Relaxation time asymmetry in stator dynamics of the bacterial flagellar motor

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The bacterial flagellar motor is the membrane-embedded rotary motor, which turns the flagellum that provides thrust to many bacteria. This large multimeric complex, composed of a few dozen constituent proteins, is a hallmark of dynamic subunit exchange. The stator units are inner-membrane ion channels that dynamically bind to the peptidoglycan at the rotor periphery and apply torque. Their dynamic exchange is a function of the viscous load on the flagellum, allowing the bacterium to adapt to its local environment, although the molecular mechanisms of mechanosensitivity remain unknown. Here, by actively perturbing the steady-state stator stoichiometry of individual motors, we reveal a stoichiometry-dependent asymmetry in stator remodeling kinetics. We interrogate the potential effect of next-neighbor interactions and local stator unit depletion and find that neither can explain the observed asymmetry. We then simulate and fit two mechanistically diverse models that recapitulate the asymmetry, finding assembly dynamics to be particularly well described by a two-state catch-bond mechanism.

INTRODUCTION

The bacterial flagellar motor (BFM) is the rotary molecular motor, which, in many bacterial species, provides the thrust necessary for motility, chemotaxis, biofilm formation, and infection. The BFM is large (~11 MDa), dynamically self-assembled, membrane-spanning, and composed of more than a dozen different proteins. The rotor, which extends into the cytoplasm, is rotated by up to around a dozen peptidoglycan (PG)-bound stator units, each one powered by the electrochemical gradient across the cell membrane. This rotation is coupled to the extracellular flagellar filament, which can spin at speeds of up to hundreds of hertz.

Historically, it was imagined that, once assembled, the composition of the BFM was static. However, thanks to a multitude of studies over the last decade showing dynamic exchange of multiple motor components, the BFM has become a hallmark of dynamic subunit exchange in multimeric protein complexes (1). This phenomenon has been most well characterized for the stator units, which exchange between an active, motor-bound, torque-producing population and a pool of inactive membrane-diffusing units (2). While the purpose and mechanism of this exchange have not yet been fully elucidated, the stator units are mechanosensitive, with larger viscous loads on the flagellum leading to higher stator occupancy (3). Stator unit exchange occurs on time scales of seconds to minutes, allowing the motors of individual cells to quickly and dynamically adapt to changes in their environment.

The dynamics of stator remodeling was first quantitatively modeled with a simple reversible Hill-Langmuir adsorption model (2, 5). When this model was applied to motors driving various viscous loads, it was observed that the stator unbinding rate was load dependent and that the stator units behaved in a manner consistent with a catch-bond mechanism. Despite these results, the particular mechanism in control of the stoichiometry dynamics remains elusive.

Recently, Wadhwa et al. (6) proposed a more complex model which conjectures that, in addition to a load-dependent unbinding rate, both the binding and unbinding rates are also dependent on the speed of the motor. In contrast to the Hill-Langmuir model, this model presents an intriguing and testable prediction: The characteristic relaxation time scale toward steady state depends on the initial stator stoichiometry.

In this work, by using a variety of experimental methods, we explore different starting stoichiometries of individual motors and measure their relaxation dynamics. To shed light on the mechanistic nature of the resulting timescales, we compare the experimental results with the model of Wadhwa et al. and with other models compatible with a relaxation time depending on the initial stoichiometry. In particular, we propose an alternative model based on a two-state catch bond, and we discuss the implications of these results with respect to stator remodeling of the BFM and more generally for dynamic protein complexes.

RESULTS

Measurements of BFM relaxation to steady state

Previously, we used magnetic tweezers to reversibly increase the load on individual motors, transiently perturbing the stator stoichiometry from steady state, and then quantified stator dynamics during the relaxation back to steady state (5). In this work, we started by performing similar measurements. Cells of a nonswitching strain of Escherichia coli lacking flagellar filaments were immobilized to a coverslip, and streptavidin-coated superparamagnetic particles were attached to an endogenously biotinylated hook. The position of the particles was tracked and used as a proxy for the angular position of the motor, yielding motor speed and torque. Following a steady-state measurement, two permanent magnets were brought near the sample. Here, the torque exerted by the magnets on the particle counters and balances the torque exerted by the BFM upon the particle, thereby stalling motor rotation. This resulted in the recruitment of additional stators, priming the system to a state of higher stoichiometry than that of steady state. Following 10 min of motor stall, the magnets were rapidly removed, and the speed and...
torque of the motor were measured as the system relaxed back to steady state.

