

Theory of single-molecule controlled rotation experiments, predictions, tests, and comparison with stalling experiments in F_1 -ATPase

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A recently proposed chemomechanical group transfer theory of rotary biomolecular motors is applied to treat single-molecule controlled rotation experiments. In these experiments, single-molecule fluorescence is used to measure the binding and release rate constants of nucleotides by monitoring the occupancy of binding sites. It is shown how missed events of nucleotide binding and release in these experiments can be corrected using theory, with F_1 -ATP synthase as an example. The missed events are significant when the reverse rate is very fast. Using the theory the actual rate constants in the controlled rotation experiments and the corrections are predicted from independent data, including other single-molecule rotation and ensemble biochemical experiments. The effective torsional elastic constant is found to depend on the binding/releasing nucleotide, and it is smaller for ADP than for ATP. There is a good agreement, with no adjustable parameters, between the theoretical and experimental results of controlled rotation experiments and stalling experiments, for the range of angles where the data overlap. This agreement is perhaps all the more surprising because it occurs even though the binding and release of fluorescent nucleotides is monitored at single-site occupancy concentrations, whereas the stalling and free rotation experiments have multiple-site occupancy.

F_1 -ATPase | biomolecular motors | single-molecule imaging | nucleotide binding | group transfer theory

Single-molecule manipulation techniques, including stalling and controlled rotation methods or “pulling” force microscopies, have been used to augment imaging experiments in biomolecular motors (1–4). In F_1 -ATPase, for example, beyond observing the kinetics of stepping rotation resolved into $\sim 80^\circ$ and $\sim 40^\circ$ substeps (5–7), the manipulation of the rotor shaft by magnetic tweezers recently opened up the possibility of directly probing the dynamical response of the system to externally constraining the rotor angle θ . In tandem with the experimental tools of X-ray crystallography (8) and ensemble biochemical methods (9), these experiments provide added insight into the processes in chemomechanical energy transduction (7, 10–13). The kinetic pathway along which concerted substeps occur in free rotation has been established (14), whereby binding of solution ATP to an empty subunit is initiated at $\theta = 0^\circ$, and the release of hydrolyzed ADP from the clockwise neighboring subunit occurs simultaneously as the θ completes the $\sim 80^\circ$ rotation step (Fig. 1). Using the detailed knowledge of individual substeps, stalling (3, 15) and controlled rotation (3) experiments provide an estimate of the rate constants of nucleotide binding and other processes as a function of θ . In particular, binding and release of ATP and analogs can be externally controlled to occur at angles other than 0° .

In the controlled rotation experiments (1, 4) we consider here, a slow constant angular velocity rotation of the shaft was produced by magnetic tweezers. A magnetic bead was attached to the rotor shaft protruding from the stator ring with a constant magnetic dipole moment pointing in the plane of the ring, the

latter fixed to a microscope coverslip. An external magnetic field was created via permanent magnets and the magnetic bead aligned itself to the direction of this field. The direction of the external field was rotated in the plane of the stator ring, and the resulting change in the nucleotide occupancy was monitored using fluorescent ATP and ADP analogs, Cy3-ATP and Cy3-ADP. To permit individual observations, the solution was diluted in the nucleotide, resulting in a low site occupancy during single-molecule trajectories (4). Events whereby the occupancy σ changed between 0 and 1 were then analyzed; any higher occupancy events were excluded from the analysis. The number of binding ($0 \rightarrow 1$) and release ($1 \rightarrow 0$) events in narrow observation intervals of width $\Delta\theta$ was used to estimate forward $k_f(\theta)$ and backward $k_b(\theta)$ rate constants of nucleotide binding, respectively, also yielding the equilibrium constant $K(\theta) = k_f(\theta)/k_b(\theta)$.

