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Using Solutes and Kinetics to Probe Large Conformational Changes in the Steps of Transcription Initiation

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Summary

Small solutes are useful probes of large conformational changes in RNA polymerase (RNAP)-promoter interactions and other biopolymer processes. In general, a large effect of a solute on an equilibrium constant (or rate constant) indicates a large change in water-accessible biopolymer surface area in the corresponding step (or transition state), resulting from conformational changes, interface formation, or both. Here, we describe nitrocellulose filter binding assays from series used to determine the urea dependence of open complex formation and dissociation with *Escherichia coli* RNAP and λP_R promoter DNA. Then, we describe the subsequent data analysis and interpretation of these solute effects.

Keywords

bacterial RNA polymerase; open complex formation; kinetics; mechanism; solute effects; conformational changes

1. Introduction

1.1 Solute Interactions and Solute Effects

Small solutes are emerging as useful probes and perturbants of intermediates and transition states and of overall changes in surface area in transcription initiation and other protein and nucleic acid processes. In general, large amounts of protein and nucleic acid surface are buried or exposed when interfaces are made or disrupted or in coupled conformational changes. The effect of a solute on an equilibrium constant (or rate constant) is determined by its favorable or unfavorable interactions with the functional groups on the surface buried or exposed in the corresponding step (or transition state).

These solute effects vary widely for different solutes and different surfaces. For example, urea interacts favorably with most protein and nucleic acid functional groups. High concentrations (typically more than 4 M) of urea denature proteins and nucleic acids at 25 °C because urea interacts favorably with the amide and hydrocarbon groups exposed in

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unfolding proteins and with the nucleobase groups exposed in melting nucleic acid helices (1). At lower concentrations, these same interactions cause urea to disfavor steps in transcription initiation that bury RNA polymerase (RNAP) or promoter DNA surface, and to favor the reverse direction of these steps (described below). Glycine betaine (GB) and proline, on the other hand, interact unfavorably with many protein functional groups and with nucleic acid phosphates and therefore stabilize proteins (though not nucleic acids) against denaturation (1, 2) and favor the early and late steps in transcription initiation that bury RNAP and promoter DNA surface ((3) and unpublished data for proline). Replacement of KCl by potassium glutamate (KGlu) has a qualitatively similar effect to replacing urea by GB in that this replacement favors protein folding and assembly (but not DNA helix formation), and therefore favors the early and late steps in transcription initiation (4).

Solute effects are analyzed and interpreted using the slope of a plot of the logarithm of the observed equilibrium constant ($\ln K_{\text{obs}}$) or rate constant ($\ln k_{\text{obs}}$) versus solute concentration ([solute]). These plots are typically linear up to at least 1 M [solute]. Their slopes, closely related to what are called solute *m*-values, can be analyzed at several levels, starting with the qualitative level discussed above, where the sign of the effect reports on whether surface is buried or exposed in the step (when $\ln K_{\text{obs}}$ is analyzed) or the formation of the transition state (when $\ln k_{\text{obs}}$ is analyzed) and the magnitude of the effect reports on how much surface is involved. As examples of this technique, details of the use of urea and GB to probe conformational changes and interface formation in the steps of forming and stabilizing the open promoter complex by *E. coli* RNAP are provided in this article (3–6).

1.2 Mechanism of Formation and Stabilization of the Open Promoter Complex in Transcription Initiation

The mechanism of transcription initiation by *E. coli* and T7 phage RNAP have been extensively investigated and are model systems for transcription initiation. Both polymerases form the open complex by a “bind-bend-melt” mechanism (7, 8). Kinetic studies using *E. coli* RNAP (R) and the P_R promoter (P) of phage λ show that a minimum of four steps and three kinetically-significant intermediates (designated I_1 , I_2 , I_3) are involved in formation of the transcriptionally-competent open complex (RP_o) by the bacterial polymerase: **[Mechanism 1 goes here]** Immediately after mixing RNAP and λP_R promoter DNA under conditions typically used to study transcription initiation, relatively unstable closed complexes designated I_1 are formed (9). For λP_R , I_1 does not appear to be a single species but rather an ensemble (i.e a mixed population) of relatively unstable closed complexes which dissociate rapidly to free RNAP and promoter DNA and therefore equilibrate with each other and with free promoter on the time scale required to convert to I_2 . Likewise in a λP_R dissociation kinetics experiment, the relatively unstable open intermediates I_3 and I_2 convert rapidly back to the stable open complex (RP_o) on the time scale required for I_2 to convert to I_1 . The interconversion $I_1 \rightleftharpoons I_2$, opening and closing the 13 base pair transcription bubble (9), is the rate-determining step in both directions for λP_R , with the highest free energy transition state (TS) in Mechanism 1. While RP_o is relatively well-characterized, much less is known about these intermediates and TS (8). This information is needed to determine the mechanism of operation of RNAP as a molecular DNA-opening machine. The use of solutes as probes of large conformational changes and

interface formation in these intermediates and transition state, as illustrated in this chapter, provides information that is not readily obtained by other methods.

1.3 Examples of Solute Effects on Steps of Transcription Initiation and their Interpretation

Solutes can be used as probes for the change in surface area in a step or the formation of a transition state. As an example from transcription initiation, the equilibrium constant and both rate constants for the interconversion of I_1 and I_2 , the step which includes opening/closing the 13 base pair initiation bubble, are independent of urea and of the choice of anion (Cl^- versus Glu^-) and only weakly dependent on salt concentration (3–5). This contrasts greatly with the situation for DNA melting in solution, where the equilibrium constant for opening DNA oligomers of this length is a strong function of urea and salt concentration, and provides one of several lines of evidence that DNA opening occurs in the active site cleft of RNA polymerase in an environment very different from that of the solution.

As a second example of the use of solutes as probes, the dissociation rate constant k_d is a strong function of urea, GB, and salt concentration, and is profoundly affected by replacing Cl^- by Glu^- (3–5). Effects of urea and KCl are entirely in the equilibrium constant K_3 for the conversion of the initial open complex I_2 to the stable open complex RP_o and provide strong evidence for folding and assembly of approximately 120 residues of downstream mobile elements (DME) (6) of WT RNAP on duplex DNA as part of stabilizing the λP_R open complex by a factor of about 10^5 (4, 5)

In addition to their use as probes, solutes can also be used as selective perturbants. As one example, because urea and KCl are found to destabilize RP_o greatly with respect to I_2 , but do not affect the rate of conversion of I_2 to I_1 , rapid increases in concentration of these solutes create a transient, majority population (called a burst) of the otherwise-undetectable intermediate I_2 for characterization by DNA footprinting (9). In another study, GB was used to increase the I_1 binding constant and hence increase the size of the burst population of I_1 , and to reduce the rate of its conversion to open complexes, to footprint I_1 in manual mixing experiments (10). The original papers should be consulted for details of these experiments.

In what follows, we describe the methods used to perform nitrocellulose filter binding assays to study the urea dependence of the kinetics of RP_o formation and of RP_o dissociation into free RNAP and promoter DNA. We then explain the data analysis required to determine the solute concentration dependences of the individual steps of Mechanism I, and describe the conclusions we have reached using such analyses in our studies of the mechanism with λP_R .