Following relaxation, and using the exact same set of motors, we perturbed the motors away from steady-state stoichiometry, but in the opposite direction, toward lower stator stoichiometries. We introduced an ionophore to collapse the proton motive force (PMF) and waited 8 min for the stators to fully dissociate from the motor. We then flushed out the ionophore and measured motor speed and torque as the stators re-incorporated to reach steady state, a process we refer to as "resurrection." All measurements were performed for three viscous loads ($\gamma_{1300}$, $\gamma_{500}$, and $\gamma_{300}$) by using beads of 1300, 500, and 300 nm in diameter. Details are given in Materials and Methods and as described previously (5).

From these different experiments we obtained, for each individual motor, traces in time for three different initial stoichiometry conditions: steady state, release from stall, and resurrection. Using a step detection algorithm and knowledge of the torque per stator from individual traces, we calculated stator stoichiometry trajectories in time (5). Figure 1A shows an example of a single motor measurement, Fig. 1B shows a schematic of the evolution of stoichiometry, and Fig. 1 (C and D) shows motor torque and stator stoichiometry as a function of time, where individual motor measurements are
shown in color, and the average behavior over all motors is shown in black.

**An asymmetry in the characteristic relaxation time**

We previously proposed a reversible Hill-Langmuir adsorption model to describe stator assembly kinetics (5). In this model, the rotor is surrounded by \( N_{\text{max}} \) fixed, independent, and noninteracting binding sites. Stator units freely diffusing in the inner membrane can bind to an empty site on the rotor with rate constant \( k_{\text{on}} \), bound stator units can dissociate with rate constant \( k_{\text{off}} \), and the average number of bound stator units, \( \langle N \rangle \), evolves according to

\[
\frac{d \langle N \rangle}{dt} = k_{\text{on}} (N_{\text{max}} - \langle N \rangle) - k_{\text{off}} \langle N \rangle \tag{1}
\]

We assume the concentration of freely diffusing stators is large enough to be considered constant. The analytical solution

\[
\langle N \rangle(t) = \langle N \rangle(0) + (\langle N \rangle(0) - \langle N \rangle(\infty)) e^{-(k_{\text{on}} + k_{\text{off}}) t} \tag{2}
\]

relaxes from stator occupancy \( N_0 \) at \( t = 0 \) to the average steady-state value, \( \langle N \rangle(\infty) = N_{\text{max}}/(1 + k_{\text{off}}/k_{\text{on}}) \), with a characteristic relaxation time \( t_c \equiv 1/(k_{\text{on}} + k_{\text{off}}) \).

Equation 2 was fit to the average over all motors for each experimental relaxation (shown in Fig. 2A), with the exception of release from stall for \( \gamma_{1300} \), wherein the number of stators is not notably affected by motor stall. The resulting rate constants and characteristic relaxation times are shown in Fig. 2B. In accordance with earlier work, we observe that \( t_c \) is dependent on load and thus on the torque delivered by a single stator unit: The smaller the single stator unit torque, the faster the relaxation. Unexpectedly, we observed a new feature that becomes apparent only when the system is prepared into different starting stoichiometries: The time scale to approach steady state is asymmetric; resurrection traces are faster than release from stall traces. We note that the resurrection behavior we measure is a convolution of two processes: recovery of the PMF following removal of the ionophore and assembly of the stator units. We assume the former is rapid compared to the latter. This is supported by the fact that transient perturbations to the PMF, which are brief enough to avoid stator disassembly, show rapid recovery dynamics (7). Moreover, sodium motive force (SMF) resurrections of the Na\(^+\)-driven hybrid motor of *E. coli* have shown that the speed contribution of the first stators to bind is equal to that of the last to bind, suggesting that the SMF recovers before incorporation of the first stator (8). In our experiments, if the PMF were to recover more slowly than assumed, then we would expect the relaxation time of resurrection to be longer than that of relax from stall; this is the opposite of what we observe.

This asymmetry (dependence of \( t_c \) on the starting stoichiometry) is not compatible with the previously proposed Hill-Langmuir adsorption model and opens the door to understanding the mechanisms that control the feedback between load and stoichiometry dynamics. Consequently, we proceeded by interrogating various potential mechanisms in search of the source of this observed asymmetry.