In a previous article (16), we formulated a theory for treating the θ -dependent $k_f(\theta)$, $k_b(\theta)$, and $K(\theta)$ in stalling experiments and compared the predictions with the experimental data. In these experiments the rotor was stalled at some θ then released after a predetermined time, rather than rotated at a constant angular velocity. For the controlled rotation experiments, we consider several questions:

- i) Are the results of stalling experiments and controlled rotation experiments consistent with each other and with a chemomechanical theory (16) of group transfer in the angular range where the two experiments overlap?
- ii) Are the time resolution limitations of single-molecule fluorescence techniques used to monitor these events significantly leading to missed events, thus altering the outcome

Significance

The investigation of nucleotide binding and release dynamics vs. rotor shaft rotation in the F_1 -ATPase enzyme is necessary to reveal biological function. We elucidate the mechanism of the exponential-like change of binding and release rate (and thus the equilibrium) constants when probed against the rotor angle at the single-molecule level. We extend our group transfer theory proposed for the stalling experiments to treat controlled rotation experiments. The model correctly predicts the controlled rotation data on fluorescent ATP without any adjustable parameters. The theory provides a framework able to treat the binding and release of various nucleotides. In the process we also learn about the properties of the fluorescent nucleotide Cy3-ATP.

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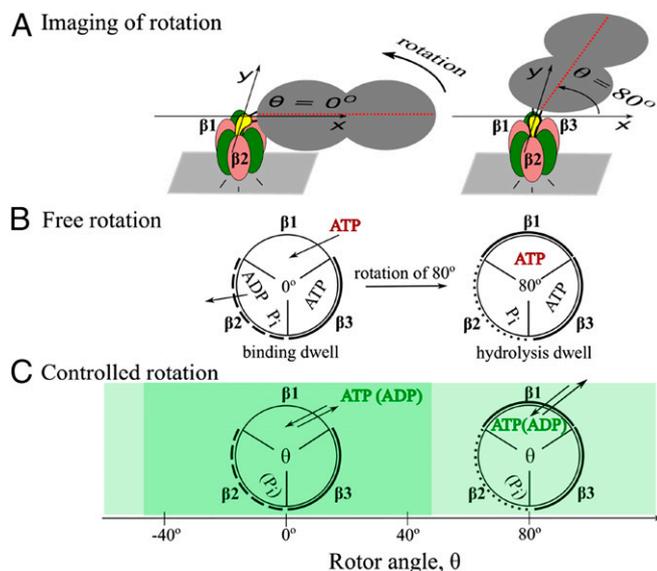


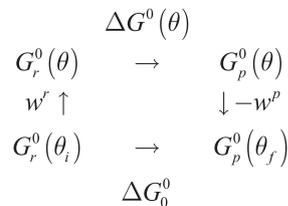
Fig. 1. Binding processes in F_1 -ATPase imaged using a bead-duplex (A) for wild-type nucleotides in free rotation (B) and for fluorescent nucleotides in controlled rotation (C) experiments. The rotor (yellow γ subunit) is linked to the bead duplex with its major geometric axis (red dashed line) that defines the rotor angle θ relative to the x axis of the laboratory xy coordinate plane. Looking at the F_1 -ATPase from the top (F_o side), θ increases counterclockwise. The coverslip (gray areas in A) to which the stator ring (green and pink α and β subunits) is fixed is in this xy plane. The range of $-50^\circ < \theta < 50^\circ$ is treated (dark shaded background in C) in which stalling experiments overlap with controlled rotation data (light shaded background). The species occupying the pockets of ring β subunits 1, 2, and 3 are shown at the dwell angles (0° and 80°), and the arrows indicate the displacement of the nucleotides during the 80° rotation. Thick arcs represent a closed subunit structure, and dashed and dotted lines indicate various degrees of openness.

- of the rate measurements? If so, can one correct for such effects using theory?
- iii) Is an approximation made in the analysis of the experiment of replacing the time spent in nonoccupied sites by the total trajectory time a significant approximation at any rotor angle value? If so, can one use theory to correct for this approximation?
- iv) Can the theory predict the binding and release rate constants and their dependence on the rotor angle in F_1 -ATPase, with no adjustable parameters, when corrections are made for the differences in the nucleotide species in the experiments, even though the occupancy in the ATPase in the controlled rotation experiment is at most one whereas that in the stalling experiment is two or three?
- v) Can a structural elasticity of the ATPase be extracted from the equilibrium constant vs. rotor angle data for various nucleotides?

