Case Study: Actin-binding Nebulin Fragments

Nebulin binds to the actin/tropomyosin/troponin thin filament of skeletal muscle

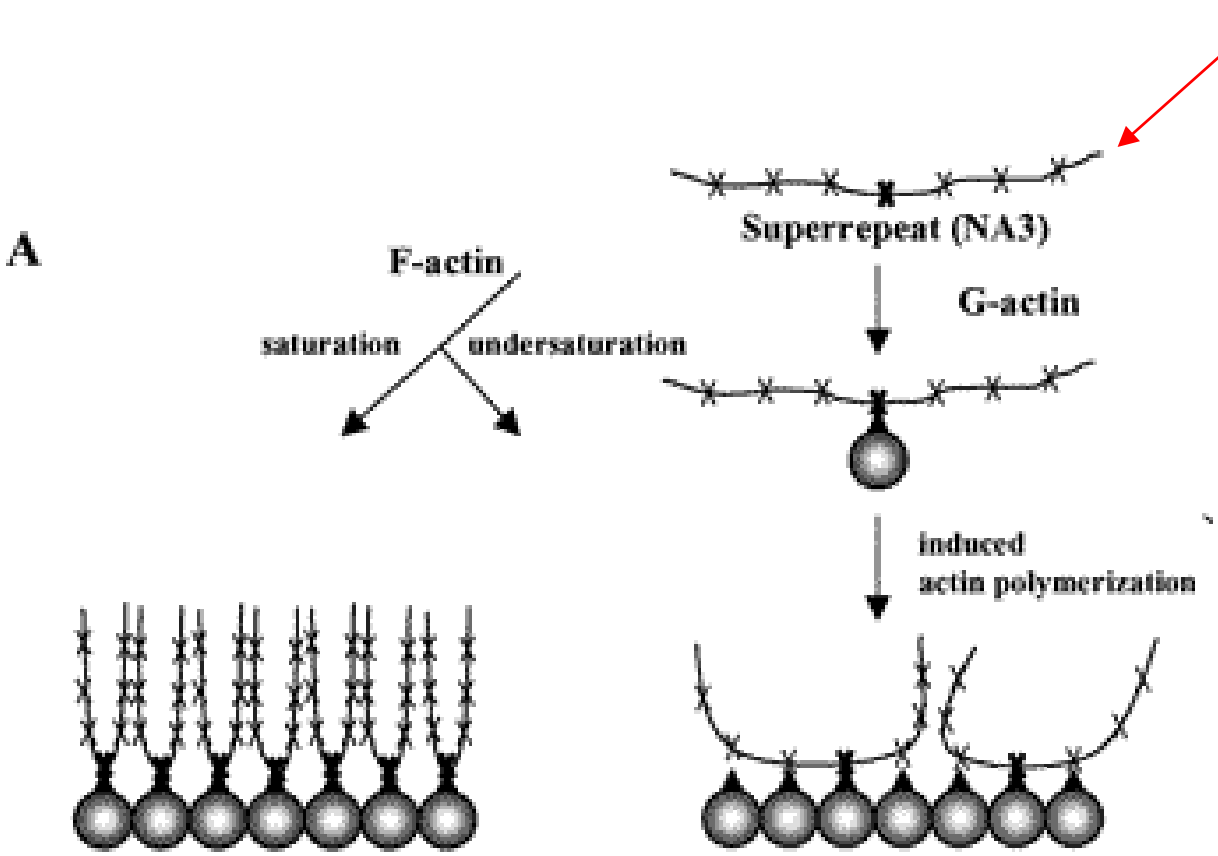
Multiples of repeated sequences arranged in modules

Fragments containing 2 - 15 modules retain actin binding function

Two fragments: NA3 and NA4

Question: Are NA3 and NA4 aggregated in solution?

Model of Nebulin binding to actin



Sedimentation equilibrium of nebulin fragments Using the Beckman Airfuge

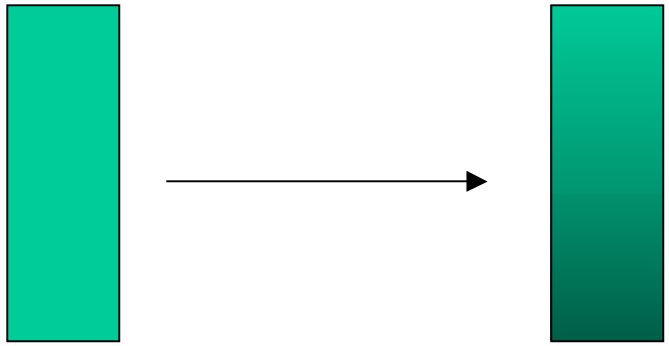
Conditions:

0.1 mg/ml protein

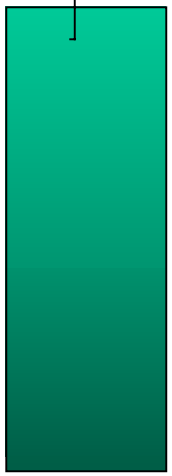
1 mM Ca^{++}, pH 7 buffer

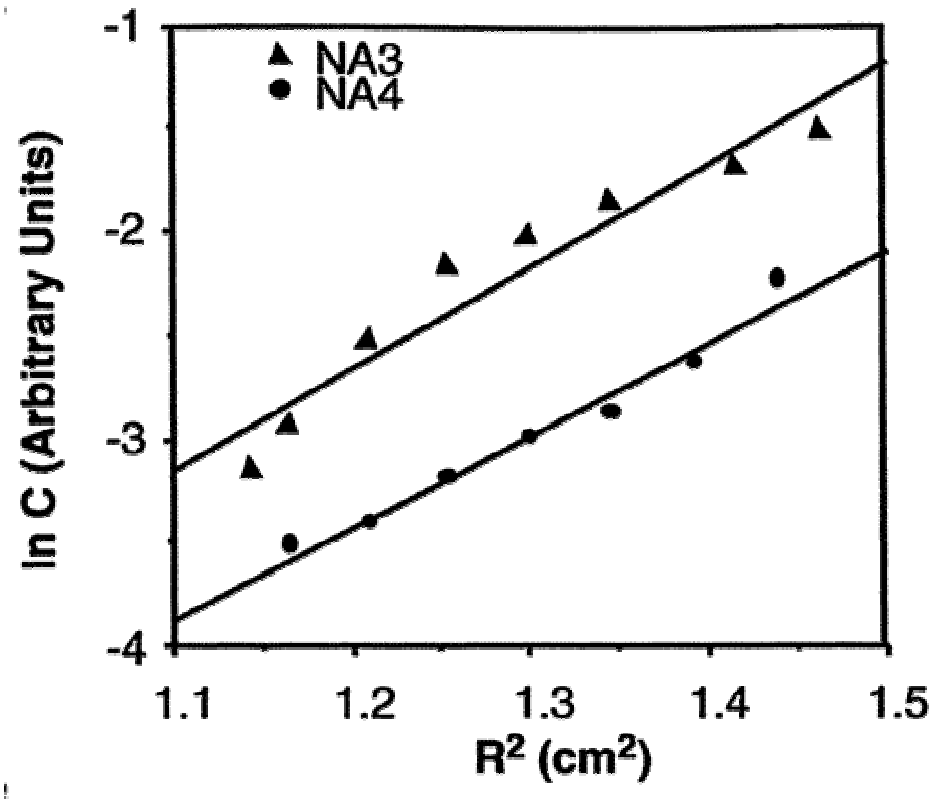
20% sucrose to stabilize the protein distribution during rotor deceleration

