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Mechanically driven ATP synthesis by F_1 -ATPase

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ATP, the main biological energy currency, is synthesized from ADP and inorganic phosphate by ATP synthase in an energy-requiring reaction¹⁻³. The F_1 portion of ATP synthase, also known as F_1 -ATPase, functions as a rotary molecular motor: *in vitro* its γ -subunit rotates⁴ against the surrounding $\alpha_3\beta_3$ subunits⁵, hydrolysing ATP in three separate catalytic sites on the β -subunits. It is widely believed that reverse rotation of the γ -subunit, driven by proton flow through the associated F_0 portion of ATP synthase, leads to ATP synthesis in biological systems^{1-3,6,7}. Here we present direct evidence for the chemical synthesis of ATP driven by mechanical energy. We attached a magnetic bead to the γ -subunit of isolated F_1 on a glass surface, and rotated the bead using electrical magnets. Rotation in the appropriate direction resulted in the appearance of ATP in the medium as detected by the luciferase-luciferin reaction. This shows that a vectorial force (torque) working at one particular point on a protein machine can influence a chemical reaction occurring in physically remote catalytic sites, driving the reaction far from equilibrium.

When isolated F_1 hydrolyses ATP, its central γ -subunit rotates anticlockwise⁴ when viewed from above in Fig. 1a, with an efficiency of chemical-to-mechanical energy conversion approaching 100% (ref. 8). The purpose of this study was to show that the chemo-mechanical coupling in the F_1 motor is completely reversible, and that reversal is achieved by manipulating a single variable—that is, the rotary angle of the γ -subunit. Any molecular machine would be reversible if one could manipulate all constituent atoms at will. Whether one or a few thermodynamic handles exist in a chemo-mechanical molecular machine such that its operation can be controlled through that handle in both directions is an important but unresolved issue. For example, whether one can synthesize ATP by pulling back a linear molecular motor such as myosin or kinesin—and if so, where to pull—is unknown. Reversal of the whole ATP synthase is well documented^{8,9}, including the demonstration of γ -subunit reorientation under synthesis conditions¹⁰, but whether or not the γ -subunit angle serves as a single handle for F_1 reversal has not been tested.

To prove this reversibility, we used $\alpha_3\beta_3\gamma$, the minimal subcomplex of F_1 that shows ATP-catalysed rotation⁴. The subcomplex was attached to a glass surface through histidine residues engineered at the amino terminus of the β -subunits, and a magnetic bead coated with streptavidin was attached to the γ -subunit, which had been biotinylated at two engineered cysteines (Fig. 1a). The beads were rotated with magnets (Fig. 1b-d) in a medium containing ADP and phosphate as substrates and the luciferin-luciferase system^{11,12}, which emits a photon when it captures and hydrolyses ATP. The initial idea was to count these chemiluminescent photons (Fig. 1b):

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volume of medium per active F_1 molecule had to be small.

First, we tried to reduce the volume by making microdroplets in oil (Fig. 2a). Figure 2b shows data from a 4×4 array of droplets in one chamber. The beads in droplets were rotated at 10 Hz alternately for 5 min each in the direction of hydrolysis (anticlockwise when viewed from top in Fig. 1a) and synthesis (clockwise). As seen in Fig. 2b, 14 out of 16 droplet curves showed the M-shaped pattern of photon counts expected for the sequence of rotation direction when the overall decline was taken into account. The decline was due to the gradual disappearance of the aqueous phase into oil: although we saturated the oil with water before the experiment, droplets tended to shrink over time. For random photon counts, the probability of observing a slanting M shape is 8^{-1} . The probability of observing 14 or more M shapes out of 16 is 2×10^{-11} . The data set shown in Fig. 2b thus strongly indicates mechanical synthesis. The experiment is extremely difficult (at most a few beads rotate in each droplet), however, and we have obtained only a few more data sets that contained several M-shaped patterns.

We thus tried to increase the number of rotating beads in an ordinary observation chamber (Fig. 1c) by infusing a concentrated solution of beads carrying F_1 . To allow F_1 to rotate in the proper direction, we derivatized only the bottom surface of the chamber with nickel nitrilo-triacetic acid (Ni^{2+} -NTA), which would specifically bind the β -histidines. In control experiments done in 4 mM ATP (no ADP) and without magnets, we found in the field of view of $1.0 \times 10^5 \mu m^2$ as many as $480 \pm 70 F_1$ molecules rotating anticlockwise at the bottom (three chambers). However, the high density of beads resulted in nonspecific binding to the ceiling, where 100 ± 20 beads rotated clockwise (as viewed from above the chamber). We also tested in the ATP medium whether forced rotation by external magnets would damage F_1 . After confirming ATP-driven rotation, we turned on the magnets and applied several bursts of hundreds of revolutions at 10 Hz in both directions. When

the magnets were turned off, ATP-driven rotation resumed.

