Reading for lecture 10

1. "Medline" for references in biological science:

   http://www.ncbi.nlm.nih.gov/PubMed/

- Key references are included *in italics* at the relevant points in the notes.

[Reading for lecture 10] (1)

It is now possible to study molecular motors one at a time.

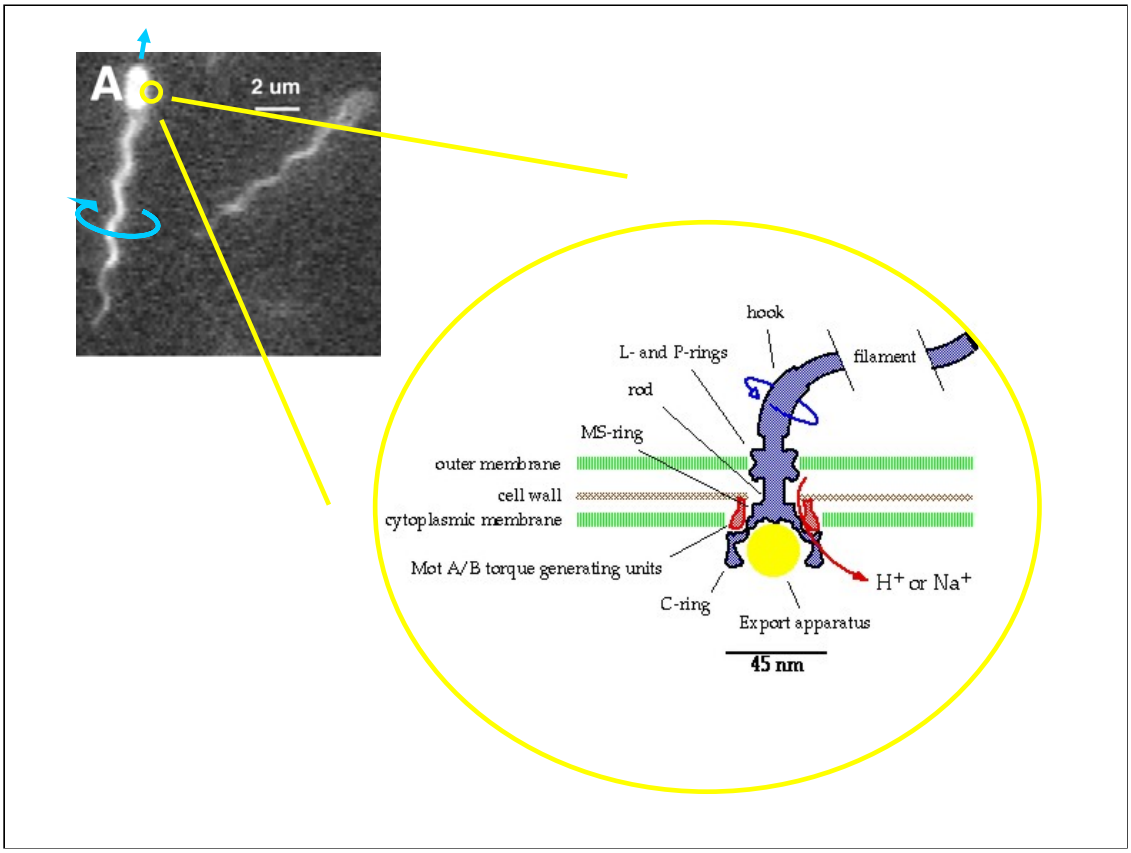
Started with bacterial flagellar motor – tethered cells, mid 1970s.

Optical tweezers first used in biophysics ~ 1990 – piconewton forces and nanometre displacements of micron polystyrene spheres can be measured.

Myosin and kinesin first studied with optical tweezers, 1994. Single mechanochemical steps observed in single motors.

F_1-ATPase rotation first observed 1997. Single mechanochemical steps and sub-steps.

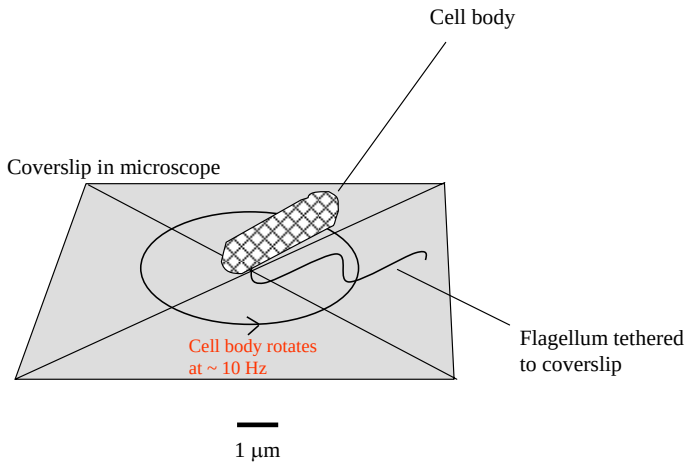
Flagellar motor single mechanochemical steps observed in 2005.



Bacterial Flagellar Motor

[Flagellar motor – reminder from lecture 7] (2)

Tethered cells



Silverman, M. and Simon, M. (1974). Flagellar rotation and the mechanism of bacterial motility. Nature 249, 73-74.

> *Demonstrated rotation of flagella. This result ruled out the alternative theory that flagella do not rotate, but propagate a helical wave.*

•Originally, flagella were attached to the glass using antibodies – molecules made by the immune system of a mammal (rabbit usually) that specifically bind to the flagellar filament.

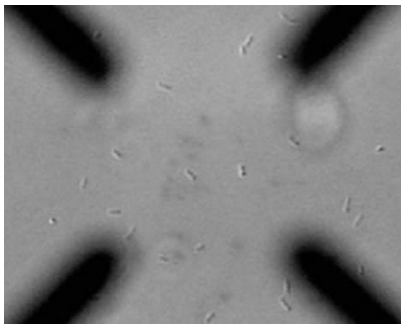
•More recent experiments use a mutant filament protein that sticks spontaneously to glass.

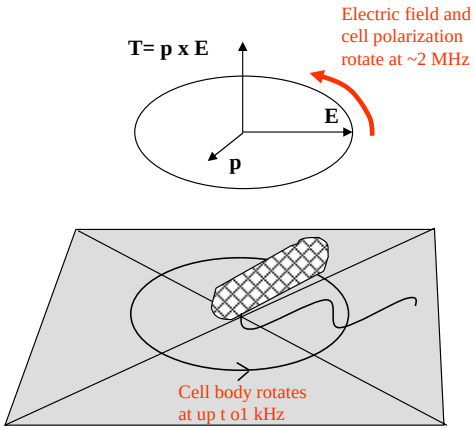
•Cell body has a high drag coefficient compared to flagella, so tethered cells rotate much slower than flagella in swimming bacteria. "High-load, low speed regime".