Spin solution for 80 h at 54,000 rpm ($\omega = 5700\ s^{-1}$)

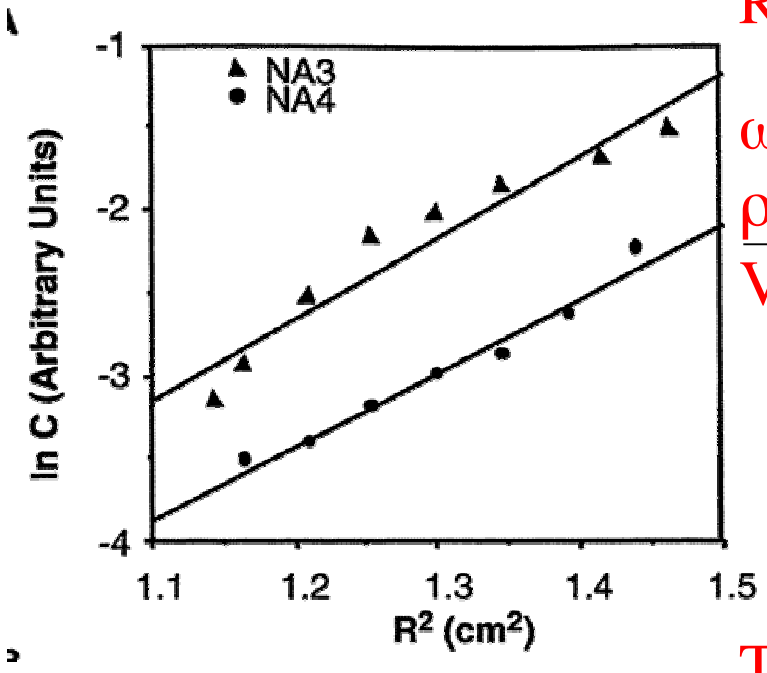


Using a syringe, remove liquid from the top of the tube and measure protein concentration for each fraction





$$M = d \ln C / dr^2 (2 R T / (\omega^2 (1 - \bar{V}\rho)))$$



R (gas constant) = 8.3×10^7 g cm^2 $s^{-2} mol^{-1} K^{-1}$

$\omega = 5700\ s^{-1}$

$\rho = 1.08$ g cm^{-3} density of 20% sucrose

$\bar{V}$ = partial specific volume of nebulin fragments in 20% sucrose.
Calculated from amino acid sequence
0.739 for NA3
0.745 for NA4

T = 300 °K

NA3: M = 37 kDa (monomer = 31kDa)
NA4: M = 35 kDa (monomer = 25 kDa)

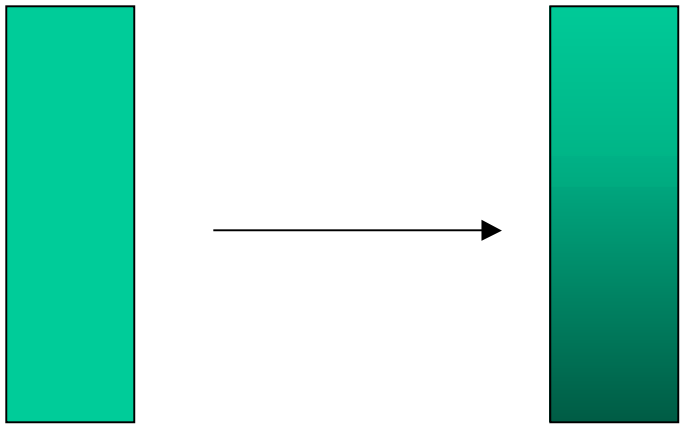
Conclusion:
Nebulin fragments
Are not aggregated

What is the shape of the nebulin fragment?

Sedimentation velocity in a Beckman L5-50 preparative ultracentrifuge: SW41 swinging bucket rotor

Conditions:

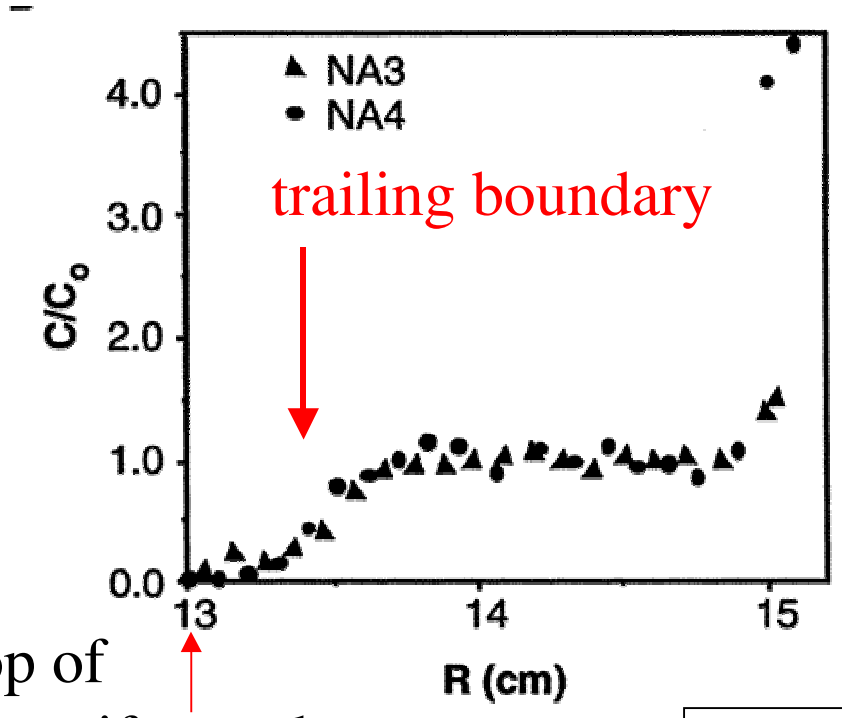
0.05 mg/ml protein concentration
1 mM Ca^{++}, pH 7 buffer
20% sucrose



Remove sample from the top and measure protein to determine the profile of the trailing boundary.

