T7 Lysozyme Represses T7 RNA Polymerase Transcription by Destabilizing the Open Complex during Initiation

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Running title: Regulation of Transcription by T7 lysozyme

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Summary

Bacteriophage T7 lysozyme binds to T7 RNA polymerase and inhibits transcription initiation and the transition from initiation to elongation. We have investigated each step of transcription initiation to determine where T7 lysozyme has the most effect. Stopped-flow and equilibrium DNA binding studies indicate that T7 lysozyme does not inhibit the formation of the pre-initiation open complex (open complex in the absence of initiating nucleotide). T7 lysozyme however does prevent the formation of a fully open initiation complex (open complex in the presence of the initiating nucleotide). This is consistent with the results that in the presence of T7 lysozyme the rate of G-ladder RNA synthesis is about 5-fold slower and the GTP $K_d$ is about 2-fold higher, but T7 lysozyme does not inhibit the initial rate of RNA synthesis with a premelted bulge-6 promoter (bubble from $-4$ to $+2$). Neither the RNA synthesis rate nor the extent of promoter opening is restored by increasing the initiating nucleotide concentration indicating that T7 lysozyme represses transcription by interfering with the formation of a stable and a fully open initiation bubble or by altering the structure of the DNA in the initiation complex. As a consequence of the unstable initiation bubble and/or the inhibition of the conformational changes in the N-terminal domain of T7 RNAP, T7 lysozyme causes an increased production of abortive products from 2-mer to 5-mer that delays the transition from the initiation to the elongation phase.
Abbreviations:

RNAP, RNA polymerase; 3’-dGTP, 3’-deoxyguanosine-triphosphate; ss, single stranded; ds, double stranded; 2-AP, 2-aminopurine; bp, base-pair; nt, non template; t, template; nt, nontemplate.
INTRODUCTION

The regulation of transcription in bacteriophage T7 RNA polymerase (RNAP) as in all RNA polymerases is dependent on the efficiency of each step of transcription, such as binding promoter DNA, binding nucleotides, overcoming abortive synthesis, RNA synthesis, and termination. Each of these steps is therefore a potential target for regulation. T7 transcription regulation begins with the gradual entry of the phage DNA into a host *Escherichia coli* cell (1) followed by the ability of the T7 RNAP, without the aid of auxiliary factors, to recognize and transcribe the phage promoters (2;3). The phage then makes T7 lysozyme, which represses transcription by T7 RNA polymerase. Unlike the LacI or the LexA repressors, which sterically block promoters from their polymerases, T7 lysozyme binds the T7 RNAP, not the DNA, forming a tertiary complex with the polymerase and DNA (4-7). T7 lysozyme binds to the T7 RNAP at a site distal to the polymerase active site as confirmed by both biochemical data and a crystal structure of the T7 RNAP-T7 lysozyme complex (8-10).

Previous studies have shown that T7 lysozyme inhibits transcription initiation and promoter clearance, but not elongation (6;7). However, the exact mechanism by which these processes are inhibited is not known. We have therefore investigated T7 lysozyme inhibition under pre-steady state conditions enabling us to more fully dissect the steps that are altered during transcription. Transcription initiation occurs with a minimum of three steps (Reaction 1). In the first step, RNAP (E) binds to the promoter DNA (D) to form a closed complex, EDc, which isomerizes to form the pre-initiation open complex,
EDo. The pre-initiation open complex binds the initiating nucleotides (N) to form the initiation complex, EDoNN.

\[ E + D \leftrightarrow E D_c \leftrightarrow E D_o \leftrightarrow E D_o N N \]  

Reaction 1

A stopped-flow kinetic study of DNA binding showed that T7 lysozyme has a very small effect on the observed rate of formation of the pre-initiation open complex. In the presence of T7 lysozyme the initial RNA synthesis rate is slow even with saturating initiating nucleotide. The 2-AP fluorescence of the DNA indicates that the promoter in the initiation complex is not fully open. This is consistent with the results that T7 lysozyme affects the rate of initiation on a duplex promoter, but not on a permanently open promoter DNA or a promoter DNA with even a single mismatch in the initiation region. The destabilization or the alteration of the structure of the initiation complex by T7 lysozyme is responsible for the inhibition of initial RNA synthesis and could also be the cause for the formation of more abortive products, which in turn delays the transition from initiation to elongation.
**EXPERIMENTAL PROCEDURES**

*Synthetic DNA and other Materials*

The oligodeoxynucleotides (unmodified and 2-AP-modified) were synthesized by Integrated DNA Technologies (Coralville, IA) and supplied as desalted samples. As described previously (11), the oligodeoxynucleotides were further purified by polyacrylamide gel electrophoresis, electroelution, and ethanol precipitation. The 3′-dGTP was purchased from TriLink Biotechnologies (San Diego, CA).

