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On the application of optical tweezers and electro-rotation to study rotational dynamics of flagellar motors

The bacterial flagellar motor is a rotary molecular engine driven by an electrochemical gradient; which turns a helical filament generating the thrust enabling the bacteria to swim. The mechanism of torque generation has been described by different models, each of them predicting a different relationship between speed and torque. In order to rule out the less effective models in favor of more plausible ones, Berg and Berry focused on one particular aspect of the rotational dynamics of flagellar motors, i.e. whether or not there is a barrier to the backwards rotation of the motor. Such a barrier, if present, should manifest itself in the form of a higher value of torque when the motor is driven against its natural direction of rotation beyond some limiting speed (Berry and Berg, Torque generated by the Flagellar motor of Escherichia coli while driven backward). This was indeed the observation made in 1993 by Berg and Turner when they used the technique of electro-rotation (to be described further below), to study the process. However, in 1996 and 1999, Berg and Berry demonstrated that the observed doubling of torque for backwards rotation was not intrinsic to the motor, but merely a consequence of the particular experimental technique they were using. This claim was strengthened further when they employed a different technique i.e. optical tweezers (Berry and Berg)to show that the motor generated the same torque regardless of its direction of rotation. In this paper, we give a critical review of their approach towards concluding that there is no barrier to backwards rotation of the motor, and what implications their study has in arriving at particular functional models. . Major physical models predicting mechanistic details of torque generation are reviewed and the best possible model based on predictions made by a simple three step kinetic model is selected.

Flagellar motors of E. coli and S.tryphimurium are driven by inward directed proton flux, powered by the proton motive force. This power input of proton flux is divided among eight distinct proton channels comprising of the proteins MotA and MotB- referred to as the stator Site directed Mutational studies have identified an electrostatic interaction between C terminal domains of MotA and FliG protein (Lloyd and Blair).Similar studies have also implicated a MotB Aspartate residue (Asp32) , located at the cytoplasmic end of the proton channel as a proton acceptor (Zhou, Lloyd and Blair).Thus, it appears that the motor output, torque, is generated through a protonation event at Asp32 , while its deprotonation modulates the interaction of a specific charged region in C-terminal MotA and a complementary charged region in FliG, possibly mediating a conformational change.

The various models elucidating torque generation mechanism in BFM (bacterial flagellar motor), can be broadly classified into two categories - based on the degree of coupling between proton transit and rotation.

A) Tight coupling- where for each revolution of the BFM a passage of defined no. of protons is required. According to this mechanism there can be two possibilities- channel type, where the proton interacts simultaneously with both stator and rotor components (Meister and Berg)or cross-bridge type, where the proton interacts only with the stator, which couples to the rotor in a conformation-dependent manner (Lauger and Kleutch) . Since, all experimental data supports this model category, both of these cases will be explained in detail.

B) Loose coupling, when the rotor can turn without proton transfer or protons can flow without motor rotation (Oosawa and Hayashi).

A fundamental question that arises at this stage is how the flagellar motor generates torque? Or how does the inward motion of one or more ions through a torque generating unit, cause it to advance circumferentially along the periphery of the rotor? Studies have shown that each torque generation unit (MotA-MotB complex) contributes equally to the total torque generated by the motor, such that the expression of an additional unit provides an exact increment. Hence, in order to dissect the torque generation mechanism in detail, the torque –speed relation of the motor in either direction needs to be studied, considering a single torque generating unit.

