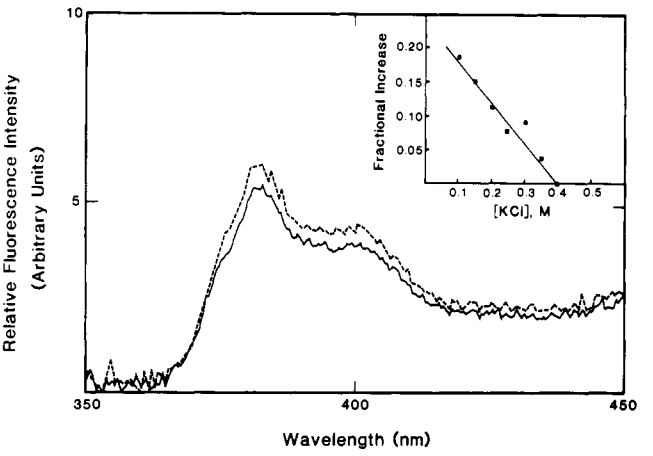

FIGURE 2: Effect of poly[d(A-T)] on the fluorescence spectrum of pyrenyl RNA polymerase at 5 °C. The fluorescence spectrum of pyrenyl RNA polymerase in HEPES buffer is given by the solid line. The dashed line is the spectrum in the presence of poly[d(A-T)]. The spectrum in the presence of nucleic acid has been corrected for dilution. The excitation wavelength was 330 nm. The dependence of this perturbation on salt concentration is given in the inset. A solution containing poly[d(A-T)] in a complex with pyrenyl RNA polymerase at 5 °C was titrated with a stock solution of 2 M KCl. The change in fluorescence was monitored at 383 nm. All values have been corrected for dilution.

The approximate expression valid for $(q/n)^{1/2} \gg 1$ and for $0.2 \leq \theta \leq 0.8$ for cooperative binding is given by

$$(2\theta - 1)/\sqrt{\theta(1-\theta)} = \sqrt{q/n}(K_{obs}C_A - 1) \qquad (4)$$

Thus, a plot of $(2\theta - 1)/[\theta(1-\theta)]^{1/2}$ versus C_A (e.g., Figure 7) has an intercept on the ordinate equal to $-(q/n)^{1/2}$; the reciprocal of the intercept on the abscissa gives the observed association constant K_{obs} which is equal to qK_{int}.

An alternate method for determining the values of the various parameters is that of McGhee and von Hippel (1974). In this method, all the data are fitted to a model for the binding of large ligands to an infinite one-dimensional lattice. A nonlinear least-squares fitting routine written in BASIC for an IBM PC was used for this purpose. This program was kindly provided by Dr. D. P. Loven from the Department of Radiation Biology Oncology at East Carolina University School of Medicine. With this program, the best fit for the intrinsic association constant (K_{int}) and the cooperativity parameter (q) was determined for various values of the lattice number (n). The initial estimates for these three parameters were obtained from the analysis of the data using the graphical method of Schwarz and Watanabe (1983). In the actual fitting of the data, the value for the lattice number was held constant, and the other two values were allowed to vary. The overall best fit of the experimental data was determined by a comparison of the values of the residual sum of the squares obtained at different fixed values of the lattice number.

RESULTS

Interaction of Synthetic Polynucleotides with Pyrenyl RNA Polymerase Holoenzyme. The initial studies on the interaction of pyrenyl RNA polymerase holoenzyme with synthetic polynucleotides were conducted at 25 °C in buffer A + 0.1 mM DTT. There was a 17–19% decrease in the magnitude of the fluorescence spectrum of pyrenyl RNA polymerase (500 mM) at 383 nm upon the addition of poly[d(A-T)] (500 μM) (Figure 1). In stark contrast, poly[d(G-C)] at a concentration of 500 μM had no discernible effect on the fluorescence spectrum of pyrenyl RNA polymerase holoenzyme at 25 °C.

It has been reported previously that the formation of the open complex is temperature dependent; that is, the closed complex is the predominant form at low temperatures (Chamberlin, 1974). Thus, it was of interest to monitor the interaction of pyrenyl RNA polymerase with poly[d(A-T)] and poly[d(G-C)] under conditions in which the open complex does not form. Furthermore, because most enzymatic studies are performed at 37 °C, the interaction of pyrenyl RNA polymerase with these synthetic polynucleotides was also monitored at this temperature. These studies were conducted using a HEPES buffer system (10 mM HEPES, pH 8.0, 0.1 M KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT, and 10% glycerol) due to the fact that Tris, but not HEPES, displays a relatively large dissociation constant shift with temperature. First, it should be noted that there were alterations in the fluorescence spectrum of pyrenyl RNA polymerase as a function of temperature. As the temperature was increased from 5 to 37 °C, there was a progressive decrease in the fluorescence intensity. These observed changes in the relative magnitudes of the fluorescence spectrum of pyrenyl holoenzyme as a function of temperature indicate that, as the temperature increases, there are structural alterations in the enzyme resulting in a greater exposure of the pyrene molecules to the aqueous environment.

