CELL MECHANICS

1. Introduction

2. Physical principles

2.1. Forces at molecular and cell level

2.2. Thermal forces, diffusion, and chemical forces

2.3. Motor proteins (types, working principles)

3. Mechanics of the Cytoskeleton and Mechnaotransduction (6h)

3.1. Cytoskeleton structure

3.2. Force generation by the cytoskeleton and cell motility

3.3. Cellular mechanotransduction (basic principles and examples)

5. Experimental techniques to study cell mechanics (10 h)

5.1. Optical, magnetic and acoustic tweezers

5.2. Super-resolution optical microscopy techniques (STED, PALM)

5.3. Lab visit and experimental optical tweezers – cell mechanics session at CNR-IOM

Remember Lecture 5

- Chemical equilibria and the effect of force on chemical equilibria
  - Conformational and structural states of a protein
  - Concentration – probability – free energy – force influence – example mechanotransduction in hair cells

- Rate theory of chemical reactions and the effect of force on the chemical rate constant
  - Arrhenius equation , Eyring and Kramers rate theories
  - How to determine the dissociation rate of a ligand – receptor bond using mechanical force – model and example of experimental implementation

All the models are wrong

but

some of them are useful !

G. Box

Lecture 6

- Use the force to measure unbinding forces and dissociation rate k_{off} for Ligand-Receptor bond

How it works in practice ?

- Use the force to investigate folding and unfolding of proteins at single molecule level

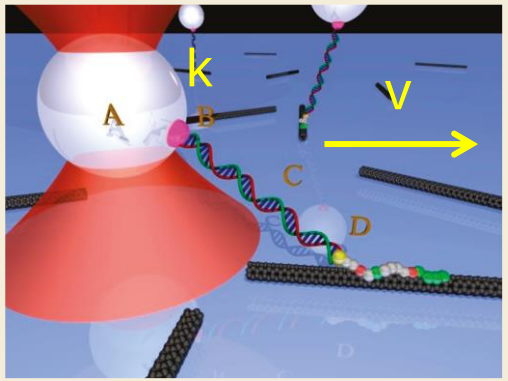
Example: giant muscle protein Titin – AFM vs OT

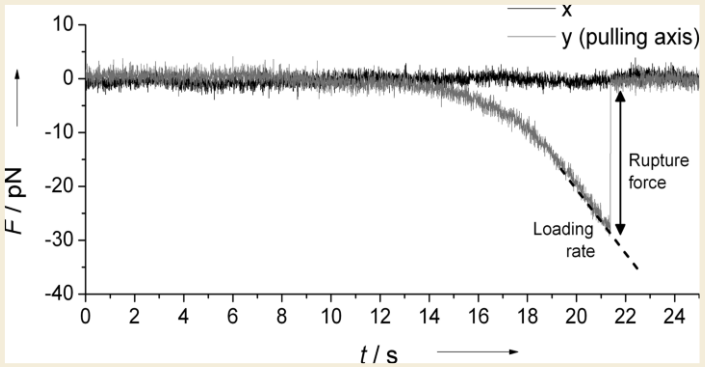
- OT experimental configurations for force spectroscopy; examples

- OT – physical principles and properties

Use the force to measure unbinding forces and dissociation rate k_{off} of Ligand to Receptor

How it works in practice ?





1. We need a tool to exert force: AFM, OT, MT, AT; the choice depends on the strength of the bond.
2. We need linkers to connect the ligand with the probe (e.g. OT bead, AFM tip)

* Measurement procedure:

1. Pull the "construct" (ligand + linker) with a force F:

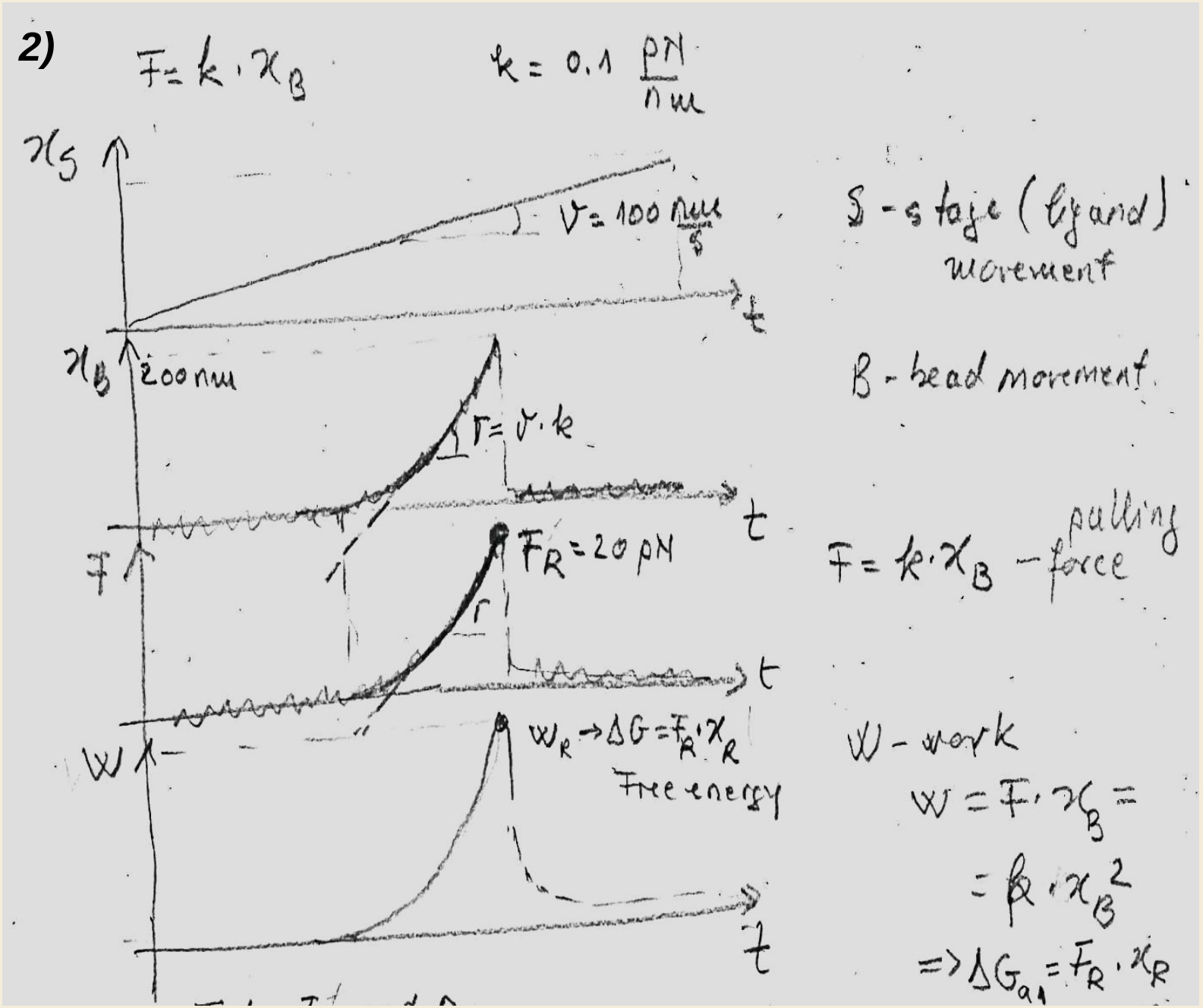
$F = r \cdot t$, r - load rate $\left[\frac{pN}{s}\right]$, t - time [s]

eg. for OT: stiffness of the trap $k_{OT} = 0.001 - 0.5 \left[\frac{pN}{nm}\right]$ $F = k \cdot x_B$

Force is applied by moving the ligand (fixed on substrate) with a constant velocity v; $v = 20 - 2000 \left[\frac{nm}{s}\right]$

The load rate, r, will be then $r = v \cdot k$

r= 0.02 – 1000 [pN/s]



Force ramp approach

The force F increases with time t:
F(t)= r t
Force is calculated measuring the displacement xB: F(xB) = k xB

The load rate r = v k and is the slope of the tangent to the measured displlacement of the bead.

3) Take F_R and r:

→ One measurement is not enough (stochastic behavior)

→ $N \gg 50$ measurements necessary → repeat:

$$\Rightarrow \{F_R^i\}_{i=1-N}, r_1$$

plot the probability distribution $P(F)$ and determine F^*

k_{off} and x as free parameters

$$P(F) = \frac{k_{off}}{r} \cdot \exp\left\{\frac{Fx}{kT} + k_{off}\frac{kT}{R}\Big/\left(1-\exp\frac{Fx}{kT}\right)\right\}$$

Note: k_{off} here is in presence of load ($F \neq 0, > 0$)

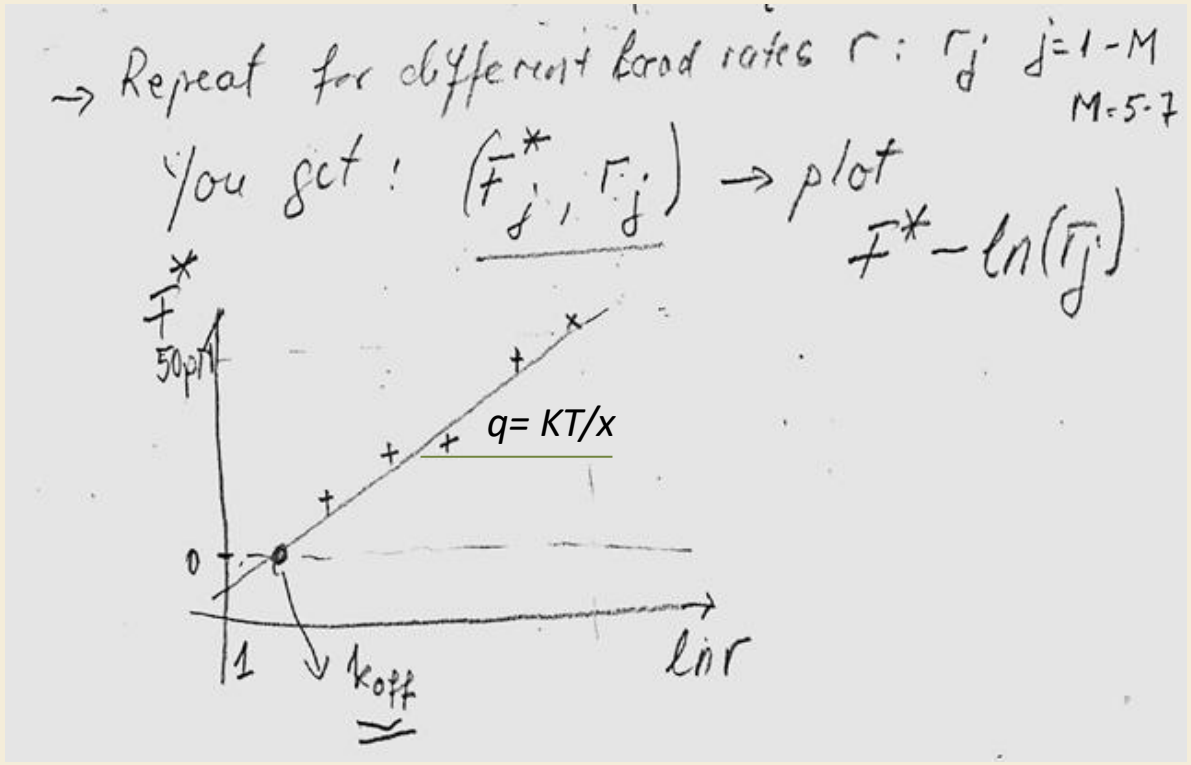
The most probable unbinding/dissociation force

$$F^* \simeq \frac{kT}{x}\ln\left(\frac{x}{kT}\frac{r}{k_{off}}\right) \qquad \frac{kT}{x} = 2$$

$$k_{off}(F^*) = k_{off} \cdot \exp(F^* x / KT)$$

Having more than one dataset at different load rates ***r*** it's crucial to extrapolate the value of k_{off} in absence of load F=0, (i.e. natural thermal off-rate), that is the most relevant parameter the assay can return.

4)



$$F^* = 2\ln\left(\frac{r}{2\cdot k_{off}}\right) = 2\ln r - 2\ln(2\cdot k_{off})$$

F^* linear with $\ln r$

The slope is q= KT/x

$F^*=0 \rightarrow$ $\quad k_{off} = r_0 / q$

r_0 – *extrapolated load rate r for F*=0*

Data set	r [nm/s]	$\bar{F}^*$ (pN)	x_{a1} [nm]	k_{off} (F) [1/s]
1	10	20	0.3	0.13
⋮	⋮	⋮	⋮	⋮
j	40	50	0.2	0.21
⋮	⋮	⋮	⋮	⋮
M	80	70	0.15	0.34

fit F^* parameters k_{off}, x_{a1}

$$k_{off}(F^*) = k_{off}\cdot\exp(F^* x/KT)$$

model prediction

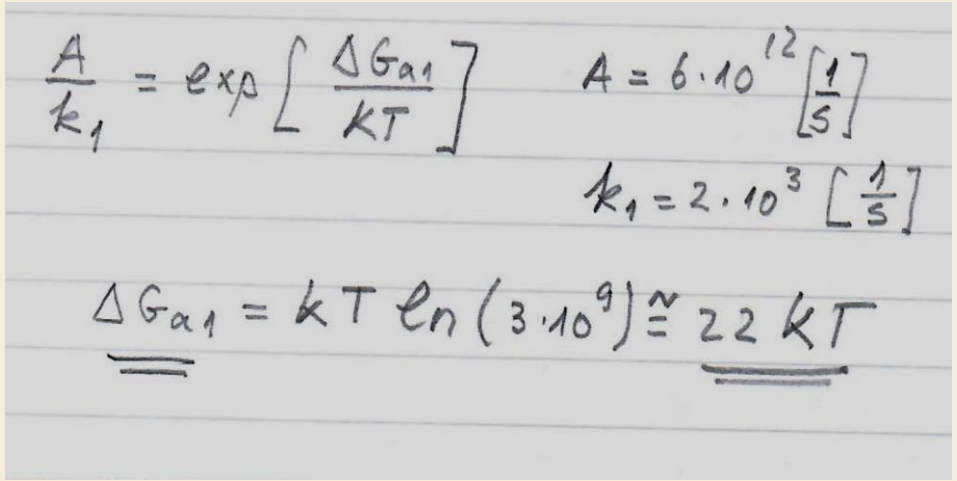
In the **Eyring rate theory,**

the reaction is assumed to correspond to the breakdown of a single quantum-mechanical vibration of the protein.

The frequency factor is ***A~ KT/h*** ≈ 6 x10^{12} [s^{-1}], where h is the Planck constant.

E.g. A reaction with a rate constant k_1= 2 x 10^3 s^{-1} , would have an activation energy : $\Delta Ga_1 = 22\ KT$

$$k_1 = A\ exp\left[-\frac{\Delta G_{a1}}{KT}\right]$$



The **Eyring theory** is expected to apply to **covalent changes of proteins and their ligands** but it is not expected to apply to global conformational changes of proteins in which a large number of bonds are made and broken, because in this case the reaction does not correspond to a single mode of vibration of the protein.

$$k_1 = A\ exp\left[-\frac{\Delta G_{a1}}{KT}\right]$$

The Eyring and Kramers rate theories represent two extreme views of the mechanism of global conformational changes of proteins.