*Protein*

T7 RNAP was over-expressed in *E. coli* BL21/pAR1219 (12). The enzyme was purified as described previously (13-15) with the exception that the CM-Sephadex separation step was eliminated. The purified enzyme was stored at −80 °C in 20 mM sodium phosphate, pH 7.7, 1 mM trisodium EDTA, 1mM dithiothreitol, 100 mM sodium chloride and 50% (v/v) glycerol. The enzyme concentration was calculated from its absorbance at 280 nm and molar extinction coefficient of 1.4x10⁵ M⁻¹cm⁻¹ (16). T7 lysozyme was purified according to a reported procedure (10). The purified enzymes were checked for the lack of DNA exonuclease activity.

*Presteady State kinetics of RNA synthesis*

The pre-steady state kinetic experiments were carried out on a rapid chemical quenched-flow instrument (KinTek Corp., Austin, TX). As a general protocol, T7 RNAP and DNA
preincubated either in the presence or absence of T7 lysozyme in buffer X (50 mM Tris-acetate, pH 7.5, 100 mM sodium acetate, 10 mM magnesium acetate, 5 mM DTT) were mixed rapidly with NTPs containing [γ-32P] GTP in buffer L (50 mM Tris-acetate, pH 7.5, 10 mM magnesium acetate, 5 mM DTT). Additional magnesium acetate was added to the NTP solution to maintain a constant amount of free Mg2+. The temperature was maintained at 25 °C using a water bath in all pre-steady state experiments. After predetermined time intervals the reaction was quenched by rapidly mixing 1N HCl from a third syringe. Chloroform was then added and the reactions neutralized by the addition of base (0.25 M Tris base and 1 M sodium hydroxide). The RNA products were resolved by electrophoresis on a highly cross-linked (23 % polyacrylamide/ 3 % bis-acrylamide/ 3 M urea) gel at 55 °C (110 W) on a BioRad sequencing gel apparatus (0.25 mm spacers and comb). The gels were exposed to a phosphor screen, scanned on a Typhoon instrument (Molecular Dynamics), and the RNA products quantified using the ImageQuaNT program.

Stopped-Flow Kinetics

The stopped-flow experiments were carried out at 25 °C using a SF-2001 spectrophotometer from KinTek Corp (Austin, TX) equipped with a photomultiplier detection system. The kinetics of GTP binding was monitored by mixing increasing concentrations of GTP in buffer A (50 mM Tris acetate, pH 7.5, 50 mM sodium acetate, 10 mM magnesium acetate, 5 mM DTT) from one syringe with a preincubated solution of T7 RNAP and dsDNA with or without T7 lysozyme in buffer A from the second syringe. The dsDNA contained a single 2-AP residue at position nt(+4) on the template.
strand. Additional magnesium acetate was included with the GTP solution to maintain a constant concentration of free Mg$^{2+}$. As the two solutions (30 µl from each syringe) were rapidly mixed (flow rate of 6.0 ml s$^{-1}$) the 2-AP was excited at 315 nm. The progress of the reaction was monitored by measuring the intensity of the fluorescence emission using a cut-on filter $>$360 nm (WG360, Hi-Tech Scientific, serial no. 273129). Multiple traces (3-10) were averaged to optimize the signal. The KinTek stopped-flow kinetic software was used to fit the stopped-flow kinetic traces to Eqn 1 describing single or multiple exponential changes,

$$F = \sum A_n \times \exp(-k_{obs,n} \times t) + C \quad \text{(Eq. 1)}$$

Where $F$ is the fluorescence intensity at time $t$; $n$ is the number of exponential terms; $A_n$ and $k_{obs,n}$ are the amplitude and the observed rate constant of the $n$th term, respectively; and $C$ is the fluorescence intensity at $t=0$. The observed rate constant ($k_{obs}$) was plotted as a function of GTP and the dependency was fit by non-linear regression analysis to the hyperbolic equation 2 (17) using SigmaPlot.

$$k_{obs} = \frac{k_{conf} \times [GTP]}{K_d + [GTP]} \quad \text{(Eq. 2)}$$

Where $k_{conf}$ is the rate of a conformational change upon GTP binding and $K_d$ is the equilibrium dissociation constant of GTP.
Equilibrium Fluorescence Measurements

T7 RNAP (1 µM) was mixed with 0.5 µM DNA containing a single 2-AP residue at t(-4) in Buffer A in a 200 µl quartz cuvette at 25 °C. The sample was excited with 315 nm light (2.5 nm band-width) and the resulting fluorescence intensity at 370 nm (2.5 nm band-width) was measured on a FluoroMax-2 spectrofluorometer (Jobin Yvon-Spex Instruments S.A., Inc) using the DataMax software program. Fluorescence was then measured after the addition of T7 lysozyme (10 µM). Similar experiments were carried out in the presence of +2 NTP (500 µM 3’-dGTP, 1 mM ATP, or 4 mM GTP). Additional magnesium acetate was included with the GTP (4 mM) to maintain a constant amount of free Mg²⁺ in solution. The fluorescence of T7 RNAP and T7 lysozyme was subtracted out by carrying out a control experiment with the non-fluorescent DNAs. The corrected fluorescence is \( F = F_f - F_{nf} \) where \( F_f \) and \( F_{nf} \) are the fluorescence intensities of samples containing 2-AP modified and non-fluorescent DNA, respectively.