![Fig. 2. Stator unit stoichiometry, rates, and characteristic times for release from stall and resurrection experiments.](image)

**Fig. 2. Stator unit stoichiometry, rates, and characteristic times for release from stall and resurrection experiments.** (A) Evolution of stator stoichiometry for release from stall (light blue) and resurrection (purple). Viscous loads, from the top to bottom are \( \gamma_{1300}, \gamma_{500}, \) and \( \gamma_{300} \). Light blue and purple lines show the average of multiple traces, i.e., \( \langle N \rangle(t) \), shading shows SD, and the orange line shows \( \langle N \rangle(t) \) of steady-state measurements. The black line shows the best fit of the Hill-Langmuir model (Eq. 2) to each time series (with the exception of release from stall for \( \gamma_{1300} \)). (B) The rates (top) and characteristic times (bottom) as a function of single stator torque at steady state, \( \tau_{ss} \). Error bars on \( \tau_{ss} \) are the SD of all measurements. Error bars on rates and \( t_c \) represent the 90% high-density interval from ABC inference.
Neither depletion nor cooperativity explains the observed asymmetry

We investigated whether either next-neighbor unit-unit interactions or local depletion of unbound stator units surrounding the motor could explain the observed asymmetry in relaxation time. We first used a grand canonical Hamiltonian description of a one-dimensional short-range lattice gas (SRLG) with periodic boundary conditions to rigorously explore the effects of stator cooperativity (see the Supplementary Materials for more details). Our Glauber Monte Carlo simulations based on this model show that the characteristic relaxation time increases quickly with the (dimensionless) unit-unit interaction energy $\beta f \geq 0$ (where $f$ indicates the stator-stator coupling, $\beta = (k_BT)^{-1}$, with the Boltzmann constant $k_B$ and $T$ the temperature), and the correlation length, the characteristic distance over which the occupancy of one binding site influences that of its neighbors, grows as well (fig. S1) (9–11). Furthermore, the characteristic relaxation time for positive values of $\beta f$ depends on the average occupancy at steady state ($\langle N \rangle$). Note that when the (dimensionless) interaction energy $\beta f$ equals zero, the SRLG is equivalent to the Hill-Langmuir adsorption model, and the following relation between the effective (dimensionless) chemical potential of the reservoir, $\mu' = \mu_\gamma - \beta \epsilon$, and the Hill-Langmuir constant rates is satisfied: $\mu' = \log(k_{on}/k_{off})$. Here, $\mu_\gamma$ is the chemical potential of the reservoir and $\epsilon$ is the binding strength.

Simulation results do exhibit different relaxation times to steady state between release from stall and resurrection, $t^{rel}$ and $t^{res}$, respectively. The cooperativity-induced time asymmetry cannot, however, reasonably explain the experimentally measured ones, although the former increases with the nearest-neighbor (dimensionless) interaction energy, $\beta f$. For biologically reasonable values of the coupling constant, $\beta f \sim 0$ to 5, the simulations predict too small a difference between the relaxation times when compared with the experimental data. Figure S2 shows that for relatively high values of the interaction, $\beta f = 5$, cooperativity predicts a relative difference in the relaxation times

$$\Delta \equiv \frac{(t^{rel} - t^{res})}{(t^{rel} + t^{res})}$$

of 20% at most. A simple extrapolation from these results shows that we would need biologically unreasonable values, $f \sim 19 k_BT$, to obtain values comparable with the experimental ones ($\sim 63\%$ for $\gamma_{300}$ and $\sim 82\%$ for $\gamma_{300}$; see Fig. 2B).

Second, taking inspiration from a recent study of depletion effects (12), we constrained the SRLG to interact with a finite reservoir of stator units. We introduced a new parameter, the number of available stator units per cell $n_{tot}$, thus fixing the total number of stator units in the system (see the Supplementary Materials for more details). The steady-state occupancy increases with $n_{tot}$ and tends asymptotically from below to the value of the SRLG model without depletion (fig. S3). Simulations conducted under high depletion ($n_{tot} = N_{max} = 13$) predict that release processes relax more rapidly than resurrection ones (fig. S1), in evident contrast with the experimental results.

In conclusion, all reasonable interpretations of the above findings suggest that neither interactions between neighboring bound stator units nor finite reservoir effects are directly responsible for the observed asymmetry of relaxation times. Nevertheless, cooperativity and depletion sensibly affect both the steady-state occupancy and the relaxation time. Thus, further theoretical and experimental investigations are needed to ascertain their role in the stator recruitment mechanism.

