Peculiar 2-Aminopurine Fluorescence Monitors the Dynamics of Open Complex Formation by Bacteriophage T7 RNA Polymerase*

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Rajiv P. Bandwar and Smita S. Patel‡
From the Department of Biochemistry, Robert Wood Johnson Medical School, Piscataway, NJ 08854

The kinetics of promoter binding and open complex formation in bacteriophage T7 RNA polymerase was investigated using 2-aminopurine (2-AP) modified promoters. 2-AP serves as an ideal probe to measure the kinetics of open complex formation because its fluorescence is sensitive to both base-unpairing and base-unstacking and to the nature of the neighboring bases. All four 2-AP bases in the TATA box showed an increase in fluorescence with similar kinetics upon binding to the T7 RNA polymerase, indicating that the TATA sequence becomes unpaired in a concerted manner. The 2-AP at −4 showed a peculiarly large increase in fluorescence upon binding to the T7 RNA polymerase. Based on the recent crystal structure of the T7 RNA polymerase-DNA complex, we propose that the large fluorescence increase is due to unstacking of the 2-AP base at −4 from the guanine at −5, during open complex formation. The unstacking may be a critical event in directing and placing the template strand correctly in the T7 RNA polymerase active site upon promoter melting for template directed RNA synthesis. Based on equilibrium fluorescence and stopped-flow kinetic studies, we propose that a fast form of T7 RNA polymerase binds promoter double-stranded DNA by a three-step mechanism. The initial collision complex or a closed complex, ED01, is formed with a Keq of 1.8 μM. This complex isomerizes to an open complex, ED02, in an energetically unfavorable reaction with an equilibrium constant of 0.12. The ED02 further isomerizes to a more stable open complex, ED03, with a rate constant around 300 s⁻¹. Thus, in the absence of the initiating nucleotide, GTP, the overall equilibrium constant for closed to open complex conversion is 0.5 and the net rate of open complex formation is nearly 150 s⁻¹.

The bacteriophage RNA polymerases, such as the T7 RNA polymerase, are single subunit enzymes capable of catalyzing all the processes such as initiation, elongation, and termination of transcription. The structural simplicity and the template specificity of the phage RNA polymerases make them attractive for exploring the mechanism of transcription and for understanding transcription regulation at the level of protein-DNA interactions. Various crystal structures of T7 RNA polymerase as a complex with the promoter DNA and initiating nucleotides are available, and these inspire detailed studies to understand the dynamics of the transcription initiation and elongation mechanisms (1, 2).

The initiation of transcription is a multistep process that directs the polymerase to the promoter region, where RNA synthesis is initiated. After the RNA polymerase recognizes a consensus DNA sequence, the specific binding energy is used to melt a region of the promoter, part of which serves as a template for the initiation of RNA synthesis (3). Several studies including the crystal structures show that T7 RNA polymerase recognizes a consensus sequence that extends from −17 to +4 relative to the transcription start site. In the absence of the initiating nucleotide, GTP, T7 RNA polymerase melts −4 to +2 region, which includes the TATA sequence, and in the presence of GTP, the unpaired region extends from −4 to +4 (4). Our goal in these studies was to determine the kinetic and thermodynamic parameters that govern the formation of the closed and open complexes. These studies provide the framework to understand how the efficiency of initiation can be regulated by protein-DNA interactions during initiation.

Previously, we have used the fluorescent adenine analog 2-AP, which was incorporated in the promoter DNAs, to monitor the dynamics of T7 RNA polymerase interactions with the T7 promoters (5). These studies were carried out with the promoter DNAs in which all five adenines in the −4 to +4 region were substituted with 2-AP. These previous studies had indicated that T7 RNA polymerase exists in two forms (5). The fast form binds dsDNA at close to a diffusion-limited rate and melts the promoter at a fast rate (5). The slow form is observed only under excess DNA concentrations, and the kinetics of open complex formation are limited by the conversion of the slow form to the fast form. At that time, other studies reported the kinetics of open complex formation in T7 RNA polymerase using 2-AP fluorescence (6, 7). Our conclusions were similar to one study in the literature (7), and it was proposed that open complex formation occurred very rapidly, but a second study indicated much slower rates of open complex formation (6). In the present paper, we have systematically substituted each of the adenines in the −4 to +4 region with 2-AP, and measured the kinetics of the fast form of the T7 RNA polymerase with these DNAs. The fluorescence stopped-flow studies have provided important structural insights and a more detailed multistep mechanism of the initiation process, including the rate constant and equilibrium constant of promoter opening and the associated conformational changes.

MATERIALS AND METHODS

Protein—T7 RNA polymerase was isolated and purified according to established procedures as described in our earlier papers (5, 8, 9). The

The abbreviations used are: 2-AP, 2-aminopurine; 17/40, 17-mer non-template/40-mer template; bp, base pair(s); ds, double-stranded; ss, single-stranded; p-ds, partially double-stranded; nt, non-template; t, template.
purified T7 RNA polymerase was found to be >95% pure by Coomassie staining of protein resolved by SDS-polyacrylamide gel electrophoresis. The purified T7 RNA polymerase was stored at −80°C in a buffer consisting of NaPO₄ (20 mM) pH 7.7, Na₃-EDTA (1 mM), diethiothreitol (1 mM), NaCl (100 mM), and glycerol (50% v/v). The protein concentration was determined by measuring the absorbance at 280 nm in 8 M urea using the molar extinction coefficient of 1.4 × 10⁴ M⁻¹ cm⁻¹.

