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# Unravelling the mechanism of RNA-polymerase forward motion by using mechanical force

Philippe Thomen, Pascal J. Lopez<sup>‡</sup>, François Heslot\*

*Laboratoire Pierre Aigrain, Ecole Normale Supérieure, 24 rue Lhomond, 75005 Paris, France*

<sup>‡</sup> *Laboratoire Organismes Photosynthétiques et Environnement,*

*Ecole Normale Supérieure, 46 rue d'Ulm, 75005 Paris, France*

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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 [1]. 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 (RNAP) 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 [2–5], is a prototype of this protein class. After stages of promoter recognition and initial transcription of a few bases, conformational changes occur [2, 3], allowing the protein to enter elongation mode [6] 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 NTP hydrolysis (see [7] 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 was identical for each nucleotide and ranged from about 30  $\mu\text{M}$  up to 590  $\mu\text{M}$ . The measurements are performed using a feedback mode, where the applied force  $F$  is maintained constant. The force  $F$  ranged from 5 pN to 15 pN.

Fig. 2 shows examples of measurements performed with different NTP concentrations at 5 pN. We measured over at least 8 seconds the extension of DNA *vs.* 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 [12]. T7 RNAP appears to behave differently in this respect than the *Escherichia coli* enzyme, for which the

occurrence of many pauses in the range 1 to hundreds of seconds has been documented [13]. We observe a large variability in the measured velocities (estimated standard deviation: 30 %). A high variability is a common feature of single molecule experiments [14]. In the force range studied, we have not observed stalling, *i.e.* a smooth lowering of velocity *vs.* 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).

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 second 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). Fig. 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  $\delta$  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 lines, (excluding the points at the lowest concentration [15]), a feature com-

patible 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 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

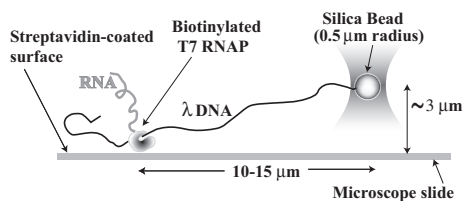


FIG. 1: Experimental configuration: the DNA template is lambda phage DNA (48502 base pairs, about  $16 \mu\text{m}$  in length) in which a T7 promoter is introduced at one end [9]. The other end of the DNA carries a digoxigenin and is attached to a microscopic bead coated with an antibody directed against digoxigenin. The transcribing polymerase is biotinylated at its N-terminus [10] 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 4 types of nucleotide 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 [11]. 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 piezo-electric 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 16 bit analog to digital conversion at a rate of 100 Hz and are stored onto a hard disk. The buffer, sample holder and the objective are temperature regulated at  $27^\circ\text{C}$ . 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.

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 [17]. 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. 4a. Provided that the step following binding is slow with respect to  $k_{-2}$ , the expression for  $K_M$  given by this model is:  $K_M = K_{diss} (1 + k_{-1} / k_1)$ , with  $K_{diss} = k_{-2} / k_2$ . Assuming that the force  $F$  acts essentially on the translocation step, *i.e.* on the  $k_{-1}$  and  $k_1$  constants, one gets:  $K_M(F) = K_{diss} [1 + K \exp(F\delta / kT)]$ , where  $\delta$  is the enzyme step size. The experimental  $K_M$  values are obtained from linear fits in Fig. 3, giving  $K = 0.27$  and  $K_{diss} = 124 \mu\text{M}$  (see Fig. 3 inset). The corresponding free energy difference  $\Delta G_0$  for the forward motion is  $kT \ln(K) = -1.3 kT$ . 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 [19] and references therein) considers that a Brownian particle can undergo directional movement by alternating between two states: in state 1 the particle essentially pinned and in state 2 (flat potential landscape [20]) diffusion can occur. However,  $\Delta G_0 < 0$  entails that  $E_{post}$  is energetically favored with respect to

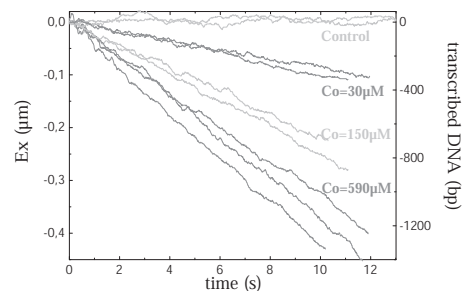


FIG. 2: Examples of experimental recordings: the measurements were performed at forces near 5 pN. Extension (Ex), *i.e.* the distance between the polymerase and the bead, is plotted vs. time. Transcription is revealed by the decrease of Ex. For each curve, extension and time have been shifted for comparison. Each curve corresponds to a different polymerase, the NTP concentrations are indicated on 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 seconds) is extracted from those curves.

$E_{pre}$ , *i.e.* the potential is not flat.

