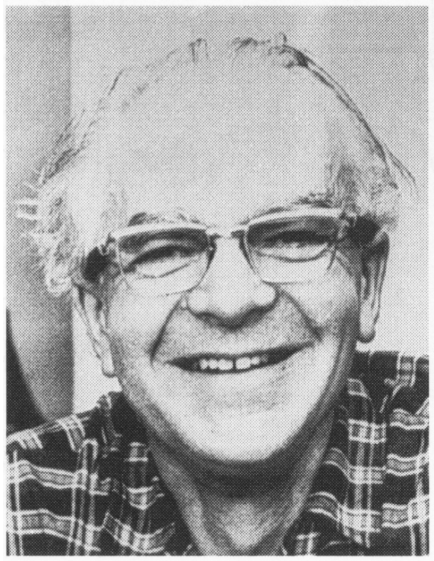
BioChem 330 - Course Outline

- **Metabolism and Bioenergetics (II)**
  - ENZYME CATALYSIS:
    - kinetic constants k_{cat}, K_m
    - Catalytic strategies, the serine proteases
  - CATABOLISM (*breakdown*)
    - Carbohydrates
      - Glycolysis
      - Tricarboxylic Acid Cycle
      - Electron Transport
      - Chemiosmosis and ATPase
    - Fatty acids and amino acids

Overview for Today, November 17, 2011

- Coupling proton pumping to ATP production through the electrochemical potential

- Smallest little motor in the world, the F_0F_1ATPase



Peter Mitchell
1920–1992

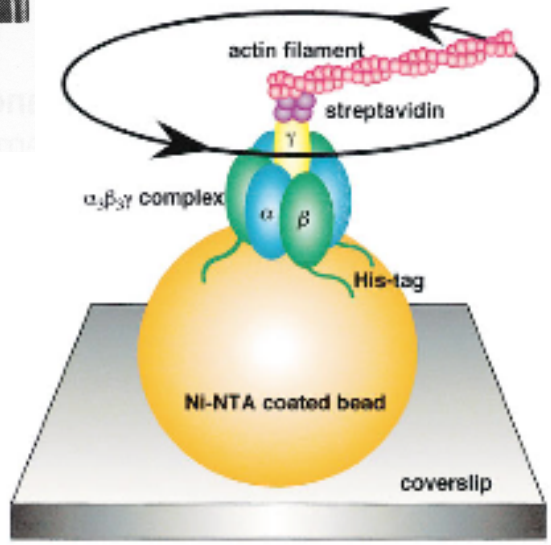
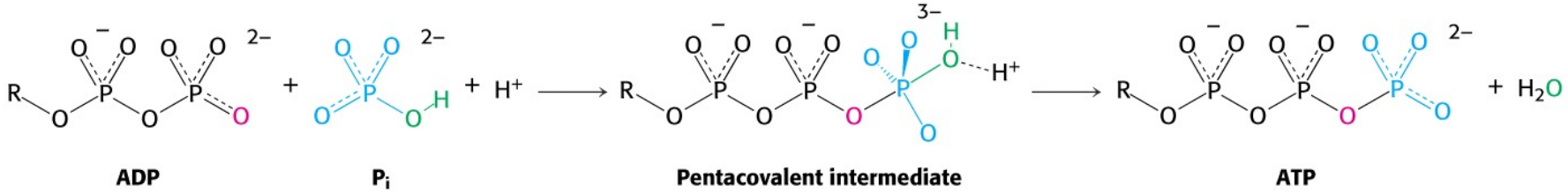


Figure 1. Experimental System (Not to Scale)

ET coupled to ATP synthesis

- ET chain induces electrochemical potential gradient by pumping protons across energy transducing inner mitochondrial membrane against proton and voltage gradient:
  - *ΔpH = 1.4* *ΔV = 0.14 V*
  - $\Delta G = 2.303\ RT\ \Delta\ pH + Z\ F\ \Delta V =$

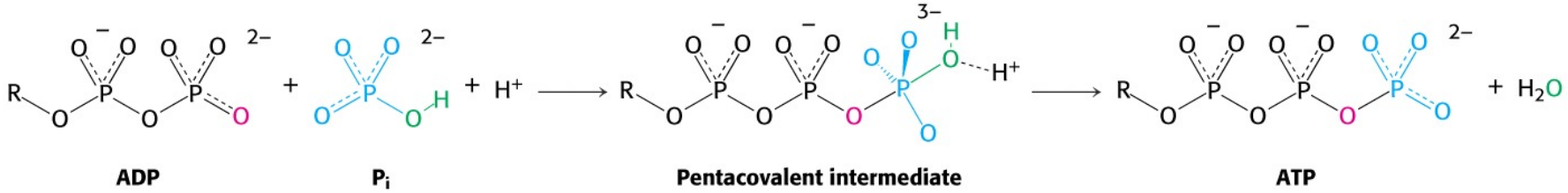
- ATP synthesis takes those protons pumped by ET and allows them to flow back into the cell through a clever little motor known as ATP synthase, catalyzes:



ET coupled to ATP synthesis

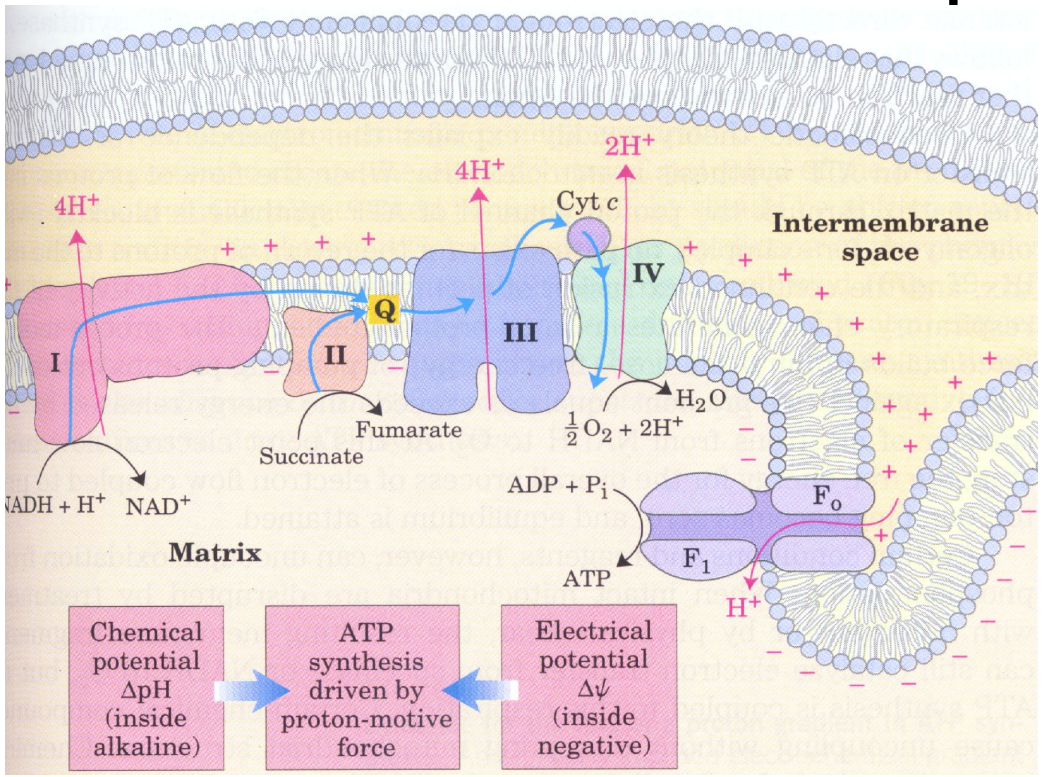
- ET chain induces electrochemical potential gradient by pumping protons across energy transducing inner mitochondrial membrane against proton and voltage gradient:
  - *ΔpH = 1.4* *ΔV = 0.14 V*
  - $\Delta G = 2.303\ RT\ \Delta\ pH + Z\ F\ \Delta V =$ 5.2 kcal/mole H

- ATP synthesis takes those protons pumped by ET and allows them to flow back into the cell through a clever little motor known as ATP synthase, catalyzes:



ΔG°' = + 7.3 kcal/mole

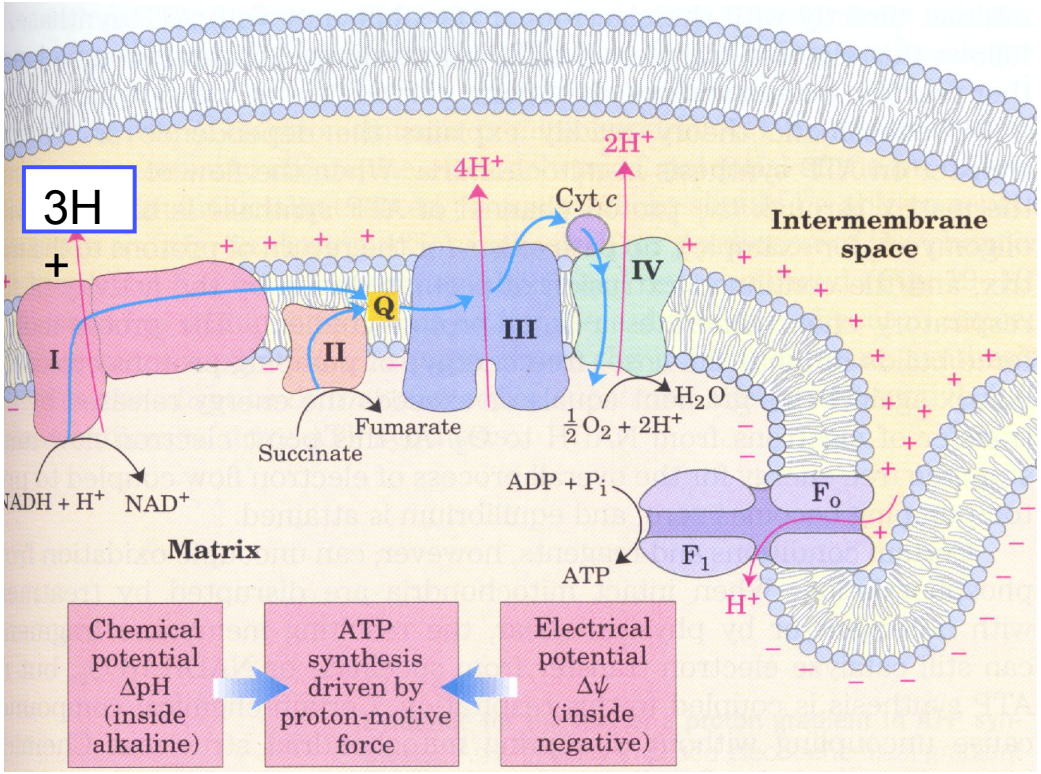
Chemiosmosis: the picture



- Understanding the detailed book-keeping of H+ stoichiometry has changed over time, your text's accounting at odds with newer texts which cite $3H^+$ through Complex I