**Synthesis and Purification of 2-AP Incorporated Oligonucleotides**—Oligodeoxynucleotides of T7 φ10 promoter sequence containing 2-AP were custom synthesized by Integrated DNA Technologies (Coralville, IA) and supplied as desalted samples. The desalted oligonucleotides were further purified by denaturing 16% polyacrylamide gel electrophoresis. Upon electrophoresis, the major band corresponding to the DNA of interest was visualized by UV shadowing and excised. The DNA was extracted from the gel by electrophoresis using an ELUTRAP® electro-separation system from Schleicher & Schuell. The electroeluted DNA was precipitated using ethanol, dried, and resuspended in deionized water. The concentration of the stock solution was determined by measuring the absorbance at 260 nm and using the integrated extinction coefficients of the bases at 260 nm: da = 15,200, dt = 8,400, dg = 12,010, dc = 7,050, and 2-AP = 1,100 M⁻¹ cm⁻¹. The template and non-template ssDNAs were annealed as previously reported (8), to yield the synthetic T7 promoter dsDNA containing only one 2-AP either on a template or non-template strand.

**Absorption Spectrophotometric Studies**—The absorption spectra were measured using an HPS452A diode-array spectrophotometer. The solutions (1 μM) of the free base 2-AP riboside and DNA (both normal and single 2-AP incorporated, single-stranded and double-stranded) were prepared in the buffer (50 mM Tris acetate, pH 7.5, 50 mM sodium acetate, 10 mM magnesium acetate, 5 mM dithiothreitol).

The fluorescence excitation and emission spectra were measured at 25°C on a FluorMax-2 spectrofluorometer (Jobin Yvon-Spix Instruments S.A., Inc.) using the DataMax program for spectra acquisition and analysis. The samples were excited at 315 nm and the fluorescence emission spectra were collected in the wavelength range 325–425 nm. The fluorescence intensity of each of the singly 2-AP incorporated ssDNA (1 μM), dsDNA (1 μM), and ssDNA (1 μM) with T7 RNA polymerase (4 μM) equilibrated for 90 s, was measured at 370 nm upon excitation at 315 nm. The fluorescence of the p-dsDNA with 2-AP at t (−4) and t (−2) was also measured in a similar manner. The fluorescence intensity of T7 RNA polymerase (4.0 μM) was also measured in the same assay. After subtracting the fluorescence contribution of the background fluorescence, and protein fluorescence, and the fluorescence of each ED species in the DNA of interest was fit to a single exponential.

**RESULTS**

**Fluorescent Properties of the 2-AP-Containing φ10 Promoter DNAs**—2-Aminopurine was incorporated at specific positions in the template (t) or the non-template (nt) strands of the 40-bp DNA, with the consensus sequence of the φ10 promoter from −21 to +19 (Fig. 1). Five DNAs were prepared in which the individual adenines at positions nt (−1), nt (−3), nt (+4), t (−2), t (−4) were substituted with 2-AP. The absorption and emission spectra of these DNAs were measured both in the ssDNA and dsDNA, and compared with one another and to the free 2-AP riboside. The absorption spectra of the DNAs were identical, exhibiting a strong peak at 260 nm, corresponding to the absorption of normal bases, and a shoulder in the region 305–315 nm, corresponding to 2-AP absorption. The absorbance ratio (A₃15/A₂60) was consistent with the presence of a single 2-AP in the 40-mer ssDNA. Upon excitation at 315 nm, the 2-AP containing ssDNAs showed a fluorescence spectra, with a peak at 370 ± 1 nm. Each of the ssDNAs showed varying emission intensities (Fig. 1, lower left panel), and the fluorescence was fit (WG 360, serial no. 273129 from Hi-Tech Scientific). The photomultiplier tube high voltage was kept constant throughout the experiment, and the kinetic traces from 10–15 experiments were collected and averaged to optimize the signal.

The purifying T7 RNA polymerase was found to be >95% pure by Coomassie staining of protein resolved by SDS-polyacrylamide gel electrophoresis. The purified T7 RNA polymerase was stored at −80°C in a buffer consisting of NaPO₄ (20 mM) pH 7.7, Na₃-EDTA (1 mM), diethiothreitol (1 mM), NaCl (100 mM), and glycerol (50% v/v). The protein concentration was determined by measuring the absorbance at 280 nm in 8 M urea using the molar extinction coefficient of 1.4 × 10⁴ M⁻¹ cm⁻¹.

