Thermodynamic and Kinetic Measurements of Promoter Binding by T7 RNA Polymerase[†]

Andrea Újvári and Craig T. Martin*

Department of Chemistry, University of Massachusetts, Amherst, Massachusetts 01003-4510

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ABSTRACT: Previous steady state kinetic studies of the initiation of transcription by T7 RNA polymerase have shown that melting of the DNA helix near the transcription start site is not rate limiting [Maslak, M., & Martin, C. T. (1993) *Biochemistry 32*, 4281−4285]. In the current work, fluorescence changes in a nucleotide analog incorporated within the promoter are used to monitor changes in the DNA helix associated with polymerase binding. The fluorescence of 2-aminopurine has been previously shown to depend on the environment of the base, with fluorescence increasing in the transition from a double-stranded to a single-stranded environment [Xu, D., Evans, K. O., & Nordlund, T. M. (1994) *Biochemistry 33*, 9592−9599]. Fluorescence changes associated with polymerase binding to promoters incorporating 2-aminopurine at positions −4 through −1 support a model which includes melting, in the statically bound complex, of the region of the promoter near the start site. Equilibrium titrations at 25 °C with label at position −2 provide a thermodynamic measure of the dissociation constant (K_d = 4.8 nM) for promoter binding, while stopped-flow kinetic assays measure the apparent association ($k_1 = 5.6 \times 10^7$ M^{-1} s^{-1}) and dissociation ($k_{-1} = 0.20$ s^{-1}) rate constants for simple promoter binding (the ratio k_{-1}/k_1 = 3.6 nM, in good agreement with the thermodynamic measurement of K_d). These results suggest that binding is close to the diffusion-controlled limit and helix melting is extremely rapid. In studies of structurally altered promoters, a base functional group substitution at position −10 is shown to significantly decrease k_1, with little effect on k_{-1}. In contrast, removal of the nontemplate strand from position +1 downstream results in a large decrease in k_{-1}, with no significant effect on k_1.

T7 RNA polymerase represents an ideal model system in which to study the fundamental aspects of transcription. The single-subunit enzyme requires no protein cofactors for the highly specific and rapid initiation of transcription. It recognizes a promoter which extends only seventeen base pairs upstream from the +1 start site for transcription. Using oligonucleotide-based promoters in *in vitro* steady state kinetic assays, we have previously provided very strong evidence supporting a two-domain model for the promoter (Chapman & Burgess, 1987). The base pairs upstream of about position −5 are recognized in a duplex form, the enzyme making contacts across the major groove, extending from position −11 to about position −5 (Maslak et al., 1993; Schick & Martin, 1993, 1995; Li et al., 1996). From about position −4 to the start site, the enzyme interacts primarily with the template strand (Maslak & Martin, 1993). Indeed, complete removal of the requirement for melting at the start site, via the construction of a partially single-stranded promoter, results in only a 2-fold increase in the steady state rate for transcription initiation (Maslak & Martin, 1993). This result suggests that the consensus promoter presents a very low barrier to helix melting.

Traditional measures of DNA binding as found in filter binding or gel shift assays require that the protein−DNA complex be kinetically inert under the conditions of separation (bound to a filter or trapped within a polyacrylamide gel matrix, respectively). Evidence suggests that this requirement is not met in the case of the complex between T7 RNA polymerase and its promoter DNA (Muller et al., 1988). Furthermore, these approaches cannot be used to measure complex formation in real time.

Fluorescence from the nucleotide analog 2-aminopurine has been shown to depend on the environment of the 2-aminopurine base; specifically, the fluorescence increases in the transition from duplex to single-stranded DNA (Lycksell et al., 1987; Nordlund et al., 1989; Xu et al., 1994). This property has been exploited previously to measure the kinetics of nucleotide incorporation and excision by DNA polymerase (Bloom et al., 1993, 1994; Hochstrasser et al., 1994; Frey et al., 1995) and to measure DNA unwinding by a helicase (Raney et al., 1994). In the current study, we have incorporated 2-aminopurine at a variety of locations within the promoter to provide further evidence for regions of the promoter which are single stranded in the bound complex. Fluorescence from a 2-aminopurine reporter at one of these positions (−2) is then used as a monitor of changes in helix structure, or more generally, of enzyme binding. This approach allows for straightforward thermodynamic assays of binding, via simple titrations of enzyme into labeled DNA. It also allows for real time measurement of the kinetics of complex association and dissociation. Finally, this approach can be exploited to probe the effects of specific modifications elsewhere in the promoter on the interaction between RNA polymerase and its promoter.

EXPERIMENTAL PROCEDURES

RNA Polymerase. T7 RNA polymerase was prepared from *Escherichia coli* strain BL21 carrying the overproducing

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plasmid pAR1219 (kindly supplied by F. W. Studier), in which RNA polymerase is expressed under inducible control of the *lac* UV5 promoter (Davanloo et al., 1984; Morris et al., 1986). The enzyme was purified as described in (King et al., 1986). The enzyme was determined to be greater than 95% pure as judged by denaturing polyacrylamide gel electrophoresis.

Oligonucleotides. Oligonucleotides were synthesized by the phosphoramidite method on a Milligen/Biosearch Cyclone Plus DNA synthesizer. Reagents were from Glen Research, CPG, Biogenex, and Prime Synthesis. Detritylation was monitored throughout each synthesis to verify the efficiency of coupling. Single strands from a 1 μmol scale synthesis were purified trityl-on using an Amberchrome reverse phase resin as described previously (Schick & Martin, 1993). The analog 2-aminopurine was incorporated using standard coupling procedures but was coupled off-line to conserve reagents, using a procedure communicated to us by Hugh Mackey of Glen Research (Schick & Martin, 1995).