- The intermembrane space is essentially in equilibrium with the cytoplasm, which is buffered at pH higher than the mito, and so, added protons don't change the pH of the cytoplasm but have profound effect on the pH of the matrix (like adding a few drops of acid to the ocean would have no effect, but adding it to a thimble would have a large effect).

Chemiosmosis: the picture



- $\Delta G = 2.3RT\,\Delta pH + F\,\Delta V$
- $F = 23.06$ kcal/mole/V

- **Mitochondria:**
  - $\Delta pH = 1$ (alkaline inside)
  - $\Delta V = 0.1\text{-}0.2$ V (inside negative)

- Electron flow is accompanied by proton transfer across the mitochondrial membrane, producing both a chemical (ΔpH) and an electrical (ΔV) gradient
- The proton-motive force that drives protons back into the matrix provides the energy for ATP synthesis, catalyzed by the F1 complex associated with Fo.

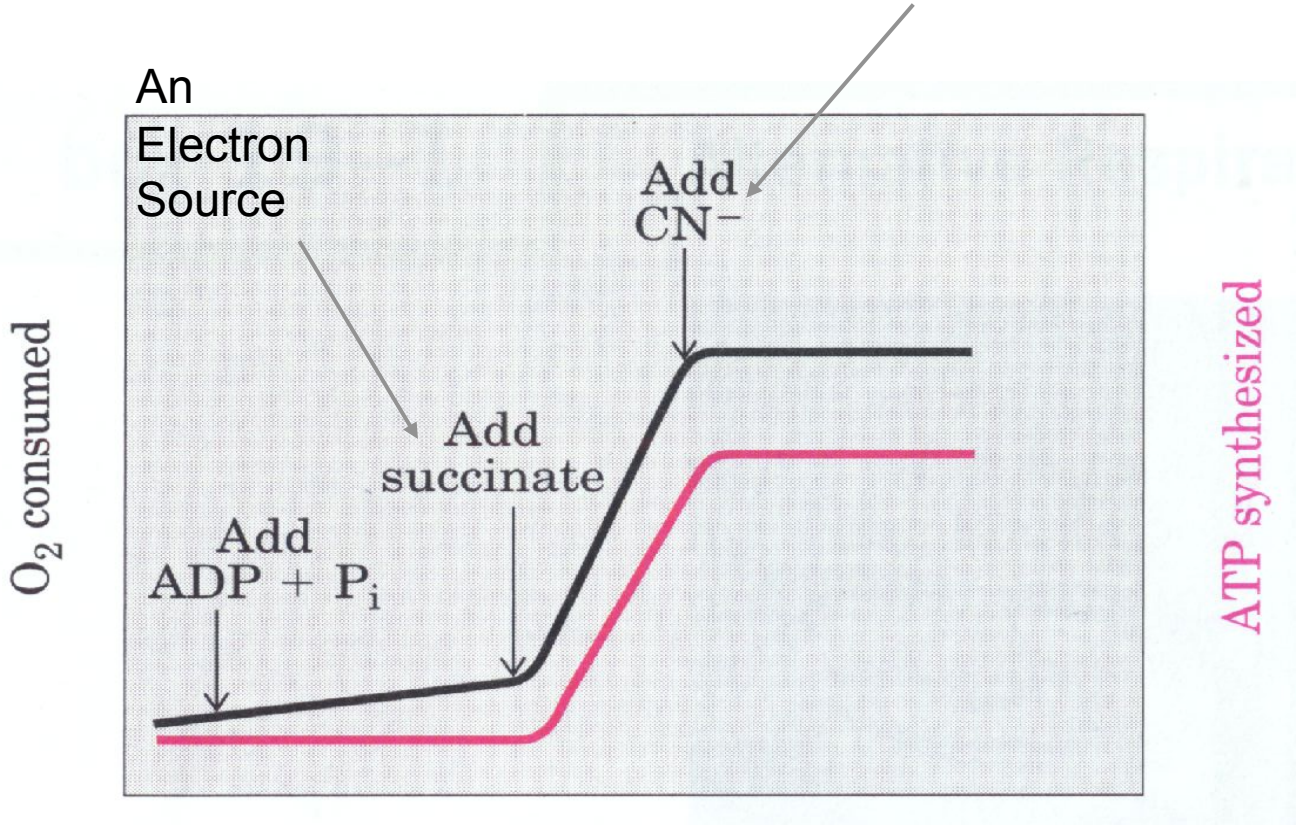
Basic evidence

- Electron transport is *coupled* to ATP synthesis (more detail in a moment)
- Coupling requires an intact membrane
- Electron flow through individual complexes results in proton pumping
- Proton carriers *uncouple* electron transport and ATP synthesis

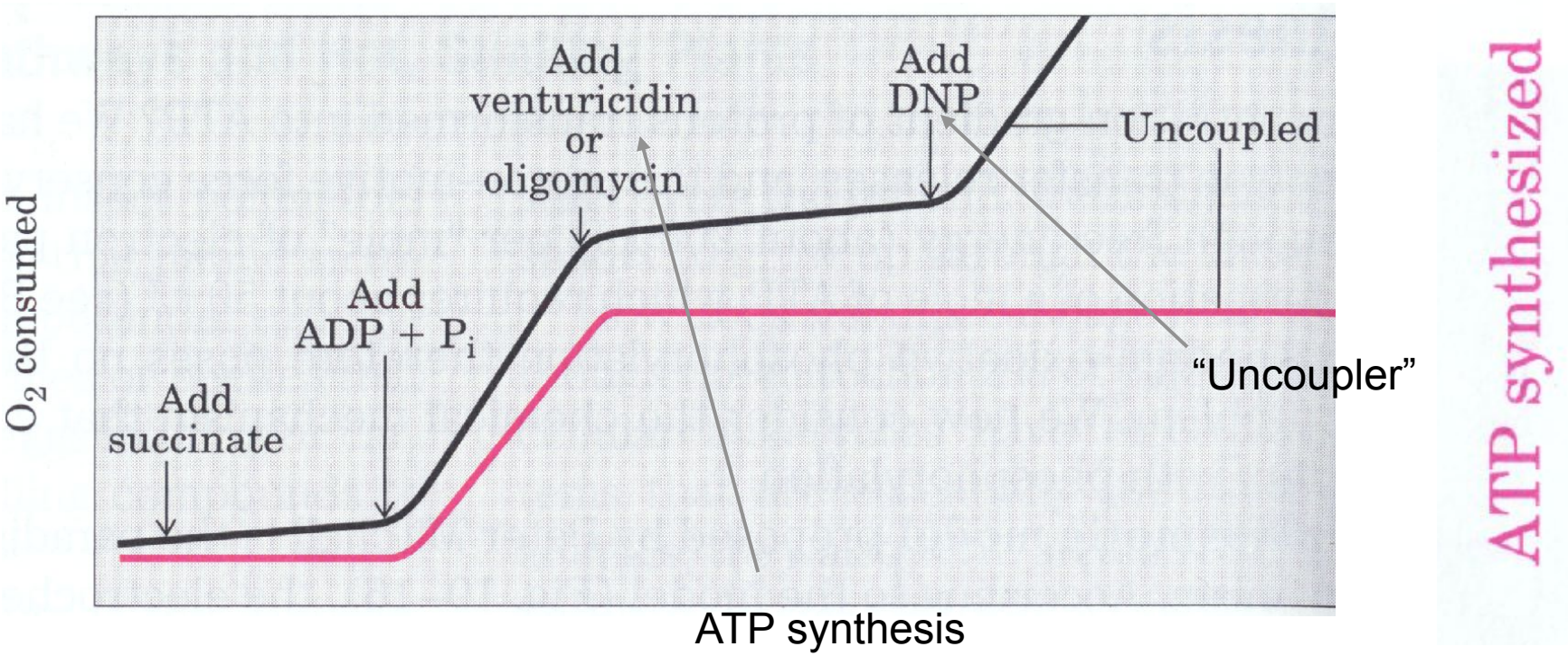
What experiments look like…

Coupling:

An inhibitor
Of electron transport
(Binds to cytochrome oxidase)