F = A*exp(−kₐ₂*ₐ) + C

(Eq. 3)

F is the fluorescence signal (observed at λ ≥ 360 nm) at time t (seconds); A and kₐ₂*ₐ correspond to amplitude (i.e. the change in fluorescence intensity from the beginning to the end of the phase), and observed rate constant of the phase respectively, and C indicates the background fluorescence intensity at t = 0. The pseudo-first-order rate constants thus obtained were plotted as a function of the T7 RNA polymerase concentration, and the corresponding plots were fit by non-linear regression analysis, using SigmaPlot from Jandel Scientific.

In order to interpret the stopped-flow fluorescence kinetic data in terms of a DNA binding mechanism, a global nonlinear least-squares fitting was performed using the software Scientist from MicroMath Scientific Software (Salt Lake City, UT). A DNA binding model was selected, and a set of differential equations was written for each of the kinetic species in the mechanism. One such set of equations was written for every concentration of the T7 RNA polymerase and DNA. During the global fitting, individual sets were distinguished by a letter suffix for each T7 RNA polymerase concentration. The stopped-flow fluorescence kinetic traces were fit directly by assigning the observed fluorescence, F(t) (the dependent variable) at any given time (the independent variable), as the sum of the background fluorescence, Fₒ, which is free DNA and protein fluorescence, and the fluorescence of each ED species in the mechanism (EDᵢ), as shown in Equation 4.

F(t) = ∑Fᵢ*EDᵢ(t) + Fₒ

(Eq. 4)

Fᵢ is the specific fluorescence of each ED species, Fₒ is the background fluorescence due to buffer and free protein, EDᵢ is the amount of each T7 RNA polymerase-DNA species at any given time, which changes during the time course of DNA binding. The initial estimates for the rate constants (as parameters) during global fitting of the kinetic data were obtained from the quantitative analysis of the observed rate constants versus T7 RNA polymerase concentration. The process of global fitting involved first, fixing only one set of intrinsic rate constants and determining the specific fluorescence values (Fᵢ) (as parameters) for various sets of T7 RNA polymerase concentrations. Subsequently, one of the parameter sets (either rate or fluorescence value) was fixed, and global fitting was used to optimize the other floating parameter set. Eventually, all the parameters were floated to fit the data to a single mechanism. The fitting process was governed by a modified Marguard-Lever algorithm making use of the analytical Jacobian matrix. The quality of the fit was judged by visual examination of overlays of the fitted curves and the data as well as inspection of the residuals.

**RESULTS**

Fluorescent Properties of the 2-AP-Containing φ10 Promoter DNAs—2-Aminopurine was incorporated at specific positions in the template (t) or the non-template (nt) strands of the 40-bp DNA, with the consensus sequence of the φ10 promoter from −21 to +19 (Fig. 1). Five DNAs were prepared in which the individual adenines at positions nt (−1), nt (−3), nt (+4), t (−2), t (−4) were substituted with 2-AP. The absorption and emission spectra of these DNAs were measured both in the ssDNA and dsDNA, and compared with one another and to the free 2-AP riboside. The absorption spectra of the DNAs were identical, exhibiting a strong peak at 260 nm, corresponding to the absorption of normal bases, and a shoulder in the region 305–315 nm, corresponding to 2-AP absorption. The absorbance ratio (A₃15/A₂60) was consistent with the presence of a single 2-AP in the 40-mer ssDNA. Upon excitation at 315 nm, the 2-AP containing ssDNAs showed a fluorescence spectra, with a peak at 370 ± 1 nm. Each of the ssDNAs showed varying emission intensities (Fig. 1, lower left panel), and the fluorescence was

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quenched when the ssDNAs were annealed to the complementary strand. On an average, the fluorescence of free 2-AP base was quenched ~95% upon incorporation into ssDNA, and an additional 30–90% quenching was observed when the ssDNA was converted to dsDNA (Fig. 1, lower left panel). Therefore, the 2-AP fluorescence is sensitive to the structure of the DNA, with the fluorescence of 2-AP being higher in the ssDNA versus the dsDNA.

Our studies also showed that the 2-AP fluorescence in ssDNA was dependent on the neighboring bases. The fluorescence intensity of 2-AP was the least at position nt(4) greatest at nt(3) and nt(2) positions, and intermediate at nt(1) and nt(3) positions (Fig. 1, lower left panel). Upon examination of the DNA sequence (Fig. 1), we found that when the 2-AP base was flanked by two guanines, the fluorescence was the least (at position nt(4)). When 2-AP was adjacent to one guanine, the fluorescence was intermediate (at position nt(1) and nt(4)), and when the flanking bases were not guanine, then the fluorescence was the highest (at position t(2) and nt(3)). Thus, guanine residues quench the fluorescence of 2-AP, especially when the 2-AP is stacked next to the guanine.

