

Integration of F₁ ATPase kinetic data and thermodynamics reveals a hidden tri-site intermediate

Corresponding Author: Dr Shintaroh Kubo

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

In the manuscript "Integration of F₁ ATPase kinetic data and thermodynamics reveals a hidden tri-site intermediate" Kubo et al. propose a "minimal" model for angle-dependent rotation kinetics (i.e., angle-dependent rotation rates) seen in F₁-ATPase single-molecule experiments. A claim of the authors is that the model is thermodynamically consistent, and thus more complete than previously published models in the field. This manuscript is relevant and important because it advances the theory-experiment interaction in the field of single-molecule imaging of the rotary F₁-ATPase motor. Another major result is that their calculations are consistent with the presence of a 3 nucleotide occupancy state. The latter is however a proposal already published in the literature and so not novel for the current manuscript (see comment A below).

Major comments and questions:

A. In the Introduction on page 4 the authors "introduce an extended model that includes a tri-site intermediate as a transient state so as to meet the thermodynamic energy balance of ATP hydrolysis." The authors seem to be unaware that this 3 occupancy state was already introduced several years ago (2019) in Ref. 17. So, the statement needs to be corrected and the authors should give credit to Ref. 17 for introducing the short-lived 3-site intermediate.

B. In Table 1 the authors list the angle-dependent rate constant for the processes (ATP binding, ATP hydrolysis, Pi release) treated in their calculations. These are based on previous stalling and controlled rotation single molecule experiments (refs. 8-10). In these references, there was a difficulty in accurately determining the angle-dependent Pi release rate (in ref 8 it is essentially inferred from only 2 widely separated data points). How are the outcomes of the calculations on Fig. 5 affected by this uncertainty in the Pi release rates vs angle? The authors claim that their results calculated using these angle-dependent rates are thermodynamically consistent. Does this conclusion stand if Pi release rates are changed in the calculations, or the results are relatively insensitive to the rates of Pi release?

C. The authors use a friction coefficient of $2.0 \times 10^{-8} \text{ s} \cdot \text{pN/nm}$. The friction coefficient is not directly measured in the single-molecule experiments, rather estimated using the Stokes law applied to a spherical probe in water. However, the probe is likely "dragging" along the surface of the microscope slide on which the F₁-ATPase is also anchored. Consequently, this friction is likely an underestimate. How sensitive are the results from Figs. 4-6 to an increase in γ ? What are the overall uncertainties in these results?

D. It seems reasonable to assume that the stall torque is dependent on the value of the friction coefficient γ . Can the authors verify this? If so, a larger friction coefficient would result in a different stall torque. What is the uncertainty of the calculated stall torque, when the uncertainty in γ is included?

E. In a recent manuscript (Toyabe, S., & Wang, Z. (2026). BBA Bioenergetics, 149583) torque-dependent forward and back rotation statistics was studied and the stall torque was determined as 31 pN.nm. The authors could compare their ATP concentration dependent results on the stall torque with the results of Toyabe and Wang.

Minor remarks:

A. Tables 1 and 2: some quantities have missing units.

B. Some references need to be corrected. E.g., in ref. 22 the title is all-capital letter and it has missing/ill formatted words.

Reviewer #2

(Remarks to the Author)

The authors present a phenomenological model for the ATP driven rotation of the FoF₁ ATPase. As such, the paper is well

written and clear. What I do not see is the predictive value of this model - the authors, relying on observation that the motor rotates in a particular direction insert rate constants consistent with this observation but make no attempt to address the fundamental question of why it rotates one direction and not another. The authors claim "Together, these results point to simple design rules for F1's near-lossless chemical-to-mechanical energy conversion". OK, so what are these design rules?

There is a shocking gap in the bibliography of this paper - nowhere is the work of Warshel and colleagues mentioned. But it is precisely that body of work that calls into question many of the tacit assumptions made in the paper. Figure three c) for example conflates the energy of a molecule - a well defined concept - with chemical potential, defined only as an ensemble quantity. It is meaningless to refer to the chemical potential of a state without specifying the state probabilities of other states. chemical potential difference is well defined, but chemical potential (like any other potential) is not. The authors must discuss what exactly they mean in order for the figure to have any value. We know that the free-energy of a molecule depends on its chemical and rotational state, and we know that the probability to make a full 360 degree transition in a clockwise direction is greater than the probability to make a full 360 degree transition in the counterclockwise direction ONLY if the chemical potential difference between ATP and ADP and Pi is different than zero. I do not see how the authors model reflects this truth, nor whether the model predicts which way the rotor will rotate when there is a lot of ATP and very little ADP.