Therefore, the basic aim in all of the biophysical experiments conducted was to obtain torque-speed relationships in the form of plots of frequency of rotation against the motor torque. Any non-linearity in the observed relationship – e.g. a sharp rise in motor torque for negative frequencies - may then be analyzed and interpreted as a barrier to rotation or vice versa. In the electro-rotation experiments, this approach is implemented in the following manner. Using microelectrodes, a rotating electric field is applied to polarize a tethered E. Coli cell. "The electrodes are arranged in a cross, with a gap of about 40 microns in the middle. Sin(wt) is applied to one opposite pair, cos(wt) to the other, so that the resulting field rotates. The frequency is about 2 MHz." (Oxford Molecular Motors) Due to a phase lag between the rotating electric field and the induced dipole moment in the cell, a torque is exerted.

$$\tau = p \; X \; E \qquad\qquad\qquad (1)$$

Using this principle, it is not only possible to spin the cell in both directions at speeds of the order of kHz, but also estimate the relative motor torque on a tethered cell by balancing it with the hydrodynamic viscous drag acting on a rotating cell body (Jin, Yasuo and Syoyu). A rigorous analysis in the light of the aforementioned balancing of torques yielded a relationship between the relative motor torque and the rotation speed. The figure below gives the results of an experiment in which the cell speed was averaged over successive periods of 1/6s during a run in which P (the electro-rotation strength) was steadily increased from 0 to -5%.
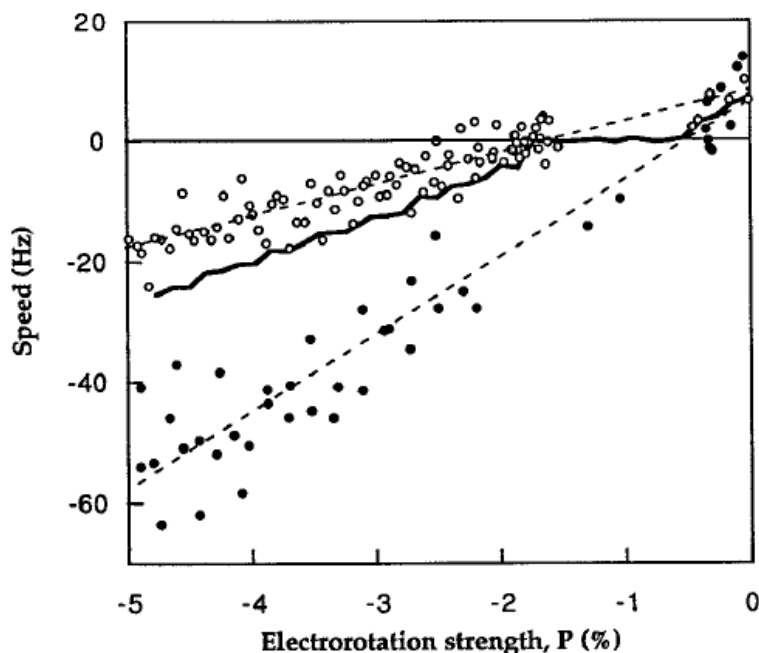
Fig. 1. *The solid line is an indication of the conclusions made in 1993 in favor of a barrier to backwards rotation. As shown in the figure "for backward rotation, motor torque rose steeply at speeds close to zero, peaking, on average, at about 2.2 times the stall torque. For forward rotation, motor torque remained approximately constant up to speeds of about 60% of the zero-torque speed. Then the torque dropped linearly with speed, crossed zero, and reached a minimum, on average, at about -1.7 times the stall torque."* (Berg and Turner)*In other words, "almost twice as much torque is required to make the motor rotate backward as is sufficient to stop its rotating forwards."* (Berry and Berg)

The above results are indicative of a tightly coupled 'thermal ratchet model' proposed by Meister et al.1989. In this model backward or forward rotation takes place only when certain sites on the torque generating assembly are protonated or de-protonated , i.e. to say a fixed proton transit is mandatory to carry the motor through one revolution. In Meister et al's model, a Torque generating unit (TGU) will move forward once a proton-accepting site on the rotor in contact with a channel leading to the periplasm is protonated while an adjacent site in contact with a channel leading to cytoplasm is not. The total probability to reach this state will be large if the motor is energized as in this case the pH of the first site is smaller than its pK while in comparison the pH is higher. Hence, for the TGU to rotate backwards, a protonation event has to take place on the second site, probability for which is low. To overcome this energy penalty for this model and for backward rotation to take place a large torque is required. This is because as the larger the externally applied torque, the more difficult it becomes for the element to move forward, so the net backward rate increases. Although this model adequately explains the case of a barrier for backward rotation (in accordance with the results of electro-rotation); it fails to explain a constant torque with a positive forward speed up to at least 300Hz (as shown in figure 5c).