Synthesis was shown in the ADP-luciferase medium by accumulating chemiluminescence photons over a series of 5-min intervals in which the magnetic field was rotated at 10 Hz in either direction or turned off. All series produced the expected pattern (Fig. 3a): higher photon counts during clockwise rotation (S, synthesis) than during anticlockwise rotation (H, hydrolysis) or no rotation (N). This graph compiles all data taken in consecutive experiments (about half of the experiments failed at some point, for example during chamber preparation or because of a large focus drift, and did not produce data). Mechanical synthesis in the flat chamber was reproducible, although variation among data was still large.

We note that in most curves shown in Fig. 3a including the total counts, counts during anticlockwise rotation (H) are higher than those at no rotation (N). This is due to the presence of F_1 at the ceiling of the chamber as stated above. For these upside-down F_1 molecules, anticlockwise bead rotation will drive ATP synthesis. Indeed, when we flipped the chamber upside down after obtaining the unbroken blue line in Fig. 3a, the count pattern was reversed, as shown by the broken blue line. Another reason for the higher counts during anticlockwise rotation was that luciferase did not consume all of the newly synthesized ATP in 5 min: as shown by the unbroken lines in Fig. 3b, the luminescence at no rotation was high after ATP synthesis at the arrows. Luminescence decay after ATP mixing was shown to involve a component with a lifetime of about 3 min (see Supplementary Information). Taking all of the above points into account, we consider that mechanical synthesis has been conclusively demonstrated.

Figure 4 shows the effect of rotary speed on the efficiency of ATP synthesis. The synthesis rate apparently saturated above 3 Hz (Fig. 4b). This was because larger beads or bead aggregates failed to rotate at high speeds, as confirmed by direct observation (uncoupling between magnet and bead rotations). Calibration of the

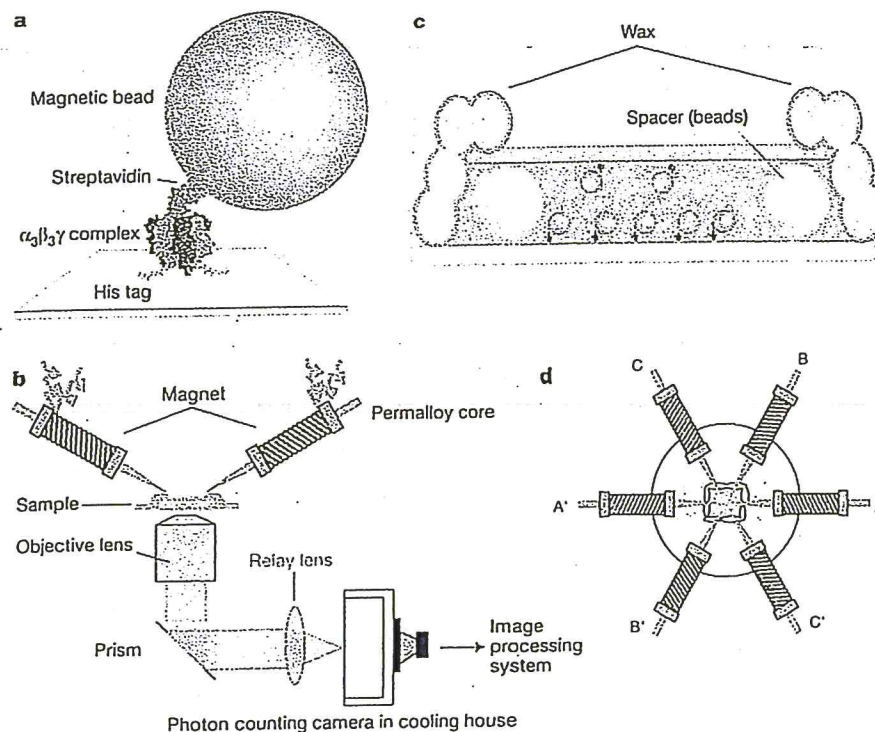


Figure 1 Experimental set-up. a, Basic design. The structures of F_1 and streptavidin are from ref. 21 and ref. 22, respectively. The bead is not to scale and the orientation of

Ni^{2+} -NTA was applied only to the bottom surface, but some F_1 -conjugated beads rotated on the ceiling. d, Top view of the magnets.