I am sorry to say that at present the paper seems more confusing than edifying.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have satisfactorily responded to my questions and remarks. However, regarding remarks/questions B, D and E: while they provided "rebuttals" in their answer they did not make any improvements in the manuscript text (such as added or modified sentences) in response to these remarks. I would strongly encourage the authors to make such changes to further improve the quality of the manuscript.

Reviewer #2

(Remarks to the Author)

This paper is a well written description of kinetic modeling of experimental data - nothing more. The authors have failed to take up the challenge of explaining why the F1 turns clockwise rather than counterclockwise. The sequential parabolas serve as *trompe l'oeil* to fool the eye into thinking the motion could not be otherwise, but this, as has been shown in many papers, is not correct. When one actually solves the reaction diffusion equations with rate constants that are consistent with both microscopic reversibility and with Curie symmetry constraints, the surprising result is that the shape of the periodic potential used in the modelling has little or nothing to do with determining the preferred direction of motion. The essential feature that determines the direction is kinetic asymmetry - at what angle binding/release of ATP is fast and at what angle is release of Pi fast, etc.

The authors failed to address this key concern that I expressed in my original report, and instead simply reiterated that their kinetic model is based on and driven by the experimental data. I will leave it up to the editors to decide whether this is a sufficient contribution to justify publication, in my opinion it is not.

Reviewer #3

(Remarks to the Author)

The authors have convincingly addressed my comments, and I have no further requests. I think this is a very interesting study.

Florian E.C. Blanc

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Reviewer #1:

Major comments and questions:

A. In the Introduction on page 4 the authors “introduce an extended model that includes a tri-site intermediate as a transient state so as to meet the thermodynamic energy balance of ATP hydrolysis.” The authors seem to be unaware that this 3 occupancy state was already introduced several years ago (2019) in Ref. 17. So, the statement needs to be corrected and the authors should give credit to Ref. 17 for introducing the short-lived 3-site intermediate.

We thank the reviewer for this comment. We would like to clarify that we do not claim the discovery of tri-site intermediate itself. In fact, as stated in the preceding paragraph, we explicitly acknowledge the previous work that proposed this state: “Advanced theoretical analyses of these datasets also predicted a previously unseen tri-site state—after ATP binding and before ADP release—where all three β subunits are nucleotide-bound (17).”

The novelty of our study lies not in proposing tri-site state, but in systematically comparing models with and without this intermediate and demonstrating that, while tri-site intermediate is not strictly required to reproduce ATP hydrolysis, it plays a critical role in achieving thermodynamically consistent ATP synthesis. To make this point clearer and to properly emphasize the contribution of Ref. 17, we have revised the text as follows:

“To resolve this limitation, we introduce an extended model that includes a tri-site intermediate as a transient state (predicted by (17)) so as to meet the thermodynamic energy balance of ATP hydrolysis.”

In addition, following a comment from Reviewer 3, we have expanded the Introduction to explain in more detail how Ref. 17 inferred the tri-site intermediate from angular-jump statistics in single-molecule rotation trajectories.

B. In Table 1 the authors list the angle-dependent rate constant for the processes (ATP binding, ATP hydrolysis, Pi release) treated in their calculations. These are based on previous stalling and controlled rotation single molecule experiments (refs. 8-10). In these references, there was a difficulty in accurately determining the angle-dependent Pi release rate (in ref 8 it is essentially inferred from only 2 widely separated data points). How are the outcomes of the calculations on Fig. 5 affected by this uncertainty in the Pi release rates vs angle? The authors claim that their results calculated using these angle-dependent rates are thermodynamically consistent. Does this conclusion stand if Pi release rates are changed in the calculations, or the results are relatively insensitive to the rates of Pi release?

We thank the reviewer for raising this important point regarding the uncertainty in the angle dependence of Pi release.