Results

Elastic Chemomechanical Group Transfer Theory. In our previous study (16) the binding and release of nucleotides were treated in F_1 -ATPase based on a formalism originally proposed for electron transfers (17) and adapted to other transfers (18), including proton (19) and methyl cation (20) transfers. In the theory a thermodynamic driving force that determines the rate and equilibrium constants in the experiments for any reaction step, including nucleotide binding, is the change in the relevant Gibbs free energy of reaction for that step. A thermodynamic cycle (Scheme 1) (16) provides a basis for relating the free energies of a change accompanying nucleotide binding in

free rotation, ΔG_0^0 (Fig. S1), to the binding free energy $\Delta G^0(\theta)$ at a constant rotor angle θ . In the present treatment we consider a quasistatic approximately constant θ in any observation interval—quasistatic because the rotor shaft is rotated slowly during the controlled rotation.



Scheme 1.

In Scheme 1, $G_r^0(\theta)$ and $G_p^0(\theta)$ denote the free energies of the system in its “reactant” and “product” states (unbound and bound ATP states in the present θ range) when the magnetic tweezers hold the rotor at an angle θ . The system is relaxed at the initial and final dwell angles $\theta_i = 0^\circ$ and $\theta_f = 80^\circ$. As before (16), it is assumed that rotary motors exhibit a harmonic response to twisting torques described by an effective stiffness κ (15, 21, 22), and so in Scheme 1 we recall from ref. 16 that $w^r = \kappa/2(\theta - \theta_i)^2$ and $w^p = \kappa/2(\theta - \theta_f)^2$. For the θ -dependent $k_f(\theta)$ and $k_b(\theta)$ in Eqs. 3 and 4 given later a quadratic group transfer theory relation is used. This relation, given in ref. 16 as equation 10, relates $\Delta G^0(\theta)$ in Scheme 1 and the free energy barrier $\Delta G^\ddagger(\theta)$ that the nucleotide needs to overcome during binding when it transfers from solution into the pocket (16–18).

Application of the Theory to Cy3-Nucleotides. In the analysis (16) of the stalling experiments linear $\ln k_f$, $\ln k_b$, and $\ln K$ vs. θ were predicted for the θ range treated experimentally. Given the similarities between the probed binding/release processes and the exponential-like rate vs. rotor data in the controlled rotation experiments (Fig. S2) compared to those in stalling experiments, in the present article we apply the chemomechanical group transfer theory (16) to the processes of nucleotide binding and release in controlled rotation experiments. In the present treatment we consider a quasistatic approximately constant rotor angle in any observation interval j of duration t on Fig. S3—quasistatic because the rotor shaft is rotated slowly during the controlled rotation. Although controlled rotation experiments provide binding and release events over nearly the complete 360° range (4), in the present article we compare the experimental results with the theoretical predictions in the angular range of $(-50^\circ, 50^\circ)$, where the current stalling and controlled rotation experiments overlap. We note that according to the notation adopted in single-molecule experiments (1, 4, 14), $\theta = 0^\circ$ is set at the ATP binding dwell.

For Cy3-ATP available data from the stalling and other experiments are used to predict the absolute values for the $k_f(\theta)$, $k_b(\theta)$, and $K(\theta)$ in the controlled rotation experiments, including therefore the slopes ($\partial \ln k_f / \partial \theta$ and $\partial \ln k_b / \partial \theta$) and the values at $\theta = 0$, $\ln k_f(0)$ and $\ln k_b(0)$ [and hence $\ln K(0)$]. The θ -dependent rate and equilibrium constants are determined, as discussed below, by the following quantities: the relevant torsional stiffness of the structure κ , the change of the locally stable rotor angle during rotation $\theta_f - \theta_i$, the “reorganization energy” λ , the Brønsted slope at $\theta = 0$, $\alpha(0)$, and the binding and release rate constants for Cy3-ATP at $\theta = 0$, $k_f(0)$, and $k_b(0)$. The procedure to use the theory together with prior independent experimental data first involves deducing functional forms for $k_f(\theta)$, $k_b(\theta)$, and $K(\theta)$, then providing the values of the quantities that appear in their expression, as follows.