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 catalytic cycle, and appear in Fig. 4b (in bold face). The structural data suggest that the post-translocated state  $E_{post}$ , where the enzyme is waiting for NTP, is a stable state. The negative estimate of  $\Delta G_0$  (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 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 pre-translocated with PPI bound, and  $E_{post}$ , the enzyme post-translocated waiting for NTP. We propose a mechanism of RNA polymerase which is consistent with published crystal structures, but differs from the

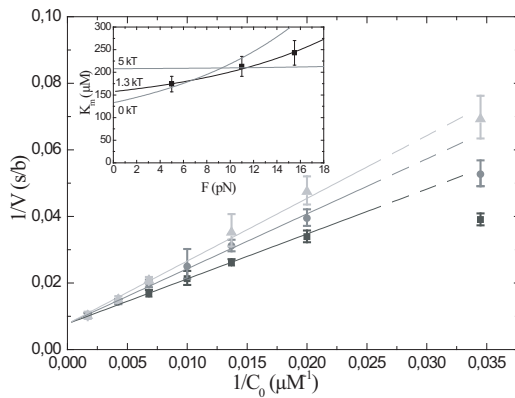


FIG. 3: Lineweaver-Burke plot of the experimental data: the inverse of the average velocity  $1/\langle V \rangle$  is plotted as a function of  $1/C_0$  for three forces:  $5 \pm 0.7$  pN (squares),  $11 \pm 0.7$  pN (circles) and  $15.5 \pm 1.5$  pN (triangles). The error bar displayed is the "error of the mean", *i.e.* (*standard deviation*). $(N)^{-1/2}$  where  $N$  is the number of data points taken to obtain the average value. Restricting to  $1/C_0 \leq 0.02 \mu\text{M}^{-1}$ , we have plotted linear fits to the data [15]. We obtained  $V_{max} = 129 \pm 8$  b/s, and  $K_M(5 \text{ pN}) = 174 \pm 17 \mu\text{M}$ ,  $K_M(11 \text{ pN}) = 213 \pm 22 \mu\text{M}$  and  $K_M(15.5 \text{ pN}) = 243 \pm 27 \mu\text{M}$ , these results are consistent with previous bulk measurements [16]. Number of points: 395; number of enzymes: 96; time of transcription: 1 h 50 min (1h10 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):  $K_M(F) = K_{diss} (1 + K \cdot \exp[F \delta / kT])$ , where  $\delta$  is enzyme step size, a fixed parameter equal to one base pair. The black line is the best fit: one gets  $K=0.27$  and  $K_{diss}=124 \mu\text{M}$ . 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.

mechanism put forward in [4], 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 post-translocated 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 [18], 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 [4]) 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  $\Delta G_0$  is about -1.3 kT.

What other energetic contributions could there be? The structural data indicates 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 an "helicase action" [4] 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  $\Delta G_1$  that we estimate to be on the average of the order of  $\approx 3$  kT [11]. Along this view, one arbitrarily separates the energetic cost of the translocation step of the transcribing complex in two

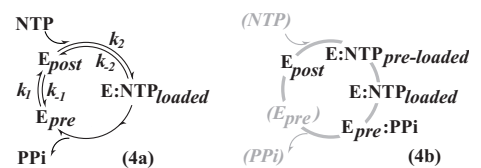


FIG. 4: Reaction cycles. (4a): model inspired by Guajardo and Sousa [17];  $E_{pre}$ : pre-translocated enzyme after hydrolysis and PPI release, unable to bind NTP;  $E_{post}$ : post-translocated enzyme waiting for NTP binding;  $E:NTP_{loaded}$ : enzyme with NTP bound in proper position in the catalytic pocket; PPI: pyrophosphate [18]. (4b): states identified from published crystal structures;  $E_{post}$  [2, 3], and  $E:NTP_{loaded}$  [4] designate the same states as in 4a;  $E:NTP_{pre-loaded}$ : post-translocated complex with NTP pre-loaded [5];  $E_{pre}$ :PPI: enzyme after nucleotide hydrolysis, but before PPI release [4].  $E_{pre}$ , corresponds to a pre-translocated transient state (without corresponding crystal structure, see text).

parts, respectively the energy  $\Delta G_2$  associated with the conformational change of the protein itself (induced after PPI has left), and the energetic change  $\Delta G_1$  in DNA conformation. Then  $\Delta G_2 = -\Delta G_1 + \Delta G_0 \approx -4$  kT, which is a sizeable fraction of the energy of hydrolysis. Nevertheless,  $\Delta G_0$  appears 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  $\Delta G_0$  is small, as compared to the free energy of hydrolysis [7]. Therefore, translocation ( $E_{pre}$  to  $E_{post}$ ) occurs by thermal motion only slightly biased (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 [21] 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 [22–24] 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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\* heslot@lpa.ens.fr

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