•For higher, more "natural" speeds, either use smaller load or add external torque..

> •Electrorotation is a technique that allows tethered cells to be rotated at high speeds…

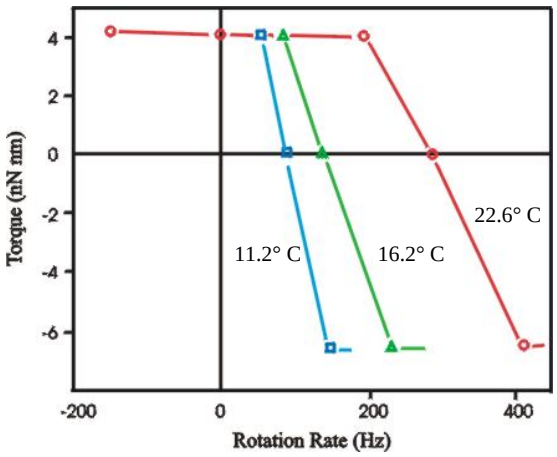
Electrorotation

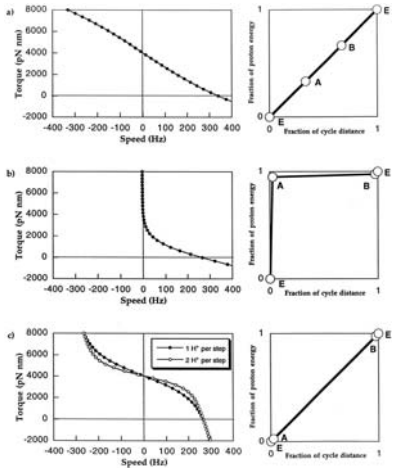




• Torque is due to the phase lag between the electric field and the polarization of the particle that it causes.

•This method is difficult, as it requires high electric fields at MHz frequencies in the sample. The cells have to be moved relative to micro-electrodes that are in the microscope sample.





(Comparison to theory, lecture 9)

[Torque vs speed in Bacterial Flagellar Motor]

Berg HC and Turner L. Torque generated by the flagellar motor of Escherichia coli. Biophys J. 1993 Nov;65(5):2201-16

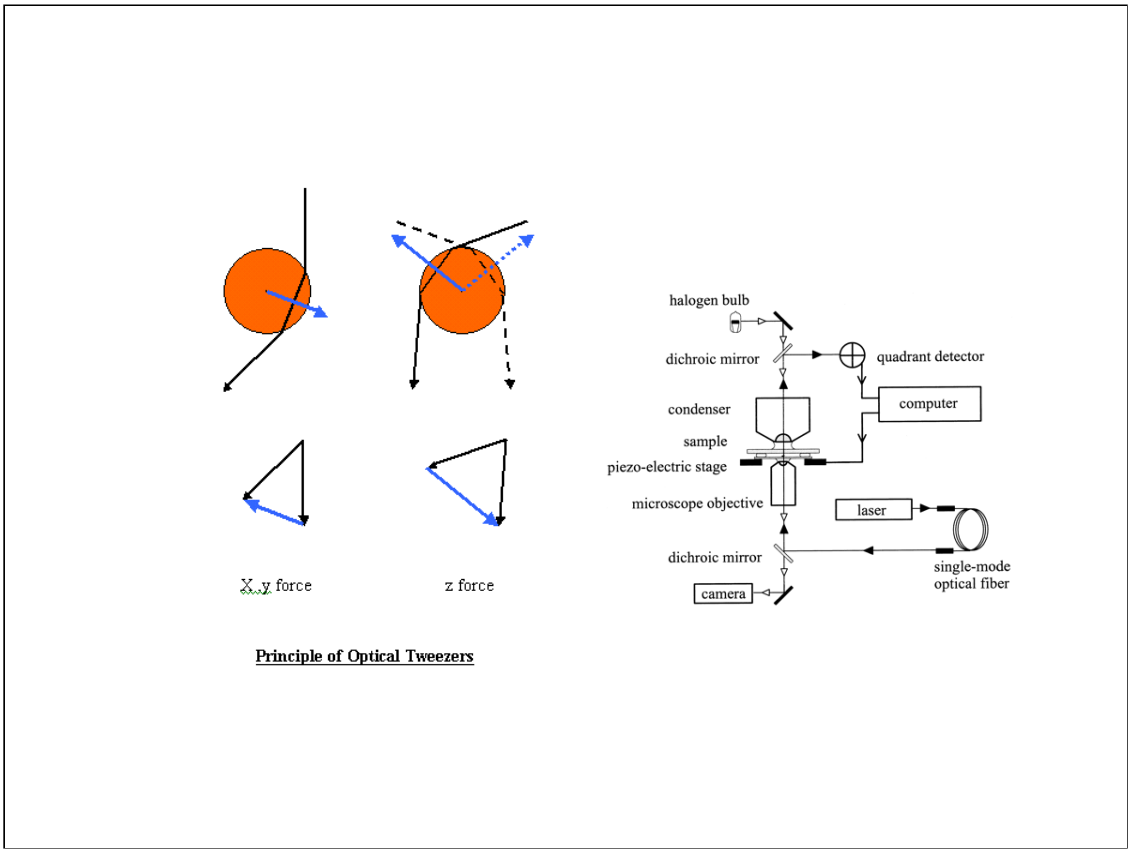
> *Torque and speed measured by electrorotation of tethered cells. Note that this paper claims that there is a barrier to backwards rotation of the motor, later shown not to be the case.*

•Concave-down torque-speed curve matches powerstroke but not thermal ratchet models.

•At low speeds torque is approximately constant. The motor is limited mechanically, and the chemical steps can always "keep up" to generate maximum torque.

•At high speeds the torque falls, eventually to zero. (Faster in Na+ motors).

- •Chemical steps are now unable to keep up with the rotation of the motor, and the motor finds itself "stuck" in states at angles where they no longer generate maximum torque.
- •At zero torque speed, all of the proton free energy is being dissipated in the "channels", and there is no work done.
- •The speed at which torque begins to fall depends upon temperature, consistent with the theory of chemical steps being thermally activated transitions with rates proportional to *exp{-DG / kT}.*



Principle of Optical Tweezers

The absolute torque generated by the flagellar motor at speeds very close to zero (stall) has been measured with “optical tweezers”

(confirming the shape of the torque-speed curve of slide 6, near zero speed)

Optical tweezers

•Optical tweezers use a focussed laser beam to trap transparent spheres with radiation pressure.

