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Evidence for a Tyrosine–Adenine Stacking Interaction and for a Short-lived Open Intermediate Subsequent to Initial Binding of *Escherichia coli* RNA Polymerase to Promoter DNA

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Bacterial RNA polymerase and a "sigma" transcription factor form an initiation-competent "open" complex at a promoter by disruption of about 14 base pairs. Strand separation is likely initiated at the highly conserved −11 A–T base pair. Amino acids in conserved region 2.3 of the main *Escherichia coli* sigma factor, σ^{70}, are involved in this process, but their roles are unclear. To monitor the fates of particular bases upon addition of RNA polymerase, promoters bearing single substitutions of the fluorescent A-analog 2-aminopurine (2-AP) at −11 and two other positions in promoter DNA were examined. Evidence was obtained for an open intermediate on the pathway to open complex formation, in which these 2-APs are no longer stacked onto their neighboring bases. The tyrosine at residue 430 in region 2.3 of σ^{70} was shown to be involved in quenching the fluorescence of a 2-AP substituted at −11, presumably through a stacking interaction. These data refine the structural model for open complex formation and reveal a novel interaction involved in DNA melting by RNA polymerase.

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Introduction

In bacteria, sigma-type transcription initiation factors bind to the core RNA polymerase (RNAP; subunit composition $\alpha_2\beta\beta'\omega$) to assemble the holoenzyme that can specifically recognize promoters and form the "open" complex competent to initiate RNA synthesis. In this complex, a promoter region of about 14 base pairs is melted, extending from the highly conserved −11A in the −10 promoter element to +3 downstream of the start site of transcription. This process requires no external source of energy and involves several intermediates.[1] The −11A of promoter DNA is a vital element in the formation of the strand-separated complex.[2–4] Promoter DNA melting is thought to be initiated by rotating the −11A out of the DNA helix and into a hydrophobic pocket on the sigma σ^{70}.[2,5] Such base flipping has been demonstrated by X-ray crystallography for enzymes involved in DNA repair and maintenance and for some enzymes by 2-aminopurine (2-AP) fluorescence (e.g., Refs. 6–9). However, its role in nucleating DNA opening by RNAP has not yet been directly shown.

The main sigma factor in *Escherichia coli*, σ^{70}, is responsible for transcription of housekeeping genes and as such is essential for growth of the cell. Although it was discovered almost 40 years ago,[10] major gaps remain in our knowledge of the mechanism by which σ^{70} participates in specific recognition of promoter DNA and in the strand separation process. Amino acids in conserved

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Abbreviations used: 2-AP, 2-aminopurine; RNAP, RNA polymerase; WT, wild type; EMSA, electrophoretic mobility shift assay; FBB, Fork binding buffer; EDTA, ethylenediaminetetraacetic acid; UpA, uridylyl (3′-5′) adenosine.

region 2.4 of σ^{70} are responsible for recognition of the −10 promoter element.[11] However, the specific amino acids involved have only been identified for interaction with the −12 base pair.[12] Residues of region 2.3 participate in promoter DNA strand separation,[13–15] but the relevant interactions have not yet been elucidated.[16] Recent evidence of the importance of the sigma factor in facilitating DNA opening has been provided by the observations that a subassembly of σ^{70} and β' fragments forms an open complex at an extended consensus −10 promoter (TGxTATAAT) on negatively supercoiled DNA[17] and that key residues in region 2 are required for efficient DNA opening at low temperature.[18] Other subunits of RNAP are likely also involved in the strand separation process.

To study the kinetic mechanism by which promoters form heparin-stable (i.e., long-lived) complexes with both wild-type (WT) RNAP and RNAP containing various substitutions in region 2.3 of σ^{70}, the electrophoretic mobility shift assay (EMSA) was employed. To study the fate of particular bases in the promoter DNA subsequent to RNAP binding, we introduced the fluorescent A-analog, 2-AP, at the −11, −8 and −4 positions in the nontemplate strand. Unstacking of 2-AP in double-helical DNA due to DNA melting or other distortions in B-form DNA structure results in increased fluorescence.[19] Analysis of the kinetics of the RNAP-induced increase in the fluorescence of 2-AP substituted at positions −4, −8 and −11 of promoter DNA revealed rate constants that are significantly greater than those for formation of stable RNAP–promoter complexes. These results provide evidence for a novel intermediate in which the bases in the region to be melted are unstacked, but the final open complex has not yet been established. With RNAP containing substitutions for Y430, but not for four other region 2.3 amino acids tested, a greatly enhanced −11 2-AP signal was observed compared to that seen with WT RNAP. It is

concluded that −11 2-AP fluorescence is quenched to nearly similar extents by stacking in double-helical promoter DNA as by interactions with Y430 in promoter complexes formed by WT RNAP. This work identifies Y430 as the key aromatic amino acid residue of σ^{70} responsible for capturing the extruded −11 A. This function was anticipated for residues in region 2.3 but not established until this study.

Results

Promoter DNA and RNAP

To examine DNA opening without complications due to effects on initial binding of RNAP, promoter DNA substrates have the consensus core promoter sequences at the −35 and extended −10 regions (Fig. 1a).[20–22] This 65-bp DNA oligomer was designed to favor tight binding, compensating for any disruptive substitutions of 2-AP and/or amino acids in region 2.3 of σ^{70}, as shown below. The holo RNAP contained either WT σ^{70} or σ^{70} with the single alanine substitutions for F427, T429, Y430, W433 and W434, region 2.3 residues that play a role in the RNAP-induced promoter strand separation.[14–16,21,23] RNAP containing multiple substitutions in σ^{70} were also investigated: YW has both the Y430A and W433A substitutions, FYW additionally has F427A, and FYWW additionally has W434A. RNAP containing substitutions for Y430 other than alanine were also studied (see Fig. 3a and b).