Kinetics Assays. Assays of transcription initiation (Martin & Coleman, 1987) were carried out in a total volume of 20 μL. Previous kinetic assays have been carried out in 30 mM HEPES,[1] pH 7.8, 15 mM magnesium acetate, 100 mM potassium glutamate, 0.25 mM EDTA, 1 mM DTT, 0.1 mg of *N*,*N*-dimethylated casein/mL (Sigma), 0.05% TWEEN-20 (Calbiochem, protein grade), 0.8 mM GTP, and 0.4 mM ATP. We have previously shown that for optimal activity dithiothreitol is not necessary, carrier protein is not required in the presence of detergent, and 25 mM potassium glutamate is sufficient for optimal transcription (Maslak & Martin, 1994). In the current study, kinetic assays were measured (with the addition of GTP and ATP, as above) in the buffer used for fluorescence: 30 mM HEPES, pH 7.8, 15 mM magnesium acetate, 25 mM potassium glutamate, 0.25 mM EDTA, and 0.05% TWEEN-20 (Calbiochem, 10% protein grade). For each template, reaction velocities were measured at various enzyme and DNA concentrations. The error in each velocity was approximated as the higher of 0.1 μM/min or the *t* distribution 80% confidence interval of the fitted slope for the three time points. Velocity data were then fit as previously described (Martin & Coleman, 1987) to the exact solution of the steady state equation, using a weighted nonlinear least-squares minimization algorithm based on the Gauss−Newton method (Johnson et al., 1981). Ranges in the values represent a 67% joint confidence interval of the fitted parameters. As a result of the nonlinear nature of the velocity equation and the potential interdependence of the fit parameters, increases in K_m typically have more confidence than do decreases (Johnson, 1983). Similarly, small changes in k_{cat} (less than a factor of 2) should be interpreted with caution.

Fluorescence Measurements. All fluorescence experiments were carried out in the fluorescence buffer (lacking nucleoside triphosphates) described above. Fluorescence titrations of single-stranded oligonucleotide onto the complementary strand indicate that at the lowest concentrations, the promoter constructs were greater than 95% duplex.

Static fluorescence experiments were performed using a QM-1 PTI (Photon Technology International) T-format fluorimeter with a 75 watt arc lamp and both emission and excitation monochrometers. The static fluorescence intensity was measured in a scan from 365 to 385 nm, with excitation at 320 nm (in order to minimize excitation of protein groups). The average fluorescence intensity was calculated by integrating the emission data in the scan from 365 to 385 nm. Excitation and emission slits were set to 2 and 9 nm for the single-point measurements (Figure 1) and to 4 and 5 nm for the titration (Figure 2). In experiments measuring the fluorescence intensity of the different labeled constructs, 500 μL of 0.5 μM 2-aminopurine-labeled single- or double-stranded DNA was used, and 9 or 30 μL of 36 μM enzyme stock solution was added to reach a final enzyme concentration of 0.64 or 2.1 μM, respectively. Fluorescence titrations were started in an initial volume of 500 μL containing 0.1 μM 2-aminopurine-labeled double-stranded DNA. Increasing amounts of a 3 or 10 μM T7 RNA polymerase stock solution were added up to a final volume of 550 μL (0.47 μM final enzyme concentration).

At a 1:1 enzyme:DNA ratio, intrinsic fluorescence from the enzyme contributes approximately 10% to the total fluorescence signal. To correct for this, in all static and kinetic fluorescence measurements a control experiment was carried out using an unlabeled oligonucleotide under otherwise identical conditions. These data were then subtracted from the experimental data. This corrects for changes not only in the fluorescence from the protein (and the remainder of the DNA), but also corrects for any possible changes of protein (or unlabeled DNA) fluorescence during the experiment. During the titration of unlabeled DNA with enzyme, minimal changes in protein fluorescence are observed.

Rapid kinetic measurements were performed using an SFM-4 BioLogic stopped flow module coupled by fiber optics to the PTI fluorimeter. The detection system was activated from an external synch-out pulse from the BioLogic stepper-motor controlling unit, and data acquisition began 10 ms before sample mixing. Excitation and emission wavelengths were 320 and 370 nm, with slit widths of 4 and 9 nm, respectively. Multiple stopped-flow measurements (typically 7) were averaged to obtain acceptable signal to noise ratios. Signal was sampled and filtered post-acquisition as indicated in the figure legends. Kinetic fits were always carried out on unfiltered data.

For the determination of forward rate constants, a solution containing 0.5 μM oligonucleotide was mixed with one containing 2.4 μM enzyme to result in final concentrations of 0.38 μM DNA and 0.6 μM enzyme. Data were collected for 10 s with a 6.8 ms dead-time. In a control experiment, the oligonucleotide solution was mixed with a buffer solution to measure the fluorescence of free DNA after mixing.

To measure dissociation rate constants, DNA containing the 2-aminopurine label was preequilibrated at the given temperature with an equimolar amount of enzyme, to yield a final concentration of 0.5 μM. This was then mixed with an 8 μM unlabeled DNA solution to yield a final solution containing 0.38 μM enzyme, 0.38 μM labeled DNA, and 2 μM trap DNA. In this case, fluorescence signal was collected for 60 s. The partially single-stranded 2-aminopurine-labeled DNA was also preequilibrated with equimolar enzyme to a final 0.5 μM concentration, but was mixed with an equal volume of 0.5 μM unlabeled partially single-stranded DNA. For the partially single-stranded construct at 37 °C and for the fully duplex DNA at 10 °C, data were

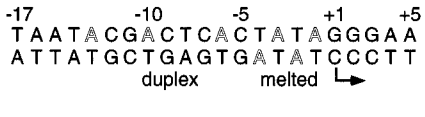
[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

collected for 10 min. Over this time course, significant photobleaching was observed. To avoid photobleaching during the long data acquisition process, after data collection for 1 min, the excitation shutter was manually closed for 40 s in every 1 min.