Ashkin A. (1997) “Optical trapping and manipulation of neutral particles using lasers”. Proc Natl Acad Sci U S A. 94(10):4853-60.

By the inventor of optical tweezers.

•The technique has revolutionized the study of molecular motors

Mehta AD, Rief M, Spudich JA, Smith DA, Simmons RM. (1999) “Single-molecule biomechanics with optical methods.” Science 283(5408):1689-95.

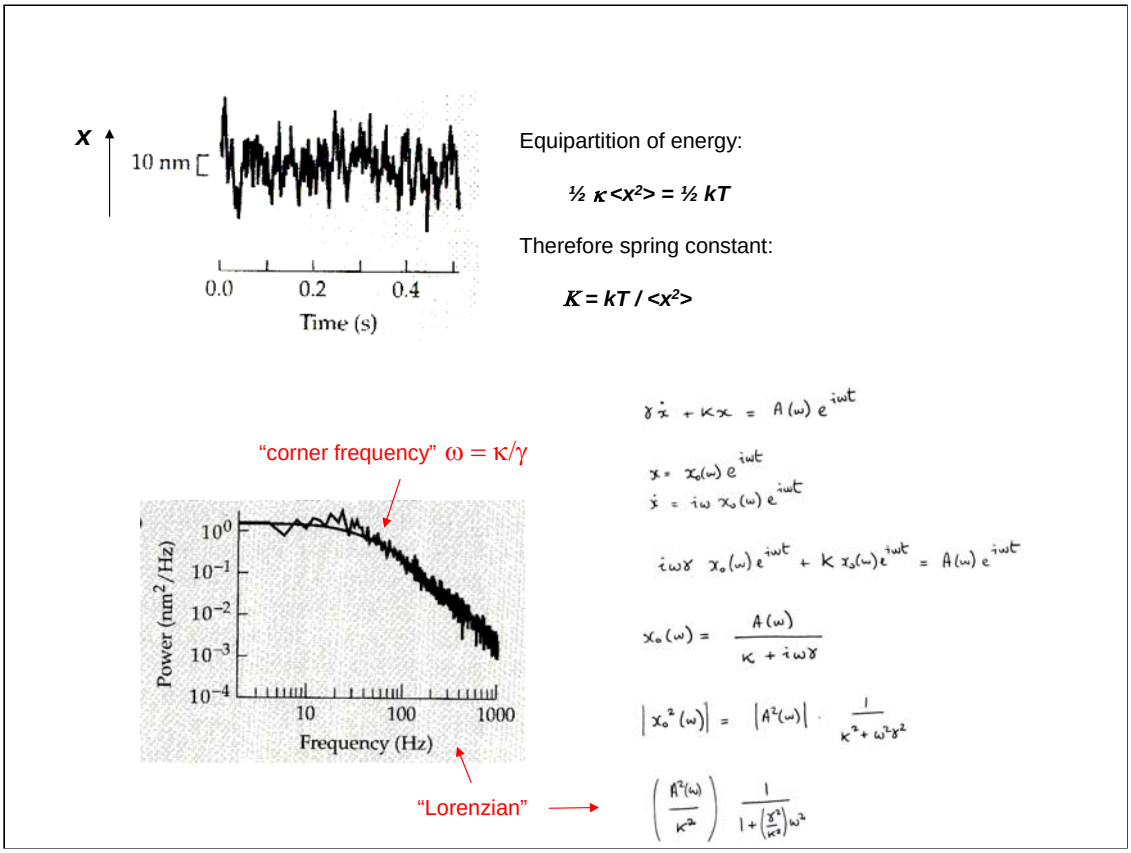
One of many reviews of molecular motors experiments using optical tweezers.

•Forces are in the range of several piconewtons (pN, 10-12 N), typical of biological molecular motors.

•Tweezers can also measure nanometre displacements of the trapped particle, typical of the distances that molecular motors move.

•For small displacements, force is proportional to displacement – the trap behaves like a linear spring.

•The lateral deflection of the laser beam by the trapped particle is a measure of the momentum exchanged, and thus of the force and the displacement.



[Brownian motion in an optical trap]

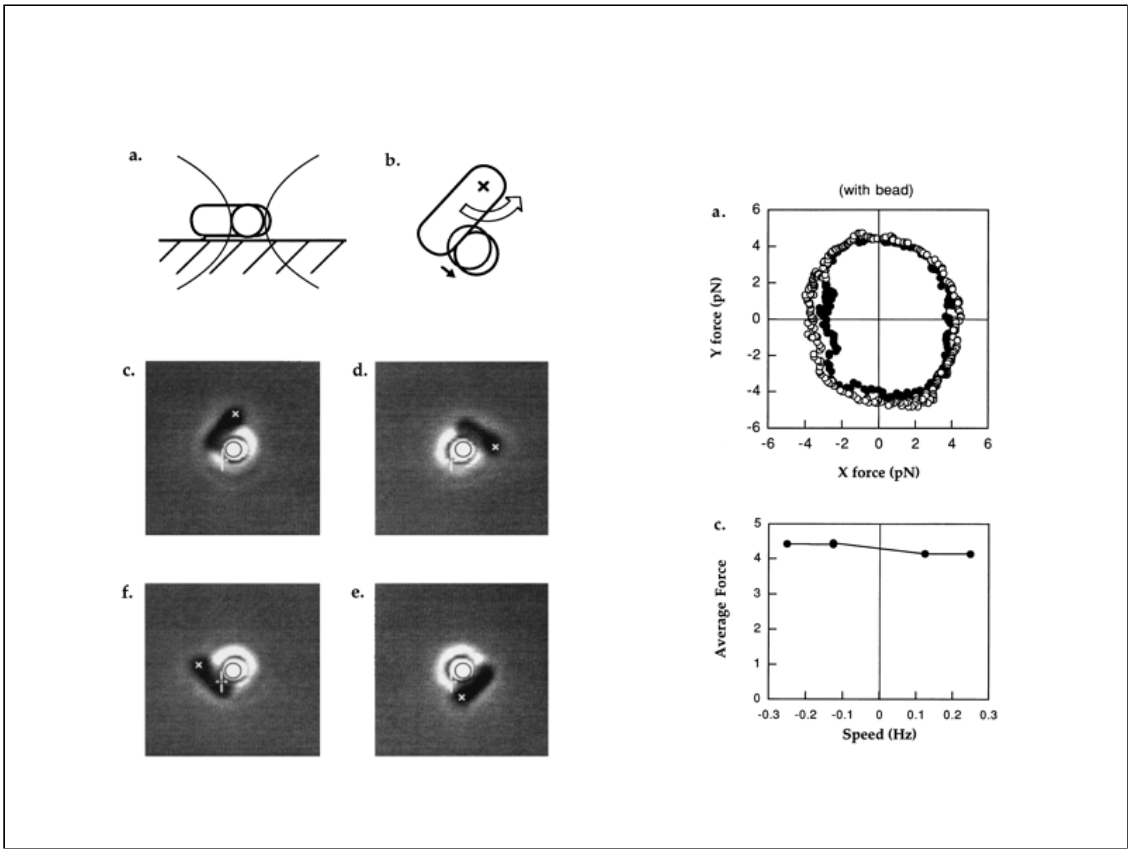
Equipartition of energy gives the stiffness of the trap from the absolute displacement.