At 5 °C, the alterations in the fluorescence spectrum of pyrenyl holoenzyme (500 nM) in the presence of either poly[d(A-T)] or poly[d(G-C)] (500 μM) were comparable. Figure 2 shows the experimental data for the interaction of pyrenyl RNA polymerase with poly[d(A-T)]. There was a 16-19% increase in the fluorescence at 383 nm. At 25 °C, there was a 16–18% decrease in the magnitude of the fluorescence of pyrenyl RNA polymerase in the presence of poly[d(A-T)] (data not shown). This is comparable to that observed in Tris buffer. At 37 °C, there was a 15–17% decrease in the pyrene fluorescence at 383 nm in the presence of poly[d(A-T)] (data not shown). For poly[d(G-C)] (500 μM), there was little if any difference in the fluorescence spectrum relative to the spectrum in the absence of template at either 25 or 37 °C. Thus, pyrenyl RNA polymerase displays

a different set of perturbations as a function of temperature with poly[d(A-T)] and poly[d(G-C)].

Salt Dependency of the Interaction of Pyrenyl RNA Polymerase with Synthetic Polynucleotides. The interaction of poly[d(A-T)] (500 μM) with pyrenyl RNA polymerase holoenzyme (500 nM) as monitored by fluorescence spectroscopy at 25 °C is salt dependent. There was a progressive decrease in the magnitude of the perturbation in the fluorescence at 383 nm as the salt concentration was increased (Figure 1, inset). Even at salt concentrations as high as 0.55 M KCl, there was appreciable interaction between the enzyme and polynucleotide.

The salt dependency of the interaction of pyrenyl holoenzyme (500 nM) with poly[d(A-T)] and poly[d(G-C)], respectively, at 5 °C was also investigated; polynucleotide concentration was 500 μM. In both cases, there was a linear decrease in the magnitude of the perturbation at 383 nm as a function of salt concentration. The data obtained for poly[d(A-T)], which are nearly superimposable with those for poly[d(G-C)], are given in the inset of Figure 2. The perturbation in the fluorescence spectrum of pyrenyl holoenzyme upon interaction with either polynucleotide was completely eliminated at a salt concentration of 0.4 M KCl.

It should be noted that no alteration in the fluorescence spectrum of pyrenyl RNA polymerase was observed at either 5 or 25 °C over the salt concentration range of 0–0.55 M.

Effect of Heparin on the Interaction of Pyrenyl RNA Polymerase with Synthetic Polynucleotides. The effect of the polyanion heparin on open and closed complexes has been well documented (Zillig et al., 1971; Pfeffer et al., 1977). As such, heparin has been used extensively to characterize the interaction between RNA polymerase and polynucleotides. Thus, to establish a correlation between the observed changes in the fluorescence spectrum of pyrenyl RNA polymerase and the formation of open and closed complexes, the effect of heparin on the interaction of pyrenyl RNA polymerase with poly[d(A-T)] and poly[d(G-C)] was investigated. Heparin over a concentration range of 1–100 μg/mL had no discernible effect on the fluorescence spectrum of pyrenyl RNA polymerase holoenzyme at either 5 or 25 °C. No alteration in the fluorescence spectrum of pyrenyl RNA polymerase holoenzyme was observed when the enzyme (500 nM) was incubated at 25 °C with heparin (20 μg/mL) for 10 min prior to the addition of poly[d(A-T)]. Furthermore, there was no time-dependent change in the fluorescence spectrum over a period of 60 min. Upon the addition of heparin (20 μg/mL) to a solution containing pyrenyl RNA polymerase holoenzyme (500 nM) and poly[d(A-T)] (500 μM) in a complex at 25 °C, there was a biphasic decrease in the magnitude of the perturbation as a function of time (Figure 3). There was a rapid decrease in the magnitude of the perturbation in the first 3 min of the reaction; this was followed by a much slower decrease over a period of approximately 90 min. The first part of this reaction is probably over in considerably less time than 3 min; the 3-min time span was simply the time required to scan from 350 to 383 nm. Analysis of the second portion of the curve indicated that it obeyed first-order kinetics (Figure 3, inset). The value of the first-order rate constant for this process was found to be 3.8×10^{-2} min^{-1}. Extrapolation of this line to zero time results in a value of 0.52 for the fraction of pyrenyl RNA polymerase bound to the polynucleotide. Thus, approximately 52% of the pyrenyl RNA polymerase molecules were slowly displaced by heparin.

Incubation of pyrenyl RNA polymerase (500 nM) with heparin (20 μg/mL), at 5 °C, prior to the addition of either

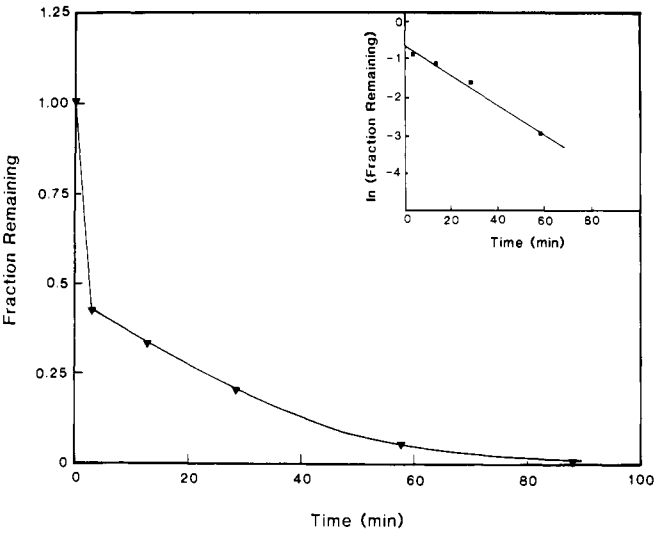

FIGURE 3: Kinetics of heparin disruption of pyrenyl RNA polymerase–poly[d(A-T)] complexes as monitored by fluorescence spectroscopy. Pyrenyl RNA polymerase was incubated with poly[d(A-T)] at 25 °C in buffer A + 0.1 mM DTT for 15 min. Heparin was then added, and the spectrum was repeatedly scanned from 350 to 450 nm over a period of 90 min. The fraction of polymerase remaining was calculated by dividing the original decrease in the fluorescence observed at 383 nm in the absence of heparin into the values obtained at the corresponding time points in the presence of heparin. In the inset is a first-order plot of the experimental data.