As described in Text S3 (iii), we did not directly use the angle-dependent Pi release rate equation proposed by Watanabe et al. The experimental determination of the Pi release rate is indeed

associated with substantial uncertainty because it was inferred from a limited number of data points. Therefore, we re-parameterized the Pi release kinetics using not only the measurements reported by Watanabe et al. and Adachi et al., but also a more robust estimate from Yasuda et al. (Nature, 2001), which showed that Pi release occurs on a timescale of approximately 1 ms. The resulting rate function was defined so that the Pi release rate reaches this experimentally supported timescale within the angular region where Pi release is expected to occur.

More importantly, the thermodynamic consistency of the model does not critically depend on the precise form of the Pi release rate. As discussed in [Text S4](#), modifying the angular dependence of the Pi release rate primarily affects the position of the Pi-state potential minimum and the angular region in which Pi release is favored. However, the overall thermodynamic balance can still be maintained.

As further described in [Text S5](#), the ADP binding and ADP release processes provide sufficient flexibility to compensate for changes in the Pi release kinetics. Consequently, although quantitative details of the potential landscape may vary, the central conclusion that a thermodynamically consistent kinetic scheme requires the tri-site intermediate remains robust against reasonable variations in the Pi release rate parameters.

C. The authors use a friction coefficient of $2.0 \times 10^{-8} \text{ s} \cdot \text{pN/nm}$. The friction coefficient is not directly measured in the single-molecule experiments, rather estimated using the Stokes law applied to a spherical probe in water. However, the probe is likely “dragging” along the surface of the microscope slide on which the F1-ATPase is also anchored. Consequently, this friction is likely an underestimate. How sensitive are the results from Figs. 4-6 to an increase in γ ? What are the overall uncertainties in these results?

We thank the reviewer for raising this important point. Changing the friction coefficient γ has two distinct effects in our simulations. One is the expected physical effect of changing the viscous resistance. The other is a numerical effect, because the angular coordinate is propagated using Langevin dynamics, in which the magnitude of the stochastic displacement depends on the ratio dt/γ . When the timestep was kept fixed at $1.0 \times 10^{-8} \text{ s}$ and γ was reduced from the reference value of 2.0×10^{-8} to $1.0 \times 10^{-8} \text{ s} \cdot \text{pN/nm}$, the stochastic angular displacement became excessively large and the simulation became numerically unstable. This indicates the existence of a numerical stability limit when γ is reduced without adjusting the timestep.

We therefore examined several values of γ that remained numerically stable under the same timestep (1.5×10^{-8} , 1.8×10^{-8} , 2.2×10^{-8} , and $2.5 \times 10^{-8} \text{ s} \cdot \text{pN/nm}$). Under both the hydrolysis-favoring conditions and the synthesis-favoring conditions with external torque, the simulated rotation rates were very similar to those obtained with the reference value ([new Fig. S2B](#) and [Fig. S2D](#)). Furthermore, the angular distributions of the nucleotide states were essentially unchanged across the

tested γ values (new Fig. S2C and Fig. S2E). These results indicate that, within the range of numerically stable values examined here, the principal conclusions of the model are not strongly sensitive to the precise value of γ . We therefore conclude that the uncertainties associated with the friction coefficient do not substantially affect the mechanistic conclusions presented in this study. A summary of this analysis has been added to the Computational System section.

D. It seems reasonable to assume that the stall torque is dependent on the value of the friction coefficient γ . Can the authors verify this? If so, a larger friction coefficient would result in a different stall torque. What is the uncertainty of the calculated stall torque, when the uncertainty in γ is included?

We thank the reviewer for this comment. We agree that, in a dynamical simulation, the apparent torque at which net rotation stops can depend on the friction coefficient γ , because γ affects the rotational mobility and the time scale of angular relaxation. However, the torque discussed in this manuscript is not the dynamically observed stall torque. Instead, it corresponds to the thermodynamic crossover point at which the preferred direction of rotation changes, i.e., where the mechanical work per 120° step balances the chemical free energy of ATP hydrolysis. Therefore, γ affects the kinetics of how rapidly the rotor moves on the energy landscape, but it does not change the thermodynamic torque required to balance the chemical free-energy difference. So, we have clarified this distinction in the revised manuscript to avoid confusion between the dynamically observed stall condition and the thermodynamic stall torque defined by energy balance as follows:

The resulting crossover torque is therefore determined by the free-energy difference between reactants and products rather than by the rotational friction coefficient γ . While γ affects the rotational kinetics and the time required to reach steady-state behavior, it does not alter the thermodynamic crossover torque itself.

