Force Spectroscopy

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Noninvasive Measurement of the Mechanical Force Generated by Motor Protein EF-G during Ribosome Translocation**

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The mechanical force generated by motor proteins plays an important role in biological processes.[1] These processes couple the chemical energy of ATP or GTP into the directional forces that are necessary to maintain the organizations and functions of life. Most force information has been obtained by atomic force microscopy and optical trap techniques.[2–4] In these experiments, motor protein kinetics is studied under varied loading of external forces. However, use of external forces makes data interpretation challenging and could introduce artifacts.[2,5] In addition, the associated technical difficulties such as complicated molecular manipulation could impose limitation on the motor proteins that can be studied.[6]

One motor protein of major interest is the elongation factor G (EF-G) involved in ribosome translocation. Ribosome translocation proceeds in two steps via an intermediate state.[7,8] The first step is the spontaneous formation of the hybrid state from the classical state.[9–13] In the hybrid state, the two subunits rotate relative to each other, and the tRNAs adopt the "hybrid sate" configuration, in which the acceptor ends bound at the A- and P-sites relocate to the P- and E-sites, respectively, while the anticodon stem loops remain bound at the A- and P-sites. The second step is the reset of the ratcheted conformation back to the non-ratcheted state concurrently with the translocation of the mRNA and the tRNAs in the small subunit.[14] EF-G stabilizes the ratcheted conformation sampled in the first step,[7,11–13,15] and GTP hydrolysis dramatically accelerates the second step.[16] EF-G catalyzes the ribosome movement with speed and accuracy at the cost of the GTP hydrolysis.[17,18]

An intriguing question regarding the mechanical–chemical coupling is how EF-G couples GTP to translocation: whether the energy is used to bias the intrinsic Brownian motion of the ribosome, termed Brownian ratchet mechanism, or to generate a directional force that drives the mRNA in the forward direction, termed power stroke mechanism.[19–22] The key difference between the two models is whether a mechanical force is generated. It is difficult to distinguish the two mechanisms using conventional methods, such as X-ray,[23] kinetics,[24] and single molecule fluorescence methods.[25] Recently, we demonstrated a force-induced remnant magnetization spectroscopy (FIRMS) technique that uses centrifugal or shaking forces to measure the binding forces of antibody–antigen interactions and DNA duplexes.[26] A force resolution of 1.8 pN has been achieved, which is sufficient for distinguishing DNA duplexes with a single-basepair difference.[27]

Here, we show a method that uses internal force references to precisely determine the intrinsic force generated by EF-G in the process of ribosome translocation. This method uses the binding forces of a series of DNA–RNA duplexes as internal force references. The mechanical force produced by the motor protein EF-G is used to dissociate the duplexes, unlike the shaking or centrifugal forces used in previous FIRMS setups.[26–28] As a result, the power stroke of motor proteins can be obtained during their biological functioning.

Figure 1 a shows a schematic of the FIRMS method with internal force references for measuring the EF-G power stroke. The ribosome pre-translocation complex is tethered to the surface via a duplex between an immobilized DNA strand and the mRNA molecule. The mRNA 5′-end is attached with a magnetic bead. Typically, tens of thousands of such

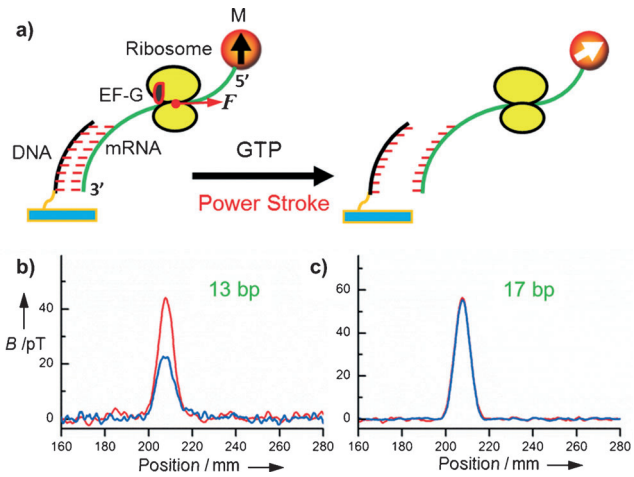

Figure 1. Measuring the EF-G power stroke with internal force references. a) Schematic of the method. b,c) Magnetic signal of the 13 bp and 17 bp DNA–mRNA duplexes before (red trace) and after (blue trace) application of EF-G·GTP, respectively.