A~ KT/h

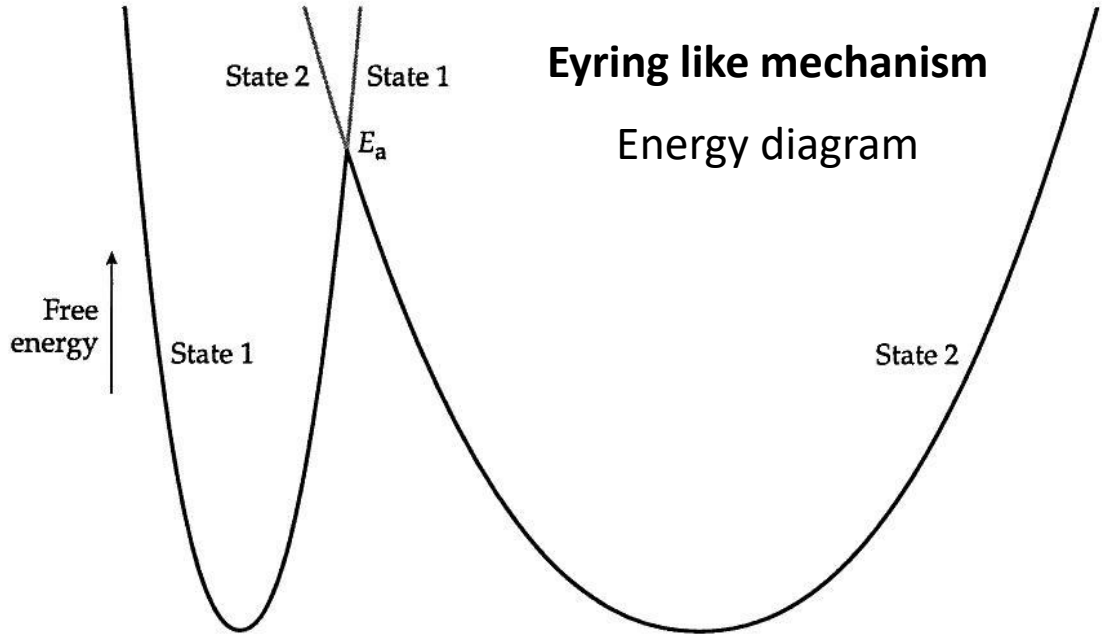
In the **Eyring model**, a sudden, local chemical change (such as the binding of a ligand or the chemical change in a bound ligand) creates a highly strained protein that then relaxes into a new stable conformation. The relaxation is along the quadratic energy curve and has time constant τ= ɣ /k .

In the **Kramers** theory, the protein undergoes a global diffusion into the activated state.

When a sufficiently large conformational change has been achieved, the protein converts to the final state.

In the extreme, the protein diffuses all the way to the final state, which is then locked in by a subsequent chemical change.

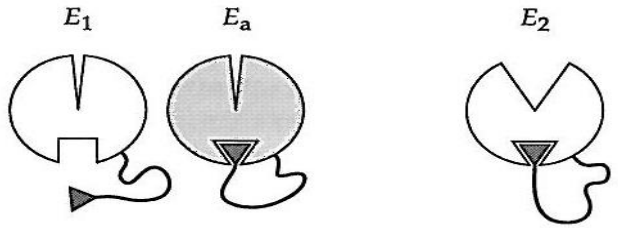
$A \approx \mathbf{1/\tau}\ \sqrt{(\Delta \boldsymbol{Ga1/KT})}$ with $\tau = \gamma/k$



Eyring like mechanism

Energy diagram

The reaction coordinate is the extent of opening of the cleft.



Monomolecular model

The model of the working stroke of myosin.

After myosin has bound to actin in its pre-powerstroke state, the phosphate rapidly disocciates, leaving the protein in a highly strained post-powerstroke state.

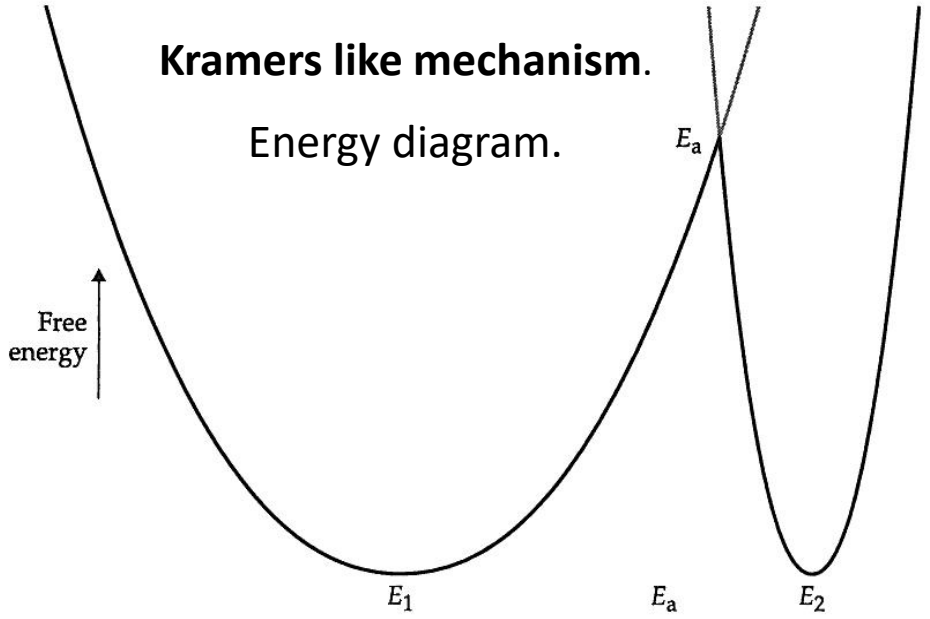
The relaxation of this highly strained state drives the sliding of the filaments and the shortening of the muscle. If the filaments are prevented from sliding, the strained state will maintain the tension in the muscle.

The principle for proteins is that global structural or 'physical' changes of proteins are much slower than local chemical changes, because structural changes are slowed by protein and solvent viscosity.

The fast local changes leave the protein in an unstable global conformation which then relaxes more slowly into a new stable state.

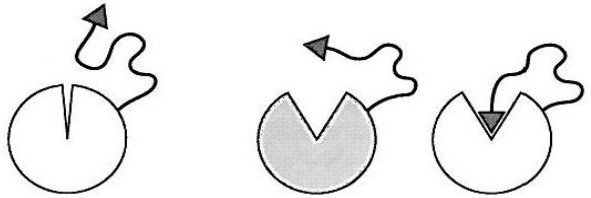
Kramers like mechanism.

Energy diagram.



The reaction coordinate is the extent of opening of the cleft.

Monomolecular model



Thermal ratchet model for motor proteins.

The protein undergoes a global diffusion into the activated state and then converts to the final state.

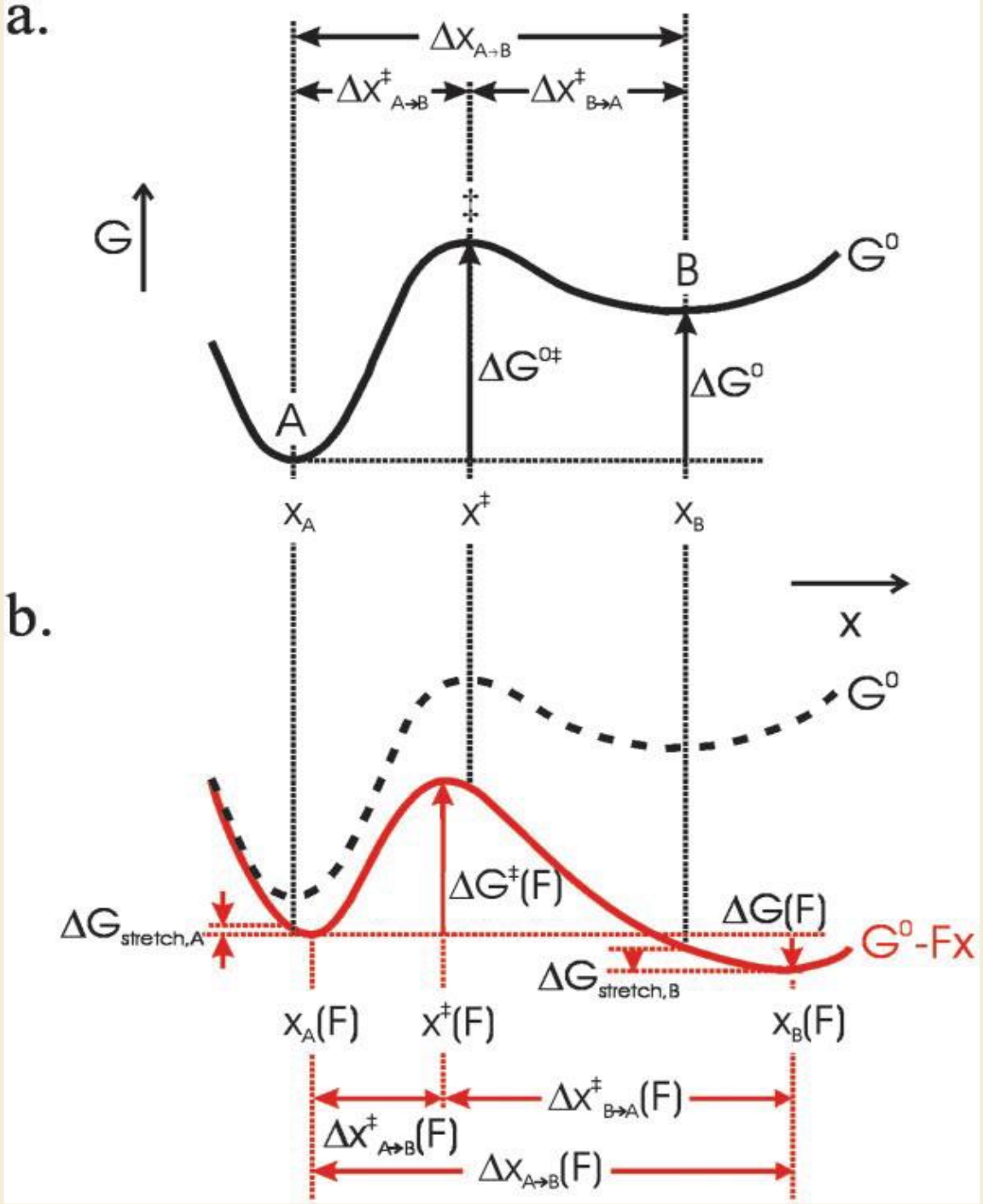
Consider a motor protein with k= 4 pN/nm and radius r= 4 nm. The free energy available from ATP hydrolysis is 25 KT. The drag coefficient ɣ= 60 pN s/m, and the relaxation time. τ = ɣ/k= 15 ns

It takes about 10 s to pick up 20 KT of energy by a purely diffusive process:

$$k_1 = A\, exp\left[-\frac{\Delta G_{a1}}{KT}\right] \qquad \boldsymbol{A \approx \frac{eff}{\tau}\sqrt{\frac{\Delta Ga_1}{KT}}}$$

But for myosin, the complete ATP hydrolysis reaction only takes about 0.05s. Therefore, if the ATP hydrolysis reaction has an efficiency of 80% (20KT/25KT), such a diffusive step could not be on the myosin's reaction pathway. However, a more reasonable efficiency is 50 %, and the corresponding pick up time becomes only t_k= 7 ms. The kinetics of myosin becomes thus consistent with Kramers like mechanism.

More details on the mechanisms in force generation in motor proteins – Lectures for 2. 3 Motor proteins



The effect of force on the free energy of a two-state system, where x represents the mechanical reaction coordinate.

(a) No applied force.

(b) Red curve: positive applied force.

The application of force lowers the energy of both the transition state ‡ and state B relative to state A, which increases the rate of the forward reaction and the population of state B, respectively. The positions of the free energy minima (*xA* and *xB*) and maximum (*x*≠) shift to longer and shorter x, respectively, with a positive applied force. Their relative shifts in position depend on the local curvature of the free energy surface.

The free energy change of states A and B upon stretching is Δ*Gstretch*;

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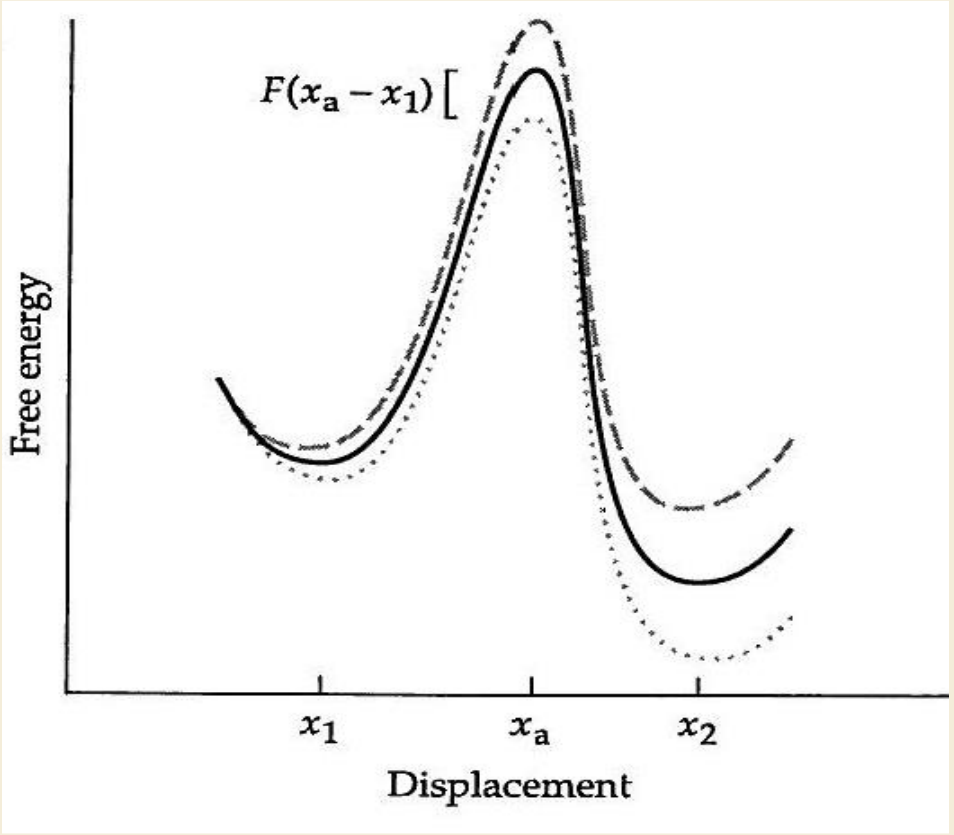
If the state transitions $E_1 \rightarrow E_a \rightarrow E_2$ are associated with displacements x1, xa, and x2 in the direction of the force, F, then the energies of the states will be decreased by Fx1, Fxa, and Fx2, respectively.