Characterization of the EMSA assay for determining heparin-stable complex formation between RNAP and promoter DNA

To compare the kinetics of initiation-competent, open RNAP–promoter complex formation with the kinetics of RNAP-induced changes in DNA structure,
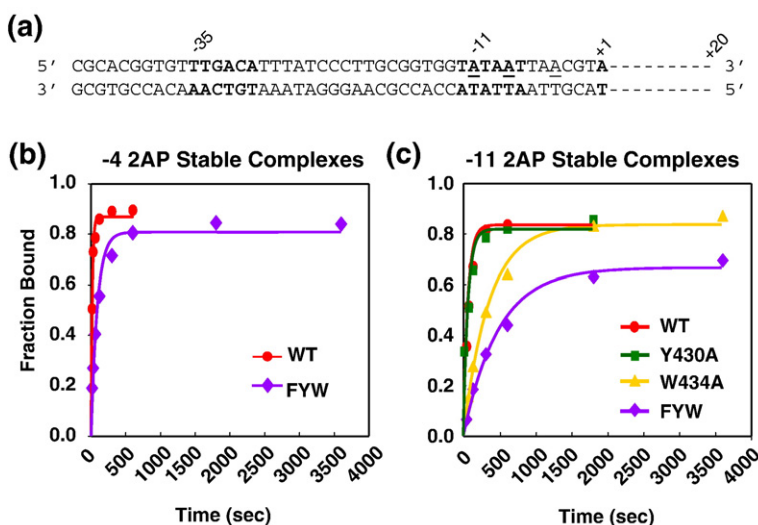

(a)

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              -35                              -11        +1          +20
5' CGCACGGTGTTTGACATTTATCCCTTGCGGTGGTATAATTAACGTA--------- 3'
3' GCGTGCCACAAACTGTAAATAGGGAACGCCACCATATTAATTGCAT--------- 5'
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Fig. 1. The promoter DNA used in this work; kinetics of stable complex formation. (a) The 65-bp promoter DNA sequence, spanning positions from −45 to +20. DNA sequences downstream of the +1 are not shown. Consensus −35 and −10 elements are shown in bold, as is the +1 transcription start site. The −11, −8 and −4 sites where 2-AP was substituted for A on the nontemplate (top) strand are underlined. (b) Plot of the fraction of RNAP bound to −4 2-AP DNA over time, as determined by EMSA. The first four time points were at 15, 30, 60 and 120 s. After a 2-min challenge with heparin, the reactions were loaded onto a nondenaturing gel. DNA and RNAP concentrations were 10 and 50 nM, respectively. Lines represent single-exponential fits to the data. (c) Plot of the fraction of RNAP bound to −11 2-AP DNA over time, determined as in (b) above. The first four time points were at 30, 60, 120 and 300 s for WT, Y430A and W434A RNAP and at 30, 120, 300 and 600 s for FYW RNAP.

we first examined the relationship between the formation of heparin-resistant complexes, detected by EMSA, and of initiation-competent complexes. Heparin is a polyanion that binds free RNAP irreversibly, eliminating any complexes that dissociate on a subminute time scale from detection. The complexes that survive the challenge by heparin (200 μg/ml) for 2 min are also referred to here as "stable." In Supplementary Fig. 1a, the amount of shifted complexes is shown, following a 15-s incubation of 50 nM RNAP (WT or with various substitutions) and 10 nM promoter containing 2-AP at −11. The quantified extents of stable complex formation (normalized with respect to WT RNAP) are shown in Supplementary Fig. 1b, and the extents of abortive RNA synthesis following the same time interval, in Supplementary Fig. 2a and b. Comparison of the normalized data presented in Supplementary Figs. 1b and 2b demonstrates that the relative amounts of stable complexes and of abortive RNA synthesis are the same, within uncertainty, for seven out of nine RNAPs tested (W434A and YW are the exceptions). For reactions where the incubation times of RNAP and promoter DNA were 10 min, the extents of binding and of abortive RNA synthesis were greater for all the RNAPs studied (data not shown). Although the differences between the various RNAPs were less pronounced (formation of stable complexes is complete for most RNAPs studied), similar patterns were observed. We conclude from these data that the EMSA monitors the formation of heparin-resistant, abortive-initiation-competent complexes.

Kinetics of complex formation detected by EMSA

Representative curves of the kinetics of stable complex formation as detected by EMSA are shown in Fig. 1b and c. RNAP containing either WT σ^{70} or region 2.3 substituted σ^{70} was mixed with 2-AP substituted promoter DNA. In all experiments, RNAP was in excess over promoter DNA. At various times, aliquots of the reaction mixtures were challenged with heparin for 2 min and then loaded onto a nondenaturing polyacrylamide gel. The kinetics of formation of EMSA-detected complexes can be monitored by manual mixing; even for WT RNAP, three data points define the early part of the curve prior to reaching the plateau (cf. Fig. 1b, WT RNAP). All curves are well fit by a single exponential. While double-exponential fits (not shown) described some experiments more accurately based on the residuals, the fit rate constants have large errors, possibly due to the relatively small number of points. Without a basis for choosing the double-exponential fit as superior, we present the rate constants and amplitudes from the single-exponential fits in Table 1.

Substitutions in region 2.3 of σ^{70} significantly affect the amount of open complexes formed at equilibrium as well as the rate of their formation. While most RNAP bind >80% of the −11 2-AP promoter in a heparin-resistant complex, W434A, T429A, FYW and FYWW disfavor conversion to

Table 1

A. Stable complexes, −4 2-AP subtitution[a]

	A[b]	$k\ (h,\ -4)\ (s^{-1})$
WT	0.87±0.01	0.057±0.002
FYW	0.81±0.01	0.011±0.001

B. Stable complexes −11 2-AP substitution[a]

	A[b]	$k_1\ (h,\ -11)\ (s^{-1})$
WT	0.84±0.01	0.016±0.003
T429A	0.58±0.05	0.0014±0.0000
F427A	0.81±0.04	0.008±0.001
Y430A	0.82±0.08	0.016±0.002
W433A	0.80±0.07	0.011±0.001
W434A	0.84±0.03	0.003±0.000
YW	0.81±0.01	0.015±0.002
FYW	0.67±0.01	0.002±0.000
FYWW	0.10±0.01	0.0016±0.0000

[a] Complex formation was determined by EMSA. The DNA concentration was 10 nM, and the RNAP concentration was 50 nM. Stable complexes of RNAP and promoter DNA (2-AP substituted at the positions indicated) are those that survive a 2-min challenge with heparin.
[b] All binding data were fit to a single exponential; A is the fraction of total labeled DNA migrating with a reduced mobility compared to the free DNA.