RESULTS

In previous studies, filter binding, gel shift, and footprinting assays have provided qualitative measures of static promoter binding by T7 RNA polymerase (Oakley et al., 1975; Gunderson et al., 1987; Chapman et al., 1988; Muller et al., 1988). Steady state kinetic assays of the synthesis of a 5-base runoff transcript have provided a quantitative measure of the effects of promoter modifications on the initiation of transcription by T7 RNA polymerase (Martin & Coleman, 1987; Maslak & Martin, 1994). In the steady state assays, the parameter K_m has been argued to approximate the static dissociation constant K_d and therefore to reflect changes in promoter binding (Martin & Coleman, 1987; Maslak & Martin, 1994); however, a more direct and thermodynamically correct assay for the simple binding of the enzyme to its promoter would provide a valuable complement to kinetic assays.

In contrast to the normal DNA bases, the base analog 2-aminopurine exhibits a substantial fluorescence signal, and the intensity of the fluorescence changes with the environment of the base (Ward et al., 1969; Nordlund et al., 1989; Xu et al., 1994). This fluorescence change has been used to follow helix melting by DNA polymerase during elongation (Hochstrasser et al., 1994) and by *Eco*RI binding to its restriction target (Lycksell et al., 1987; Nordlund et al., 1989). In the current study, fluorescence changes of 2-aminopurine incorporated into an oligonucleotide-based T7 promoter are used to probe for local structural changes in the promoter accompanying polymerase binding, to provide a true thermodynamic measure of promoter binding, and to measure the kinetics of promoter association and dissociation in the absence of nucleotide triphosphates.



Incorporation of 2-Aminopurine Minimally Perturbs Promoter Function. The adenine analog 2-aminopurine can form two Watson−Crick hydrogen bonds with thymine and so, to a first approximation, can replace adenine within the promoter sequence with a minimal perturbation of the overall promoter structure (although locally, the major and minor grooves are significantly perturbed). It is likely that, in regions of the DNA in which the polymerase directly contacts major or minor groove functional groups, incorporation of a reporter analog could disrupt binding and/or the initiation of transcription. To test for this, steady state kinetic assays were carried out for the constructs containing 2-aminopurine at positions −13, −10, −6, −3, and −2. The results summarized in Table 1 reveal a previously unidentified contact at position −13 (K_m increases substantially). From this single substitution, the exact nature of the contact cannot be identified; however, the duplex binding model predicts a minor groove contact. At position −10, although recognition appears to be via a major groove interaction with the adenine 6-amino group (Schick & Martin, 1995), the replacement of adenine leads to no significant change in K_m. This result is readily compared with that from the incorporation of purine at this position, which in the previous study led to a very small increase in K_m, presumably due to the positioning of water to mimic the 6-amino group. Finally, the large increase in K_m accompanying the substitution at position −6 is consistent with previous studies which implicate major groove recognition at this position (Li et al., 1996).

Table 1: Steady State Kinetics of Promoters Containing 2-Aminopurine[a]

construct	K_m (nM)	k_{cat} (s^{-1})
native	10 (6−16)	0.60 (0.57−0.65)
-13_N	88 (55−160)	0.25 (0.20−0.30)
-10_N	4.1 (2.3−7.5)	0.72 (0.63−0.82)
-6_N	66 (34−133)	0.42 (0.32−0.52)
-3_N	1.9 (0.7−4.5)	1.0 (0.93−1.1)
-2_T	8.7 (4.5−17)	0.48 (0.45−0.55)

[a] Steady state assays of the initiation of transcription at 37 °C, carried out in the fluorescence buffer, as described in Experimental Procedures.

At position −3, where the thymine methyl on the template strand has been identified as the primary recognition contact (Maslak et al., 1993; Ho, Maslak, and Martin, unpublished results), incorporation of 2-aminopurine results in no change in K_m, while k_{cat} increases slightly. The increase in k_{cat} is consistent with the fact that, in general, the base pair between 2-aminopurine and thymine is weaker than an A-T pair (Lycksell et al., 1987; Nordlund et al., 1989; Xu et al., 1994), and with our previous observation that complete removal of the barrier to melting leads to a 2-fold increase in k_{cat} (Maslak & Martin, 1993).

Probe of Melting in Various Regions of the Promoter. To a first approximation, the fluorescence of 2-aminopurine is low in duplex DNA and higher in single-stranded DNA (Nordlund et al., 1989; Xu et al., 1994). This property can be used to test a previously developed model for promoter recognition (Chapman & Burgess, 1987; Li et al., 1996). In this model, DNA upstream of about position −5 remains duplex in the bound complex, while DNA downstream of position −5 is melted. The placement of 2-aminopurine at various positions within the promoter provides a simple test of this model. The results summarized in Figure 1 compare the fluorescence of 2-aminopurine substituting for adenine at positions -13_N, -10_N, -6_N, -4_T, -3_N, -2_T, and -1_N (the subscript denotes incorporation into the nontemplate or template strand). For each construct in which 2-aminopurine replaces a consensus sequence adenine, three fluorescence measurements are reported. The first, labeled "ssDNA", represents the fluorescence from the appropriate single-stranded oligonucleotide (either template or nontemplate strand), to control for sequence context effects. The second measurement, labeled "dsDNA", represents fluorescence from the same construct annealed to its complementary strand. Finally, the measurement labeled "dsDNA+enzyme" represents the fluorescence of the RNA polymerase-promoter complex (0.64 μM polymerase and 0.50 μM DNA). Although protein fluorescence is relatively small for fluorescence excitation at 320 nm with observation near 370 nm, in these and the experiments which follow, as a control, we subtract the measurement for a parallel experiment lacking the 2-aminopurine label.

