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Fast state detection in F₁-ATPase rotation enhanced by theory of mixed states and external torque

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Abstract

During brief 120° transitions between long catalytic dwells, single F₁-ATPase molecules exhibit angular jumps that vary with rotation angles. Using the angular jump profile enables the detection of fast states in the mechano-chemical scheme of the enzyme, states that are difficult to capture from single-molecule trajectories due to the fluctuations of the imaging nanoprobe. In a previous work, a short-lived, three occupancy state was postulated from a multi-state, probabilistic theory to explain the mean angular jump profile. An assumption in the theory was that the ‘mixing’ of chemical states is negligible during jumps. In a mixing event, two subsequent angular positions recorded by the imaging apparatus belong to two different chemical states of the motor enzyme due to fast reactions within a recording frame. In this paper, we provide an enhanced method for the detection of fast states. On one hand, we show using Langevin simulations that state mixing leads to faster mean angular jump, shifting up the profile. Consequently, the improved method provides a correction to the angular position and lifetime of the postulated three-occupancy metastable state. On the other hand, we show that when F₁-ATPase is subject to torques opposing rotation in hydrolysis direction, the torques shift down the dwell angles without affecting the angle-dependent reaction rates. The torques improve detection capability for the fast state by increasing dwell times which is made evident by the flattening of the mean angular jump profile within 40°–60° from the catalytic dwell. In the three-occupancy state release of ADP occurs in concert with the binding of ATP to a different site in the F₁-ATPase. Similarly, in the full ATP synthase when torques are created by the proton gradient in the FO region, the release of the product ATP is presumably accelerated by the binding of ADP to a different site in the F₁ domain.

1. Introduction

Single molecule studies of F₁-ATPase, a water-soluble part of the ATP synthase, reveal the intricate relation between reaction rates and rotation of γ shaft which is indirectly observed via probes [1, 2]. The kinetic coupling scheme of chemistry and mechanics in the ATP-hydrolysis fueled rotation of F₁-ATPase has been suggested to be the reverse of the kinetics governing the synthesis of ATP by ATP synthase [3]. A revolution consists of three cycles, each associated with a 120° rotation, a behaviour which is closely related to pseudo-threefold symmetry of the α₃β₃ pockets in F₁-ATPase. Accordingly, the rotation trajectories of the thermophilic bacillus F₁-ATPase at millimolar ATP concentration comprise of long catalytic dwells separated by fast 120° transitions. A 120° transition occurs in two subsequent substeps of 40° and 80° induced by Pi release and ATP binding, respectively [4, 5].

When F₁-ATPase spontaneously rotates in the hydrolysis direction and an optical nano-probe is attached to the rotor shaft, a high time resolution camera can capture the probe rotation during the transitions [6, 7]. The single-molecule recording yields discrete rotation angle versus time trajectories, such as the one
Figure 1. (a) Illustration of a 120° transition between two long dwells in a single-molecule imaging trajectory. F1-ATPase undergoes transitions across 4 states: (1) pre-Pi release (purple), (2) pre-ATP binding (blue), (2∗) metastable state (magenta) and (0,3) pre-catalysis state (orange). (b) Structure of F1-ATPase [10–12] with gold nanobead attached (side view above, top view below) and the four-state kinetic scheme showing the evolution of the 3 active β subunits in F1-ATPase (circles) and the position of the central rotor shaft (yellow arrow). States 0 and 3 are the long dwells, sandwiched between them is the transition of interest. The dashed boxes in (a) contain adjacent data points, where state mixing occurred during a recording frame $\Delta t$: the system has undergone a switch from one state to the next state, and the switch may have been detectable, if higher time resolution $dt$ was used for observation. In experiment, each data point (circles) is an average from all movements during $\Delta t$.

illustrated in figure 1(a), in which the angular position jumps according to the fluctuations picked up by the probe. Analysis of brief transitions in single-molecule trajectories has revealed that the mean angular jump varies with rotation angle [8]. One explanation suggests that a non-constant angular profile of the jumps arises from an intricate combination between fast, angular dependent reaction rates, and a slow, visco-elastic response of the nano probe, ultimately causing the overlap of multiple chemical states at the same angle [9]. A theoretical angular jump profile, constructed in our previous work, yields a simple expression for the jumps and leads to the discovery of a short-lived state with triple nucleotide occupancy. The approach assumes that states do not ‘mix’ during jumps, i.e. that there is no change in the chemical state within an experimental imaging time step of $\Delta t = 10 \mu s$ (camera time resolution).

In the current paper, we consider two effects. On the one hand, better detection of the lifetime and rotation angle of the triply occupied meta-stable state is done by taking into account the mixing of states in each time step. In a time step, the imaging apparatus collects (‘bins’) photons scattered by the probe, and so the state mixing is similar to ‘time binning’ effects ubiquitous in various single-molecule imaging experiments [13, 14]. The finite time step in single-molecule imaging also affects the apparent distribution of thermal fluctuations, thus the effective torsional stiffness of the rotor, when extracted from these fluctuations [15], requires correction. A systematic theoretical solution to these two effects is proposed, for the first time to the best of our knowledge, in the present paper.