Fluorescent Properties of the T7 RNA Polymerase-DNA Complex at Equilibrium—Each of the five dsDNA promoters (1 μM) containing 2-AP at the various positions was incubated with T7 RNA polymerase (4 μM), under these conditions, most of the dsDNAs should be bound to the T7 RNA polymerase. Upon complex formation, an increase in the fluorescence intensity of 2-AP was observed with all dsDNAs, except the dsDNA with the 2-AP at nt(+4) (Fig. 1). Because the increase in the 2-AP fluorescence is most likely due to base-unpairing or base-unstacking or both, these results indicate that the +4 position is not melted in the open complex formed in the absence of the initiating GTP. The fluorescence of 2-AP at nt(−1), nt(−3), and nt(−2) positions was 1.2–1.7 times lower than the respective ssDNA fluorescence. Thus, either the bases at these positions were not completely unpaired in the open complex or, if they were completely unpaired, their fluorescence was quenched in the ED complex. A particularly large increase in the fluorescence of 2-AP at t(−4) was observed in the ED complex. The fluorescence of 2-AP at t(−4) increased nearly 4 times higher than the free ssDNA fluorescence. This indicated a unique conformation of the 2-AP base at t(−4) in the ED complex.

The peculiar increase in the fluorescence of 2-AP at t(−4) was also observed in the p-dsDNA (Fig. 4). The p-dsDNA was prepared by annealing a 17-mer non-template strand (−21 to −5) to the 40-mer template strand (−21 to +19), with 2-AP at either t(−4) or t(−2) position. Although the 2-AP at t(−4) is already unpaired in the p-dsDNA, an 8-fold increase in fluorescence was observed when the p-dsDNA was bound to T7 RNA polymerase. We have rationalized these results based on the crystal structure of the ED complex and the fluorescent properties of the 2-AP base. The crystal structure showed that the adenine at t(−4) was flipped out and unstacked from the adjacent guanine residue at t(−5) in the ED complex (1). Because the guanine quenches the fluorescence of the stacked 2-AP base, the unstacking of 2-AP from the guanine results in a large increase in fluorescence relative to its fluorescence in the uncomplexed dsDNA. Thus, the increase in fluorescence of 2-AP at t(−4) in the complexed dsDNAs is both due to base-unpairing and base-unstacking. Whereas in the p-dsDNA promoter that is premelted, the increase in fluorescence must be due to base-unstacking alone.

Stopped-flow Kinetics Reveals the Pathway of Open Complex Formation—Previous stopped-flow kinetic studies have shown that there are two forms of T7 RNA polymerase (5). The fast form binds and melts the dsDNA promoter with a relatively tight Kd and with fast kinetics. At high DNA concentrations and under limiting polymerase conditions, the slow form converts to the fast form at a relatively slow rate. However, under conditions of excess polymerase over DNA, the DNA binding kinetics from the slow form is not observed. We are still investigating the significance of the two forms of the polymerase; hence, the experiments in this paper were designed to measure the kinetics and equilibrium of only the fast form of T7 RNA polymerase.

To measure the kinetics of DNA binding and strand separation at each of the four positions in the TATA box, the stopped-flow kinetic studies were carried out with the four 2-AP-modified dsDNAs. T7 RNA polymerase (0.4 μM) was mixed with the 2-AP dsDNA (0.1 μM), and the fluorescence at λ = 360 nm was monitored as a function of time with continuous excitation at 315 nm. All four 2-AP-modified dsDNA promoters showed a time-dependent increase in fluorescence that fit to a single exponential (Fig. 2a). The observed rate of the fluorescence increase was the same regardless of the position of the 2-AP within the TATA sequence. The amplitudes were different and followed the same trend as observed in the equilibrium fluo-

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**Fig. 1. Positional effect of the 2-AP fluorescence in the T7 φ 10 promoter and the T7 RNA polymerase-DNA complex.** The 2-AP base was incorporated at the indicated positions in the 40-bp dsDNA T7 φ10 promoter (consensus sequence from −21 to +19). The number in parentheses refers to the base position relative to the transcription start site at +1, shown in boldface letters. The fluorescence coefficients of the singly 2-AP modified DNAs in the ssDNA, dsDNA, and in the T7 RNA polymerase-DNA complex (ED) are listed in lower left panel, and the corresponding values are plotted in a bar chart (lower right panel).
rescence measurements (Fig. 2b). A large fluorescence increase at t(−4) was observed, and successively smaller changes were observed at t(−3), t(−2) and t(−1) positions.

To dissect each step in the kinetic pathway of DNA binding, the stopped-flow experiments were conducted at varying T7 RNA polymerase concentrations, with each of the dsDNA promoters. The resulting time courses fit to a single exponential, and the observed rates increased with increasing T7 RNA polymerase concentration in a hyperbolic manner. In the previous studies, we had observed only a linear increase because higher T7 RNA polymerase concentrations were not employed (5). The rate versus T7 RNA polymerase concentration dependences were similar for each of the four positions, and a representative kinetic trace and the polymerase concentration dependence is shown for the dsDNA with 2-AP at t(−4) (Fig. 3, a and b).