•The mod-squared of the Fourier transform of the bead position is called the Powerspectrum. It quantifies the magnitude of bead motion in the frequency domain.

•The frequency response of a trapped particle is a "Lorenzian" curve – a simple result for an overdamped harmonic oscillator.

•The thermal driving force is "white noise", so its amplitude *A(w)* is independent of *w*. Thus the Lorenzian curve also describes the powerspectrum of a trapped bead.

•Finding the "corner frequency" gives the trap stiffness if the drag coefficient is known, or vice versa. (The absolute displacement does not need to be known.)

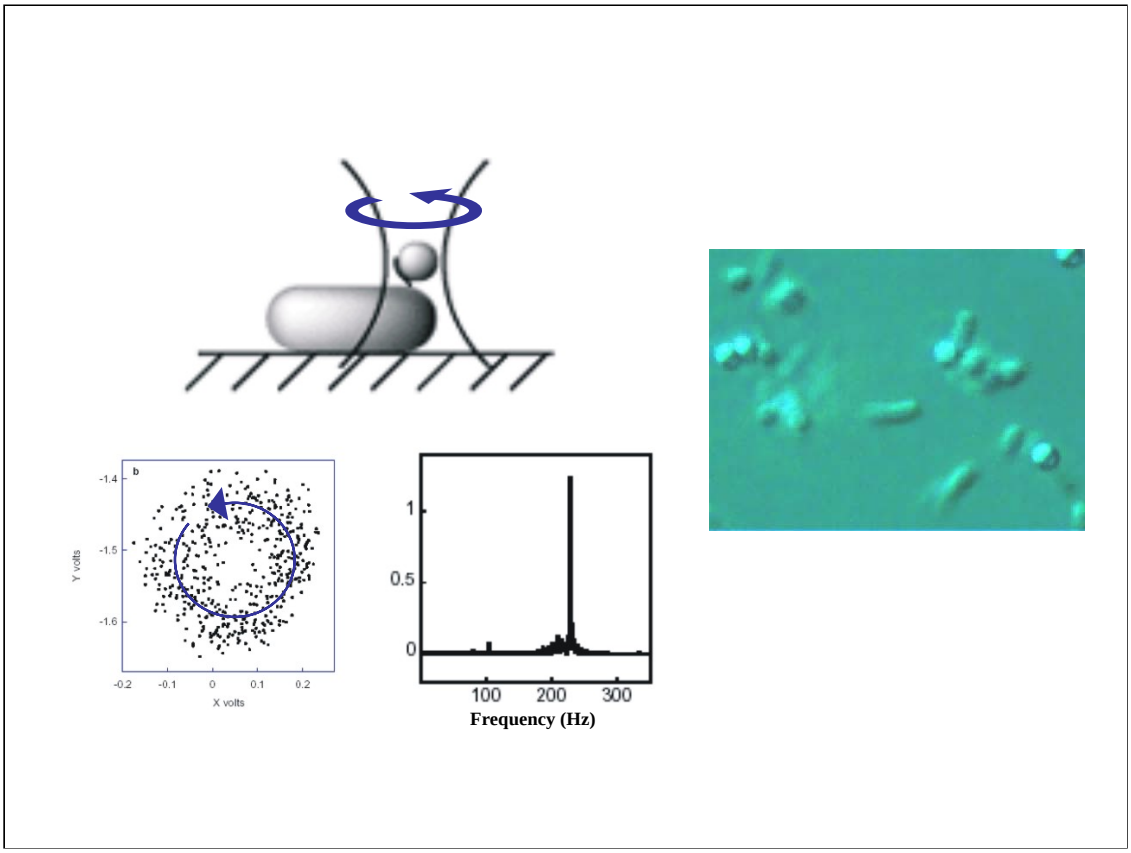


[Measuring the torque generated by the flagellar motor near stall with optical tweezers.]

•Force is measured in x and y as optically trapped bead pushes the motor slowly through one revolution backwards, or allows it slowly to make one revolution forwards.

•Similar force is required to push motor backwards as to make it rotate forwards very slowly – torque-speed curve is continuous through zero speed.

•Confirms that the motor is not like a thermal ratchet (Other molecular motors are, eg kinesin requires much more force to push backwards than to stop it moving forwards.)



[High-speed measurements of flagellar rotation using smaller viscous loads.]

•Polystyrene beads 0.3 – 2 microns in diameter stick to (mutated) flagella

•Reduce viscous drag coefficient by mechanically breaking off most of the filament

•Measure position of the bead using a weak optical trap

•Power-spectrum of the bead signal $z = x + iy$ gives speed and direction (note for a circular bead orbit $x + iy = exp(+/- iwt)$, so the Fourier transform is a delta function at +/- w).