stable complexes. Combined with the 2-AP substitution at the highly conserved −11 position in the −10 hexamer, T429A, FYW and FYWW bind 58%, 67% and 10% promoter DNA in long-lived complexes, respectively (Table 1). More than a 10-fold difference in rate constants is observed: WT and Y430A exhibit the greatest values [$k\ (h,\ -11) = 0.016\ s^{-1}$] and FYWW and T429A the smallest [$k\ (h,\ -11) = 0.0016\ s^{-1}$ and $k\ (h,\ -11) = 0.0014\ s^{-1}$, respectively]. (Rate constants for stable complex formation are designated $k\ (h,\ -x)$ where the h denotes the formation of heparin-resistant complexes and $-x$ is the position of 2-AP substitution.) However, all open complexes, once formed, are very stable: no dissociation is detected during a 60-min heparin challenge (data not shown).

In previous experiments with other templates, Y430A and other single substitutions in region 2.3 clearly had deleterious effects on stable complex formation.[14,23] However, such effects are not observed with the consensus promoter studied here. We also measured the kinetics of stable complex formation of the WT or FYW RNAP with the promoter DNA bearing 2-AP substitutions at less conserved positions in the −10 region (−8; Supplementary Table 2) or downstream (−4; Table 1). The rate constants for WT RNAP are fourfold greater with DNA containing −4 or −8 2-AP than that with −11 2-AP, and more RP_o forms at equilibrium (Table 1; Supplementary Table 2). These results are consistent with prior data indicating that 2-AP substitution at −11 disfavors open complex formation.[4,5,16]

Evidence for rapid saturation of an early short-lived complex at the consensus promoter

Kinetic studies of open complex formation performed in excess RNAP over promoter DNA

typically exhibit single-exponential kinetics where k_{obs} reaches a plateau with increasing RNAP concentration. Such data are well described by a mechanism of open complex formation involving at least one short-lived intermediate formed after an initial binding step. This intermediate rapidly equilibrates with reactants on the time scale of its conversion to a stable complex (cf. Refs. 24 and 25). When the RNAP concentration is sufficiently high so that all free promoter DNA is rapidly converted to this heparin-sensitive intermediate, k_{obs} becomes independent of [RNAP] and equal to the first-order rate constant for the subsequent conformational change. The dependence of k_{obs} on [RNAP] was investigated for the doubly substituted YW RNAP binding to the −11 2-AP promoter DNA. No effect of YW RNAP concentration on k_{obs} was observed from 5 to 50 nM RNAP in either the EMSA or stopped-flow fluorescence assay below (data not shown). We deduce that all promoter DNA is rapidly converted to an initially bound complex at the beginning of either experiment and that a binding step does not contribute to the observed kinetics.

Previous studies show that the T429A,[21] Y430A and W433A[26] substitutions (and likely also F427A, although it was not specifically tested) do not affect initial DNA binding by RNAP. Since promoter DNA is rapidly saturated with YW RNAP under these conditions, we infer that (1) WT RNAP also exhibits first-order k_{obs} in these experiments and (2) any effects of the substitutions reported here are exerted subsequent to initial complex formation. A possible exception is the W434A RNAP, in view of its binding defect.[13,27]

Kinetics of RNAP-induced increase in fluorescence of 2-AP promoter DNA

To study the conformational changes that occur in the steps after formation of the early complex between RNAP and promoter DNA, we monitored the change in the fluorescence of 2-AP substituted at various positions in the −10 region and downstream region of the promoter as a function of time after mixing. In B-form DNA, base stacking of 2-AP causes near-complete quenching of its fluorescence.[19,28] Changes in stacking interactions such as structural distortions of the double helix by DNA kinking or sharp bending,[29,30] melting[31–34] or flipping of the 2-AP out of the helix[7,9,35] are known to increase the 2-AP fluorescence. To probe the proposed critical role of the −11A in nucleating strand separation, 2-AP was incorporated at this position on the NT strand in the consensus promoter. DNA unstacking downstream of −11 was monitored by 2-AP substitutions at −8 and −4 on the NT strand. In control experiments with 2-AP substitutions on the NT strand at positions outside the initiation bubble (−13 and +7) we observed no changes in 2-AP fluorescence in open complex formation. Moreover, mixing of core RNAP (lacking σ^{70}) with promoter DNA does not affect the fluorescence signal of 2-AP at −4, −8 and −11 (data not shown). We conclude that the observed fluorescence increases at −11, −8 and −4 reflect specific interactions of the holo RNAP with the consensus promoter.

Detected kinetics of − 4 2-AP: large, relatively rapid, biexponential fluorescence increases for WT and variant RNAP

The kinetics of the RNAP-induced increase in fluorescence of 2-AP substituted at −4 on the nontemplate strand are shown in Fig. 2a. Similar amplitudes (averaged as indicated in Materials and Methods) are seen regardless of the σ^{70} substitution (see Table 2). The notable exception is FYWW, which has a greatly reduced signal, commensurate with its impaired ability to form open complexes.[21,36,37] The
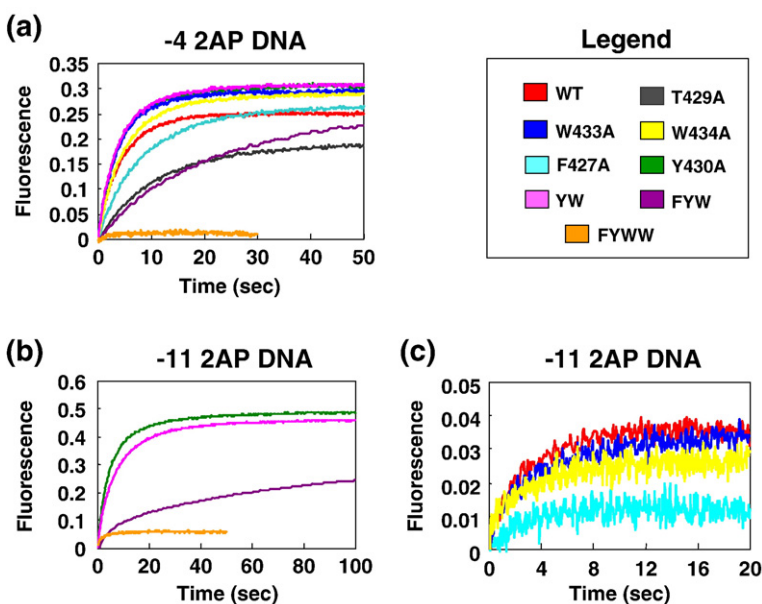
Fig. 2. Kinetics of change in 2-AP fluorescence as measured by stopped-flow analysis. DNA and RNAP concentrations were the same as for Fig. 1b and c. Complexes were not challenged with heparin; representative curves are shown. (a) Change in fluorescence (arbitrary units) after mixing of RNAP and −4 2-AP DNA. (b and c) Change in fluorescence after mixing of RNAP and −11 2-AP DNA. The different RNAPs were plotted in two groups (note differences in the *y*-axis scales between (b) and (c) due to the large differences in amplitude of the fluorescence signal.