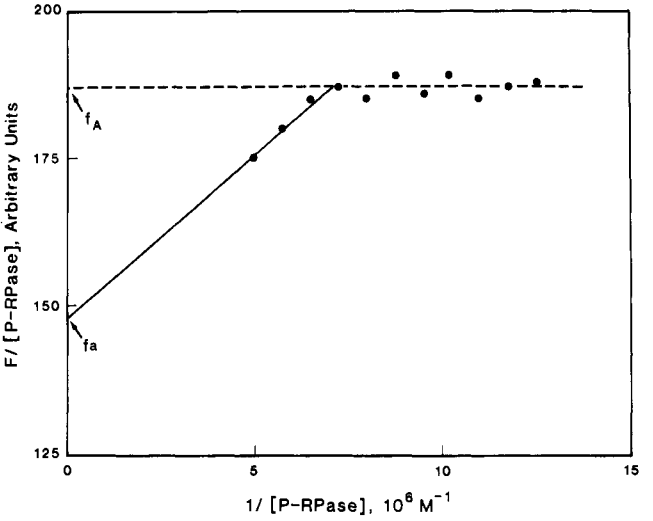

FIGURE 4: Fluorescence intensity per pyrenyl RNA polymerase concentration versus reciprocal protein concentration. The sample contained a constant molar ratio of poly[d(A-T)] to enzyme of 3250 nucleotides/protein. The dashed horizontal line indicates the fluorescence of unbound protein (f_A), and the solid line extrapolated to infinite protein concentration gives the fluorescence of bound protein (f_a). (Buffer: buffer A1 + 0.1 mM DTT.) The fluorescence was monitored at 383 nm.

poly[d(A-T)] or poly[d(G-C)] at a concentration of 500 μM resulted in no alteration in the spectrum of pyrenyl RNA polymerase. (These studies were conducted in the HEPES buffer system.) Upon the addition of heparin (20 μg/mL) to a solution containing either polynucleotide in a complex with pyrenyl holoenzyme, the alteration in the fluorescence spectrum was eliminated in less than 3 min. Once again, this reaction is probably over in considerably less time than 3 min.

Stoichiometry and Binding Parameters for the Interaction of Pyrenyl Holoenzyme with Poly[d(A-T)]. As can be seen in Figure 1, there is a decrease in the fluorescence of pyrenyl RNA polymerase upon the binding of poly[d(A-T)]. The maximum decrease in the fluorescence was determined as

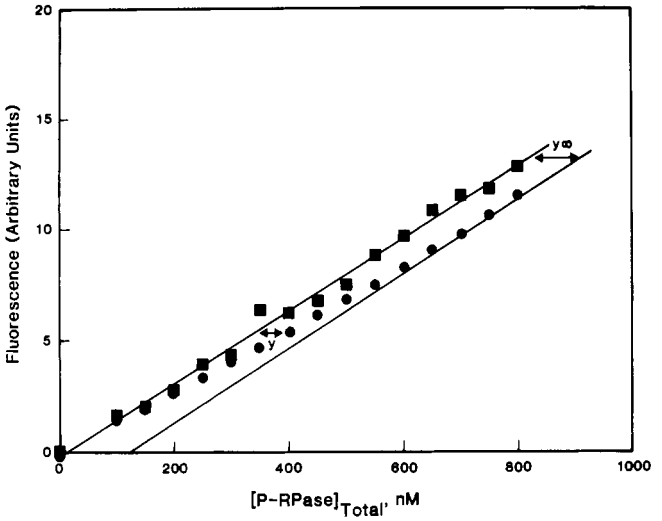

FIGURE 5: Fluorescence intensity of pyrenyl RNA polymerase in the absence and presence of poly[d(A-T)] as a function of protein concentration. Variation in the fluorescence intensity at 383 nm in the absence of nucleic acid is given by (■). Variation in the fluorescence at 383 nm in the presence of nucleic acid (50.4 μM) is given by (●). All values have been corrected for dilution. The temperature was 25 °C. (Buffer: buffer A1 + 0.1 mM DTT.)

Table I: Estimates of Binding Parameters for the Interaction of Pyrenyl RNA Polymerase with Poly[d(A-T)] Assuming 100% Saturation[a]

	method	
	Schwarz–Watanabe	McGhee–von Hippel
K_{obs} ($\times 10^7$ M^{-1})	1.4 ± 0.5	1.1 ± 0.3
	1.1 ± 0.1	0.9 ± 0.1
K_{int} ($\times 10^4$ M^{-1})	7.0 ± 2.8	5.2 ± 1.0
	6.3 ± 1.1	7.2 ± 1.2
q	197 ± 36	211 ± 42
	174 ± 27	124 ± 17
n'	43 ± 7	36
	39 ± 4	35

[a] The first entry for each parameter was determined at a poly[d(A-T)] concentration of 50.4 μM, whereas the second entry was determined at a concentration of 39.8 μM. Binding parameters are apparent values.