This implies that:

$$k_1 = A\exp\left[-\frac{\Delta G_{a1} - F\Delta x_{a1}}{kT}\right] = k_1^0 \exp\left[\frac{F\Delta x_{a1}}{kT}\right]$$

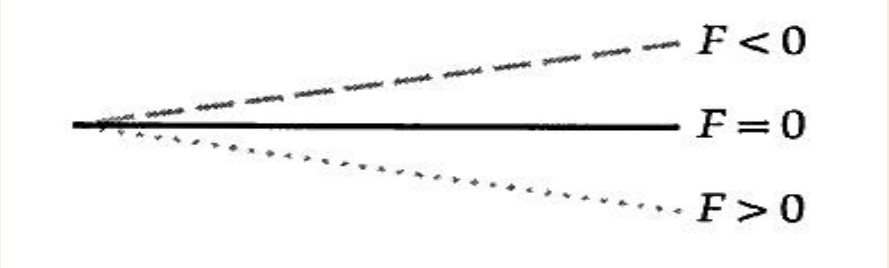
where $\Delta G_{a1} = G_{a1} - G1$ and $\Delta x_{a1} = X_{a1} - x_1$.

An analogous expression holds for k_{-1}.



The effect of force on the reaction rates:

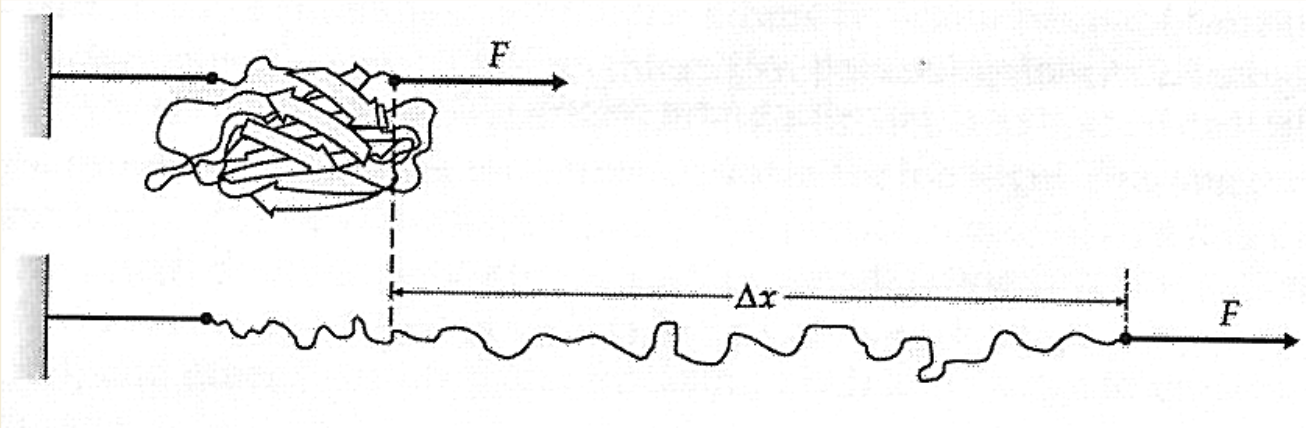
tilting the free energy diagram.



Problems home work

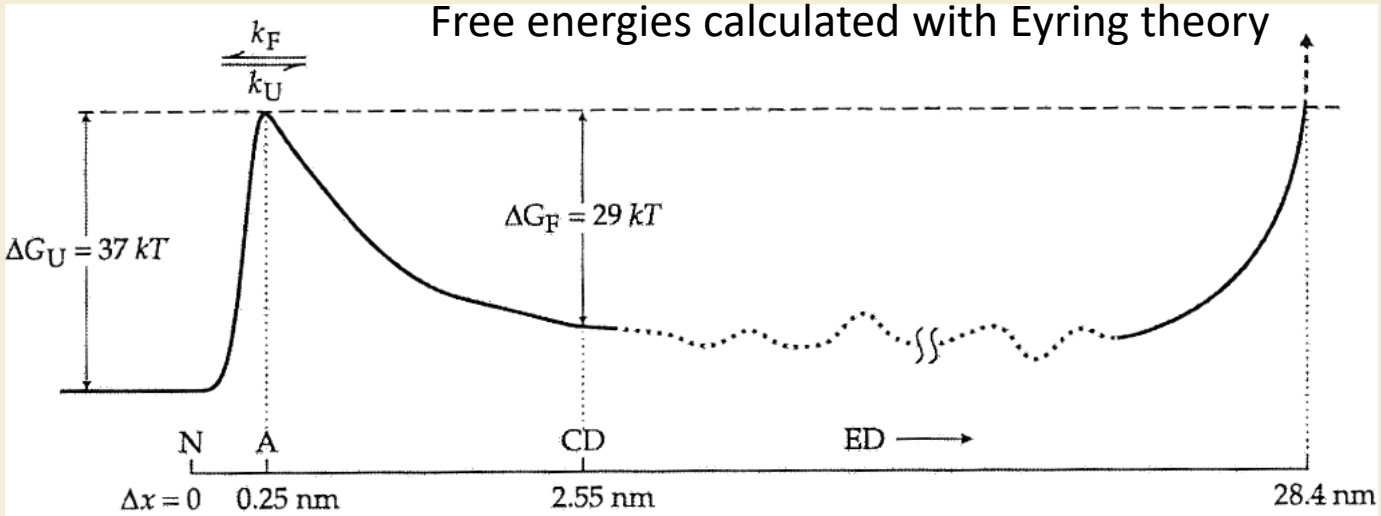
1 Suppose that one could pull directly on the gate of an ion channel, and that the gate swings through 2 nm as it goes from the closed to the open position. If, in the absence of force, the channel spends half its time open and half its time closed, how much force is needed to increase the open probability to 0.9?

2 Suppose that a protein has a stiffness of 2 pN/nm in state 1 and a stiffness of 1 pN/nm in state 2, but that the two states have the same resting length (the length in the absence of a force). If there is initially a very low probability of being in state 2, how much force is needed to increase the open probability *e*-fold?



energy profile

Free energies calculated with Eyring theory



N = native state, A = activated state,
CD = compact disordered state, ED = extended state

AFM was used to reversibly unfod immunoglobulin modules domain, ~120 amino acid domains found in a variety of proteins including the muscle protein titin.

The unfolding rate depends only weakly on force (e-fold increase per 16 pN), indicating that the transition state for unfolding is of similar length to the folded state ($\Delta x = KT/F = 0.25$ nm).

On the other hand, the folding is strongly dependent on force (e-fold slowing per 1.6 pN).

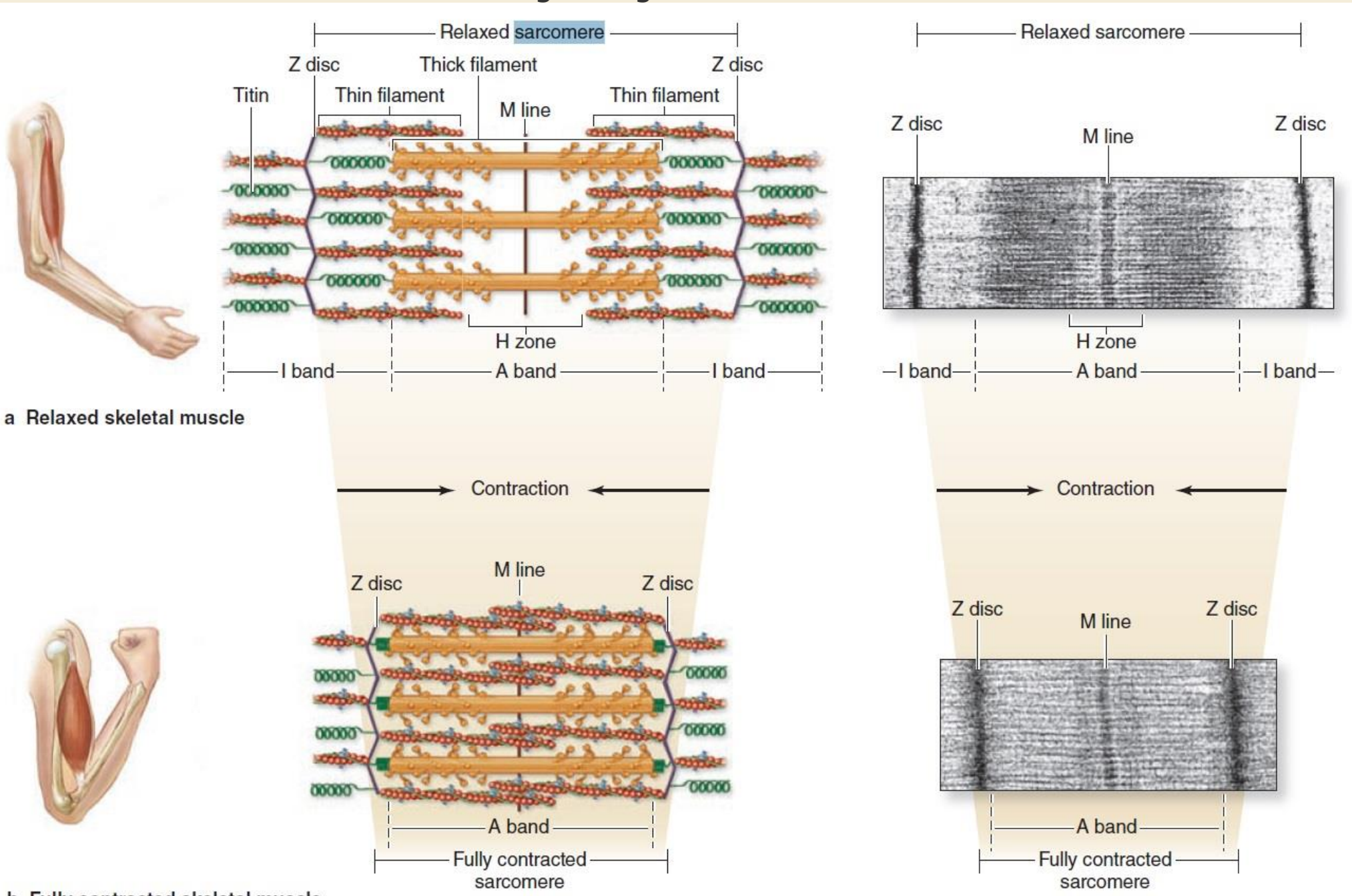
A small strain of 0.25 nm, about 5% of the length of the folded protein (5.1 nm), is enough to completely destabilize the structure and lead to unfolding.

The folding of the protein requires the formation of a nearly fully folded transition state.

Carrion-Vasquez et al, PNAS 1999

Mechanical and chemical unfolding of a single protein a comparison

Sarcomere shortening during skeletal muscle contraction.

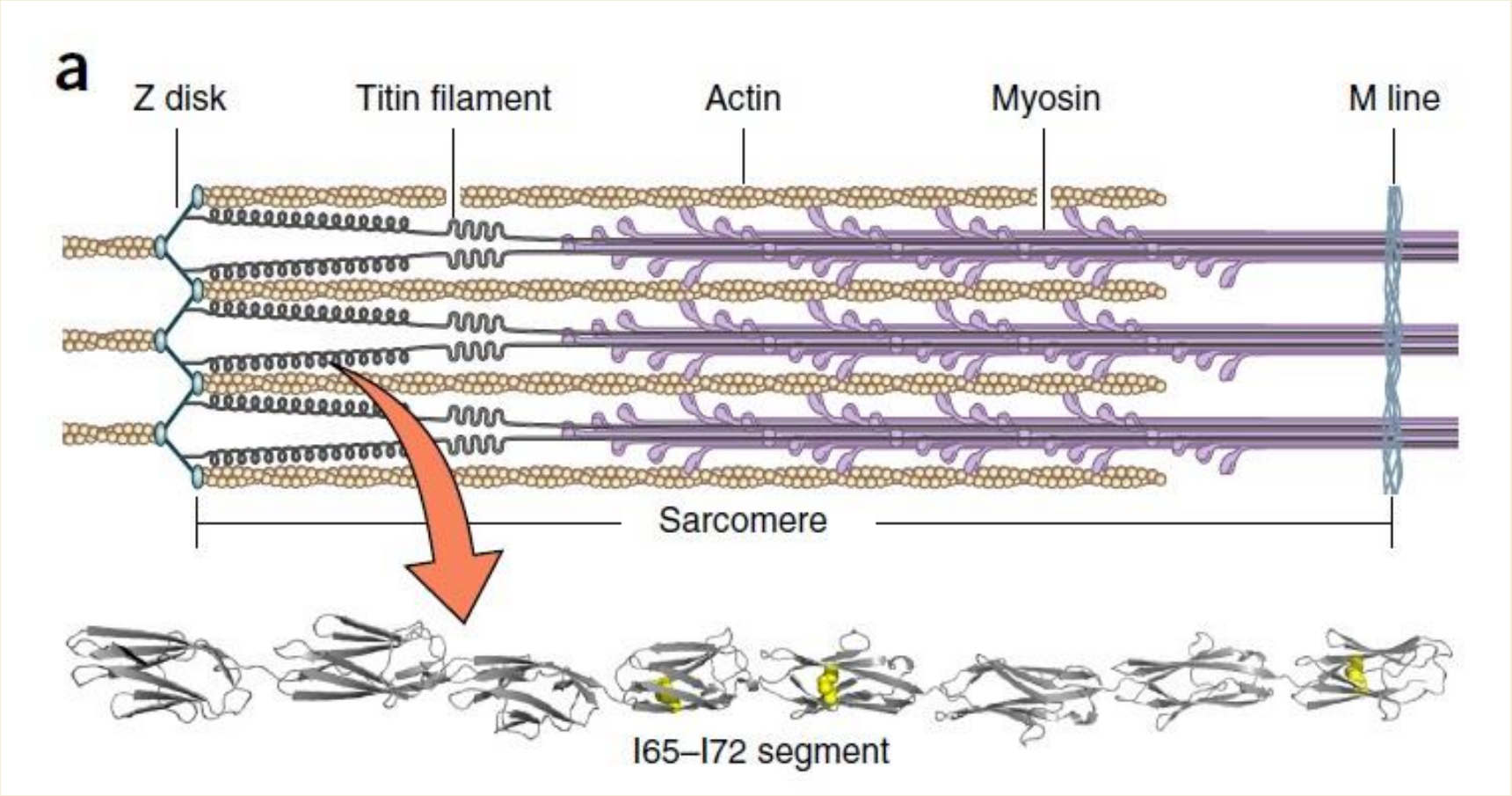


Sarcomere shortening during skeletal muscle contraction.

(a) In the relaxed state the sarcomere, I band, and H zone are at their expanded length. The springlike action of titin molecules, which span the I band, helps pull thin and thick filaments past one another in relaxed muscle.