Spin for 18 h at 35,000 rpm at 20° C

Sedimentation Velocity of Nebulin Fragments



$$S_o = (\ln r_b - \ln r_m)/\omega^2 t$$

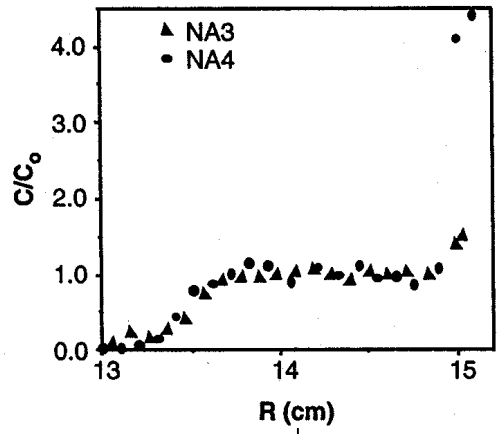
t = 64,800 sec

$\omega = 3665\ s^{-1}$

r_b = boundary position

r_m = meniscus position

Sedimentation velocity of nebulin fra



Must correct for the fact that the measurement is done in 20% sucrose:

1. Density is 1.08 (+sucrose) instead of 1.00 g/cm^3

2. Viscosity is 0.0195 P (+ sucrose) instead of 0.01 P

3. Partial specific volumes are different

   V = 0.739 (+ sucrose) instead of 0.728 cm^3/g for NA3

   V = 0.745 (+ sucrose) instead of 0.733 cm^3/g for NA4

$$S_{w,20} = S_o \frac{\eta(1 - \bar{V}_w\rho_w)}{\eta_w(1 - \bar{V}\rho)}$$

Measured in 20% sucrose

Results:

$S_{w,20}$ = 1.1 S for both NA3 and NA4

Interpretation of the Sedimentation of nebulin fragments

We can now calculate the Diffusion coefficients:

From sedimentation equilibrium

$$D_{w,20} = S_{w,20} RT/(M(1 - \bar{V}_2\rho)$$

(Note: use values of η, ρ and $\bar{V}$ that apply to the protein in water)

Results: NA3 $D_{w,20} = 3.2 \times 10^{-7}\ cm^2s^{-1}$

NA4 $D_{w,20} = 3.6 \times 10^{-7}\ cm^2s^{-1}$

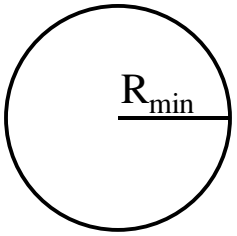
f/f_{min}

experimental $\quad D = \frac{kT}{f} = \frac{kT}{6\pi\eta R_S}$ $\quad$ since $f = 6\pi\eta R_s$

$$\mathbf{R_S = (kT/D_{w,20})/6\pi\eta}$$

Anhydrous sphere

$$\text{vol} = [\bar{V}_2 \bullet \frac{M}{N}] = \frac{4}{3}\pi R^3_{min}$$



$$\mathbf{R_{min} = (3V_2M/4\pi N)^{1/3}}$$

:

Define: $f_{min} = 6\pi\eta R_{min}$

measured

$$\left(\frac{R_s}{R_{min}}\right) = \left(\frac{f}{f_{min}}\right) = \frac{(kT/D_{w,20})/6\pi\eta}{(3V_2M/4\pi N)^{1/3}}$$

anhydrous sphere

Interpretation of the Sedimentation of nebulin fragments

Calculate f/f_{min}:

From sedimentation equilibrium

$$f/f_{min} = (kT/D_{w,20})(6\pi\eta(3M\bar{V}_2/4\pi N)^{1/3})$$

Results: NA3 $D_{w,20} = 3.2 \times 10^{-7}\ cm^2s^{-1}$ $f/f_{min} = 3.27$

NA4 $D_{w,20} = 3.6 \times 10^{-7}\ cm^2s^{-1}$ $f/f_{min} = 2.96$

Nebulin fragments are highly asymmetric

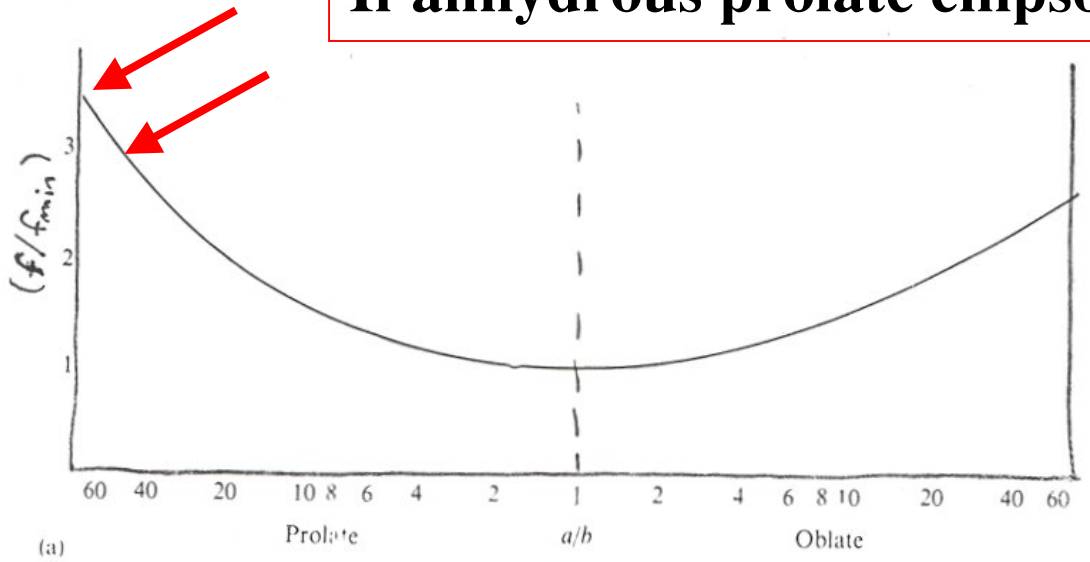
NA3

$f/f_{min} = 3.27$

NA4

$f/f_{min} = 2.96$

If anhydrous prolate ellipsoids: a/b = 50/1 to 60/1



about 600 Å long

Gel filtration chromatography

F_{ap} = solvent flow

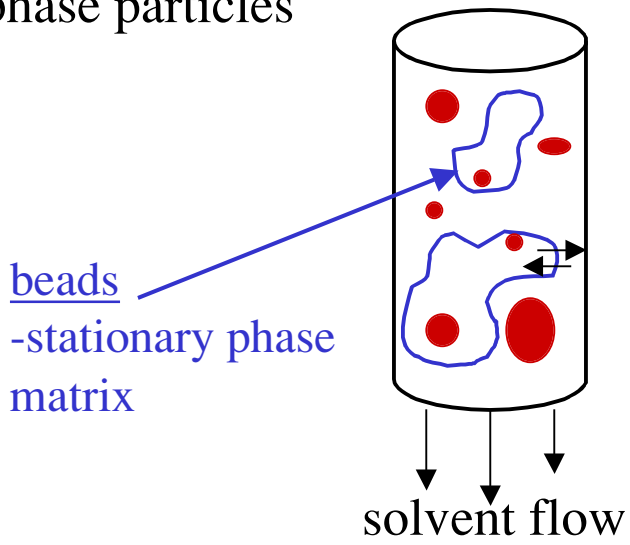
F_{ret} = partitioning into stationary phase particles

small molecules partition into beads

- This retards their progress down the column

Large particles are excluded from the bead interior

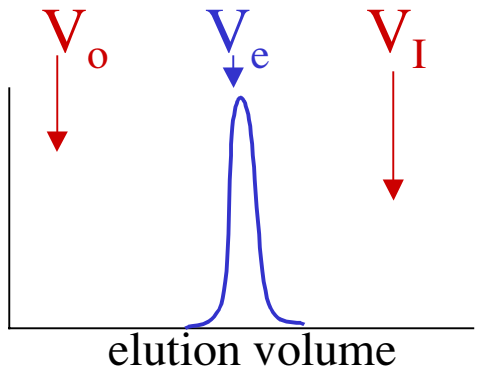
-elute faster



Measure elution volume, V_e, or σ

V_o = elution volume of totally **excluded** molecules

V_I = elution volume of totally **included** molecules



$$\sigma = \frac{V_e - V_o}{V_I - V_o}$$

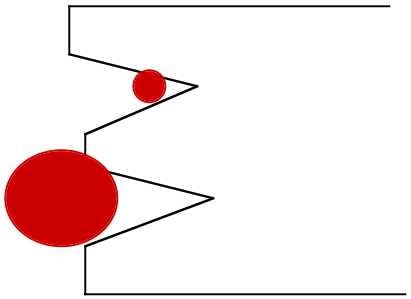
measure $\frac{\text{particle velocity}}{\text{solvent velocity}}$

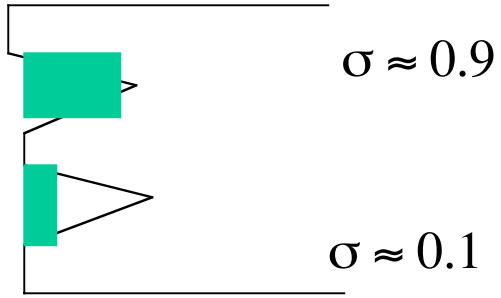
Gel Filtration Chromatography

σ depends on size and shape-

small molecule

large molecule





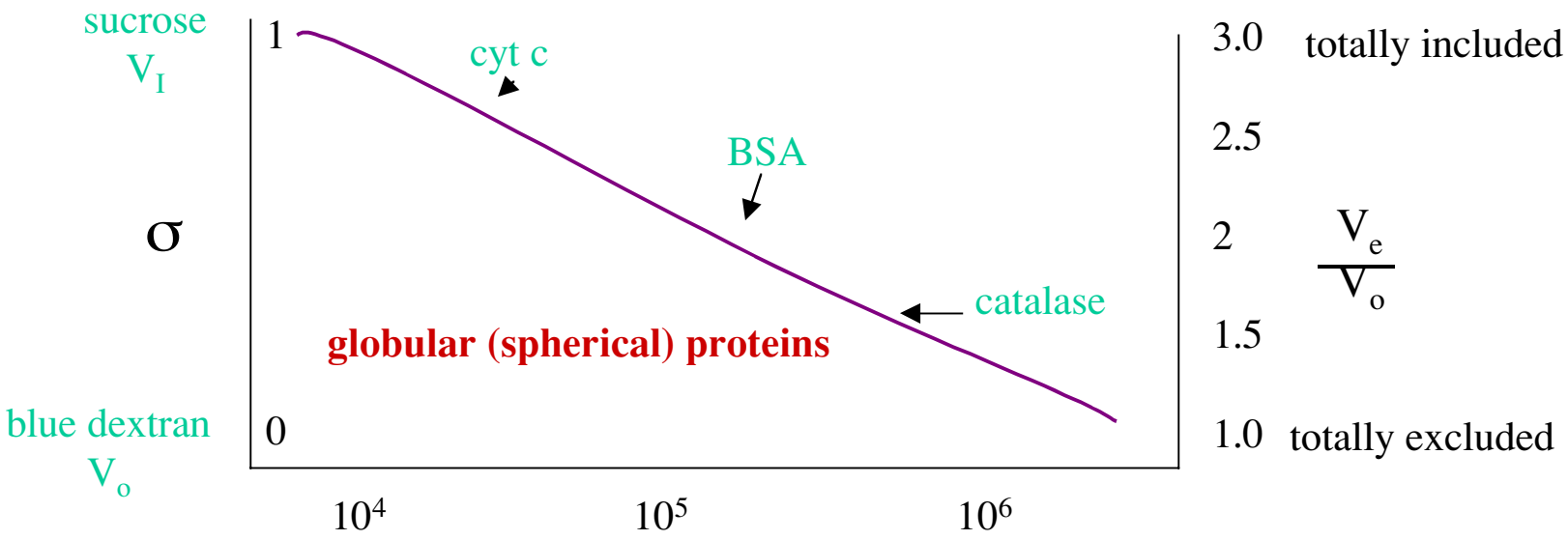
accessible volume within the matrix

$$\sigma = \frac{V_e - V_o}{V_I - V_o}$$

Example:
Elution of native globular protein using Sephadex G-200

Experimentally, the retardation of macromolecules in gel filtration chromatography correlates very well with the Stokes radius measured by Diffusion