RESULTS

Promoter DNAs

Several synthetic promoter DNAs were used in these studies (Table 1). The dsDNA is a fully duplex promoter that contains the T7 φ10 promoter consensus sequence from –21 to +19 and initiates RNA synthesis at +1 with the sequence GGG. The p-dsDNA and bulge-6 DNAs are considered mimics of an opened promoter. To monitor open complex
formation and nucleotide binding the fluorescent adenine analog 2-AP was incorporated at position –4 in the template or at position +4 in the non-template strand relative to the transcription start site. Altered promoters with the initiation sequence (GAC) were used to distinguish binding of the +1 and +2 initiating nucleotides.

_T7 lysozyme does not affect promoter binding or the rate of pre-initiation open complex formation_

Previous studies have shown that T7 lysozyme inhibits both transcription initiation and the transition from initiation to elongation (7). T7 lysozyme does not reduce transcription by preventing the promoter DNA from binding to the T7 RNAP or by decreasing the affinity of the promoter for the RNAP (6;7;18). As this was determined using indirect method, we sought to verify the observation with a more direct assay. The kinetics of promoter binding and the formation of the pre-initiation open complex were measured in real time using a promoter DNA that was modified with the fluorescent adenine analog 2-AP (Table 1). During open complex formation, the –4 to +2/+3 region of the promoter is converted from a duplex to a single stranded region. If 2-AP is substituted for the adenines in the melted region, open complex formation is accompanied by an increase in 2-AP fluorescence, which can be easily monitored. Any adenine in the melting region may be substituted with 2-AP. The greatest increase in fluorescence upon binding to T7 RNAP is observed when the 2-AP is positioned at the –4 position of the template strand, t(–4) (19). This is because the adenine at t(–4) undergoes a large structural change becoming both unpaired and unstacked from its neighboring guanine (20), and it is the latter process that gives the large fluorescence increase(21).
We have verified that the insertion of 2-AP does not affect promoter binding or transcription (13;14).

The steps of DNA binding and the formation of the pre-initiation open complex were measured with the dsDNA promoter containing 2-AP at t(-4), as described previously (19). T7 RNAP in the presence or the absence of T7 lysozyme was mixed with the promoter DNA in a stopped-flow apparatus, and the increasing fluorescence intensity was monitored as a function of time. We used 12 µM of T7 lysozyme, which is above the $K_d$ of T7 lysozyme (7), as higher concentrations increased the background fluorescence interfering with signal detection. The hyperbolic fit of the observed rate versus [DNA] provided $K_{1/2}$ and an observed rate of pre-initiation open complex equal to $0.9 \pm 0.2$ µM and $116 \pm 6$ s$^{-1}$ and $1.1 \pm 0.5$ µM and $87 \pm 10$ s$^{-1}$ in the absence and presence of T7 lysozyme, respectively (supplemental material). The similar rates of DNA binding with and without T7 lysozyme indicates that T7 lysozyme has very little effect on the rate of pre-initiation open complex formation.

*T7 lysozyme decreases the pre-steady state rate of RNA synthesis, but does not significantly affect the $K_d$ of the initiating nucleotide*

Subsequent to the steps of promoter DNA binding and the formation of a pre-initiation open complex are the steps of initiating nucleotide binding to form the initiation complex that is ready to make the RNA. By monitoring the steady state kinetics of abortive synthesis, it has been determined that the $K_m$ of the initiating nucleotides GTP increases in the presence of T7 lysozyme (18). The steady state $K_m$ however is a kinetic
constant whose value is influenced by the formation as well as the dissociation rates of the abortive products, and thus may not reflect the $K_d$ of GTP, which may be greater than, less than, or equal to the $K_m$ (17). Therefore, pre-steady state kinetic experiments were used to measure the rate of RNA synthesis with increasing [GTP] with and without T7 lysozyme. The dsDNA promoter was preincubated with T7 RNAP in the presence or the absence of T7 lysozyme and transcription was initiated by the rapid addition of [$\gamma^{32}$P] GTP. The reactions were quenched after millisecond time intervals with the aid of a rapid chemical quenched flow apparatus and the RNA products quantitated. In the presence of GTP alone, we see the production of pppGpG and pppGpGpG RNA products and also some pppGpGpGpG at longer times (Figure 1a). The G-ladder production increased linearly with time up to 0.25 s and the slope provided the initial rate. The initial rate was plotted as a function of [GTP] and the results indicated that the rate at maximal [GTP] is reduced approximately 5-fold (from 7 s$^{-1}$ to 1.5 s$^{-1}$) when T7 lysozyme is present (Figure 1b). Due to the low signal, we were unable to get an accurate value of the GTP $K_d$ from this radiometric assay in the presence of T7 lysozyme. A 20-fold reduction in catalytic efficiency was calculated from the initial slope of rate versus [GTP] dependence (11.6 ± 2.5 s$^{-1}$mM$^{-1}$ without T7 lysozyme and 0.56 ± 0.1 s$^{-1}$mM$^{-1}$ in the presence of T7 lysozyme).

