Unravelling the Mechanism of RNA-Polymerase Forward Motion by Using Mechanical Force

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Polymerases form a class of enzymes that act as molecular motors as they move along their nucleic acid substrate during catalysis, incorporating nucleotide triphosphates at the end of the growing chain and consuming chemical energy. A debated issue is how the enzyme converts chemical energy into motion [J. Gelles and R. Landick, Cell **93**, 13 (1998)]. In a single molecule assay, we studied how an opposing mechanical force affects the translocation rate of T7 RNA polymerase. Our measurements show that force acts as a competitive inhibitor of nucleotide binding. This result is interpreted in the context of possible models, and with respect to published crystal structures of T7 RNA polymerase. The transcribing complex appears to utilize only a small fraction of the energy of hydrolysis to perform mechanical work, with the remainder being converted to heat.

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Polymerases form a class of essential enzymes that carry out transcription, replication or repair of nucleic acids. RNA polymerases (RNAPs) carry out an essential step in gene expression, the synthesis of a RNA copy from the DNA template. T7 RNAP, a single unit polymerase with known crystal structures [1–4], is a prototype of this protein class. After stages of promoter recognition and initial transcription of a few bases, conformational changes occur [1,2], allowing the protein to enter elongation mode [5], during which synthesis of RNA occurs by processive motion of RNAP along the DNA template. The standard textbook description of transcription is that the motor is ''powered'' by the chemical energy of Nucleotide triphosphate (NTP) hydrolysis (see [6] for an estimation of this energy). In order to examine this view, we have performed single molecule measurements to study the influence of mechanical force on the kinetic properties of T7 RNAP transcription.

In the present work the elongation mode of the T7 RNAP is investigated. The configuration is presented in Fig. 1. For the experiments presented here, the concentration C_0 of ATP, GTP, CTP, and UTP (the four types of NTP) was identical for each nucleotide and ranged from about 30 μM up to 590 μM. The measurements are performed using a feedback mode, where the applied force F is maintained constant. The force F ranged from five to 15 pN.

Figure 2 shows examples of measurements performed with different NTP concentrations at 5 pN. We measured over at least 8 sec the extension of DNA versus time for a given enzyme at a given force, and calculated the average velocity V by a linear fit. Within the apparatus accuracy and within this time scale, we only very seldomly observed pauses and excluded the few unambiguous pauses encountered for velocity determination [7]. T7 RNAP appears to behave differently in this respect than the *Escherichia coli* enzyme, for which the occurrence of many pauses in the range one to hundreds of seconds has been documented [8]. We observe a large variability in the measured velocities (estimated standard deviation: 30%). A high variability is a common feature of single molecule experiments [9]. In the force range studied, we have not observed stalling, i.e., a smooth lowering of velocity versus force, with stall occurring when the force is raised further. Reversible and irreversible stalling has been reported for *E. coli* RNAP in a range of forces above 20 pN. With the T7 RNAP, however, we observe that the enzyme under force may abruptly stop transcribing. This may be irreversible, or in some cases a reversible event, i.e., polymerization resumes when the force is lowered (unpublished work).

We analyze the influence of force on the enzyme under steady-state transcription elongation conditions. Because it is convenient to analyze enzyme kinetics, we present the data using the Lineweaver-Burke plot where $1/\langle V\rangle$ (in seconds per base, related to the time taken by the enzyme to perform a catalytic cycle) is plotted as a function of $1/C_0$ (related to the time taken for the catalytic site of the enzyme to encounter a nucleotide). Figure 3 shows the $1/\langle V\rangle$ vs $1/C_0$ plots at different force levels. At low $1/C_0$ (i.e., large C_0), nucleotide binding is not limiting the kinetics and the velocity is thus limited by some intrinsic minimal time lapse necessary for the enzyme to perform a catalytic cycle (this time is $\delta/V_{\max}$, where $V_{\max}$ is the maximum velocity of the enzyme and δ is the enzyme step per cycle assumed to be of one base pair). In this regime, the force is observed to have no significant effect on the average velocity. At large $1/C_0$ (i.e., small C_0), the velocity is limited by the availability of nucleotides and their binding kinetics. In this regime, we observe that the effect of force is to lower the average velocity. The data are well fit by line, (excluding the points at the lowest concentration [10]), a feature compatible with standard Michaelis-Menten kinetics. The fitted lines have identical Y intercepts (identical $V_{\max}$) but varying slopes (corresponding to $K_M/V_{\max}$, with K_M being the apparent nucleotide binding constant). This reveals that the applied load

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acts as a competitive inhibitor of NTP binding: the effect of an increasing force is an increase in K_M but $V_{\max}$ stays constant.