2. Materials

2.1. Reagents

1. RNAP: Prepare using standard protocols, such as described in (**reference chapter in this volume describing this prep**). Dialyze into RNAP Storage Buffer (see below), divide into 100 μ L aliquots, and store at -80°C . Store a 100 μ L aliquot at -20°C while in use.

2. Radiolabeled DNA: Use T4 Polynucleotide Kinase (New England Biolabs) and γ ^{32}P -labeled ATP (Perkin Elmer) to ^{32}P -label one primer, then use this primer to amplify the fragment containing the promoter via PCR. Purify PCR fragment from an agarose gel. Store at 4°C (*see* Note 1).
3. 8× Transcription Buffer Salts: 320 mM Tris (pH 8.5 at room temperature), 960 mM KCl, 80 mM MgCl_2 . Dissolve 3.876 g Tris HCl, 7.157 g KCl, and 1.626 g MgCl_2 into about 90 mL deionized H_2O . Adjust pH to 8.5 using HCl. Bring to 100 mL with deionized H_2O in a volumetric flask. Store at 4°C.
4. 4× Transcription Buffer Salts: 160 mM Tris (pH 8 at assay temperature), 480 mM KCl, 40 mM MgCl_2 . Combine 25 mL 8× Transcription Buffer Salts with about 20 mL deionized H_2O . Equilibrate at intended assay temperature and adjust the pH to 8.0 using HCl. Bring to 50 mL in a volumetric flask using deionized H_2O . Store at 4°C.
5. 1 M dithiothreitol (DTT). Mix 0.154 g DTT with 1 mL deionized H_2O . Vortex to dissolve. Filter using a 0.45 μm syringe filter. Store at −20°C in 50 μL aliquots.
6. 50 mg/mL bovine serum albumin (BSA). Mix 100 mg with 2 mL deionized H_2O . Filter using a 0.45 μm syringe filter. Store at −20°C in 50 μL aliquots.
7. 50 mg/mL heparin. Mix 100 mg with 2 mL deionized H_2O . Filter using a 0.45 μm syringe filter. Store at −20°C in 50 μL aliquots. (*See* Note 2).
8. RNAP Storage Buffer: 50% v/v glycerol, 10 mM Tris (pH 7.5 at 4°C), 100 mM NaCl, 0.1 mM DTT, 0.1 mM Na_2EDTA . Dissolve 0.061 g Tris HCl and 0.292 g NaCl in about 20 mL deionized H_2O . Add 10 μL 0.5 M Na_2EDTA (pH 8.0 at room temperature) and 5 μL 1 M DTT. Equilibrate at 4°C in a water bath and adjust pH to 7.5 using HCl. Bring to 25 mL with deionized H_2O in a volumetric flask. Bring to 50 mL with glycerol in a volumetric flask. Store at −20 °C.
9. 6 M Urea in 1× Transcription Buffer Salts (*see* Note 3): 43 mm (millimolar) Tris (pH 8 at assay temperature), 129 mm KCl, 10.7 mm MgCl_2 , 6 M urea. Combine 12.5 mL 4× Transcription Buffer Salts and 6.25 mL RNAP Storage Buffer. Bring to 50 mL with deionized H_2O in a volumetric flask. Dissolve 18.018 g dry urea into a small amount of this solution of 1× Transcription Buffer Salts. Transfer this solution into a 50 mL volumetric flask and add additional 1× Transcription Buffer

¹RNA polymerase binds well to nicked DNA and overhanging ends, so complete fill-in of the PCR fragment is essential. We use Vent DNAP (NEB) for PCR and a plasmid as our PCR template when possible, and we observe the best results with DNA fragments extending to at least −120 on the upstream end and +40 on the downstream end, relative to the transcription start site +1.

²Possible competitors include unlabeled promoter DNA, single stranded DNA, or the polyanion heparin. No matter which competitor is used, it is necessary to test whether it is performing as intended. Heparin, typically used at concentrations in excess of 10 $\mu\text{g}/\text{mL}$, is an effective and inexpensive competitor that makes dissociation of WT RNAP from the λP_R promoter irreversible. For WT RNAP and the λP_R promoter, the dissociation rate constant k_d is independent of heparin concentration above this threshold concentration, indicating that the mechanism of action of heparin as a competitor is to bind free RNAP and not to displace promoter DNA from open complexes. However, at some other promoters (e.g., T7 phage promoters (22)) and for some RNAP mutants (e.g., deletions of $\sigma_{1.1}$ (Amanda Drennan, unpublished data), k_d is observed to increase with increasing heparin concentration. In these situations, heparin must also bind to the open complex, displacing promoter DNA. Extrapolation of k_d to zero heparin concentration provides an estimate of the unperturbed k_d , which can also be determined using unlabeled full-length λP_R promoter DNA (added in over 5–10-fold excess over RNAP) as a fast-binding, non-displacing competitor.

³We use a 6 M urea stock here as a convenient example. Other concentrations can be used, up to the solubility limit of urea (approximately 10 M).

Salts to bring the final volume to 50 mL (*see* Note 4 and **Section 3.1**). Store at 4 °C.

10. Filter Binding Wash Buffer: 10 mM Tris (pH 8.0 at room temperature), 100 mM NaCl, 0.1 mM Na₂EDTA. Dissolve 5.844 g NaCl and 1.211 g Tris in about 950 mL deionized H₂O. Add 200 µL 0.5 M Na₂EDTA stock (pH 8.0 at room temperature). Adjust pH using HCl. Bring to 1 L with deionized H₂O. Store at 4 °C.

2.2 Supplies and equipment

1. 0.45 µm nitrocellulose filters (Schleicher and Schuell). Soak in deionized H₂O at least 30 minutes prior to assay. Store at 4 °C in deionized H₂O.
2. 10-place filter-holding vacuum manifold capable of achieving pressures of 15–30 inches of Hg (Hoefer Scientific, San Francisco, CA).
3. Three-syringe Rapid Quench-Flow Fast Mixer (Model RQF-3; KinTek Co. Austin, TX) with temperature-regulated water bath for rapid-mix, rapid-quench experiments.
4. Approximately 20 1.5 mL microcentrifuge tubes with holes made in the top. (*see* Note 5).
5. Scintillation counter (Hewlett-Packard Tri-Carb 2100 TR).
6. Laboratory timer for manual mixing experiments.

3. Methods

This section provides details of nitrocellulose filter binding kinetics experiments that detect long-lived competitor-resistant (CR) open complexes and serve to determine the dependences of equilibrium and rate constants of individual steps in Mechanism I on solute concentration. For λP_R under the wide range of solution conditions investigated, the kinetics of open-complex dissociation are single exponential, as are the kinetics of association when one reactant (typically RNAP) is in excess. Under such conditions, overall rate constants for dissociation (k_d) and association (β_{CR} or α_{CR} ; *see* Equations 2–4 **below**) are directly determined from filter binding experiments. From the dependence of α_{CR} on RNAP concentration, the equilibrium constant K_1 and rate constant k_2 in Mechanism 1 are determined. A different assay, capable of detecting I_1 directly, is necessary to obtain individual rate constants k_1 and k_{-1} in addition to K_1 . Rapid destabilization of RP_o using high concentrations of salt or urea yields a burst of I_2 , allowing one to determine k_{-2} directly and therefore dissect k_d to obtain K_3 (and in favorable cases k_3 and k_{-3} (5)).