If standards and the unknown have the same shape then- and only then- does gel filtration chromatography give a good estimate of molecular weight

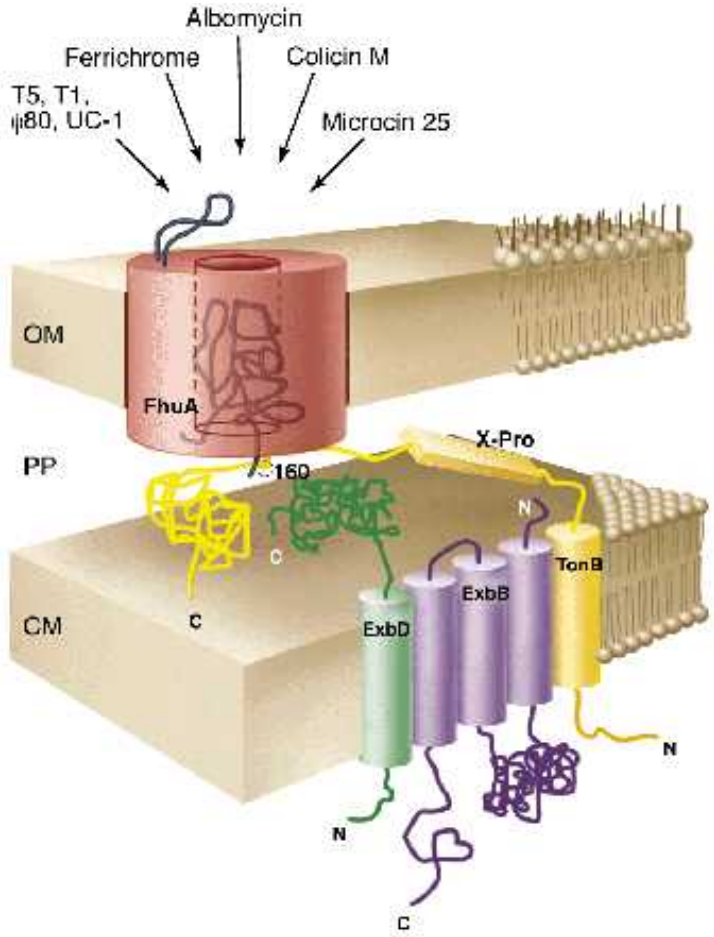


Active Transport in *E. coli* Mediated by outer membrane proteins and TonB

Example: the **FhuA** transport
Protein from *E. coli*

-active transport system coupled
To **TonB** in the cytoplasmic membrane

-required for ferric ion transport
via ferrichrome complex



Science (1998)282,2202-

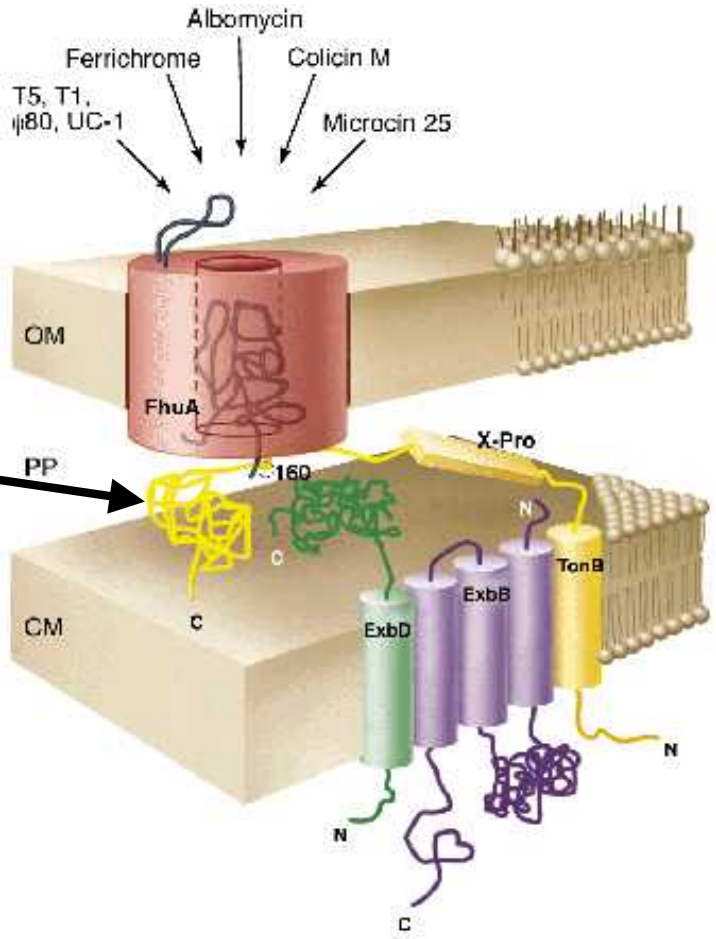
Express and characterize TonB without the N-terminal membrane anchor