(b) During muscle contraction, the Z discs at the sarcomere boundaries are drawn closer together as they move toward the ends of thick filaments in the A band. Titin molecules are compressed during contraction.

Scheme of one half of the sarcomere from Z disk to M line



There are three main sarcomeric proteins: actin, myosin and titin. The segment encompassing domains I65–I72 from the elastic part of titin is shown.

Why TITIN ?

Titin has nearly 250 Ig immunoglobulin-like and fibronectin domains arranged in a linear fashion, for a total molecular weight of almost 3 MDa. It is big ! (length up to 1 um).

This morphology greatly facilitates its grabbing and tethering between the surface of two beads in an optical tweezers instrument, or between the tip of an AFM cantilever and a surface, making it possible for the first time to investigate the response of a protein to mechanical denaturation.

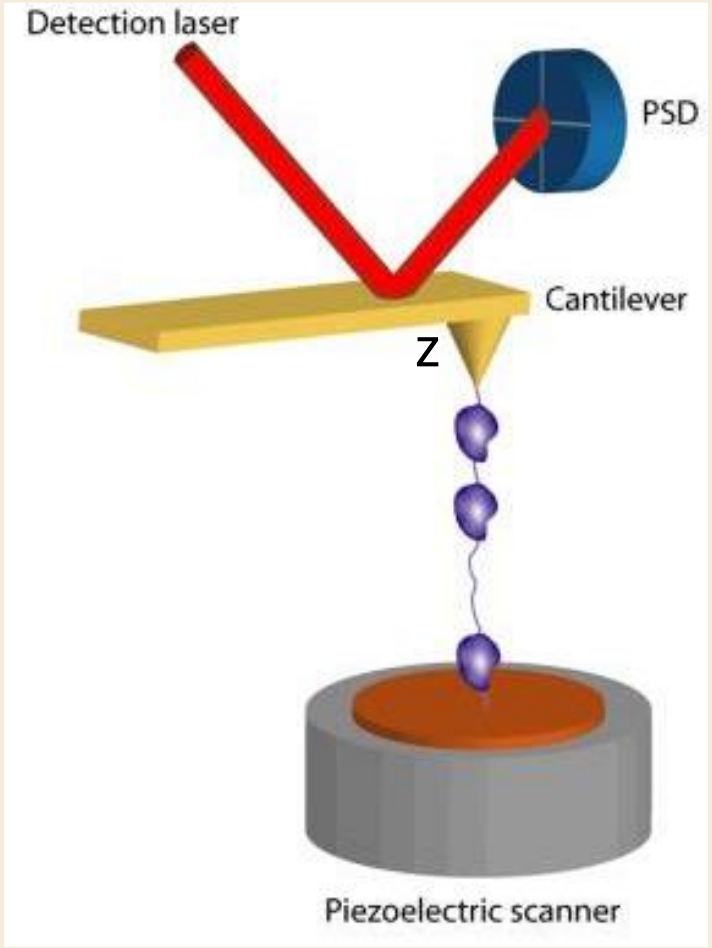
Rief M, Gautel M, Oesterhelt F, Fernandez JM, Gaub HE. 1997.
Reversible unfolding of individual titin immunoglobulin domains by AFM.
Science 276:1109–12

!! Published in the same issue of Science

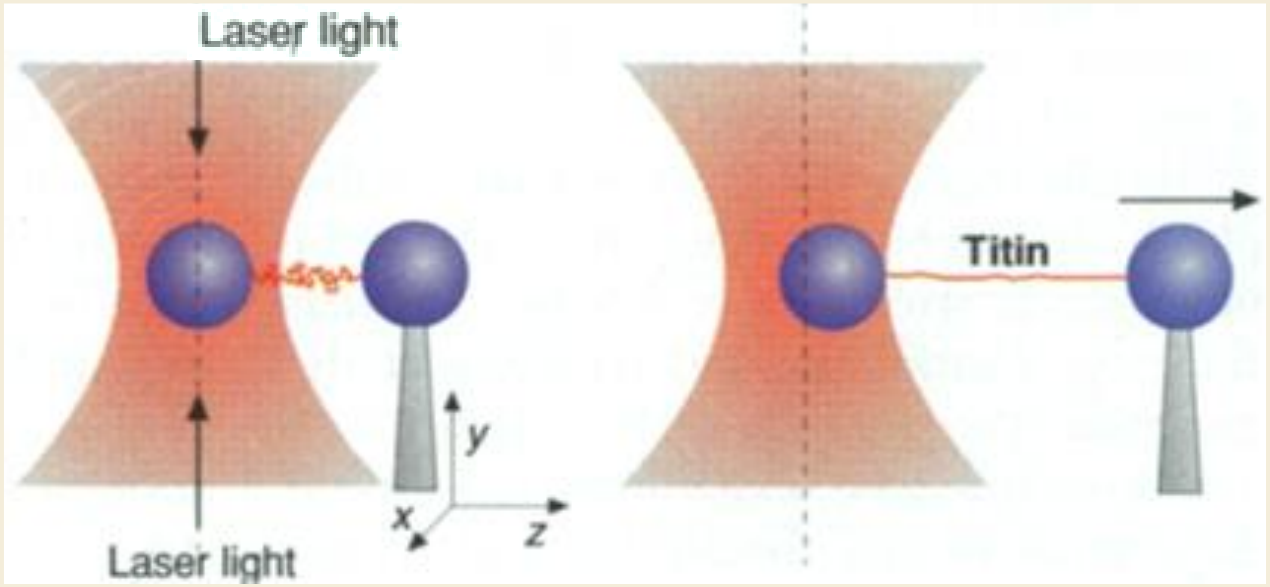
M. S. Z. Kellermayer, S. B. Smith, H. L. Granzier, C. Bustamante,
Folding-Unfolding Transitions in Single Titin Molecules Characterized with Laser Tweezers, Science 276, 1112–1116 (1997).

Tskhovrebova L, Trinick J, Sleep JA, Simmons RM. 1997.
Elasticity and unfolding of single molecules of the giant muscle protein titin. AFM
Nature 387:308–12

AFM



Laser /Optical Tweezers



$F = k\,z$

Spatial resolution / displacement detection

$\delta x \sim 1nm$ for both

Stiffness or spring constant

$k_{AFM} >> k_{OT}$

↓

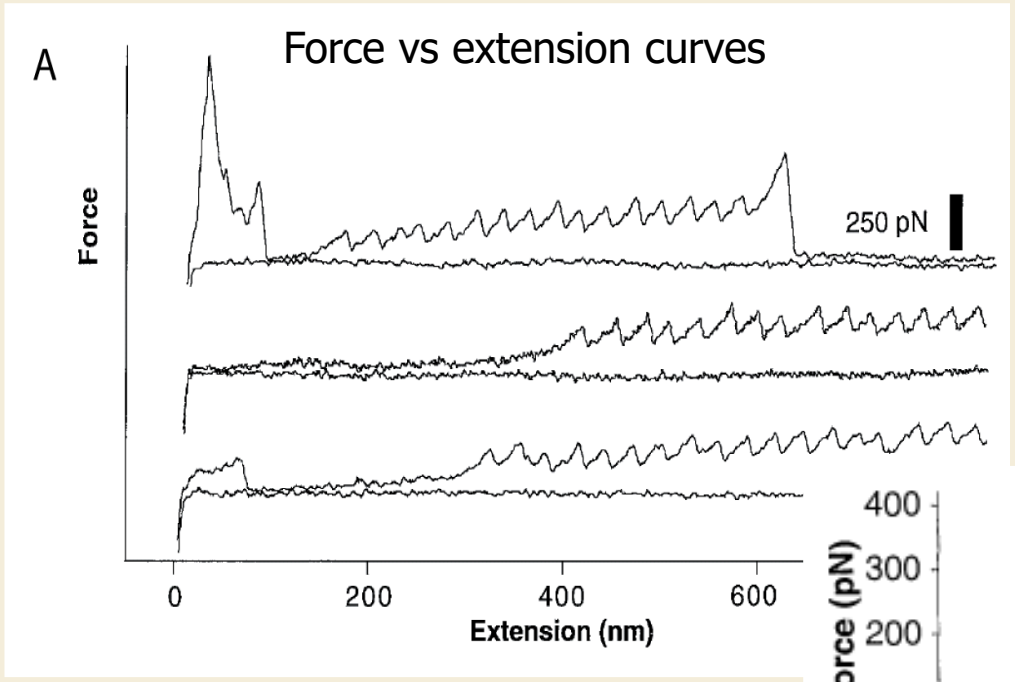
Force resolution: AFM < OT

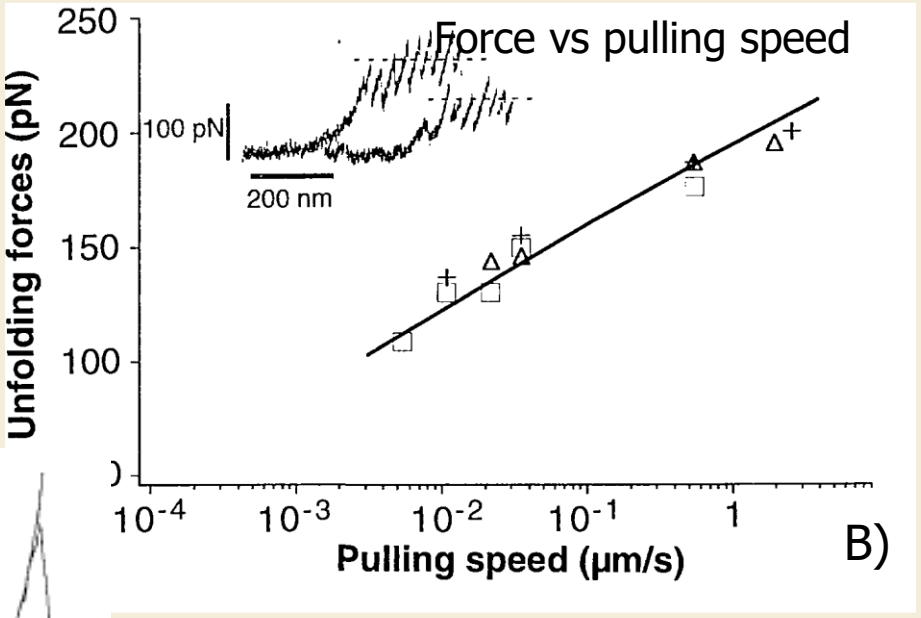
$\delta F_{AFM} >> \delta F_{OT}$

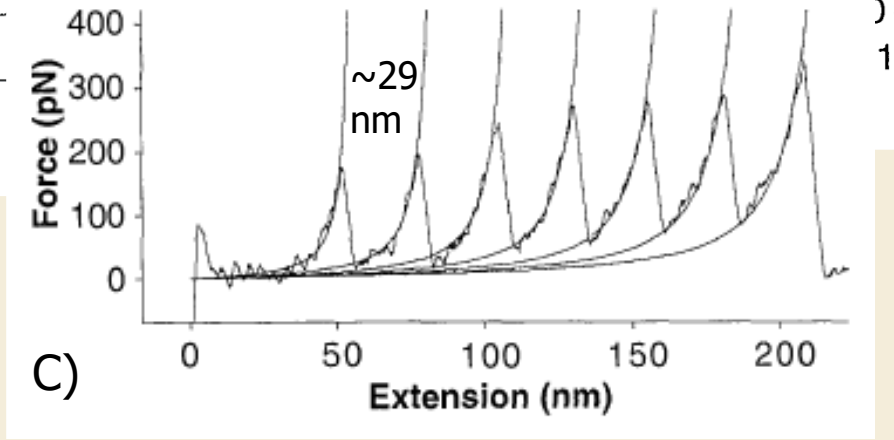
Load rate = probe stiffnes x pulling speed

Load rate AFM > Load rate OT for the same pulling speed

First experiments – unfolding single molecule TITIN AFM







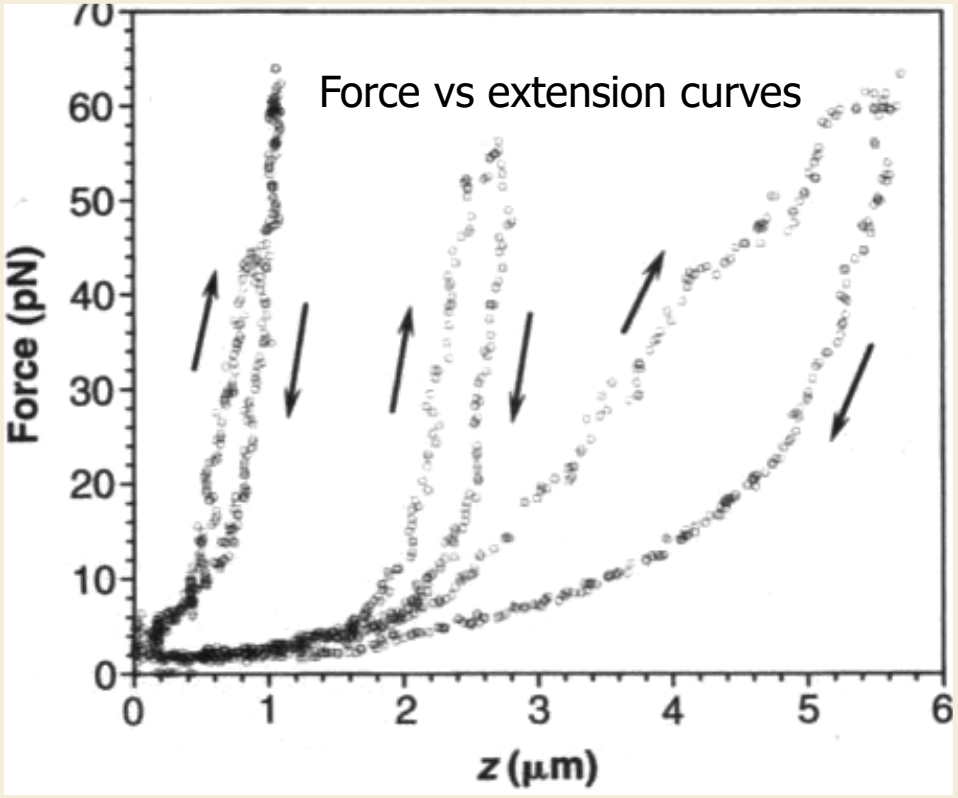
A) Individual titin molecules were repeatedly stretched, and the applied force was recorded vs elongation.
Unfolding of individual Ig domains was observed (shark tooth pattern). AFM tip attachment to the protein is at random points (the observation starts at different extensions in the three curves shown).

