

Photosynthesis and Bioenergetics

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PHOTOSYNTHESIS AND BIOENERGETICS

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Preface

This book covers some of the most recent advances in the fields of Bioenergetics and Photosynthesis presented at an international workshop held in the Institute of Advanced Studies (IAS), Nanyang Technological University (NTU), Singapore, from 21st to 23rd March, 2016. The meeting was held as a tribute to Jan Anderson FRS who passed away in August 2015 and to celebrate the 75th birthdays of Leslie Dutton FRS and John Walker FRS, Nobel Laureate. Contributions to the workshop and to this book, are from outstanding scientists which includes the Nobel Laureate Rudolph Marcus (Chapter 2 by Marcus and Volkán-Kacsó) who created a theory of electron transport reactions. Marcus first provided the kinetic and thermodynamic description of the movement of electrons between molecules — a chemical processes at the very heart of the biological electron transfer in the major classes of bioenergetic systems such as bacterial cells, mitochondria and chloroplasts. Not surprisingly, the theme of electron transport reactions in the key redox enzymatic complexes of mitochondria and chloroplasts is covered in the majority of chapters of this book. Indeed, Chapters 3 (Wikström) and 4 (Rich) are dedicated to cytochrome *c* oxidase — a complex that terminates the respiratory electron transport chain producing water as a by-product. The most recent advances in studies of cytochrome *c* oxidase, photosystem II (PSII), that splits water and produces oxygen, are thoroughly reviewed in Chapters 5 (Kaucikas *et al.*) and 6 (Barber). The recent structural advances in understanding photosystem I that functions in series with PSII, are covered in Chapter 7 (Nelson). In modern times the science of bioenergetics attained an extraordinary progress with development and applications of state-of-the-art techniques, such as femtosecond crystallography (Chapter 5, Kaucikas *et al.*); single molecule microscopy and spectroscopy,

(Chapter 2, Marcus and Volkán-Kacsó); combination of electron and confocal fluorescence microscopy (Chapter 9, Ruban; Chapter 10, Koochak *et al.*). A significant pool of chapters is dedicated to the regulatory processes that take place in the photosynthetic electron transport chain owing to the dynamic nature of the photosynthetic membrane organisation reviewed in Chapter 10 by Koochak *et al.* These mechanisms include regulation of the photosynthetic light harvesting (Chapter 9, Ruban), cyclic electron transport (Chapter 12, Kou *et al.*; Chapter 13, Hanke and Schreibe and Chapter 14, Larkum *et al.*) and the whole oxygenic electron transport chain (Chapter 11, Järvi *et al.*). Chapter 8 by Shivhare and Mueller-Cajar, summarises the recent progress in understanding the protein *rubisco activase* that regulates the most abundant enzyme, Rubisco (ribulose-1,5-bisphosphatecarboxylase/oxygenase) in the biosphere and is responsible for carbon fixation. And finally, the ideas and the most recent developments in the field of applications of the knowledge of Bioenergetics and Photosynthesis are discussed in Chapter 1 (Ennist *et al.*) and Chapter 6 (Barber).

We recommend this book to specialists in Bioenergetics and Photosynthesis, researchers and postgraduate students who are working and studying various aspects of respiratory and photosynthetic electron transport chains as well as dynamic regulation of the light harvesting and electron transport events in oxygenic photosynthesis.

We would like to acknowledge IAS and NTU for supporting and hosting the workshop in March 2015 and encouraging us to produce this book.

J. Barber and A. V. Ruban
London, 7 June 2017

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Chapter 2

Free, Stalled, and Controlled Rotation Single Molecule Experiments on F_1 -ATPase and their Relationships

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We show how an elastic group transfer theory can be used to interpret and treat free, stalling, and controlled rotation single molecule experiments on F_1 -ATPase. It is shown how predictions can be made and tested within this class of experiments using our recent theoretical treatment of rotor angle dependent rate constants for this biological motor. The theory is also used to suggest an additional type of analysis of single molecule trajectories involving dwell angles.

1. Introduction

It is a pleasure to participate in this volume honoring our colleagues John Walker and Les Dutton. This chapter includes some of the results that we presented at their joint 75th birthday symposium. It touches on both their works, since it is on studies of F_1 -ATPase, single molecule studies that rely heavily on John's structural discoveries, and it uses the functional form of a rate equation that expresses the activation free energy of each reaction step in terms of the standard free energy and "reorganization energy" λ for that step. The equation was originally derived for electron transfers, a field in which Les has made many contributions, some of them using this equation. This equation has now been adapted, as in earlier work

[e.g. Marcus, 1968], to treat transfer reactions [Sutin, 1968; Murdoch, 1983; Lewis and Hu, 1984] that are unrelated to electron transfers.

The chapter is organized as follows: In Section 2 some of the experimental background in single molecule experiments is summarized, in particular, the free rotation, stalling and controlled rotation experiments. In Section 3 the functional form of the free energy of activation *versus* standard free energy of reaction for the various processes is described and used to obtain an equation for the various rotor angle dependent rate constants. Experimental results are described in Section 4. A theory of the free rotation experiments used by us [Volkán-Kacsó and Marcus, 2015] is described in Section 4, leading to Eq. (13), that was used to extract a key quantity (ΔG_0^0) that is used in predicting angle dependent rate constants in other experiments. The derivation is based on fluctuations from a “dwell” angle and angle dependent rate constants.

In Section 4.3 the dwell angles and substeps are discussed. For concreteness and self consistency we use in this article the dwell angles estimated in single molecule experiments, but since the equations are expressed in terms of the symbols θ_i and θ_f other values can be used instead. It is shown in Section 4.3 how an approximate difference in dwell angles for two successive reaction steps can be predicted from kinetic studies. It is applied to the ATP binding step and to the hydrolysis step. The results illustrate how incomplete or sparse data, in this case for the hydrolysis step, can be used and is particularly helpful when the existing kinetic data are very sparse, as they are for the hydrolysis step.