Previous experiments suggest that the region from position −13 to −6 is double stranded in the recognition complex

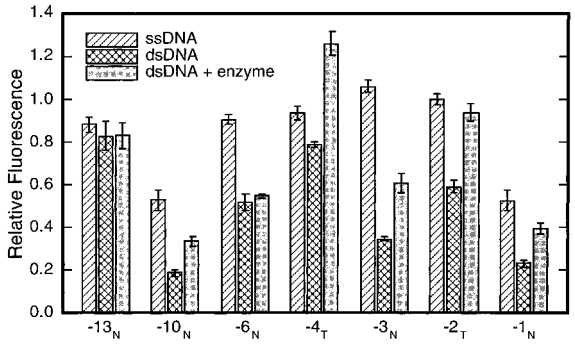


FIGURE 1: Relative fluorescence intensity for 2-aminopurine placed at various positions within the T7 RNA polymerase promoter, measured at 25 °C. For each construct, fluorescence was measured for the single-stranded oligonucleotide (ssDNA), for the corresponding duplex (dsDNA), and for the enzyme−promoter construct (dsDNA+enzyme). Each value represents an average of three measurements, and the error bars represent the simple standard deviation of those measurements. Concentrations of DNA and enzyme were 0.50 and 0.64 μM, respectively. The nomenclature describing each modification indicates the position and the strand containing 2-aminopurine. The sequence of the 22-base oligonucleotide is given in the text.

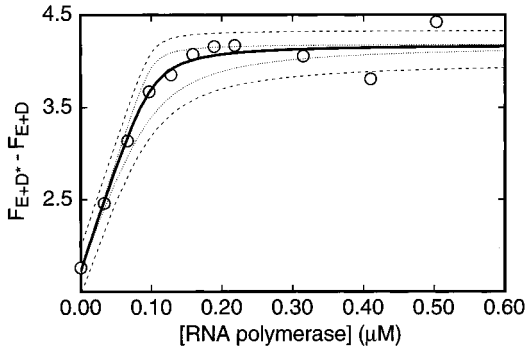


FIGURE 2: Equilibrium titration of T7 RNA polymerase onto duplex promoter at 25 °C. Fluorescence from 2-aminopurine incorporated on the template strand at position −2 is excited at 320 nm and monitored from 365 to 385 nm, as described in Experimental Procedures. Concentration of promoter is 0.10 μM. The fluorescence of unbound and fully bound DNA were fit simultaneously with the value for K_d = 4.8 (1.2−15) nM, shown as a solid line. The dotted lines represent the 67% confidence interval, varying only K_d, while the dashed lines represent the 67% joint confidence interval for all three parameters.

(Li et al., 1996). The current fluorescence results show only small changes in the fluorescence associated with enzyme binding to double-stranded promoter containing 2-aminopurine at positions -13_N, and -6_N. Assuming that the steady state K_m represents an upper limit for the dissociation constant K_d, the complex should be greater than 75% formed under these conditions. Nevertheless, measurements were carried out at higher concentrations (2.1 μM enzyme), where greater than 95% occupancy is expected. No change was observed for the substitution at position -13_N, while a small increase was observed for the construct containing 2-aminopurine at position -6_N. At position -10_N, where previous studies point strongly to major groove recognition, a significant increase in fluorescence is observed accompanying complex formation. These results may suggest a perturbation of the DNA helix associated with binding.

Large changes in fluorescence are associated with enzyme binding to promoter containing 2-aminopurine at positions -4_T, -3_N, -2_T, and -1_N. The increase in fluorescence associated with enzyme binding to promoter containing 2-aminopurine at positions −4 to −1 is consistent with previous studies which predict that the "TATA" bases immediately adjacent to the start site should be melted in the complex. Consistent with the two-domain model, this demonstrates that in the statically bound complex, the polymerase is melted into the DNA in this region. Incorporation of 2-aminopurine at position −2 has no measurable effect on the steady state kinetics but shows a large increase in fluorescence accompanying polymerase binding, making this an ideal site for the incorporation of a reporter probe.

Thermodynamic Measurements of Promoter Binding. Changes in the fluorescence of 2-aminopurine at position −2 accompanying titration of enzyme into a solution of the promoter provide a direct measure of the binding thermodynamics. The data presented in Figure 2 show such a titration at 25 °C, with a best-fit binding curve for K_d = 4.8 (1.2−15) nM [values in parentheses represent a joint confidence interval of the nonlinear fit (Johnson, 1983)]. Measurements with 2-aminopurine at position −3 give very similar results, yielding K_d = 13 (7−24) nM, further confirming the observations.

These thermodynamic titrations are generally consistent with the steady state kinetic assays, which show K_m in the range from 1 to 6 nM at temperatures from 22 to 42 °C (Maslak & Martin, 1993). They are also in reasonable agreement with quantitative footprinting studies which reported values of K_d ranging from 10 to 25 nM (Gunderson et al., 1987). This basic agreement further supports the conclusion from the above steady state kinetic assays that incorporation of 2-aminopurine at these positions does not substantially alter the interaction between RNA polymerase and the promoter.

Kinetic Measurements of Promoter Binding. The presence of a fluorescent probe also allows measurement of the *kinetics* of association and dissociation in a stopped-flow assay. The association time course presented in Figure 3A shows the change in fluorescence of 2-aminopurine at position −2 of the promoter, after mixing of enzyme and DNA. The data are fit to the simple equation

$$E + D^* \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ED^*$$

where D* represents fluorescently labeled promoter DNA. A fit of the data (at 25 °C) to this kinetic scheme results in an apparent association rate constant k_1 = 5.6 (4.6−6.9) × 10^7 M^{-1} s^{-1} (in this experiment, k_{-1} is underdetermined and cannot be fit well), revealing that the on-rate for promoter binding is very fast. Under the current experimental conditions, binding is complete within a very short observation time, such that with the current level of signal-to-noise, the value for k_1 should be considered a lower limit. Also note that the fluorescence change must arise from changes in base stacking (presumably from melting) of the base pair containing the 2-aminopurine, which must occur after or simultaneous with polymerase binding. It is possible therefore that binding in a closed complex occurs on an even more rapid time scale.