The fluorescence increase during the time course of DNA binding indicates that the ED species has a higher fluorescence relative to the uncomplexed dsDNA. The single exponential increase indicates that only one ED species is populated. However, the hyperbolic dependence of the observed rate versus T7 RNA polymerase concentration indicates that there are at least two steps in the DNA binding pathway and two ED species that are formed sequentially, as shown.

\[ E + D \rightleftharpoons \text{ED}_c \rightleftharpoons \text{ED}_{c1} \]

REACTION 1

E is the T7 RNA polymerase, D is the dsDNA promoter, and EDc and EDc1 are the two ED complexes. The values of the intrinsic rate constants for the above mechanism were obtained by fitting the dependence to the hyperbolic equation: \( k_{obs} = k_{-2} + k_{2}(\text{polymerase})/(K_d + \text{polymerase}) \), which provided a \( K_d = 1.9 \pm 0.4 \mu M, k_{-2} = 152 \pm 11 \text{ s}^{-1}, \) and \( k_{2} = 6 \pm 3 \text{ s}^{-1}. \) Similar values for the \( K_d \) and the maximum rate constant \( k_2 \) were observed for all the dsDNA promoters (data not shown). The kinetics indicate that the EDc complex is in rapid equilibrium with free E and D. The EDc1 that results from the isomerization of EDc has a higher fluorescence than the free dsDNA and makes a major contribution to the observed fluorescence change. The observed rate, at low concentrations of E, is limited both by the amount of EDc and by the rate of EDc to EDc1 conversion (i.e., \( k_2 \)). At high concentrations of E, the observed rate is limited by the rate of EDc to EDc1 conversion. Hence, the maximum rate \( (152 \pm 11 \text{ s}^{-1}) \) provides an estimate of the intrinsic rate constant, \( k_2 \) for EDc to EDc1 conversion, the intercept of \( 6 \pm 3 \text{ s}^{-1} \) provides an estimate of \( k_{-2} \), and the \( K_d \) an estimation of the \( K_d \) for the EDc complex.

To understand the nature of the EDc and EDc1 complexes, we conducted stopped-flow studies with the 17/40 p-dsDNA promoter. The p-dsDNA is pre-melted in the −4 to −1 region, and we believe that the fluorescence increase in the p-dsDNA with 2-AP at t(−4) originates mainly from base-unstacking. Thus, the kinetics of fluorescence change with the p-dsDNA promoter should provide the rate of base-unstacking and enable us to isolate this step from the strand separation or the base-unpairing step. A constant amount of 17/40 p-dsDNA (0.05 μM) was mixed with varying concentrations of T7 RNA polymerase (0.1–4.0 μM). The resulting increase in fluorescence with time was fit to a single exponential (Fig. 3c). The observed rates increased in a hyperbolic manner as the T7 RNA polymerase concentration was increased (Fig. 3d). The hyperbolic fit provided a maximum rate constant, \( k_d = 303 \pm 31 \text{ s}^{-1}, K_d = 1.3 \pm 0.6 \mu M, \) and \( k_{-2} = 17 \pm 23 \text{ s}^{-1}. \) The observed kinetics are consistent with the following two-step mechanism for DNA binding.

\[ E + pD \rightleftharpoons \text{EpD}_c \rightleftharpoons \text{EpD}_{c1} \]

REACTION 2

pD represents p-dsDNA promoter. The above mechanism indicates that the EpDc is in rapid equilibrium with E and pD with a \( K_d \) similar to the dsDNA, indicating the similar nature of the collision complexes with the two types of promoters. The EpDc isomerizes to EpDc1 in the p-dsDNA promoter at a rate close to 300 s−1, which is 2 times faster than the isomerization rate of the dsDNA promoter. Although the TATA sequence in the p-dsDNA promoter is pre-melted, the template strand of the p-dsDNA still needs to bind within the active site pocket of the T7 RNA polymerase in the manner shown by the crystal structure and in Fig. 6 (1). The isomerization therefore represents a conformational change in the collision complex that results in the open complex, EpDc1. This conformational change is likely to be the base-unstacking step in the p-dsDNA promoter, whereas, in the dsDNA promoter, the conformational change likely involves...
both base-unpairing and base-unstacking reactions.

Extent of Open Complex Formation—The overall equilibrium constant of the two-step DNA binding mechanism (shown in Reaction 1) derived from the stopped-flow kinetic studies indicated that most of the closed complex, EDc, should be converted to the open complex, ED o1, at equilibrium. We therefore expected the dsDNA fluorescence to increase to the same level or higher than the fluorescence of p-dsDNA in the ED complex. When we compared the fluorescence values of the complexed dsDNA and p-dsDNA promoters, we observed that the fluorescence of the complexed dsDNA promoter was 3–3.5 times lower than the fluorescence of complexed p-dsDNA (Fig. 4). The lower fluorescence of complexed dsDNA can be explained in several ways. First, the template strand in the premelted p-dsDNA may be in a conformation that is different from the “polymerase-induced” conformation of the melted dsDNA promoter. To see a larger fluorescence of the p-dsDNA in the ED complex, the bases in the p-dsDNA would have to be unstacked to a larger extent relative to the melted bases in the dsDNA. Note that our previous studies have shown that the dsDNA and p-dsDNA promoters have similar pre-steady state rates of RNA synthesis, suggesting that they form kinetically similar open complexes (9). The second reason for the lower fluorescence of the complexed dsDNA may be that the EDc complex is not converted stoichiometrically to the open complex, ED o1. In other words, the complexed dsDNA may exist as a mixture of closed and open structures. Such an idea has been suggested for T7 RNA polymerase based on steady state analysis of RNA synthesis.
The averaged kinetic traces at various concentrations of T7 RNA polymerase (constant [DNA] = 0.05 μM) are represented as dotted traces, and the solid lines show the global least squares fit to the kinetic mechanisms, shown in Table I. The time axis is logarithmic. a. Each kinetic trace (total of 500 points) was collected on a linear time scale in two time windows, with 350 points in the first time window (0–0.1 s) and 150 points in the second time window (0.1–5 s). The least squares fit for the binding of dsDNA to the three-step mechanism provided the following rate constants:

\[
K_d = 1.8 \, \mu\text{M}, \quad k_2 = 208 \pm 15 \, \text{s}^{-1}, \quad k_3 = 1719 \pm 132 \, \text{s}^{-1}, \quad k_5 = 322 \pm 8 \, \text{s}^{-1}, \quad k_{-3} = 54 \pm 1 \, \text{s}^{-1}.
\]

b. The kinetic traces were collected on a logarithmic time scale. The global least squares fit for the binding of p-dsDNA to the two-step mechanism provided the following rate constants:

\[
K_d = 0.2 \, \mu\text{M}, \quad k_2 = 222 \pm 47 \, \text{s}^{-1}, \quad k_{-2} = 0.2 \pm 51 \, \text{s}^{-1}.
\]

We propose that the equilibrium constant governing the closed to open complex transition can be determined by comparing the fluorescence values of the complexed p-dsDNA and dsDNA promoters. We assume that the complexed p-dsDNA promoter is converted fully to the open complex, and hence the fluorescence value of complexed p-dsDNA is a measure of the maximum amount of open complex in the reaction. Equilibrium fluorescence measurements were carried out with DNAs containing the 2-AP at t(−4) or t(−2) position in the p-dsDNA and dsDNA promoters, and the fluorescence values are listed in Fig. 4 (lower left panel). Each of these positions provided a ratio between 29% and 36%, indicating that about 33% of the complexed dsDNA promoter was in the open form. The fact that both t(−2) and t(−4) provided a similar ratio of open to closed form indicates that this phenomenon is not peculiar to position t(−4). These data provide a ratio of open to closed form equal to 0.33/0.67 = 0.5, which is a measure of the overall equilibrium constant for the ED1 to ED2 conversion in the dsDNA promoter.

**Table I**

|                      | Kinetic mechanism for the binding of φ10 promoter DNAs to T7 RNA polymerase |
|----------------------|--------------------------------------------------|
| **A. Three-step mechanism for double-stranded DNA binding:** |                                                   |
|                      | \[
| E + D \underset{k_2}{\overset{k_3}{\rightleftharpoons}} EDc \overset{k_{-2}}{\rightarrow} EDo1 \overset{k_{-3}}{\rightarrow} EDo2 |
| \(K_d\) | \(k_2\) | \(k_{-2}\) | \(k_3\) | \(k_{-3}\) |
| (μM) | s\(^{-1}\) | s\(^{-1}\) | s\(^{-1}\) | s\(^{-1}\) |
| 1.8 | 208 ± 15 | 1719 ± 132 | 322 ± 8 | 54 ± 1 |
| **B. Two-step mechanism for partially double-stranded DNA binding:** |                                                   |
|                      | \[
| E + pD \underset{k_2}{\overset{k_3}{\rightleftharpoons}} EDc \overset{k_{-2}}{\rightarrow} EDo1 \overset{k_{-3}}{\rightarrow} EDo2 |
| \(K_d\) | \(k_2\) | \(k_{-2}\) |
| (μM) | s\(^{-1}\) | s\(^{-1}\) |
| 0.2 | 222 ± 47 | 0.2 ± 51 |

EDc is a closed complex in equilibrium with the first open complex EDc1 that isomerizes to EDc2. Using numerical integration methods, we were able to globally fit the stopped-flow kinetic data at various T7 RNA polymerase concentrations to the three-step mechanism, as shown by the solid lines in Fig. 5a. The method of global fitting involved writing the differential equations of the three-step model, which were solved by numerical methods using the program Scientist, as described in more detail under “Materials and Methods.” A single set of rate constants was obtained from global fitting and shown in Table I. The derived rate constants indicated that the dsDNA promoter forms a closed complex EDc1 with a \(K_d\) of 1.8 μM. The closed complex isomerizes to an open complex EDo1 with a forward rate constant \((k_2)\) of 208 ± 15 s\(^{-1}\) and a reverse rate constant \((k_{-2})\) of 1719 ± 132 s\(^{-1}\). Hence, there is an unfavorable equilibrium of 0.12 for EDc to EDc1 conversion. The EDc1 then isomerizes to a more kinetically stable EDc2 species with a forward rate constant \((k_3)\) of 322 ± 8 s\(^{-1}\) and a reverse rate...
constant \((k_{-2})\) of \(54 \pm 1 \text{ s}^{-1}\). The same treatment of the p-dsDNA binding kinetics (Fig. 5b) showed that the data fit only to a two-step mechanism (Reaction 2), and the kinetic constants derived from the global fit are shown in Table I. The p-dsDNA (pD) forms an EpDc collision complex with a \(K_c\) of 0.2 \(\mu\text{M}\), the EpDc isomerizes to EpDc2 with a forward rate constant \((k_f)\) of \(222 \pm 47 \text{ s}^{-1}\) and a reverse rate constant \((k_{-2})\) of \(0.2 \pm 51 \text{ s}^{-1}\).