The biological function of the F_0F_1 -ATP synthase is to regenerate, from ADP and Pi, “spent” ATP molecules used as a “fuel” in a multitude of cellular processes. [Boyer, 1993] ATP synthesis being energetically uphill, it is achieved by coupling a unidirectional mechanical rotation of a central γ shaft in the F_0 ring, prompted by a local proton transfer between offset ion channels in the F_0 (E.g., [Junge and Nelson, 2015]), and the chemistry in the F_1 ring. Single-molecule studies have been thus far focused on the study of the water-soluble F_1 -ATPase, [e.g., Noji *et al.*, 1997; Yasuda *et al.*, 2001; Adachi *et al.*, 2012; Sielaff *et al.*, 2008; Spetzler *et al.*, 2009; Watanabe *et al.*, 2010] a complex formed by the F_1 ring and the γ shaft, which functions in a reverse direction and converts the free energy of the inverse process, ATP hydrolysis, to unidirectional rotation in the opposite direction.

Single molecule techniques have thus been providing quantitative data on the dynamics of the component F_1 of F_0F_1 -ATP synthase, complementing ensemble biochemical and structural experiments [Braig *et al.*, 2000; Boyer, 1993; Senior, 2007; Weber, 2010]. In treating research data, typically in the hydrolysis direction of the F_1 -ATPase motor, the goal is that the mechanism for the enzyme ATP synthase can be elucidated, assuming reversibility in the chemo-mechanical rotation Scheme [Adachi *et al.*, 2007, 2012].

In the present article we analyze three types of single molecule studies: stalling, controlled rotation and free rotation experiments using a chemo-mechanical theory. Controlled rotation data were predicted using the stalling data [Volkán-Kacso and Marcus, 2016]. We derive in Section 3.1 an equation, Eq. 2, that we assumed previously in treating the free rotation experiments. This equation is key to relating the properties of the latter system to the quasistatic rate constants extracted from the stalling and controlled rotation experiments.

We review in Section 3 a chemical-mechanical theory [Volkán-Kacsó and Marcus, 2015] that we formulated to treat several types of single molecule experiments on a biomolecular motor [Adachi *et al.*, 2007, 2012]. The theory is adapted from a theory of electron and other transfers in solution [Marcus, 1968; Marcus and Sutin, 1985; Marcus, 1993] and in addition contains the torsional elasticity effect [Sielaff *et al.*, 2008] in the motor in the various physical and chemical processes, such as ATP binding and ATP hydrolysis.

2. Description of Three Single-Molecule Experiments

2.1. Free rotation experiments: Stepping rotation and concerted kinetics

By attaching the F_1 “stator” ring subunit to a microscope slide the rotation of the “rotor” shaft has been monitored by dark field and fluorescent single molecule microscopy. In one version of this experiment a microfilament was attached to the rotor which rotates under heavy viscous load due to friction in the solution [Noji *et al.*, 1997]. When the viscous drag was reduced by replacing the probe with a submicron-sized bead, a counter-clockwise stepping rotation (when viewed from the F_o side) with dwell angles 120° apart was resolved [Adachi *et al.*, 2000]. Later, substeps of 40° and 80° were resolved using a lower ATP concentration [Yasuda *et al.*, 2001].

A chemo-mechanical scheme shown in Figure 1 has been established by a series of single-molecule experiments, in which ATP binding and ADP release (from a different β subunit) are concerted events (80° substep) and ATP hydrolysis and Pi release (again, from a different β subunit) occur during a 40° substep [Nishizaka *et al.*, 2004; Adachi *et al.*, 2007; Senior, 2007; Watanabe *et al.*, 2010]. In the scheme, when following the events in the same subunit, ATP binding occurs during the 80° step following a binding dwell set to 0° [Yasuda *et al.*, 2001]. The ATP does not undergo hydrolysis until much later in the rotation, during the 200° to 240° step, a 40° step. ADP release follows during the subsequent 80° step, from 240° to 320° . Finally, Pi release occurs during the last 40° step, from 320° to 360° .

The timescale of rotation of the γ subunit the biochemically active ATP synthase is comparable to that occurring in the free rotation experiments (ms).

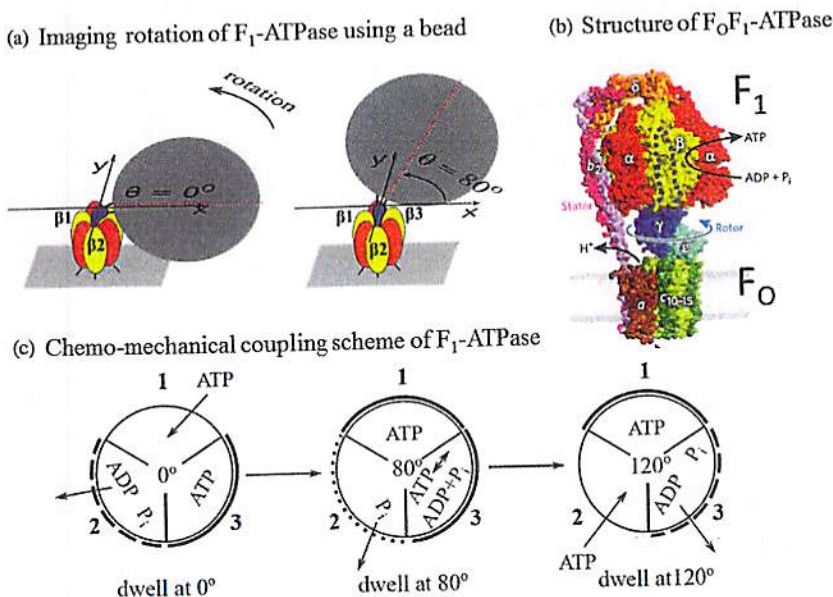


Figure 1. (a) Imaging rotation of F₁-ATPase with rotor fixed to the microscope slide and imaging bead attached to the rotor. The example shows a step of 80°. (b) Structure of the complete membrane embedded F₀F₁-ATPase, seen upside-down relative to the F₁-ATPase in a, reproduced from Weber [2010] with journal permission. (c) Scheme of coupled processes in F₁-ATPase during free rotation. The dwell angle increases in the counter clockwise direction. The species occupying the pockets of ring β subunits 1, 2 and 3.