On the other hand, movement of F1-ATPase under opposing torque is important because the natural function of the complete ATP synthase occurs under conditions of a torque acting in the clockwise (synthesis) direction. Efficient rotation of the F1-ATPase under constant external torque has been demonstrated in electro-rotation experiments [16, 17], in turn prompting the need to investigate the effect
Figure 2. (a) Illustration of the assay which observes rotation of F1-ATPase through the attached probe. (b) The free energy of each chemical state assumes harmonic potential where the bead experiences the elastic force via linker and may be subjected to external torque. (c) The free energy profile for the multi-state model with 4 states: (1) pre-Pi release (purple), (2) pre-ATP binding (blue), (2\textsuperscript{*}) metastable state (magenta) and (3) pre-catalysis state (orange). Transitions between chemical state are dictated by reaction rates depending on the bead’s rotation angle.

of external torque on the dwell times and on the angular jumps in the transitions. Arguably, the analysis of the angular jump profiles under torque is a necessary step towards understanding the high mechano-chemical efficiency in F1-ATPase [18].

To address the two effects, we use Langevin simulation, noting that the method has been previously successfully employed to model dynamics of F1-ATPase [19] and other biomolecular motor systems [20, 21], in particular for validating theoretical models [22]. The method enables tracking of chemical states and the probe’s angular position during the time evolution of F1-ATPase, as observed in imaging experiments. The method is used here for investigation of the chemical state mixing effect on the angular jump profiles.

2. Theory for single-molecule rotation jumps

2.1. F1-ATPase’s multi-state rotation model

During the ATP hydrolysis cycle, F1-ATPase undergoes a repetitive sequence of four chemical states comprising of a pre-Pi release state (i = 1), an empty (pre-ATP binding) state (i = 2), a three occupancy state resulting in ADP release (i = 2\textsuperscript{*}), and a state of pre-ATP hydrolysis (i = 3). In single-molecule experiments the rotation of F1-ATPase is observed indirectly via a probe (nanoparticle or bead) attached to the \(\gamma\) rotor shaft [23], as illustrated in figure 2(a). Due to the elastic nature of the linkage, in each state \(i\), the imaging probe fluctuates in a parabolic potential centered at a specific dwell angle \(\theta_i\), as depicted in figure 2(b). The probe is also subjected to viscous friction of the watery environment. The potential randomly switches to the next state when a chemical reaction occurs, according to a rate constant that depends on the angular position \(\theta\) of the probe, as illustrated in figure 2(c).

The bead undergoes torsional Brownian fluctuations [24], while it is elastically connected to F1-ATPase’s \(\gamma\) shaft. The quantities \(\gamma\), \(\kappa_r\), and \(\theta_i\) are the bead’s viscous friction, the elastic rotational (torsional) spring constant and dwell angle for chemical state \(i\), respectively. The bead’s angular relaxation time in the harmonic potential from figure 2(b) is \(\tau = \gamma / \kappa_r\). Then, the rotation monitored with the bead under constant, opposing torque \(N > 0\) is described by an overdamped Langevin equation,

\[
\frac{d\theta}{dt} = -\kappa_r (\theta - \theta_i) - N + \eta. \tag{1}
\]

The negative sign of \(N\) represents its opposition to spontaneous rotation of the bead in the ATP hydrolysis direction (when ATP is in the solution), as illustrated in figure 2(a). Clearly, torque-free rotation is
recovered when \( N = 0 \). The bead’s motion is subjected to white noise with properties \( \langle \eta(t) \rangle = 0 \) and \( \langle \eta(t) \eta(t') \rangle = 2\gamma k_B T \delta(t - t') \).

Interplay between chemistry and mechanics of F\(_1\)-ATPase manifests in angle-dependent reaction rates seen in stalling and controlled rotation experiments [25, 26]. In these experiments, the bead is either stalled or rotated by magnetic tweezers at a significantly slow speed compared to the chemical reaction transition times in the F\(_1\)-ATPase active subunits. The chemical transitions occur so fast that the bead remains quasi-stationary, with virtually no change in its angular position, while the system crosses the energy barrier of the transition state [27]. In free rotation (without magnetic tweezers), there is enough viscous load on the bead that it is assumed to rotate in a similar quasi-static manner [9]. A free-energy model of molecular transfer yields exponential angle-dependent forward rate constants and backward rate constants [28],

\[
\begin{align*}
    k_{fi}(\theta) &= k_{0i}^f \exp \left[ a_{fi} \left( \theta - \frac{\theta_i + \theta_{i+1}}{2} \right) \right], \\
    k_{bi}(\theta) &= k_{0i}^b \exp \left[ -a_{bi} \left( \theta - \frac{\theta_i + \theta_{i+1}}{2} \right) \right].
\end{align*}
\]

In the model, the exponential coefficients were given [28] as \( a_{fi} = \beta \alpha_i \kappa_i s_i \) and \( a_{bi} = \beta(1 - \alpha_i) \kappa_i s_i \), where \( \beta = (k_B T)^{-1} \), \( s_i = (\theta_{i+1} - \theta_i) \) is the step size, \( \alpha_i \) is the Brønsted slope and \( \kappa_i \) is the spring constant for the elastic coupling between rotation and \( \alpha \beta \) pockets where chemistry occurs in the reaction from state \( i \) to state \( i + 1 \). We will show in a later section that these exponential rate constant expressions, while originally derived for stalling and torque-free experiments, are unaffected by the presence of the external torque \( N \) in electro-rotation experiments.