One would like now to interpret why force acts as a competitive inhibitor, somehow inhibiting NTP binding. One might propose that application of force inhibits a conformational change in the polymerase that is required for NTP binding to occur. While it has been proposed that NTP binding is associated with a conformational change in the RNAP, there is *a priori* no justification for believing that application of force in a manner depicted in Fig. 1 would either favor or disfavor such a conformational change. On the other hand, it is easy to see how the application of force would inhibit forward motion and thus inhibit NTP binding: the polymerase must move forward after completion of the bond formation step to clear the 3′ end of the RNA out of the binding site to make room for the next incoming NTP. For this motion, the enzyme performs mechanical work against a load. A simple model using a classic kinetic description in which the enzyme transits stochastically through a sequence of states has been proposed by Guajardo and Sousa [11]. It consists of decomposing the binding step into two substates: (1) after completion of the bond formation step the polymerase is able to move forward by random thermal motion even against a load; then (2) an incoming NTP binds. This is followed by hydrolysis (3), but the corresponding liberated energy produces heat rather than mechanical work. Step (3) is assumed to be irreversible, so that no backwards motion is possible. Such a model predicts that force should be a competitive inhibitor of nucleotide binding. The corresponding scheme is described in Fig. 4(a). Provided that the step following binding is slow with respect to k_{-2}, the expression for K_M given by this model is: $K_M = K_{\rm diss}(1 + k_{-1}/k_1)$, with $K_{\rm diss} = k_{-2}/k_2$. Assuming that the force F acts essentially on the translocation step, i.e., on the k_{-1} and k_1 constants, one gets $K_M(F) = K_{\rm diss}[1 + K\exp(F^*\delta/{\rm kT})]$, where δ is the enzyme step size. The experimental K_M values are obtained from linear fits in Fig. 3, giving $K = 0.27$ and $K_{\rm diss} = 124\ \mu$M (see Fig. 3 inset). The corresponding free energy difference ΔG_0 for the forward motion is ${\rm kT}\ln(K) = -1.3$ kT. Our data support a view approaching that proposed by Guajardo and Souza, although in their model they had $\Delta G_0 \geq 0$. The corresponding "flashing ratchet" or "two state model" Brownian motor (see [12] and references therein) considers that a Brownian particle can undergo directional movement by alternating between two states: in state one the particle is essentially pinned and in state two (flat potential landscape [13]) diffusion can occur. However, $\Delta G_0 < 0$ entails that $E_{\rm post}$ is energetically favored with respect to $E_{\rm pre}$, i.e., the potential is not flat.

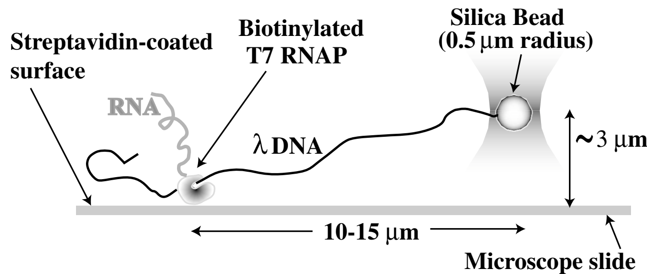

FIG. 1. Experimental configuration: the DNA template is lambda phage DNA (48502 base pairs, about 16 μm in length) in which a T7 promoter is introduced at one end [20]. The other end of the DNA carries a digoxygenin and is attached to a microscopic bead coated with an antibody directed against digoxygenin. The transcribing polymerase is biotinylated at its N terminus [21] and binds to a streptavidin coated solid surface. To detect tethered beads a flow is created. After selecting a tethered bead under the microscope, the four types of nucleotides are added to the sample. The enzyme pulls the bead as it transcribes the DNA, thereby shortening the tether. The bead is held in an optical tweezers equipped with force measurement [15]. Measurements are performed in a feedback mode where a constant force can be maintained on the DNA: the voltage corresponding to the measured force is fed back to a piezoelectric translation stage that laterally displaces the sample while the trap position is fixed. In this mode, the translocation of the enzyme along DNA is reflected by the displacement of the sample. The electronic time constant of the force feedback loop is 20 ms. The position and force data are filtered through an antialiasing filter at 44 Hz, sampled with a 16 bit analog to digital conversion at a rate of 100 Hz and are stored onto a hard disk. The buffer, the sample holder, and the objective are temperature regulated at 27 °C. In order to avoid potential photodamage of the polymerase by the optical tweezers, we have taken the precaution of preventing the laser from impinging on the enzyme. This is possible because of the length of the DNA template.