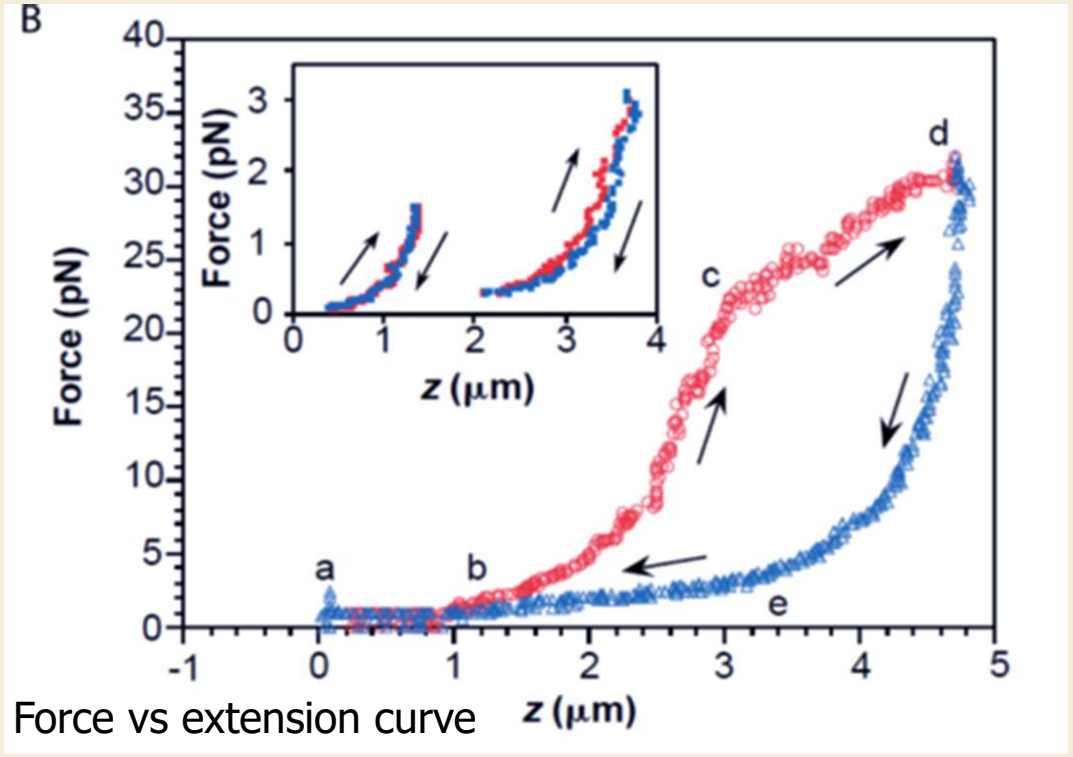
B) Unfolding forces depend on the pulling speed . If we consider a tip stiffness k= 10 pN/nm, the range of the corresponding load rate is: 0.1 – 10 nN/s .

C) Fitting with the WLC model for each Ig domain unfolding

First experiments – unfolding single molecule TITIN

OT





Force vs extension curve

A) Stretch and release of single titin molecules at a load rate of max 0.01 nN/s. Hysteresis is observed (the rate of stretch is different from the rate of release). The differences between the three curves are assigned to the different contact point on the titin.

B) The points at the beginning and at the end of the transistions are highlighted. At the beginning of the stretch5-4% of the the molecule is already unfolded. a-c : WLC model ; at higher force (after c) tansistions fold – unfold ; d-e WLC model, no refolding; e-b refolding; much of the shortening appear at about 2.5 pN. Inset : curves for experiments where the stretch of the release of titin was stopped short or entering the stretch or release transition (points c and e)

Proteins Unfolding - Single molecule

Force denaturant vs chemical or temperature denaturant

Advantages of studying protein folding by using force, one molecule at a time.

1. the direction along which the force is applied in a mechanical unfolding experiment establishes a well-defined reaction coordinate. Along this privileged spatial direction, parameters of the reaction such as the distance to the transition state, the height of the energy barrier, and the energy difference between the initial (folded) and the final (unfolded) states can be determined.
2. unlike its bulk counterparts (urea, temperature), force is a selective denaturant capable of acting on one part of the molecule without directly affecting another. This locality has made it possible to investigate, for example, the energetic coupling during the folding or unfolding of different regions of a protein.
3. the use of force as a denaturant, which typically requires studying the folding process at the single-molecule level, makes it possible to avoid complications such as aggregation that often plague folding studies in bulk.
4. Studies in bulk – average the parameters.

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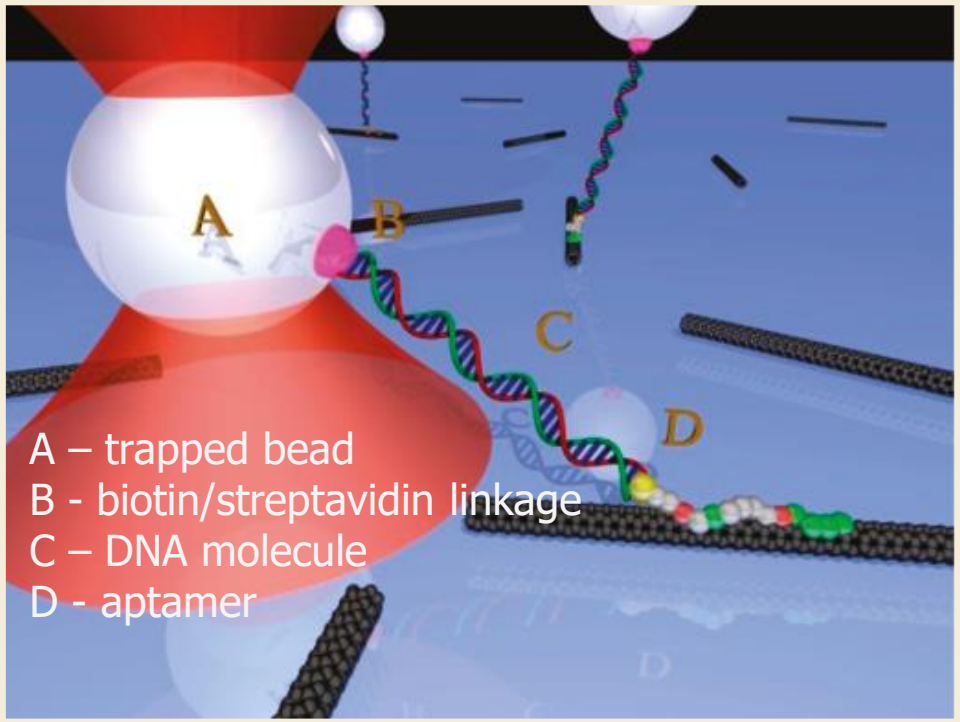
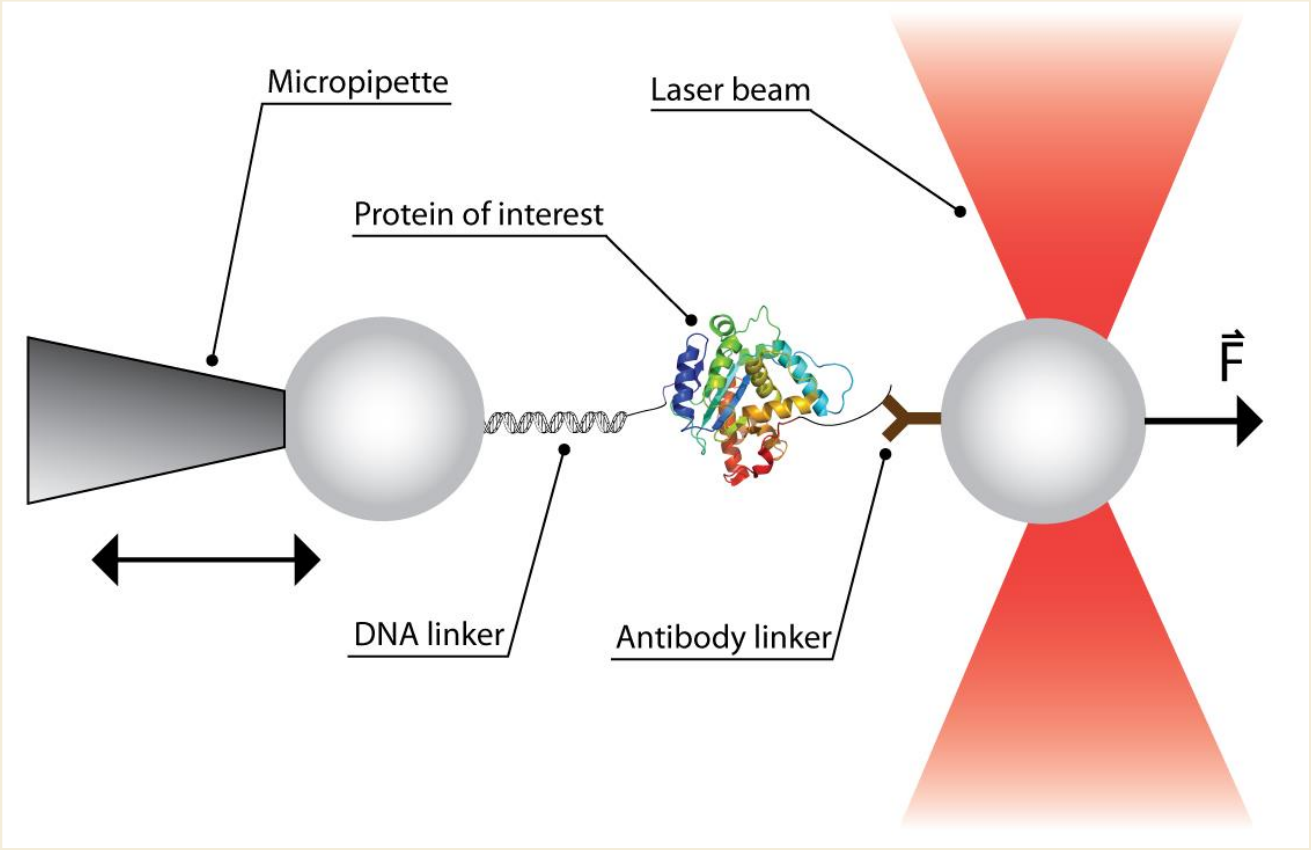
Examples of OT configuration for force spectroscopy

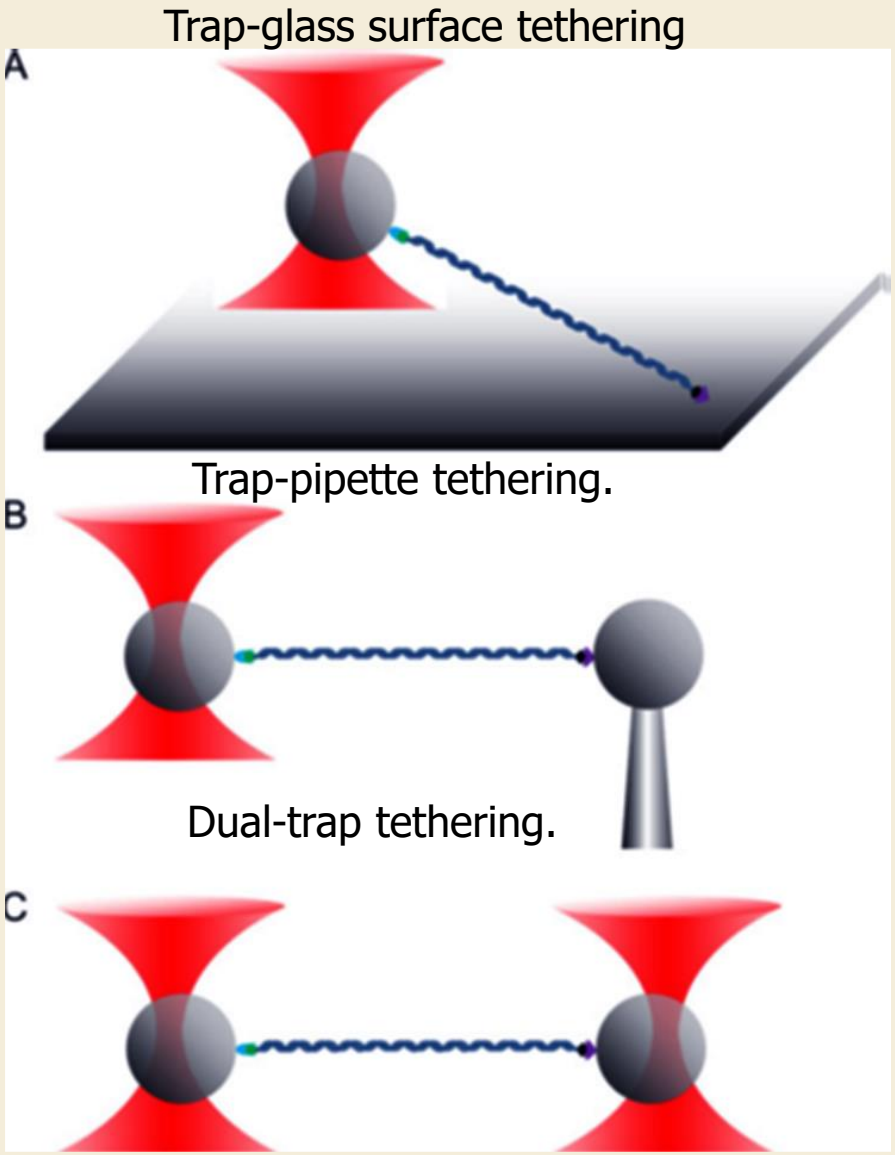




Substrate → Bead- micropipette.

F= k x – for the moment we consider OT as a Hook spring with elastic constant k= 0.001 – 0.5 pN /nm (lower than thar of AFM cantilever

OT configurations for force spectroscopy



Trap-glass surface tethering

Trap-pipette tethering.

Dual-trap tethering.

Weak trap

Stiff trap

Most frequent: Single or two beads.