For the $K(\theta)$, from equation 5 of ref. 16 it follows that

$$kT \ln K(\theta) = kT \ln K(0) + \theta \kappa (\theta_f - \theta_i). \quad [1]$$

To calculate the $k_f(\theta)$, we first introduce the value of the Brønsted slope $\alpha(\theta) = \partial \ln k_f(\theta) / \partial \ln K(\theta)$ (18, 23, 24), at the angle $\theta = 0$,

$$\alpha(0) = [\partial \ln k_f(\theta) / \partial \ln K(\theta)]|_{\theta=0}. \quad [2]$$

From our previous treatment (16), $k_f(\theta)$ as a function of θ is given by

$$kT \ln k_f(\theta) = kT \ln k_f(0) + \theta \alpha(0) \kappa (\theta_f - \theta_i) - \theta^2 \kappa^2 (\theta_f - \theta_i)^2 / 4\lambda. \quad [3]$$

For the release of the nucleotide, $k_b(\theta) = k_f(\theta) / K(\theta)$, and so from Eqs. 1–3,

$$kT \ln k_b(\theta) = kT \ln k_b(0) - \theta [1 - \alpha(0)] \kappa (\theta_f - \theta_i) - \theta^2 \kappa^2 (\theta_f - \theta_i)^2 / 4\lambda. \quad [4]$$

We consider next the effect of changing from the wild-type nucleotide ATP to fluorescent species Cy3-ATP, as well as the condition of single-site occupancy. If κ describes the effective stiffness of rotor, the β lever arm, and to some extent the bonding network with the nucleotide, we can presume that it is not significantly affected by changing the substrate to Cy3-nucleotide, because the Cy3 part remains outside the pocket because of the linker, as discussed in a following section.

Next, we describe a procedure to provide the values for the quantities $\theta_f - \theta_i$, κ , λ , $\alpha(0)$, $k_f(0)$, and $k_b(0)$, also listed in Table 1, which appear in Eqs. 1, 3, and 4 and are used to construct the theoretical plot. A part of the procedure given below relies on a procedure described previously in ref. 16.

- i) Based on the previous description, we use the $\kappa = 16$ pN · nm/rad² found earlier (16) from the stalling experiments.
- ii) The angular changes of 80° and 40° in the stepping rotation have been reported to remain unchanged when ATP is replaced by Cy3-ATP in free rotation experiments (1), so we continue to use $\theta_f - \theta_i = 80^\circ$.
- iii) The reorganization energy λ appears explicitly in the quadratic term in Eqs. 3 and 4, and for its value we use $\lambda = 68$ kcal/mol. It was calculated in equation 18 of ref. 16 for the stalling experiments using a work (W^r) and two free energy (ΔG_0^f and ΔG_0^b) terms provided in table 3 of ref. 16.* A simpler procedure based on the method given in ref. 16 to estimate these three quantities is described in *Supporting Information, Fig. S1*.
- iv) For the Brønsted slope, an $\alpha^{\text{Cy3-ATP}}(0) \cong 0.5$ (Table 1) can be inferred for Cy3-ATP binding from the $\alpha^{\text{ATP}}(0) = 0.48 \cong 0.5$, calculated in our previous treatment of stalling experiments using equation 12 of ref. 16 with the same W^r , ΔG_0^f , and ΔG_0^b terms given in *Supporting Information*, and the quantities from steps i and ii above.