Table 2. Fluorescence, −4 2-AP substituted promoter DNA

	A_1[a]	$k_1\ (f, -4)$ (s^{-1})[a]	A_2[a]	$k_2\ (f, -4)$ (s^{-1})[a]
WT	0.07±0.01	0.53±0.08	0.20±0.01	0.15±0.03
T429A	0.22±0.04	0.08±0.005	NF[b]	NF[b]
F427A	0.07±0.01	0.29±0.04	0.21±0.02	0.08±0.01
Y430A	0.15±0.04	0.37±0.01	0.17±0.05	0.11±0.00
W433A	0.15±0.03	0.39±0.03	0.16±0.05	0.14±0.01
W434A	0.12±0.03	0.35±0.02	0.18±0.04	0.11±0.00
YW	0.17±0.01	0.36±0.05	0.15±0.02	0.12±0.015
FYW	0.06±0.01	0.12±0.04	0.21±0.04	0.03±0.01
FYWW	0.018±0.001	0.34±0.07	NF[b]	NF[b]

The time-dependence of 2-AP fluorescence as determined in a stopped-flow instrument. The final DNA and RNAP concentrations were 10 and 50 nM, respectively. Six hundred points were taken over times ranging from 20 to 300 s (depending on the RNAP mutant used).
[a] The data were fit to a double-exponential equation. The amplitudes (A_1 and A_2) are in arbitrary units.
[b] NF indicates that the data could not be fit to a double-exponential equation. Single-exponential fits are given in Supplementary Table 1.

data were best fit to a double exponential (see Supplementary Fig. 3), with exception of those for the T429A and FYWW RNAP, which could only be fit to a single exponential. We surmise that for these RNAPs the slower phase was not resolved due to the low signal-to-noise ratio. The parameters of the fits are collected in Table 2. The rate constants determined by monitoring fluorescence are given in the text as $k\ (f, -x)$ where $-x$ is the position of 2-AP substitution in the promoter. For double-exponential fits, the rate constants for the fast and slow phases have subscripts of 1 or 2, respectively.

The rate constants determined by 2-AP fluorescence (whether the 2-AP is at −4, −8 or −11), are at least sevenfold greater than those detected by EMSA for heparin-resistant complex formation, regardless of whether fit to single or double exponentials. Thus some or all of the DNA conformational changes responsible for the fluorescence signal *precede* formation of the EMSA-detected complexes. In the case of WT RNAP, the $k_1\ (f, -4)$ was an order of magnitude greater than the corresponding rate constant for the formation of the EMSA-detected stable complex. The rate constants for the fast phase determined with the 2-AP substituted promoters are similar for RNAP containing WT, F427A, Y430A, W433A, W434A and YW σ^{70} (see Tables 2 and 3 and Supplementary Table 2); here, the average $k_1\ (f, -4)$ =0.38±0.08 s^{-1}. For the FYW RNAP, $k_1\ (f, -4)$ is smaller than that of WT RNAP by about a factor of 4. The rate constants for the slower phase [$k_2\ (f, -4)$] are all about threefold smaller than the $k_1\ (f, -4)$.

Detected kinetics of −11 2-AP: evidence for interaction between −11A and σ^{70} Y430

In Fig. 2b and c, the kinetic curves for the interaction of WT and variant RNAP with −11 2-AP substituted promoter DNA are shown. The parameters are collected in Table 3. For the interactions of

WT RNAP with −11 2-AP promoter DNA, the observed fluorescence increase is very small (low amplitude), in sharp contrast to the amplitudes observed for WT and variant RNAP interactions with the −4 2-AP substitution (Fig. 2a). Much larger fluorescence increases are observed for interactions of all RNAP containing the Y430A substitution and −11 2-AP promoter DNA, ranging from 2-fold greater than WT RNAP for the FYWW RNAP to almost 20-fold for the single Y430A substitution. The latter is 1.5-fold greater than the largest effect observed for the interaction of RNAP with the −4 2-AP promoter. The kinetics of the fluorescence increase with the Y430A, YW and FYW RNAP (variants for which the amplitudes of the fluorescence increase are largest) are double exponential (see Supplementary Fig. 4). The combined average of $k_1\ (f, -11)$ and $k\ (f, -11)$ is 0.36±0.08 s^{-1} for WT, F429A, Y430A, W433A, W434A and YW σ^{70}. For the WT RNAP and other RNAPs containing the Y430 residue, the amplitudes are small. Together these observations demonstrate that Y430 participates directly or indirectly in quenching the −11 2-AP fluorescence.