Filter binding studies of the effects of different solutes and solute concentration on the observable rate constants k_d and β_{CR} or α_{CR} are analyzed similarly to determine which

⁴Dissolving urea is highly endothermic and occurs slowly. For high concentration stocks, it may be best accomplished by vortexing or stirring continuously in a water bath. Buffers containing urea can be stored at 4 °C for approximately one week before the urea begins to hydrolyze significantly.

⁵Using microcentrifuge tubes with holes punched in the top ensures that the expelled mixture does not splash out of the tube, as could be the case for a tube with an open cap. We find it convenient to produce a hole with an ideal diameter using a soldering iron.

individual rate or equilibrium constants are affected by that solute, and by how much. Methods described below are for the destabilizing solute urea, which favors the exposure of RNAP and DNA surface and hence destabilizes both binding and folding steps. In most cases these methods are easily adapted to study protein-stabilizing solutes like GB and proline, effects of which on protein folding are comparable in magnitude but opposite in direction to urea. (See Note 6.)

It is important to note that solute-as-probe experiments typically are performed in the solute concentration range 0 – 1 M. Higher concentrations of protein-stabilizing solutes can be used if needed to detect small effects, but higher concentrations of urea and other destabilizing solutes should generally be avoided. In experiments using urea as a perturbant to rapidly destabilize RP_o (5), concentrations of urea in the molar range can be used because RNAP in promoter complexes is stabilized against subunit dissociation and unfolding by binding free energy.

Below, we describe assays utilizing both manual mixing and rapid quench-flow fast mixing with a three syringe mixer. For best results, fast mixing should be used for reactions with half-lives shorter than about 100 seconds. For λP_R these include studies of the kinetics of RP_o formation at temperatures above 20 °C and of RP_o dissociation at high urea or salt concentrations. However, slower reactions can be easily (and more efficiently) assayed using manual mixing (see (3–6, 11)). In fast mixing experiments, each time point is an individual reaction that proceeds for a defined amount of time and then is stopped with a quench solution. In manual mixing experiments, individual aliquots are taken from the same large continuous reaction at different times and quenched.

In general, for fast mixer reactions, approximately 19 μ L of each reactant in sample ports A and B are combined at time $t = 0$ in the instrument's reaction loop. At a later time t , approximately 150 μ L (consisting of the combined reaction mixtures and the buffers from push syringes A and B used to drive the reaction mixtures together) are combined with half this volume of the quenching reagent loaded into push syringe C and immediately expelled through the exit line (total volume: approximately 220 μ L). Only minimal instructions are given here for use of the RQF-3 Rapid Quench-Flow Fast Mixer. For more comprehensive instructions, the instrument manual should be consulted.

3.1 Preparation of Reaction Mixtures

Filter binding assays are performed in 1 $\times$ Transcription Buffer, which consists of the following concentrations in the absence of urea: 120 mM KCl (129 mm (millimolal)), 10 mM (10.7 mm) $MgCl_2$, 40 mM (43mm) Tris pH 8.0 at assay temperature, 1 mM DTT, 100 μ g/mL bovine serum albumin (BSA), and (from storage buffer) 12.5 mM (13.3 mm) NaCl and 6.25% v/v (855 mM, 913 mm) glycerol. This solution can be prepared by mixing one-fourth the final volume 4 $\times$ Transcription Buffer Salts, one eighth the final volume RNAP

⁶Note that the protein-stabilizing solute glycerol, used at high concentration (50% v/v) in RNAP storage buffer, is of necessity present at moderately high concentration (typically 0.7 – 0.9 M) and must be held constant in series where concentrations of RNAP and other solutes are varied.

Storage Buffer, 1/1000th the volume 1M DTT stock, and 1/500th the volume 50 mg/mL BSA stock with deionized H₂O.

As described in **Section 2.1**, we add dry urea to 1× Transcription Buffer to make our urea stock solutions. This procedure maintains the molal concentrations of the other components constant, and does not significantly change the molar concentrations of other species at final urea concentrations less than 1 M, though the favorable interaction of urea with KCl reduces the effective salt concentration (i.e., the salt activity (1)).

In general, reaction volumes should be large enough to allow for the production of 12–18 time points, which should be sufficient to determine a time course of competitor-resistant open complex formation or dissociation (*see* Note 7). All volumes described below have been chosen to achieve this number of data points.

All solutions used should be equilibrated at the assay temperature 5–15 minutes prior to initiation of an experiment.

3.2 Nitrocellulose Filter Binding (*see* Note 8)

1. Use a pair of tweezers to place a nitrocellulose filter over a manifold port under vacuum. Place one of the provided stainless steel chimney weights over the filter (leaving the center of the filter accessible via the hole in the weight). Open the port to remove water from the filter, close the port, and cover filter with 200–500 μ L Filter Binding Wash Buffer.
2. To bind an experimental sample, open the port to pull through the Wash Buffer. Apply the sample to the center of the filter. Wash with 200–500 μ L Wash Buffer. Close the port, and remove the filter from the manifold.
3. Dry the filter completely. Insert the filter into a scintillation vial and determine its radioactive counts per minute (cpm) using a scintillation counter (*see* Note 9).

⁷For accurate nonlinear fitting, at least half the time points on the time course should be before the first half life, and at least 3–4 time points should be used to define the plateau in counts as t approaches ∞ . If the plateau cpm value is less than that expected for complete reaction (i.e. reversible, $\theta_{t=\infty} < 1$ (*see* **Section 4.1**)), as is the case for WT RNAP- WT λ PR experiments at low T, especially at low concentrations of excess RNAP, accurate plateau determination is particularly important.

⁸Nitrocellulose filter binding is a highly quantitative kinetic assay on both long and short timescales which yields data readily compared to a large body of previous work. However, this method is not continuous, and not all promoter and RNAP variants form filter-retainable complexes. Use of a brief heparin challenge allows filter binding to distinguish between short-lived (competitor-sensitive, typically closed) complexes and long lived (competitor-resistant (CR), typically open) complexes. To test whether CR complexes are open, the kinetics of forming permanganate-reactive complexes are compared with the kinetics of forming CR complexes (Amanda Drennan and ER, unpublished data). These filter binding methods cannot determine individual rate constants k_1 and k_{-1} for I_1 , as stated in **Section 3**. Alternative bulk kinetic methods include intrinsic tryptophan fluorescence (23) and the recently developed RNAP beacon assay (24). Both of these methods are still in development for quantitative use with long DNA fragments and are limited to relatively short assay times, but we are excited about their potential for fast collection of kinetic data. Single-molecule methods for analyzing kinetics of open complex formation and dissociation have also recently become available, including colocalization single-molecule spectroscopy (CoSMoS) (25).

⁹We find it effective to dry filters by laying them flat onto a sheet of aluminum foil under a heat lamp. When quantifying filter cpm, we find that we obtain adequate signal detection by placing a dried filter into a standard borosilicate scintillation counter vial; however, one can also add scintillation fluid into the vial to amplify the signal.