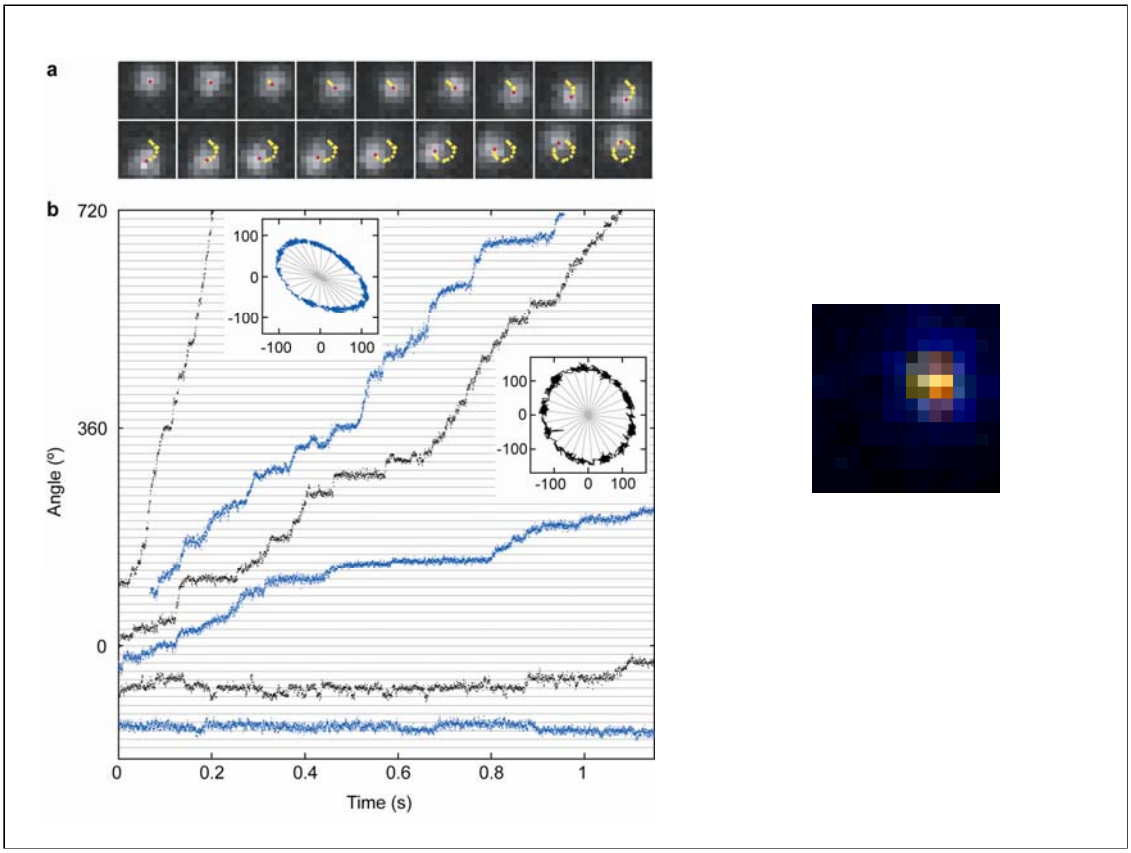
•Torque-speed curves can be measured by varying the viscous drag coefficient of the bead, either via viscosity of solution or bead size.

Work = torque x angle

Torque = d(work) / d(angle)

Low Reynolds number:

Torque = viscous drag coefficient x angular velocity



[steps in flagellar rotation]

Sowa Y, Rowe AD, Leake MC, Yakushi T, Homma M, Ishijima A, Berry RM. (2005) Direct observation of steps in rotation of the bacterial flagellar motor. Nature. 437:916-919.

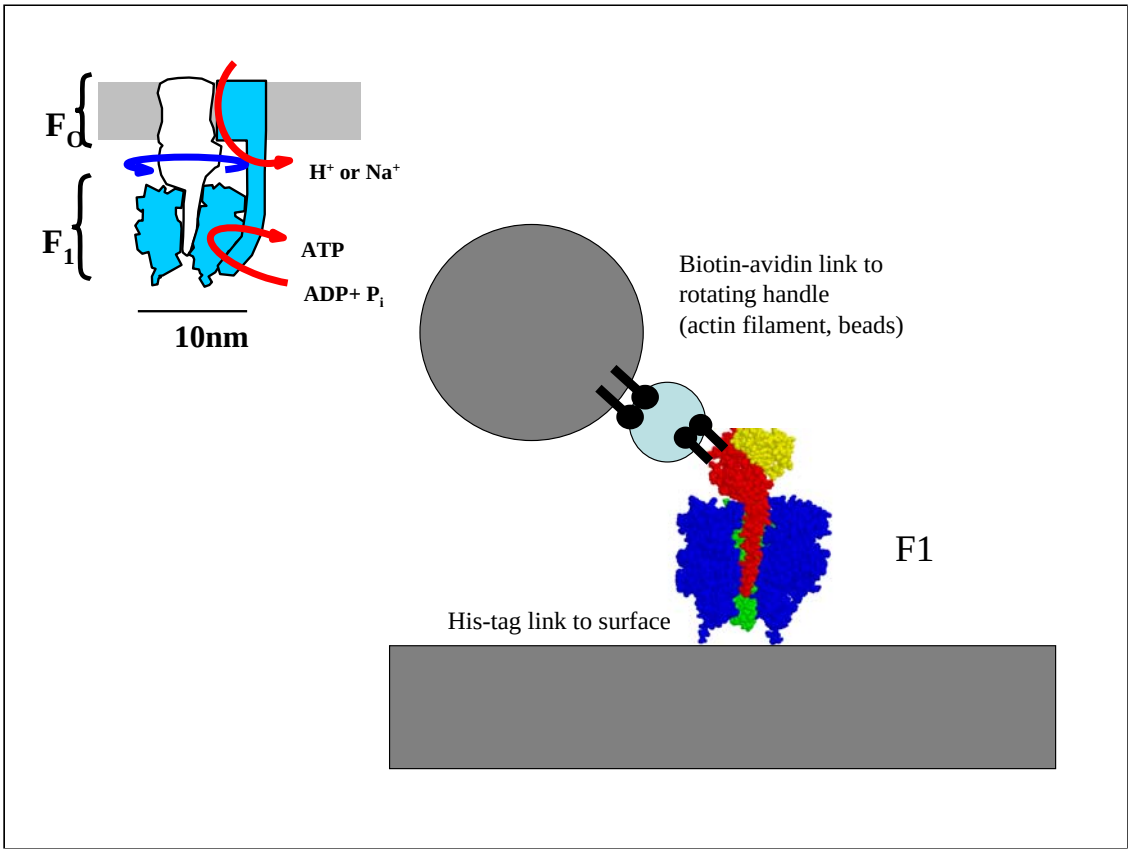
•26 steps per revolution (~ 14° per step)

•Compare to 26-fold periodicity of inner part of the flagellar rotor – is each step one mechanochemical cycle on a 26-fold track?

•Energetics: the free energy of one proton crossing the membrane is NOT ENOUGH to rotate 14° at the measured torque of a single-stator motor.

•2 ions per step, or something more complicated? We still don't know.