### 2.2. Angular jumps

#### 2.2.1. Jumps in a Langevin description

Jumps that involve a change of state leading to mixing of states are automatically included in the analysis when using equation (1). To solve this equation computationally, the angle \( \theta \) of the bead is updated following a standard integration scheme [29] described in appendix A. In addition, the angle-dependent rate constants dictate the switching between the potentials in state \( i \) and \( i \pm 1 \). Since chemical reactions occur instantaneously, the dwell angle \( \theta_i \) of the chemical state \( i \) can change during each integration step according to the multi-state model scheme in figure 2(c). For trajectories from both single-molecule experiments and Langevin simulations the angular jump is calculated, for a given time step \( \Delta t \), as

\[
\frac{\Delta \theta}{\Delta t} = \frac{\theta(t + \Delta t) - \theta(t)}{\Delta t}.
\]

The mean angular jump at each angle \( \langle \Delta \theta / \Delta t \rangle |_{\theta(t)} \) is calculated by averaging over many trajectories, according to the procedure used to extract mean angular jump from experimental data [9, 30].

#### 2.2.2. Jumps in a Fokker–Planck description

In a description previously for F\(_1\)-ATPase rotation experiments with no external torque, \( \rho_i \) denoted the angle and time dependent probability distribution probability of the bead’s angle in state \( i \) [9]. During transition between long dwells, under physiologically relevant conditions, the F\(_1\)-ATPase forward rates are much faster than backward rates [1, 9, 23]. Then, if \( D = k_B T / \gamma \) is the rotational diffusion constant of the bead attached to the rotor shaft via the elastic linker, the rotary time evolution of the bead can be described by a Fokker–Planck equation,

\[
\frac{\partial \rho_i}{\partial t} = D\beta \frac{\partial}{\partial \theta} \left[ \kappa_i (\theta - \theta_i) \rho_i + N(\theta) \rho_i \right] + D \frac{\partial^2 \rho_i}{\partial \theta^2} - k_{fi}(\theta) \rho_i + k_{bi}(\theta) \rho_{i-1}.
\]

At millimolar ATP concentrations the catalytic dwells last much longer than transitions, so we assume that \( k_{i,0} \approx k_{i,1} \approx 0 \). The probability of the F\(_1\)-ATPase being in state \( i \) at rotation angle \( \theta \) is expressed via an angle-dependent probability \( P_i(\theta) \) as

\[
P_i(\theta) = \int_0^T dt \rho_i(\theta, t) / \left( \sum_i \int_0^T dt \rho_i(\theta, t) \right).
\]

When state mixing events are neglected, there is a vanishing probability distribution of an angular jump \( \Delta \theta \) during which the system switches states, \( \rho_{i \pm 1}(\Delta \theta | \theta) \approx 0 \), then, the mean angular jump at an angle \( \theta \) is
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![Diagram](image)

**Figure 3.** Thermodynamic cycle of F1-ATPase under opposing torque \( N \) which reduces dwell angles by \( N/\kappa_r \). When the bead is located at angle \( \theta \), the free energies of F1-ATPase at chemical state \( i \) and \( i + 1 \) are denoted as \( G_i(\theta), G_{i+1}(\theta) \) respectively. \( E_i \) and \( E_{i+1} \) denote the elastic energy required to rotate the bead to angle \( \theta \) where chemical reaction occurs with angle dependent rate \( k_s(\theta) \).

Calculated by averaging over all chemical states \( i \) during an interval time step of \( \Delta t \) (imaging camera time resolution),

\[
\frac{1}{\Delta t} \sum_i (\theta - \bar{\theta}_i) (1 - e^{-\Delta t/\tau_f}) P_i(\theta).
\]  

(7)

2.3. Angle-dependent free energies and rate constants in the presence of torque

When F1-ATPase is in a dwell of chemical state \( i \), from equation (1) follows that an external torque opposing the rotation produces a shift in the dwell angle to \( \theta_i - N/\kappa_r \). Downshift of the dwell angle is observed in electro-rotation experiments, where dwell angles decrease by 40° when F1-ATPase is forced to rotate in the synthesis direction, compared to the force-free rotation in the hydrolysis direction [16]. Meanwhile, there is no change in the angular step size of 120° between two catalytic dwells since the same shifting applies to all states.

When F1-ATPase transitions from state \( i \) to \( i + 1 \) with free energy drop \( \Delta G_0^i \), it will spend a part of \( \Delta G_0^i \) to do work \( W = \frac{\pi}{\kappa_r} N (\theta_{i+1} - \theta_i) \) against the opposing torque, while the remaining free energy \( -\Delta G_0^i (N) = -\Delta G_0^i - W \) is used to drive the chemical reaction [31]. A thermodynamic cycle, similar to one previously used to treat angle dependent rates of ATP binding [28], can be used to investigate the effect of torque on reaction rates, as shown in figure 3. For a reaction occurring at an angle \( \theta \), thermal fluctuations from the surrounding environment provide contributions \( E_i \) and \( E_{i+1} \) to the reaction free energy. These energies are needed for the twisting of \( \gamma \)-shaft and other structural elements connecting the bead to the reaction site in order for the motor-bead system to be changed from the relaxed state in the dwell angles \( i \) and \( i + 1 \), respectively, to angle \( \theta \). The contributions of the twisting of the structure to the free energy are assumed to be harmonic functions with some rotational spring constant \( \kappa_c \); the mechanical rotation of the bead is harmonically coupled to the free energy by the mechanical-chemical ‘coupling’ constant \( \kappa_c \). The value of \( \kappa_c \) may be different from \( \kappa_r \), leading to the modified torque as \( \frac{\kappa_c}{\kappa_r} N \). The net elastic energy is the difference between energies \( E_{i+1} \) and \( E_i \) [28],