3.3 Examples of Assays to Determine Solute Effects on Kinetics of Open Complex Formation

3.3.1 Manual Mixing Association Experiment at 0.5 M urea

1. Prepare Solutions:

- a. DNA Mix (800 μ L total): Combine 66.7 μ L 6 M Urea in 1 $\times$ Transcription Buffer Salts, 183 μ L 4 $\times$ Transcription Buffer Salts, 0.80 μ L 1 M DTT, 1.60 μ L 50 mg/mL BSA, 91.6 μ L RNAP Storage Buffer, X μ L of radiolabeled DNA (final concentration 50–500 nM; *see* Note 10), and (456 – X) μ L of deionized H₂O.
 - b. RNAP Mix (750 μ L total): Combine 62.5 μ L 6 M Urea in 1 $\times$ Transcription Buffer Salts, 172 μ L 4 $\times$ Transcription Buffer Salts, 0.75 μ L 1 M DTT, 1.50 μ L 50 mg/mL BSA, Y μ L RNAP in Storage Buffer (to obtain a final concentration twice as large as that desired in the association reaction), (85.9 – Y) μ L Storage Buffer, and 427 μ L deionized H₂O (*see* Note 11).
 - c. Competitor Mix (500 μ L, for 50 μ g/mL heparin in assay): Combine 41.7 μ L 6 M Urea in 1 $\times$ Transcription Buffer Salts, 115 μ L 4 $\times$ Transcription Buffer Salts, 0.5 μ L 1 M DTT, 1.0 μ L 50 mg/mL BSA, 57.3 μ L RNAP Storage Buffer, 3 μ L 50 mg/mL heparin, and 285 μ L dH₂O. Aliquot 20 μ L of this mix into one tube per filter for the experiment.
2. Before initiating the reaction, remove 50 μ L of DNA Mix and combine it with 50 μ L of 1 $\times$ Transcription Buffer and 20 μ L Competitor Mix. Filter 100 μ L of this to determine the background filter retention of DNA (cpm_{bkgd}; *see* section 4.1).
 3. At time 0, combine 750 μ L DNA Mix and 750 μ L RNAP Mix. Vortex gently or pipet to mix.
 4. At various time points, remove a 100 μ L aliquot of the DNA-RNAP reaction mixture, quench by combining it with 20 μ L of Competitor Mix in a separate tube for 10 s, and filter 100 μ L of the quenched solution. Take enough long time points to accurately define the plateau cpm value (*see* Note 7).
 5. At the end of the assay, remove two separate aliquots of 100 μ L, and combine each with 20 μ L Competitor Mix. Filter 100 μ L of one of the 120 μ L samples 10 seconds after mixing to determine the cpm value for this population of 100% CR complexes (cpm_{100% CR}, used in determining filter efficiency; *see* section 4.1). Remove 100 μ L from the other 120 μ L sample and divide it into 20 μ L aliquots. Spot these aliquots onto previously dried filters to determine the total cpm in each filtered aliquot (cpm_{TOT}, used in determining the fraction of competitor-resistant filter retainable complexes, θ ; *see* section 4.1).

¹⁰The amount of DNA required will vary with the number of cpm filtered, the reversibility of association, etc. It should be adjusted empirically such that at equilibrium the filters retain enough cpm for easy detection and accurate data fitting, while the molar concentration of DNA does not exceed 1/5 the molar concentration of RNAP (so that RNAP is in sufficient excess for pseudo-first order kinetics).

¹¹RNAP should be added to buffer as close to the beginning of the experiment as possible to prevent damage to the enzyme from oxidation.

3.3.2 Rapid-Mix Rapid-Quench Association Experiment at 0.5 M Urea

1. Prepare solutions:
 - a. DNA Mix (500 μ L total) and RNAP Mix (500 μ L): Same proportions of reagents as in the corresponding mixes detailed in **Section 3.3.1.1**.
 - b. Push Buffer for DNA and RNAP Mixes (10 mL total): Combine 833 μ L 6 M Urea in 1 $\times$ Transcription Buffer, 2290 μ L 4 $\times$ Transcription Buffer Salts, 10 μ L 1 M DTT, 20 μ L 50 mg/mL BSA, 1150 μ L RNAP Storage Buffer, and 5700 μ L deionized H₂O. Remove 100 μ L for determination of cpm_{bkgd} and cpm_{TOT}.
 - c. Competitor Mix (5 mL total, for 50 μ g/mL heparin in assay): Combine 417 μ L 6 M Urea in 1 $\times$ Transcription Buffer Salts, 1.15 mL 4 $\times$ Transcription Buffer Salts, 5 μ L 1 M DTT, 10 μ L 50 mg/mL BSA, 573 μ L RNAP Storage Buffer, 15 μ L 50 mg/mL heparin, and 2.83 mL deionized H₂O.
2. Load Push Buffer into the left (A) and right (B) vertical drive syringes of a KinTek RQF-3 Rapid Quench-Flow Fast Mixer. Load Competitor Mix into the center (C) drive syringe.
3. Load the DNA Mix into the instrument's left (A) sample port. Load Push Buffer (set aside in step 1c) into the right (B) sample port. Perform two reactions, mixing DNA Mix with Push Buffer in the reaction loop for 1 second. Filter one of the expelled samples to determine cpm_{bkgd}. Apply the other expelled sample onto a set of dry filters to determine cpm_{TOT}. Clean sample port B and load RNAP Mix.
4. Generate time points for the association of RNAP and DNA by allowing individual reactions to proceed for defined times prior to being quenched and expelled. Take enough long time points to accurately define the plateau cpm value (*see* Note 7). Collect the expelled volumes by inserting the exit line on the fast mixer into a 1.5 mL microcentrifuge tube into which a hole has been made in the top (*see* Note 5). Filter the entire expelled sample as described in **Section 3.2**.

3.4 Examples of Assays to Determine Solute Effects on Kinetics of Open Complex Dissociation

3.4.1 Manual Mixing Dissociation Experiment at 2 M urea

1. Prepare solutions:
 - a. RP₀ Mix (810 μ L total): Combine 233 μ L 4 $\times$ Transcription Buffer Salts, 0.93 μ L 1 M DTT, 1.9 μ L 50 mg/mL BSA, X μ L of radiolabeled DNA solution (final concentration 50–500 nM (*see* Note 8)), and (578 – X) μ L deionized H₂O (resulting in a volume of 814 μ L). Remove 105 μ L of this mix and combine with 15 μ L Storage Buffer (resulting in 120 μ L). To the remaining 709 μ L, add 101 μ L RNAP in Storage Buffer. Incubate at 37°C for 30–60 minutes to form open complexes.
 - b. Urea/Competitor Mix (750 μ L total, for 50 μ g/mL heparin in assay): Combine 500 μ L 6 M Urea in 1 $\times$ Transcription Buffer Salts, 62.5 μ L 4 $\times$

Transcription Buffer Salts, 0.75 μ L 1 M DTT, 1.5 μ L 50 mg/mL BSA, 31.3 μ L RNAP Storage Buffer, 1.5 μ L 50 mg/mL heparin, and 153 μ L deionized H₂O.

2. Before initiation of dissociation reaction, remove 60 μ L from RP_o Mix and incubate at 37 °C to ensure the association reaction proceeds to completion (an additional 5–10 half-lives). Combine with 60 μ L of 1 $\times$ Transcription Buffer containing 100 μ g/mL heparin, incubate for 10 seconds, and filter 100 μ L to determine cpm_{100%CR}. To the 120 μ L sample from Section 1.a. above, add 120 μ L Urea/Competitor Mix. Filter 100 μ L of this to determine cpm_{bkgd}. Remove 100 μ L and spot onto a set of dry filters to determine cpm_{TOT}.
3. To the remaining 750 μ L of RP_o Mix, add 750 μ L of Urea/Competitor Mix at time 0. At various time points, remove 100 μ L of the dissociation reaction and filter as described in **Section 3.2**.