However, as shown by Berg and Berry in 1996 and 1999, the apparent non-uniformity in the relationship between motor torque and speed is because "the electro-rotation strength required to stall the motor varies with cell angle." (Berry and Berg, Torque generated by the Flagellar motor of Escherichia coli while driven backward)
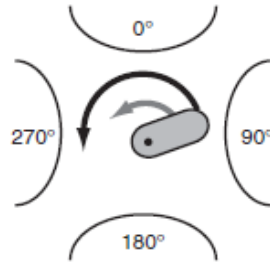
Fig. 2. Torque on a polarized cell at various angles (Berry and Suwa, Bacterial Flagellar Motor)

Essentially, the motor-torque and the external torque (applied by electro-rotation) either reinforce or cancel each other depending on the angle of the cell.
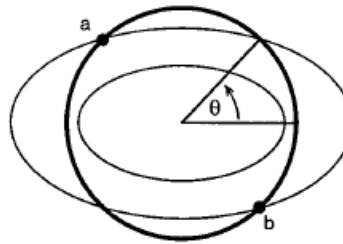
Fig. 3. The amplitude of the torque exerted on the body of a tethered cell by its flagellar motor (circle) or by an externally applied high frequency rotating electric field (ellipses) shown as a function of the angular position of the cell. (Berry and Berg)

"If the externally applied torque opposes motor torque, then the cell slows down. Its speed is proportional to the algebraic sum of the radii of the circle and the ellipse, and this varies with angle. When the externally applied torque opposes motor torque and its strength is sufficiently large, the cell stops or turns backward. For a strength corresponding to the larger ellipse in Fig. 2, the cell stops at *a* or *b*, where motor torque and externally applied torque balance." (Berry and Berg).

In light of this information, the data was separated into subsets by grouping according to cell angles. As shown by the dotted lines in the graph, "at each angle the relationship between speed and applied torque is actually linear. Evidently, the externally applied torque was larger when the cell was oriented between 120° and 180° (*filled circles, steeper slope*) than it was when it was oriented between 240° and 300° (*open circles*)." (Berry and Berg, Torque generated by the Flagellar motor of Escherichia coli while driven backward)