Detected kinetics of −8 2-AP: low amplitudes and single exponentials

The results obtained with consensus promoter DNA containing 2-AP at −8 are summarized in Supplementary Table 2. Low amplitudes were observed for all the RNAPs tested, while no measurable signal was detected for FYWW RNAP. All curves fit well to a single exponential. Interestingly, the amplitudes for the RNAP with the W433A and T429A substitutions were about threefold greater than those for the WT RNAP. While these are considerably smaller effects than that observed for Y430A and the −11 2-AP promoter, they may still reflect proximities of W433 and T429 to the −8 base

Table 3. Fluorescence, −11 2-AP substituted promoter DNA

	A_1[a]	$k_1\ (f, -11)$ (s^{-1})[a]	A_2[a]	$k_2\ (f, -11)$ (s^{-1})[a]
WT[b]	0.03±0.01	0.39±0.02		
T429A	NF[c]	NF[c]	NF[c]	NF[c]
F427A[b]	0.014±0.001	0.48±0.01		
Y430A	0.27±0.06	0.29±0.01	0.21±0.05	0.07±0.07
W433A[b]	0.024±0.004	0.27±0.04		
W434A[b]	0.019±0.001	0.29±0.01		
YW	0.17±0.05	0.34±0.05	0.26±0.02	0.065±0.001
FYW	0.094±0.001	0.13±0.04	0.27±0.01	0.008±0.001
FYWW	0.05±0.01	0.36±0.02		

The time-dependence of 2-AP fluorescence as determined in a stopped-flow instrument. The final DNA and RNAP concentrations were 10 and 50 nM, respectively. Six hundred points were taken over times ranging from 20 to 500 s (depending on the RNAP mutant used).
[a] The data were fit to a double-exponential equation. The amplitudes (A_1 and A_2) are in arbitrary units.
[b] The data could only be fit to a single exponential.
[c] NF indicates that the data could not be fit to either a single- or a double-exponential equation due to a low signal.

in an intermediate complex. The average k $(f, -8)$ is 0.34 ± 0.08 s^{-1} for WT, F427A, Y430A, W433A, W434A and YW σ^{70}. The fluorescence increase for FYW RNAP is considerably slower than that for any of the other RNAPs tested with the -8 2-AP substituted promoter DNA.

Effects of different amino acid substitutions at σ^{70} Y430 on the environment of the nontemplate base at -11

When Y430 is changed to alanine, the fluorescence signal for 2-AP at -11 increases dramatically (more than 15-fold; Fig. 2b and c). This effect is not seen for the Y430A substitution and promoter DNA containing 2-AP at -8 or -4. Possible interpretations are that, after it is flipped out of the helix, the -11 2-AP stacks with Y430 leading to fluorescence quenching and/or that Y430's role is to place the -11 2-AP in a pocket where other interactions lead to fluorescence quenching. To determine whether stacking or

Fig. 3. Effects of various amino acid substitutions at position 430 of σ^{70} on -11 2-AP fluorescence. (a) The substitutions at 430 do not have large effects on stable complex formation. EMSA experiments were performed by incubating 5 nM of -11 2-AP DNA with 25 nM of WT or mutant RNAP for 15 s. The reaction mixes were then loaded onto a nondenaturing gel after a 2-min heparin challenge. The fraction of RNAP-bound DNA for various substitutions, normalized to that for WT RNAP, was plotted as a function of the particular RNAP investigated. (b) The substitutions at Y430 affect fluorescence amplitudes, but not the rate constants. Changes in fluorescence over time upon mixing of RNAP and -11 2-AP promoter DNA, as measured by a stopped-flow analysis. The concentrations of RNAP and DNA are the same as in (a); no heparin challenge was used for these experiments.

another type of binding interaction quenches the fluorescence signal of the 2-AP at -11, we changed Y430 to the aromatic residues F or W to a long aliphatic side chain L, or to H. To assess whether these substitutions affect open complex formation under the conditions of the stopped-flow experiment, we determined extents of formation of heparin-resistant complexes after a 15-s incubation of RNAP with the -11 2-AP consensus promoter (see Fig. 3a). In this short time interval, amounts of heparin-resistant complexes formed by RNAP σ^{70} variants are either the same as for WT RNAP (Y430A, Y430W and Y430F) or somewhat greater (Y430H and Y430L). Stable complex formation was also determined following a 10-min incubation; after this longer time, similar extents of binding were seen for all RNAPs (data not shown).

The results of kinetic studies with -11 2-AP substituted promoter DNA are shown in Fig. 3b. As seen for the change to alanine, substitutions of H, L and F for Y430 all increase the fluorescence of -11 2-AP relative to the low signal for WT RNAP. Strikingly, the amplitude of the signal obtained for Y430W RNAP is smaller than that for WT RNAP, too low for the data to be fit. The data for the H, L, F and A substitutions were best fit to a double-exponential equation. Other than Y430W, the substitutions do not greatly affect either k_1 $(f, -11)$ (average of five values is 0.49 ± 0.06) or k_2 $(f, -11)$ (average of four values is 0.12 ± 0.01) (data not shown). Apparently the W430 residue quenches the -11 2-AP fluorescence to an even greater extent than did the WT residue, Y430. On the other hand, other substitutions at 430 tested rendered RNAP unable to quench the -11 2-AP fluorescence. F and A have similar effects and H and L result in larger increases in the fluorescence of the 2-AP substituted at -11 in the nontemplate strand. The lack of fluorescence quenching by the Y430F RNAP accentuates the importance of the tyrosine hydroxyl group.
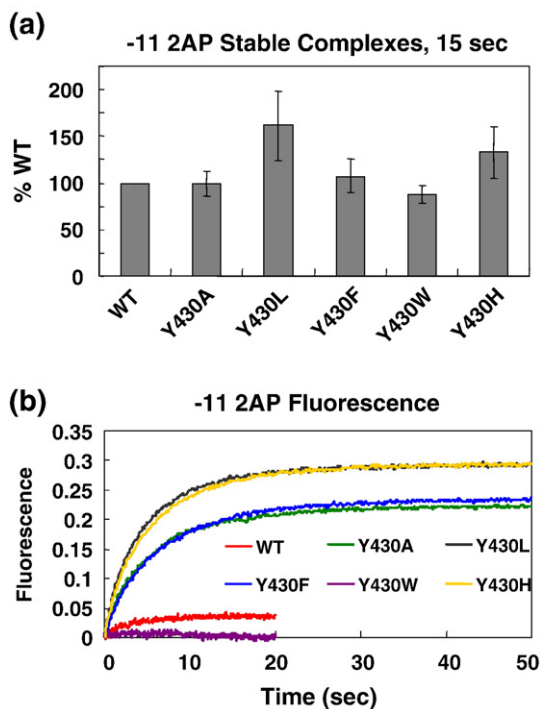
Discussion