3.4.2 Rapid-Mix Rapid-Quench Dissociation Experiment at 2 M urea

1. Prepare solutions:
 - a. RP_o Mix (500 μ L total): Combine 155 μ L 4 $\times$ Transcription Buffer Salts, 0.61 μ L 1 M DTT, 1.24 μ L 50 mg/mL BSA, X μ L of radiolabeled DNA solution (final concentration 50–500 nM (*see* Note 10)), and (386 – X) μ L deionized H₂O (resulting in a volume of 543 μ L). Remove 105 μ L of this mix and combine with 15 μ L Storage Buffer (resulting in 120 μ L). To the remaining 438 μ L, add 62.5 μ L RNAP in Storage Buffer. Incubate at 37°C for 30–60 minutes to form open complexes.
 - b. Urea/Competitor Mix (5 mL total): Same proportions of reagents as in the corresponding mixes detailed in **Section 3.4.1.1**.
 - c. Push Buffer for RP_o Mix (10 mL total): Combine 2.50 mL 4 $\times$ Transcription Buffer Salts, 10 μ L 1 M DTT, 20 μ L 50 mg/mL BSA, 1.25 mL RNAP Storage Buffer, and 6.22 μ L deionized H₂O.
 - d. Quench Buffer (10 mL total): Combine 2.5 mL 4 $\times$ Transcription Buffer Salts, 10 μ L 1 M DTT, 20 μ L 50 mg/mL BSA, 1.25 mL RNAP Storage Buffer, 10 μ L 50 mg/mL heparin, and 6.21 mL dH₂O.
2. Load 5 mL Push Buffer for RP_o Mix into the left (A) vertical drive syringe of a KinTek RQF-3 Rapid Quench-Flow Fast Mixer. (Set aside 5 mL Push Buffer for RP_o Mix for later determination of cpm_{100%CR}.) Load Urea/Competitor Mix into the right (B) drive syringe. Load Quench Buffer into the center (C) drive syringe.
3. Load mixture containing DNA in 1 $\times$ Transcription Buffer (removed before addition of RNAP in production of RP_o Mix) into the instrument's left (A) sample port. Load Urea/Competitor Mix into the right (B) sample port. Perform two reactions, mixing DNA with Urea/Competitor Mix for 1 second. Filter one of the expelled samples to determine cpm_{bkgd}. Apply the other expelled sample onto a set of dry filters to determine cpm_{TOT}. Clean sample port A and load RP_o Mix.

4. Generate time points for the dissociation of RP_o by allowing individual reactions to proceed for defined times prior to being quenched and expelled (*see* Note 12). Collect and filter the expelled volumes as described in **Section 3.3.2**.
5. After all experimental time points are collected at the desired temperature, bring the temperature of the water bath connected to the fast mixer to 37 °C. Replace the urea solutions with Push Buffer for RP_o . Mix and filter several samples with very short incubation times to determine $cpm_{100\%CR}$.

4. Data analysis

4.1 Determination of θ_t

The first step in the analysis of filter binding kinetic data is to calculate θ_t , the fraction of promoter DNA in competitor-resistant (CR) complexes at time t . CR complexes are operationally defined as those which survive a 10 s challenge with an inert (non-displacing) competitor like heparin (for λP_R ; *see* Note 2) or unlabeled promoter DNA. For λP_R , because RP_o is generally much more stable than I_3 and I_2 , it is usually the dominant competitor-resistant open complex, though I_3 may also be significant at lower temperatures. Values of θ_t are calculated by dividing the background-corrected counts per minute (cpm) for a time point filter by the total cpm per sample bound and correcting for filter efficiency (E), defined as the fraction of labeled promoter DNA retained on the filter under conditions where 100% of promoter DNA is bound in a CR complex in solution ($E = cpm_{100\%CR} / cpm_{TOT}$; *see* Notes 13 and 14).

$$\theta_t = (1/E)(cpm_t - cpm_{bgd}) / cpm_{TOT} \quad (1)$$

In RP_o formation kinetics experiments, if the long-time plateau value of θ_t (called $\theta_{t=\infty}$) is significantly less than 1, then RP_o formation is reversible and the kinetics are for the approach to an equilibrium distribution of RP_o , intermediate complexes and free promoter DNA. If $\theta_{t=\infty} = 1$, then RP_o formation is irreversible.

4.2 Determination of the Observed Rate Constant for RP_o Formation

In excess RNAP, single-exponential kinetics of formation of CR complexes at λP_R (primarily RP_o) are observed under all conditions investigated. In these experiments, the

¹²Combination of the dissociation reaction with the Quench Buffer at this point results in a dilution of the urea concentration, which slows down the rate of dissociation. The quench should be designed to slow down the rate of dissociation enough such that effectively no additional dissociation occurs while the solution is being collected and filtered. For λP_R and wild-type RNAP, the quenched mixture should have concentrations of 0.08–0.12 M KCl and less than 1.5 M urea in order to effectively stop the dissociation reaction at room temperature. For other systems, the dilution of destabilizing solutes required should be empirically determined. It may be necessary to further dilute the reaction beyond what is achieved within the fast mixer itself. In such a case, samples can be further diluted with additional Quench Buffer already present in the microcentrifuge tube into which it is expelled. Where large volumes of Quench Buffer are required, we find it convenient to use 5 mL tubes.

¹³For good quality DNA, background retention should be less than 5% of total counts.

¹⁴Filter efficiency can vary considerably. Our standard of known high filter efficiency is 37°C in transcription buffer, where it varies from about 70% to about 100% depending on wash volume, vacuum pressure, pretreatment of filters, amount of free ³²P label in the solution, etc. High concentrations of some solutes (e.g. over 0.24 M KCl or 1.5 M urea (5)) reduce filter efficiency, as does low salt concentration. Efficiency of retention of open complexes is not significantly temperature dependent (11). Under reversible conditions, it is best determined based in an irreversible assay performed side-by-side, or by removing 100 µL from the reaction mixture, incubating it at 37°C for one hour, and then subjecting to a heparin challenge.

observed rate constant for formation of these CR (open) complexes is called β_{CR} , and is determined by fitting θ_t versus time to the first order rate equation

$$\theta_t = \theta_0 + (\theta_{t=\infty} - \theta_0)(1 - e^{-\beta_{CR}t}) \quad (2)$$

where θ_0 is the fitted value of θ_t at $t = 0$ (typically θ_0 less than 0.1) and $\theta_{t=\infty}$ is the long-time (plateau, equilibrium) value of θ_t (11, 12). Equivalently, β_{CR} can be fit using the decay to equilibrium equation

$$\Delta\theta_t = \Delta\theta_0 e^{-\beta_{CR}t} \quad (3)$$

where $\theta_t = \theta_{t=\infty} - \theta_t$ and $\theta_0 = \theta_{t=\infty} - \theta_0$. Representative kinetic data for the formation of RP_0 using wild-type RNAP and λP_R DNA are shown in Fig. 1.A., plotted as θ_t versus t .