The overall equilibrium constant for ED1 to ED2 conversion was calculated from the derived rate constants of the three-step mechanism. This value of 0.7 is in close agreement with 0.5, which was calculated by comparing the fluorescence values of complexed dsDNA and p-dsDNAs. The percentage of EDc complexes (EDc1 + EDc2) in the dsDNA predicted from the three-step mechanism is 35%, in close agreement to the amount (29–36%) calculated from the relative fluorescence values of the complexed dsDNA and p-dsDNA, under similar experimental conditions. Note that the two methods for obtaining the equilibrium constant are independent. The three-step mechanism also predicts that the open complex EDc1 does not accumulate to a significant level. Therefore, the formation of EDc1 was not observed as a separate phase in the stopped-flow experiments. Based on the available data, we speculate that the EDc1 represents a complex in which only the TATA sequence is melted and the EDc2 may represent a complex in which the t(−4) base is unstacked, the melted region extends to +2, and the template strand is directed into the T7 RNA polymerase active site making interactions with the active site residues.

**DISCUSSION**

The transient-state fluorescence measurements have provided the detailed kinetics of the T7 RNA polymerase-promoter interactions. The interactions were measured by following the fluorescence of the 2-AP base, an analog of adenine that can be incorporated at specific positions in the promoter DNA. The normal bases in the DNA have very short decay times, typically a few picoseconds, and as a result a very weak intrinsic fluorescence of DNA (11, 12). This hampers the use of normal DNA for studying DNA-protein interactions to monitor real-time changes in the DNA within the confines of the protein-DNA complex. However, substitution of a normal base by its modified structural analog with fluorescent properties, without significantly altering the native structure of DNA, provides an important handle to study real time changes in the DNA. The 2-AP is a structural analog of adenine that forms a stable Watson-Crick type base pair with thymine. The 2-AP-T base pair is only marginally weaker (0.5 kcal/mol) than a normal A:T pair as reported for a decamer (13). The 2-AP substitution does not destroy the B-helical structure of the dsDNA, and does not affect the specific recognition by proteins, in most cases (14–16). The 2-AP can be selectively excited at longer wavelengths (310–320 nm), in the presence of tryptophan and tyrosine protein residues, and the 2-AP fluorescence is highly sensitive to the structure of the DNA.

We have substituted four adenines in the TATA box preceding the initiation site of the T7 φ10 promoter and one adenine in the coding region individually to 2-AP. The absorption and fluorescence emission studies indicated that, although the 2-AP at all positions absorbed equally, their fluorescence intensity was dependent on the neighboring bases in the DNA sequence. The fluorescence intensity was the minimum when the 2-AP was flanked by guanines. The 2-AP fluorescence quenching by guanine has been reported (17). The results in the literature showed that the guanine caused static quenching of 2-AP fluorescence, when it was immediately adjacent to 2-AP, and the quenching was reduced when the guanine was placed at longer distances from 2-AP. Most effective quenching occurred through strong π-stacking when G was located on the same strand and adjacent to 2-AP. These results are in agreement with our observations that the 2-AP fluorescence in the DNA is dependent on the neighboring G residue. The fluorescence of 2-AP was also sensitive to the structure of the DNA. The fluorescence of H-bonded 2-AP at t(−4) position, for example, in the dsDNA promoter was less than one-tenth the fluorescence in the ssDNA (Fig. 1). Therefore, 2-AP can serve as a sensitive and site-specific probe to monitor local melting of the dsDNA. In fact, 2-AP has been employed successfully to monitor local melting of DNA bound to the Klenow fragment of DNA polymerase I (18) and T7 RNA polymerase (6, 7) and base-flipping/unstacking in EcoRI DNA methyltransferases (19), and bacteriophage T4 DNA polymerase (20).

**Peculiar Increase of 2-AP Fluorescence at t(−4)—**The fluorescence of all the 2-AP bases in the TATA box increased when the dsDNA promoter formed a complex with T7 RNA polymerase. The 2-AP at t(−4), however, showed a large increase in fluorescence that was 4-fold higher than the free ssDNA. This unusually large fluorescence increase of 2-AP at t(−4) was noted previously (7), but the recent crystal structure of the T7 RNA polymerase-DNA complex provides a rationale for the large fluorescence increase specifically at position t(−4) (1). The crystal structure of the T7 RNA polymerase-DNA open complex shows that adenine at t(−4) is in a unique conformation relative to other adenines. As shown in Fig. 6, the adenine at t(−4) is completely unstacked from the neighboring guanine at t(−5), and such unstacking is expected to cause a drastic increase in 2-AP fluorescence when placed at this position. This proposal is also supported by the fact that the t(−4) 2-AP shows an increase in fluorescence even in the p-dsDNA promoter. Since the 2-AP in the p-dsDNA promoter is premelted, the fluorescence increase most likely arises from base-unstacking.