The role of σ^{70} Y430

The Y430A substitution in σ^{70} leads to a large increase in the 2-AP signal upon binding of RNAP to promoter DNA substituted at -11, but not at -8 or -4. Based on these data, we propose that Y430 plays a role in quenching the fluorescence of the -11 2-AP. Y430 could stack with the -11 base after it is flipped out of the helix, or it could facilitate placing the -11 base in an RNAP pocket where its fluorescence would be quenched due to interactions with additional amino acid side chains. Because the -11 2-AP fluorescence increases for four out of the five substitutions tested, we surmise that it is unstacked in these RNAP–promoter complexes and not in a fluorescence-quenching environment. No change in fluorescence is observed with the Y430W RNAP, even though the kinetics and extent of formation of stable -11 2-AP complexes are indistinguishable

from that of WT RNAP. Our experiments are most consistent with direct capture by Y430 of the −11 2-AP after it is flipped out of the helix. In the 6.5 Å resolution open complex model,[38] the Y430 is in close proximity to the −11A. We had previously concluded from studies of interactions between RNAP and a consensus promoter truncated at +1 that −11A and Y430 did not interact.[16] While the results presented here supersede that simple interpretation, our previous data still indicate that Y430 plays a role beyond its interaction with −11A. This role may be more critical for promoters lacking downstream DNA.

Studies of base flipping by other enzymes on DNA where 2-AP was substituted for the extruded base found that fluorescence quenching was suppressed if an alanine was substituted for the aromatic amino acid residue to which the 2-AP stacks following its removal from the helix.[9,35] For both WT and mutant RNAP–promoter complexes, the −11 2-AP apparently remains extrahelical once flipped. Since the observed rate constant [k_1 (f, −11)] is similar for WT and Y430A RNAP (see Table 3), i.e., independent of whether the −11 base is captured or not, we propose that another region of the RNAP facilitates its extrusion from the DNA double helix and that capture does not determine the rate of this step. Likely candidates are amino acid side chains in the upstream lobe of the beta subunit. Located directly across from region 2 of σ[70] at the entrance of the active-site channel,[39,40] these residues appear well-positioned to induce the bend that puts the downstream DNA in the active-site cleft in an early intermediate and to flip bases in the −10 region out of the DNA helix.

Evidence for an intermediate species on the pathway to formation of an initiation-competent open complex

The events monitored by 2-AP fluorescence are first order for all RNAPs studied here, indicating that they occur subsequent to RNAP binding to promoter DNA. It is likely that this is the case for all single substitutions in σ[70] studied here as well as for DNA with 2-AP substitutions at −4 and −8. The values of the larger rate constants (k_1 or k) are about 0.36 s^{−1}, independent of the positions of the 2-AP substitutions (see Results). These values are at least an order of magnitude greater than the EMSA-detected rate constants for stable complex formation [k (h, −11) =0.016 s^{−1} for WT RNAP; see Table 1B]. The k_2 (f, −11) for Y430A and YW were similar at about 0.07 s^{−1}, fourfold greater than the values for stable complex formation. Similarly, for FYW RNAP the k_2 (f, −11) was fourfold greater than the k_{obs} for stable complex formation [k_2 (f, −11)=0.008 s^{−1} versus k (h, −11)=0.002 s^{−1}, respectively; see Tables 1 and 3].

Our results provide compelling evidence for at least one fluorescent intermediate formed subsequent to the early (quenched) complex and on the pathway to formation of the stable open complex. What might be the nature of these changes in the

DNA? The observed increase in 2-AP fluorescence likely results from unstacking as a consequence of DNA melting or other distortions of DNA structure.[29,30] For the 2-AP at −11, we observed large effects of substitutions at residue 430 on the amplitude of the −11 2-AP fluorescence signal. The simplest explanation of this result is that flipping of the −11 base out of the helix (and thus local disruption of base stacking at −11) takes place; for WT RNAP, this base is then captured by Y430. The interaction with Y430 appears to quench −11 2-AP fluorescence nearly as effectively as its stacking interactions in promoter duplex DNA. As no early, transient increase in 2-AP fluorescence is observed, we conclude that the transit time of the −11 2-AP from being stacked in the DNA helix to interacting with the Y430 is very short compared to the time scale of our experiments. The lack of an observable effect of the Y430A substitution in the experiments described here is likely due to the use of a very strong promoter (see Fig. 1a).

As the rate constants of the fluorescence increases for the 2-AP at −4 and at −8 are equal to that of the 2-AP at −11, it is likely that base pairs in the region from −11 to −4 are melted concurrently, likely in response to introduction of a bend across this region. The positioning of sigma on RNAP requires that promoter DNA bends sharply at −11/−12 to enter the active-site channel. We and others proposed that (i) the bend occurs in an early intermediate (I_1 at the λP$_R$ promoter) and (ii) this ~90° bend places the start site (+1) DNA in the active-site cleft and likely nucleates melting.[40,41] A bending event was proposed to occur at the T7A1 promoter with a rate constant similar to that observed here for k_1 (f, −4, −8 or −11): 0.4 to 1.4 s^{−1}, depending on the model used to analyze the data.[42] Indeed, for T7 RNAPs concurrent promoter DNA bending and melting was observed.[33,43] Additionally, in base flipping by uracil DNA glycosylase, DNA bending has been found to be crucial in promoting the extrusion of the base out of the helix.[8]

Comparison of the fluorescence signals obtained with promoter DNA bearing 2-AP substitutions at −11, −8 and −4

The amplitudes of the fluorescent signals obtained with WT RNAP and promoter DNA containing the 2-AP at −4 are much higher than for the substitutions at both the −11 and −8 positions (a large signal for the −4 2-AP was previously noted).[31] Interestingly, the higher signal observed with WT RNAP and the promoter DNA containing the substitution at −4 is comparable to (within 65% of) that seen with Y430A RNAP and the −11 2-AP. We infer that there is little or no quenching of the −4 2-AP once it is displaced out of the DNA helix (compare Tables 2 and 3). The signals obtained with WT RNAP interacting with promoter DNA bearing the 2-AP at −11 and −8 are similar (compare Table 3 and Supplementary Table 2). These results likely mean that the fluorescence of the −8 2-AP, just like that of

the −11 2-AP, is still partially quenched once it is out of the DNA helix. The residue(s) responsible for quenching the −8 2-AP are as yet unknown.