The above data are very sensitive to k_1, but insensitive to k_{-1}. A separate experiment, however, *can* provide a determination of the dissociation rate k_{-1}. In this case, enzyme

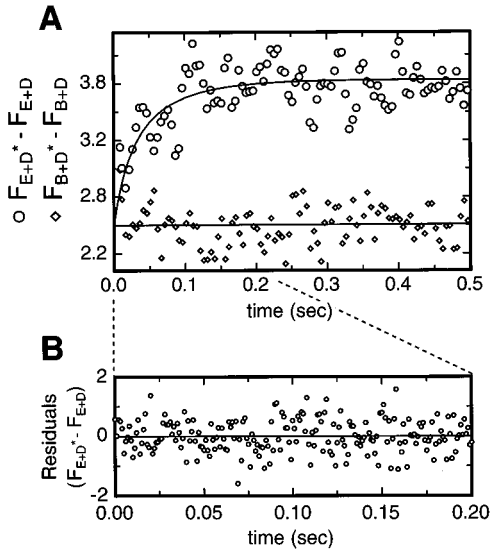


FIGURE 3: Stopped-flow kinetics of association for the polymerase−promoter complex at 25 °C. Fluorescence from the duplex promoter containing 2-aminopurine at position −2 was monitored at 370 nm, with excitation at 320 nm. For the association reaction (○) data presented in panel A, 0.5 μM DNA was rapidly mixed with 2.4 μM RNA polymerase to yield final concentrations of 0.38 and 0.60 μM, respectively. A control (◇) was run containing only buffer in place of enzyme, and the zero intercept was used as the zero point in the fit of the association reaction. The best fit curve for second-order association is shown with $k_1 = 5.6$ (4.6−6.9) $\times 10^7$ M^{-1} s^{-1}. The fluorescence signal was sampled at a rate of 1000 s^{-1}; for the presentations above, the data were averaged over 10 points and every fifth point is shown. Panel B shows the residuals for the fit in the early part of the time course (in this case, the scatter of the unfiltered data is presented).

is pre-incubated with promoter DNA containing 2-aminopurine at position −2, and then unlabeled "trap" DNA (containing adenine at position −2) is added in a stopped-flow experiment. In this experiment, it is impractical to add a sufficiently large excess of unlabeled DNA so that the coupled equilibrium can be ignored. Consequently, the data are fit to the exact solution of the coupled rate equations:

$$ED^* \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} E + D^* \qquad E + D \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ED$$

where D* refers to promoter DNA containing the fluorescent 2-aminopurine at position −2 and D refers to the unlabeled trap DNA. This treatment assumes only that the rate constants are unaffected by the presence of 2-aminopurine at position −2 and assumes appropriate starting conditions based on the pre-equilibrium of labeled promoter. The final concentrations are defined by the solution of the coupled equilibrium, while the kinetics are described by a double exponential, with apparent decay constants which are functions of k_1, k_{-1}, $[D]_t$, and $[E]_t$ (Johnson, 1992).

The data presented in Figure 4 are best-fit by an apparent dissociation rate constant of $k_{-1} = 0.20$ (0.18−0.21) s^{-1} (in this experiment k_1 is highly underdetermined). Combining this kinetic result with the previous, we calculate that the apparent equilibrium dissociation constant is $K_d = k_{-1}/k_1 =$ 3.6 (2.6−4.6) nM, consistent with the binding measurements above and with the K_m determined by steady state kinetic analyses (Maslak & Martin, 1994). This off-rate is also consistent with unpublished results from our laboratory and with results from the laboratory of McAllister and co-workers (Diaz et al., 1996), in which preincubation of enzyme and promoter, followed by a 1 min incubation with an excess of a challenge promoter, results in no detectable RNA formation from the preincubated promoter.

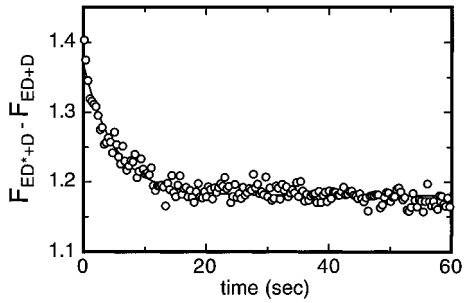


FIGURE 4: Stopped flow kinetics of dissociation for the polymerase-promoter complex at 25 °C. Fluorescence from the duplex promoter containing 2-aminopurine at position −2 was monitored at 370 nm, with excitation at 320 nm. Dissociation was measured by use of a nonfluorescent trap promoter. DNA containing the 2-aminopurine label was preequilibrated at 25 °C with an equimolar amount of enzyme, to yield a final concentration of 0.5 μM. This was then mixed with an 8 μM solution of unlabeled DNA to yield a final solution containing 0.38 μM enzyme, 0.38 μM labeled DNA, and 2.0 μM trap DNA. Data were sampled at a rate of 150 s^{-1}; in the presentation above, data were averaged over 50 points and every fiftieth point is shown. The best fit curve for first-order dissociation, as described in the text, is shown, with $k_{-1} = 0.20$ (0.18−0.21) s^{-1}.

These data are summarized in Table 2, which also includes measurements at 37 and 10 °C. It is clear that the on-rate k_1 shows a significantly weaker temperature dependence than does the off-rate k_{-1}. Previous studies of the temperature dependence of k_{cat} show good Arrhenius behavior between 22 and 37 °C, with an apparent activation energy of 26 kcal mol^{-1} (Maslak & Martin, 1993). More limited Arrhenius analysis of the current data for k_{-1} lead to an apparent activation energy of 15 kcal mol^{-1}.