We note that a high-speed rotation assay using gold nanorods as probes attached to the shaft [Spetzler *et al.*, 2009] permitted the study of the angular velocity of the rotor during the transition between dwells (timescales of μ s). In this article we focus instead on free rotation experiments (ms) that display the stepping kinetics and yield trajectories from which the angle-dependent rate constant data were extracted from the dwell times. More will undoubtedly be learned from the short timescale fast rotation on the physiological timescale.

2.2. Stalling experiments

In these experiments the imaging beads were replaced by double beads possessing a permanent magnetic dipole moment [Adachi *et al.*, 2012]. When these micro-magnets were attached to the rotor (Figure 2a) and an external magnetic field was applied, the rotor aligned itself to the direction of the controlling external magnetic field. The setup, termed *magnetic tweezers*, can be used to set an arbitrary rotor angle θ .

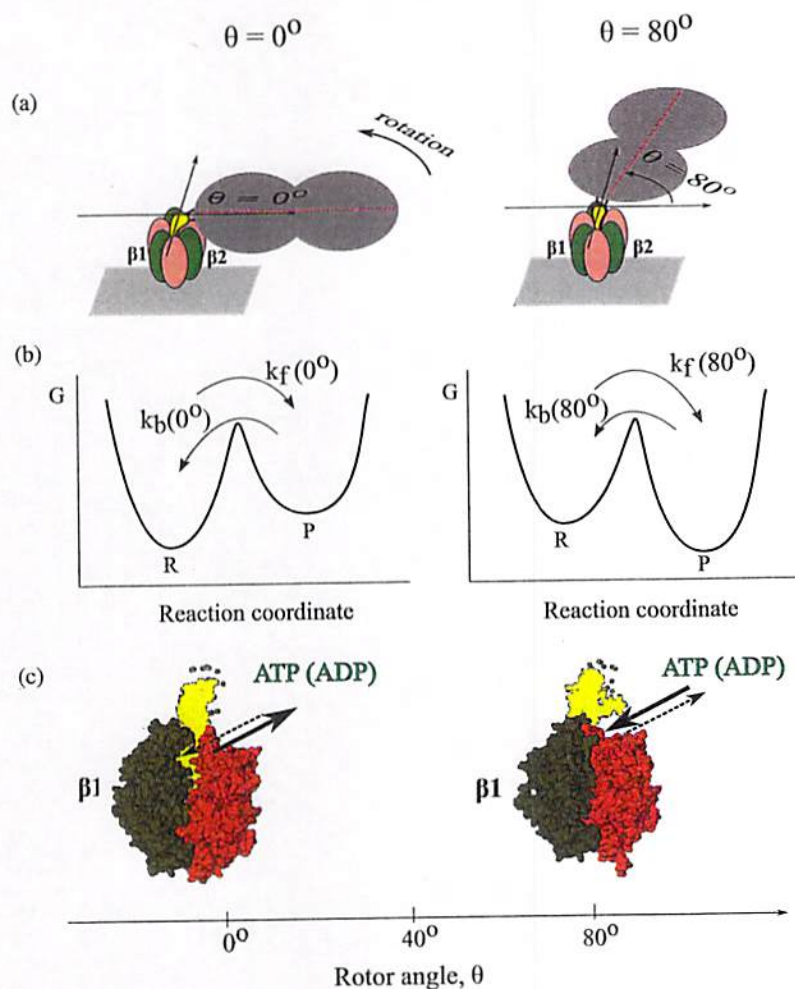


Figure 2. (a) F₁-ATPase in single molecule imaging and controlled rotation experiments at two rotor angles: 0° and 80° . A double-bead is attached to the γ rotor shaft (in yellow) and rotated against the stator ring (active subunits β_1 - β_3). (b) Free energy profile for nucleotide binding (k_b) and release (k_f) rate constants at the two angles. (c) Open-to-close changes in the nucleotide binding β_1 subunit as a function of rotor angle.

Noji and coworkers have devised an ingenious stalling technique for using the magnetic tweezers to extract rate constant data [Watanabe *et al.*, 2010]. In these stalling experiments, a freely rotating shaft was allowed to reach a dwell angle (e.g., the binding dwell), then the tweezers were immediately activated to set the rotor to an arbitrary angle θ . The rotor was then stalled at θ for a given time, after which it was released and it either moved back to the original dwell or forward to the next dwell.

After repeating these experiments for a series of dwell times, Adachi *et al.* [2012] interpreted the statistics of forward and back rotations in terms of a two-state model. They assumed that (1) during the stall time, the system can undergo several forward and backward processes, and (2) the rotations (forward or back) upon release depended on the state of the underlying process (*e.g.*, for ATP binding either ATP-bound or empty) at the moment of release. The observed relative number of forward and back events as a function of stall time yielded the forward (k_f) and backward (k_b) rate constants of ATP binding, or other processes. The model of elastic transfer of nucleotides was proposed to explain this rate constants *versus* θ data in terms of the effect of the elastic response of the rotor structure on the free energy surface (Figure 2b) [Volkán-Kacsó and Marcus, 2015].

2.3. Controlled rotation experiments

By replacing the “wild-type” nucleotides, ATP or ADP, by a modified nucleotide that fluoresces, single-molecule fluorescence microscopy was used to monitor individual binding and release events [Adachi *et al.*, 2007]. In particular, a fluorescent Cy3 moiety was attached to the nucleotides *via* a flexible alkane tether. In the experiments, when the Cy3-nucleotide bound to the F_1 -ATPase the Cy3 moiety emitted a fluorescence signal but when it was in the solution the fluorescence was quenched.

Adachi *et al.* [2012] employed the fluorescence Cy3-nucleotide in conjunction with the rotation microscopy and magnetic tweezers using the micro-bead probes. To do so, the shaft was rotated at a constant rate and the individual binding and release events of fluorescent nucleotides, and so the site occupancy, were directly monitored [Adachi *et al.*, 2012]. By keeping a low ATP concentration, events of occupancy changing between 0 and 1 were determined and analyzed (Figure 2c). Using a grouping criterion of binding and release times, the number of $(0 \rightarrow 1)$ and $(1 \rightarrow 0)$ events state yielded forward and reverse rate constants as a function of rotor angle. The feature that is especially informative is comparing the results of such low occupancy experiments with the physiologically relevant experimental results where the occupancy is 2 or 3.