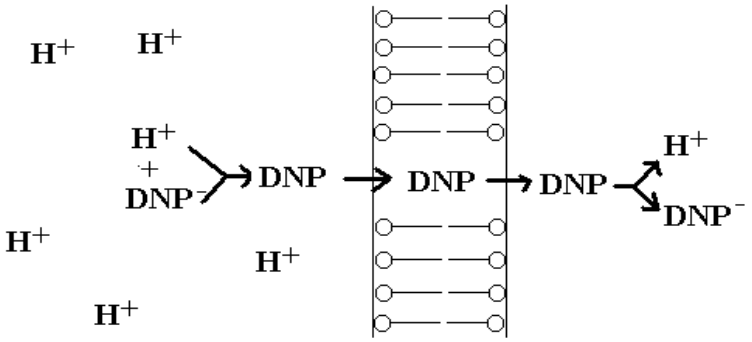


Or, more subtly…

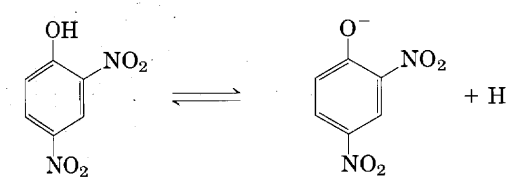


“Uncouplers”

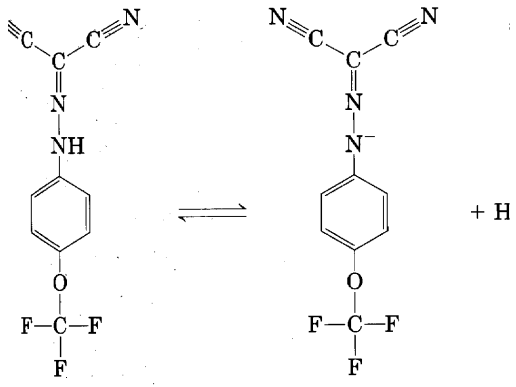
What they do…



What they look like…

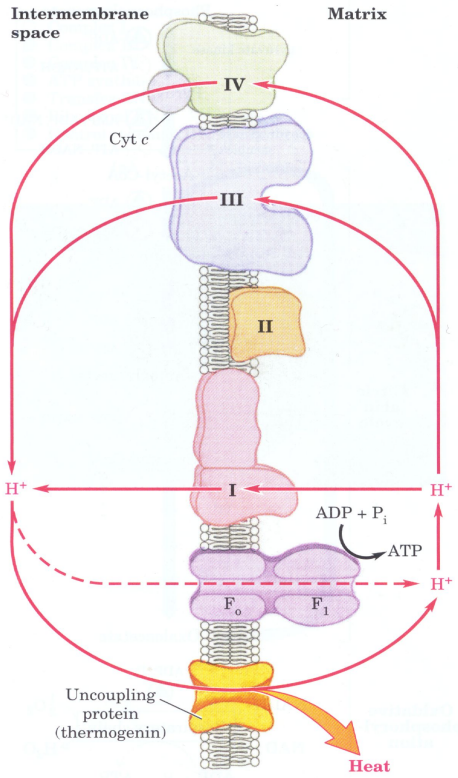


2,4-Dinitrophenol
(DNP)

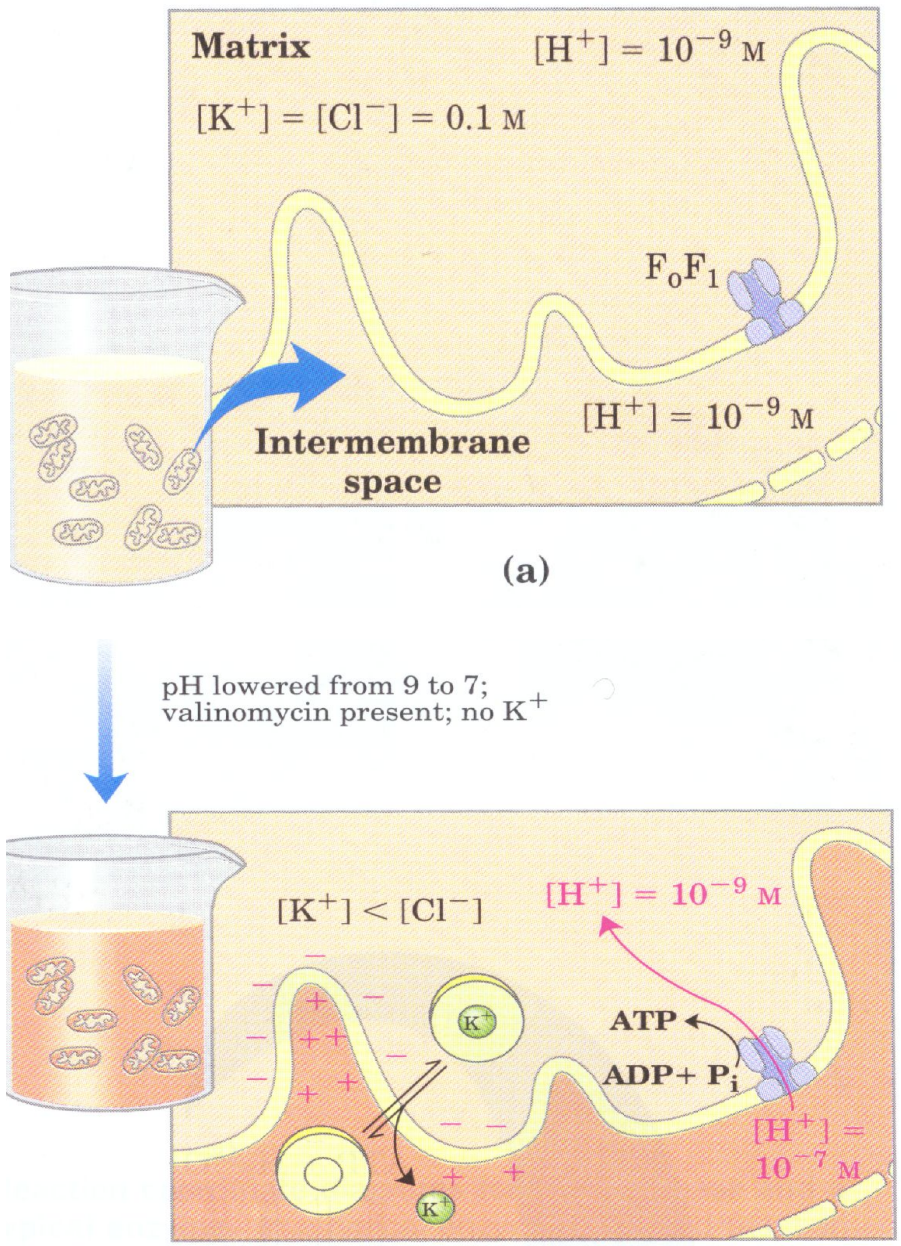


Carbonylcyanide-*p*-
trifluoromethoxyphenylhydrazone
(FCCP)

A natural version of these things in brown fat…



Creation of an electrochemical gradient is sufficient…



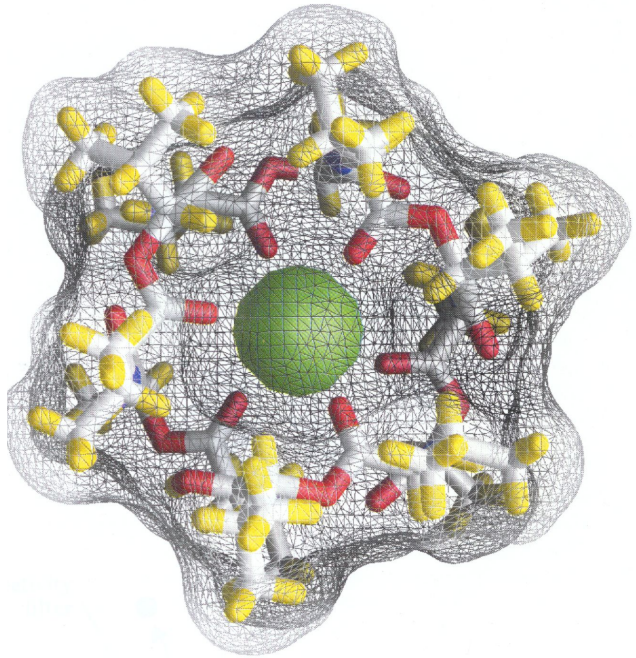
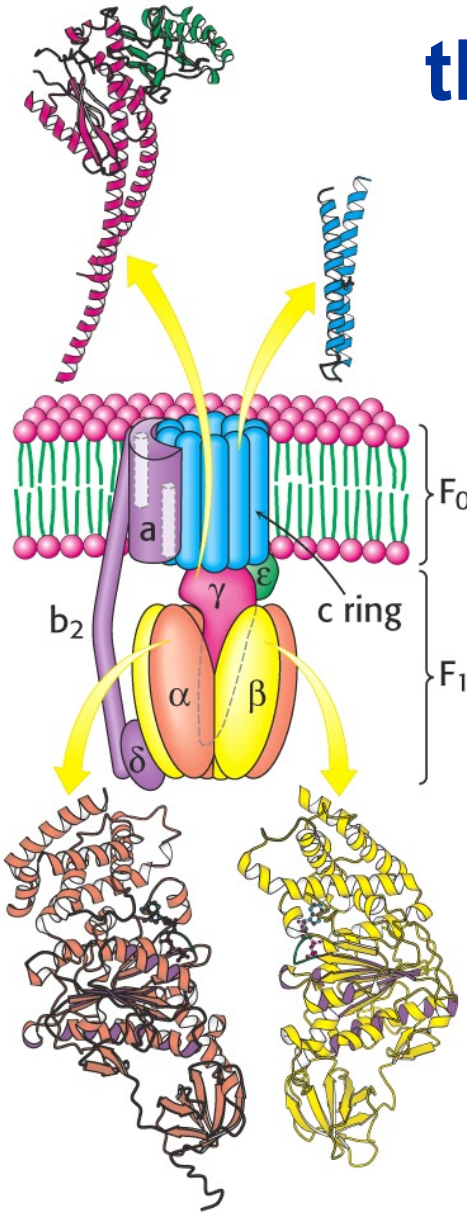


figure 12–37
Valinomycin, a peptide ionophore that binds K^+. In this image, the surface contours are shown as a transparent