•Individual mechano-chemical steps in F1-ATPase are better understood…



F1-ATPase

[measuring rotation of F1]

Noji H, Yasuda R, Yoshida M, Kinosita K Jr. Direct observation of the rotation of F1-ATPase. Nature. 1997 Mar 20;386(6622):299-302

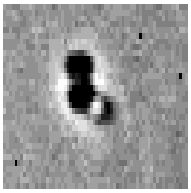
The first direct observation of rotation of F1, using actin filaments

•A visible handle must be attached to the rotating g-subunit if rotation ins to be detected.

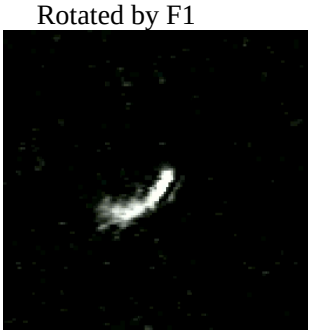
•Numerous techniques are available for making specific attachments to the right parts.

- •Biotin is a small vitamin that is tightly bound by streptavidin, both are available commercially.
- •A derivative of biotin is used that make covalent bonds with only the –SH groups of genetically engineered cysteine residues into the g-subunit. All other surface cysteines are removed, again genetically.
- •Multiple histidines are added to the end of the b-subunit by genetic engineering. This “his-tag” binds Nickel ions that are lined to the surface by a molecule called “NTA”.
- •(His-tags are commonly used to purify the protein that is encoded by a selected gene.)

0.5 micron beads
Rotated by F1

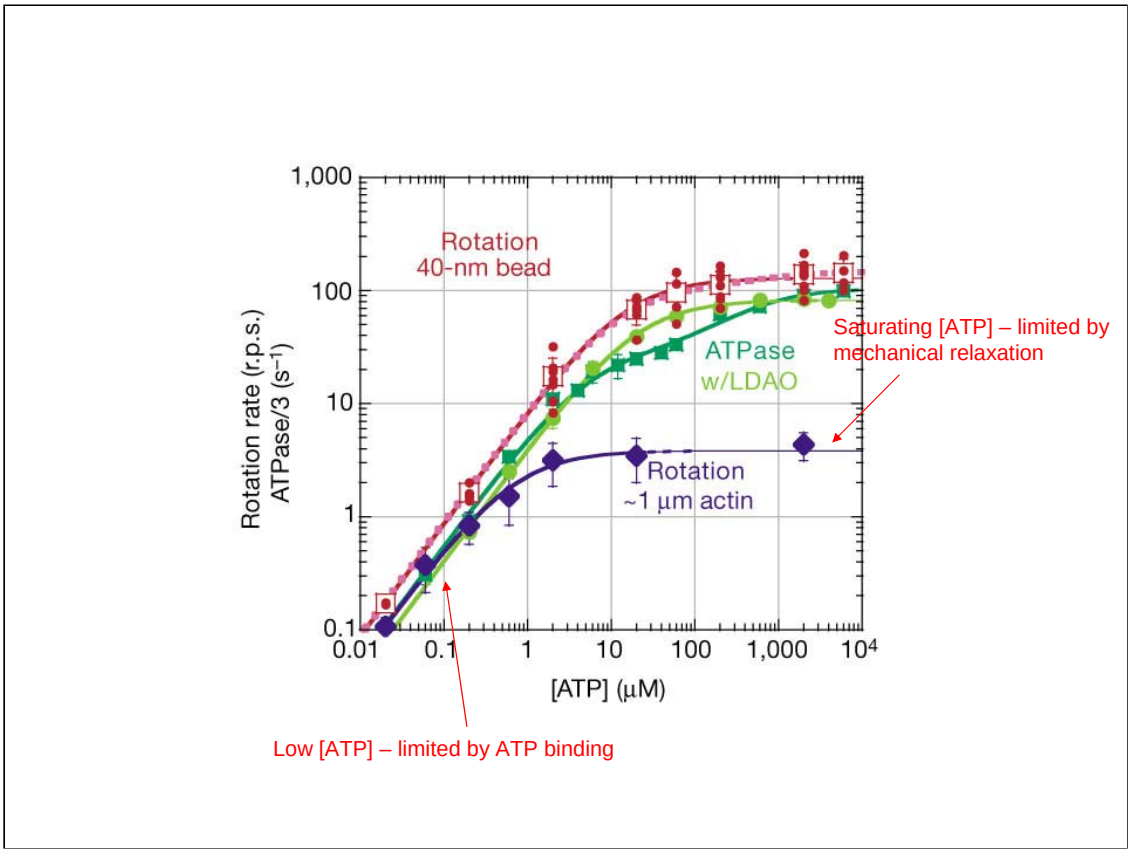


Fluorescent actin filament
Rotated by F1



(movie: Wolfgang Junge)

F1 definitely rotates!