3. Group Transfer Theory in Stalling and Controlled Rotation Experiments

We give in this section an overview of the group transfer theory first proposed to treat the stalling experiments [Volkán-Kacsó and Marcus, 2015] and then used to predict controlled rotation experiment data [Volkán-Kacsó and Marcus, 2016], and later including composite events to extract hydrolysis and synthesis data from the trajectories [Volkán-Kacsó and Marcus, 2017]. Then, using the theoretical

framework, the average rate constants extracted from dwell distributions in the free rotation experiments were then calculated.

3.1. Angle dependent rate constants and free energies

In a transition-state picture, the activation free energy barrier ΔG^* for a reaction determines the rate constant of the process. The rate constant k is given by

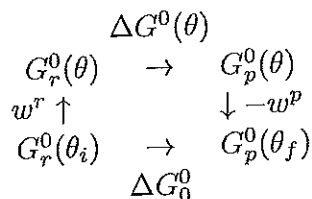
$$k = A \exp(-\Delta G^*/kT) \quad (1)$$

where A depends on the reaction order and the details of the transition state of the process. For a bimolecular process the A can be expressed in terms of a collision theory [Volkán-Kacsó and Marcus, 2015] in treating ATP binding that involves a weak binding site of an empty β subunit. The pre-exponential term A is a collision frequency Z , and a term related to an ATP binding outside of the β subunit. For the binding and release of ATP there are extensive single molecule data obtained by different methods [Yasuda *et al.*, 2001; Spetzler *et al.*, 2009; Watanabe *et al.*, 2010; Braig *et al.*, 2000; Watanabe *et al.*, 2012; Adachi *et al.*, 2012]. In the ATP binding an ATP first forms a collision complex with the F_1 -ATPase, followed by an ATP binding in an 80° step in the overall hydrolysis direction [Oster and Wang, 2000].

To treat the free energy barrier in Eq. (1), we note that the equations of the “weak-overlap” theory of electron transfer reactions were adapted to treat “strong overlap” reactions, such as the transfer of an atom in a reaction [Marcus, 1968; Marcus and Sutin, 1985; Marcus, 1993]. We have applied this idea to treat the transfer of an even larger species, such as a nucleotide, ATP or ADP, during binding to the F_1 -ATPase, while the rotor angle θ is fixed, *e.g.*, as in the stalling experiments. Taking into account a work term W^r for attaching the ATP from solution to the exterior of the F_1 -ATPase (a weak binding site), to the binding pocket (strong binding site) the well-known quadratic equation is written as a function of the rotor angle θ .

$$\Delta G^*(\theta) = W^r + [\lambda + \Delta G^0(\theta)]^2/4\lambda \quad (2)$$

In Eq. (2) ΔG^0 denotes the standard free energy reaction of the transfer process, a function of θ , and λ denotes the “reorganization energy” for the reaction. The quadratic formalism has been tested experimentally in its adaptation to strong overlap processes [Sutin, 1968; Lewis and Hu, 1984] and theoretically [Murdoch, 1983]. A related approach leading to a related equation is based on a bond energy-bond order model (BEBO) [Marcus, 1968 and reference cited therein] in which the sum of the bond orders of the reactant and product state bonds is assumed constant during the reaction. It served to explain why the quadratic equation given in Eq. (2) derived initially for weak overlap electron transfer reactions, can be



Scheme 1.

applied to other transfer reactions. In the BEBO model the activation energy of the transfer is roughly 10% of the total bond energy. It is easier to break a chemical bond if at the same time a new chemical bond is also forming, in this case the process is 10 times easier in terms of energetics. This cooperation is assumed when applying Eq. (2) to the physical processes of binding a nucleotide and release, which involve the breaking and forming of bonds such as hydrogen bonds and “hydrophobic” bonds.

To calculate the θ dependence of the standard free energy of reaction $\Delta G^0(\theta)$ a thermodynamic cycle (Scheme 1) was used [Volkán-Kacsó and Marcus, 2015]. The energy balance around the cycle provides a relationship between the free energies of a ligand binding in free rotation, ΔG_0^0 , and the binding free energy $\Delta G^0(\theta)$ at a constant rotor angle θ .

In Scheme 1, $\Delta G_r^0(\theta)$ and $\Delta G_p^0(\theta)$ are the free energies of the system in the reactant and product states when the rotor is at an angle θ , while $\Delta G_r^0(\theta_i)$ and $\Delta G_p^0(\theta_f)$ are the reactant and product free energies in the relaxed system at dwell angles θ_i and θ_f , respectively. We assume an elastic coupling first suggested by Junge and coworkers [Panke *et al.*, 2001; Sielaff *et al.*, 2008] between the rotor angle and the stator subunit conformation [Volkán-Kacsó and Marcus, 2015] involved in each of these steps. Accordingly, the system, while in the reactant state, can be rotated from a dwell angle θ_i to an arbitrary angle θ by elastically distorting the structure, by doing a work $w_r(\theta)$ provided externally by the external magnetic field of the magnetic tweezers. To achieve a rotation from θ_i to θ , parts of the structure (mainly the rotor and to some extent the lever arm of the β subunit) undergo an elastic twisting deformation of stiffness κ .

Analogously, the F_1 -ATPase in the product state, can be rotated from an angle θ to the relaxed state at θ_f by doing a work $w_p(\theta)$, a work done by the system. To achieve this rotation an elastic twisting deformation of stiffness κ must occur. The elastic work terms then are

$$w^r = \frac{\kappa}{2}(\theta - \theta_i)^2 \quad \text{and} \quad w^p = \frac{\kappa}{2}(\theta - \theta_f)^2. \quad (3)$$