Structure of F_oF_1 ATPase:
the littlest motor in the world

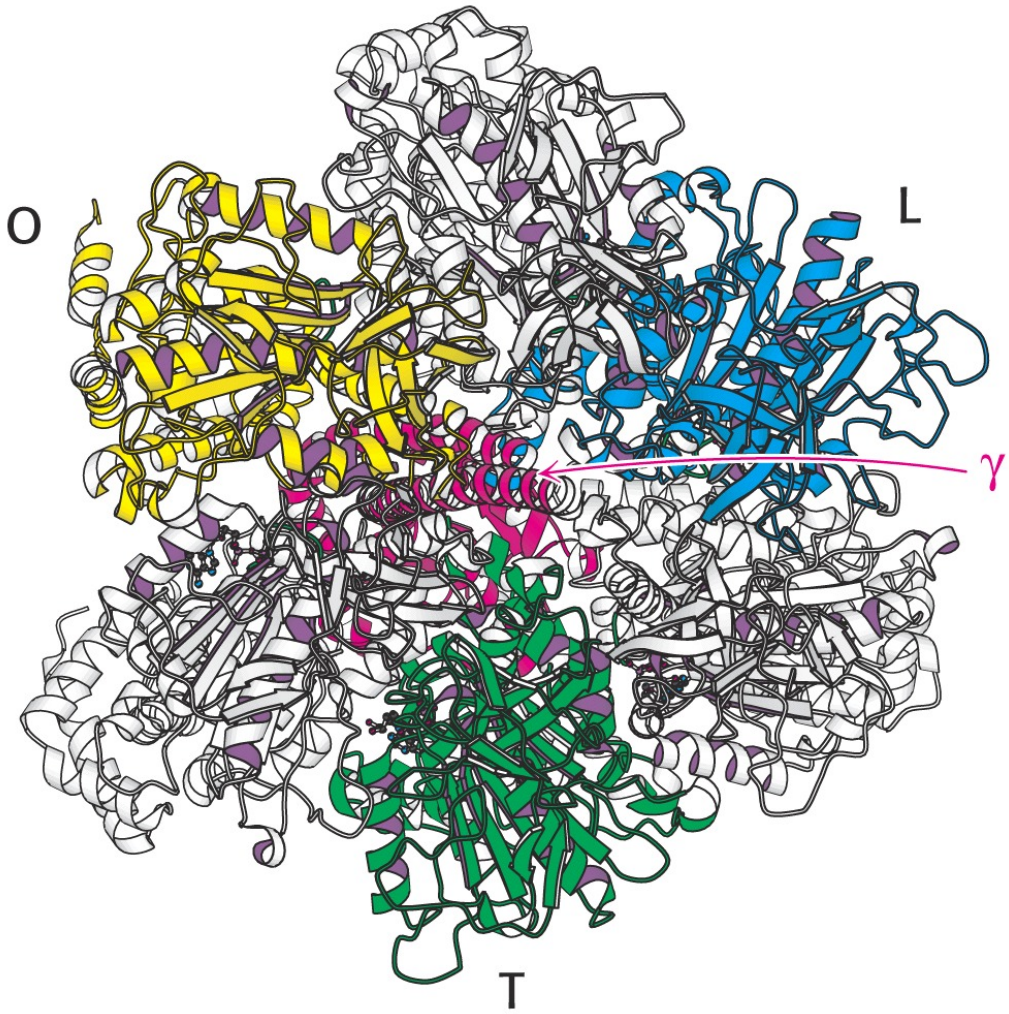


1. F_o unit: proton conducting unit, integral membrane proteins, proton channel formed by a ring of 10-14 **c** subunits and a single **a** subunit which binds to outside of **c** ring.

2. F_1 catalytic unit ($\alpha_3\beta_3\gamma\delta\varepsilon$) which consists of a hexamer of alternating α and β subunts (only β is active), a central stalk of γ and ε long α-helical proteins, which secure F1 to Fo. Assymetry of γ is key to activity

3. b subunit of Fo interacts with F1 through d.

Looking down through the top of F1



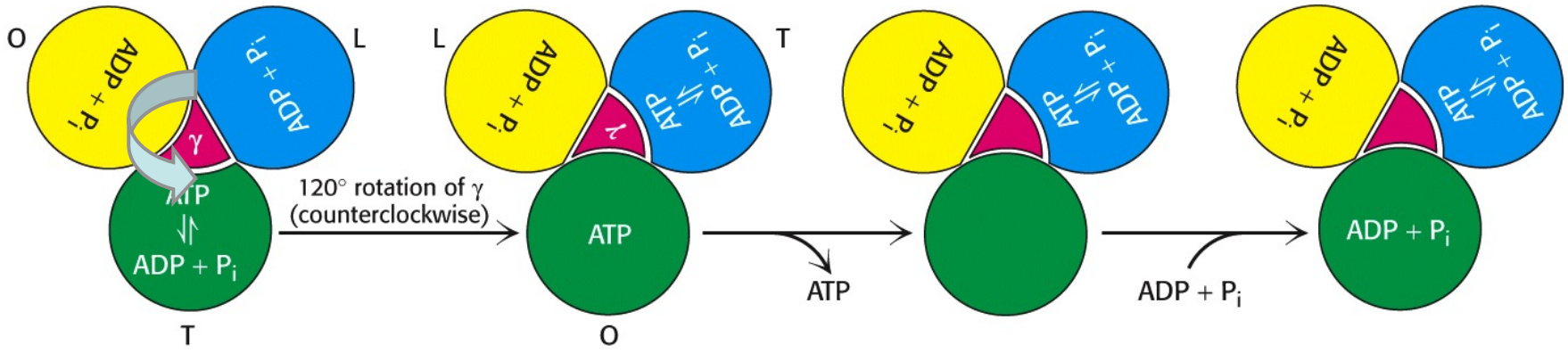
* γ subunit breaks the symmetry, interacting differently with each of the β subunits (color),

•ATP binds differently to each conformer:

- •L is loose (ADP and Pi bound),
- •T is tight (ATP made),
- •O is open, (bound ATP released).

•Intact assembly, no proton gradient, ATP is formed, but not released.

Binding Change Model for coupling



Perspective from matrix above F1 : rotation of γ counterclockwise ATP synthesis

1. Each **counterclockwise step** of the red wedge (γ subunit) by 120^o changes β conformation: O -> L or L->T, or T -> O
2. Each full cycle of γ (3x120^o steps) makes 3ATP(1 from each β)
3. Catalysis only happens in T
4. Motor can go backwards for hydrolysis (ATPase) in bacteria, but only ATP synthesis in mitochondria and chloroplasts.

F1 the first **single molecule experiments**

Reporter gene assays. THP-1 cells were transfected with DEAE-Dextran[26] and RAW 264.7 cells with calcium phosphate[27]. Cells were transfected with a β-galactosidase expression vector pCMV-β, with the reporter plasmid pG5E1-bLuc, with an expression vector encoding a GAL4–MEF2C fusion protein or GAL4(1–147) or GAL4–MEF2C mutants, and with the expression vector encoding a constitutively active form of MKK6b(E) or with empty vector pcDNA3. In some experiments the cells were also transfected with increasing amounts of DNA (0, 2, 4, 8, 12 or 16 μg) from an expression plasmid for p38(M). The total amount of DNA for each transfection was kept constant using pcDNA3. In studies using FHPI, the inhibitor was added (3 μM) 36 h after transfection for 1 h; and the cells were then treated with or without LPS for 8 h. LPS (5 μg ml^{-1} and 10 ng ml^{-1}) was used to stimulate THP-1 and RAW 264.7 cells, respectively. The relative luciferase activities presented were normalized by dividing the luciferase activity by β-galactosidase activity.

Phosphoamino-acid analysis and phosphopeptide mapping. These methods were performed as described[18]. GAL4–MEF2C fusion protein and mutants from [^{32}P]orthophosphate-labelled permanently transfected RAW 264.7 cells (1 mCi ml^{-1}, 2 h) treated with or without LPS (10 ng ml^{-1}, 2 h) were immunoprecipitated with anti-GAL4 DNA-binding domain monoclonal antibody RK5C1 (Santa Cruz).

Received 18 November 1996; accepted 21 January 1997.