outlined under Materials and Methods, and as illustrated in Figure 4. The initial concentrations of pyrenyl RNA polymerase and poly[d(A-T)] were 200 nM and 650 μM, respectively. This solution (2 mL) was titrated with 0.25-mL aliquots of buffer A1 + 0.1 mM DTT (this buffer is identical with buffer A except that it contains 0.2 M KCl), the fluorescence spectrum was recorded after each addition, and the magnitude at 383 nm was determined. The value of Z calculated from eq 1 for this data is 0.21 ± 0.02. In the determination of the binding parameters for the interaction of pyrenyl RNA polymerase with poly[d(A-T)], the fluorescence was monitored in the presence of polynucleotide as the concentration of enzyme was increased from 0 to 800 nM. A direct determination of DNA concentration based on the UV spectrum was performed prior to the titration. In the absence of DNA, there was a linear increase in the fluorescence at 383 nm as a function of protein concentration (Figure 5). However, in the presence of poly[d(A-T)], there was deviation from linearity (Figure 5). The change in the fluorescence at 383 nm in the presence of DNA was monitored until the binding curve became linear. This indicates that saturation of nearly all of the available DNA binding sites has occurred. The lattice number (n) was calculated by using eq 2 and the value of C_A^0 determined by extrapolation of the final linear portion

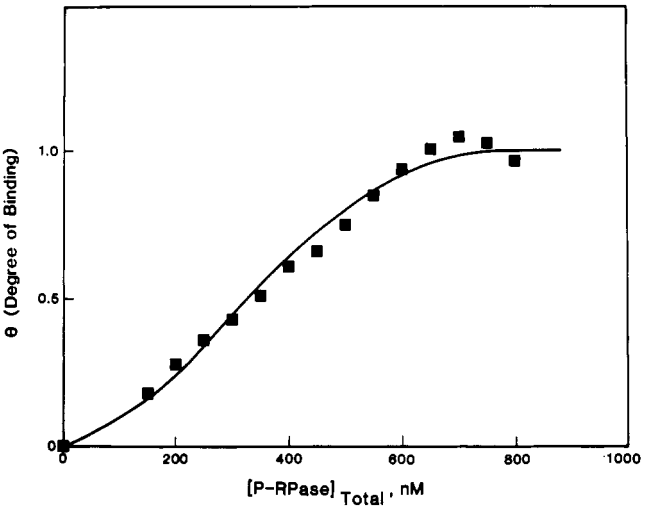

FIGURE 6: Titration of poly[d(A-T)] with pyrenyl RNA polymerase. Plot of lattice saturation (θ) versus total protein concentration for data given in Figure 5. The curve drawn through the data points is for illustrative purposes and does not represent a theoretical curve based on the calculated binding parameters.

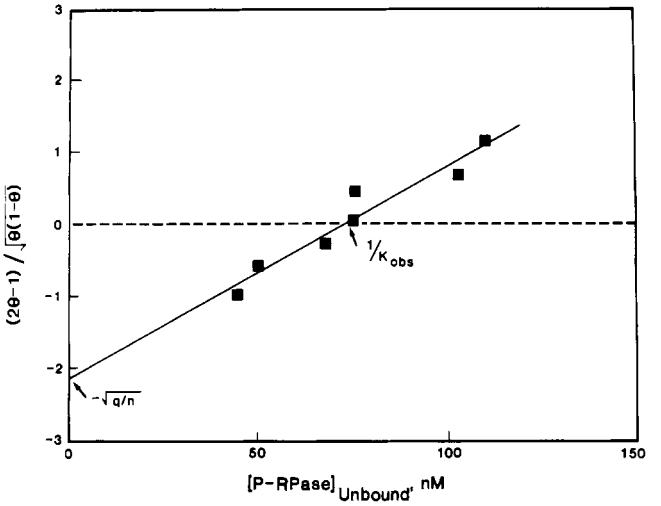

FIGURE 7: Binding data from the titration of a constant amount of poly[d(A-T)] by pyrenyl RNA polymerase. Graphical analysis of the data given in Figure 5 as described under Materials and Methods. Linear regression using the points from $\theta = 0.28$ to 0.75 yields a line crossing the abscissa at $C_A = 1/K_{obs}$ and the ordinate at $(2\theta - 1)/[\theta(1 - \theta)]^{1/2} = -(q/n)^{1/2}$.

of the curve to zero fluorescence. Estimates of the lattice number in terms of base pairs are listed in Table I. For the data shown in Figure 5, a value of 43 ± 7 was obtained assuming that 100% saturation occurred between an enzyme concentration of 650 and 800 nM.

For the calculation of the other binding parameters, the fraction of DNA sites occupied ($\theta = y/y_\infty$) at different protein concentrations was determined as indicated under Materials and Methods, and the amount of unbound pyrenyl RNA polymerase was calculated by using eq 3. A plot of lattice saturation (θ) versus total protein concentration for the binding data is given in Figure 6. The values of the cooperativity parameter (q), K_{obs}, and K_{int} were determined by the graphical method of Schwarz and Watanabe (1983) from the data for $0.20 \leq \theta \leq 0.80$. A plot of $(2\theta - 1)/[\theta(1 - \theta)]^{1/2}$ versus C_A is given in Figure 7. Estimates for the various parameters are listed in Table I. For the binding data shown in Figure 5 and assuming 100% saturation between 650 and 800 nM, analysis of the data yielded values of (1.4 ± 0.5) × 10^7 M^{-1},