put a His-tag on the N-terminus
to facilitate one-step purification

H_6-'TonB

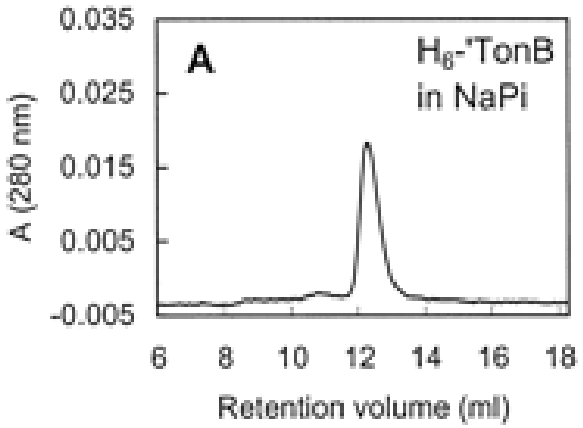
Protein is soluble

Calculated molecular weight: 24,880



Size Exclusion Chromatography of H_6-TonB

use Superose 12 column



Total volume: 21 ml

measured by the elution of $NaNO_3$

Void Volume: 7.3 ml

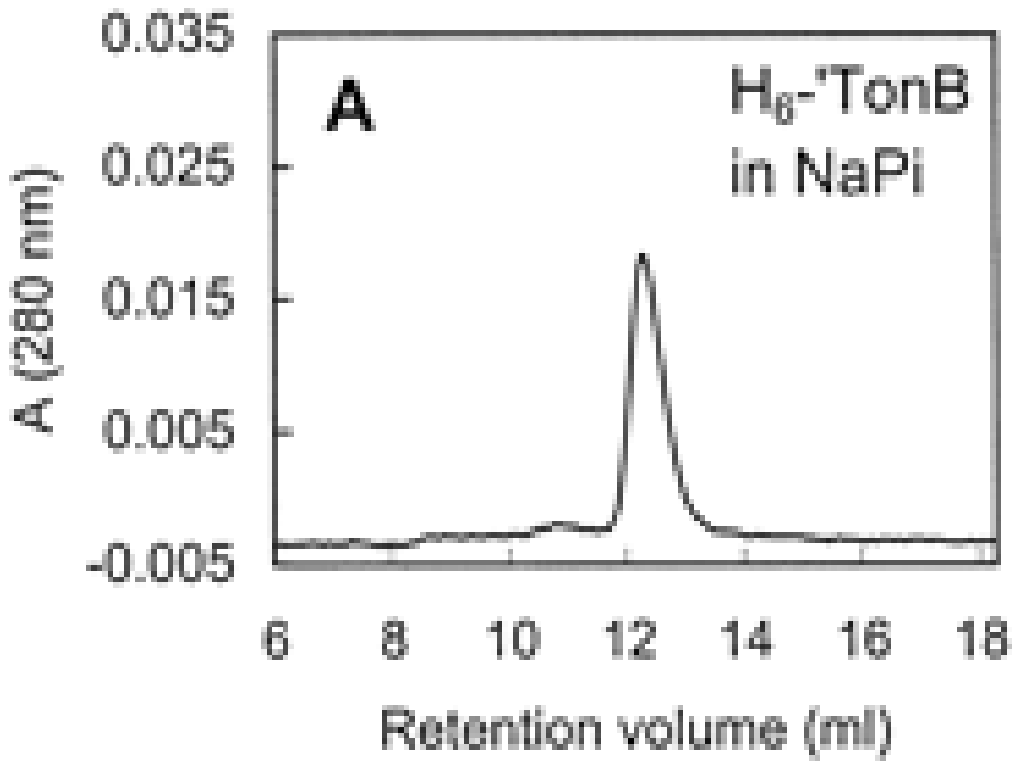
measured by the elution of Dextran blue 2000

Calibrate vs R_s

thyroglobulin R_s = 8.6 nm; V_e = 8.8 ml
ferritin R_s = 6.3 nm; V_e = 10.7 ml
catalase R_s = 5.2 nm; V_e = 11.7 ml
aldolase R_s = 4.6 nm; V_e = 12.0 ml
bovine serum albumin R_s = 3.5 nm; V_e = 12.5 ml
ovalbumin R_s = 2.8 nm; V_e = 13.4 ml
chymotrypsinogen R_s = 2.1 nm; V_e = 14.9 ml
RNase R_s = 1.75 nm; V_e = 15.5 ml

Journal of Bacteriology, May 2001, p. 2755-2764, Vol. 183, No. 9

Size Exclusion Chromatography of H_6-TonB



R_s = 4.1 nm for H_6-TonB

Journal of Bacteriology, May 2001, p. 2755-2764, Vol. 183, No. 9

Sedimentation Velocity of H_6-TonB

Measure $S_{20,w}$ = 1.4 S

Calculate M

ρ = 1.007 g/ml

η = 1.04 Poise

$\bar{V}$ = 0.7376 ml/g (from sequence)

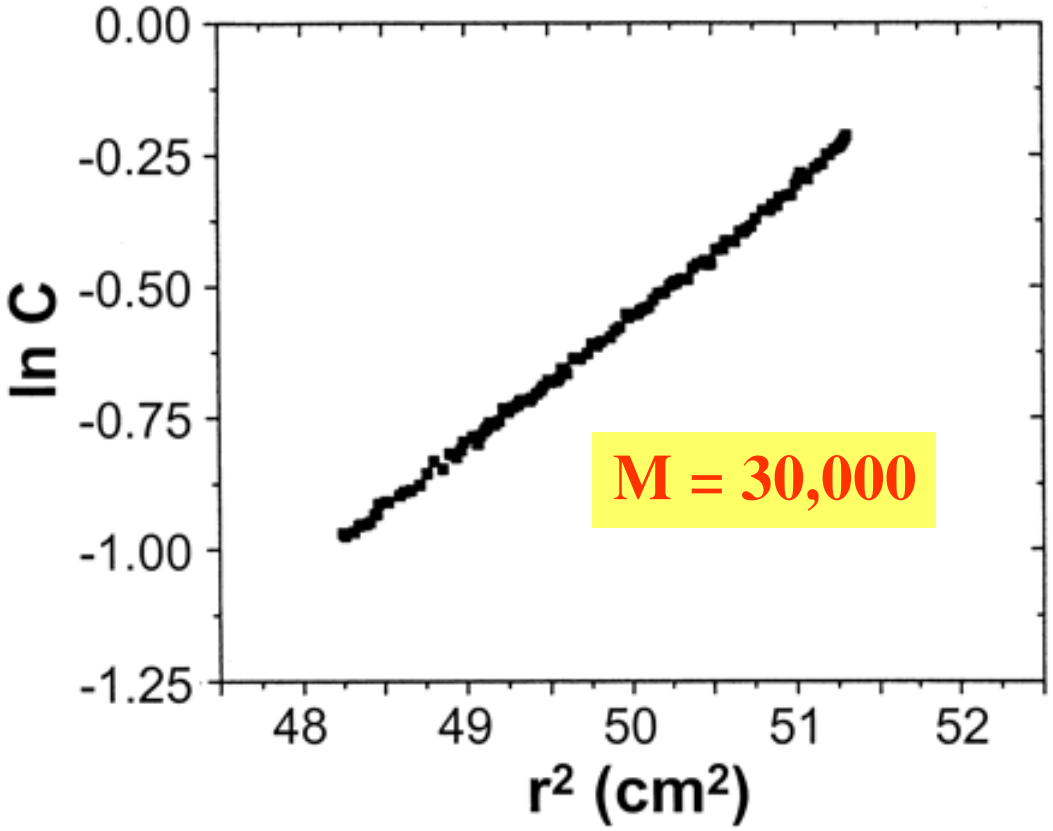
R_s = 4.1 nm (from gel filtration)

$$R_s = M(1 - \bar{V}\rho)/6\pi\eta N S_{20,w}$$

M = 28,000

Conclude that H_6-TonB is a monomer in solution

Sedimentation Equilibrium of H_6-TonB



0.8 mg/ml protein
18000 rpm
overnight 20°C

Slope = $M(1-\bar{V}\rho)$
ρ = 1.007 g/ml
$\bar{V}$ = 0.7376 ml/g

Conclusion: H_6-TonB is a monomer in solution consistent with gel filtration/Sed. velocity