E. In a recent manuscript (Toyabe, S., & Wang, Z. (2026). BBA Bioenergetics, 149583) torque-dependent forward and back rotation statistics was studied and the stall torque was determined as 31 pN.nm. The authors could compare their ATP concentration dependent results on the stall torque with the results of Toyabe and Wang.

We thank the reviewer for this suggestion. We have now included a comparison with the recent work of Toyabe and Wang in the revised manuscript. Toyabe and Wang reported a stall torque of 30.7 ± 2.9 pN·nm/rad under conditions of [ATP] = 0.4 μ M, [ADP] = 4 μ M, and [Pi] = 1 mM. For the same nucleotide concentrations, the thermodynamic stall torque predicted from the chemical free-energy difference in our framework is 34.7 pN·nm/rad. Furthermore, simulations using our model yielded a stall torque of approximately 35 pN·nm/rad (new Fig. S2A). The close agreement between the experimental value, the thermodynamic prediction, and the simulation result provides additional

support for the validity of the model.

Minor remarks:

A. Tables 1 and 2: some quantities have missing units.

B. Some references need to be corrected. E.g., in ref. 22 the title is all-capital letter and it has missing/ill formatted words.

Units have now been added to all quantities in Tables 1 and 2 where they were previously omitted. We also carefully checked and corrected the reference list for formatting inconsistencies and typographical errors. Regarding the example mentioned by the reviewer, we believe there may have been a misunderstanding. The title of Ref. 25; "Rosing, J. & Slater, E. C. THE VALUE OF ΔG° FOR THE HYDROLYSIS OF ATP. Biochim. Biophys. Acta 267, 275–290 (1972)," appears in all capital letters because this is the format used in the original publication. We have therefore retained the original capitalization for this reference while correcting other formatting issues where appropriate.

Reviewer #2:

The authors present a phenomenological model for the ATP driven rotation of the FoF1 ATPase. As such, the paper is well written and clear. What I do not see is the predictive value of this model - the authors, relying on observation that the motor rotates in a particular direct insert rate constants consistent with this observation but make no attempt to address the fundamental question of why it rotates one direction and not another. The authors claim "Together, these results point to simple design rules for F1's near-lossless chemical-to-mechanical energy conversion". OK, so what are these design rules?

We thank the reviewer for this thoughtful comment. We would first like to clarify that our model does not impose directional rotation by construction. The rate constants used in the model were not chosen to reproduce a predetermined direction of rotation. Rather, they were derived from experimentally measured angle-dependent kinetic rates. Starting from these experimentally determined rates, the model naturally reproduces the observed directional rotation of F1-ATPase. In this sense, the directionality is an emergent property of the model rather than an assumption built into it.

We agree, however, that the present work does not directly address the more fundamental question of why the experimentally observed kinetic rates themselves possess the particular directional bias that gives rise to unidirectional rotation. Addressing the molecular origin of these rate constants would require a more microscopic description of the chemical transitions and structural dynamics of the catalytic sites, which is beyond the scope of the present study.

The primary goal of this work is instead to establish a quantitative framework that integrates experimentally measured angle-dependent kinetics with thermodynamic constraints and reproduces

the observed rotational behavior of F₁-ATPase.

By “design rules,” we do not mean a mechanistic explanation of the molecular origin of directionality. **Rather, we refer to the minimal requirements that a kinetic model must satisfy in order to reproduce both the experimentally observed rotational behavior and thermodynamic consistency.**

In particular, our analysis demonstrates that (i) experimentally measured angle-dependent rates alone are insufficient to achieve thermodynamically consistent ATP synthesis, (ii) inclusion of a transient tri-site intermediate resolves this inconsistency, and (iii) the balance between chemical free energy and mechanical work determines the crossover between hydrolysis- and synthesis-dominated rotation.

More broadly, we anticipate that the framework developed here can be applied to other ATPase systems. Although the present model is parameterized using kinetic measurements from TF₁, the same procedure could be used for other species once comparable angle-dependent kinetic measurements become available. Likewise, the framework should be extendable to V₁-ATPases. Such comparative analyses may ultimately reveal which kinetic processes are responsible for species-specific differences in motor performance. It is in this sense that we refer to the extracted principles as “design rules.”