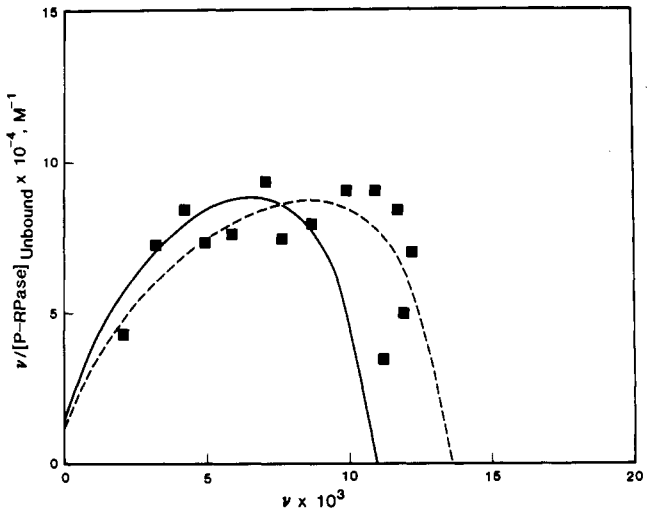

FIGURE 8: Scatchard plot for the interaction of pyrenyl RNA polymerase with poly[d(A-T)]. Scatchard plot of data given in Figure 5. ν is defined as moles of bound enzyme per total moles of poly[d(A-T)]. The solid line is a theoretical curve generated from eq 15 of McGhee and von Hippel (1974) by using the binding parameters determined by the graphical method of Schwarz and Watanabe (1983), whereas the dashed line is the theoretical curve generated by using the binding parameters determined by the best fit of the data according to McGhee and von Hippel (1974) analysis. The data and the values of constants used to generate the curves are in terms of total nucleotides for polynucleotide concentration.

197 ± 36, and (7.0 ± 2.8) × 10^4 M^{-1} for the observed association constant, the apparent cooperativity parameter (q), and the apparent intrinsic association constant, respectively. These values are in terms of base pairs for DNA concentration. As outlined under Materials and Methods, for the relationship $(2\theta - 1)/[\theta(1 - \theta)]^{1/2} = (q/n)^{1/2}(K_{obs}C_A - 1)$ to be valid, the expression $(q/n)^{1/2}$ must be very much greater than 1. For the interaction of poly[d(A-T)] with pyrenyl RNA polymerase, it is apparent that the condition $(q/n)^{1/2} \gg 1$ is not rigorously met. However, this approach is still useful for obtaining initial estimates of the various parameters to be used in the curve-fitting method. A Scatchard plot for the interaction of pyrenyl RNA polymerase with poly[d(A-T)] is given in Figure 8. The theoretical curve represented by the solid line was generated by using eq 15 of McGhee and von Hippel (1974) and the values of the binding parameters that were obtained by the graphical method of Schwarz and Watanabe (1983). The theoretical curve lies below the data at high binding density. A better fit of the data was obtained by using a nonlinear least-squares fitting routine to fit the entire binding isotherm to the McGhee and von Hippel theory (1974). In this procedure, the value of n was held constant, and the best fit for K_{int} and q was determined. A range of values for n was used, and the relative goodness of fit was determined by a comparison of the values of the residual sum of squares. The initial estimates for these parameters were obtained from the analysis of the data using the graphical method of Schwarz and Watanabe (1983). The values obtained by using this method are listed in Table I. The dashed line in Figure 8 is the theoretical line generated by using these parameters and eq 15 of McGhee and von Hippel (1974). It should be noted that although the values obtained by using the nonlinear least-squares fitting routine provide a better fit to the data, this procedure still has its limitations. Under conditions where the magnitude of q is comparable to that of the lattice number n, it has been demonstrated that it is difficult to accurately determine unique values of K_{int} and q (Kowalczykowski et al., 1986). This is due to the tight coupling of the parameters used for fitting

Table II: Estimates of Binding Parameters for the Interaction of Pyrenyl RNA Polymerase with Poly[d(A-T)] Assuming 81% Saturation[a]

	method	
	Schwarz–Watanabe	McGhee–von Hippel
K_{obs} (×10^7 M^{-1})	1.1 ± 0.5	1.2 ± 0.3
	0.9 ± 0.1	0.9 ± 0.2
K_{int} (×10^4 M^{-1})	4.8 ± 2.4	5.0 ± 1.0
	7.8 ± 1.6	7.6 ± 1.2
q	228 ± 46	228 ± 43
	115 ± 20	116 ± 16
n'	35 ± 6	37
	31 ± 3	35

[a] The first entry for each parameter was determined at a poly[d(A-T)] concentration of 50.4 μM, whereas the second entry was determined at a concentration of 39.8 μM. Binding parameters are apparent values.

and the inability to obtain an independent estimate of q. Although in the interaction of pyrenyl RNA polymerase with poly[d(A-T)] the magnitude of q is comparable to that of n, it can be seen in Table I that there is reasonably good agreement in the estimated values for q and K_{int} for the two data sets.