The above results are considered to be evidence of the fact that barrier to backward rotation was merely an artifact of the technique of electro-rotation. To establish this further, the bacterial flagellar rotation was probed with the help of a different technique i.e. optical tweezers. Optical tweezers essentially exploit the principle of radiation pressure to exert forces on a bead or single molecules in such a way as to trap them between two opposing laser beams. In this experiment, a tethered cell was stalled by a bead held in the optical trap. The displacement of the laser beam on a quadrant photodiode, generated signals that were fitted to a grid calibrated for force on a bead. The cells rotational speed was varied with the help of a piezo-electric stage that employed non-sinusoidal voltages to move the tether in circles of various radii at different frequencies. The magnitude of force on the bead was obtained at both negative and positive frequencies (figure below).The motor torque can be directly calculated from the force by deriving the viscous drag coefficient of the bead and multiplying it by the angular speed of rotation.
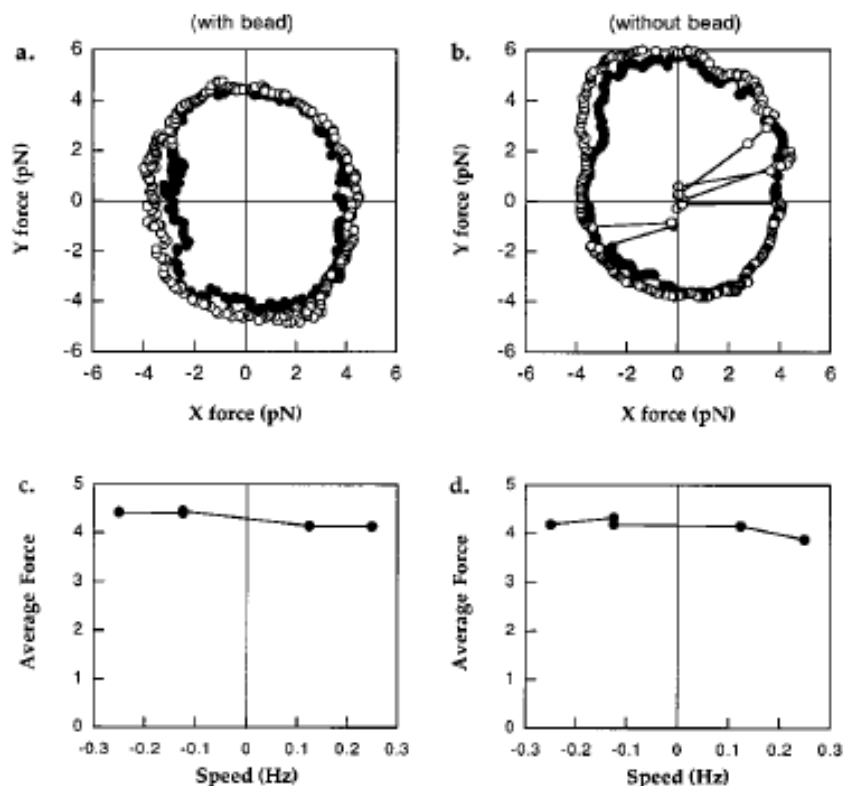
Fig. 4. (a) Forces exerted by a tethered cell on a trapped bead entirely.
when allowed to rotate slowly forwards (filled symbols, counterclockwise,
+ 1/8 Hz) or pushed slowly backwards (open symbols, clockwise, -1/8 Hz). The circles started from the bottom right,
where extra points were recorded while the cell was stalled before and after rotations. (b) Forces as in a, recorded
with the same cell, only now held directly in the trap without a bead. Note how the cell escaped the trap on several

occasions during the backwards push. (c) Mean force vs. speed for this cell with the bead. (d) Mean force vs. speed for this cell without the bead (Berry and Berg)

The above results show that the average force on the bead remains roughly uniform, irrespective of the speed and direction of rotation, that in turn rule out those models which predict a barrier to the backwards rotation of the motor; in which, in addition to rotation being tightly coupled to ion flux, rate of transit of ions against their chemical potential is highly limited.

These revised experiments show a constant torque at speeds up to 100 Hz in either direction. In order for a tightly coupled model to predict this result, there must exist a rate limiting step in the torque generation cycle, such that the rate change is extremely sensitive to torque. This is illustrated by a simple kinetic model (Fig 5a). Fig shows the schematic of the mechano-chemical cycle of a single TGU (Berry and Berg, Torque generated by the Flagellar motor of Escherichia coli while driven backward).The unit moves between state E and states A or B (steps 1 and 3, respectively) by exchange of protons with either the cytoplasm (Hi) or the periplasm (Ho), and any processes that occur while a proton is within the motor are summarized by the transition between states A and B (step 2). Tight coupling between proton flux and rotation is assumed. Thus torque against which the motor is acting can be specified the corresponding proton flux and speed can be determined from the net rate at which the kinetic cycle: E to A to B to E is performed. For each step in the cycle there exists a chemical free energy change Ui (i= each step)and a simultaneous rotation of the rotor through an angle φi. Where the work done by each TGU and the free energy change in each step is given by:

$$\mathbf{W_i = \beta_i \; \Gamma_i \varphi_i.} \qquad\qquad \textbf{(2)}$$

$$\mathbf{U_i = \alpha_i n e \Delta p} \qquad\qquad \textbf{(3)}$$

Where β_i is the fraction of the distance moved in step i. Γ is the torque while α_i is the measure of available free energy of protons dissipated in each step. N is the no. of protons with e being its charge and Δp = pmf.
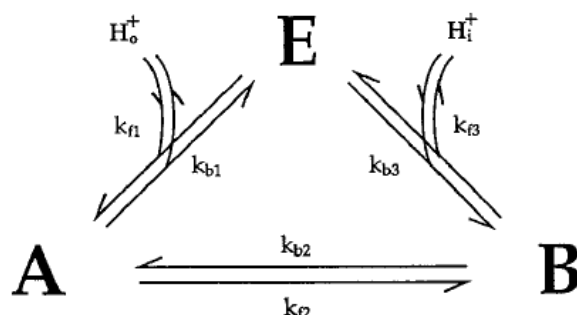
Fig. 5.(a) A simple kinetic model for the mechanochemical cycle of
torque-generating units in the bacterial flagellar motor. The cycle is reduced to three steps. Steps 1 and 3 involve exchange of protons between
the motor and the periplasm or cytoplasm respectively, while step 2 incorporates all events that occur while protons are within the motor. Rate constants for steps leading to forwards and backward cycles are labeled k_{fi} and k_{bi} respectively, where *i* indicates the step. (Berry and Berg)