The energy balance in Scheme 1 requires that

$$\Delta G^0(\theta) = \Delta G_0^0 + w^p(\theta) - w^r(\theta) = \Delta G_0^0 - \kappa(\theta_f - \theta_i)(\theta - \theta_c), \quad (4)$$

where the notation $\theta_c = (\theta_f + \theta_i)/2$ was applied and ΔG_0^0 is the free energy change corresponding to the difference between the free energy minima in the final dwell state *versus* that in the initial dwell state. Using Eqs. (1)–(5) we have shown [Volkán-Kacsó and Marcus (2017)] that the theory provides a symmetric expression for the rate constant *versus* rotor angle dependence,

$$\begin{aligned} kT \ln k_f(\theta) &= kT \ln k_f(\theta_c) + (1/2 + \Delta G_0^0/2\lambda)\Delta\theta_r - (\Delta\theta_r)^2/4\lambda, \\ kT \ln k_b(\theta) &= kT \ln k_b(\theta_c) + (-1/2 + \Delta G_0^0/2\lambda)\Delta\theta_r - (\Delta\theta_r)^2/4\lambda, \end{aligned} \quad (5)$$

where $\Delta\theta_r = \kappa(\theta_f - \theta_i)(\theta - \theta_c)$ and $\theta_c = (\theta_i + \theta_f)/2$, as defined in Volkán-Kacsó and Marcus [2015]. The quadratic term is small in the case of the ATP binding step (less than 8% contribution). We note that for the equilibrium constant this dependence is predicted to be exponential,

$$kT \ln K(\theta) = kT \ln K(\theta_c) - \kappa(\theta_f - \theta_i)(\theta - \theta_c). \quad (6)$$

3.2. Application to ATP binding in the overlapping θ -range

The stalling and controlled rotation experiments are complementary in several respects. Both experiments provide the θ -dependent rate constants and equilibrium constants for the steps in the overall process. There is a region of angles θ roughly -45° to $+45^\circ$ where the controlled rotation and stalling data overlap. Important differences are that in the former a fluorescent species, Cy3-ATP and Cy3-ADP, is used instead of ATP and ADP. Further, the nucleotide concentration in the controlled rotation experiments is kept low such that the occupancy is at most 1, while in the stalling experiments the system has the physiologically relevant occupancy 2 to 3 by nucleotides. Due to the finite time resolution of the single molecule fluorescence signal, in the controlled rotation experiments, there are certain missed events when the rate of nucleotide release is very fast. These missed events were calculated using the theory embodied in the previous equations [Volkán-Kacsó and Marcus, 2016].

Turning now to the quantitative analysis of the rate constant *versus* rotor angle data in these experiments, we first note that the $\ln K(\theta)$ seen in Eq. (7) to be linear in θ , in agreement with the experimental data [Volkán-Kacsó and Marcus, 2015]. With data from other ensemble [Boyer, 1993; Weber and Senior, 1997] and single molecule [Yasuda *et al.*, 2001; Adachi *et al.*, 2012; Spetzler *et al.*, 2009] experiments Eqs. (5)–(6) were used to predict [Volkán-Kacsó and Marcus, 2015] for the stalling experiments on ATP binding the Bronsted slope $\alpha = \partial \ln k_f / \partial \ln K$ of 0.47,

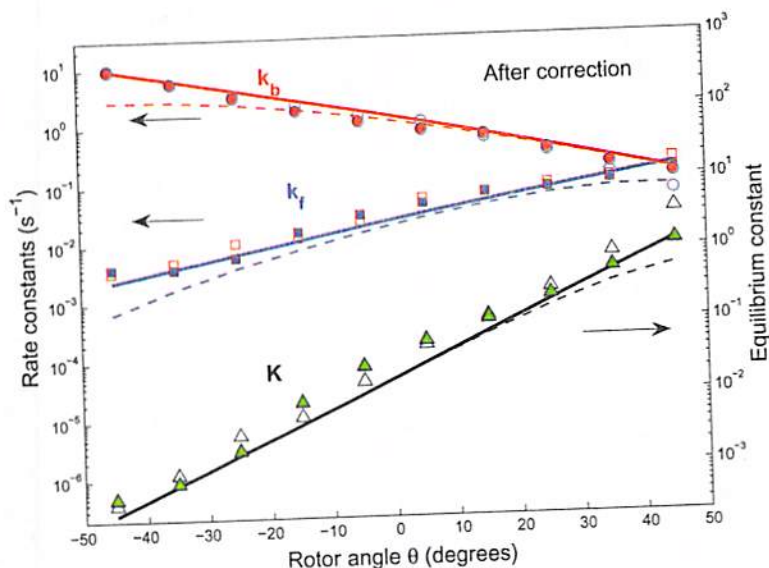


Figure 3. Corrected binding and release rate and equilibrium rate constants versus θ angle for Cy3-ATP in the presence (solid squares, circles and triangles) and absence of Pi (open symbols) in solution adapted from Volkán-Kacsó and Marcus [2016]. The experimental data of Adachi *et al.* [2012] corrected for missed events (and an error due to replacing the time spent in the empty state by total time of a trajectory) are compared with their theoretical counterparts (solid lines). Dashed lines show the data without corrections.

which compares with the value of 0.48 in the stalling experiment for the rate of ATP binding and release over the θ range studied. For the ATP binding rate k_f the predicted slope of the $\ln k_f(\theta)$ versus θ was found to be in reasonable agreement ($\sim$ within 10%) with experiment [Volkán-Kacsó and Marcus, 2015]. For the spring constant κ of the rotor that appears in Eq. (3) the value of $\kappa = 16$ pN nm rad $^{-2}$ obtained from the stalling experiments was used.

The stalling data, supplemented by ensemble data, was used to predict, using Eq. (5), the rate constants in the controlled rotation experiments with no adjustable parameters. The comparison is given in Figure 3, in the θ -range of overlap. Good agreement was found between these experiments and our calculations, seen there, where the points (symbols) are experimental and the solid curves theoretical data.

3.3. Turnover, near symmetry and long binding events in the controlled rotation experiments

To explain a turnover in binding seen in controlled rotation data seen in Figure 4, it was suggested [Volkán-Kacsó and Marcus, 2016] that a slow diffusion process occurs after the binding to the outside of the enzyme: the diffusion of ATP in a channel at the interface of an α and β subunit, is slowed by the partially closed subunit.