**A model incorporating speed- and torque-dependent rates**

Wadhwa et al. (6) have proposed a model of stator assembly dynamics, which uses a statistical physics approach, explicitly incorporating the dependence of the rates of the motor speed and thus stator stoichiometry (see Fig. 3B). In this model, which we henceforth refer to as the “speed-rate” model, the binding of a single stator unit to the rotor changes its free energy $\epsilon(\omega) - \mu_\gamma$, where we recall that $\mu_\gamma$ denotes the chemical potential of the reservoir, and here, the binding energy $\epsilon(\omega)$ is dependent on the torque produced by the stator. In line with our previous model (5), they hypothesize that torque production lowers the free energy difference depending on the torque, which leads to the following rate ratio (6)

$$\frac{k_{on}}{k_{off}} = e^{\beta(\mu_\gamma - \epsilon(\omega))}$$

For this general starting point, Wadhw et al. found that their data are best fit by a model in which $k_{off}$ is torque dependent and $k_{on}$ is speed dependent, and to satisfy Eq. 4, $k_{off}$ must also incorporate the same speed dependence. The rates are thus

$$k_{on} = \kappa(1 - e^{-\kappa/\omega}), \quad k_{off} = \kappa(1 - e^{-\kappa/\omega}) e^{\beta(\tau(\omega) - \mu_\gamma)}$$

where $\kappa_0$ and $\kappa$ are constants, and $\omega$ is the rotation speed of the motor. Because the speed of the motor is proportional to its stoichiometry for a given value of the load ($\gamma$) (13,14), the rates of Eq. 5 can also be written in terms of the stoichiometry $\kappa/\omega \equiv \alpha_t/N$, where $\alpha_t$ is a multiplicative constant dependent on the load. This allows us to proceed similarly to the Hill-Langmuir model Eq. 2 and write an ordinary differential equation (ODE) for the evolution of the average stoichiometry

$$\frac{d(N)}{d\tau} = \langle k_{on}(N) (N_{max} - N) - k_{off}(N) N \rangle$$

$$= \kappa_0(\langle 1 - e^{-\alpha_t/N} \rangle (N_{max} - N) e^{\beta(\tau(\omega) - \mu_\gamma)} - 1^{N})$$

Given the nonlinearity of the rates of Eq. 5 with the stoichiometry, the speed-rate model does not give a closed analytical differential equation for the evolution of the average stoichiometry $\langle N \rangle$, as proposed previously (6), since $\langle f(N) \rangle \neq f(\langle N \rangle)$ for any nonlinear function $f(\cdot)$. As a consequence, calculating the average stoichiometry trajectories for any given set of parameters requires us to computationally solve the corresponding master equation (see the Supplementary Materials for more details). To infer the optimal parameters of the speed-rate model given the experimental data, we use approximate Bayesian computation (ABC) (15), comparing the resulting average stoichiometry dynamics of the model with the experiments (see the Supplementary Materials). The results are shown in Fig. 3A, and the parameters are given in table S3 and fig. S5. The use of a Bayesian framework not only allows us to perform a global search, reporting the range and correlations of the parameters compatible with the experimental data, but also allows for quantitative comparison of the credibility of various theoretical models (see the Supplementary Materials).
**A two-state catch-bond model**

We also consider an alternative kinetic model based on previous models of two-state catch bonds (16–18). Our previous work observed that the lifetime of the stator in the motor complex increases as the stator applies more torque upon the rotor, and by reaction, as the stator pulls with higher force on the PG (5). This behavior is characteristic of a catch bond. A widely applied phenomenological model to describe catch-bond behavior is a two-state model in which the thermodynamic stability between two bound conformational states is mediated by force (16, 19). So, we propose a model, depicted in Fig. 3B, in which the stator can bind to the PG with low affinity or high affinity, mechanical force regulates the transition between these conformational states, and the stator applies torque to the rotor in both bound states. For simplicity, and to minimize the number of free parameters, we assume that the energy barrier between the high affinity (strong, s) and unbound (u) state is sufficiently high, such that the stator can only transit to and from the unbound state from the low affinity (weak, w) state.