In the analysis of the binding data by the method of Schwarz and Watanabe (1983), it was assumed that 100% saturation was attained between a protein concentration of 650 and 800 nM. If, however, the final portion of the curve in Figure 5 is asymptotically approaching 100% saturation, then an overestimation of the lattice number (n) will result, as well as errors in the estimates of K_{int}, q, and K_{obs}. On the basis of the site size (39–43) and the observed association constant [(6–7) × 10^4 M^{-1}] obtained for the interaction of pyrenyl RNA polymerase with poly[d(A-T)] by using the method of Schwarz and Watanabe (1983), a simple mass action calculation was carried out for DNA and final protein concentrations in the range employed in these studies. For both sets of data, this calculation predicts approximately 81% saturation of the DNA by the protein. Reanalysis of the data in Figure 5 based on the assumption that the extrapolated line represents 81% saturation results in an apparent lattice number of 35 ± 6 instead of 43 ± 7, an apparent intrinsic association constant of (4.8 ± 2.4) × 10^4 M^{-1} instead of (7.0 ± 2.8) × 10^4 M^{-1}, an apparent cooperativity parameter of 228 ± 46 instead of 197 ± 36, and an observed association constant of (1.1 ± 0.5) × 10^7 M^{-1} instead of (1.4 ± 0.5) × 10^7 M^{-1}. These values as well as those obtained for the second data set assuming 81% saturation are listed in Table II. As can be seen from the values listed in Tables I and II, assuming 81% rather than 100% saturation does not drastically alter the values of the binding parameters. However, in the case of the binding parameters obtained by using the method of Schwarz and Watanabe, a better fit to the binding isotherm was obtained assuming 81% rather than 100% saturation (Figure 9). This was not the case with the binding parameters obtained with the McGhee–von Hippel method (1974); i.e., the fits obtained assuming either 81% and 100% saturation were comparable. Other assumptions of lattice saturation between 81% and 100% saturation might produce comparable fits, but it is unlikely that the values of the binding parameters will differ drastically.

Discussion

It has been demonstrated previously that RNA polymerase forms predominantly closed complexes with DNA at low temperatures (Chamberlin, 1974). Thus, the increase in the fluorescence spectrum of pyrenyl RNA polymerase enzyme molecules at 5 °C in the presence of either poly[d(A-T)] or

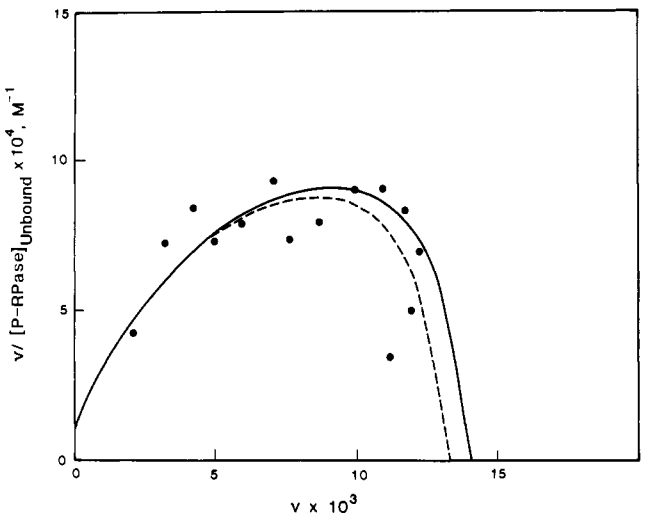

FIGURE 9: Scatchard plot for the interaction of pyrenyl RNA polymerase with poly[d(A-T)] assuming 81% saturation. The solid line is a theoretical curve generated from eq 15 of McGhee and von Hippel (1974) by using the binding parameters determined by the graphical method of Schwarz and Watanabe (1983) and assuming 81% saturation of DNA binding sites at a protein concentration of 800 nM. The dashed line is the theoretical curve generated by using the binding parameters determined by the best fit of the data according to McGhee and von Hippel (1974) analysis assuming 81% saturation at a protein concentration of 800 mM. The data and the values of the constants used to generate the curves are in terms of total nucleotides for polynucleotide concentration.

poly[d(G-C)] must reflect the change in the conformation of RNA polymerase upon the formation of closed complexes. The ability to totally eliminate the interaction of pyrenyl RNA polymerase with either polynucleotide at high salt concentrations suggests that the predominant force stabilizing these closed complexes is electrostatic in nature. This is consistent with the studies of deHaseth et al. (1978), Strauss et al. (1980), and Shaner et al. (1983) which demonstrated that nonspecific interior DNA binding and 0 °C (closed) promoter complex formation have major electrostatic components to the free energy of binding, but disagrees with the results of Kadesch et al. (1980a) which indicated that nonspecific interactions of RNA polymerase with DNA are relatively insensitive to salt concentration. Although the evidence suggests that the observed perturbation in the fluorescence spectrum of pyrenyl RNA polymerase in the presence of either poly[d(A-T)] or poly[d(G-C)] at 5 °C is due to a change in the conformation of the enzyme molecules upon the formation of closed complexes, it is not clear whether a comparable increase in the fluorescence would be observed for the transient formation of closed complexes at higher temperatures. Confirmation of this must await stopped-flow experiments.

At 25 °C as well as 37 °C, it was observed that there was a decrease in the fluorescence of pyrenyl RNA polymerase in the presence of poly[d(A-T)]. Because the extent of modification of the holoenzyme by *N*-(1-pyrenyl)iodoacetamide was shown not to be reduced in the presence of calf thymus DNA (Johnson et al., 1991), it is unlikely that there is a direct interaction between the ligand and fluorophore resulting in quenching, but rather there is a conformational change in the enzyme that alters the microenvironment of the fluorophore. Reisbig et al. (1979) demonstrated that a short region of DNA involving about 10 base pairs per enzyme molecule is melted out upon the binding of RNA polymerase to poly[d(A-T)] at 25 °C. Thus, it is likely that the decrease in the fluorescence of pyrenyl RNA polymerase observed in this study in the presence of poly[d(A-T)] is due to a conformational change in RNA polymerase molecules upon the formation of open complexes. There was no alteration in the fluorescence spectrum of pyrenyl RNA polymerase in the presence of poly[d(G-C)] at either 25 or 37 °C. The lack of a perturbation comparable to that observed in the case of poly[d(A-T)] may be a reflection of the fact that poly[d(G-C)] is poorly transcribed by RNA polymerase. The results obtained here suggest that RNA polymerase is not able to assume a highly transcriptionally active conformation with poly[d(G-C)].