To determine the effect of T7 lysozyme on the $K_d$ of the initiating GTP, a more sensitive fluorescence assay was used. In this assay, GTP binding and a subsequent conformational change is monitored by following the changes in the 2-AP modified promoter DNA (14;22). Using a stopped-flow apparatus, T7 RNAP and the dsDNA containing 2-AP at nt(+4) with or without T7 lysozyme was rapidly mixed with a
solution of GTP (Figure 2a). This resulted in a time dependent increase in fluorescence (Figure 2b), and the observed rate was plotted against GTP concentration (Figure 2c). The hyperbolic fit provided an average $K_d$ of the +1 and +2 GTP as $435 \pm 276$ µM and $742 \pm 233$ µM in the absence and the presence of T7 lysozyme, respectively. Note that in the absence of T7 lysozyme, the GTP $K_d$ is consistent with the values previously reported (11;22). Thus, T7 lysozyme doubles the apparent $K_d$ of GTP and causes over a three-fold decrease in the maximal rate of a conformational change occurring upon GTP binding ($k_{\text{conf}}$).

*T7 lysozyme does not inhibit the rate of initial RNA synthesis on a “premelted” promoter.*

The experiments thus far show that T7 lysozyme inhibits the rate of RNA synthesis during initiation. Both the stopped-flow and radiometric assays show that the inhibition cannot be overcome by increasing the initiating GTP concentration. We next measured transcription in the presence of all rNTPs to observe the effect of T7 lysozyme on the synthesis of longer RNA. T7 RNAP and dsDNA in the absence or the presence of T7 lysozyme was mixed with four rNTPs (1 mM of GTP and 500 µM other NTPs) and [$\gamma$-$^{32}$P] GTP in a rapid chemical quenched-flow instrument. The reaction was quenched after various times and the products resolved on a sequencing gel are shown in Figure 3a.

In the presence of T7 lysozyme, the pre-steady state rate of RNA synthesis is 3-fold slower (Table 2) that is evident from the decrease in the initial burst phase with T7 lysozyme shown in Figure 3b. The abortive products from 2-mer to 5-mer on the other hand are produced in greater amounts with T7 lysozyme (Figure 3c). Hence, the steady
state rate of RNA synthesis is actually higher in the presence of T7 lysozyme (Table 2). The 19-mer runoff product is produced with a longer delay in the presence of T7 lysozyme (Figure 3d).

Similar measurement of the pre-steady state kinetics of RNA synthesis on a “premelted” DNA was carried out to determine whether T7 lysozyme inhibits the chemistry step. Bulge-6 is considered a mimic of a melted promoter as it contains 6 non-complementary bases to the template strand in the initiation region (−4 to +2). This substrate was chosen over the p-dsDNA as the bulge-6 contains the non-template strand, which is capable of interacting with T7 RNAP. The initial rate of total RNA synthesis with the bulge-6 promoter is unaffected by the presence of T7 lysozyme (Table 2), as evident from the similar burst phase with and without T7 lysozyme in Figure 4a. The bulge-1 DNA shows a similar behavior as bulge-6 (Figure 4d). This indicates that T7 lysozyme does not alter the chemical step of RNA synthesis as long as the DNA is premelted or easily melted. Even though T7 lysozyme had little effect on the RNA synthesis rate, the production of abortive products from 3-mer to 5-mer with the bulge-6 and 2-mer to 5-mer with the bulge-1 was increased in the presence of T7 lysozyme (Figure 4b and 4e). This results in a higher steady state rate with T7 lysozyme (Table 2).

*T7 lysozyme affects the formation of the open complex in the presence of the initiating nucleotide*

Our results indicate that the decrease in the rate of initiation cannot be attributed to a defect in the formation of the pre-initiation complex or a defect in chemistry or solely due to an increase in the GTP $K_d$. Increased abortive synthesis in the presence of T7
lysozyme appears to be due to an effect on the stability of the open complex during initiation. To explore this idea, we have used 2-AP fluorescence to investigate the nature of the open complexes in the pre-initiation and initiation stages with and without T7 lysozyme.

The crystal structure of T7 RNAP-promoter complex, which likely represents the structure of the pre-initiation complex, shows that the promoter DNA is unpaired from -4 to +2 and the -4 base is unstacked from the -5 base (21). A promoter DNA containing 2-AP at t(-4) when bound to T7 RNAP has a higher fluorescence relative to the fluorescence of the free 2-AP modified DNA, mainly because the t(-4) base is unstacked in the open complex. To investigate the nature of the open complex with and without T7 lysozyme, we compared the 2-AP fluorescence of E·p-dsDNA and E·dsDNA (23). The fluorescence of E·dsDNA is 30% of E·p-dsDNA (Figure 5a) as measured with the (GAC) promoter in the absence of the initiating nucleotide. The fluorescence of E·dsDNA·ATP however is close to that of E·p-dsDNA·ATP, indicating as previously reported that the addition of the +2 nucleotide NTP drives open complex formation (23).