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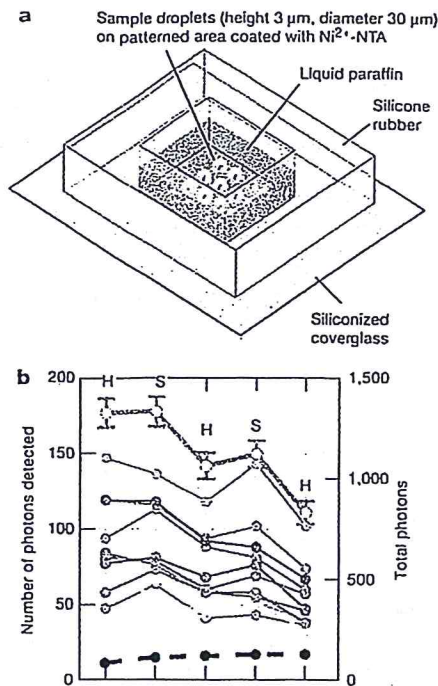


Figure 2 Rotational synthesis in microdroplets. **a**, Observation chamber. On the silanized bottom coverglass, 4×4 spots separated by $\sim 50 \mu\text{m}$ and measuring $20\text{--}30 \mu\text{m}$ were derivatized with Ni^{2+} -NTA, which rendered the spots hydrophilic. An oil layer with a thickness of $\sim 3 \text{mm}$ was placed on top. Using a glass micropipette, we formed a droplet of $\sim 1 \text{pl}$ containing F_1 on each spot and then replaced the solution with one containing beads, ADP, phosphate and the chemiluminescence system. **b**, Simultaneous observation of 16 droplets. Thick magenta curve with open circles represents the sum of all unbroken curves; error bars represent $\pm 2\sigma$, where σ is the expected s.d. for photon statistics (square root of the total count). The broken curve at the bottom represents a control in which an area outside the droplets was imaged. See Supplementary Information for details.

photon-to-ATP ratio (see Supplementary Information) indicated a synthesis rate of about five molecules of ATP per second under rotation at 3 Hz, as compared with the nine molecules of ATP per second expected for the one ATP molecule per 120° scheme⁸.

Although Fig. 4 represents our best data so far and variation among chambers is large (Fig. 3a), we anticipate that the coupling between mechanical rotation of the γ -subunit and chemical synthesis is tight, at least at low speeds. The significant synthesis at low speeds (Fig. 4), which were much lower than the maximal rotary speed of 130 Hz of this motor during ATP hydrolysis¹³, merits attention. Because the slow rotation was at a constant speed, it is likely that chemical reactions were at quasi-equilibrium at all angles. Rotation by ATP hydrolysis can also be made slow and at a constant speed by attaching a long actin filament to the γ -subunit⁸, again suggesting quasi-equilibrium. The implication is that ATP synthesis in F_1 proceeds as a straightforward reversal of the hydrolysis reaction, tracking the same reaction pathway in the opposite direction. On that pathway, both hydrolysis and synthesis reactions are controlled by one mechanical handle, the rotary angle of the γ -subunit. The situation contrasts with the more complex rotary motor of bacterial flagella, where rotational directions can be switched without reversing the proton motive force¹⁴.

ATP synthesis is a chemical reaction that is energetically uphill, requiring $80\text{--}100 \text{pN nm}$ of energy under physiological conditions². In the experiments shown here, contaminant ATP amounted to about 1nM , implying that roughly 30pN nm of free energy was needed per molecule of ATP synthesized (see Supplementary