1. Morrison, D. C. *et al.* Bacterial endotoxins and pathogenesis of Gram-negative infections: current status and future direction. *J. Endotoxin Res.* **1,** 71–83 (1994).
2. Blumer, K. J. & Johnson, G. L. Diversity in function and regulation of MAP kinase pathways. *Trends Biochem. Sci.* **19,** 236–240 (1994).
3. Derijard, B. *et al.* JNK1: A protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell* **76,** 1025–1037 (1994).
4. Han, J., Lee, J.-D., Bibbs, L. & Ulevitch, R. J. A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. *Science* **265,** 808–811 (1994).
5. Zhou, G., Bao, Z. Q. & Dixon, J. E. Components of a new human protein kinase signal transduction pathway. *J. Biol. Chem.* **270,** 12665–12669 (1995).
6. Lee, J.-D., Ulevitch, R. J. & Han, J. Primary structure of BMK1: A new mammalian MAP kinase. *Biochem. Biophys. Res. Commun.* **213,** 715–724 (1995).
7. Lee, J. C. *et al.* Identification and characterization of a novel protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature* **372,** 739–746 (1994).
8. Raingeaud, J. *et al.* Pro-inflammatory cytokines and environmental stress cause p38 MAP kinase activation by dual phosphorylation on tyrosine and threonine. *J. Biol. Chem.* **270,** 7420–7426 (1995).
9. Beyaert, R. *et al.* The p38/RK mitogen-activated protein kinase pathway regulates interleukin-6 synthesis in response to tumour necrosis factor. *EMBO J.* **15,** 1914–1923 (1996).
10. Martin, J. F., Schwarz, J. J. & Olson, E. N. Myocyte enhancer factor (MEF) 2C: A tissue-restricted member of the MEF-2 family of transcription factors. *Proc. Natl Acad. Sci. USA* **90,** 5282–5286 (1993).
11. McDermott, J. C. *et al.* hMEF2C gene encodes skeletal muscle- and brain-specific transcription factors. *Mol. Cell. Biol.* **13,** 2564–2577 (1994).
12. Leifer, D. *et al. Proc. Natl Acad. Sci. USA* **90,** 11546–1550 (1993).
13. Durfee, T. *et al.* The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit. *Genes Dev.* **7,** 555–569 (1993).
14. Martin, J. F. *et al.* A Mef2 gene that generates a muscle-specific isoform via alternative mRNA splicing. *Mol. Cell. Biol.* **14,** 1647–1656 (1994).
15. Molkentin, J. D., Black, B. L., Martin, J. F. & Olson, E. N. Mutational analysis of the DNA binding, dimerization, and transcriptional activation domains of MEF2C. *Mol. Cell. Biol.* **16,** 2627–2636 (1996).

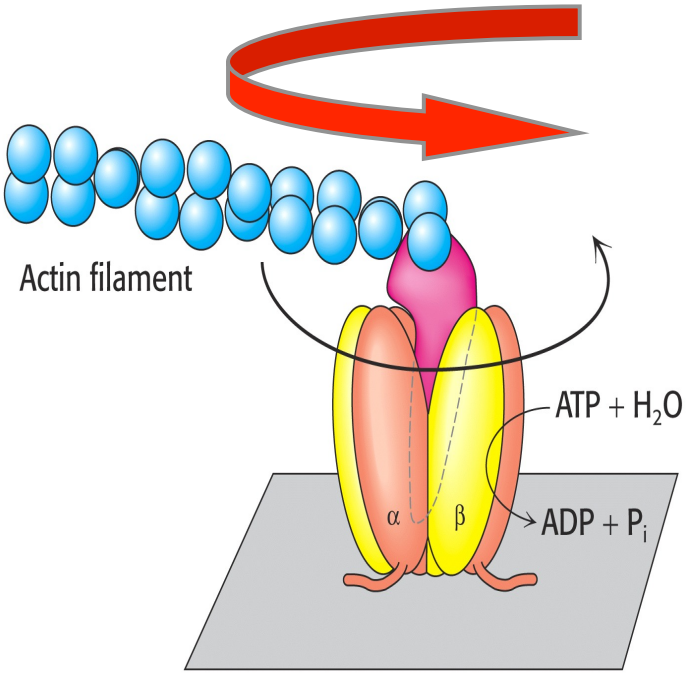
Direct observation of the rotation of F_1-ATPase

Hiroyuki Noji*, Ryohei Yasuda†, Masasuke Yoshida* & Kazuhiko Kinosita Jr†

* *Research Laboratory of Resources Utilization, Tokyo Institute of Technology, Nagatsuta 4259, Midori-ku, Yokohama 226, Japan*
† *Department of Physics, Faculty of Science and Technology, Keio University, Hiyoshi 3-14-1, Kohoku-ku, Yokohama 223, Japan*

Cells employ a variety of linear motors, such as myosin[1–3], kinesin[4] and RNA polymerase[5], which move along and exert force on a filamentous structure. But only one rotary motor has been investigated in detail, the bacterial flagellum[6] (a complex of about 100 protein molecules[7]). We now show that a single molecule of F_1-ATPase acts as a rotary motor, the smallest known, by direct observation of its motion. A central rotor of radius ~1 nm, formed by its γ-subunit, turns in a stator barrel of radius ~5 nm formed by three α- and three β-subunits[8]. F_1-ATPase, together with the membrane-embedded proton-conducting unit F_0, forms the H^+-ATP synthase that reversibly couples transmembrane proton flow to ATP synthesis/hydrolysis in respiring and photosynthetic cells[9,10]. It has been suggested that the γ-subunit of F_1-ATPase rotates within the α3β3-hexamer[11], a conjecture supported by structural[8], biochemical[12,13] and spectroscopic[14] studies. We attached a fluorescent actin filament to the γ-subunit as a marker, which enabled us to observe this motion directly. In the presence of ATP, the filament rotated for more than 100 revolutions in an anticlockwise direction when viewed from the 'membrane' side. The rotary torque produced reached more than 40 pN nm^{-1} under high load.

In the crystal structure of mitochondrial F_1-ATPase[8], rigid[15] coiled-coil α-helices of the γ-subunit penetrate the central cavity of the $\alpha_3\beta_3$ and extend into the stalk region that links F_1-ATPase to the F_0 portion. The amino terminus of the β-subunits is on the side opposite the stalk region of the γ-subunit. To fix the $\alpha_3\beta_3\gamma$ subcomplex on a glass plate, the subcomplex derived from a thermophilic bacterium was expressed in *Escherichia coli*, with ten histidines (His tag) linked to the N terminus of each β-subunit. The



ATP Hydrolysis:
Energy Wasted

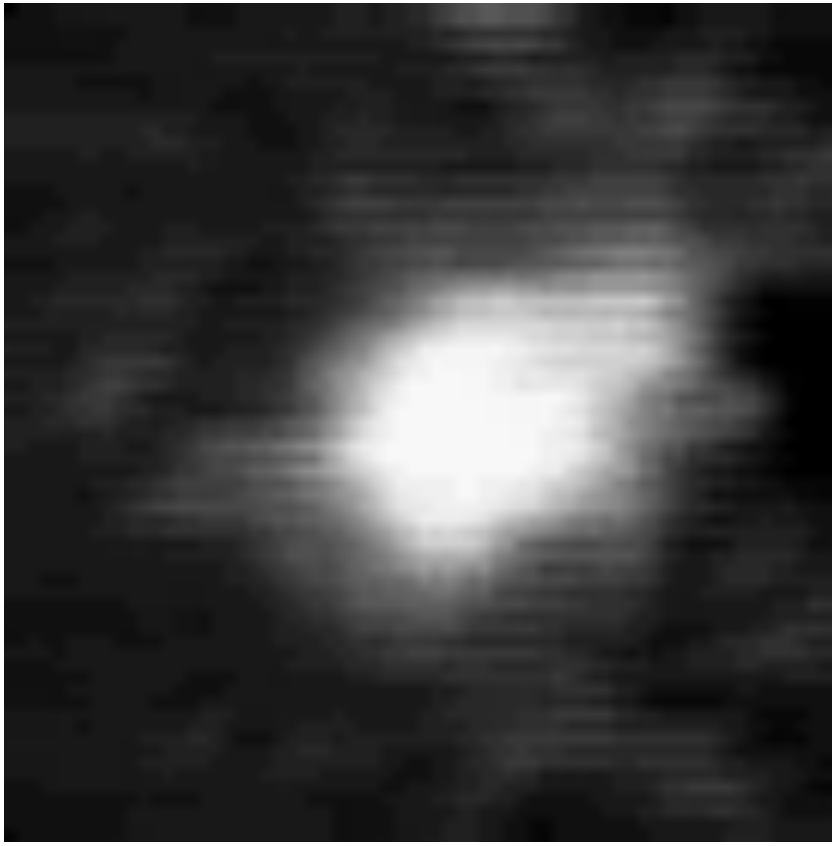
H.Noji*, **R.Yasuda***, M. Yoshida, and K. Kinosita, Jr. (1997). *Nature* 386:299-302
Equally contributed