The number of weakly bound stators, w(t), and strongly bound stators, s(t), follows inherently stochastic dynamics. Similarly to the Hill-Langmuir model (see Eq. 2), we can write the master equation for the stochastic process that allows us to obtain a pair of ODEs describing the time evolution of the expected values \( \langle w \rangle(t) \) and \( \langle s \rangle(t) \)

\[
\frac{d\langle w \rangle}{dt} = k_{sw} \langle N_{\text{max}} \rangle - \langle w \rangle - \langle s \rangle - (k_{wu} + k_{ws1}) \langle w \rangle + k_{sw} \langle s \rangle
\]

\[
\frac{d\langle s \rangle}{dt} = k_{ws} \langle w \rangle - k_{sw} \langle s \rangle
\]

that can be solved to obtain an analytical expression for the stator stoichiometry in time

\[
\langle N \rangle(t) = D_s e^{\lambda_s t} + D_w e^{\lambda_w t} + \langle N_{\text{in}} \rangle
\]

where prefactors \( D_s \) depend on the rates and the initial stoichiometry condition, while the steady-state \( \langle N_{\text{in}} \rangle \) and the relaxation rates \( \lambda_{\pm} \) only depend on the stator rates \( k \) (derivation and explicit expressions in the Supplementary Materials). Note that, in contrast to the Hill-Langmuir dynamics (Eq. 2), the two-state model naturally predicts two different relaxation time scales in line with the experimental observations.

We hypothesized that the dependence on torque appears in the rates of exiting the intermediate weak state, and we henceforth refer to this as the “two-state catch-bond” model. Nevertheless, to generally and more extensively explore two-state models, we also examined cases in which rates exiting the strong state, and binding rates are also allowed to depend on torque. As in the speed-rate model, we used ABC to infer distributions of parameters compatible with the observed experimental trajectories, with the fit shown in Fig. 3A, parameters listed in table S3, and the posterior parameter distributions shown in figs. S6 and S7.

**Model comparison**

Despite both dynamical models—the speed-rate model and the two-state catch-bond model—being able to reproduce relaxation asymmetry, the two-state catch-bond model returned average relaxation asymmetries comparable to the experimental ones (Fig. 3A), resulting in trajectories that better fit the data (fig. S4). This result was anticipated since the two-state catch-bond model involves a larger set of parameters (8) compared to the speed-rate model (5). To assess whether we can select one model over the other, we calculated the Bayes factor comparing the posterior marginal probabilities of each model by making use of the ABC inferred distributions (20).
that naturally incorporate a penalty associated with the dimensionality of the models (see Materials and Methods and the Supplementary Materials). Results show that, despite having more parameters, the fit of the two-state model is good enough to have the highest Bayes factor (see Fig. 3C), although the difference over the speed-rate model is small enough to be considered anecdotal (Bayes factor <3). On the other hand, the difference over other variations of the two-state model (10 and 12 parameter models) is high enough to select the eight-parameter two-state catch-bond model shown in Fig. 3B as the most credible two-state model, although the fitting was better for the high-dimensional extended and general two-state models (see table S3).

**DISCUSSION**

We measured the torque of individual BFMs and calculated the temporal evolution of stator stoichiometry under three sequential conditions: at steady state, after motor stall and subsequent stator recruitment, and during resurrection starting from an empty rotor. These experiments were performed for three viscous loads. We observed that, on average, the relaxation time to steady state is faster for smaller viscous loads, in agreement with our previous work (5). However, we also observed a subtle but unexpected effect that, for each viscous load tested, the relaxation time from "below" (during resurrection) was faster than from "above" (release from stall). This effect is not predicted by our previous simple Hill-Langmuir reversible adsorption model of stator dynamics (5).

We have theoretically explored the effects of interactions between neighboring adsorbed stator units and finite stator unit reservoir effects, and we confirm that neither of these mechanisms can account for the degree of relaxation time asymmetry observed. Such effects may, nonetheless, prove relevant to the description of stator assembly dynamics and are worthy of further exploration.

On the basis of previous studies that show that stators dissociate from the motor upon ion motive force (IMF) collapse within ~1 min, we have assumed that after 8 min of ionophore-induced PMF collapse, all stator units have dissociated from the rotor and the system is at steady-state zero occupancy (7, 8, 21). This is supported by recent work that estimates the difference in effective free energy of bound and unbound stator units and concludes that the binding of stator units to the motor at zero torque is unfavorable (6).

A recent model of stator dynamics, depicted in Fig. 3B, described by Eqs. 5 and 6, and referred to here as the speed-rate model, proposes that the rate of stator assembly depends nonlinearly on both the rotor speed and the torque generated per stator unit (6). Because of the speed dependency, this model predicts that, when perturbed from steady-state stator stoichiometry, the relaxation time depends on the direction of perturbation. Our data exhibit this perturbation direction–dependent asymmetry in $t_{c}$ (Fig. 2). Nevertheless, as shown in Fig. 3A and fig. S4, a global fit of this model to our entire dataset is still not capable of recapitulating the observed differences in time scales and the two-state catch-bond model.