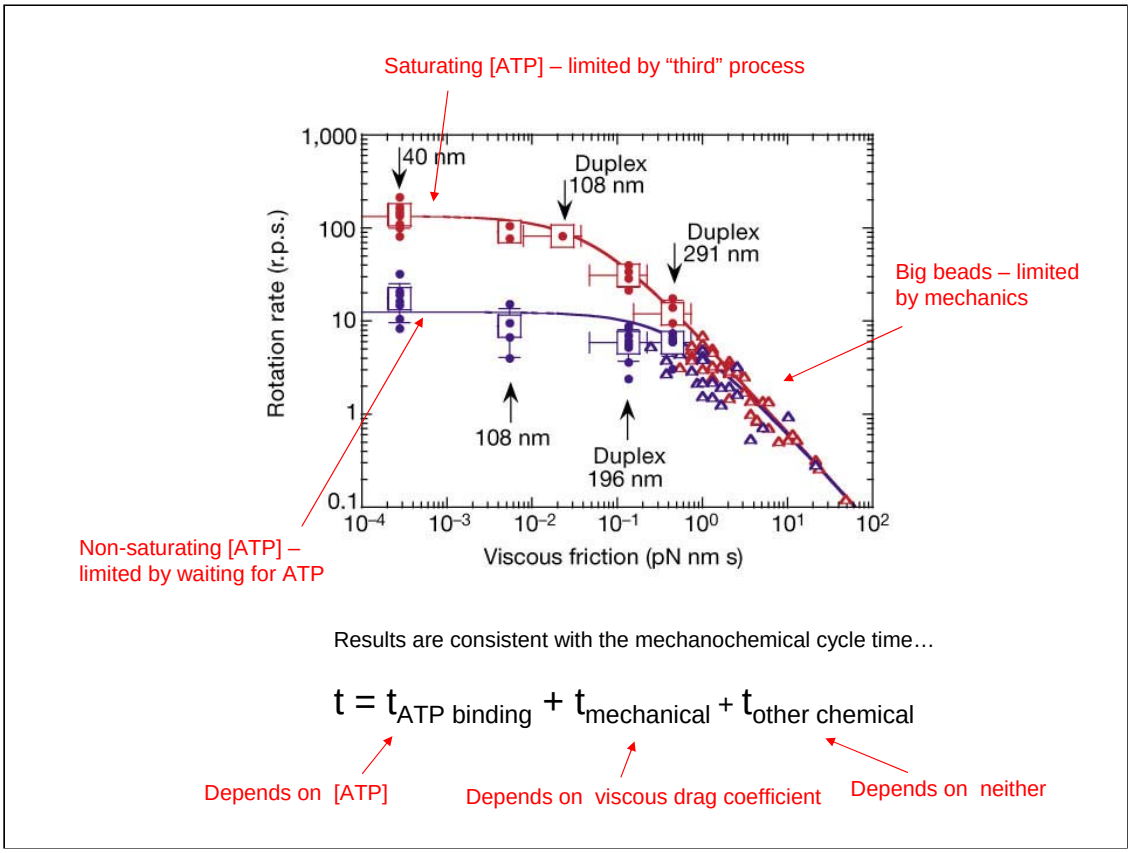


[rotation rate vs ATP]

•Rotation rates (top and bottom curves for small and larger handles respectively) obey Michaelis Menten kinetics (see lecture 5)…

- •Maximum speed, at saturating [ATP], is faster for smaller handles. Thus for the larger handles at least, mechanical relaxation of the motor is rate-limiting at high [ATP].
- •At low [ATP], the speed is determined by waiting for ATP to bind, and speed is proportional to [ATP].

•Analyse by distinguishing between steps that depend on ATP binding and those that don't.

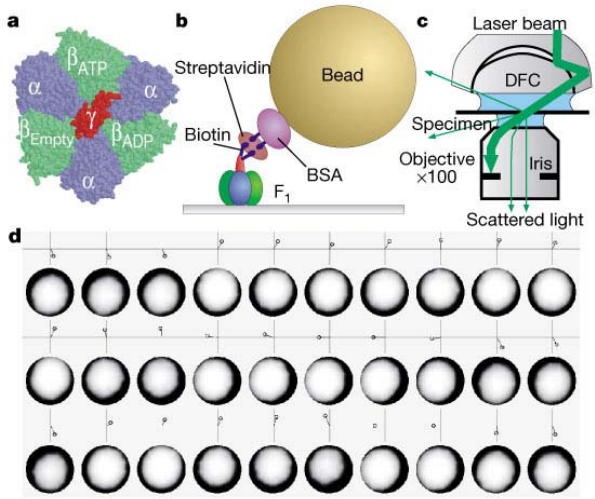


[rotation rate vs handle size]

•Analyse by distinguishing between steps that depend on viscous drag (ie. mechanical relaxation to the energy minimum of the new state) and those that don't.

•At saturating [ATP], making handles smaller than 100 nm does not make the motor go faster.

- •In this case, mechanical relaxation, is no-longer rate-limiting, even at saturating [ATP]. Something else must be rate-limiting here…
- •Thus a 3rd type of step is identified, that depends on neither viscous drag nor ATP binding.



All becomes clear when individual steps in the mechanochemical cycle can be seen…

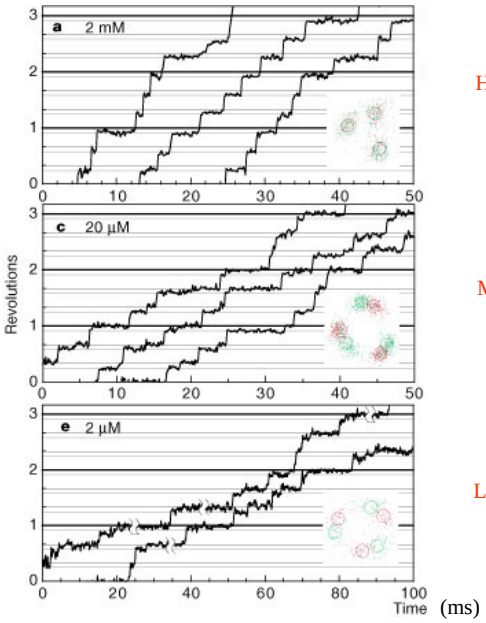
[rotation with 40 nm gold particles]

Yasuda R, Noji H, Yoshida M, Kinosita K Jr, Itoh H. Resolution of distinct rotational substeps by submillisecond kinetic analysis of F1-ATPase. Nature. 2001 Apr 19;410(6831):898-904

> *Measurement of rotation with Increased time-resolution, allowing sub-steps to be seen.*

•The link between the handle and the gamma-subunit has torsional stiffness k, and therefore the handle filters rotation of the gamma-subunit with a time constant k/f, where f is the rotational drag coefficient of the handle (compare optical tweezers, slide 10.)

- •f can be calculated for a sphere, and depends on radius cubed. Therefore smaller handles reveal faster events, larger handles smooth them out.
- •40 nm gold particles can be seen using dark-field microscopy.



High [ATP] : no wait before 90° step

Medium [ATP] : ~ms before 90° step

Low [ATP] : long wait before 90° step

All [ATP] : ~ms before 30° step

[Steps in rotation of F_1]

•The motor rotates in 120° steps, each corresponding to hydrolysis of 1 ATP.

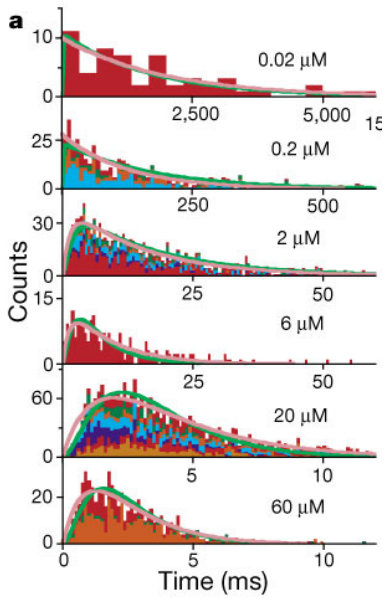
•Sub-steps of 90° and 30° are resolved when 40 nm gold handles are used to give high time-resolution.

Key results:

•The time interval before a 90° step is inversely proportional to [ATP] – this is the ATP waiting step.

•The time interval before a 30° step is about 2 ms, independent of [ATP] – this is ATP hydrolysis and product release steps.

•The time interval during any step is very short, ~0.25 ms, close to the time-resolution limit of the technique – this is the mechanical relaxation, and is consistent with a torque of about 40 pN nm, equal to the torque at stall, during the powerstroke.