\[
E = E_{i+1} - E_i
= \int_{\theta_{i+1} - \frac{\pi}{\kappa_r}}^{\theta_i} \left[ \kappa_r (\theta' - \theta_{i+1}) + \frac{\kappa_c}{\kappa_r} N \right] d\theta' - \int_{\theta_i - \frac{\pi}{\kappa_r}}^{\theta_{i+1}} \left[ \kappa_r (\theta' - \theta_i) + \frac{\kappa_c}{\kappa_r} N \right] d\theta'
= -\kappa_r (\theta_{i+1} - \theta_i) (\theta - \frac{\theta_i + \theta_{i+1}}{2}) - \frac{\kappa_c}{\kappa_r} N (\theta_{i+1} - \theta_i)
\]  

(8)

The free energy driving the chemical reaction at angle \( \theta \) can be expressed as a function of \( \theta \), as

\[
\Delta G(\theta) = \Delta G_0(N) + E
= \Delta G_0^i - \kappa_r (\theta_{i+1} - \theta_i) (\theta - \frac{\theta_i + \theta_{i+1}}{2})
\]  

(9)

Noting that the free energy change going from state \( i \) to \( i + 1 \) is defined as \( \Delta G(\theta) = -k_B T \ln \left[ \frac{k_s(\theta)}{k_b(\theta)} \right] \), the forward and backward rate constants from equations (2) and (3) satisfy equation (9), thus obeying the principle of ‘local detailed balance’ (see, e.g. references [32–34]). Microscopic reversibility is satisfied for each transition between the ‘mesoscopic’ states in the kinetic scheme in figure 1(b). We also note that equation (9) yields a free energy difference of F1-ATPase which is
identical to the free energy under conditions in stalling experiments [28]. Therefore, the molecular group transfer theory, proposed originally for stalling experiment, can be applied to associate the standard free energy of reaction with the energy barrier of reaction [28], which determines the rate constants for both forward and backward reactions. From equation (9), these rate constants are identical to those from equations (2) and (3), namely, $k_f(N, \theta) = k_f(\theta)$ and $k_b(N, \theta) = k_b(\theta)$. In other words, we conclude that the rate constants have the same angle dependence in single-molecule experiments, whether they are constant torque, stalling, controlled rotation or free rotation experiments.

3. Results

3.1. Mean jump distribution as a function of rotation angle

The angle-dependent mean jump plotted on figure 4 was extracted from the transitions in the experimental trajectories, i.e. rotation angle versus time data series. On the figure we also show simulated jump profiles using three and four-state models, with the latter being clearly the better match to the experiment. We note that all quantities used for the three-state model simulations were extracted from independent experimental data, thus the three-state curve is a prediction with no adjustable parameters. The 4th state was postulated in order to explain the more flattened appearance of the profile in the $60^\circ–80^\circ$ range.

The simulation requires, as input, the relaxation time $\tau$ of the probe and elastic spring constant $\kappa$. These constants are extracted from the analysis of the fluctuations in the dwells. To do so, one needs to consider that in single-molecule imaging experiments the CCD camera effectively captures the angular position of the bead averaged over the interval time step $\Delta t$, which may bias quantities extracted from a trajectory. Our analysis in appendix B indicates that the bead’s relaxation time $\tau$, extracted from the angular time autocorrelation function $\langle \theta(t_0)\theta(t_0 + t) \rangle$, is not affected by the time step $\Delta t$. Meanwhile, the torsional stiffness $\kappa$, which is extracted from the angle histograms of the fluctuations in the dwells [15], requires multiplying by a correction factor which in equation (A13) is seen to depend on the ratio of the relaxation time and time step.

3.2. Mixing effects at long interval time step and comparison with experiment

The estimated lifetime and dwell angle of the postulated metastable state using the previous approach which neglected mixing of states when two adjacent data points belong to different chemical state of F$_1$-ATPase [9] require adjustment to include the state mixing effect. With these adjustments, the updated parameters in the model produce a very good match to experimental data, as depicted in figure 4. In particular, the present more accurate calculation shows somewhat longer lifetime of the postulated three-occupancy state ($14 \mu s$ compared to $12.5 \mu s$) and smaller dwell angle ($72^\circ$ compared to $76^\circ$), as summarized in table 1. The three-occupancy state is part of a kinetic scheme proposed to describe the behaviour of F$_1$-ATPase in stalling experiments, so this state is consistent with experimental data under the conditions of stalling experiments as well (appendix C).
Table 1. Physical quantities in the four-state model which takes into account of chemical state mixing. The values are based on [9] and references therein.

| State $i$ | Substep $i \rightarrow i + 1$ | $\theta_i$ (deg) | $k_i(\theta_i)$ (ms$^{-1}$) | $a_i$ (deg$^{-1}$) | $\kappa_i$ (pN nm) | $D_i$ (deg$^2$ $\mu$s$^{-1}$) |
|-----------|--------------------------------|------------------|-----------------------------|------------------|------------------|-------------------------------|
| 0, 3      | Hydrolysis                     | 0.116            | 1                           | 0                |                  |                               |
| 1         | Pi release                     |                  | 3                           | 3$^b$            | 0.117            | 56$^b$                       |
| 2         | ATP binding (4 mM)             | 36               | 40                          | 0.045            |                  |                               |
| 2*        | ADP release                    | 72               | 71                          | 0.045            |                  |                               |

$^a$Corrected for finite time resolution (appendix B).