The interaction of pyrenyl RNA polymerase with poly[d(A-T)] was investigated further. Heparin studies carried out at 25 °C indicate that approximately 52% of the enzyme molecules present were capable of forming complexes with poly[d(A-T)] that were slowly displaced from the template. This slow displacement by heparin is consistent with the formation of stable open complexes by this population of the enzyme molecules. The other 48%, which presumably represents inactive enzyme molecules, were still capable of binding to poly[d(A-T)] and giving rise to a perturbation in the fluorescence signal. This suggests that they also underwent conformational changes and presumably formed open complexes. However, for some inexplicable reason these complexes were rapidly displaced by heparin.

The open complexes formed between pyrenyl RNA polymerase and poly[d(A-T)] were relatively resistant to high salt concentrations. Even at a salt concentration of 0.55 M KCl, there was appreciable interaction between pyrenyl RNA polymerase and poly[d(A-T)]. This is consistent with the results of Revzin and Woychik (1981) which indicated that complexes form between poly[d(A-T)] and RNA polymerase at salt concentrations as high as 0.45 M NaCl. Thus, there appears to be either a large nonelectrostatic contribution to the stabilization of this complex or a greater electrostatic interaction than that observed at 5 °C.

In the detailed analysis of the interaction of pyrenyl RNA polymerase with poly[d(A-T)] at 25 °C, moderate positive cooperativity was observed in the binding. A possible explanation for this may be due to the nature of the template used in these studies. Promoters reside in A-T-rich regions of the genome (Botchan, 1976; Jones et al., 1977; Dasgupta et al., 1977; Tong & Battersby, 1979). Thus, to a first approximation, poly[d(A-T)] can be considered to be a contiguous array of promoter sites. The results presented above indicate that the interaction of pyrenyl RNA polymerase with poly[d(A-T)] led to the formation of open complexes. Because poly[d(A-T)] has a relatively low melting temperature, propagation of alterations in the structure of this polynucleotide along its length is more likely than in the case of poly[d(G-C)] or native templates containing a more random distribution of bases. Thus, the binding of a molecule of RNA polymerase to poly[d(A-T)] followed by the formation of an open complex may perturb the structure of the DNA such that the region contiguous to the bound polymerase molecule has a greater affinity for the enzyme than some distant site. An alternative explanation for the observed cooperativity in the binding of pyrenyl RNA polymerase to poly[d(A-T)] is protein–protein interactions. RNA polymerase is capable of forming dimers (Berg & Chamberlin, 1970; Shaner et al., 1982). The extent of dimerization is dependent on both salt and enzyme concentrations. On the basis of eq 7 from Shaner et al. (1982), the amount of enzyme in the dimeric form can be calculated. At the salt concentration used in the binding studies presented here (i.e., 0.2 M KCl), the amount of enzyme in the dimeric form varied from 0 to 10% over an enzyme concentration range

of 100–800 nM. Although the percent of enzyme in the dimeric form varied from 0 to 10%, there is still a linear variation in the fluorescence intensity as a function of protein concentration over this range. The independence of fluorescence intensity on dimerization of RNA polymerase is even more dramatically illustrated at a salt concentration of 0.1 M KCl. At this salt concentration, the amount of enzyme in the dimeric form varies from 25 to 60% as the enzyme concentration varies from 100 to 800 nM. However, there is still a linear variation in the fluorescence as a function of protein concentration from 100 to 800 nM (Johnson, unpublished results). Thus, the formation of dimers does not alter the conformation of the enzyme such that the microenvironment of the fluorophore is perturbed. The propensity of RNA polymerase to form dimers leads one to speculate that the observed cooperativity in the interaction of pyrenyl RNA polymerase with poly[d(A-T)] may be due to protein–protein interactions. On the basis of other studies, however, the former explanation for the observed cooperativity seems more reasonable. In most other binding studies using native templates, positive cooperativity in the binding of the enzyme to the template has not been observed (Kadesch et al., 1980a,b; Revzin & Woychik, 1981). In the case of restriction fragments that lack promoters or tight binding sites, it is likely that the vast majority of the complexes formed with RNA polymerase are closed. Thus, if the positive cooperativity observed in the current study is due to the propagation of conformational changes along the length of the polynucleotide upon the formation of open complexes, then positive cooperativity would not be expected in the interaction of RNA polymerase with a group of templates that form predominantly closed complexes with the enzyme. If the observed cooperativity is due to protein–protein interactions, then one would expect these interactions to occur even if only closed complexes are formed. In the case of DNA fragments containing promoters or tight binding sites, it is likely that the G-C content of the area surrounding these sites prevents the propagation of conformational changes along the length of the DNA chain upon the formation of open complexes. Thus, once again, positive cooperativity would not be expected, if deformation of the DNA is the mechanism for the observed cooperativity. If protein–protein interactions give rise to the cooperativity, then cooperativity of protein binding should be observed with these DNA fragments. It should be noted that up to seven RNA polymerase holoenzyme molecules can bind cooperatively to the promoter region of TyrT in vitro at 37 °C (Travers et al., 1983; Travers, 1987). This has been postulated to be due to the presence of multiple initiation sites in this region, that is, a primary site directing transcription initiation, a weaker secondary site upstream, and multiple weak tertiary sites further upstream. It should also be noted that Reisbig et al. (1979) conducted a set of experiments using a combination of CD and UV difference spectroscopy to investigate the interaction of poly[d(A-T)] with RNA polymerase. These studies were carried out under conditions in which stoichiometric binding was observed, that is, a salt concentration of 5 mM. Thus, any cooperativity which may have been present would have been obscured due to the strong binding. All the evidence appears to indicate that RNA polymerase is capable of matrix-dependent cooperativity in its binding to DNA; that is, with poly[d(A-T)] or regions containing multiple contiguous promoter sites, it displays cooperativity of binding, whereas with random DNA it does not. A similar phenomenon has been observed for the isolated DNA binding domain of the Lex A repressor (Hurstel et al., 1990). Poly[d(A-T)] is homologous to the specific consensus binding domain for Lex A. Cooperativity of binding is observed for the interaction of the DNA binding domain with poly[d(A-T)], but not with random DNA.