Crystallographic structures of the T7 RNA polymerase have been obtained with various substrates, yielding four different structures. Those structures may be interpreted as snapshots of various states of the enzyme during the cata-

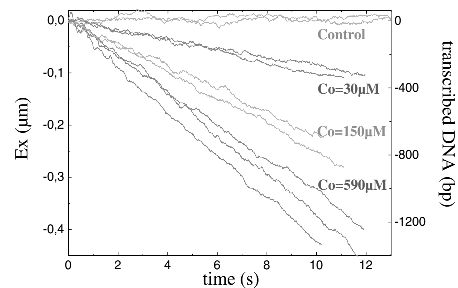

FIG. 2. Examples of experimental recordings: the measurements were performed at forces near 5 pN. Extension (Ex), i.e., the distance between the polymerase and the bead, is plotted vs time. Transcription is revealed by the decrease of Ex. For each curve, extension and time have been shifted for comparison. Each curve corresponds to a different polymerase; the NTP concentrations are indicated in the figure. As a control we plot two Ex(t) curves obtained with a simple DNA construction attached between the surface and the bead. The average velocity of the enzyme (over at least 8 sec) is extracted from those curves.

lytic cycle, and appear in Fig. 4(b) (in bold face). The structural data suggest that the posttranslocated state E_{post}, where the enzyme is waiting for NTP, is a stable state. The negative estimate of ΔG_0 (the free energy difference between E_{pre} and E_{post} in our model) is consistent with this. The crystal data also indicate that neither movement of the enzyme nor significant conformational change occur upon Pyrophosphate (PPi) cleavage. Force being a competitive inhibitor is also consistent with this finding. From structural analysis, it has been argued that translocation occurs between two states: E_{pre}:PPi where the enzyme is pretranslocated with PPi bound, and E_{post}, the enzyme posttranslocated waiting for NTP. We propose a mechanism of RNA polymerase which is consistent with published crystal structures, but differs from the mechanism put forward in [3], where it is assumed that (i) the disassociation of PPi is directly coordinated with translocation and that (ii) a large free energy change is associated with translocation. The structural data suggest that a force opposing forward motion will push the enzyme backward from the posttranslocated state. We propose that there is a transient state in the catalytic cycle where PPi is just disassociated, but the enzyme has not yet moved forward (noted E_{pre} in Fig. 4). In the absence of PPi [14], a force opposing the forward motion would favor such a transient state. Then a coherent picture emerges, compatible both with structural and kinetic (single molecule) data: competitive inhibition by force derives from the force-induced state-occupancy bias between E_{pre} (unable to bind NTP) and E_{post} (able to bind NTP). The enzyme advances after PPi release, that (as a charged molecule as argued in [3]) modifies the preferred state of the enzyme when leaving the active site, in part because the electrostatic interactions are modified: this induces a forward motion. From our single molecule measurements (see Fig. 3), and using the above model, this change of energy ΔG_0 is about -1.3 kT.

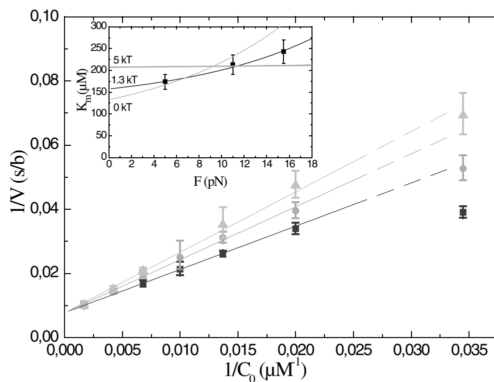

FIG. 3. Lineweaver-Burke plot of the experimental data: the inverse of the average velocity $1/\langle V\rangle$ is plotted as a function of $1/C_0$ for three forces: 5 ± 1 pN (squares), 11 ± 0.7 pN (circles) and 15.5 ± 1.5 pN (triangles). The error bar displayed is the "error of the mean", i.e. (standard deviation) $\times \langle N\rangle^{-1/2}$, where N is the number of data points taken to obtain the average value. Restricting to $1/C_0 \leq 0.02\ \mu\mathrm{M}^{-1}$, we have plotted linear fits to the data [10]. We obtained $V_{\mathrm{max}} = 129 \pm 8$ b/sec, and $K_M(5\ \mathrm{pN}) = 174 \pm 17\ \mu\mathrm{M}$, $K_M(11\ \mathrm{pN}) = 213 \pm 22\ \mu\mathrm{M}$, and $K_M(15.5\ \mathrm{pN}) = 243 \pm 27\ \mu\mathrm{M}$; these results are consistent with previous bulk measurements [22]. Number of points, 395; number of enzymes, 96; time of transcription, 1 h 50 min (1 h 10 min at 5 pN, 30 min at 11 pN, and 10 min at 15.5 pN). Inset: Experimental apparent K_M vs force: the K_M values are deduced from linear fits. The data is fit by the following expression (see text): $K_M(F) = K_{\mathrm{diss}}(1 + K \exp[F\delta/\mathrm{kT}])$, where δ is enzyme step size, a fixed parameter equal to one base pair. The solid line is the best fit: one gets $K = 0.27$ and $K_{\mathrm{diss}} = 124\ \mu\mathrm{M}$. To show the effect of a change in the parameter K, the other lines are fits obtained when imposing respectively $\ln(K) = 0$ and $\ln(K) = -5$, situations where E_{post} would either have the same free energy as E_{pre}, or be more stable by 5 kT.