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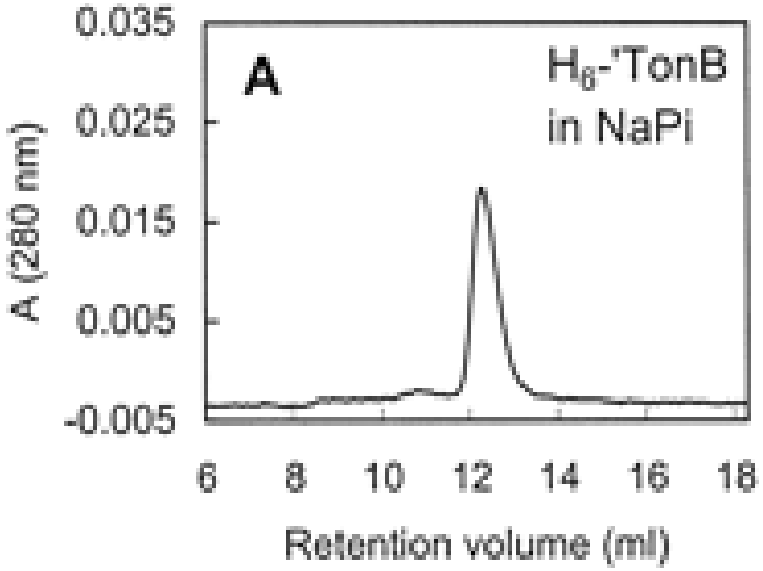
Size Exclusion Chromatography of H_6-TonB



R_s = 4.1 nm for H_6-TonB

Calculate $R_{min} = (3MV/4\pi\bar{N})^{1/3}$ = 1.9 nm

for anhydrous sphere with

M = 24,900 from sequence

Conclude: molecule is highly asymmetric

R_s/R_{min} = 2.1

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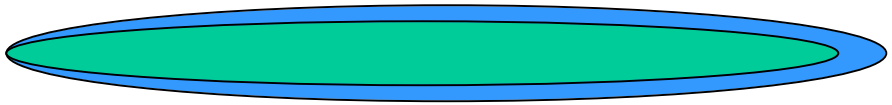
One can re-calculate R_{min} assuming hydration of 0.3 g H_2O/g protein

R_{min} = 2.0 nm

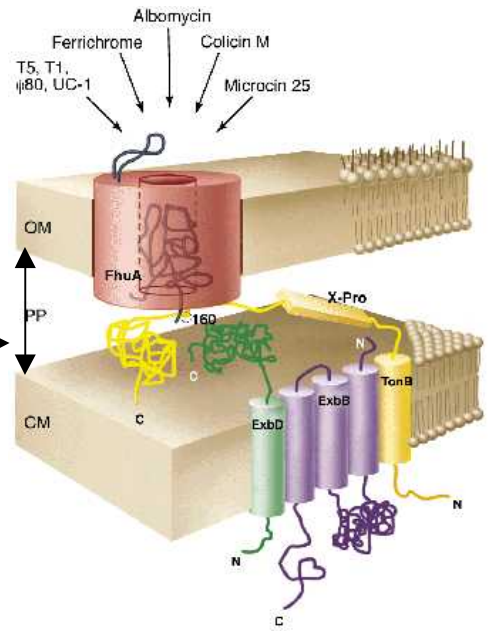


so R_s/R_{min} = 2

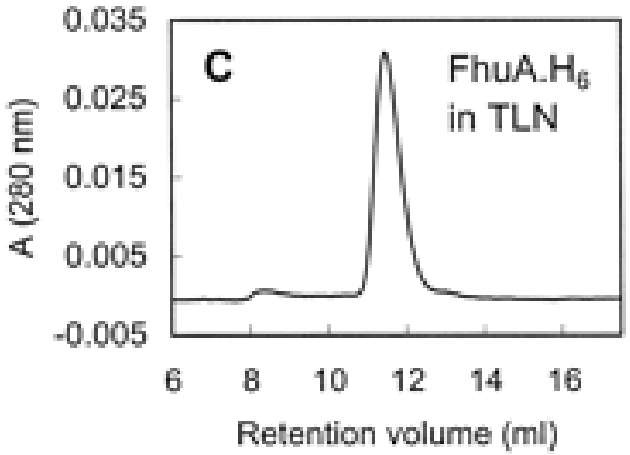
Consistent with an ellipsoid with an axial ratio of 15:1

240 Å x 16 Å

TonB goes from the inner to the outer bacterial membrane



Size Exclusion Chromatography of FhuA



R_s = 4.8 nm for FhuA

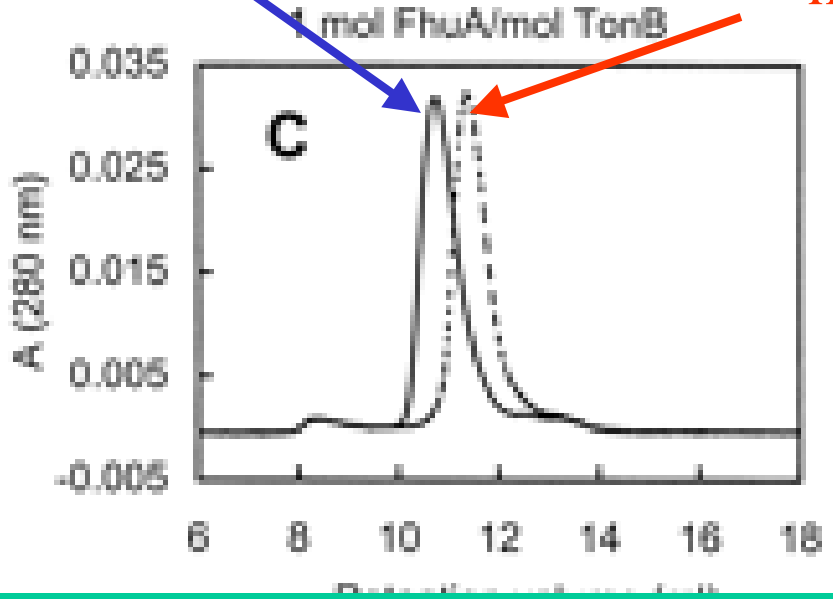
TLN buffer contains detergent,some of which is bound to the membrane protein to maintain the protein in solution

consistent with protein plus bound detergent: M_r = 185,000

Mixture of FhuA and TonB Elutes at a Smaller Volume in the presence of the FhuA Fe ligand

plus ferricrocin: R_s = 6.2 nm

no ferricrocin



Assuming a 1:1 Complex, R_{min} can be estimated as 4.4 nm so R_s/R_{min} = 1.4 for the presumed complex. This is more typical of globular proteins and much less asymmetric than TonB alone