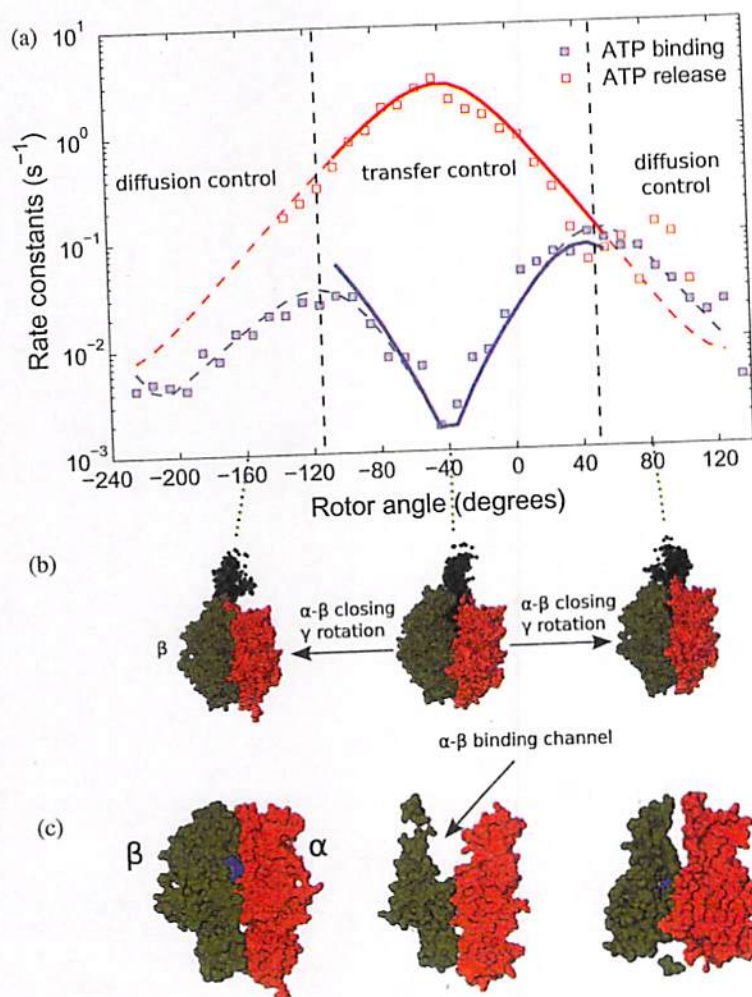


Figure 4. (a) Reported binding and release rate constants *versus* controlled rotation angle for fluorescent ATP in the presence of Pi in solution. The reported uncorrected experimental data (squares) are compared with theoretical counterparts (solid lines) by calculating missed events and also correcting for an error due to replacing the time in the empty state T_0 by the total time T . Dashed lines show a fit to the experimental data. (b) F₁-ATPase structure at three different rotor angles with β subunits in green, α subunits in red and the γ subunit in black. (c) Cutaway of the three structures revealing the binding channel at the α - β interface and its narrowing as the rotor angle is changed.

Structurally, the turnover indicates that the closing of the binding channel continues as the angle is rotated beyond 80°. The turnover is consistent with structural data showing a continuum of open-to-close states between -40° and 140°. A closure of the binding channel (Figure 4c) as the rotor is moved beyond 80° (Figure 4b) retards the diffusion process and it becomes the bottleneck during binding. In a diffusion-reaction scheme [Marcus, 1960; Noyes, 1961], the transfer reaction is the bottleneck in the θ -region from -120° to +45°.

In Figure 4c the β subunit is the most open at -40° and the most closed presumably at $+140^\circ$ (0° is defined as the binding dwell angle at which the β subunit in question is empty and is about to undergo ATP binding associated with a subsequent 80° substep). The minimum at -40° does not correspond to a specific dwell angle. Diffusion reaction treatments are given in Noyes [1961] and Marcus [1960].

Recently, this theory of elastic group was further extended [Volkán-Kacsó and Marcus, 2017] to treat the experimentally observed lifetime distribution of long binding events in the controlled rotation. Using these distributions the long binding events in the experiments can be calculated and the rate constants for the hydrolysis and synthesis reactions occurring during these events extracted. As discussed in a later section a near symmetry of the data about the angle of -40° and a “turnover” in the binding rate data *versus* rotor angle for angles greater than about 40° was interpreted using diffusion-reaction kinetics.

4. Application of the Theory to Free Rotation

4.1. Rate constant of a free rotation experiment

In this subsection we derive a key equation Eq. (10), used to obtain some of the quantities, including ΔG_0^0 , used [Volkán-Kacsó and Marcus, 2015] to predict the controlled rotation results from the stalling data. In a free rotation experiment we suppose that the system jumps from θ_i to θ_f during some fluctuation from its initial value θ_i . The probability of a fluctuation in θ is proportional to $\exp[-C(\theta)/kT]$ where

$$C(\theta) = \kappa(\theta - \theta_i)^2/2. \quad (6)$$

At this θ the free energy of activation for binding for an ATP attached to the outside of an ATPase β -subunit is (apart from a W^r term in Volkán-Kacsó and Marcus [2015] that we include later as part of a factor A). Using Eq. (2) for the forward rate constant $k_f(\theta)$ one finds that

$$k_f(\theta) = A \exp(-\Delta G^*(\theta)/kT), \quad (7)$$

where A is given in Volkán-Kacsó and Marcus [2015] and $\Delta G^*(\theta)$ is given by Eq. (2). The average value of the forward rate constant in a free rotation is then given by

$$\langle k_f \rangle = \int_{\theta_L}^{\theta_H} A e^{-(\Delta G^*(\theta) + C(\theta))/kT} d\theta / \int_{\theta_L}^{\theta_H} e^{-C(\theta)/kT} d\theta. \quad (8)$$

We note that the effective integration limits enclose a peak angle θ^* of the integrand, so $\theta_L < \theta^* < \theta_H$, and are determined by the steepness of the integrand about its maximum. If one neglects the term quadratic in $\Delta G^0(\theta)$ in Eq. (4) (valid, in the case of ATP binding, as seen in the closeness of the Bronsted slope α to the value

0.5) experiment one finds from Eqs. (4)–(7) that the minimum value of exponent in the numerator occurs when θ is given by

$$\theta^* - \theta_i \approx (\theta_f - \theta_i) / 2. \quad (9)$$

The integrand of the exponent in the numerator of Eq. 8 is peaked at this angle $\theta = \theta^*$. Using the saddle point method to evaluate the integral, in the first approximation we find that

$$\langle k_f \rangle \approx k_f(\theta^*), \quad (10)$$

a result used in Volkán-Kacsó and Marcus [2015] to obtain the value of the ΔG_0^0 from the experimental data.