F1 the first **single molecule experiments**

1. Experimental witness to rotation of γ subunit with single molecule spectroscopy.

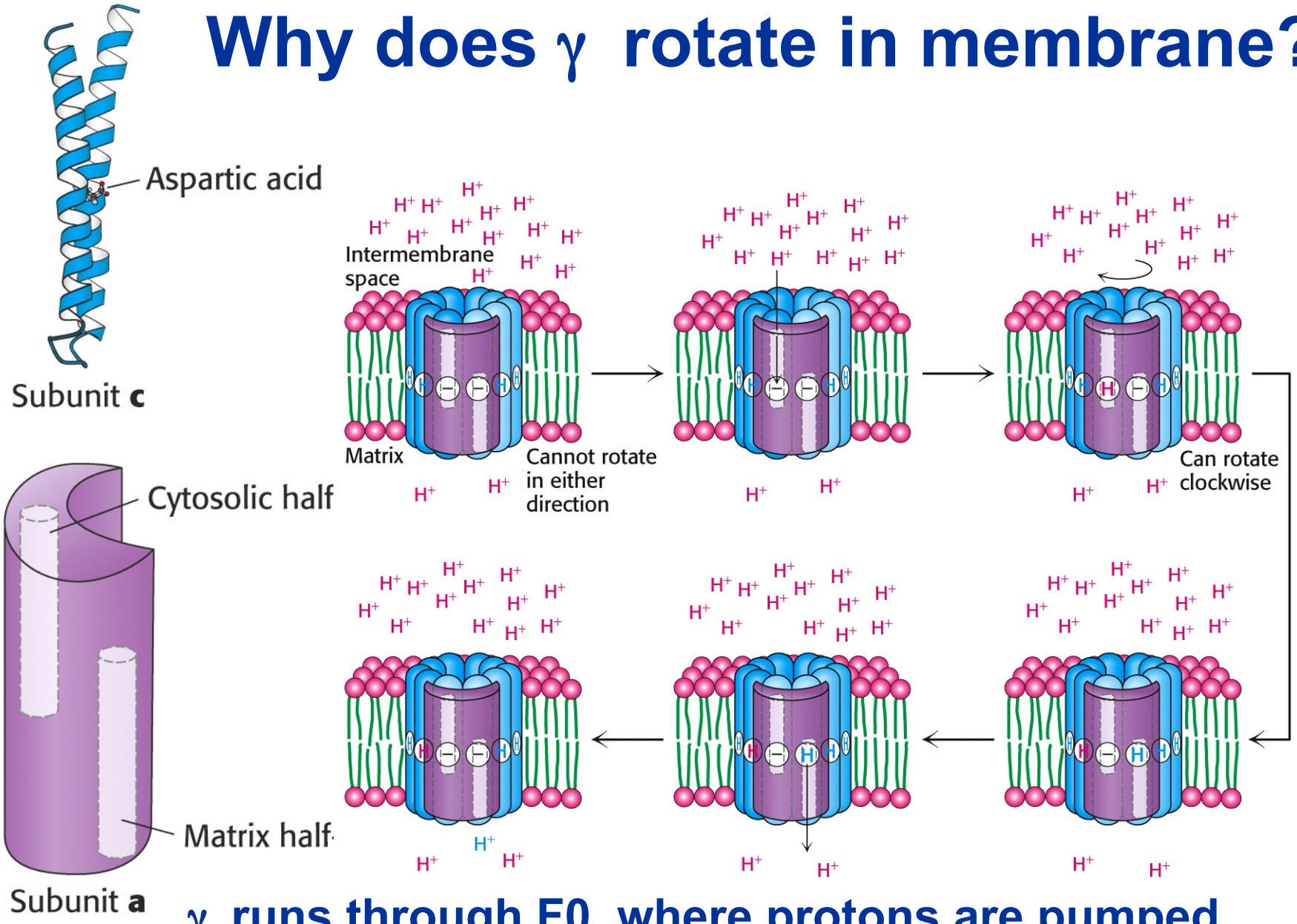
2. NO Fo subunit, DRIVING FORCE IS ATP HYDROLYSIS

3. When ATP is hydrolyzed, shaft goes in the opposite direction as before L → O; T → L: O → T releasing ADP product



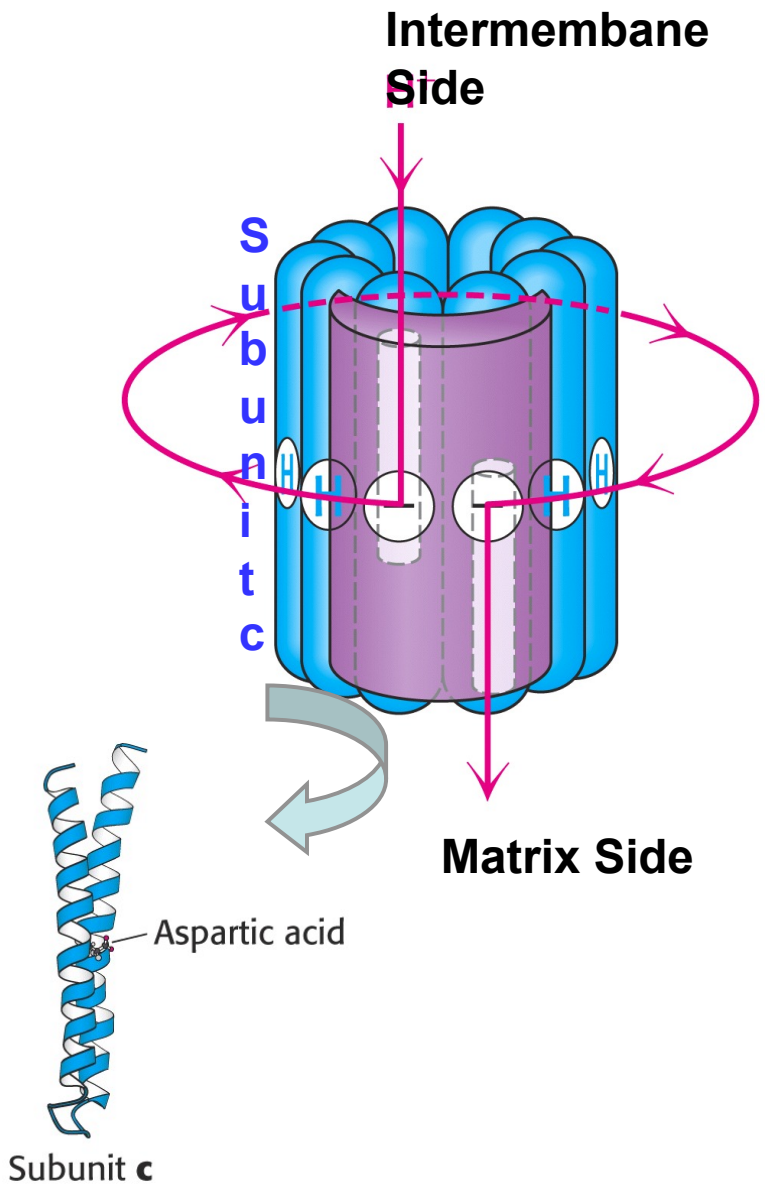
Rotation is in 120° steps
Rotor speed is 100 Hz, 3 ATP/cycle
Rate is 100 ATP/s/subunit,

Why does γ rotate in membrane?



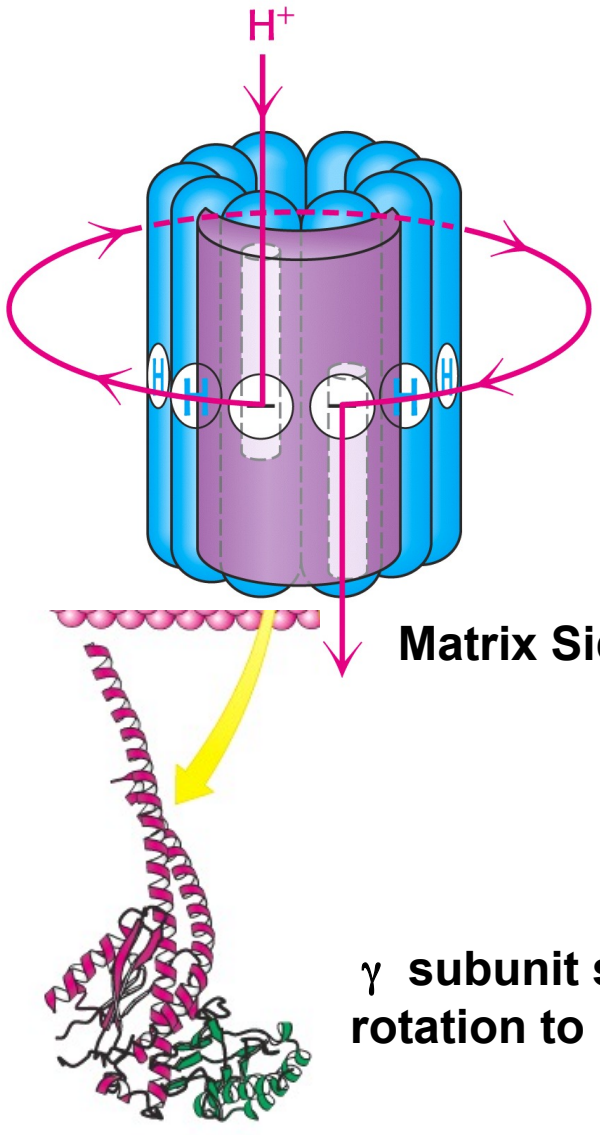
γ runs through F0, where protons are pumped

Coupling of Fo to F1



Unlike what seems to be illustrated in this image, it is not the a subunit that rotates, but the c subunits. The c subunits each contain an aspartic acid group centrally located within the helix within the membrane. When subunit a is in association with this subunit c, this asp is connected through a half channel to either the matrix space or the inter-membrane space. When the channel connects to the matrix space, the asp deprotonates because of the H+ gradient. This deprotonation is followed by a conf change which ratchets the newly deproto-nated c into alignment with the second half chan-nel, in which it reprotonates from the intermem-brane space. Meanwhile, the next c has just deprotonated to matrix, and the assembly ratchets again, moving the newly protonated asp out of alignment with any channel. it will undergo a full rotation around the assembly, driven by the suc-cessive protonation/ratchet events, until it comes full circle to the a subunit and the matrix channel, where it once again, deprotonates, ratchets and protonates.