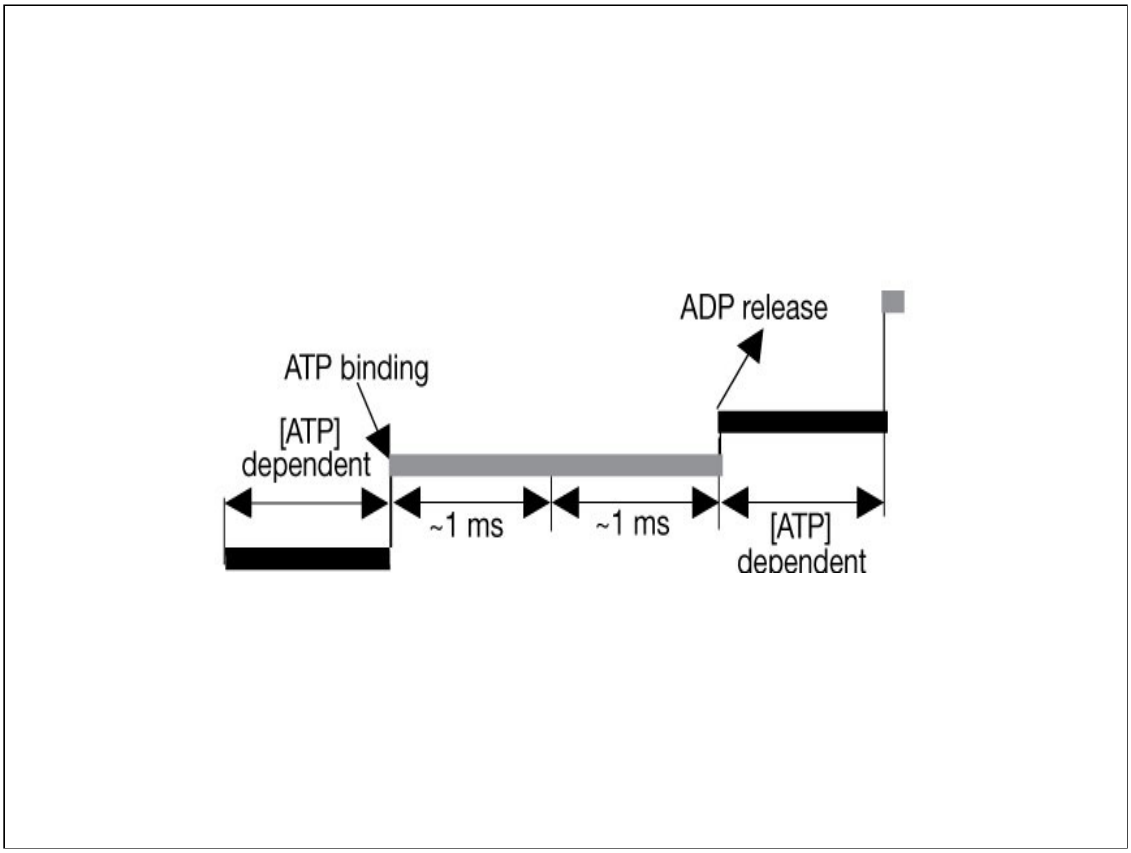
Low [ATP] :
- 90° step rate-limiting
- exponential distribution
- single step

High [ATP] :
- 30° step rate-limiting
- peaked distribution
- double (or more) step

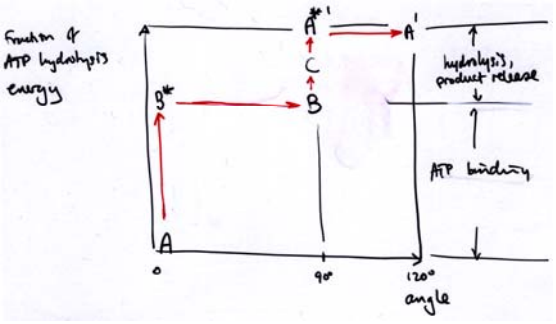
[Interval length distributions]

•When the ATP waiting step is rate-limiting, distributions are single-exponential – ATP binding is a single transition.

•When the 30° step is rate-limiting, distributions have a peak – this step is actually at least two sub-steps with similar rates. See lecture 5 slide 18 for derivation of this distribution.



[Summary of F1 rotation results]



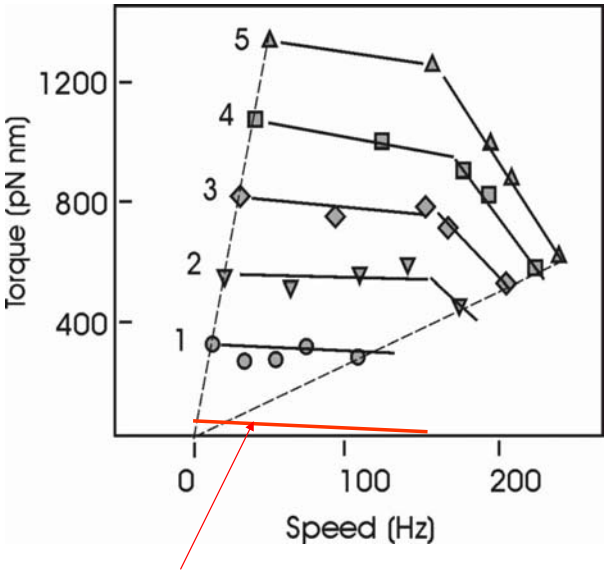
$$\tau = \tau_{AB^*} + \tau_{BA^{*\prime}} + \tau_{B^*B} + \tau_{A^{*\prime}A}$$

$\propto 1/[ATP]$ $\sim 2ms$ $\propto f$

(drag coefficient, γ)

[Chemical and mechanical steps]

•Expressing these results in terms of the model of lecture 9, we see that F_1 can be classed as a 2-powerstroke motor.



Average torque vs speed for F_1

[Average torque vs speed]

•The data of slide 16 can be used to find average torque vs speed for comparison with the flagellar motor and also with the simple kinetic model…

- •Linear torque vs speed, as expected from model.
- •MUCH less power than flagellar motor. Power is torque times speed, or area on the torque-speed plot. (Flagellar motor torque speed curves are for motors with 1 to 5 independent torque generators, measured using the method of slide 10. Each generator is orders of magnitude more powerful than F1.)
- •Power and high speed of the flagellar motor may be possible because it runs on the pmf rather than ATP hydrolysis.

•Next lecture: Single molecule experiments on Myosin with optical tweezers.