The analysis of the reverse process, ATP release, can be performed in an analogous manner. An important difference is that the initial state for the release, the dwell at 80° , is beyond the interval covered in the stalling experiments. Controlled rotation data can be used to predict the step size and dwell angles, by taking into account the effect of the Cy3 moiety attached to the ATP on binding and release in both controlled and free rotation. Using a functional form for the release rate constant $k_b = A \exp(-\Delta G^b(\theta)/kT)$, where $\Delta G^b(\theta)$ is the barrier for the ATP release step that can be extracted from the controlled rotation data, the analogue of Eq. (8) is

$$\langle k_b \rangle = \int_{\theta_L}^{\theta_H} A e^{-[\Delta G^b(\theta) + C(\theta)]/kT} d\theta \bigg/ \int_{\theta_L}^{\theta_H} e^{-C(\theta)/kT} d\theta. \quad (12)$$

According to Eq. (5) and as described for Pi release in Volkán-Kacsó and Marcus [2015], and for nucleotide release in Volkán-Kacsó and Marcus [2016] the $\Delta G^b(\theta)$ is an approximately linear function of the rotor angle. If the saddle point method is used, Eq. (12) yields in first approximation the analogue of Eq. (10),

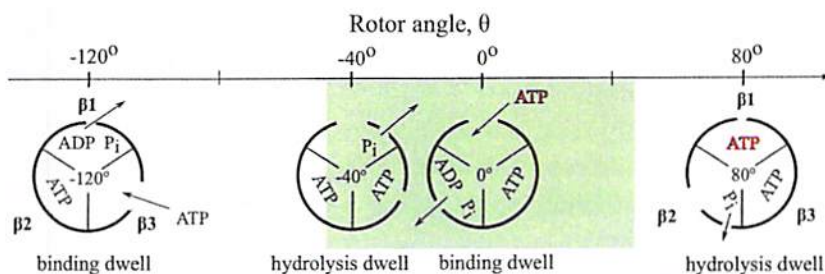
$$\langle k_b \rangle \approx k_b(\theta^*), \quad (13)$$

and the maximum will be at $\theta^* = \theta_c = 40^\circ$.

4.2. Relation between controlled and free rotation experiments

The structural changes of opening and closing are consistent with the trends seen in the slopes of the binding and release rate *versus* rotor angle data and with spontaneous stepping rotation. How can the features revealed in stalling and controlled rotation data in Figures 3 and 4 be connected with the rotational kinetics

(a) Stalling experiments and dwell angles



(b) Controlled rotation at low ATP (ADP) concentration

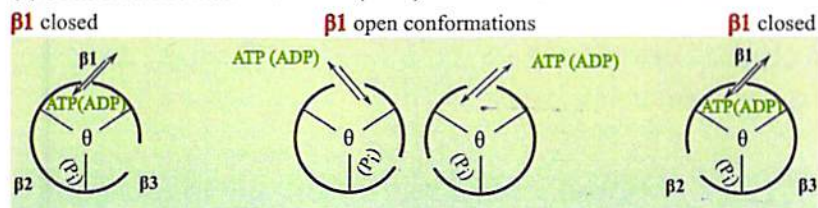
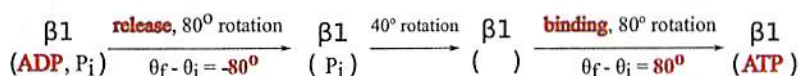
(c) Free stepping rotation and $(\theta_f - \theta_i)$ for nucleotide binding in $\beta 1$ 

Figure 5. Shaft rotation, nucleotide binding/release activity and conformational changes of a ring subunit in single molecule experiments of stalling (a), controlled rotation (b) and free stepping rotation (c). In (a–c) the system is represented at four different values of the rotor angle, corresponding to dwell angles at -120° , -40° , 0° and 80° . θ is defined relative to subunit 1, i.e. $\theta = 0$ for the binding dwell with an empty β subunits.

(Figure 1c) and structural changes? The correspondence between the results of these three experiments, given in the present analysis, based on the theoretical treatment, are summarized in Figure 5.

On the one hand, displacing the rotor to the left or right starting from -40° both accelerate the rate of binding in the controlled rotation experiments. Such displacements of the rotor invariably induce a closing of the β subunit, as seen in the structure in Figure 4c. These effects are consistent with the spontaneous stepping forward rotation induced by binding of ATP in free rotation experiments occurring in a θ -range to the right of -40° (from 0° to 80°).

On the other hand, rotating the rotor towards -40° from the left or from the right both induce an opening of the β subunit which accelerates nucleotide release. This effect, in turn, is consistent with the spontaneous forward rotation induced by the release of ADP in free rotation experiments occurring in a θ -range to the left of -40° (from -120° to -40°).

4.3. Use of the rate constant versus rotor angle data to predict the step size and dwell angles in free rotation

Here we show how the single molecule free rotation kinetics, including step size ($\theta_f - \theta_i$) and dwell angles θ_i and θ_f of the free system, which cannot be resolved in free rotation assays, can be predicted in the rare case that $\ln K(\theta)$ versus θ is linear over the entire range ($\theta_f - \theta_i$). It also presents an example of combining different types of sparse data.

The theory has provided simple mathematical relations between the dwell angles, the spring constant and $\ln k_f$, k_b or K versus rotor angle slopes [Volkán-Kacsó and Marcus, 2015; Volkán-Kacsó and Marcus, 2016, 2017]. To predict dwell angles, an independent estimate of the spring constant κ is needed, *e.g.*, from fluctuation experiments. [Sielaff *et al.*, 2008] Using the expression from Eq. (6) for the slope $kT \partial \ln k(\theta) / \partial \theta = K(\theta_f - \theta_i)$ of angle-dependent rate constant, obtained from controlled rotation experiments, the predicted step size in free rotation experiments, ($\theta_f - \theta_i$) is given by

$$\theta_f - \theta_i = \frac{kT}{\kappa} \frac{\partial \ln K(\theta)}{\partial \theta}. \quad (14)$$

In the present application of Eq. (14) for the hydrolysis step the linearity spans an angular range of (150°, 250°) [Watanabe *et al.*, 2012] with $\theta_i = 200$. From these data and Eq. (14) one obtains $\theta_f = \theta_i + 15^\circ = 215^\circ$.