Coupling of Fo to F1



γ subunit shaft communicates c rotation to F1

…….Meanwhile, the rotation of the c subunits within Fo transfer this rotation to the γ subunit which acts as a shaft connecting Fo to F1. Rotation of γ about the hexamer of α3β3 converts b subunits successively from O to T to L.

Happy motoring with ATP synthase

Alan E Senior & Joachim Weber

ATP synthase research has reached a milestone as single-molecule techniques are used to examine the direction and stepping of the proton gradient–driven rotation, to determine the effect of forced rotation on ATP synthesis and to synchronously monitor rotation and nucleotide kinetics.

Purposeful generation and utilization of energy define living organisms. The central molecule of biological energetics is ATP, and the enzyme responsible for the majority of ATP synthesis in organisms from bacteria to man is ATP synthase. A rush of excitement ran through this field in 1997 when Noji *et al.*[1] discovered that ATP synthase is not just an enzyme, but also a tiny motor. One part of the enzyme rotates, at ~100 Hz, and rotation is integral to the mechanism—as you read this article, billions of these 'nanomotors' are turning at this speed in your tissues and cells. This striking discovery revolutionized experimentation: it was evident that a field that had been previously dominated by membrane biochemistry and enzymology would need to import technologies of physics and biophysics. There

gradient through the enzyme, transducing gradient energy into chemical energy by converting ADP and phosphate (P_i) into ATP.

The ATP synthase from *Escherichia coli*[5] is composed of two major domains (Fig. 1). The membrane-embedded part (F_o) provides a specific H^+ conduction path, between the *c* subunit ring and the *a* subunit. The catalytic sites, three in number, are located at α/β subunit interfaces of the $\alpha_3\beta_3$ hexagon in the membrane-external F_1. The 'rotor' consists of γ and ε subunits firmly fastened to the *c* ring. A helical region of the γ subunit passes through the center of the $\alpha_3\beta_3$ hexagon, providing communication with the catalytic sites. At the periphery, the 'stator' ($b_2\delta$) prevents co-rotation of catalytic sites with the rotor due to viscous drag. Visualization of

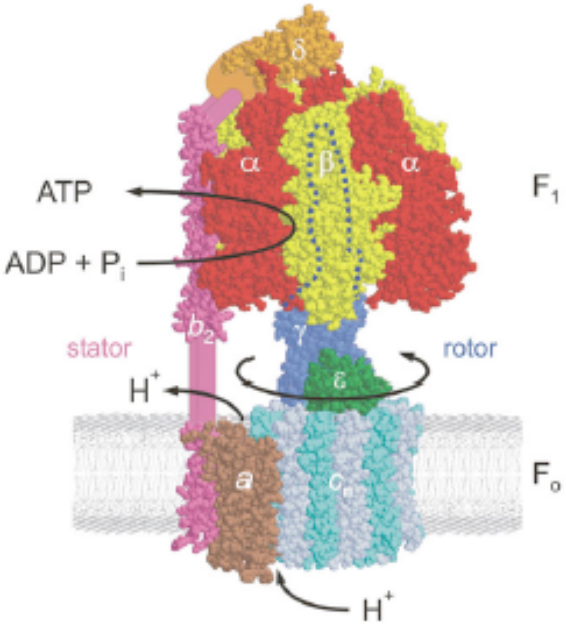


Figure 1 Structure of ATP synthase. The enzyme

Nature Struct. & Mol. Bio. 11 Feb 2004 110-112

At low dilution, only one ATP synthase containing liposome will migrate into irradiation volume at a time

ARTICLE

Nature Structural & Molecular Biology **11**, 135 - 141 (2004)
Published online: 18 January 2004 | doi:10.1038/nsmb718

Proton-powered subunit rotation in single membrane-bound F_OF_1-ATP synthase

Manuel Diez[1], Boris Zimmermann[1], Michael Börsch[2], Marcelle König[3], Enno Schweinberger[3], Stefan Steigmiller[1], Rolf Reuter[1], Suren Felekyan[3], Volodymyr Kudryavtsev[3], Claus A M Seidel[3] & Peter Gräber[1]

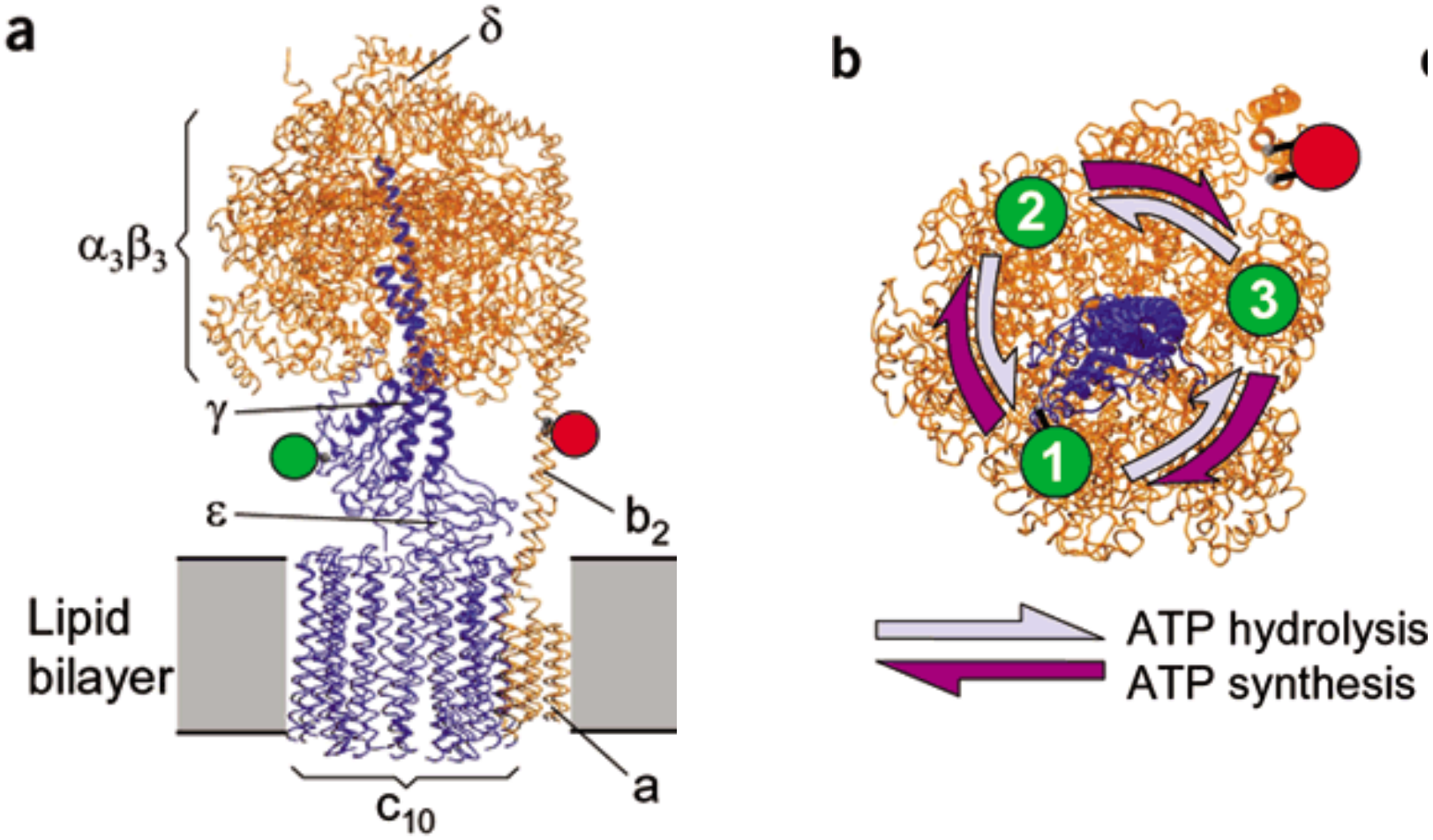
[1] Institut für Physikalische Chemie, Albert-Ludwigs-Universität Freiburg, Albertstrasse 23 a, 79104 Freiburg, Germany.

[2] 3. Physikalisches Institut, Universität Stuttgart, Pfaffenwaldring 57, 70569 Stuttgart, Germany.

[3] Max-Planck-Institut für Biophysikalische Chemie, Am Fassberg 11, 37077 Göttingen, Germany

Correspondence should be addressed to Michael Börsch
michael.boersch@physchem.uni-freiburg.de

Synthesis of ATP from ADP and phosphate, catalyzed by F_0F_1-ATP synthases, is the most abundant physiological reaction in almost any cell. F_0F_1-ATP synthases are membrane-bound enzymes that use the energy derived from an electrochemical proton gradient for ATP formation. We incorporated double-labeled F_0F_1-ATP synthases from *Escherichia coli* into liposomes and measured single-molecule fluorescence resonance energy transfer (FRET) during ATP synthesis and hydrolysis. The γ subunit rotates stepwise during proton transport–powered ATP synthesis, showing three distinct distances to the b subunits in repeating sequences. The average durations of these steps correspond to catalytic turnover times upon ATP synthesis as well as ATP hydrolysis. The direction of rotation during ATP synthesis is opposite to that of ATP hydrolysis.



Green sphere is fluorescence donor on γ (which rotates) and red sphere is fluorescence acceptor on stator, which doesn't move. In a FRET experiment, whenever the donor (green) comes close to the acceptor (red) energy will be transferred to acceptor.