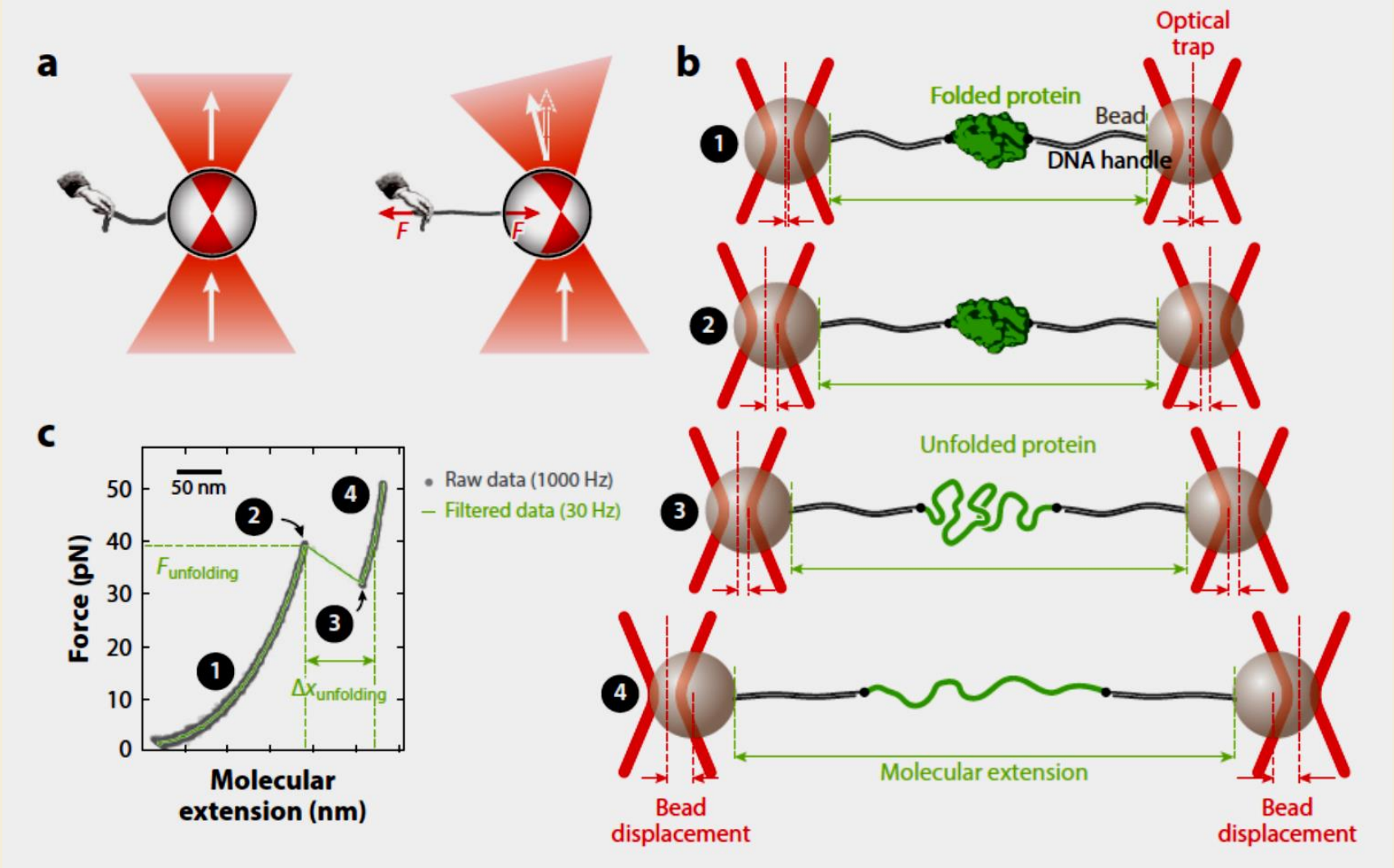
Three beads is also proposed.

Each of these geometries can be implemented as:

- Force ramp (F(t)= r*t)
- Force clamp (F(t)= ct)
- Position clamp (x(t)= ct)

Using feedback system to adapt the position of the bead / trap

Manipulation of single molecules using dual OT.



Manipulation of single molecules using optical tweezers.

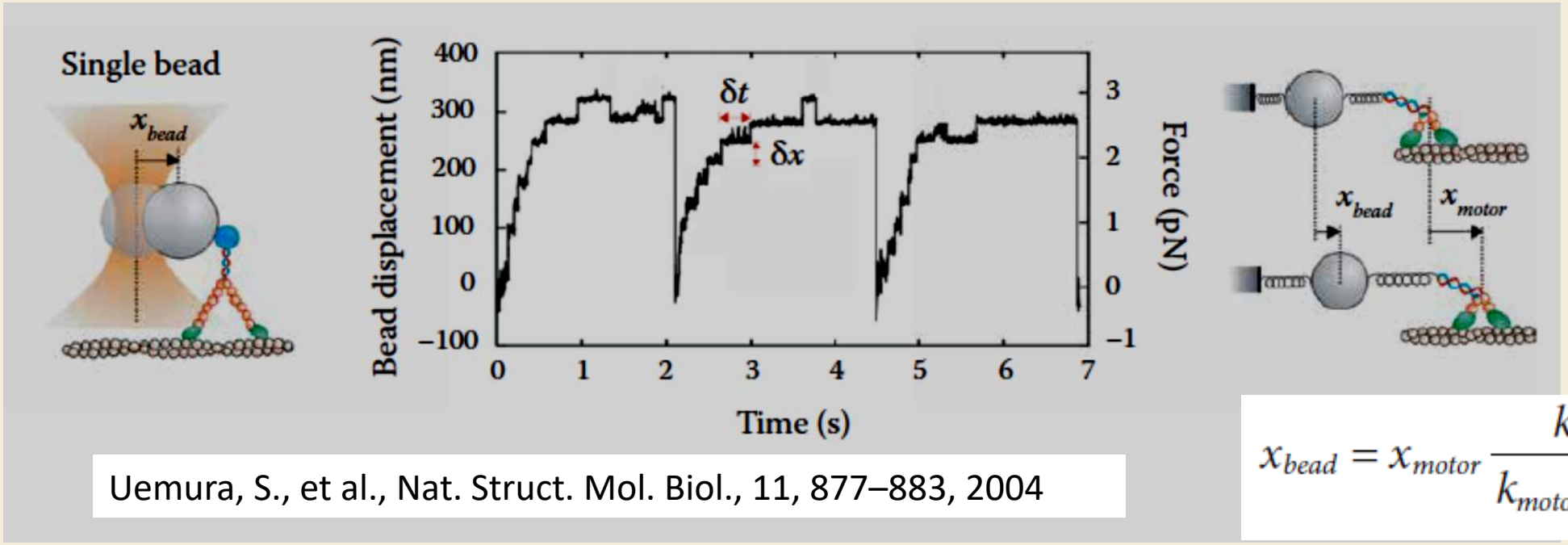
a) Light from a tightly focused laser beam (*red*) passes through a bead. When the center of the bead is aligned with the laser focus (*left*), all rays hit the bead surface at a right angle, and the light does not change direction. When the center of the bead is not aligned with the laser focus (*right*), the change in momentum of the light elicits an equal and opposite change in momentum of the bead, resulting in a force *F* that attracts the bead to the laser focus.

a) Schematic of a protein tethered between two trapped beads via DNA handles. At low forces, the protein remains in the folded state. However, an increase in force results in stretching of the DNA handles, increasing the molecular extension of the assembly between the beads. Unfolding of the protein results in a further increase in extension . Further increasing the force results in stretching of the DNA and the unfolded protein Note that the bead displacement is proportional to the applied force, because the traps behave as harmonic springs.

b) Example of a typical force–extension curve, generated by applying a continuously increasing force to a tethered protein; gray dots represent data at 1,000 Hz, and the green curve represents data filtered to 30 Hz. The numbers are as in panel *b*. The curvature is due to the entropic elasticity of the DNA (region 1) and DNA plus unfolded protein. Unfolding of the tethered protein is apparent as a discontinuity in the curve (rip; from point 2 to point 3).

2020 Annual Review of Biochemistry
Single-Molecule Studies of Protein Folding with Optical Tweezers
Carlos Bustamante et all Review

Sngle-trap geometry. Example.



Uemura, S., et al., Nat. Struct. Mol. Biol., 11, 877–883, 2004

$$x_{bead} = x_{motor} \frac{k_{motor}}{k_{motor} + k_{trap}}$$

A myosin V molecule is attached to a trapped bead which proceeds along an actin filament stuck on the coverslip surface.
The optical trap position is fixed and the bead displacement x_{bead} measures protein displacement.

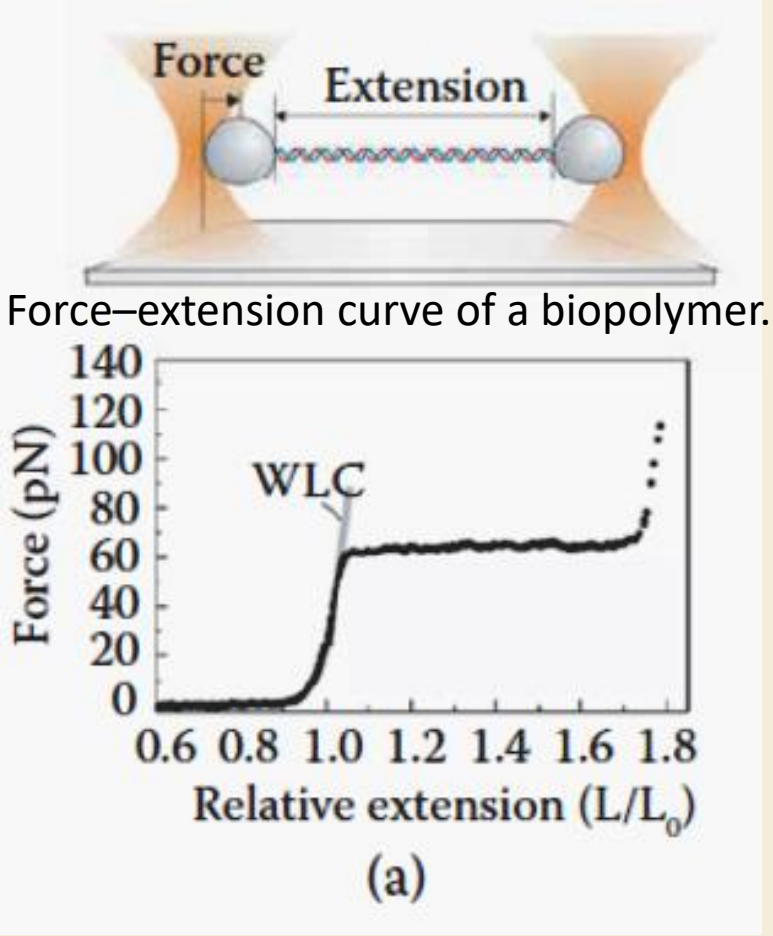
Example showing stepwise movement of a single myosin Va motor in a single-bead assay. Consecutive 36 nm stepwise movements (δx) and dwell times between steps (δt) are clearly visible.
Backward steps are also visible at high force.
The force was calculated as: $F = k\, x_{bead}$
Trap stiffness k=0.009 pN/nm
Stall force is about 2.5 pN.

Measured bead displacements (x_{bead}) do not correspond to motor displacements (x_{motor}) but depend on the motor protein displacement and on the values of the trap and protein stiffness.

Capitanio, M . Book Chapter 2017

More flexible and precise

Double-trap assay.



Force–extension curve of a biopolymer.

Example

Left trap is stationary and measures the force applied to the polymer.

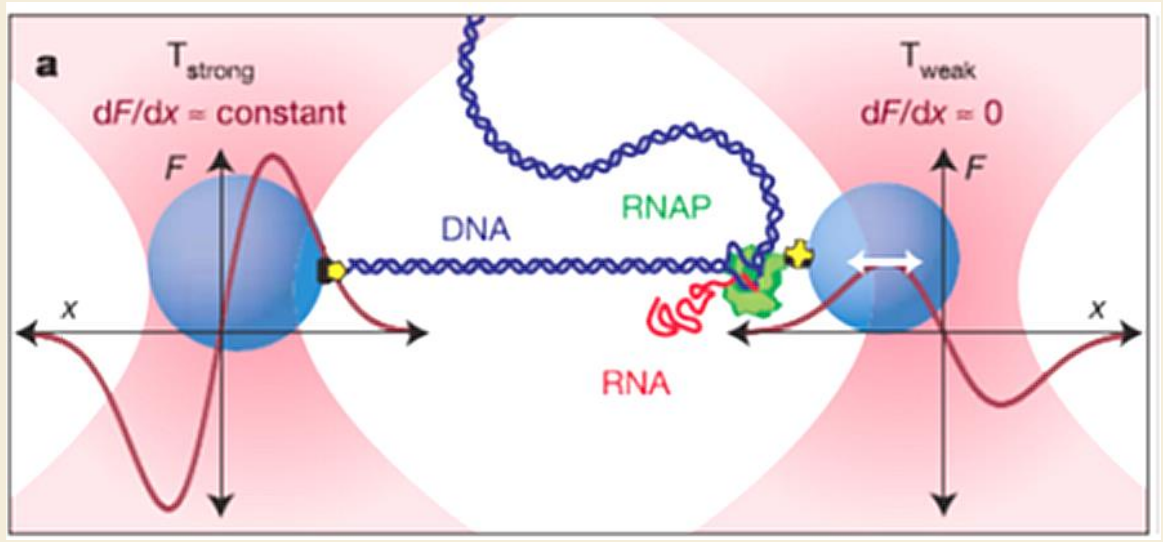
The right bead moves in steps or ramps and, for each displacement, the force applied to the polymer and its extension are measured.

The elastic properties of DNA below the overstretching force of 65 pN are well described by the extensible worm-like chain (WLC) model (gray line).

At 65 pN, the DNA molecule undergoes the overstretching transition, during which the intrinsic contour length of the DNA increases from 100% to about 170%.

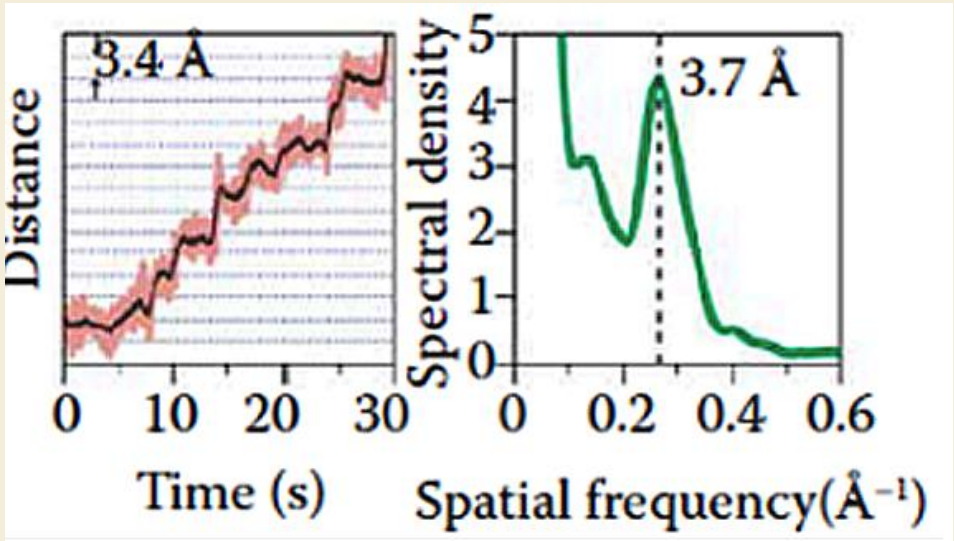
van Mameren, J., et al., Proc. Natl. Acad. Sci. U S A, 106, 18231–18236, 2009

Double-trap assay.



Example: Dynamics of DNA-processing enzymes.

Single, transcriptionally active molecule of RNA polymerase (RNAP, green) attached to a bead held in a trap and tethered via the upstream DNA to another trapped bead. During elongation, the DNA tether lengthens and the beads move apart.