Even in the presence of T7 lysozyme (L), the fluorescence of EL·dsDNA is 30% of the EL·p-dsDNA. Thus, T7 lysozyme has little effect on the formation or the stability of the pre-initiation open complex. However, the fluorescence of EL·dsDNA·ATP is only 42% of EL·p-dsDNA·ATP. The addition of GTP, the +1 nucleotide, along with the ATP produced no further increase in fluorescence (data not shown). The inability of the +2 ATP to drive open complex formation to completion in the presence of T7 lysozyme was not due to subsaturating concentrations of ATP. The $K_d$ of the +2 nucleotide was
obtained from fluorescence equilibrium titrations. The pre-initiation E·dsDNA complex of the (GAC) promoter in the absence or the presence of T7 lysozyme was titrated with ATP and the increase in fluorescence measured. The ATP dependencies fit to a hyperbolic equation provided an ATP $K_d$ of $89 \pm 12 \, \mu M$ in the absence and $134 \pm 64 \, \mu M$ in the presence of T7 lysozyme (data not shown). The +2 nucleotide $K_d$ is only slightly affected by T7 lysozyme. The fact that the fluorescence of EL·dsDNA·ATP does not reach the level of the fully open promoter even with a high concentration of the initiating nucleotide indicates that T7 lysozyme affects the structure of the initiation complex rather than the equilibrium constant of the initiation complex.

Similar experiments were carried out with the (GGG) promoter to eliminate the possibility that the effect of T7 lysozyme on the initiation complex was due to the use of the nonconsensus (GAC) promoter. As shown in Figure 5b, the results are similar to that of the (GAC) promoter. The nucleotide 3′-dGTP was substituted for GTP to avoid complications from RNA synthesis (14;22;23). The results indicate that the pre-initiation open complex is not affected by T7 lysozyme, but the initiation complex is perturbed by T7 lysozyme.

Since, the 2-mer RNA synthesis rate was not affected by T7 lysozyme with an already or readily melted promoter, one would predict that T7 lysozyme would not affect the formation of the initiation complex with these promoters. This is indeed the case as shown in Figure 5c. The fluorescence of bulge-6 and bulge-1 in complex with T7 RNAP is similar to that of the p-dsDNA. Upon addition of GTP, the dsDNA complex showed the characteristic increase in fluorescence from 30% to 100% but no change was
observed in the bulge DNAs. In the presence of T7 lysozyme (10 µM), a slight decrease in the fluorescence of bulge DNA pre-initiation complexes was observed, but this was overcome by the addition of nucleotide. Thus, the initiation complex of bulge-6, bulge-1 or p-dsDNA was unaffected by the presence of T7 lysozyme. The fluorescence of EL-dsDNA with GTP was greater than with 3′-dGTP (65% versus 40%) most likely due to the stabilizing effects of the newly synthesized RNA.

DISCUSSION

The experiments presented here were carried out to investigate the mechanism by which T7 lysozyme inhibits T7 RNAP transcription. It is already known that unlike many other transcriptional repressors, which sterically block promoters from their polymerases, T7 lysozyme does not bind DNA. Rather it directly binds to T7 RNAP to form a tertiary complex with the polymerase and DNA (7). More specifically, a crystal structure of the T7 lysozyme-T7 RNAP complex revealed that T7 lysozyme binds to a site distal to the polymerase active site and causes little change in the overall T7 RNAP structure with the exception of the extreme C-terminus. These last four residues (FAFA883) are disordered in the complexed structure, whereas in the T7 RNAP-DNA complex structure they are located below the polymerase active site (8). Furthermore, biochemical data revealed that mutations in any of these four C-terminal residues results in decreased T7 RNAP activity (24) and that the C-terminus is more sensitive to proteolysis in the presence of T7 lysozyme (25). It has been proposed based on this
information and steady state transcription assays that showed an increase in the NTP apparent $K_m$ during initiation, that T7 lysozyme inhibits T7 RNAP by stabilizing the conformation of the T7 RNAP with an altered C-terminus. This model predicts that inhibition can be overcome by the addition of nucleotide (18;25). We have further investigated this model for T7 lysozyme inhibition of T7 RNAP transcription initiation using pre-steady state techniques.