$^b$For simulation, 90 ms$^{-1}$ was used because 120$^\circ$ rotation occurs at the end of catalytic dwells where there are a few number of data points associated with Pi release which can be approximated with faster rate [9].

Figure 5. Predicted mean angular jump profile under different opposing torque simulated by the Langevin method ($dt = \Delta t = 1 \mu s$). The dots show dwell angles of ATP hydrolysis shifted by the opposing torque $N$, while the angular step size of 120$^\circ$ is independent of torque. (d) The average lifetime of the metastable, three-occupancy state depends on the external torque for different exponent coefficient $a_{21}$ of the metastable state.

3.3. Angular jump profile under constant opposing external torque

The effect of opposing torque on angular jump profile is shown on figure 5(a). The bead can rotate forward despite a large load torque because of a huge thermodynamic driving force with forward rates being significantly faster than backward rates. The average angular jump reduces with increasing torque due to downshift of the dwell angles. The downshift of dwell angles is clearly observed in catalytic state (i.e. hydrolysis): the circles, where the mean angular jump value is zero as shown in figure 5(a), indicate the dwell angles of the catalytic state. Under opposing torque, the bead spends more time fluctuating in each chemical state before reactions occur, i.e. there are longer dwell times which are observed in experiments [16]. Consequently, the metastable state can be detected more readily in the single-molecule data, due to its extended lifetime, as depicted in figure 5(b), especially when reaction rates depend strongly on rotation angle via a large exponent coefficient $a_{21}$. The increase in the metastable state’s lifetime can be noticed in changes of the mean angular jump profile between 40$^\circ$ and 60$^\circ$, when torque is at least 20 pN nm (short plateaus on figure 5(a)).

3.4. Consistency of two modelling methods

The two methods assume rotations of $\gamma$ axis and the beads are equivalent to each other and can reflect the chemical states of F$_1$-ATPase, which is similar to another Langevin approach [6]. Both rotations can also be treated separately with reaction rates depending on the angle of $\gamma$ axis [35]. Angular jump profiles from Fokker–Planck simulation consists of mean angular jump at each angle from equation (7) which is based on angular occurrence probabilities $p_i(\theta)$ and mean jump values at different angles for each chemical state. The mean jump values are based on the assumption that F$_1$-ATPase remains at the same chemical state during interval time step $\Delta t$ corresponding to camera time resolution. In contrast, equation (4) shows that angular jump calculated from Langevin trajectories requires no information on chemical state of F$_1$-ATPase. Besides, it is possible to compare between two methods because angular occurrence probabilities and mean jump values used by Fokker–Planck can be obtained from Langevin trajectories. There is good agreement in angular occurrence probability distribution between Fokker–Planck and Langevin simulation for all
Figure 6. Mean angular jump profiles simulated without mixing (Fokker–Planck method, dashed lines) and with mixing (Langevin method, solid lines) for a bead radius of 20 nm. Evolution time step $d_t$ and interval time step $\Delta t$ (depicted in insets) were (a) $d_t = \Delta t = 1 \mu s$ and (b) $d_t = 1 \mu s$, $\Delta t = 10 \mu s$. The insets also illustrate chemical state change of the bead during free rotation (blue to black). Parameters used for simulation are summarized in supporting information (tables A1 and A2).

Predicted effect due to state mixing on mean angular jump profiles simulated using Langevin approach for a bead radius of (c) 10 nm and (d) 40 nm.

chemical states, as illustrated in figure A2 where time step $d_t$ of 1 $\mu s$ is used for Langevin simulation. The angle dependent mean angular jumps at each chemical state also show similarity between two methods when time interval $\Delta t$ of 1 $\mu s$ is used in Fokker–Planck method, as depicted in figure A3. The consistency between two methods results in barely indistinguishable angular jump profiles, as shown in figure 6(a). The agreement can also be seen when smaller time step $d_t$ and interval time step $\Delta t$ are 0.1 $\mu s$ (figure A4). We only varied $\Delta t$ in the Fokker–Planck simulation due to the limitations of the numerical solver which automatically determines its own evolution time step. Camera with high time resolution can capture the moment when the bead's angle changes due to state switching. Since state switching occupies only a small fraction during the course of transition, shown in the inset of figure 6(a), its effect on angular jump profile is not significant, thus justifying the usage of the formula for jump values.

4. Discussion

4.1. Imaging time resolution and probe size

Lower time resolution $\Delta t = 10 \mu s$ may cause discrepancy in angular jump profiles for the two approaches, as illustrated in figure 6(b). The difference means that the assumption for mean angular jump from equation (7) does not hold for longer time steps $\Delta t$, so correction is necessary due to the presence of state-mixing events. In the absence of mixing effect, using long time steps (i.e. lower time resolution) in the theoretical calculation yields underestimated values of mean angular jump because $F_1$-ATPase switches to the next chemical state during the CCD camera frame capture time. In the analyzed trajectories, if a chemical transition occurs within 10 $\mu s$, angular jump will be larger than angular jump without no change.
in the chemical state due to switching of state angle. The resulting overall shift for profiles is captured by using Langevin simulation, as shown in figure 6(b). The 10 μs time interval also affects angular occurrence probabilities and angular jump distribution (figures A2 and A3). The mixing effect is more pronounced within the first 85° due to fast chemical rates, but is negligible beyond 85° because of the slow hydrolysis rate, resulting in the bead staying in the hydrolysis state for times much longer than 10 μs.