The pathway to open complex formation and effects of particular substitutions in region 2.3

In Scheme 1, QC refers to the most advanced quenched (silent), short-lived (heparin-sensitive) complex between RNAP and promoter DNA; QC reversibly isomerizes to a complex detectable by 2-AP fluorescence (F; also sensitive to heparin challenge) that then converts to competitor (e.g., heparin)-resistant fluorescent complexes (including the open complex RP_o), CR, detected by EMSA. Positioning of F before CR is based on the observations for both WT and sigma variant RNAP that the rate constants for increase in 2-AP fluorescence at all promoter positions examined (−11, −8 and −4) significantly exceed those for formation of EMSA-detected stable complexes.

Preliminary results from kinetic simulations based on this mechanism (Gries et al., in preparation) reveal that the double-exponential character of the fluorescence data arises from the reversibility of the step converting the silent QC to the F complex detected by 2-AP fluorescence. To observe two exponentials, the conversion of F back to QC, while not necessarily rapid, is at least significant on the time scale of converting F to CR (e.g., RP_o). There may be additional intermediates between F and CR; their detection requires investigating the dissociation of CR. The observed rate constants (Tables 1, 2 and 3) do not correspond directly to the microscopic rate constants for the individual steps in Scheme 1.

How do the intermediates in Scheme 1 relate to the two kinetically significant intermediates I_1 and I_2 that precede formation of RP_o at the λP_R promoter? λP_R deviates from consensus at each of the −10 and −35 hexamers by one position and lacks the extended −10 sequence (λP_R −15 to −7: gG-gATAAT, where consensus bases are indicated in capital letters). I_1 is sensitive to a 10-s heparin challenge and appears to be a closed complex that protects the DNA backbone to at least +20.[44,45] The highly transient nature of I_2 has precluded its structural characterization to date. I_2 is inferred to be a competitor-resistant complex that rapidly converts to RP_o on the time scale of its back conversion to I_1. Based on the similar magnitudes of the overall EMSA rate constant for converting QC to CR and the rate constant for converting I_1 to I_2 at 25 °C (~ 0.05 s^{-1})[41] and the evidence to date which indicates that I_1 is a closed complex,[44,45] we speculate that QC is similar to I_1. CR would represent both

$$R + P \quad \rightleftarrows \quad QC \quad \rightleftarrows \quad F \rightarrow CR$$

Scheme 1. Minimal mechanism for describing the EMSA and fluorescence-detected kinetic data for the formation of complexes between WT or sigma variant RNAP and 2-AP labeled consensus promoter DNA.

heparin-resistant complexes at λP_R, I_2 and RP_o. What is intermediate F?

At λP_R, interconversion of I_1 and I_2 appears to be an elementary step without intermediates. At the consensus promoter, we detect an additional step (formation of F) between the I_1-like complex QC and CR. One hypothesis is that optimal sequences in the consensus promoter lower the activation barrier between I_1 and I_2 compared to that observed for λP_R, stabilizing the I_1–I_2 transition-state complex. What promoter sequences provide enough binding free energy to convert this transition-state complex to a kinetically significant intermediate? Based on the observation that a TG sequence at −15 and −14 increases the composite isomerization rate constant k_f more than 10-fold at a galP1 derivative promoter with no effect on the initial binding constant,[46] we propose that contacts between RNAP and the extended −10 region drive key conformational changes involved in DNA opening.

Substitutions Y430A, W433A and even the double substitution YW in region 2.3 have little effect on the rate constant determined by monitoring 2-AP fluorescence k_1 (f) or k (f) (Tables 2 and 3 and Supplementary Table 2) of stable complex formation (Table 1). This observation is in contrast to their large effects on stable complex formation previously reported.[14,23] Because the consensus sequence used here is such a strong promoter, the above substitutions may not have discernible thermodynamic or kinetic effects. As a consequence, our experiments do not reveal at which steps in Scheme 1 Y430 and W433 normally exert their effects. The W434A substitution inhibits stable complex formation as measured with the −11 2-AP substituted DNA (see Table 1), but it does not affect the rate constants for the increase in the fluorescent signal from any of the three 2-AP substituted promoter DNAs tested (Tables 2 and 3 and Supplementary Table 2). W434 apparently plays an important but as yet unknown role at a late step in formation of the open complex. The single substitution, T429A, in region 2.3 of σ^{70} affects multiple steps, as does the FYW triple substitution.

Comparison of our results with those of other recent kinetic studies

The rate constants reported for early steps at the T7 A1 promoter obtained by rapid hydroxyl radical footprinting experiments[42] are considerably larger than those obtained in our stopped-flow studies. We conclude that in these early complexes, significant changes in the DNA structure surrounding −4, −8 or −11 have not yet occurred. In studies performed on promoters containing 2-AP in the melted region, just as in our work, both slow and fast phases were seen. For the lacP1 promoter (2-AP at +1), a very fast step (rate constant of about 10 s^{-1}) was observed, interpreted as immediately following closed complex formation in addition to a much slower one (rate constant of 0.003 s^{-1}). The reaction mix contained a high [RNAP] (550 nM).[47] In a study of the interaction of RNAP (100 nM) with the galP1

promoter,[31] only with 2-AP at +2 or +3 could a fast step be observed, interpreted as DNA distortion. It had a rate constant of about 0.8 s^{-1}, similar to that obtained for the fast step in our studies. In reactions at a much higher [RNAP] (830 nM), just a slower step with a rate constant of about 0.001 s^{-1}, dependent on 2-AP position, was seen, which was interpreted as strand separation. The slow steps in both these studies may be slower than in our experiments, as both the galP1 and lacP1 promoters deviate from consensus at several positions, in contrast to the DNA used here (Fig. 1a).

Conclusions

We conclude that Y430 of σ^{70} captures the base at −11 A of promoter DNA through stacking interactions once it is flipped out of the promoter DNA. We present evidence for the existence of an open, heparin-sensitive intermediate F on the pathway to formation of an open complex. In F, the DNA at positions −11, −8 and −4 (and likely also the intervening positions) is open, as judged by 2-AP fluorescence. Most single substitutions in region 2.3 of σ^{70} affect the conversion of F to the final open complex.