During T7 RNAP transcription initiation there are several potential points for regulation by T7 lysozyme. The first potential regulatory site is promoter binding. Stopped-flow experiments indicated that T7 lysozyme does not prevent T7 RNAP from binding the promoter. Similarly, the pre-initiation complex formation rate was affected only to a small extent in the presence of T7 lysozyme. We next explored the possibility that T7 lysozyme altered the binding of the initiating nucleotide to the T7 RNAP-promoter pre-initiation complex. A quenched-flow radiometric assay measuring the rate of RNA synthesis during initiation at increasing [GTP] showed that the rate of G-ladder synthesis decreased five-fold in the presence of T7 lysozyme. The rate could not be restored even by high concentrations of nucleotide (2 mM GTP). Since obtaining a reliable GTP $K_d$ value was impossible due to the difficulty in measuring the products synthesized in the presence of T7 lyszoyme, a fluorescent assay monitoring an increase in 2-AP fluorescence upon GTP binding was employed. The stopped-flow assay also indicated that the maximal rate of a conformational change occurring upon GTP binding ($k_{conf}$) was reduced approximately three-fold while the average GTP $K_d$ was increased at most two-fold in the presence of T7 lysozyme. These results are only partly in agreement with the proposed mechanism of Villemain and Sousa (18) that postulated T7 lysozyme
inhibition due to an increase in nucleotide $K_m$ values. According to this model, a high concentration of GTP should be able to restore the rate of RNA synthesis to uninhibited levels even in the presence of T7 lysozyme (18). However, the pre-steady state rate of RNA synthesis does not increase at high GTP with T7 lysozyme. The observed recovery of steady state synthesis observed by Villemain and Sousa (18) with increasing nucleotide may have been due to the increased rate of dissociation of small RNA products occurring during multiple turnovers, which is facilitated in the presence of T7 lysozyme (18).

As a defect in initiating nucleotide binding was not the sole cause for inhibition, we next examined if T7 lysozyme inhibited the inherent ability of T7 RNAP to carry out RNA synthesis. When T7 RNAP was supplied with an already melted or an easily melted promoter (bulge-6 or bulge-1), then T7 lysozyme was unable to slow the rate of initial RNA synthesis. The lack of inhibition on the open promoter is not the result of T7 lysozyme being unable to form a tertiary complex. Complex formation is evident from the greater production of abortive products in the presence of T7 lysozyme. The result that T7 lysozyme does not hinder the ability of T7 RNAP to carry out RNA synthesis but causes additional accumulation of short RNA products is consistent with the idea that T7 lysozyme inhibits a step associated with stable open complex maintenance.

The nature or the amount of the open complex formed by T7 RNAP-DNA or T7 RNAP-T7 lysozyme-DNA complexes was investigated by comparing the 2-AP fluorescence of dsDNA and p-dsDNA in complex with T7 RNAP or T7 RNAP-T7 lysozyme complex. The fluorescence of 2-AP in DNA is sensitive mainly to base-
stacking interactions (26); hence, the measurement of the fluorescence of 2-AP provides information about the degree of t(-4) unstacking. The fluorescence of E·dsDNA is 30% of E·p-dsDNA, and we have shown that the lower fluorescence of dsDNA is due to the unfavorable equilibrium constant for the formation of the pre-initiation open complex (19). The same ratio was observed in the presence of T7 lysozyme indicating that pre-initiation open complex formation is not affected by T7 lysozyme. In the presence of the +2 NTP, the fluorescence of E·dsDNA increases to nearly the level of E·p-dsDNA (23). In the presence of T7 lysozyme, the +2 NTP was unable to increase the fluorescence of 2-AP to the level of the p-dsDNA. This was not due to the fact that +2 NTP was not bound to the RNAP as the +2 NTP \( K_d \) is only 1.5 fold higher in the presence of T7 lysozyme. The fact that even saturating concentrations of the initiating nucleotide was unable to drive open complex formation indicates that T7 lysozyme affects the structure of the open DNA in the initiation complex rather than the equilibrium constant for open complex formation.

T7 lysozyme interacts with parts of the palm, finger and the N-terminal domain of T7 RNAP, and this mode of binding can lock the protein or affect protein flexibility to prevent a conformational change that is required to form a fully open initiation complex. There are numerous protein-DNA interactions that are required to maintain the transcription bubble and since T7 lysozyme binds at a site remote from these interactions, the action of T7 lysozyme is allosteric. The allosteric effect of T7 lysozyme results in the destabilization of the transcription bubble, which may happen due to long range perturbation of T7 lysozyme binding on the interactions of T7 RNAP with the nontemplate or the template strand. This hypothesis is consistent with the finding that the
G235D and R231H mutations in the intercalating beta hairpin, which is involved in maintaining the open bubble (27;11) have been identified as T7 lysozyme sensitive mutants (28). Similarly, the higher sensitivity of the class II \( \phi 3.8 \) promoter to T7 lysozyme (18) (data not shown) is consistent with the fact that the \( \phi 3.8 \) promoter is less efficiently opened by T7 RNAP than a consensus promoter (22).