Kinetic Studies of a Modified Promoter. As an initial test of the utility of these assays in characterizing previously identified promoter contacts, we have examined the kinetics of binding of RNA polymerase to a promoter containing the substitution dAdT→dGdT at position −10. For the T7 RNA polymerase promoter, the adenine at position −10 on the nontemplate strand has been previously identified as an upstream specificity contact (Martin & Coleman, 1987; Schick & Martin, 1993, 1995). Specifically, the 6-amino group has been shown to contact Asn748 of the protein (Raskin et al., 1992). The locations of Asn748 on the protein (Sousa et al., 1993) and position −10 of the promoter place this contact clearly in the duplex binding domain of the two-domain model.

Previous steady state kinetic data have shown that for this substitution, K_m increases from 2 to 32 nM (Schick & Martin, 1995). Kinetic measurements reveal that the apparent association rate constant at 25 °C ($k_1 = 3.6 \times 10^6$ M^{-1} s^{-1}) is much slower for the mutant promoter than for the native promoter. This 16-fold reduction in k_1 compares well with the 16-fold increase in K_m (at 37 °C) and confirms a role for this base in promoter binding. Measurement of the dissociation kinetics (in a challenge with native promoter, as above) yields $k_{-1} = 0.25$ s^{-1}, revealing that this substitution has little effect on the apparent off-rate of the enzyme−DNA complex.

Local Helix Formation Influences the Dissociation Rate. In previous studies, we have shown that removal of the requirement for helix melting near the start site, by the use

Table 2: Association/Dissociation Kinetics[a]

temp (°C)	k_1 ($\times 10^7$ M^{-1} s^{-1})	k_{-1} (s^{-1})	k_{-1}/k_1 (nM)	K_m^{calc} (nM)	K_m (nM)	k_{cat} (s^{-1})
Fully Duplex						
37	3.9 (3.0−4.8)	0.48 (0.46−0.49)	12	25	2.0 (0.8−4.2)	0.47 (0.45−0.49)
25	5.6 (4.6−6.9)	0.20 (0.18−0.21)	3.6	6	5.2 (1.6−15)[b]	0.13(0.11−0.14)[b]
10	3.4 (3.0−3.9)	0.05 (0.046−0.056)	1.5	2	na	0.009[c]
Partially Single-Stranded Promoter[d]						
37	5.7 (4.5−7.3)	0.0071 (0.0066−0.0075)	0.12	18	1.9 (0.6−5.5)	1.03 (0.95−1.12)
Duplex Promoter Containing dAdT→dGdT at Position −10						
37	1.7 (1.2−2.1)	0.38 (0.30−0.48)	22	47	32 (20−51)	0.42 (0.37−0.48)
25	0.36 (0.35−0.38)	0.25 (0.24−0.26)	69	na	na	na

[a] Kinetic parameters k_1 and k_{-1} are derived from the fluorescence change in a promoter construct containing 2-aminopurine at position −2. The steady state kinetic parameters K_m and k_{cat} were reported previously (Maslak & Martin, 1993; Schick & Martin, 1995), under the standard reaction conditions, which vary slightly from the conditions for the fluorescence measurements, as described under Experimental Conditions. The value for K_m^{calc} is calculated from the reported values for k_1, k_{-1}, and k_{cat}, as described in the text. [b] Measured at 27 °C. [c] k_{cat} at 10° C is extrapolated from Arrhenius behavior (Maslak & Martin, 1993). [d] This 22-base construct consists of the consensus nontemplate strand from position −17 to −1, and the corresponding template strand (with 2-aminopurine at position −2) reaches from position −17 to +5.

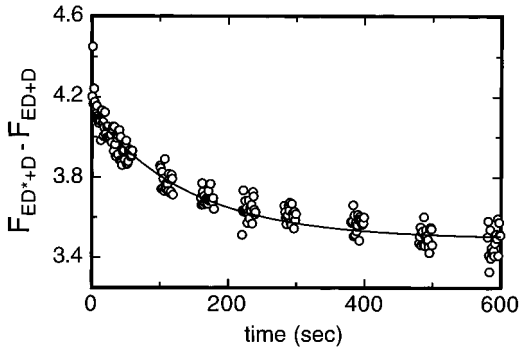


FIGURE 5: Stopped-flow kinetics of dissociation at 37 °C for a polymerase−promoter complex in which the promoter lacks the nontemplate bases from position +1 through +5. Conditions are similar to those for Figure 3, except that final concentrations of enzyme, labeled DNA, and unlabeled trap DNA were 0.25, 0.25, and 0.25 μM, respectively. Note that in order to avoid photobleaching, the excitation shutter was kept closed during part of the reaction time course. Data were sampled at a rate of 15 s^{-1}; in the presentation above, data were averaged over 15 points and every fifteenth point is shown. The best fit curve for dissociation is shown, with $k_{-1} = 0.0071$ (0.0066−0.0075) s^{-1}.

of a partially single-stranded promoter construct, leads to a 2-fold increase in the observed steady state parameter k_{cat}, with only a slight effect on K_m (Maslak & Martin, 1993). These data were interpreted in terms of a model in which helix melting plays only a small role in the formation of an initiation competent complex. In order to ask whether local helix structure plays a role in binding, we can now directly measure the association and dissociation rates for one of the previously characterized partially single stranded promoter constructs. The data in Figure 5 and Table 2 compare k_1 and k_{-1} for binding of T7 RNA polymerase to a promoter construct analogous to the others used in this study, but which is double stranded from position −17 to −1 and contains only the template strand from position +1 to +5 (the 2-aminopurine fluorescent label is at position −2). The results show that removal of the requirement for melting downstream of the start site has no significant effect on the apparent association rate constant, consistent with the idea that helix melting does not limit binding. In contrast, removal of the nontemplate strand leads to a 70-fold reduction in the apparent dissociation rate. This suggests a role for the nontemplate strand in the mechanism of dissociation.