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magnetically labeled ribosome complexes are tethered to the surface. The overall magnetic signal is measured by an atomic magnetometer.[26] If the power stroke generated by the EF-G is sufficient to dissociate the DNA–mRNA duplexes, the overall magnetic signal will decrease. In contrast, if EF-G only biases the ribosome thermal motions, as indicated by the Brownian ratchet model, the DNA–mRNA duplexes will remain intact, and the magnetic signal will not change. The magnitude of the power stroke can be deduced by varying the number of complementary basepairs of the duplex to adjust the binding force.

Figure 1b and c compare the results from using 13 base-pair (bp) and 17 bp duplexes as the force references. The sequences are shown in Figure S1 in the Supporting Information.[29] For each case, the magnetic signals before and after adding EF-G·GTP were measured using a scanning detection method,[26] in which the x-axis shows the relative position between the sample and the detector (Figure S2). For the 13 bp experiment, the magnetic signal decreased by 22 pT or 46% of the original signal. No signal change was observed for the 17 bp case. Therefore, the 13 bp duplex could be dissociated by the mechanical force exerted by the EF-G, but the 17 bp duplex had a stronger binding force than the EF-G force. The typical error in magnetic signal measurements was ± 1 pT (Figure S3). Because this value was much greater than the variations among repeating measurements of a given sample, it was used as the overall uncertainty.

Figure 2a shows that the dissociation could only be caused by EF-G·GTP. The magnetic signals were obtained when the 13 bp duplex and the pre-translocation complex were incubated with either plain buffer or apo-EF-G. The signal did not change in either case. When the mRNA direction was reversed such that the 5′-end formed the duplex and the 3′-end was bound to a magnetic bead, no dissociation was observed either (green trace in Figure 2a, sequence in Figure S1). These results indicate that the force generated by EF-G·GTP is unidirectional, consistent with the direction of the ribosome translocation. Two additional experiments are shown in Figure S4. One was using EF-G·GDPCP instead of EF-G·GTP, which did not cause a magnetic signal decrease. This result means that the power stroke by EF-G can only come from the energy of GTP hydrolysis. The other experiment involved shifting the duplex by 3 nucleotides toward the 3′-end ("13 bp shifted" in Figure S1) while retaining a 13 bp duplex. The percentage of signal decrease was the same as that of the original duplex; thus, the duplexes used as the force references were not affected by the locations.

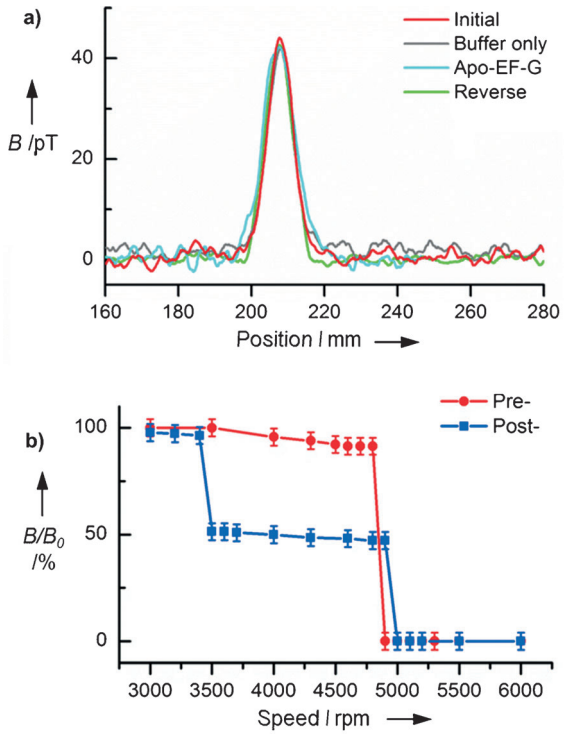

Figure 2. Confirmation of the force-translocation correlation. a) Magnetic signals of the 13 bp duplex at various control conditions. b) Binding forces of the 17 bp duplex with the pre- and post-translocation ribosome complexes, respectively.

The reactivity of the ribosome complex was verified by using FIRMS to measure the binding force of the 17 bp duplex before and after the addition of EF-G·GTP (Figure 2b). A centrifugal force was used to induce the duplex dissociation. Before the addition of EF-G, only one transition was observed at 4900 rpm (revolutions per minute). But after the addition of EF-G, two transitions were observed: one at 3500 rpm and the other at approximately 4900 rpm. The uncertainty was 100 rpm, the smallest speed increment for the centrifuge. The binding forces can be calculated by $f = m\omega^2 r$, where m is the buoyant mass of the magnetic particles, ω is the angular velocity, and r is the distance between the sample and the center of the centrifuge. Based on $m = 4.6 \times 10^{-15}$ kg and $r = 8$ cm, the binding forces were calculated to be 50 ± 3 and 97 ± 4 pN for 3500 and 4900 rpm, respectively.[30,31] The weaker binding force of 50 pN was attributed to the duplex with less number of complementary basepairs that resulted from ribosome translocation. The relative amplitude of 50% verifies the ribosome reactivity to be approximately 50%, consistent with the signal decrease in Figure 1b. This shows that even though EF-G could not dissociate the 17 bp duplex, it nevertheless promoted the translocation. The reactivity of the ribosome complex was also confirmed by single molecule FRET, during which the ribosome complex was immobilized onto the surafce (Figure S5).