The state mixing effect also causes slight reduction in the mean angular jump for Langevin simulation, as shown in figures 6(a) and (b). The reduction is predicted to be more significant when bead size is reduced, as depicted in figure 6(c), because a smaller bead will rotate faster with shorter relaxation time and more fluctuation. In contrast, increasing bead size will alleviate mixing effects because of slower rotation, as shown in figure 6(d).

4.2. Improved detection of life time of the three-occupancy state
When using a method that takes into account switching of states during a jump, the short lived state \( i = 2^* \) can be detected with enhanced accuracy. The four-state model angle-dependent mean jump profile on figure 6 provides a very good fit to the experimental profile when a life time of \( 1/k_{f2^*} = 14 \) μs is used at the dwell angle of \( \theta_{f2^*} = 72° \). Our results indicate that ADP is released about 5 orders of magnitude faster when ATP binds to another pocket, as opposed to spontaneous release, when the occupancy of the F1-ATPase is low. Likely, an opening of the structure around the pocket is associated with the ADP transition through the binding channel during release (cf figure 7). Full atomistic simulations can be used to test whether these two processes occur in a concerted way [36].

The triply occupied state is short-lived and cannot be detected by traditional approach, such as plotting the angular histograms. We speculate that the reason the current jump distribution based method can detect fast states is due to the fast velocity relaxation time of the probe: the jumps are approximating of the instantaneous angular velocity of the rotor shaft. A fast switch of the potential reverses the velocity and the jump distribution of the system responds to this change. In contrast, the probe angular relaxation time is slower, so the histogram is weakly affected by the presence of a short-lived state during a transition.

4.3. Predicted average reaction rates in the presence of external torque
We have argued that the angle-dependent rate constants in equations (2) and (3) do not depend on the external torque. However, the average forward rate when F1-ATPase jumps from state \( i \) to state \( i + 1 \) will be torque dependent:

\[
\langle k_{fi} \rangle \approx \int_{-\infty}^{\infty} k_{fi}(\theta')P_{eq}^{\theta'}(\theta')d\theta'
= k_{fi}^0 \exp \left\{ \frac{\alpha \beta \kappa_c (\theta_{i+1} - \theta_i)}{\kappa_r} \left\{ \frac{(\alpha \kappa_c - \kappa_r) (\theta_{i+1} - \theta_i)}{2} - N \right\} \right\},
\]

where \( P_{eq}^{\theta'} \) is the equilibrium Boltzmann distribution of bead’s angle during starting dwell:

\[
P_{eq}^{\theta'}(\theta) = \sqrt{\frac{\kappa_r \beta}{2\pi}} \exp \left\{ -\frac{\beta \kappa_r}{2} \left( \theta - \theta_i + \frac{N \kappa_r}{\kappa_r} \right)^2 \right\}.
\]
The calculation is based on the postulation that F1-ATPase may experience different stiffness: $\kappa_i$ when it is idle but $\kappa_r$ when chemical reaction occurs leading to major conformational changes. Similarly, the average backward rate is calculated from equilibrium distribution from ending dwell:

$$
\langle k_{b_i} \rangle \approx \int_{-\infty}^{\infty} k_b(\theta') r_{eq}(\theta') \, d\theta' = k_b^0 \exp \left\{ \frac{(1 - \alpha)\beta \kappa_c (\theta_{i+1} - \theta_i)}{\kappa_r} \left[ \frac{(1 - \alpha)\kappa_r - \kappa_c (\theta_{i+1} - \theta_i)}{2} + N \right] \right\}.
$$

Since $\alpha \approx 0.5$ [28], the net free energy drop can be approximated as follows:

$$
k_b T \ln \left( \frac{\langle k_i \rangle}{\langle k_b \rangle} \right) \approx -\Delta G_0^0 = \frac{\kappa_c (\theta_{i+1} - \theta_i)}{\kappa_r} N.
$$

4.4. Acceleration of ATP release by ADP binding in the presence of torque in F0,F1-ATPase

In the presence of sufficient concentrations of ADP and Pi, a higher opposing torque can induce backward rotation in F1-ATPase to synthesize ATP molecules from ADP and Pi molecules and accelerate their release into solution [16]. For a 0.287 $\mu$m (in diameter) bead under the condition of 0.4 $\mu$m ATP, 4 $\mu$m ADP and 1 mM Pi, transition times between dwells are nearly 0.05 s for opposing torques within 40–49 pN nm [16]. The transition times are shorter than ATP release time (nearly 7 s [2]) obtained from stalling experiments when F1-ATPase undergoes hydrolysis direction. Besides, sluggish ATP release rate (approximately 0.14 s$^{-1}$ [2]) may not allow backward rotation (rotation in synthesis direction) to occur due to fast ADP release rate (rotation in hydrolysis direction) which may be five orders of magnitude higher (cf table 1). The observations lead to the postulation that ATP release rate (in synthesis direction) may be comparable to ADP release rate (in hydrolysis direction). The acceleration of ATP release rate induced by ADP binding may be similar to the mechanism of fast ADP release induced by ATP binding in hydrolysis direction, but now the mechanism is in reverse order. This acceleration of the synthesized ATP release in the F1 region occurs in the presence of a torque acting on the $\gamma$ shaft in the synthesis direction of rotation, and presumably in the complete F0,F1-ATPase this torque is provided by the proton gradient via the $C$-ring in the F0 region.