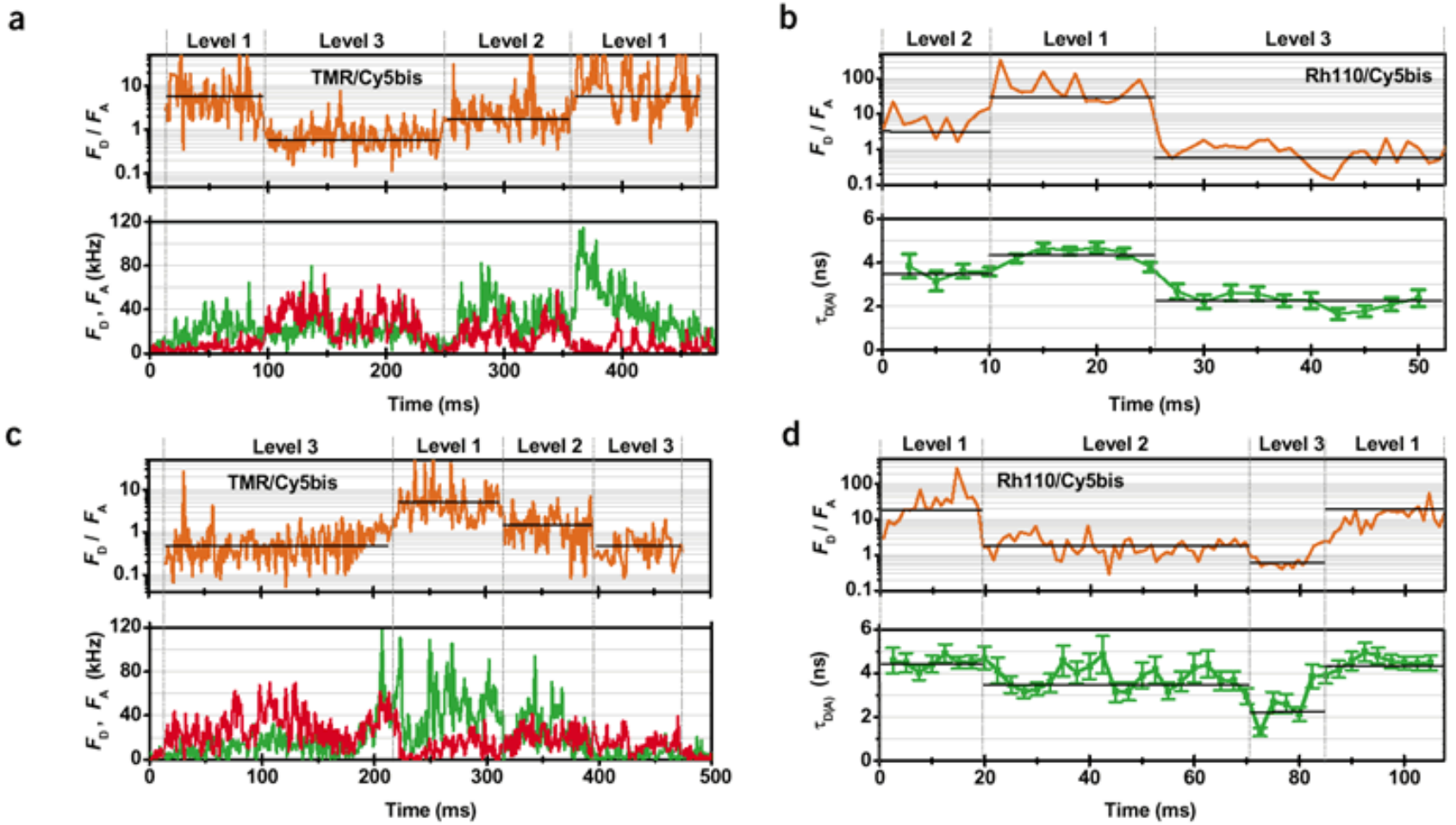


Figure 2. Photon bursts from single F0F1-ATP synthases in liposomes.
(**a**,**b**) Photon bursts during ATP hydrolysis. (**c**,**d**) Photon bursts during ATP synthesis. FRET donor is TMR in **a** and **c**, Rh110 in **b** and **d**; FRET acceptor is Cy5bis in all traces. Fluorescence intensity of the donor, *F*D, and acceptor, *F*A, are green and red, respectively, in **a** and **c** (time window 1 ms). Corrected intensity ratios *F*D / *F*A are orange in all panels (time window 1 ms), fluorescence lifetimes of the donor Rh110 are green in **b** and **d** (total time window 5 ms, shifted by 2.5 ms per data point). Three distinct FRET levels are attributed (1, 2 or 3) at top of panels.

letters to nature

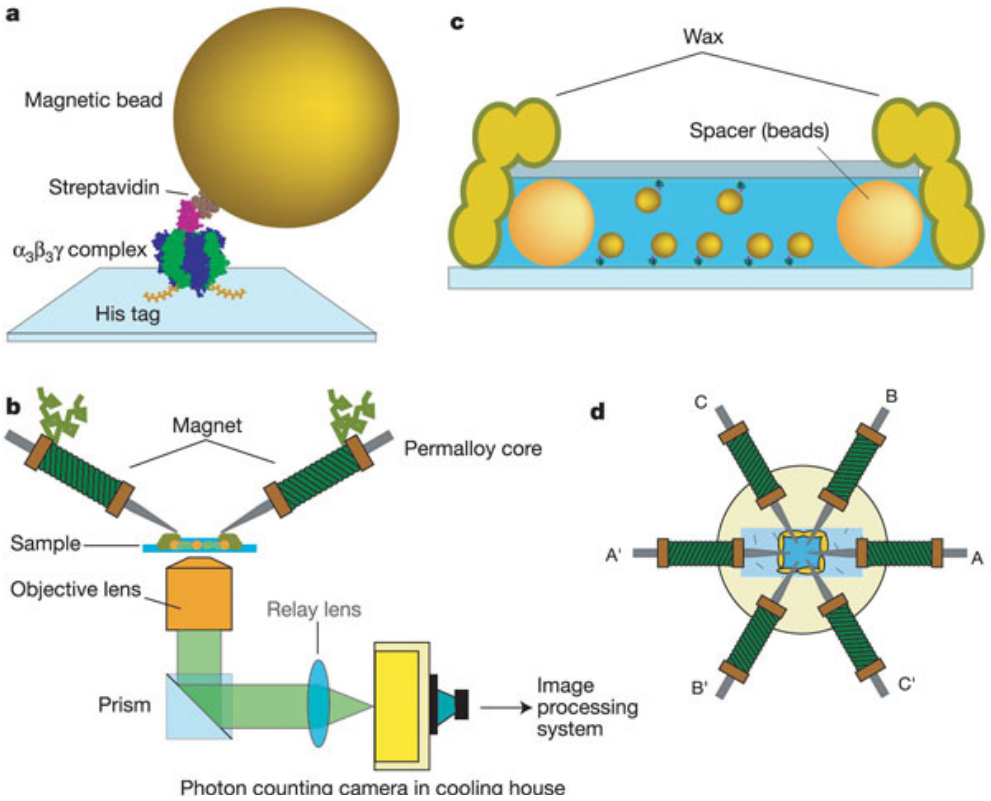
Mechanically driven ATP synthesis by F_1-ATPase

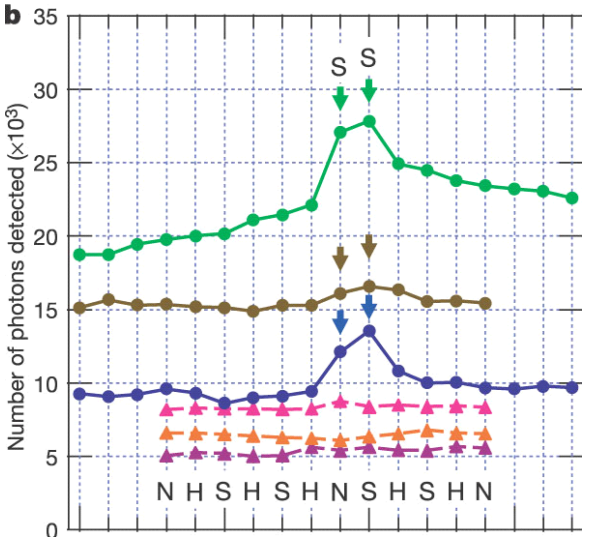
Hiroyasu Itoh[1,2], Akira Takahashi[3], Kengo Adachi[4], Hiroyuki Noji[5], Ryohei Yasuda[6], Masasuke Yoshida[7] & Kazuhiko Kinosita Jr[4]

[1]*Tsukuba Research Laboratory, Hamamatsu Photonics KK, and* [2]*CREST "Creation and application of soft nano-machine, the hyperfunctional molecular machine" Team 13*, Tokodai, Tsukuba 300-2635, Japan*
[3]*System Division, Hamamatsu Photonics KK, Joko, Hamamatsu 431-3103, Japan*
[4]*Center for Integrative Bioscience, Okazaki National Research Institutes, Okazaki 444-8585, Japan*
[5]*Institute of Industrial Science, University of Tokyo, Tokyo 153-8505, Japan*
[6]*Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724 USA*
[7]*ERATO "ATP System", 5800-3 Nagatsuta, Yokohama 226-0026, Japan*

ATP, the main biological energy currency, is synthesized from ADP and inorganic phosphate by ATP synthase in an energy-requiring reaction[1–3]. The F_1 portion of ATP synthase, also known as F_1-ATPase, functions as a rotary molecular motor: *in vitro* its γ-subunit rotates[4] against the surrounding $\alpha_3\beta_3$ subunits[5], 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_o 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





Nature Vol 427, Jan 29 2004 p465-468