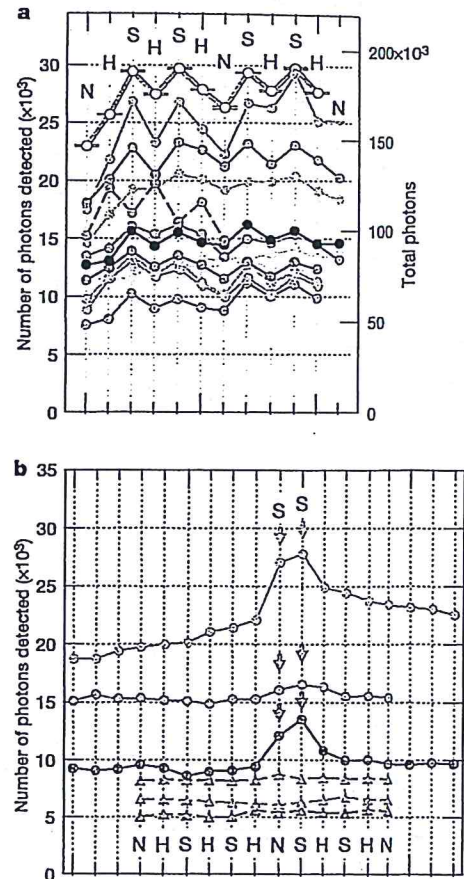


Figure 3 Rotational synthesis in a flat chamber. Each symbol shows the number of photons detected over 5 min in an image area of $7.8 \times 10^4 \mu\text{m}^2$. N, no rotation; H, 10-Hz rotation in the hydrolysis direction (for F_1 on the bottom of the chamber); S, 10-Hz rotation in the synthesis direction. **a**, Results from 12 chambers, distinguished by different colours. Broken blue curve represents results obtained after the chamber giving rise to the unbroken blue curve was flipped upside down. Thick magenta curve with open circles represents the sum of all unbroken curves; error bars represent $\pm 4\sigma$ ($>99.99\%$ confidence). For clarity, some curves have been shifted vertically within $\pm 1,000$ counts. **b**, Control experiments. Unbroken curves show results either without rotation or with imposed clockwise rotation for synthesis at 10 Hz (arrows). Broken curves show experiments carried out as in **a** except that phosphate was omitted from the medium. The counts in the broken curves are low in comparison to the others because phosphate tended to increase the background, apparently by increasing the portion of luciferase that reacted with ATP extremely slowly (see Supplementary Information).

Information). If F_1 , or the motor enzyme myosin, is mixed with high concentrations of ADP and phosphate in the absence of ATP, some ATP is spontaneously formed on the enzyme without input of energy^{15–18}. This, however, is a dead-end reaction and the ATP that has been formed is not available in the medium: release of the tightly bound ATP requires an external supply of energy^{1–13}.

Here we have demonstrated repetitive synthesis by F_1 ($\sim 10^3$ ATP molecules in 5 min), leading to appearance of the product in the medium. To our knowledge, this is the first accomplishment of artificial chemical synthesis by a vectorial force (although nature presumably has been doing this for millions of years). Pressure could also shift a chemical equilibrium by acting on substrates (and solvent); however, in our experiments the chemical equilibrium *per se* is on the side of almost complete hydrolysis, and a force on a point remote from substrates counters the hydrolysis reaction and pushes the equilibrium to the point of favouring synthesis. Because we still have to rely on nature's nanomachine, the F_1 motor, our

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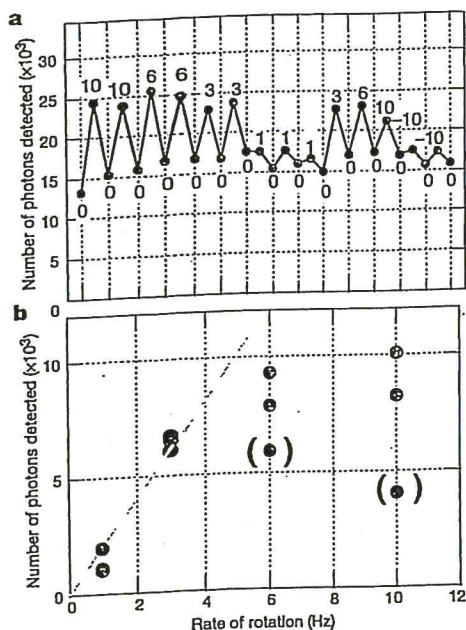


Figure 4 Dependence of synthesis efficiency on the rate of magnet rotation. a, Photons detected over an image area of $1.0 \times 10^5 \mu\text{m}^2$ in 5-min periods at various rotary speeds (shown in Hz; negative numbers indicate rotation in the hydrolysis direction). b, Photon increments during rotation. From the photon count for each synthesis rotation in a, the average of the adjacent no-rotation counts was subtracted. Symbols in the parentheses show the last two measurements for synthesis in a; low values for these presumably reflect sample deterioration.

primary goal is to understand fully how it works and thereby to exploit the mechanism in artificial ways. Improving the present system for more quantitative assays, such as the torque and speed dependence of the coupling efficiency, that can be compared with theoretical predictions^{7,19} should be our next task. The key is to obtain magnetic beads that are small and uniform in size. □

Methods

Materials

A mutant $\alpha_3\beta_3\gamma$ subcomplex (comprising C193S α -, His₁₀- β -, and S107C, I210C γ -subunits; referred to as F₁ in this paper), derived from the thermophilic *Bacillus* strain PS3, was biotinylated at the only cysteines on the γ -subunit (ref. 8). Streptavidin-coated magnetic beads (Seradyn; nominally 0.7 μm) were lightly centrifuged to remove large beads and aggregates (but elimination was incomplete). Biotinylated F₁ (400 pM) was incubated with beads (~50 pM) in buffer A (50 mM 3-(N-morpholino)propanesulphonic acid/KOH, 20 mM K₂SO₄, 4 mM MgSO₄, pH 7.6) for 10 min at 23 °C, washed with buffer A, and concentrated with a magnet. Luciferase¹² was a kind gift from Kikkoman Co. We purified ADP (K-salt; Sigma) as described²⁰ on a Poros HQ/L column (Applied Biosystems).