What other energetic contributions could there be? The structural data indicate that there is little change in structure upon PPi cleavage and phosphodiester bond formation, but this does not mean that there is no associated energy loss; actually it is likely that some fraction of the global energy loss occurs at this step. Also, it may be argued that between the states E_{pre}:PPi and E_{post} there is a "helicase action" [3] of the polymerase, because the transcription bubble is displaced forward: one DNA-DNA base pair is broken, a DNA-DNA base pair is formed, and one DNA-RNA base pair is broken, resulting in a net necessary energy input ΔG_1 that we estimate to be on average of the order of ≈ 3 kT [15]. Along this view, one arbitrarily separates the energetic cost of the translocation step of the transcribing complex in two parts, the energy ΔG_2 associated with the conformational change of the protein itself (induced after PPi has left) and the energetic change ΔG_1 in DNA conformation. Then $\Delta G_2 = -\Delta G_1 + \Delta G_0 \approx -4$ kT, which is a sizable fraction of the energy of hydrolysis. Nevertheless, ΔG_0 ap-

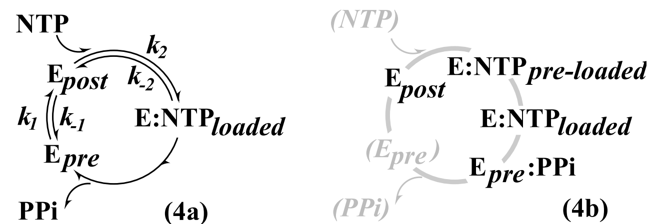

FIG. 4. Reaction cycles. (a) model inspired by Guajardo and Sousa [11]; E_{pre}: pretranslocated enzyme after hydrolysis and PPi release, unable to bind NTP; E_{post}: posttranslocated enzyme waiting for NTP binding; $E:\mathrm{NTP}_{\mathrm{loaded}}$: enzyme with NTP bound in proper position in the catalytic pocket; PPi: pyrophosphate [14]. (b) states identified from published crystal structures; E_{post} [1,2] and $E:\mathrm{NTP}_{\mathrm{loaded}}$ [3] designate the same states as in (a); $E:\mathrm{NTP}_{\mathrm{preloaded}}$: posttranslocated complex with NTP preloaded [4]; E_{pre} : PPi: enzyme after nucleotide hydrolysis, but before PPi release [3]. E_{pre}, corresponds to a pretranslocated transient state [without corresponding crystal structure (see text)].

pears as the available energy ''output'' that the transcribing complex as a whole is investing in translocation, and this is what we measure.

The value estimated for ΔG_0 is small, as compared to the free energy of hydrolysis [6]. Therefore, translocation (E_{pre} to E_{post}) occurs only slightly biased by thermal motion (by -1.3 kT at zero force). This mechanism of forward motion of the whole transcribing complex may thus be termed a ''weak'' powerstroke (i.e., in essence close to a Brownian motor description).

It has been shown [16] using the *E. coli* RNAP at high NTPs concentration (1 mM), that force (below 15 pN) does not affect the elongation rate, a result consistent with our present observations on T7 RNAP. This contrasts with other molecular motors such as kinesin, F1 ATPase, or the Phi 29 portal [17–19], for which it was shown that force slows movement or rotation, even at saturating ATP concentrations. From this point of view, this characteristic of RNA polymerases appears to be different from other molecular motors.

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[21] The T7 RNA polymerase is biotin tagged by adding the amino acid sequence MAGGLNDIFEAQ*K*MEWRLE at the N terminus of the protein (the underlined Lysine corresponds to the biotinylated amino acid). The λ DNA substrate can be transcribed by the biotinylated T7 RNAP over more than 10 kb in our setup.
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