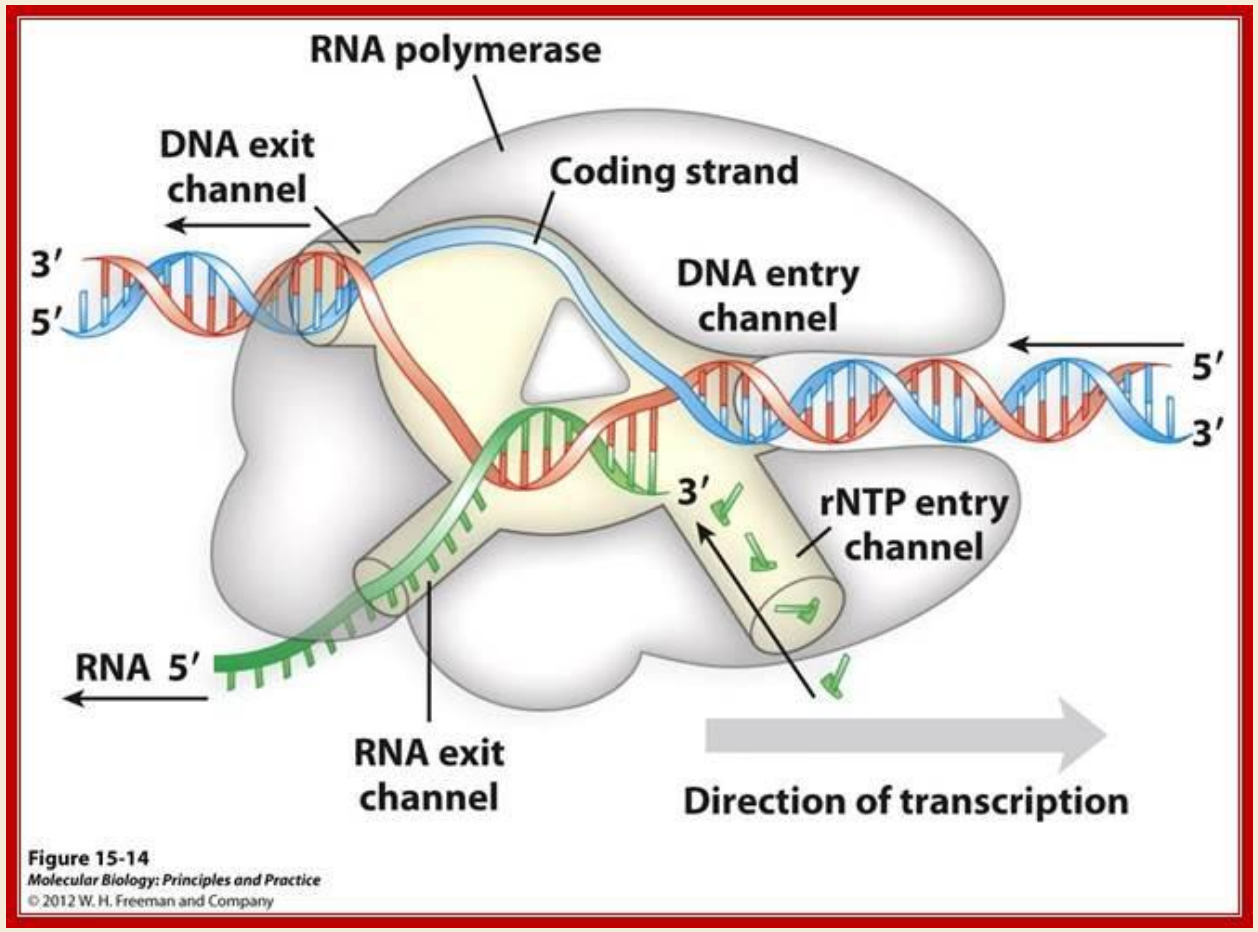


A representative record for a single RNAP molecule transcribing under 18 pN of assisting load. Horizontal lines (dotted) are spaced at 3.4 Å intervals.

The power spectrum of the average autocorrelation function derived from position histograms shows a peak at the spatial frequency corresponding to the inverse of the fundamental step size, 3.7 ± 0.6 Å.

Abbondanzieri, E.A., et al., Nature, 438, 460–465, 2005

RNA polymerases and DNA transcription



RNA polymerase is the main transcription enzyme.
It transcribes DNA into RNA

Transcription begins when RNAP binds to a promoter sequence near the beginning of a gene .

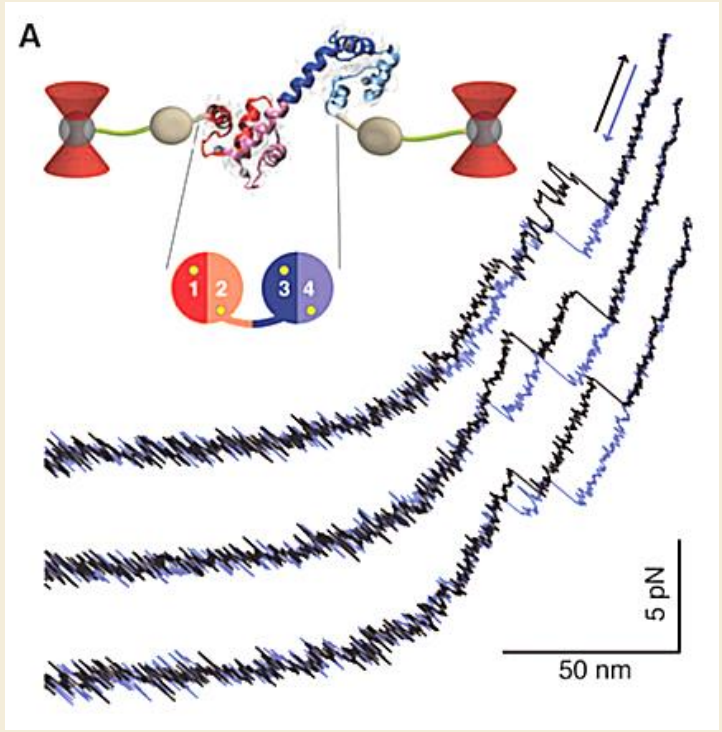
RNAP uses one of the DNA strands as a template to make a new, complementary RNA molecule.

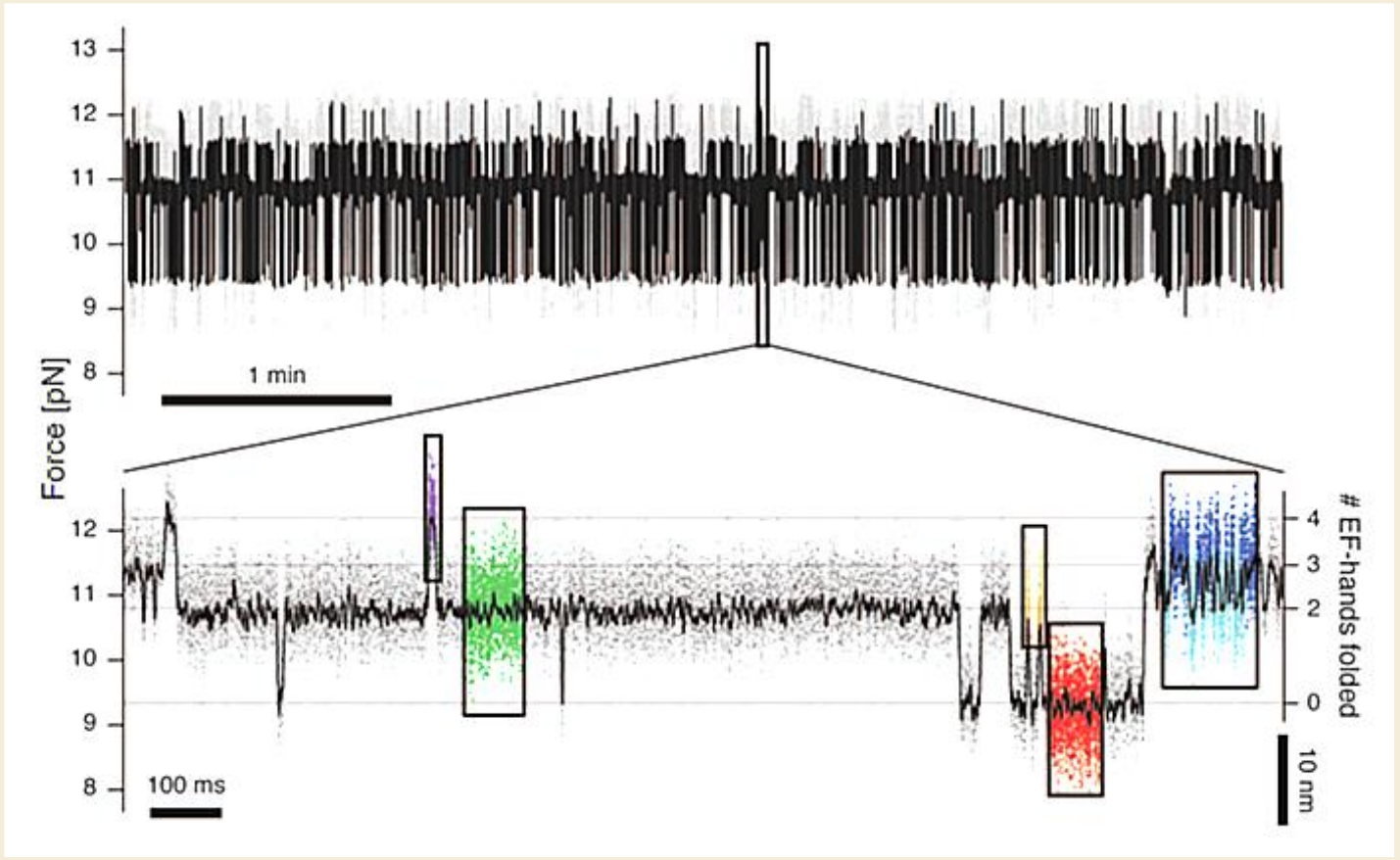
RNAP needs to have the substrate nucleoside triphosphate (**NTP**) diffuse into its deeply buried active site.

Double-trap assay. Example

Calmodulin folding-unfolding energy landscape
Stigler, J., et al., Science, 334, 512–516, 2011

Force ramp

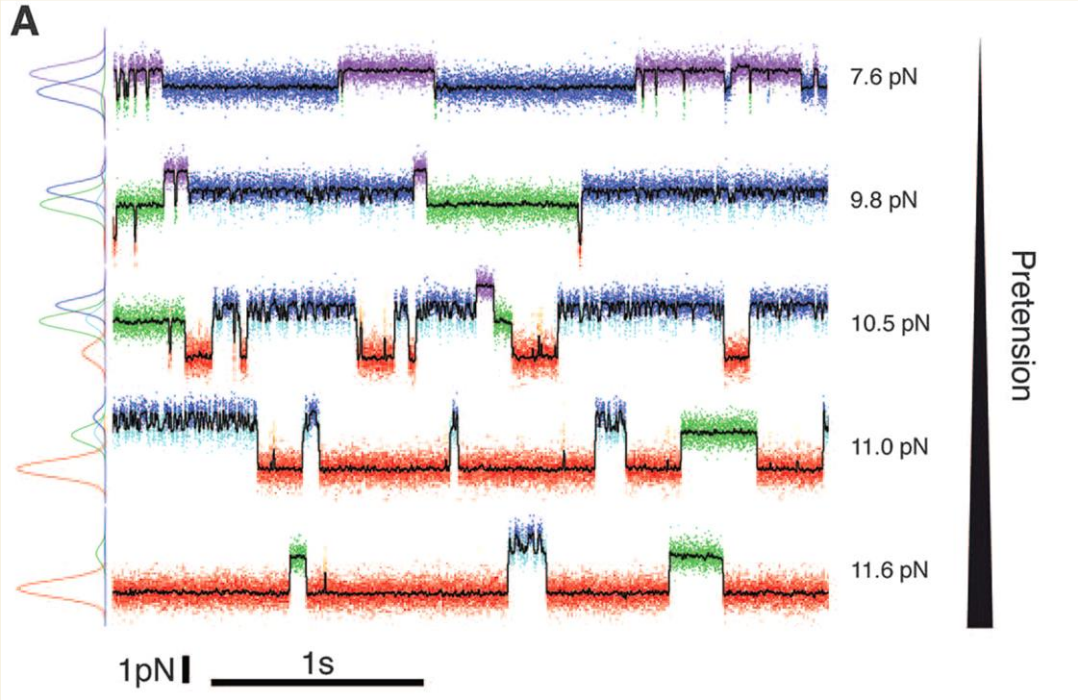




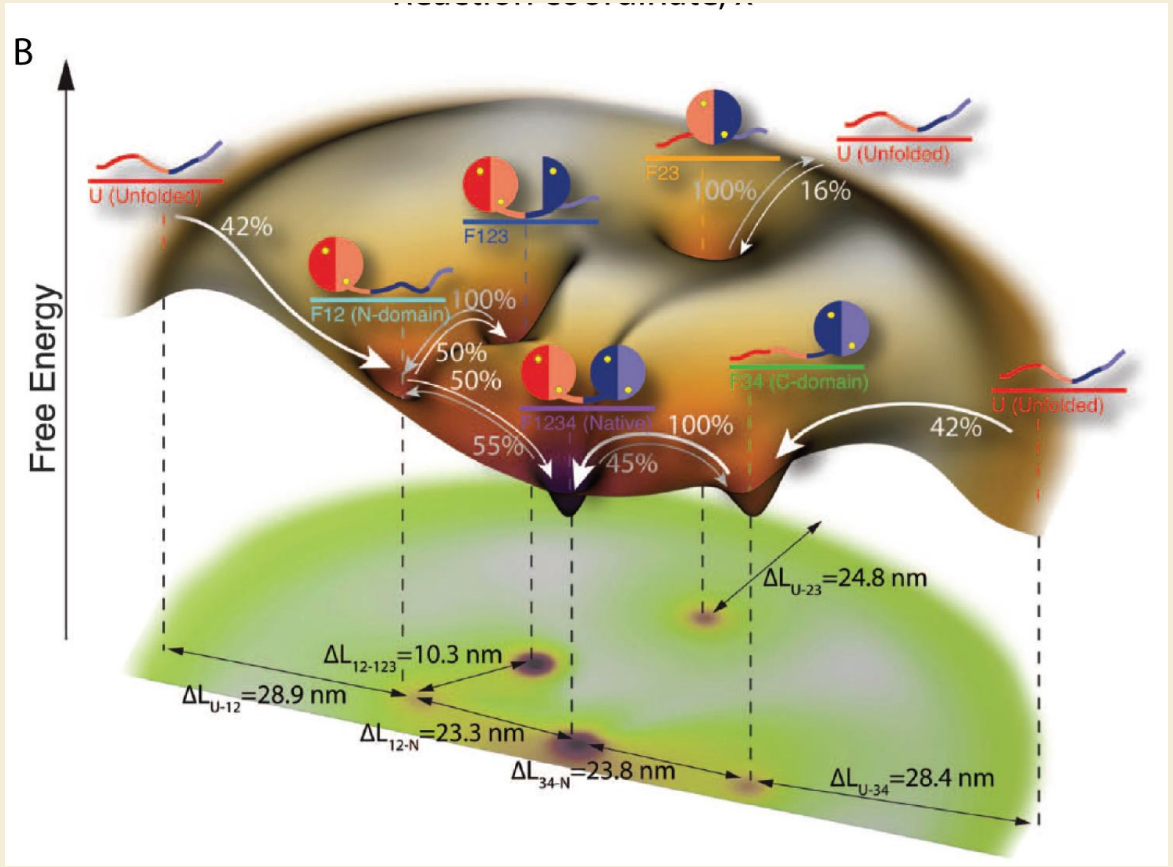
Folding and unfolding of single CaM molecules. Representative stretch-and-relax cycles for WT-CaM at velocity v = 500 nm/s. (Inset) Sketch of the experimental setup with the protein linked with ubiquitin-DNA handles to functionalized silica beads. (Expanded section) Cartoon representation of CaM with the EF hands numbered.