*Correction for ref. 16: on page 4, column 2 “ $\Delta G_0^b - W^r = 14.1$ kcal/mol” should read “ $\Delta G_0^b - W^r = 11.3$ kcal/mol.” This change causes the two λ values in table 3 and table 4, “56” and “55,” to be changed to “68” and “67,” respectively. Because α for ATP and GTP binding is close to 1/2, $\Delta G_0^b \ll 2\lambda$, according to equation 12, α is relatively insensitive to changes in a quadratic term $\Delta G^b/4\lambda$ that has a small contribution to the deviation of α from 0.5. The new α for ATP binding, 0.476, rounds off to “0.48” instead of “0.47” in table 3. All other numbers involving the comparison of theory and experiment remain unchanged, and so no conclusions are affected.

Table 1. Summary of effective quantities and comparison between theoretical predictions and experiment on the angle-dependent rate constants for Cy3-ATP binding

Properties used in theory*			Controlled rotation, rates vs. θ		
Quantity	Value*	Source [†]	Quantity	Theory [‡]	Experiment [‡]
$\theta_f - \theta_i$	80°	F (1)	$k_f(0)$	0.9	~1.2
λ	68	EFS (16)	$d \ln k_f / d\theta$	0.49	~0.48
κ	16	S (3, 16)	$k_b(0)$	1.3	~1.0
$\alpha(0)$	0.5	EFS (16)	$d \ln k_b / d\theta$	-0.49	~-0.48
$k_f^{\text{ATP}}(0)$	9.2	S (3)			
$k_b^{\text{ATP}}(0)$	0.13	S (3)			
$k_{f,0}^{\text{Cy3-ATP}} / k_{f,0}^{\text{ATP}}$	0.1	E (25)/F (1)			
All of the above. EFS (1, 3, 4, 16, 25)			k_f^{rep} , k_b^{rep} , and K^{rep} ; Fig. S4A		
All of the above. EFS (1, 3, 4, 16, 25)			Actual k_f , k_b , and K ; Fig. 2		

* k_f and k_b are in units of (micromoles seconds)⁻¹ and seconds⁻¹, respectively; $d \ln k_f / d\theta$ and $d \ln k_b / d\theta$ are in 1/10°; κ is in piconewtons-nanometer/rad²; and λ is in kilocalories per mole.

[†]Properties were extracted from single-molecule [free rotation (F) and stalling (S)] and ensemble (E) experiments.

[‡]The values in this column are approximate.

- v) Changing from ATP to Cy3-ATP changes the $kT \ln k_f(0)$ term. To calculate $k_f(0)$, we first note that theory (16) relates it to the binding rate constant in free (unconstrained) rotation $k_{f,0}$ by a relation deduced from equations 5–9 of ref. 16, $kT \ln k_f(0) = kT \ln k_{f,0} - \alpha(0) \kappa (\theta_f^2 - \theta_i^2) / 2 + (\theta_i + \theta_f)^2 \kappa^2 (\theta_f - \theta_i)^2 / 16\lambda$. Because, according to steps i–iv, the last two terms are unchanged if ATP is changed to Cy3-ATP, the ratio of $k_f(0) / k_{f,0}$ will not change either, and so

$$k_{f,0}^{\text{Cy3-ATP}} / k_{f,0}^{\text{ATP}} = k_f^{\text{Cy3-ATP}}(0) / k_f^{\text{ATP}}(0). \quad [5]$$

From stalling experiments (3), $k_f^{\text{ATP}}(0) = 9.2 \cdot 10^6$ M⁻¹ s⁻¹, the experimentally (1) measured ratio of $k_f^{\text{ATP}} / k_f^{\text{Cy3-ATP}} \cong 10$, reported in Table 1, yields $k_f^{\text{Cy3-ATP}}(0) = 0.9 \cdot 10^6$ M⁻¹ s⁻¹.

- vi) To provide a value for $k_b(0)$ that appears in Eq. 4, we note that for the $\alpha(0) \cong 0.5$ a suppression by some factor in the forward binding rate corresponds to an enhancement by the same factor in the backward rate. In particular, because from ref. 3 $k_b^{\text{ATP}}(0) = 0.13$ s⁻¹, we calculate $k_b^{\text{Cy3-ATP}}(0) = 1.3$ s⁻¹. Finally, from Eq. 1 and steps iv and v we deduce $K(0) = k_f(0) / k_b(0) = 0.7 \times 10^6$ M⁻¹.