DISCUSSION

RNA polymerases efficiently catalyze the template-dependent, processive synthesis of polynucleotides. In addition, they must first recognize a specific site within the DNA, facilitate opening of the helix, and then initiate the unprimed synthesis of RNA. A simple model for such functioning might separate initial binding of the enzyme to a closed duplex (as observed for repressors and other regulatory proteins) from helix melting and subsequent catalysis. Indeed, results from numerous studies of the RNA polymerase from *E. coli* suggest that the enzyme binds first to a closed duplex and subsequently melts into the DNA (Buc & McClure, 1985; McClure, 1985). The RNA polymerase from the bacteriophage T7 is highly specific for a relatively short consensus promoter and initiates synthesis rapidly and with high fidelity. As such, it represents an ideal model system in which to study fundamental mechanisms in sequence specific initiation of transcription. A previous steady state kinetic study has shown that in this system, helix melting presents only a small barrier to the initiation of transcription.

2-Aminopurine As a Probe of Polymerase Binding. The fluorescence spectrum of 2-aminopurine in solution is characterized by an excitation maximum at 305 nm and an emission maximum at 370 nm (Gräslund et al., 1987). Incorporation of the 2-aminopurine nucleoside into an oligonucleotide results in a reduction in the emission intensity (Nordlund et al., 1989; Guest et al., 1991; Xu et al., 1994). The fluorescence is reduced still further on formation of duplex DNA, making 2-aminopurine an ideal probe of local helix environment (Nordlund et al., 1989; Evans et al., 1992; Xu et al., 1994). We have exploited this property to develop a new approach to studying DNA binding and helix melting by T7 RNA polymerase. In this initial survey, 2-aminopurine is substituted in place of adenine at several positions within the consensus T7 RNA polymerase promoter. Fluorescence measurements are combined with steady state kinetic assays to provide information on the polymerase−promoter complex.

The results in Figure 1 demonstrate that at all but position −13, the fluorescence decreases significantly on formation of the DNA duplex, as expected. The large increase in K_m associated with incorporation of 2-aminopurine at position −13 provides information on a region of the promoter not

previously probed by base analog substitution. This result combined with the observation that the fluorescence from 2-aminopurine at position −13 does not decrease on formation of duplex promoter may indicate an unusual structural role for this region, perhaps involving unusual base stacking (Nordlund et al., 1994).

Surprisingly, at position −10, where extensive studies have shown exclusive recognition of major groove functional groups (Jorgensen et al., 1991; Schick & Martin, 1993, 1995), the 2-aminopurine fluorescence increases with DNA binding. At this position, Asn748 in the protein makes a hydrogen bond contact to the adenine 6-amino group (Raskin et al., 1992). In the case of purine, as for 2-aminopurine, it has been argued that water can mediate an interaction between Asn748 and the exocyclic 6-hydrogen (Schick & Martin, 1995). The change in fluorescence may reflect a change in the stacking of 2-aminopurine with neighboring bases necessary to optimize the exocyclic contact (Nordlund et al., 1994).

At position −6, previous studies identified the 5-methyl group of thymine in the major groove as a recognition element (Maslak et al., 1993). The increase in K_m associated with the incorporation of 2-aminopurine suggests a second contact at this position. The adenine 6-amino group is the most likely contact, although the introduction of an amino group into the minor groove at this position could interfere with interactions in the minor groove, and previous studies have identified a potential minor groove contact at the adjacent position −5 (Li et al., 1996).

At all four positions within the TATA sequence, the fluorescence of the label then increases significantly on subsequent binding of RNA polymerase, consistent with formation of an open complex. At position −4, the fluorescence from 2-aminopurine increases to a level higher than that of the single-stranded control. This behavior has previously been proposed to arise from the loss of base stacking, without complete melting of the duplex (Nordlund et al., 1994). In principle, the excitation spectrum of 2-aminopurine can also provide information on the environment of the base (Evans et al., 1992; Nordlund et al., 1994). Such an analysis in this system is made difficult by the intrinsic fluorescence of the protein.

Thermodynamic Measure of Promoter Binding. The above results suggest some interesting new aspects to recognition within the upstream, duplex region of the promoter. The results from the placement of 2-aminopurine in the downstream TATA region are completely consistent with melting of this region in the enzyme−DNA complex (Maslak & Martin, 1993). In any case, regardless of the precise details leading to the fluorescence change associated with enzyme binding in this region, the change in fluorescence can be used as a measure of binding and/or helix melting in various thermodynamic and kinetic assays. The steady state kinetic assays using promoters containing 2-aminopurine at position −3 or −2 reveal no significant perturbation resulting from the substitution.

The titration of T7 RNA polymerase onto a promoter containing 2-aminopurine at position −2, presented in Figure 2, demonstrates simple formation of 1:1 RNA polymerase: promoter complex. The apparent dissociation constant, K_d = 4.8 nM at 25 °C, is consistent with previous steady state kinetic and quantitative footprinting measurements (Gunderson et al., 1987; Martin & Coleman, 1987; Maslak & Martin, 1994). A similar titration for a promoter construct containing 2-aminopurine at position −3 yields K_d = 13 nM at 25 °C, which lies within the uncertainties of the measurement. It appears that this approach provides a thermodynamically valid measure of static binding and will prove a valuable complement to kinetic assays in assessing specific contributions to recognition. In particular, the steady state kinetic assays are restricted to promoter constructs and enzyme variants which are capable of the initiation of transcription.

Kinetics of Promoter Association. Using the construct with 2-aminopurine at position −2, the kinetics of association and dissociation can be readily determined. More specifically, at this position the change in fluorescence almost certainly arises from the local melting of the DNA helix. It is possible, therefore, that binding to a closed duplex occurs first, followed by a slower conversion of the complex to an open form. In this case, the fluorescence change would monitor the latter step and the measured rate constant would represent a lower limit for simple association. However, the kinetics of the fluorescence change observed in Figure 3A, with $k_1 = 5.6\ (4.6-6.9) \times 10^7\ M^{-1}\ s^{-1}$, represent binding at close to the diffusion limit (Berg & von Hippel, 1985; von Hippel & Berg, 1989). Thus, helix melting must occur either coincident with or very shortly after DNA binding but is extremely rapid in any case.