There is a shocking gap in the bibliography of this paper - nowhere is the work of Warshel and colleagues mentioned. But it is precisely that body of work that calls into question many of the tacit assumptions made in the paper. Figure three c) for example conflates the energy of a molecule - a well defined concept - with chemical potential, defined only as an ensemble quantity. It is meaningless to refer to the chemical potential of a state without specifying the state probabilities of other states. chemical potential difference is well defined, but chemical potential (like any other potential) is not. The authors must discuss what exactly they mean in order for the figure to have any value. We know that the free-energy of a molecule depends on its chemical and rotational state, and we know that the probability to make a full 360 degree transition in a clockwise direction is greater than the probability to make a full 360 degree transition in the counterclockwise direction ONLY if the chemical potential difference between ATP and ADP and Pi is different than zero. I do not see how the authors model reflects this truth, nor whether the model predicts which way the rotor will rotate when there is a lot of ATP and very little ADP.

We have now added several representative theoretical studies by Warshel and co-workers in the context of previous mechanistic models of F₁-ATPase.

We sincerely appreciate the reviewer for pointing out the use of the terminology “chemical potential”. In the present modeling, we deal with free energies of several chemical states of F₁ in the environment of given ATP, ADP, and Pi concentrations. Since this is different from the standard free energy that uses 1M concentrations for all quantities, we tempted to use different terminology and called it

“chemical potential curves” in the original manuscript. We admit that this terminology is inappropriate and also confusing. Following the classic textbook of Terrell Hill, we decided to use the terminology “basic free energy” for this quantity. We have explained it in the revised manuscript both in the main text and in the supporting text and changed words and notations throughout.

Reviewer #3:

Major comments:

The authors hypothesize a functional form for the angle-dependent rates, because experimental values are not available over the full angular range. Although the functional forms chosen by the authors seem very reasonable, I am wondering to what extent this choice determines the outcome of the analysis. For example, would a rate function omitting the constant part give a different mechanism? Same question with the estimates of the wild-type catalytic constants; Overall the authors should conduct a sensitivity analysis by trying other (simple) functional forms and values for the parameters, and assess the extent to which their conclusions are robust under these changes.

We thank the reviewer for this important suggestion. We are testing several alternative functional forms, including 180° rise/180° fall, 120° rise/240° fall, and 120° rise/120° plateau/120° fall profiles. The results, especially 180° rise/180° fall and 120° rise/120° plateau/120° fall, are summarized in new Fig. S1CD and discussed in the revised manuscript.

Briefly, the 180° rise/180° fall and 120° rise/240° fall profiles failed to maintain a constant free-energy change over a full rotation cycle and therefore violated thermodynamic consistency. The 120° rise/120° plateau/120° fall profile remained thermodynamically consistent, but was inconsistent with the experimentally observed angular sensitivity. These results support the triangular profile adopted in the manuscript as the simplest model consistent with both thermodynamic and experimental constraints. We have added a brief discussion of these alternative profiles at the end of the paragraph describing the thermodynamic consistency of the extended model.

We also verified that the principal conclusions are robust to moderate variations in the rotational friction coefficient γ for parameter testing.

The authors state that the tri-site intermediate is "too brief to be clearly resolved in existing single molecule records" (page 8) and later estimate its lifetime to 10 μ s. Yet, they mention (page 12) that this is consistent with the single molecule data from ref. 17. This seems a bit contradictory; could the authors clarify to what extent the tri-site intermediate was/was not seen in the data from ref 17?

We thank the reviewer for this helpful comment. We have added a more detailed description of Ref. 17 in the Introduction to clarify how the tri-site intermediate was inferred from angular-jump statistics rather than directly observed as a distinct dwell state.

It would make the impact of the study even greater if the authors could suggest strategies to guide experimental characterization of the tri-site intermediate. As the authors mention, the 10 μ s lifetime of this intermediate in hydrolysis may be too short for single-molecule experiments. I could not find an estimate of the corresponding lifetime of the intermediate in synthesis mode under physiological torque. However, given that the authors convincingly show that the tri-site intermediate is necessary for ATP synthesis, is it reasonable to expect that it may be much longer lived in these conditions, and therefore detectable experimentally? If so, could the authors broadly outline the type of single-molecule experiment that could detect it?