The speed-rate model posits that, when a stator unit binds to the motor, its free energy changes by an amount dependent on the torque produced by that unit. Figure 3D shows the resulting change in free energy as a function of single stator torque, with a trend that agrees with Wadhwa et al. (6). Figure 3E shows the speed-dependent decrease in $k_{on}$, $k_{off}$, and $1/t_{c}$, with the confidence intervals given by ABC. Over the physiological range of speed for *E. coli*, this model would suggest that, as the rotor speeds up, the rate of stator unit binding decreases by about a factor of three. As Wadhwa et al. explain (6), an interaction between the stator unit and the FliG protein of the rotor, triggering a conformational change in the periplasmic region of the stator complex that enables PG binding (22–24), provides a plausible explanation for the rotor speed–dependent rate of stator binding. However, the exact mechanism and the specific role of stator-rotor interactions have yet to be illuminated [for a review, see (25)]. Curiously, this model also requires that the rate of stator unbinding decreases with increasing motor speed, by the same factor of about three. Speed-dependent disassembly is a phenomenon for which we have yet to find a compelling mechanistic explanation.

Our previous work has shown that the stator units have lower rates of unbinding for increased torque upon the rotor and thus increased force upon the PG, a behavior characteristic of a catch bond (5). Single-molecule force spectroscopy experiments have now identified a wide range of biological catch bonds, particularly among proteins with an adhesive or mechanosensory role (26, 27). While many of the underlying mechanisms that govern this behavior remain to be elucidated, a number of phenomenological and microscopic theories have been proposed (19). One of the most successful models to date is a two-state model (16, 17), which can quantitatively explain the experimental results for an impressive range of biological catch bonds, including P-selectin (16, 28), the bacterial FimH adhesive protein (18, 29), kinetochore-microtubule interactions (30), cadherin-catenin interactions (31), cell surface sulfatase and glycosaminoglycan interactions (32), vinculin-actin interactions (33), and platelet–von Willebrand factor binding (34).

The two-state catch-bond model (depicted in Fig. 3B) proposes that the stator complex has two bound and torque–producing states, a weak and strong PG affinity state, and that the conversion between these states is force dependent, with high force favoring the putative high-affinity state. Mechanical forces on the stator complex could act directly upon the PG binding domain, for example, exposing PG binding sites, in a manner similar to other mechanism-sensitive proteins (27). Alternatively, tensile forces could act allosterically via the intrinsically disordered region, which links the inner membrane domain to the PG domain (35, 36). Both experiments and simulations have suggested that allosterics plays a role in a number of biological catch bonds, wherein mechanical stress at the allosteric site is propagated along the protein to invoke rearrangements at the binding pocket (37, 27). We speculate that extension of the disordered and flexible interdomain region of MotB could similarly regulate stator-PG affinity. As seen in Fig. 3A, a global fit of this model to the experimental data produces a very good fit, reproducing the experimentally observed asymmetry in $t_{c}$. The relaxation time asymmetry would arise because, during resurrection, a stator must merely transition from unbound to weakly bound to begin applying torque, whereas during relaxation from stall, stators that are in the strongly bound state must pass through the weakly bound state and then unbind to stop producing torque. As depicted in the energy landscape schematic of Fig. 3G (see also table S3 and figs. S6 to S8), Bayesian inference in a general two-state model suggests that it is the transition out of the intermediate weak state that is torque dependent. Increasing torque reduces the barrier between the weak and strong states, in a manner consistent with traditional slip bond behavior. While it is known that the stator must undergo a large conformational change to bind to the PG (38, 36), we note that, to
date, there is no evidence for two different PG-bound conformations, and this model remains hypothetical. In any case, the success of the two-state model may point toward a more continuous (rather than bistable) or complex conformational and energy landscape for the stator.

Thus, we find that the two-state catch-bond model fits our experimental data more accurately than the speed-rate model. Nevertheless, since the catch-bond model requires a larger dimensional parameter set, we compared the likelihood of both models. While the two-state catch-bond model shows the highest posterior credibility, the difference is insufficient to exclude the speed-rate model.