Predictions of torque-speed relationships by this model are made by altering the aforementioned set of parameters. As Fig A, shows if the dissipation of proton free energy and the rotation of the rotor occur in separate steps, the model predicts a barrier to backward rotation. Whereas, If a single step couples proton free energy to rotation, the predicted torque-speed relationship shows a region of slowly varying torque (a plateau) at low speeds, and a steeper torque dependence at higher speeds (shown in Fig B). Latter result being in line with experimental evidence as mentioned earlier represents the 'power stroke mechanism' which is realized if rotation and proton transit occur simultaneously as in the half channel cross-bridge mechanism of Lauger (1988). Thus, in this case, protons can be driven out of the cell by backward rotation and steep barriers are not expected as observed. In addition as the rate limiting step is strongly torque dep., the torque speed curve has a relatively flat plateau unlike the thermal ratchet case, with tight coupling, the possibility of ion transfer against electrochemical potential is small and thus the system must wait for favorable coupling even when large external torque is applied hence predicting steep torque-speed curves.
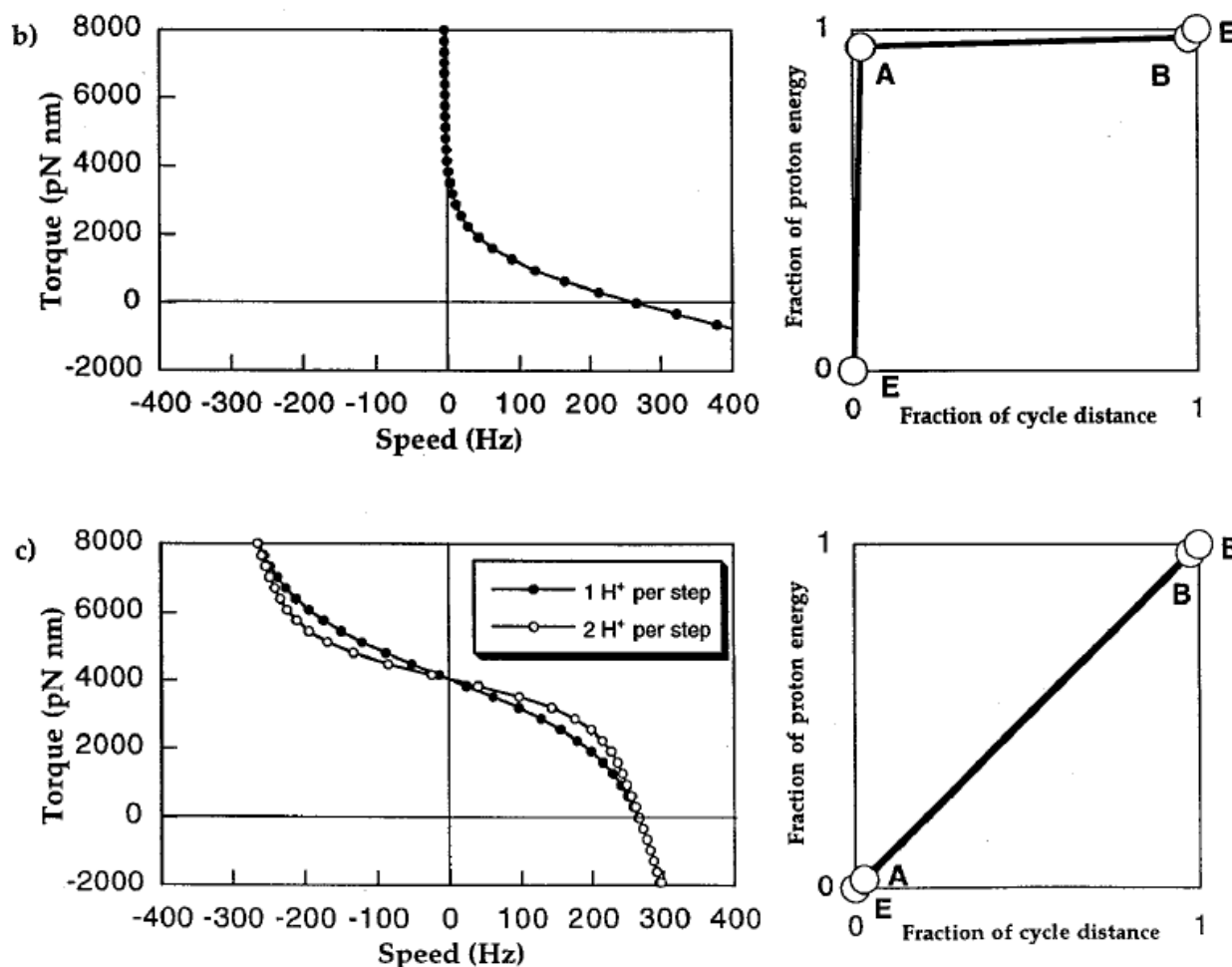
Fig. 5b,c *The relationship between torque and speed predicted by the model of Fig. 5 (left-hand panels) with different sets of parameters as described in the text. The right-hand panels are a graphic representation of the degree to which each step in the mechanochemical cycle dissipates the free energy available from proton translocation (vertical axes) and results in rotation of the rotor (horizontal axes). (b) represents a case of barrier to backward rotation. (c) prediction made in accordance with experimental results*

Recent research and experimental evidence supports the 'power stroke mechanism' similar to the tight coupling model (Meacci and Berg) with a cross bridge mechanism envisaged by Lauger (mentioned earlier). In such a scheme proton transit drives a cyclic scheme in which a proton binds to an outward-facing binding site (Asp32), followed by a power-stroke (a conformational change) driven by the proton motive force (Berry and Berg) that moves the rotor forward and transforms the binding site into an inward –facing site where proton dissociation triggers detachment of the cross-bridge from the rotor and relaxation to its original shape. Thus, the two biophysical techniques of electro-rotation and optical traps have been crucial to the study of BFM rotation and have helped us select the model that involves tight-coupling between proton transit and rotation – where the mechanism is likely to be that of power-stroke rather than the

thermal ratchet type; any steps that dissipate a significant fraction of the PMF must also involve the rotation of the motor.

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