Where association is reversible (i.e., where $\theta_{t=\infty}$ is less than 1), then β_{CR} is interpreted as the decay-to-equilibrium rate constant, while if association is irreversible ($\theta_{t=\infty} = 1$), β_{CR} is interpreted as the rate constant α_{CR} for formation of competitor-resistant complexes. In general, β_{CR} is related to α_{CR} by

$$\alpha_{CR} = \beta_{CR} - k_d = \beta_{CR} \theta_{t=\infty} \quad (4)$$

where k_d is the dissociation rate constant. Where possible, α_{CR} should be determined under conditions where RP_0 formation is irreversible ($\beta_{CR} \gg k_d$, so that $\alpha_{CR} = \beta_{CR}$). Where decay-to-equilibrium studies are necessary, the best way to determine α_{CR} from β_{CR} is to obtain an independent determination of k_d . (see Note 15).

4.3 Determination of K_1 and k_2

The quantities K_1 and k_2 in Mechanism 1 are determined from analysis of the dependence of α_{CR} on RNAP concentration in excess RNAP and at sufficiently high concentrations of DNA and RNAP that the kinetics of the initial diffusion-limited promoter binding step are not rate-determining. Single-exponential kinetics of CR complex formation indicates that all intermediates (I_1 in **Mechanism 1**) formed before the rate-determining step ($I_1 \rightarrow I_2$ in **Mechanism 1**) are in rapid equilibrium with each other and with free promoter DNA under the conditions investigated to date. For this situation, the rate constant α_{CR} for RP_0 formation is a hyperbolic function of RNAP concentration (11–13):

$$\alpha_{CR} = \frac{K_1 k_2 [RNAP]_{tot}}{1 + K_1 [RNAP]_{tot}} \quad (5)$$

where K_1 is the equilibrium constant for I_1 formation, k_2 is the rate constant for the DNA opening step that converts I_1 to I_2 , and $[RNAP]_{tot}$ is the total concentration of active RNAP (see Note 16). Nonlinear fitting of α_{CR} versus $[RNAP]_{tot}$ is used to determine K_1 and k_2 (as in Fig. 1.B).

¹⁵The most accurate association kinetic data (and hence the most accurate values of K_1 , k_2 , and their solute dependences) are obtained under conditions where the process of open complex formation is irreversible. To counteract the reversibility potentially induced by destabilizing solutes such as urea, lower salt concentrations than those described here can be used.

4.4 Determination of Observed Dissociation Rate Constant k_d

The first order kinetics of dissociation of open complexes, made irreversible by the presence of an inert (non-displacing) competitor (*see* Note 2), are analyzed by the rate equation

$$\theta_t = \theta_{t=\infty} + (\theta_0 - \theta_{t=\infty})(e^{-k_d t}) \quad (6)$$

to obtain the dissociation rate constant k_d (see Fig. 2.A.): In Equation 6, $\theta_{t=\infty}$ is the fitted value of θ_t at completion of dissociation (typically $\theta_{t=\infty}$ less than 0.15), and θ_0 is the fitted initial value of θ (typically $\theta_0 \approx 1$).

Single exponential kinetics of dissociation of RP_o are observed after addition of an inert competitor because intermediates in dissociation formed before the rate-determining step (i.e. I_3 and I_2 in **Mechanism 1**) are unstable relative to RP_o and rapidly equilibrate with one another and with RP_o before the rate-determining conversion of I_2 to I_1 . For this situation, k_d is interpreted (12) as

$$k_d \approx k_{-2}/(1+K_3) \quad (7)$$

where K_3 is the overall equilibrium constant for the conversion of I_2 to RP_o . For most conditions investigated, K_3 is much greater than 1 for competitor-initiated dissociation experiments for λP_R and therefore $k_d \approx k_{-2}/K_3$. For dissociation initiated by a rapid shift to 1.1 M KCl (or 4 M urea), making K_3 much less than 1, the observed dissociation rate constant $k_d \approx k_{-2}$. Dissociation kinetics experiments initiated by upshifts to less extreme concentrations of urea have been used to determine rate constants k_3 and k_{-3} and establish the existence of the intermediate I_3 (5).

4.5 Interpreting Effects of Solutes

Effects of solutes on biopolymer processes arise from interactions of the solute with the biopolymer surface that is buried or exposed in the process (14). If the interaction of the solute with the biopolymer surface is favorable, relative to interactions with water, then addition of the solute will favor the direction of the process that exposes the biopolymer surface to water. Urea and Hofmeister salt ions like GuH^+ , I^- and SCN^- are protein denaturants because they interact favorably (or at least not unfavorably) with the amide and hydrocarbon surface of the protein that is exposed when the protein unfolds. On the other hand, osmolytes like GB and proline and Hofmeister salt ions like Glu^- , F^- and SO_4^{2-} are protein stabilizers because their interactions with amide and aliphatic hydrocarbon surface of proteins are unfavorable relative to interactions with water. Effects of a solute on the rate constant for a step of a process are interpreted analogously in terms of interactions of the solute with the biopolymer surface that is buried or exposed in forming the high-free-energy transition state in that step (see (15)).

¹⁶We determine the active concentration of RNAP by titrating promoter DNA (at high concentration (typically over 1 nM) relative to the reciprocal of the overall equilibrium constant for RP_o formation (typically less than 0.1 nM)) with RNAP so that all active RNAP added will bind to promoter DNA, as in (13). In general, we find that activity of WT holoenzyme varies from 30 to 100%, depending on enzyme preparation. Mutant polymerases, and especially mutants of the σ subunit, tend to be less active (10–30%). Activity can decrease in storage at -20°C and should be determined regularly.

4.5.1 Interpreting Solute Effects on Equilibrium Constants—The effect of a solute on the equilibrium constant for a step is interpreted in terms of interactions of the solute with the biopolymer surface that is buried or exposed. For the conversion of reactant X to product Y, where the observed equilibrium constant $K_{\text{obs}} = ([Y]/[X])_{\text{eq}}$ at solute concentration [solute] and $K_{\text{obs},0}$ is the corresponding equilibrium constant in the absence of that solute,

$$\ln K_{\text{obs}} = \ln K_{\text{obs},0} + \alpha_{X \rightarrow Y} \Delta \text{ASA}^{X \rightarrow Y} [\text{solute}] \quad (8)$$

where $\text{ASA}^{X \rightarrow Y}$ is the change in water-accessible surface area in the conversion of X to Y and the proportionality constant $\alpha_{X \rightarrow Y}$ is the “interaction potential” quantifying the interaction of the solute with a unit area (i.e. $1 \text{ \AA}^2$) of a surface with the composition specified by $\text{ASA}^{X \rightarrow Y}$. Predicted values of $\alpha_{X \rightarrow Y}$ for interactions of urea with the predominantly hydrocarbon surfaces involved in various protein processes are generally in the range $-(2.5 \pm 0.5) \times 10^{-4} \text{ M}^{-1}$ (1). Therefore, as proposed originally by Myers *et al.* (16), urea can be used semiquantitatively as a probe of ASA in protein processes irrespective of the composition of the ASA. In particular, urea is an unambiguous probe for distinguishing steps with large changes in ASA (e.g. $I_2 \rightarrow \text{RP}_0$, which has very large urea dependence of K_{obs} , indicating a very large reduction in ASA (*see* Fig. 1.C.)) from those with small or compensating changes in ASA (e.g., $I_1 \rightarrow I_2$, which has no urea dependence of K_{obs} , indicating small or compensating changes in ASA (*see* Fig. 2.B.)).