Sample trace during 5min of the fluctuations of a singleWT-CaMmolecule at a constant trap separation. The vertical scale denotes the force acting on the molecule as measured by the deflection of the beads from the trap center.
Expanded section: Six different states (see colored regions) can be identified using hidden Markov modeling.

Force clamp



Traces of WT-CaM at different pretensions (Left) Gaussian fits to histograms of each respective state.

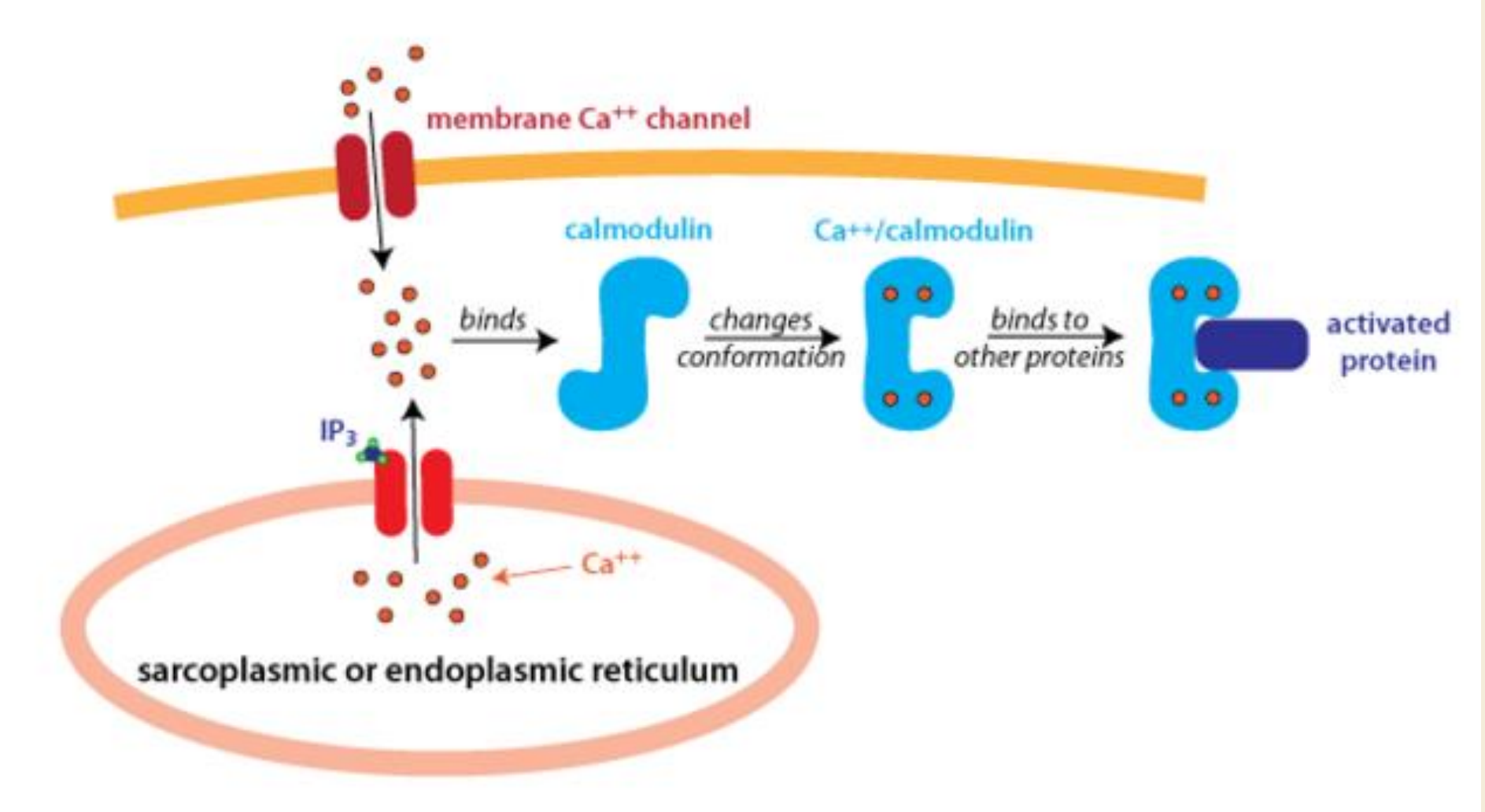


Full kinetic network of WT-CaM folding and unfolding at zero load. Arrows show all observed transitions. The percentage values provided for each transition give the fraction of transitions along the respective pathways out of each state. Distances in the lower part are differences in contour length.

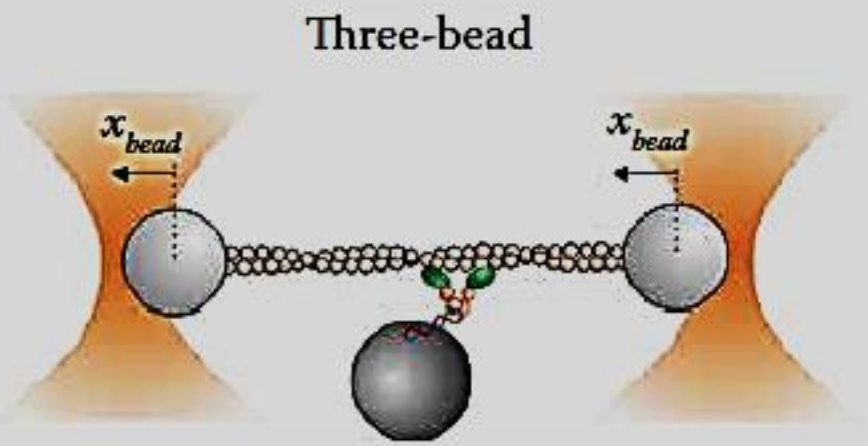
Calmodulin folding-unfolding energy landscape
Stigler, J., et al., Science, 334, 512–516, 2011

Calmodulin Pathway

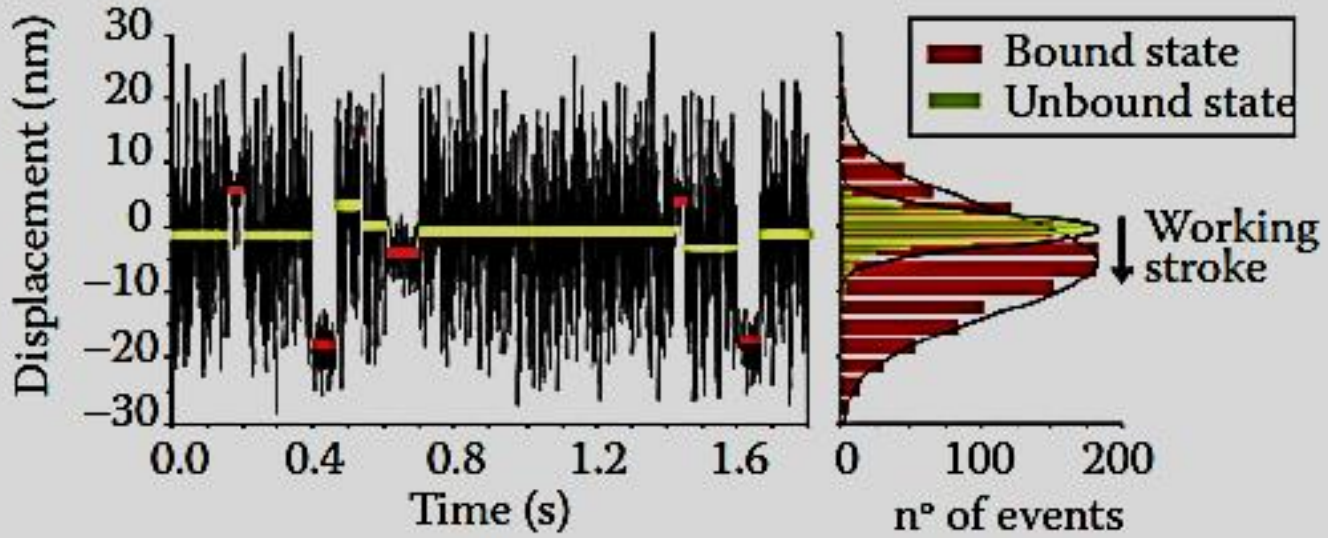
Calmodulin binds to 4 Calcium Ions and Undergoes Conformational Changes



Three-bead assay. Example: working stroke of myosin



Actin filament connected to polystyrene beads held by optical tweezers.



Position recording while myosin is interacting with actin.

A single myosin molecule is attached onto a third bead stuck to the coverslip surface.
Movements of the actin filament produced by the attached myosin are measured through bead displacements (x_{bead})

Position recording while myosin is interacting with actin.
Red and green lines indicate the average position of bound and unbound events, respectively.
Distributions of the average position of bound and unbound events of a 100 s position recording containing several hundreds of interactions.
The working stroke is obtained from the displacement between the centers of the two distributions

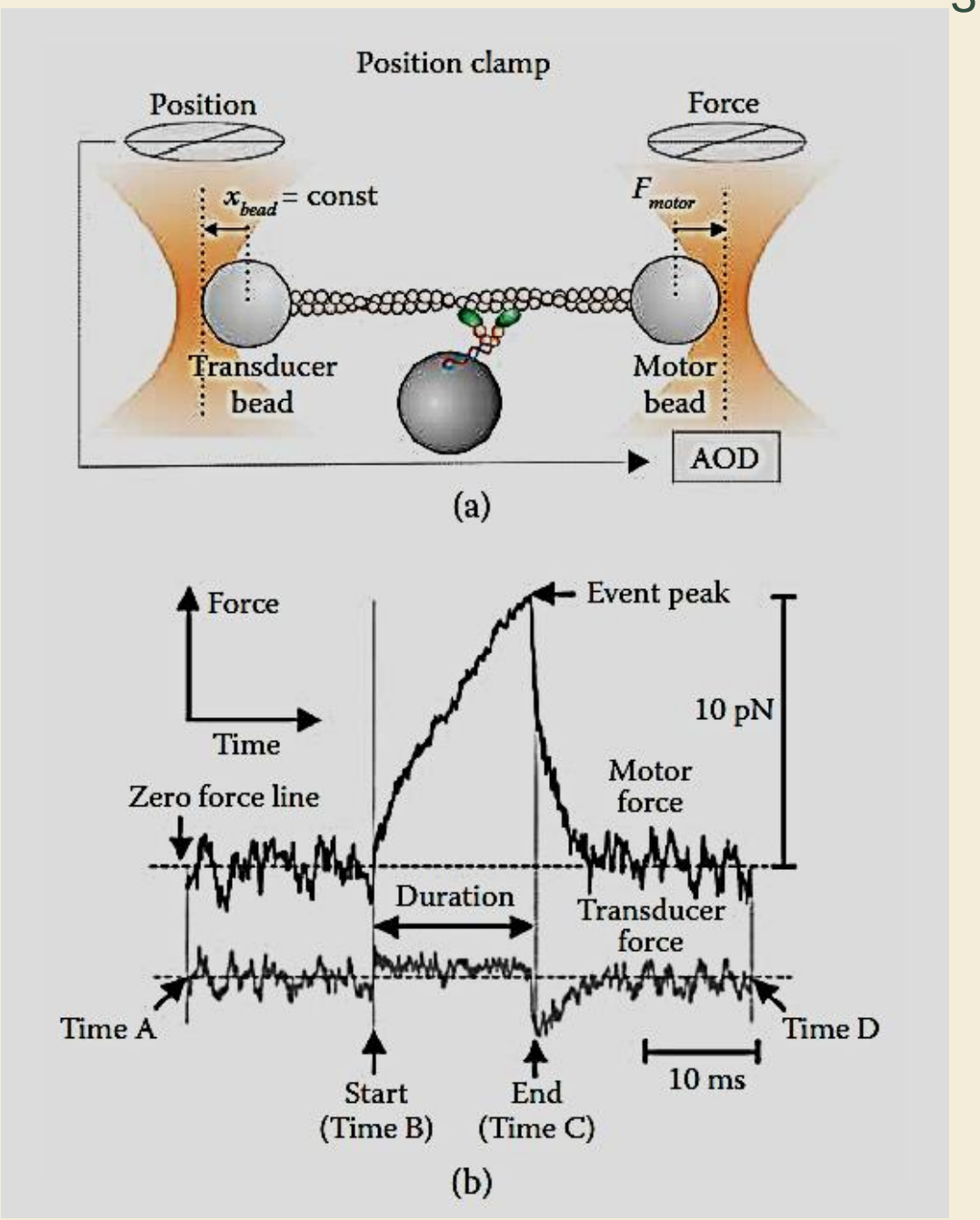
Capitanio, M., et al., Micr. Res. Tech., 65, 194–204, 2004

Position (or isometric) clamp.

(a) The left bead detects movements of the dumbbell (xbead), whereas the right bead moves using an AOD (Acoustic Optical Deflector) to oppose the detected movements. The right bead measures the force applied by the motor protein (Fmotor).

(b) A single acto-myosin interaction in the position clamp. After the force peak the force rapidly declines. The time between the start (Time B) and the point at which the rate of force declines is fastest (Time C) is defined as the duration of an episode.

Takagi, Y., et al., Biophys. J., 90, 1295–1307, 2006.



OT for single molecule spectroscopy; spatial and temporal resolution.

- measure conformational changes and displacements produced by single biological molecules.

Such movements range from several nanometers (molecular motors) down to one base pair (0.35 nm, for DNA and RNA processing enzymes) -- > **high spatial resolution** detection based on Interferometry (see next section).

Actually, position detectors do not set a limit on spatial and temporal resolution of OT.

Thermal noise sets instead fundamental limits on displacement and force measurements with single molecules.

Temporal resolution limit due to relaxation time for bead position.

When a single bead trapped in optical tweezers is perturbed from equilibrium, for example, by protein conformational changes or by trap displacements, it moves exponentially to a new equilibrium position with a time constant (relaxation time) $\tau = \gamma/k$, where γ is the viscous drag coefficient and k the stiffness of the system. Therefore, systems with higher stiffness attached to small probes exhibit fast erresponses to perturbations. If the perturbation develops faster than τ, the bead moves with the same relaxation time τ, filtering out all the movements that occur on shorter time scales.

AFM : k = 1 pN /nm → τ – microseconds us , OT : k= 0.001 pN/nm → τ - milliseconds