These results show the feasibility of using a series of DNA–mRNA duplexes as internal force references to study the power stroke in situ. Precise quantification of the EF-G power stroke was then carried out in two steps. The first step was to determine the binding forces of a series of duplexes with successive numbers of complementary basepairs. The binding forces of the 13 to 18 bp DNA–mRNA duplexes are shown in Figure 3a, as well as that of the 10 bp duplex. For all of the duplexes, the debinding occurs at a sharp transition: above each specific force threshold, the dissociation was complete (Figure S6); below the force threshold, no dissociation occurred even for a prolonged pulling attempt of 30 min. We have reported similar observations previously.[30] These force measurements are consistent with the measurements by atomic force microscopy.[32,33] In particular, Figure 3b shows the 14 bp duplexes with binding force 52 ± 3 pN. Based on this calibration, we can clearly see that the weaker

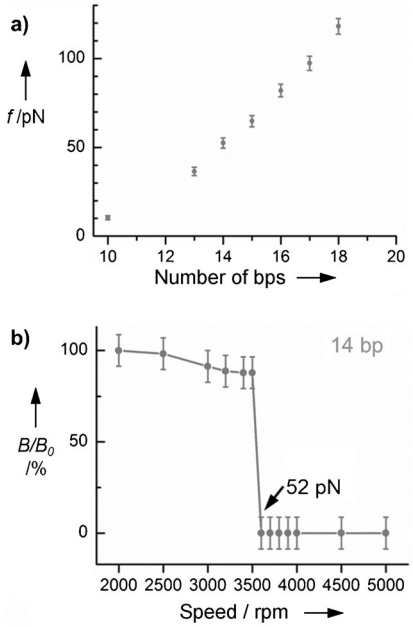

Figure 3. Calibration of the binding force of the DNA–mRNA duplexes. a) Plot of the binding force versus the number of complementary basepairs. b) FIRMS measurement for the 14 bp duplex.

transition at 50 pN in Figure 2b for the post-translocation complex corresponds to a 14 bp duplex, meaning the translocation indeed weakened the binding force by three bps. The 3 bp change indicates that the ribosome covers the mRNA at the 12th nucleotide. This value is consistent with but more precise than the literature value.[34,35] This information may provide the precise location of the helicase activity on the ribosome.

The second step was to use the characterized duplexes as internal force references for measuring the EF-G power stroke. For each duplex, the magnetic signals before and after adding EF-G·GTP were obtained. The percentages of remnant duplexes were plotted in Figure 4. Details are shown in the Supporting Information Figure S7. For the 10, 13, and 14 bp duplexes, the magnetic signals decreased by 44–47%, which were similar considering the experimental uncertainty of approximately 5%. A much smaller signal decrease was observed for the 15 bp (30%) and 16 bp (21%) duplexes, and no significant dissociation was observed for the 17 bp (2%) and 18 bp (0%) duplexes. Therefore, the magnitude of the EF-G power stroke is between the binding forces of the 16 bp and 17 bp duplexes, which are 82 and 97 pN, respectively.

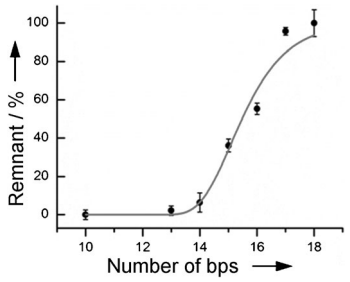

Figure 4. Plot of the remnant percentage of the duplex versus the number of complementary basepairs. The red trace is the fitted curve.

Thus, the amplitude of the power stroke should be in between the two force values, or 89 ± 9 pN.