To provide further insight into this question, removal of the nontemplate strand from position +1 downstream to the end of the template (including three GC pairs at positions +1 through +3) should significantly reduce any barrier to melting at position −2. The kinetics of the fluorescence change measured for polymerase binding to this construct do not increase significantly, supporting the proposal that the fluorescence change monitors DNA binding, rather than a slower melting step. It may also be proposed that the use of a short oligonucleotide artificially facilitates melting, specifically, that the presence of duplex beyond position +5 would increase the barrier to melting at a position seven base pairs upstream. However, previous steady state kinetic results (which have been shown to be sensitive to helix melting), show no significant change on a much longer duplex construct (Maslak & Martin, 1993).

Further evidence that helix melting is not rate determining comes from analysis of the kinetics on a promoter containing the substitution dAdT→dGdT at position −10. Previous steady state kinetic studies showed that this substitution leads to a large increase in K_m with no effect on k_{cat} (Schick & Martin, 1995). The position of this substitution ten base pairs upstream of the start site for transcription, combined with the previously identified role of the AT pair as a major groove recognition contact, makes it very unlikely that this base pair would play a direct role in helix melting but rather should be involved in initial binding. The current kinetic measurements at 25 °C show that this substitution leads to a large decrease in k_1, supporting the proposal that k_1 measures the rate-limiting step in binding.

Kinetics of Promoter Dissociation. In order to measure the kinetics of enzyme−DNA complex dissociation, a second, nonfluorescent promoter can be used to trap enzyme which dissociates from a preformed complex with labeled DNA. In this case, the fluorescence change occurs as the two DNA strands reanneal. Consequently, it is conceivable that the enzyme dissociates on a faster than observed time

scale and that the fluorescence change monitors the subsequent closure of the helix. In this case, k_{-1} would represent a lower limit for the true dissociation rate constant. Nevertheless, the apparent first-order dissociation constant measured at 25 °C, $k_{-1} = 0.20\ s^{-1}$, is completely consistent with binding and association kinetics results presented above. Specifically, the ratio $k_{-1}/k_1 = 3.6$ nM is indistinguishable from the apparent equilibrium constant, $K_d = 4.8$ nM, supporting the simple interpretation that k_{-1} is a true measure of the rate of complex dissociation.

Unlike the behavior observed for the association rate constant k_1, the dissociation rate constant k_{-1} shows a significant temperature dependence, with a preliminary apparent activation energy of 15 kcal mol^{-1}. Both the value of k_{-1} and its Arrhenius behavior are comparable to k_{cat}, which may reflect some commonality in origin; however, other aspects of these parameters are distinctly different. In particular, removal of the nontemplate strand from position +1 through +5 leads a 2-fold increase in k_{cat} (Maslak & Martin, 1993) but to a more than 50-fold decrease in the measured value for k_{-1}. In the partially single-stranded construct used here, the duplex ends only two base pairs away from the probe, such that helix dynamics following complex dissociation are unlikely to result in kinetics with a half-life of 100 s. Rather, these kinetics must measure an effect on complex dissociation. Further support for this interpretation comes from recent results in which a complex actively synthesizing a short runoff transcript is challenged with a different, trap promoter (Diaz et al., 1996). In the case of a fully duplex promoter, the enzyme switches to the trap in less than 1 min. However, for a partially single-stranded construct like that used here, the enzyme cycles on the original promoter for more than 20 min. In contrast to previous models which give the nontemplate strand in this region no role in recognition (Maslak & Martin, 1993; Li et al., 1996), these data suggest that nontemplate strand contacts and/or local helix stability play a role in the dissociation of the binary complex.

Comparison with Previous Kinetic Results. The thermodynamic and kinetics results obtained from the current work can be compared with results from steady state kinetic assays. Specifically, it has been argued that the steady state parameter K_m largely reflects changes in promoter binding (Maslak & Martin, 1994). Given the simplest steady state kinetic model

$$E + D \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ED \xrightarrow{k_{cat}} E + D + R$$

$$K_m = \frac{k_{-1} + k_{cat}}{k_1}$$

in which an initial step in the initiation of transcription is assumed to be rate determining (Martin & Coleman, 1987; Maslak & Martin, 1994), then the apparent Michaelis constant K_m can be calculated from the measured values for k_1, k_{-1}, and k_{cat}. These values are compared in Table 2 with the observed values for K_m. At 25 °C, the predicted and observed values are in complete agreement, although the level of agreement may be fortuitous; agreement at 37 °C is good only within an order of magnitude (under current conditions, the forward association constant is the least well-determined of the parameters and, as mentioned in the previous section, should be considered a lower limit). This analysis also shows that in the above equation for K_m, the parameter k_{cat} is comparable in magnitude to k_{-1} and so cannot be ignored. To the extent that the above steady state equation is valid, K_m will be highly sensitive to increases in either k_{cat} or k_{-1} but will be reduced by a maximum of about 0.5 for decreases in either parameter. Hence, the proposition that changes in K_m reflect changes in binding would be true more for increases in K_m than for decreases.

Conclusions. The current study demonstrates that the changes in fluorescence of 2-aminopurine incorporated within the promoter DNA can be effectively used to measure both the thermodynamics and kinetics of the interaction between T7 RNA polymerase and its promoter. The applicability of this approach to characterize changes in potential recognition contacts has been demonstrated with preliminary analysis of promoters containing modifications in the DNA structure. Finally, the results provide strong evidence further supporting a model for the initiation of transcription in which local melting of the helix is not rate determining.

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