5. Conclusions

Using a multi-state molecular transfer model of angular jumps in single-molecule imaging experiments, we have revealed the presence of a short-lived triple occupancy state in the rotation of the F1-ATPase. In this state, the ADP release at the end of the chemo-mechanical cycle (291$^\circ$ after ATP binding) is accelerated by the binding of an ATP to another, empty subunit in the F1-ATPase. Its lifetime was found to be 14 $\mu$s, about five orders of magnitude faster than the spontaneous ADP release, when there is no ATP binding to the empty subunit.

Applying theoretical modeling to experimental trajectories increased the effective time resolution beyond the limitations of the imaging apparatus. The improved accuracy needed for detecting the ADP release event is due to specific features of the theoretical modeling of single-molecule data, as follows:

(a) Using a Langevin implementation of the multi-state model improves the accuracy for extracting short-lived states at low time resolution, where mixing effects become important. In particular, this is the case for nano probes in single-molecule imaging, because a nano-bead’s relaxation time is fast (microseconds), but the video recording timestep is limited and can be longer than chemical reaction times.

(b) When applying theory to single-molecule data, the increased sensitivity to shorter-than-timestep events is likely due to the use of the jump distribution, since the jumps are related to the velocity and the velocity response times are much smaller ($\approx 1$ ns) than the lifetime of the metastable state.

(c) When F1-ATPase is under constant, opposing external torque, dwell angles are shifted down. The bead spends more time fluctuating in dwells until a chemical reaction occurs, causing reduction in the angular jumps. As a result, the angular jump profile is predicted to show a pronounced short plateau (within 40°–60°) due to increasing lifetime of metastable state, thus offering a way for its detection.

An implication of the present analysis for the biological function of ATP synthesis by the F0,F1-ATPase is that in the presence of torque on the rotor shaft due the ion gradient in the F0 region the release of the
Figure A1. Kinetic scheme of F$_1$-ATPase during stalling within 50° from ATP binding dwell. State A is the occupancy of F$_1$-ATPase during ATP binding dwell while state B is the postulated three occupancy, short-lived state. Long stalling time may lead to spontaneous release of ADP with rate $k_r$, resulting in state D and E. State E and C have the same occupancy but may be different in overall structural conformation. Certain approximations allow to simplify the kinetic scheme into the final, reversible two states considered by stalling experiments [2].

product ATP is likely accelerated several orders of magnitude by the binding of ADP to another subunit in the F$_1$ ring.

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Data availability statement

The data that support the findings of this study are available upon reasonable request from the authors.

Appendix A. Time evolution of the bead based on Langevin description

The angle of bead described in equation (1) is updated based on the following scheme [29]:

$$\theta(t + dt) = \mu(t) + \sigma R(0, 1), \quad (A1)$$

where $R(0,1)$ is a random number generated from normal distribution with mean of zero and variance of one and expressions of $\mu(t)$ and $\sigma$ being:

$$\mu(t) = (\theta(t) - \theta_i)e^{-\frac{\gamma t}{\kappa_r}} + \theta_i \quad (A2)$$

$$\sigma^2 = k_B T \frac{k_r}{\kappa_r} \left(1 - e^{-\frac{\gamma t}{\kappa_r}}\right). \quad (A3)$$

When F$_1$-ATPase is at chemical state $i$, the probability of having a chemical reaction at the current angle is

$$P = 1 - \exp[-(k_{fi}(\theta) + k_{fi-1}(\theta)) \, dt], \quad (A4)$$

where F$_1$-ATPase can either jump forward to the following state or jump backward to the preceding state. However, backward rates can be ignored because they are significantly smaller than forward rates in the
experimentally the discrete version of the time auto-correlation function can be estimated as a time average, measured angle be updated accordingly, followed by update of the bead’s angular position. For stationary process, this correlation function only depends on the lag \(\Delta t\), can be calculated from theory if we take into account that the angles are averages over the integration time, \(\Delta t\). Accordingly, all quantities extracted from single-molecule trajectories are subject to artifacts due to this averaging. For a given imaging frame, when the system is in a given chemical state \(i\) (in a dwell), the measured angle \(\theta_n = \overline{\theta}\Delta t\), is a time average over the frame time, \(\Delta t\), \(n\) times, \(n\) is a positive integer. For the discrete version, the measured correlation function, \(c_n(t)\), is defined as:

\[
c_n(t) = \frac{1}{\Delta t} \int_{(n-1)\Delta t}^{n\Delta t} \theta(t) \, dt.
\]

Let \(m\) and \(m + n\) be positive indexes of angle data points in the same dwell of a trajectory. Then, experimentally the discrete version of the time auto-correlation function can be estimated as a time average, i.e. using the ergodic assumption,

\[
C_n = \langle \theta_m \cdot \theta_{m+n} \rangle = \langle \theta_m \cdot \theta_{m+n} \rangle_m.
\]

For stationary process, this correlation function only depends on the lag \(n\) and it does not depend on \(m\). It can be calculated from theory if we take into account that the angles are averages over the integration time, \(\Delta t\), the system will jump to state \(i + 1\); otherwise it jumps back to state \(i - 1\). Once new state is identified, chemical reaction occurs instantaneously and the dwell angle of chemical state will be updated accordingly, followed by update of the bead’s angular position.