Observation chamber

For the chamber shown in Fig. 1c, a 32 × 24 mm² coverglass was functionalized by a silane coupling agent with an SH group (TSL 8380; GE Toshiba Silicone), and reacted first with 10 mg ml⁻¹ maleimide-C₃-NTA (Dojindo) and then with 10 mM NiCl₂. Two parallel strips of Lumirror polyester film (TORAY) were placed on the coverglass, and a 18 × 18 mm² coverglass coated with hexamethyldisilazane was placed on top to form a flow chamber. We infused ~500 pM F₁-conjugated magnetic beads in buffer A₃ (buffer A containing 3 mg ml⁻¹ bovine serum albumin (BSA)) and incubated the chamber for 30 min at 23 °C. After infusing buffer A₃ several times, buffer A containing 10 μM luciferase, 1 mM luciferin, 10 mM K₂PO₄, 200 μM ADP and 1.5 mg ml⁻¹ BSA, together with a small number of 3- μm Dynabeads M-280 beads (Dyna) that would eventually serve as spacers, was infused. The spacer strips were carefully removed and the upper coverglass was pressed to reduce the chamber height to ~3 μm , as determined by the spacer beads. The chamber was then sealed with wax and subjected to observation.

Microscopy

The chamber was placed on an inverted ICM 450 microscope (Zeiss). Luminescence was collected with an oil immersion ×60 objective, numerical aperture 1.45 (Olympus) and deflected with a prism to a factory-made side port (Fig. 1b). The beam was focused with an ED Plan ×2 objective (Nikon) onto a cooled photon-counting camera (V8070U-64-N230/C4566 equipped with an Argus 50 image processing system; Hamamatsu Photonics), which recorded centroid positions of incoming photons (counts in the dark: ~15 s⁻¹ over the whole image plane). To rotate the beads, we placed three opposing pairs of custom-made electromagnets with a Permalloy core on the chamber (Fig. 1d). The three pairs were activated with a custom circuit 120° out of phase to produce a rotating magnetic field (in either direction).

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Guiding Questions for Reading This Article

A. About the Article

1. What is the name of the first author of this research team?
2. Most of the authors of this paper work in major research laboratories in what nation? What is the name of the author affiliated with the Cold Spring Harbor Laboratory in the United States?
3. What is the name of the journal in which this article was published? This paper was published in what month and year?
4. This research was supported in part by grants from what organization? (See Acknowledgements, p. 468.)
5. The article's abstract (p. 465, first paragraph, in bold print) summarizes the major contributions of the study. Write the one sentence from the abstract that states the basic concept for which the study provides direct evidence.
6. Specialized vocabulary: Write a brief definition of each term.

chemiluminescence

hydrolysis

luciferase

photon

B. About the Study

7. Was this an *in vivo* study (in intact cells, in living organisms) or an *in vitro* study (literally, "in glass," meaning in test tubes or in laboratory containers)?
8. The action of isolated F₁ particles of ATP synthase appeared to be reversible. (a) The central subunit rotates *anticlockwise* (that is, *counterclockwise*) when what happens—A or B? (A) ATP is synthesized; (B) ATP is hydrolyzed (broken down to ADP and inorganic P). (b) The central subunit rotates *clockwise* when what happens—A or B? (A) ATP is synthesized; (B) ATP is hydrolyzed (broken down to ADP and inorganic P).
9. The researchers manipulated a single subunit of the ATP synthase complex, the γ (gamma) subunit. Prior to this study, what aspect of the γ subunit in F₁ reversal had not yet been tested?
10. One end of the F₁ test complex was attached to a glass slide by modifying two cysteine amino acids of one end of the complex. The other end of the F₁ test complex, the γ end, was attached to a magnetic bead using a specific attachment protein (streptavidin). Examine the experimental setup in Figure 1 (p. 466). Although the drawing in Figure 1a is not to scale, which is the larger element, the F₁ protein complex or the magnetic bead? How did the investigators manipulate this system to cause the γ end of the complex to rotate?
11. The investigators noted that during ATP hydrolysis, the complex rotated in one direction. What reaction did they hypothesize would occur if the complex was rotated in the opposite direction?