Values of $\alpha_{X \rightarrow Y}$ for interactions of GB or proline with different surfaces can differ by as much as an order of magnitude, making them less easily interpretable and less useful as general probes of ASA in structurally uncharacterized processes (2). However, these solutes should prove very useful in conjunction with urea for beginning to characterize the composition of the ASA, in addition to the amount. For the surface exposed in local or global protein unfolding (approximately 70% hydrocarbon, 20% amide, 10% other polar or charged), effects of GB and proline are opposite in sign and slightly larger in magnitude than those of urea (2).

4.5.2 Interpreting Solute Effects on Rate Constants—For the interconversion of reactant X and product Y via high free energy transition state TS, with single exponential kinetics and observed forward and back rate constants k_f and k_b :

$$\ln k_f = \ln k_{f,0} + \alpha_{X \rightarrow \text{TS}} \Delta \text{ASA}^{X \rightarrow \text{TS}} [\text{solute}], \text{ and } \ln k_b = \ln k_{b,0} + \alpha_{Y \rightarrow \text{TS}} \Delta \text{ASA}^{Y \rightarrow \text{TS}} [\text{solute}] \quad (9)$$

Protein folding transition states (TS) have recently been characterized using effects of denaturants (urea or guanadinium chloride) on folding and unfolding rate constants (15). The slopes of linear plots of $\ln(k)$ versus [denaturant] (closely related to kinetic m -values) for folding and unfolding of thirteen globular proteins were analyzed together with data on the temperature dependences of these rate constants (activation heat capacities) to quantify the amounts of amide and hydrocarbon ASA buried in folding to and from TS. From these data, we conclude that folding to TS preferentially buries amide surface and that the amount of amide and hydrocarbon surface buried in folding to TS in most cases exceeds that buried in forming all the native secondary structure of the protein.

For RNAP, the only rate constants that have been characterized in this way are k_2 and k_{-2} for the step that includes DNA opening (*see* Fig. 1.C. and Fig. 2.B.). For this step, neither k_2 nor k_{-2} depends on urea concentration. Additionally, k_2 is found to be independent of salt concentration and is unchanged when Cl^- is replaced by Glu^- , variables which greatly affect other steps in the mechanism of transcription initiation. We conclude that DNA opening occurs in the active site cleft, greatly reducing effects of solutes and salts from what would be observed for DNA melting in solution. Also, or alternatively, burial of RNAP surface in this step may compensate for the exposure of DNA surface upon melting. Investigation of effects of other solute probes on this step will help distinguish between these two scenarios.

4.5.3 Example Application of Solute Dependence: DME Assembly in Late Steps of RP_0 Formation

Recent work has shown the useful application of solute concentration dependence to the study of large conformational changes in the late steps of RP_0 formation. The slope of the initial linear region of a plot of $\ln(k_d)$ versus urea for RP_0 complexes at λP_R is approximately 3.0 M^{-1} (*see* Fig. 2 B); an increase in urea concentration from 0 to 1 M therefore increases k_d by a factor of e^3 , about 20-fold. In this urea concentration range, $k_d \approx k_{-2}/K_3$ (Equation 7). Dissociation experiments after an upshift to high concentrations of urea in Fig. 2B reveal that k_{-2} is independent of urea concentration, so the entire effect of urea on $\alpha_{X \rightarrow Y} \text{ ASA}^{X \rightarrow Y}$ is on the equilibrium constant K_3 for the conversion $\text{I}_2 \rightarrow \text{RP}_0$. Therefore, $\ln K_3$ decreases with increasing [urea] with a slope $\alpha_{\text{I}_2 \rightarrow \text{RP}_0} \text{ ASA}^{\text{I}_2 \rightarrow \text{RP}_0} = -3.0 \text{ M}^{-1}$. Interpreted as a protein folding or a subunit assembly process involving the DME of RNAP, using $\alpha_{\text{I}_2 \rightarrow \text{RP}_0} = (2.5 \pm 1) \times 10^{-4} \text{ M}^{-1}$ (*see* above) indicates that $\text{ASA}^{\text{I}_2 \rightarrow \text{RP}_0}$ is about $1.2 \times 10^4 \text{ \AA}^2$. Folding buries 80–100 $\text{\AA}^2$ per amino acid residue buried (*ref?*), so this $\text{ASA}^{\text{I}_2 \rightarrow \text{RP}_0}$ corresponds to 120–150 buried residues. The effect of GB on dissociation is similar in magnitude and opposite in sign to that of urea, consistent with this interpretation.

Many important questions remain about this folding transition in the late steps of the mechanism, including which regions of RNAP and promoter DNA are responsible for it, and its role in transcription initiation. Analysis of amino acid sequences of the beta and beta prime subunits with the program PONDR (Predictions of Natural Disordered Regions; (17, 18)) predicted regions of RNAP that are disordered in holoenzyme and that might fold in RP_0 formation, contributing to the massive urea dependence of the step $\text{I}_2 \rightarrow \text{RP}_0$ (3). Several such unfolded regions, located at the downstream end of the cleft, are now designated downstream mobile elements (DME) (6). Variants of RNAP with deletions in these regions are being investigated to determine their effect on K_3 and its urea dependence. For example, deletion of residues 1149–1190 of the β' subunit of RNAP removes most of a DME called the β' jaw (19, 20). This deletion (termed “JAW RNAP”) reduces the urea dependence of K_3 from -3.0 M^{-1} to -2.0 M^{-1} , reducing the predicted number of residues folded in this step from 120–150 for WT RNAP to 80–100 for JAW RNAP and suggesting that these 42 residues of the jaw are unstructured in the unbound WT RNAP holoenzyme and fold as part of the conformational changes that stabilize the initial open complex I_2 (6). We find that the urea dependence of k_d for RP_0 formed at the T7A1 promoter is similar to that seen for λP_R (ER, unpublished data), indicating that a similar folding transition occurs to stabilize the initial open complex at this promoter. Solute studies performed at other promoters, as well

as determinations of clamp width by FRET (21), RNAP-DNA crosslinking, or other methods will help to answer questions about the generality and physiological role of this massive conformational change late in the mechanism of transcription initiation.