Appendix B. Correction on \(\kappa\) extracted from single-molecule trajectories

It was suggested that the apparent fluctuations of the probe in single-molecule imaging can only be detected accurately if the time step of the imaging apparatus \(\Delta t\) is smaller than the relaxation time \(\tau\). In particular, we now calculate (1) the measured distribution of angles (standard deviation \(\sigma_m\)) and (2) the measured correlation function, \(c_m(t)\). In single-molecule imaging, the average position of the probe is detected during the imaging frame time \(\Delta t\). Accordingly, all quantities extracted from single-molecule trajectories are subject to artifacts due to this averaging. For a given imaging frame, when the system is in a given chemical state \(i\) (in a dwell), the measured angle \(\theta_n = \overline{\theta}\Delta t\), is a time average over the frame time,

\[
\theta_n = \frac{1}{\Delta t} \int_{(n-1)\Delta t}^{n\Delta t} \theta(t) \, dt.
\]

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where $\sigma^2 = k_B T/\kappa$. It should be noted that the correlation function for $n = 0$ lag must be treated differently than those of $n > 0$ where the condition is automatically satisfied,

$$C_0 = \frac{2}{\Delta t^2} \int_0^{\Delta t} \int_0^{t'} dt' C(t - t').$$  \hspace{1cm} (A9)$$

$$C_n = \frac{1}{\Delta t^2} \int_0^{\Delta t} dt' \int_{n\Delta t}^{(n+1)\Delta t} dt C(t - t').$$  \hspace{1cm} (A10)$$

The factor of 2 for zero lag is due to interchangeable between $t$ and $t'$ when both have same range from 0 to $\Delta t$. We next show that the decay rate of the correlation function extracted from trajectories yields an unbiased estimate for the relaxation time. Equation (A10) gives:

$$C_n = \frac{k_B T}{\kappa} e^{-n\Delta t/\tau} \frac{\sigma^2}{\Delta t^2} \left(1 - e^{-\Delta t/\tau}\right)\left(e^{\Delta t/\tau} - 1\right).$$  \hspace{1cm} (A11)
Figure A4. Langevin simulations show similarity in the mean angular jump profile generated from evolution time step \( dt = \Delta t = 0.1 \mu s \) and \( dt = \Delta t = 1 \mu s \).

Therefore the rate of decay of \( C_n \) is the same as that of \( C(t - t') \), suggesting that relaxation time \( \tau \) can be estimated from fitting an exponential to \( C_n \).

The variance calculated from equation (A9) is:

\[
C_0 = 2 \frac{k_B T}{\kappa} \left( \frac{\tau}{\Delta t} - \frac{\tau^2}{\Delta t^2} (1 - e^{-\Delta t/\tau}) \right).
\]  

(A12)

The variance allows relation between the true and measured value of the stiffness \( \kappa \):

\[
\kappa_m = \frac{\kappa}{r^2} = 2 \left( \frac{\tau}{\Delta t} - \frac{\tau^2}{\Delta t^2} (1 - e^{-\Delta t/\tau}) \right).
\]  

(A13)

It means that measured angles will follow Gaussian distribution \( p_m(\theta_m) \) whose width is scaled by proportionality factor \( r^2 \). Magnetic bead having diameter of 730 nm and rotation radius of 100 nm [2] will have theoretical friction of 1.29 pN nm s which is the lower limit. Since elastic stiffness is around 75 pN nm rad\(^{-2} \) [2], the bead’s relaxation time should be at least 17 ms. Combined with the camera time resolution of 2 ms, there is no much difference between true and measured value of stiffness because \( r^2 \) is 0.96. For a trajectory of \( \Delta t = 10 \mu s \) analyzed previously [9], an unbiased relaxation time of \( \tau = 14 \mu s \) was estimated from the correlation function. This value indicates that a significant correction factor of about \( r^2 = 0.80 \) should be applied to the apparent \( \kappa_m = 70 \) pN nm rad\(^{-2} \), resulting in a spring constant of \( \kappa_r = 56 \) pN nm rad\(^{-2} \).

In conclusion, the distribution of measured fluctuations is biased with factor \( r^2 \) which depends on the ratio of \( \tau / \Delta t \). It suggests that the correction in practice requires accurate estimation of the relaxation time.

Appendix C. Kinetic scheme of F\(_1\)-ATPase during 80° rotation

The postulation of metastable, three occupancy state needs putting in context with the stalling experiments [2]. The kinetic scheme of F\(_1\)-ATPase during stalling consideration is shown in figure A1. The states denote different occupancy of \( \beta \) pockets inside F\(_1\)-ATPase. In free rotation, F\(_1\)-ATPase is expected to follow a sequence of state A, B and C which represent ATP binding, metastable and catalytic state respectively. A few approximations can be made such as fast ADP release rate \( k_{ADP} \), comparable ATP bimolecular binding rate and release rate between A and B or D and E. State E and C may be in equilibrium due to fast, comparable \( k_3, k_{-3} \) rates.

Appendix D. Relevant tables and figures

See tables A1, A2 and figures A2–A4.
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