The lattice number of the interaction of pyrenyl RNA polymerase with poly[d(A-T)] determined by using the method of Schwarz and Watanabe (1983) ranged from 31 to 43 base pairs, whereas when the method of McGhee and von Hippel (1974) was used the values ranged from 35 to 37. Typical values in the range of 40–50 base pairs have been observed for the formation of specific complexes between RNA polymerase and promoters (Gilbert, 1976; Johnsrud, 1978; Oppenheim et al., 1980; Siebenlist et al., 1980) or for the nonspecific interaction of RNA polymerase with templates (Revzin & Woychik, 1981), although values as high as 70 base pairs (Hansen & McClure, 1979) and as low as 28 base pairs (Reisbig et al., 1979) have been reported for the interaction of RNA polymerase with poly[d(A-T)].

The apparent intrinsic association constant determined in these studies (at 25 °C in 50 mM Tris-HCl, 0.2 M KCl, and 10 mM $MgCl_2$, pH 8) represents the overall process for the formation of open complexes from free enzyme and polynucleotide. The values of the intrinsic association constant determined for the interaction of pyrenyl RNA polymerase with poly[d(A-T)] using two different methods were (4.8–7.8) and (5.0–7.6) $\times 10^4$ M^{-1}, respectively. Due to the moderate cooperativity observed in this system, the affinity for the binding of RNA polymerase to a site contiguous to a bound enzyme molecule in an open complex is between 116 and 228 times greater, that is, (0.9–1.1) and (0.9–1.2) $\times 10^7$ M^{-1}, respectively. These values are considerably less than those observed in studies in which the formation of an open complex between free RNA polymerase and DNA containing a promoter or a tight binding nonpromoter site was monitored. Values of 10^{12}–10^{14} M^{-1} were observed (at 37 °C, and in 10 mM Tris-HCl, 50 mM NaCl, and 10 mM $MgCl_2$, pH 7.9) by Hinkle and Chamberlin (1972) for the formation of open complexes with promoters found in bacteriophage T7 DNA using filter binding assays. Values of 10^8–10^9 M^{-1} were observed by Kadesch et al. (1980b) using electron microscopy (at 37 °C, and in 10 mM Tris-HCl, 10 mM $MgCl_2$, and 50 mM NaCl, pH 8) and also by Hinkle and Chamberlin (1972) using filter binding assays (at 37 °C, and in 10 mM Tris-HCl, 50 mM NaCl, and 10 mM $MgCl_2$, pH 7.9) for tight binding nonpromoter sites found in bacteriophage T7 DNA. Cech and McClure (1980) estimated values in excess of 10^{10} M^{-1} for the promoters of bacteriophage T7 DNA (at 37 °C, and in 40 mM Tris-HCl, 80 mM KCl, and 10 mM $MgCl_2$, pH 7.7) using an abortive initiation assay. Although these values were obtained under different experimental conditions (i.e., salt concentrations) than those used to analyze the interaction of pyrenyl RNA polymerase with poly[d(A-T)], it is clear that sequence elements found in promoter and tight binding nonpromoter sites play a crucial role in stabilizing open complexes.

Conclusions

These studies have demonstrated that the introduction of pyrene molecules into RNA polymerase provides a sensitive means of monitoring structural events in the enzyme during the process of transcription. There appear to be structurally distinct states for the enzyme in closed and open complexes. Furthermore, it has been demonstrated that there are template-specific alterations in the structure of the enzyme. Presumably these structural changes play a crucial role in the process of RNA synthesis. Moderate cooperativity was observed in the binding of pyrenyl RNA polymerase to poly[d(A-T)]. This appears to be due to deformation of the template

upon the formation of open complexes rather than to protein–protein interactions. If these cooperative interactions occur in vivo, they would appear to have to be limited to regions with high A-T content or containing multiple contiguous initiation sites as in the case of the TyrT promoter (Travers et al., 1983; Travers, 1987). More detailed studies are currently underway in an effort to further elucidate the role of these structural alterations using pyrenyl core polymerase and also well-defined templates containing promoter sites. These well-defined templates will eliminate any anomalies that may result in binding studies using a template such as poly-[d(A-T)] that may contain nicks, loops, and other unusual structures.

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