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References

1. Guinn EJ, Pegram LM, Capp MW, Pollock MN, Record MT Jr. Quantifying why urea is a protein denaturant, whereas glycine betaine is a protein stabilizer. *Proceedings of the National Academy of Science USA*. 2011; 108:16932–16937.
2. Diehl RC, Guinn EJ, Capp MW, Tsodikov OV, Record MT Jr. Quantifying Additive Interactions of the Osmolyte Proline with Individual Functional Groups of Proteins: Comparisons with Urea and Glycine Betaine, Interpretation of m-Values. *Biochemistry*. 2013; 52:5997–6010. [PubMed: 23909383]
3. Kontur WS, Saecker RM, Davis CA, Capp MW, Record MT Jr. Solute Probes of Conformational Changes in Open Complex (RP_o) Formation by Escherichia coli RNA Polymerase at the IP_R Promoter: Evidence for Unmasking of the Active Site in the Isomerization Step and for Large-Scale Coupled Folding in the Subsequent Conversion to RP_o. *Biochemistry*. 2006; 45:2161–2177. [PubMed: 16475805]
4. Kontur WS, Capp MW, Gries TJ, Saecker RM, Record MT Jr. Probing DNA Binding, DNA Opening, and Assembly of a Downstream Clamp/Jaw in Escherichia coli RNA Polymerase-IP_R Promoter Complexes Using Salt and the Physiological Anion Glutamate. *Biochemistry*. 2010; 49:4361–4373. [PubMed: 20201585]
5. Kontur WS, Saecker RM, Capp MW, Record MT Jr. Late steps in the formation of the E. coli RNA polymerase-IP_R promoter open complexes: Characterization of conformational changes by rapid [perturbant] upshift experiments. *Journal of Molecular Biology*. 2008; 376:1034–1047. [PubMed: 18191943]
6. Drennan AC, Kraemer MR, Capp MW, Gries TJ, Ruff EF, Sheppard C, et al. Key Roles of the Downstream Mobile Jaw of Escherichia coli RNA Polymerase in Transcription Initiation. *Biochemistry*. 2012; 51:9447–9459. [PubMed: 23116321]
7. Tang G-Q, Patel SS. Rapid Binding of T7 RNA Polymerase is Followed by Simultaneous Bending and Opening of the Promoter DNA. *Biochemistry*. 2006; 45:4947–4956. [PubMed: 16605262]
8. Saecker RM, Record MT Jr, deHaseth PL. Mechanism of Bacterial Transcription Initiation: RNA Polymerase-Promoter Binding, Isomerization to Initiation-Competent Open Complexes, and Initiation of RNA Synthesis. *Journal of Molecular Biology*. 2011; 412:754–771. [PubMed: 21371479]
9. Gries TJ, Kontur WS, Capp MW, Saecker RM, Record MT Jr. One-step DNA melting in the RNA polymerase cleft opens the initiation bubble to form an unstable open complex. *Proceedings of the National Academy of Science USA*. 2010; 107:10418–10423.
10. Davis CA, Bingman CA, Landick R, Record MT Jr, Saecker RM. Real-time footprinting of DNA in the first kinetically significant intermediate in open complex formation by Escherichia coli RNA polymerase. *Proceedings of the National Academy of Science USA*. 2007; 104:7833–7838.
11. Saecker RM, Tsodikov OV, McQuade KL, Schlax PE Jr, Capp MW, Record MT Jr. Kinetic studies and structural models of the association of E. coli ⁷⁰S RNA polymerase with the IP_R promoter: Large scale conformational changes in forming the kinetically significant intermediates. *Journal of Molecular Biology*. 2002; 319:649–671. [PubMed: 12054861]
12. Tsodikov OV, Record MT Jr. General method of analysis of kinetic equations for multistep reversible mechanisms in the single-exponential regime: application to kinetics of open complex formation between Es⁷⁰S RNA polymerase and lambda (R) promoter DNA. *Biophysical Journal*. 1999; 76:1320–1329. [PubMed: 10049315]

13. McClure WR, Cech CL, Johnston DE. A Steady State Assay for the RNA Polymerase Initiation Reaction. *Journal of Biological Chemistry*. 1978; 253(24):8941–8948. [PubMed: 363712]
14. Record MT Jr, Guinn EJ, Pegram LM, Capp MW. Introductory Lecture: Interpreting and predicting Hofmeister salt ion and solute effects on biopolymer and model processes using the solute partitioning model. *Faraday Discuss*. 2013; 160:9–44. [PubMed: 23795491]
15. Guinn EJ, Kontur WS, Tsodikov O, Shkel I, Record MT Jr. Probing the protein-folding mechanism using denaturant and temperature effects on rate constants. *Proceedings of the National Academy of Science USA*. 2013
16. Myers JK, Pace CM, Scholtz JM. Denaturant m-values and heat capacity changes: Relation to changes in accessible surface areas of protein unfolding. *Protein Science*. 1995; 4:2138–2148. [PubMed: 8535251]
17. Li X, Romero P, Rani M, Dunker AK, Obradovic Z. Predicting protein disorder for N-, C-, and internal regions. *Genome Informatics*. 1999; 10:30–40. [PubMed: 11072340]
18. Romero P, Obradovic Z, Li X, Garner EC, Brown CJ, Dunker AK. Sequence complexity of disordered protein. *Proteins*. 2001; 42(38–48)
19. Ederth J, Artsimovitch I, Isaksson LA, Landick R. The Downstream DNA Jaw of Bacterial RNA Polymerase Facilitates Both Transcriptional Initiation and Pausing. *Journal of Biological Chemistry*. 2002; 277:37456–37463. [PubMed: 12147705]
20. Ederth J, Isaksson LA, Abdulkarim F. Origin-specific reduction of ColE1 plasmid copy number due to mutations in a distinct region of the Escherichia coli RNA polymerase. *Molecular Genetics and Genomics*. 2002; 267(5):587–592. [PubMed: 12172797]
21. Chakraborty A, Wang D, Ebright YW, Korlann Y, Kortkhonjia E, Kim T, et al. Opening and Closing of the Bacterial RNA Polymerase Clamp. *Science*. 2012; 337:591–595. [PubMed: 22859489]
22. Cech CL, McClure WR. Characteriation of Ribonucleic Acid Polymerase-T7 Promoter Binary Complexes. *Biochemistry*. 1980; 19:2440–2447. [PubMed: 6992860]
23. Johnson RS, Chester RE. Stopped-flow kinetic analysis of the interaction of Escherichia coli RNA polymerase with the bacteriophage T7 A1 promoter. *Journal of Molecular Biology*. 1998; 283:353–370. [PubMed: 9769210]
24. Mekler V, Pavlova O, Severinov K. Interaction of Escherichia coli RNA Polymerase σ^{70} Subunit with Promoter Elements in the Context of Free σ^{70} , RNA Polymerase Holoenzyme, and the σ^{70} Complex. *Journal of Biological Chemistry*. 2011; 286:270–279. [PubMed: 20952386]
25. Friedman LJ, Mumm JP, Gelles J. RNA polymerase approaches its promoter without sliding along DNA. *Proceedings of the National Academy of Science USA*. 2013; 110:9740–9745.

Figure 1.

Data showing the determination of the urea dependences of K_1 and k_2 . A. Rate constants of formation of filter-retainable open complexes (β_{CR}) are determined at 25°C in BB_{urea}^{assoc} at 0 m urea at 38 and 1.3 nM active RNAP using Equation 2. B. Data obtained for α_{CR} are fit to Equation 5 as a function of active [RNAP] to obtain fits for K_1 and k_2 at varying concentrations of urea. C. Values of K_1 and k_2 are plotted as functions of urea and GB to determine solute concentration dependences, or “ m -values”. **Figure reproduced from (3) with permission (ACS Publications).**

Figure 2.

Data showing the determination of the urea dependences of k_{-2} and K_3 . A. Rates of dissociation of filter-retainable open complexes are determined at 10 and 37°C in DB at 0, 0.5, and 1 M urea using Equation 6. B. Data obtained for $\ln(k_d)$ are fit as a function of urea concentration. Nonlinear fits are shown with (solid lines) and without (dashed lines, using Equation 7) consideration of the urea dependence of the term k_{-2}/k_{-3} . (For detailed analysis, see (5).) Horizontal dotted lines represent values of k_{-2} , while the initial slopes of the fits determine the m -value $-\text{dln}K_3/\text{d[urea]}$. **Figure reproduced from (5) with permission (Elsevier).**