Quantitatively, the dissociation of the DNA–mRNA duplexes as internal force references by the EF-G power stroke can be interpreted by Bell's model.[36,37] The dissociation rate is given by Equation (1):

$$k_N(f) = k_N(0)\exp(f/f_\beta) \quad (1)$$

where $k_N(0) = (1/\tau)\exp(-N E_0/k_B T)$ and $f_\beta = k_B T/N\delta_0$

Here, k, N, f, τ, E_0, and δ_0 are the rate constant for the dissociation reaction, number of bps, external force, characteristic time constant of diffusion, activation energy barrier per bp, and transition state distance per bp, respectively. k_B and T are the Boltzmann constant and temperature (310 K in this work), respectively. When the dissociation of DNA–mRNA duplexes is induced by EF-G·GTP during translocation, a short-lived and invariable power stroke F induces dissociation of the duplexes with varied percentages. Equation (1) can be rewritten as Equation (2).

$$k_N(F) = (1/\tau)^*\exp(-N E_0/k_B T)^*\exp(F N\delta_0/k_B T) \quad (2)$$

The percentage of remaining duplexes then is given in Equation (3).

$$\ln(B_t/B_0)_N = -k_N(F)^* t = -A^*\exp(N^*(F\delta_0 - E_0)/k_B T) \quad (3)$$

Here, B_0 is the initial magnetic signal, B_t is the magnetic signal at time t, and A is a pre-factor. The value of δ_0 has been obtained by a detailed AFM study in the literature, which was 0.06 nm for short RNA duplexes.[37] The value of E_0 has also been reported to be $2\,k_B T$.[38] Substituting these values into Equation (3), we obtain Equation (4).

$$(B_t/B_0)_N = \exp(-A^*\exp(N^*(0.012\,F - 2))) \quad (4)$$

The red trace in Figure 4 shows the fitting by Equation (4) with $F = 89$ pN. The resulting pre-factor A was 1.3×10^6. The fitting shows the good agreement between Bell's model and our experimental results.

The interpretation that the dissociation of the DNA–mRNA duplexes was due to the EF-G power stroke was based on two assumptions. First, the magnetic beads remained static during the power stroke. Because of the much larger size and mass of the magnetic beads compared to the mRNA, the force required to move the beads would far exceed the EF-G power stroke, validating this assumption (Supporting Information). Second, there was no loop structure for the mRNA section that formed duplexes with the probing DNAs. The validity of this assumption was confirmed by using both simulation and the literature (Supporting Information).

From the force value of the EF-G power stroke, the effective distance of the power stroke, Δ, can be obtained by comparing the translocation kinetics with and without EF-G using Equation (1). Because EF-G accelerates the translocation approximately 15000-fold, $F/f_\beta = 9.6$. Substituting f_β with $K_B T/\Delta$, we have $F^*\Delta = 9.6\,K_B T$. Consequently, the power stroke of 89 pN leads to a Δ of 0.5 nm. This transition state distance is much less than the full travelling length of one

codon (2 nm), indicating that EF-G does not act as a pure Brownian ratchet. Otherwise, the transition state distance should equal to the full translocation distance.[39]

The EF-G power stroke can be correlated to the EF-G conformational changes. In the structure of EF-G bound on the post-translocation ribosome, the tip of domain IV interacts with the P-site tRNA at the anticodon stem loop, implying that these are the interaction points between EF-G and the A-site tRNA prior of translocation.[23] Overlapping the G domains of this structure and of the free EF-G[40] indicates a large-scale hinged swing of the domain IV (Figure S8). Therefore, the EF-G undergoes a large conformational change in domain IV and couples the GTP energy to generate a mechanical force. This force exerts on the A-site tRNA, which moves together with the mRNA towards the 5′-end during translocation.

In conclusion, using a noninvasive FIRMS method with internal force references, we have determined the EF-G power stroke to be 89 pN and it exerts for 0.5 nm, on the contrary to the current prevailing Brownian ratchet model. This force is large in magnitude but small in working distance compared to Myosin and Kinesin that exert small forces over large ranges.[1] The detection of the EF-G power stroke clarifies the long-standing question regarding the coupling between GTP and translocation. In addition, we have demonstrated that the EF-G-induced duplex dissociation is correlated with in situ translocation, which clearly showed that the EF-G power stroke caused the ribosome translocation. Because the internal force references have simple molecular constructs and can be adjusted precisely and broadly, our method will be suitable for detecting the mechanical forces of a wide range of motor proteins during their biological functions.

Experimental Section

Details are provided in the Supporting Information. The ribosome pre- and post-translocation complexes were prepared as previously described.[29] Biotinylated probing DNA was immobilized on the streptavidin-coated surface of a sample well. DNA–mRNA duplexes were formed when the ribosome complex in TAM_{10} buffer with 1 M NaCl was added. Streptavidin-coated magnetic beads M280 (Invitrogen) were added to label the 5′-end of the mRNA that contained biotin. The formed DNA–mRNA duplexes underwent force-induced dissociation by either the power stroke of EF-G or a centrifugal force in the case of force calibration.

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