The experimental slope for the equilibrium constant $\partial \ln K(\theta) / \partial \theta = 1.9 \text{ deg}^{-1}$ and a $\kappa = 16 \text{ pN nm/rad}^2$ yield a predicted step size of $\theta_f - \theta_i = 15^\circ$. We note that the stiffness $\kappa = 16 \text{ pN nm/rad}^2$ corresponds to the ATP binding step, and we use this value in the present analysis to approximate the κ for the hydrolysis step. Different steps likely have somewhat different κ values, *e.g.* in Volkán-Kacsó and Marcus [2016] we found for ADP binding a $\kappa = 12 \text{ pN nm/rad}^2$ which is smaller than the $\kappa = 16 \text{ pN nm/rad}^2$ of ATP binding.

A separate hydrolysis and a Pi release step was not directly resolved in single molecule trajectories, but a difference of about 10° in the stepping size in the 90°/30° and 80°/40° stepping ratio seen in different experiments lead Adachi *et al.* [2007] to speculate that this 10° difference is due to the hydrolysis step. The present calculated value of $\theta_f - \theta_i = 15^\circ$ is in reasonable agreement with this assertion, considering the sparsity of the data of Adachi *et al.* [2012]. These data also suggest a Bronsted slope close to unity for the hydrolysis step which we speculate could indicate the presence of two or more substeps during hydrolysis [Shweins and Warshel, 1996]. We note that Eq. (14) does not apply for binding since $\ln K(\theta)$ versus θ is not linear over the whole range of (θ_p, θ_b) for binding (*e.g.*, as seen in the lack of the linearity over the complete range in Figure 4a for binding).

In a crystallographic method of studying the kinetic states (dwells) in active rotation Walker and coworkers prepared stable states by using substitutions in the binding species [Bason *et al.*, 2015]. By resolving such the X-ray crystallography structures of mammalian (human and bovine) F_1 -ATPase, three stable states were found about 65° , 25° and 30° apart and it was suggested that these states correspond to dwell angles that define the stepping spontaneous rotation of the F_1 -ATPase. The 65° , 25° and 30° division is somewhat similar to the 80° , 10° and 30° division proposed from single molecule experiments by Kinoshita and coworkers for the thermophilic bacillus F_1 -ATPase [Adachi *et al.*, 2007].

5. Concluding Remarks

In the present chapter, we have illustrated how an elastic group transfer theory can be used to treat three types of single molecule experiments: stalling, controlled rotation and free rotation experiments. To do so, we first reviewed the relations for the rate constants of substeps such as ATP binding or ADP release provided using the theory to treat and predict the stalling and controlled rotation data in previous studies [Volkán-Kacsó and Marcus, 2015, 2016, 2017]. A new feature in the present book chapter is the calculation of average rate constant that can be extracted from the dwell time distributions in the free rotation experiments, Eq. (10), used in an earlier work. We also demonstrated how the difference in dwell angles for two successive reaction steps in the free rotation experiments can be predicted from rate constant *versus* rotor angle data from stalling and/or controlled rotation experiments. These predictions were applied to the ATP binding 80° substep, and the hydrolysis substep, supporting an earlier contention that the latter is a small (10°) step.

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Appendix

Evaluation of Eq. (8)

We explore here the approximation for ATP binding in more detail by evaluating the numerator and the denominator. For ATP binding $\theta_i = 0^\circ$ and $\theta_f = 80^\circ$ and we found using the present Eqs. (9) and (10) [Volkán-Kacsó and Marcus, 2015] that $\Delta G_0^0 = 6 \text{ kcal/mol} \ll 2\lambda = 136 \text{ kcal/mol}$ and $\kappa = 16 \text{ pN nm/rad}^2$.

In the numerator in Eq. (10), $\theta^* = \theta_c = 40^\circ$ and Eq. 7 is strictly applicable in the θ range covered by stalling experiments of Adachi *et al.* [2012], roughly $-45^\circ < \theta < 45^\circ$. The integrand at the lower bound of $\theta_L \approx -45^\circ$ is smaller than the maximal value by a factor of $\exp(1/8\beta\kappa c^2 - \alpha\lambda^2) / \exp(-3/4\beta\kappa c^2 - \alpha\lambda^2) \approx \exp(5.3) \approx 200$. The angle $\theta^* \approx 40^\circ$ is close to a 45° upper bound, and so an empirical functional form can be used as described in Volkán-Kacsó and Marcus [2017], to extend Eq. (7) beyond the turn-over in controlled rotation experiments. In this case, for an upper bound at $\theta_H \approx 60^\circ$, the integrand is estimated to have decayed roughly by a factor of $\exp(1/8\beta\kappa c^2) / \exp(-1/8\beta\kappa c^2) \approx \exp(2.2) \approx 9$. Using the saddle-point approximation that treats the integrand as a Gaussian in a $(-\infty, +\infty)$ interval of θ , we have

$$\int_{\theta_L}^{\theta_H} A e^{-[\Delta G^*(\theta) + C(\theta)]/kT} d\theta \approx \sqrt{2\pi / \kappa} A e^{-[\Delta G^*(\theta^*) + C(\theta^*)]/kT}. \quad (11)$$

In the denominator of Eq. (8) the Gaussian is centered at $\theta^* = 0^\circ$, the elastic approximation in Eq. (6) has been also found to apply within the range of the stalling experiments [Adachi *et al.*, 2012], although it likely applies beyond it [Panke *et al.*, 2001]. For a $\theta_L \approx -45^\circ$ and $\theta_H \approx 45^\circ$ the integrand has decayed relative to the peak by a factor of about $\exp(1.34) \approx 4$ so the Laplace approximation yields $\sqrt{2\pi / \kappa} \exp(-C(\theta^*) / kT)$.

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