We thank the reviewer for this insightful suggestion. Interestingly, our simulations do not predict that the tri-site intermediate becomes substantially longer-lived during ATP synthesis. Rather, as shown in Fig. 6E and F, the system frequently transitions back and forth between the catalytic dwell state and the tri-site intermediate under synthesis conditions. As a result, the occupancy of the tri-site state increases, even though the lifetime of each individual visit remains relatively short. This behavior can be seen in Fig. 6G, where the angle histograms of individual states show distinct peaks separated by approximately 40°. However, when experimentally observable states are combined, as in Fig. 6H, the primary effect is a broadening of the catalytic-dwell peak rather than the appearance of a clearly separated additional peak. Therefore, direct experimental identification of the tri-site intermediate may remain challenging even under synthesis conditions.

Nevertheless, our results suggest several possible experimental signatures of the tri-site intermediate. For example, increasing the ADP concentration is expected to shift the equilibrium toward the tri-site state in our model, which may enhance the broadening of the catalytic-dwell population. In addition, a more direct approach may be possible using high-resolution cryo-EM analysis of isolated F₁-ATPase. Because the tri-site state becomes more populated under synthesis-favoring conditions, a small subpopulation (potentially a few percent) of particles may be captured in the tri-site conformation and resolved through careful structural classification. These approaches may provide experimentally accessible routes for testing the existence of the proposed intermediate.

Minor comments:

The introduction states "Advanced theoretical analyses of these datasets also predicted a previously unseen tri-site state—after ATP binding and before ADP release—where all three β subunits are nucleotide-bound", citing reference 17 (Marcus et al. PNAS 2015). The authors may wish to give more concrete details on why these analyses concluded to a tri-site state, because this is important for the rest of the paper (this point can be addressed jointly with the second major point above)

We thank the reviewer for this comment. This point has been addressed together with above.

p 6 The authors mention the "known limitations" of the angle-dependent rate datasets. Are the

authors referring only to the fact that these values have not been measured across the whole rotation, which they explain right after, or are there additional limitations with these data? If it is the former, please explain these additional limitations briefly.

We thank the reviewer for pointing this out. The term “known limitations” referred to two issues: (i) the angle-dependent rate constants are available only over limited angular ranges, and (ii) ADP-binding step rate constant coupled with ATP-releasing step in the ATP synthesis have not been directly measured experimentally. We have clarified this point in the revised manuscript.

the color code on Fig1 is not always easy to follow and should be stated explicitly in the caption

We thank the reviewer for this suggestion. We have revised the figure caption accordingly.

• On Fig1C, on the orange to blue transition, $k_{\text{Pi}}^{\text{on}}$ and $k_{\text{Pi}}^{\text{off}}$ should be switched

• Ref (22) is cited as "Chun-Biu et al" in the text but as "Li et al." in the reference list; please fix this inconsistency

• page 12 missing word: "This bias arises (because?) the hydrolysis rate is slightly smaller..."

We thank the reviewer for pointing out these issues. All have been corrected in the revised manuscript.

Reviewer #1

The authors have satisfactorily responded to my questions and remarks. However, regarding remarks/questions B, D and E: while they provided "rebuttals" in their answer they did not make any improvements in the manuscript text (such as added or modified sentences) in response to these remarks. I would strongly encourage the authors to make such changes to further improve the quality of the manuscript.

We thank the reviewer for this helpful suggestion. While unclearly stated in the previous rebuttal letter, we have incorporated the raised points into the manuscript in the first revision either in main text or in SI. This time, we made them clearer.

Comment B: Regarding the discussion of the Pi binding/release rates, all written in the previous rebuttal letter is fully explained in [Text S3 \(iii\)](#) as below:

Quotation from Text S3 (iii):

For the release of Pi, Watanabe et al. reported data at rotor angles 200°, 240°, and 320°², while Adachi et al. reported values at 320° and 360°⁶, which are qualitatively consistent but differ to some extent. Watanabe et al derived a kinetic model by fitting these data. As a separate and robust data, the timescale for Pi release in the ATP-driven rotation is known to be on the order of 1 ms⁵. Adachi's data at 320° is compatible with this scale, whereas Watanabe's fit gives ≈ 10 times faster value, likely because the fit included data at 200°, way outside the expected Pi-release range (≈ 240°–360°). Here, we update the model of the Pi off rate using data at 240° and at 320°. The resulting model is

$$k_{off}^{Pi} = 10^{-9} \cdot \exp\left(4.9 \cdot \frac{\pi}{180}(\theta + 240)\right) \quad (s^{-1}).$$

To make the formula usable in the first span $0^\circ \leq \theta \leq 120^\circ$, here we include a +240° phase shift, where release occurs in the experimental convention (e.g., 0°–120°) are mapped consistently within our model's cyclic representation.