The greater production of abortive products in the presence of T7 lysozyme indicates that the RNA dissociates more frequently when T7 lysozyme is complexed to T7 RNAP. This may be because the RNA is bound less stably at the active site either due to the frequent collapse of the initiation bubble back to the duplex form or due to the disruption in the interactions of the T7 RNAP with the RNA at the active site. Nonetheless, due to the increased abortive synthesis the RNAP spends more time in the recycling mode, which causes a delay in the transition from initiation to elongation. The fact that abortive products from 3-mer to 5-mer increases with T7 lysozyme also indicates that the delay in the transition from the initiation to the elongation phase could be caused by the inhibition of some or all the conformational changes of the N-terminal domain (29;30) that are necessary to make RNA products longer than 3-mer. T7 lysozyme can therefore allosterically inhibit the rotation of the N-terminal core domain or the refolding of the N-terminal domains delaying promoter clearance.

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**FIGURE LEGENDS**

Figure 1: Pre-steady state kinetics of initial RNA synthesis at various GTP concentrations in the presence or the absence of T7 lysozyme.

(a) T7 RNAP (15 µM) was preincubated with the dsDNA (10 µM) in the absence and the presence of T7 lysozyme (30 µM) and the transcription was initiated by the addition of GTP containing [γ-32P] GTP. Gel shows the products generated in the presence of 500 µM GTP. (b) The initial rates of G-ladder synthesis at various [GTP] in the absence (●) and presence (○) of T7 lysozyme are plotted. The initial rates were calculated from the initial slopes (µM G-ladder/s) divided by [T7 RNAP-dsDNA] (10 µM).
Figure 2: Stopped-flow kinetics of GTP binding in the presence or the absence of T7 lysozyme.

(a) The stopped-flow experimental design where T7 RNAP (1.35 µM) was preincubated with the dsDNA containing a 2-AP residue at position nt(+4) (0.45 µM) in the absence or the presence of T7 lysozyme (10 µM) and rapidly mixed with various concentrations of GTP. The 2-AP DNA was excited at 315 nm and the fluorescence >360 nm monitored. (b) Representative fluorescence traces at 1 mM GTP. (c) The fluorescence time courses were fit to a single exponential and the observed rates are plotted as a function of [GTP]. The GTP dependencies fit to the hyperbolic equation 2 with $k_{conf}$ of 10 ± 1.3 s$^{-1}$ and 3.1 ± 0.2 s$^{-1}$ and GTP $K_d$ of 436 ± 277 µM and 743 ± 233 µM in the absence ($\bullet$) and presence ($\bigcirc$) of T7 lysozyme, respectively.

Figure 3: Pre-steady state kinetics of transcription with all NTPs with dsDNA in the presence and the absence of T7 lysozyme.

(a) The sequencing gel image shows the transcription products generated by T7 RNAP (10 µM) and dsDNA (10 µM) in the absence and the presence of T7 lysozyme (12 µM) with 1 mM GTP + [$\gamma^{32}$P] GTP and 500 µM each of the rest of rNTPs. (b) The pre-steady state kinetics of total RNA products, (c) 2-5mer RNA products, and (d) runoff
products in the presence (○) and the absence (●) of T7 lysozyme. The pre-steady state and steady state rates are listed in Table 2.

**Figure 4: Pre-steady state kinetics of transcription with all NTPs with bulge-6 and bulge-1 promoter DNAs in the presence and the absence of T7 lysozyme.**

Transcription with T7 RNAP (10 μM) and the bulge-6 or the bulge-1 DNA (10 μM) in the absence and the presence of T7 lysozyme (30 μM) with [γ-32P] GTP and 500 μM each of rNTPs were measured as a function of time. (a) The pre-steady state kinetics of total RNA synthesis, (b) 2-5mer RNA products, and (c) runoff products in the presence (○) and the absence (●) of T7 lysozyme for the bulge-6 DNA, and the bulge-1 DNA (d, e, f). The pre-steady state and steady state rates are listed in Table 2.