Neither model offers a specific structural explanation for the catch-bond behavior. The high-resolution structures of the stator complex have recently been resolved via cryo–electron microscopy (cryo-EM) (39, 40), opening the door to future structure-based models. Simulations may yield atomistic insights regarding the effects of force-induced structural changes on stator assembly. For example, steered molecular dynamics (SMD) simulations, seeded with the static cryo-EM structure, were used to predict force-induced allosteric structural changes in FimH (41), which were later confirmed by single-molecule atomic force microscopy experiments (42). One current limitation to such an approach is that, because of the short time scales accessible, SMD simulations require forces and loading rates much higher than those used in experiments to observe bond rupture or allosteric change (19).

In a more recent set of electrorotation experiments, Wadhwa et al. (43) revert back to the Hill-Langmuir model (5) to fit their data and find that the extracted rate constants show speed dependence in $k_{on}$, an effect which may be compatible with one of the two models described above. Recent electrorotation experiments by Ito et al. (12) also suggest a speed dependence in $k_{on}$, albeit in the opposite direction: Ito et al. observe that stator binding is enhanced by rotor speed, although only at low stoichiometry, potentially only applicable to the first stator unit to bind. While we sometimes observe long dwells at zero stoichiometry, potentially compatible with their observations, given our uncertainty in the (short) time needed to restore the PMF during our resurrection experiments, we have not attempted to quantify this effect. We nonetheless hypothesize that this observation, instead of arising from a rotor speed dependence in $k_{on}$ may be equally well explained by the two-state catch-bond model, wherein the transition to the strongly bound state is dependent on the force across the arriving stator unit, which remains at zero until the rotor begins to rotate. We also note that such an effect could arise from the first stator unit needing to recruit a putative partner [for example, FilL (44)] or if the torque from a single stator unit were insufficient to maintain motor rotation (45).

Last, we note that there is evidence for IMF-dependent stator assembly (46, 7, 47, 48) (as also shown by our resurrection experiments), suggesting that stators sense not only the mechanical environment but also the electrochemical environment. While this phenomena has yet to be robustly characterized, a successful model of stator assembly dynamics should also be able to recapitulate this effect. A catch-bond mechanism within the stator would allow it to stabilize attachment to the PG exactly when it is needed, provide resistance to large mechanical stresses, and destabilize attachment when it is no longer needed, allowing reconfiguration under small stresses, thereby conserving the PMF. This mechanism may be consistent with IMF-dependent assembly, although future experiments are needed to quantify the dependence. Future structural models may yield testable predictions linking stator structure and assembly and may even allow the bond to be engineered. Interfering with the catch-bond behavior of the stator could have grand implications for infection, biofilm formation, and disease.

**MATERIALS AND METHODS**

**Bacteria and growth**

We used *E. coli* strain MTB32, wherein the flagella have been genetically deleted and the hook is biotinylated (49). We genetically deleted cheY (5), the chemotactic response regulator. Frozen aliquots of cells (grown to saturation and stored in 25% glycerol at −80°C) were grown in Terrific Broth (Sigma-Aldrich) at 33°C for 5 hours, shaking at 200 rpm, to a final optical density at 600 nm of 0.5 to 0.6, then washed and resuspended in motility buffer [10 mM potassium phosphate, 0.1 mM EDTA, and 10 mM lactic acid (pH 7.0)]. Cells were immobilized to a poly-L-lysine (Sigma-Aldrich, P4707) coated coverslip in a custom flowslide with a parafilm spacer. Streptavidin superparamagnetic beads (1.36 μm, Sigma-Aldrich; 543 or 302 nm, Adamtech) were washed in phosphate-buffered saline, resuspended in motility buffer, and then allowed to attach to the BFM hooks.

**BFM experimental measurements**

Experiments were performed in motility buffer at 22°C. The sample was illuminated with a 660-nm light-emitting diode on a custom inverted microscope and imaged with a 100× 1.45–numerical aperture objective (Nikon) onto a complementary metal-oxide semiconductor camera (Optronics, CL600x2/M) at a frame rate of 1 kHz. Two permanent magnets were positioned above the sample at a distance that was controlled by a motorized vertical translation stage.

For a given motor, steady-state rotation was measured with the magnets far from the sample (8 min), the magnets were lowered to within ~1 mm of the sample to stall motor rotation (10 min), the magnets were raised and the motors were allowed to relax to steady state (11 min), the ionophore carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP; 20 μM) was added to the media to collapse the PMF and dissociate the stators (8 min), then CCCP was washed out with a volume of MB that was six times that of the microfluidic channel to ensure complete buffer exchange, and cells were allowed to resurrect (11 min). In some measurements of the smaller beads, torque from the magnetic tweezers was insufficient to hold the motor stalled for the entire 10 min. While this effect likely reduces the number of stators recruited during stall, it does not affect the relaxation after stall. In approximately 20% of cases, motors either failed to resurrect after treatment with CCCP or resurrected to less than 40% the steady-state speed, in these cases, the entire recording for that motor was discarded. This cycle was repeated multiple (five to eight) times per flowslide, such that the majority of measurements are from motors that had already been submitted to CCCP, and we noted no deleterious effect with multiple CCCP exposures.