For the Pi binding, re-evaluating Adachi's data at 320° and 360° together with Watanabe's data at 200° showed little systematic angle dependence. We therefore use a slightly milder slope than Watanabe's original report:

$$k_{on}^{Pi} = 22 \cdot \exp\left(1.0 \cdot \frac{\pi}{180}(\theta + 240)\right) \quad (M^{-1}s^{-1}).$$

for the range $0^\circ \leq \theta \leq 120^\circ$.

Although these experimental datasets contain considerable uncertainty, the overall timescale of Pi release is well established and provides a reliable constraint for model construction. The present formulation therefore combines this robust timescale with the angle-dependent trends inferred from

single-molecule measurements. Importantly, even if the detailed angular dependence of the Pi on/off rates is varied within the experimentally plausible range, the thermodynamic consistency of the model is preserved. As described in Text S4, this consistency is ensured by maintaining the balance between the angle-dependent Pi binding and release terms.

Comment D: Regarding the independence of the thermodynamic crossover (stall) torque from the rotational friction coefficient γ , we have clarified this point at the end of the second paragraph of "Torque-dependent conversion from ATP hydrolysis to ATP synthesis and optimal efficiency" (p. 15).

Quotation from the main text:

Of note, the resulting crossover torque is therefore determined by the free-energy difference between reactants and products rather than by the rotational friction coefficient γ . While γ affects the rotational kinetics and the time required to reach steady-state behavior, it does not alter the thermodynamic crossover torque itself.

Comment E: Regarding the comparison with the recent measurements by Toyabe et al. (2026), we have added the following paragraph at the end of the third paragraph of the same section:

Quotation from the main text:

Recently, a stall torque of 30.7 ± 2.9 pN·nm/rad under conditions of [ATP] = 0.4 μ M, [ADP] = 4 μ M, and [Pi] = 1 mM was reported²³. For the same nucleotide concentrations, our thermodynamic estimate gives 34.7 pN·nm/rad, and simulations yielded a stall torque of approximately 35 pN·nm/rad (Fig. S2A). The close agreement between experiment, thermodynamic prediction, and simulation provides additional support for the validity of the model.

Reviewer #2

This paper is a well written description of kinetic modeling of experimental data - nothing more. The authors have failed to take up the challenge of explaining why the F1 turns clockwise rather than clockwise. The sequential parabolas serve as trompe l'oeil to fool the eye into thinking the motion could not be otherwise, but this, as has been shown in many papers, is not correct. When one actually solves the reaction diffusion equations with rate constants that are consistent with both microscopic reversibility and with Curie symmetry constraints, the surprising result is that the shape of the periodic potential used in the modelling has little or nothing to do with determining the preferred direction of motion. The essential feature that determines the direction is kinetic asymmetry - at what angle binding/release of ATP is fast and at what angle is release of Pi fast, etc. The authors failed to address this key concern that I expressed in my original report, and instead

simply reiterated that their kinetic model is based on and driven by the experimental data. I will leave it up to the editors to decide whether this is a sufficient contribution to justify publication, in my opinion it is not.

We agree with the reviewer that the origin of rotary directionality is a fundamental question not addressed in the current paper. In fact, this is not our purpose in this work. In our modeling, the angle-dependent kinetic rates that were experimentally measured contain this information. While important, we do not ask why this angle-dependence comes out from structures of F_1 , in this paper. Instead, we build upon the angle-dependent experimental kinetic data together with thermodynamic consistency to obtain a unified mathematical model. In the revised paper, we made this point clearer in the “Discussion and Conclusion” in this second revision.

Quotation from the main text:

It should be stated clearly that the current modeling is not to explain the microscopic origin of unidirectional rotations or tight mechanochemical coupling, which are incorporated in experimentally measured angle-dependent kinetic rates. Instead, we show that integration of all experimental kinetic data together with thermodynamic consistency leads to a complete mathematical model of the mechanochemical coupling in F_1 -ATPase.

Reviewer #3

The authors have convincingly addressed my comments, and I have no further requests. I think this is a very interesting study.

We sincerely thank the reviewer for the positive evaluation of our work and for recognizing its potential significance. We greatly appreciate your encouraging comments.