**Fig. 6. The structure of the φ10 promoter DNA in the T7 RNA polymerase-promoter complex.** The coordinates for the DNA were obtained from the 2.4-Å crystal structure of the complex (PDB identification code 1CEZ), and shows the orientation of the bases in the template, t (−17 to −1) and non-template, nt (−17 to −3) regions. The sequence is schematically represented on the top. In the crystal structure, the template and the non-template strands are paired from −17 to −5 and melted from −4 to −1. The melted template strand is twisted between −5 and −4, and the four downstream bases (−4 to −1) are directed toward the polymerase active site in the palm. The bases −4 to −2 are stacked but not paired and also flipped compared with the dsDNA region upstream. The unpaired −4A (green) is unstacked from −5G (purple), implying that the 2-AP at −4 might be similarly unstacked from the neighboring G at −5, causing a large fluorescence change.
We rule out the possibility that specific interactions with the T7 RNA polymerase such as hydrogen bonding or hydrophobic interactions increase the fluorescence of 2-AP at t (~4), because such interactions are predicted to decrease the 2-AP fluorescence (21).

Three-step Mechanism of T7 RNA Polymerase Binding to Promoter DNA—Each of the 2-AP bases in the TATA box showed an increase in fluorescence upon binding to the T7 RNA polymerase with similar kinetics indicating that the bases in the TATA region melt in a concerted manner during open complex formation. The simplest model that explained the stopped-flow kinetic data was the two-step mechanism. In this mechanism, the initial encounter of the T7 RNA polymerase with the DNA occurs at close to a diffusion-limited rate to form a closed complex ED1, which is in rapid equilibrium with free E and D. The ED1 isomerizes at an observed rate close to 150 s⁻¹ to form an open complex. It was interesting that the kinetics of the p-dsDNA promoter, which is premelted from −4 onward, showed a two-step DNA binding mechanism. The initial T7 RNA polymerase-p-dsDNA complex was formed similar to the dsDNA, but the subsequent isomerization step (~300 s⁻¹) was 2-fold faster in the p-dsDNA.

Comparison of the fluorescence values of the p-dsDNA and dsDNA in the polymerase-DNA complex under conditions where the polymerase was in excess of the DNA suggested that only 29–36% of the complex dsDNA promoter was open. We therefore incorporated a third step after ED1 formation in the dsDNA binding mechanism. The kinetic data at various polymerase concentrations were globally fit to the three-step mechanism, and the intrinsic rate constants are listed in Table I. The kinetic constants indicate that the ED complex resulting from the initial encounter of the T7 RNA polymerase with the dsDNA promoter has a $K_d$ of 1.8 μM. This complex isomerizes to EDₙ with an unfavorable equilibrium constant of 0.12, and subsequently to ED₂ with a rate constant close to 322 s⁻¹. A schematic representation of the promoter binding and open-complex formation steps is depicted in Fig. 7. We postulate that, during the formation of ED₂, the T7 RNA polymerase makes specific interactions with the conserved promoter recognition region from −17 to −5 sequence. The ED complex then isomerizes to ED₂, in which the promoter is melted from −4 to −1, but the interactions of the template strand with the T7 RNA polymerase are not extensive to stabilize the complex. The ED₂ is therefore in equilibrium with the closed complex ED₁. The ED₁ then isomerizes to ED₂, in which the template strand is directed within the active site of T7 RNA polymerase, making more extensive interactions, as seen in the recent crystal structure of the ED complex (1). The ED₂ is therefore a preinitiation complex, in which the template bases up to +2 are melted to pair with the incoming initiating GTPs for RNA synthesis. The three-step mechanism predicts an overall $K_d$ for dsDNA equal to $K_a/(1 + K_a^{-1}K_d) = 0.98$ μM (where numbers in subscript refers to step), which is in agreement with the $K_d$ determined from fluorimetric titrations and nuclease-DNA binding experiments (data not shown). Similarly, the overall $K_d$ for p-dsDNA is $K_{a1}/(1 + K_d)$ = 0.2 μM, which is similar to the $K_d$ of 0.3 nM measured by equilibrium methods reported in our earlier paper (5).

In summary, the present studies have demonstrated a unique fluorescence change arising from unstacking of the 2-AP base at the junction of binding and melting regions of the promoter template strand, between bases −4A and −5G. This unstacking may be a critical event in directing and placing the template strand correctly in the T7 RNA polymerase active site upon promoter melting for template directed RNA synthesis. However, to keep the complexed promoter open in the absence of the transcription reaction appears to be energetically unfavorable, as seen from the incomplete conversion of the closed to the open complex. Nevertheless, the observed rate of open complex formation (~150 s⁻¹) is not rate-limiting during transcription initiation.

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