**Figure 5: Promoter opening measured by 2-AP fluorescence.**

(a) Fluorescence (excitation at 315 nm and emission at 370 nm) of dsDNA(GAC) over p-dsDNA (0.5 μM) both modified with 2-AP at t(-4) is shown after the progressive additions of T7 RNAP (1 μM) and ATP (1 mM). Measurements were repeated in the presence of T7 lysozyme (10 μM). Results shown are the average of two independent experiments. (b) Fluorescence of dsDNA(GGG) over p-dsDNA with progressive additions of T7 RNAP (1 μM) and 3’-dGTP (500 μM) without and with T7 lysozyme (10
µM). (c) Fluorescence of dsDNA (black), bulge-6 (red), and bulge-1 (green) versus p-dsDNA complexes with T7 RNAP with and without T7 lysozyme (10 µM) with the subsequence addition of GTP (4 mM).
| DNA               | Description                      | Sequence                                                                 |
|-------------------|----------------------------------|--------------------------------------------------------------------------|
| p-dsDNA           | -4 to +19 ss template            | 5' -AAATTAATACGACTCAC-3'                                                 |
|                   |                                  | 3' -TTTAATTATGCTGAGTG\text{A}T\text{A}T\text{C}CCTCTGGTGGTCGCAAAG-5'    |
| p-dsDNA(GAC)      | -4 to +19 ss template            | 5' -AAATTAATACGACTCAC-3'                                                 |
|                   |                                  | 3' -TTTAATTATGCTGAGTG\text{A}T\text{A}T\text{C}t_7GCTGTTGGTCGCAAAG-5' |
| bulge-6           | -4 to +2 mismatch                | 5' -AAATTAATACGACTCAC\text{CGCAT}GAGACCACAACGGTTTC-3'                   |
|                   |                                  | 3' -TTTAATTATGCTGAGTG\text{A}T\text{A}T\text{C}CCTCTGGTGGTCGCAAAG-5'    |
| bulge-1           | -2 AA mismatch                   | 5' -AAATTAATACGACTC\text{ATA}A\text{G}G\text{G}G\text{A}\text{C}\text{A}\text{A}\text{C}\text{A}\text{A}\text{C}\text{G}\text{G}\text{G}\text{T}\text{T}\text{T}\text{T}-3' |
|                   |                                  | 3' -TTTAATTATGCTGAGTG\text{A}T\text{A}T\text{C}CCTCTGGTGGTCGCAAAG-5'    |
| dsDNA             | 40-bp concensus                  | 5' -AAATTAATACGACTC\text{ACTA}\text{ATA}\text{G}\text{G}\text{G}\text{A}\text{C}\text{A}\text{A}\text{C}\text{A}\text{A}\text{C}\text{G}\text{G}\text{G}\text{T}\text{T}\text{T}-3' |
|                   |                                  | 3' -TTTAATTATGCTGAGTG\text{A}T\text{A}T\text{C}CCTCTGGTGGTCGCAAAG-5'    |
| dsDNA(GAC)        | +2 and +3 bases altered from consensus | 5' -AAATTAATACGACTC\text{ACTA}\text{ATA}\text{C}\text{G}\text{A}\text{G}\text{A}\text{C}\text{A}\text{A}\text{A}\text{C}\text{G}\text{A}\text{T}\text{T}\text{T}-3' |
|                   |                                  | 3' -TTTAATTATGCTGAGTG\text{A}T\text{A}T\text{C}t_7GCTGTTGGTCGCAAAG-5' |

\(\text{A}: 2\text{-aminopurine}\)
Table 2: Kinetic parameters of RNA synthesis with and without T7 lysozyme

| Promoter | Pre-steady state | Steady-State |
|----------|------------------|--------------|
|          | Rate of RNA Synthesis | Rate of RNA Synthesis |
|          | (µM⁻¹ s⁻¹)       | (µM⁻¹ s⁻¹)   |
| - T7 lysozyme | + T7 lysozyme | -T7 lysozyme | +T7 lysozyme |
| dsDNA    | 12 ± 5           | 4 ± 0.6      | 1.0 ± 0.05  | 2 ± 0.1 |
| bulge-6  | 14.5 ± 1.7       | 11 ± 0.2     | 4 ± 0.4     | 6 ± 0.2 |
| bulge-1  | 12 ± 1.2         | 14 ± 0.6     | 2 ± 0.3     | 7 ± 0.6 |
Figure 1: Stano and Patel
Figure 2: Stano and Patel
Figure 4: Stano and Patel
Figure 5: Stano and Patel
Supplemental Figure 1: Stopped-flow kinetics of open complex formation.

(a) The diagram shows the experimental design where increasing concentrations of (GGG)40bp-dsDNA promoter (t-4) were mixed with RNAP (0.15 μM), or a complex of RNAP (0.15 μM) and lysozyme (12 μM), and the resulting time dependent increase in 2-AP fluorescence was monitored (excitation 315 nm and emission >360 nm). (b) Fluorescence time traces obtained using 4 μM DNA.

(c) As reported previously (Jia, Y.P., A. Kumar, and S.S. Patel. 1996. Equilibrium and stopped-flow kinetic studies of interaction between T7 RNA polymerase and its promoters measured by protein and 2-aminopurine fluorescence changes. J.Biol.Chem. 271:30451-30458.) the fluorescence traces fit to two exponentials. The rate of the first phase increases hyperbolically with DNA concentration as shown (c) and the rate of the slow phase was over 100 times slower. The fast phase rate versus [dsDNA] was fit to a hyperbola (observed rate=V₀*[dsDNA]/(K+[dsDNA])+ y0, where K is the concentration of dsDNA where the observed rate is one-half the maximal observed rate (V₀), and y0 is the y-intercept). The solid line shows the fit to the data with V₀ (SD) 116.4 (5.6) s⁻¹ and 86.6 (9.8) s⁻¹, K (SD) 0.9 (0.2) μM and 1.1 (0.5) μM, and y-intercept (SD) 12.5 (3.7) s⁻¹ and 0 (11.6) s⁻¹ for DNA binding in the absence (black circles) and presence of lysozyme (open circles).
T7 lysozyme represses T7 RNA polymerase transcription by destabilizing the open complex during initiation
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