Materials and Methods

Materials

2-Aminopurine containing oligodeoxynucleotides were synthesized by Integrated DNA Technologies or Midland Certified Reagent Company and uridylyl (3′-5′) adenosine (UpA) by Dharmacon. [γ-^{33}P]UTP and [α-^{33}P]ATP were purchased from Perkin Elmer, DNA-modifying enzymes from either New England Biolabs or Roche and *E. coli* RNAP core from EpiCenter. All chemicals were from Sigma, Fisher, or Amresco.

Protein purification, characterization and reconstitution

E. coli σ factors were purified exactly as described.[16] Holoenzyme was reconstituted by incubating core RNAP (400 nM) and WT or substituted σ^{70} on ice for 1 h. Depending on the particular preparation, the σ^{70} was added in 5- to 15-fold excess over the core enzyme, as determined by EMSA (see below) to give optimal formation of stable complexes with promoter DNA.[16]

Promoter DNA

Both strands of promoter DNA were chemically synthesized, and the 2-AP substituted nontemplate strand was annealed to the template strand, with the latter in 1.5-fold excess.[16] For binding studies the nontemplate strand was phosphorylated with ^{33}P at the 5′ end.[16]

Electrophoretic mobility shift assay

Binding assays were carried out exactly as previously described.[21] Annealed promoter DNA (10 nM) and RNAP holoenzyme (50 nM) were incubated at 25 °C for 15 s or 10 min in Fork binding buffer [FBB: 30 mM Hepes, pH 7.5, 1 mM DTT, 0.1 mg/ml bovine serum albumin, 100 mM NaCl, 0.1 mM ethylenediaminetetraacetic acid (EDTA), pH 8, 1% glycerol]. The reactions were then challenged with 200 μg/ml of heparin for an additional 2 min to assay for formation of stable complexes. Four percent nondenaturing gels were loaded and run at room temperature. After drying, the gel was analyzed as described.[16]

Obtaining association rate constants by EMSA

The rate constants k_{obs} for stable complex formation were determined as described.[21] Reactions were set up and subjected to EMSA as described above, except that the RNAP and promoter DNA were incubated for various amounts of time prior to addition of heparin. Fractions of DNA bound were determined and plotted as a function of time and fit using Kaleidagraph version 3.52 to the single-exponential equation: $y = y_{max}[1 - \exp(-k_{obs}t)]$, where y_{max} is the amplitude and k_{obs} is the pseudo first-order rate constant for the association of RNAP and promoter DNA to form a heparin-resistant complex. Experiments with lower DNA (1 nM) and RNAP concentrations (5–50 nM), as indicated, were performed as described above. All glycerol concentrations were adjusted with storage buffer to be 10% final. For determination of the k_{off}, RNAP-promoter complexes were formed in 30-μl reaction mixtures by mixing the RNAP and DNA at the same concentrations as for the association kinetics. After a 15-min incubation at 25 °C, heparin was added to 200 μg/ml and time points were taken thereafter.

Abortive initiation assay

Reactions contained 10 nM annealed DNA in 1×FBB supplemented with 10 mM MgCl$_2$ and were started by the addition of RNAP to 50 nM (final volume 10 μl). Incubation was carried out at 25 °C for either 15 s or 10 min followed by a 2-min heparin challenge (200 μg/ml). Then 1 μl of a cocktail containing 850 μM UpA, 150 μM uridine triphosphate (UTP), and 60 μCi of [α-^{33}P]UTP was added and incubation was allowed to proceed for 25 min before addition of 10 μl of stop solution (to final concentrations of 3 M urea, 50 mM EDTA, 0.1% bromphenol blue and 0.1% xylene cyanol FF). Reactions were loaded onto a 20% denaturing PAGE gel (1×TBE [89 mM Tris-borate (pH 8.3), 2 mM EDTA], 6 M urea, 4% glycerol). Following electrophoresis, the gel was dried and the relative amounts of radiolabeled UpApU trinucleotide and free UTP were determined by Phospor-Imaging. The values obtained for the mutant RNAPs were then normalized to that obtained with the WT RNAP.

Fluorescence

For stopped-flow experiments, two (900 μl) solutions were prepared in 1×FBB, but without bovine serum albumin to reduce background signal. One solution contained 20 nM annealed 2-AP substituted promoter DNA (10 nM after mixing with RNAP) and storage buffer to keep the glycerol and salt concentrations constant for all reactions. A second solution contained 100 nM of reconstituted WT or mutant holoenzyme (50 nM after mixing with 2-AP DNA). The separate reaction mixtures were loaded into 3-ml syringes and maintained at 25 °C by

a circulating water bath. For some experiments, 1 nM 2-AP DNA was used and 5–50 nM RNAP (final concentrations), but otherwise the procedure was similar to that described above. Using an Applied Photophysics Π∗-180 stopped-flow apparatus, the two solutions were rapidly mixed into a cuvette. The dead time of mixing was 8 ms. The excitation wavelength was 310 nm (mercury–xenon lamp) and light above the 350-nm cutoff filter was monitored; the slits were set at 2.5 nm. For all reactions, the same photomultiplier voltage was used and 600 points were collected for two or more mixing events. Each experiment was carried out twice on separate days. The data were analyzed by fitting each individual trace with Applied Photophysics software to a single exponential [$y = A(1 - \exp(-k_{obs}t))$]. Only single exponentials provided adequate fits to some −11 2-AP data, possibly because the low signal to noise ratio did not allow the precise definition of the trace for the slow phase. Alternatively, as indicated, a double-exponential equation was used to fit some of the data [$y = A_1(1 - \exp(-k_{obs1}t)) + A_2(1 - \exp(-k_{obs2}t))$], if the fit was visually better than with a single exponential (e.g., see Supplementary Figs. 3 and 4). Errors represent the spread between two experiments performed on separate days. For the Y430 mutant reactions shown in Fig. 3, traces were averaged, then fit. For these experiments the reactions contained 5 nM 2-AP DNA and 25 nM RNAP.

Acknowledgements

Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2008.10.023

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