For Cy3-ADP, the controlled rotation data were simply fitted to the functional form given in Eqs. 1–4 by assuming the same $\theta_f - \theta_i = 80^\circ$ and $\lambda = 68$ kcal/mol, and adjusting the other parameters, because there were no data to predict the Cy3-ADP values from prior experiments.

Rate Constant Estimate in Experiments and Missed Events. In the present analysis, we treat the controlled rotation data of Adachi et al. (4) and use some of the procedure devised by these authors in their analysis, as follows. When a site is occupied, the nucleotide fluoresces; otherwise, fluorescence drops to a background level, giving rise to site-occupancy trajectories along which σ switches between 0 and 1. The binding and release events along the trajectories were assigned to specific sites and grouped into 36 consecutive intervals of $\Delta\theta = 10^\circ$, with a rotation time $t = 0.14$ s per interval, during which the binding and release rates were considered as approximately constant. Within each $\Delta\theta$ simple two-state kinetics was assumed with angle-dependent binding and release rate constants. The forward rates can be estimated as the number of $0 \rightarrow 1$ events divided by time T_0 spent in the $\sigma = 0$

related to charge transport (36). In that study the gating voltage is a control parameter analogous to the θ of the F₁-ATPase in our treatment.

Conclusions

The elastochemical theory of the rotary biomolecular motors described here provides an interpretation and treatment for the controlled rotation experiments on the F₁-ATPase enzyme. For these experiments the theory makes and tests predictions using independent experimental data on binding and release of fluorescent ATP given in Table 1 and Fig. 2, in the range of rotor angles θ where the controlled rotation and stalling experiments overlap. The dependence of the rate and equilibrium constants on θ from the theory are compared with experiment and are found to be in agreement. The theoretical model originally proposed to treat nucleotide binding and release in stalling experiments was found to be applicable to controlled rotation experiments on fluorescent ATP and ADP analogs, even though there is a marked difference in conditions—single vs. multiple site occupancy. By taking into account the effect of missed events in the experiments and the error due to using T instead of T_0 , the specific nature of the $\log k_f$ and $\log k_b$ vs. θ data was explained. It was found that the effective torsional spring constant is smaller for binding of ADP than of ATP, but it is not affected by the presence of the fluorescent Cy3 moiety in Cy3-ATP and Cy3-ADP. The effect of the Cy3 tethered to the nucleotide was found, not surprisingly, to shift the equilibrium constant for

binding toward release by limiting the degrees of freedom of the nucleotide in the binding pocket. In the Introduction several questions were posed. In each case, the answers are seen to be affirmative. The controlled rotation experiments also provide binding and release rate data over much of the 360° range of θ . Furthermore, one may anticipate that the present elastic group transfer theory applies to relatively small, compact domain motions and not to large changes such as folding of proteins.

Materials and Methods

Correction of Controlled Rotation Data. The theoretical counterparts of the reported k_f^{rep} and k_b^{rep} (Eqs. S1 and S2) are calculated, from the averaged values over an interval j , using the actual k_f and k_b predicted by theory (Cy3-ATP) or as fitting functions (Cy3-ADP). Then, the corrections are calculated as the differences $k_f - k_f^{\text{rep}}$ and $k_b - k_b^{\text{rep}}$. In these calculations the terms due to the missed events are evaluated. Because the denominator of k_f^{rep} (Eq. S2) T is used, the error due to replacing T_0 with T is explicitly taken into account (Supporting Information).

Fitting Procedure for Cy3-ADP. We assumed $\theta_f - \theta_b = 80^\circ$. The search for a “best fit” then involved finding a pair of $k_f(\theta)$ and $k_b(\theta)$ that remain within the scatter of the experimental data for all θ . These experimental data in Fig. 3 originated from correcting the reported data by calculating the missed events and the change due to using T instead of T_0 .

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