**Data analysis**

The $x$, $y$ position of the bead was determined via image cross-correlation analysis (50), and the angular position of the bead was calculated as $\theta = \arctan(y/x)$. The rotational viscous drag coefficient of the bead was calculated as (51)

$$
\gamma = \frac{8\pi \eta r_b^3}{1 - (1/8) (r_b/d)^3} + \frac{6\pi \eta r_v^2 r_b}{1 - (9/16) (r_b/d) + (1/8) (r_b/d)^3}
$$

(8)
where $r_b$ is the bead radius, $r_c$ is the measured radial distance to the bead’s axis of rotation, and $d$ is the distance from the bead to the cell surface, estimated to be 5 nm. Motor torque was calculated as $\tau = \gamma_0$, where $\omega$ is the rotational speed of the motor. Stator stoichiometry and single stator torque values were calculated as described previously (5). All analysis was performed with custom LabView, MATLAB, and Python scripts.

Model simulation
Mean trajectories $\langle N(t, N_0) \rangle$ for an initial motor configuration $N_0$ for the Hill-Langmuir model and the two-state model were obtained using their analytical expressions (Eqs. 2 and 7). Trajectories for the speed-rate model were obtained by solving the corresponding master equation consisting of a system of $N_{\text{max}}$ linear ODEs by diagonalizing the resulting rate matrix (see the Supplementary Materials).

The initial condition for resurrection trajectories was set to correspond with an empty motor ($N_0 = 0$), while for stall trajectories, the steady-state stoichiometry was used ($N_0 = \langle N_0 \rangle$). To reproduce the observable variability of initial conditions during release trajectories, the average trajectory $\langle N(t) \rangle$ was obtained by calculating the average over all the initial conditions $N_0^\ell$ for each observed experimental motor $\ell = 1, \ldots, L$

$$\langle N(t) \rangle = \frac{1}{L} \sum_{\ell=1}^L \langle N(t, N_0^\ell) \rangle$$

Last, for the two-state model, release trajectories require inference of the initial values of weakly and strongly bound motors $w_0$ and $s_0$ for each experimental observed value of $N_0$. This relationship was obtained by assuming that the initial configurations in the release experiment are the steady-state probability distribution of occupancy when stator detachment is forbidden $k_{wu} = 0$ (see the Supplementary Materials).

Parameter inference and model selection
To infer sets of parameters able to reproduce the experimental data, we made use of ABC. The outcome of this analysis returns distributions of credibility for the ensemble of parameters and models with which we obtain intervals and correlations between parameters and a means of model comparison. The inference was performed using sequential Monte Carlo (SMC) to obtain distributions for the credibility $P(\theta_m | d(\theta_m) < \epsilon)$ for the parameter set $\theta_m$ for each model $m$ such that the score $d$ of a given parameter set is below a certain threshold $\epsilon$ (see the Supplementary Materials). The score function $d(\theta)$ was defined as the distance between the experimental trajectories $\langle N_{\exp}(c, t) \rangle$ for a given experimental setup $c = \{\text{resurrection, steady state, release}\}$ and the corresponding predicted trajectories $\langle N_{\text{theo}}(\theta_m, t, c) \rangle$

$$d(\theta_m) = \sum_c \sum_t \left( \langle N_{\exp}(c, t) \rangle - \langle N_{\text{theo}}(\theta_m, t, c) \rangle \right)^2$$

where the subindex $i$ runs for all experimental time points $t_i$ that include information of at least three individual motors with nonzero speed and a time step of $\Delta t \equiv t_{i+1} - t_i = 1$s. The Bayes factor was calculated by resampling the ABC-SMC (20) with a distance threshold 10% above the minimal threshold for the speed-rate model and using a Gaussian kernel with covariance equal to the covariance of the original sample. All fittings and SMC simulations were performed with custom Python scripts that can be found at https://doi.org/10.5281/zenodo.5784548.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at https://science.org/doi/10.1126/sciadv.ab8112

View/request a protocol for this paper from Bio-protocol.

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