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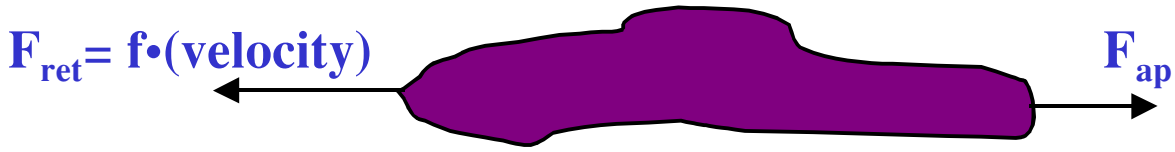
Electrophoresis

applied force: electrical field
retarding force: frictional drag
moving through solution

F_{ap}



in steady state: **f•(velocity) = Q•E**

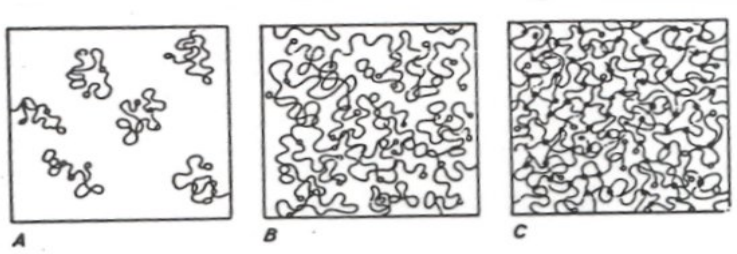
electrophoretic mobility: $U = \frac{\text{velocity}}{E} = \left(\frac{Q}{f}\right)$

depends on charge (**Q**) and $\mathbf{R_s}$

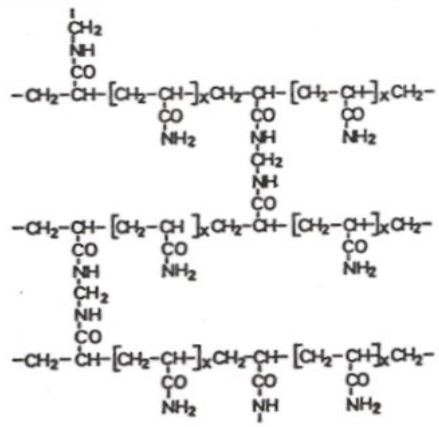
However, usually electrophoresis is done in the presence of a retarding matrix such a polyacrylamide

The ability of a macromolecule to move through the retarding matrix depends on the Stokes radius

A commonly used retarding matrix is cross-linked polyacrylamide



Schematic representation of the formation of polyacrylamide gel from random gel coils. Transition from a dilute polymer solution (A) through the concentrated solution (B) to the gel (C). –o– cross-linking agent; –●– tie-points.

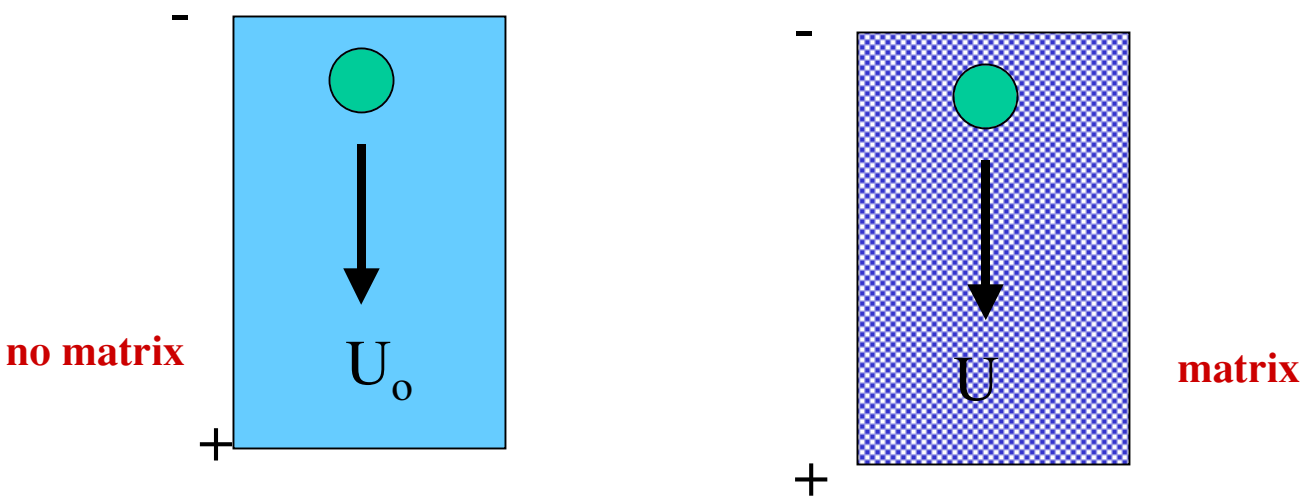


– Structure of polyacrylamide gel. Monomer: acrylamide; comonomer (cross-linking agent): N,N'-methylene-bis-acrylamide.

Electrophoresis in a retarding matrix such as agarose or polyacrylamide

$$\log U = \log U_o - K_R (\%T)$$

- measured mobility
- mobility in absence of gel (depends on **Q and R_s**)
- **retardation coefficient** Depends **only** on **R_s** (size and shape)
- percent acrylamide



Electrophoresis of Native proteins

:

$$\log U = \log U_o - K_R (\%T)$$

U_o is different for each protein

since it depends on both **Q and R_s**

Since the retardation coefficient K_R depends only on Stokes radius and not on charge, one can obtain R_s by determining K_R

Measure the electrophoretic mobility vs % acrylamide
the slope gives K_R which can be calibrated in terms of Stokes radius

this is called a Ferguson Plot

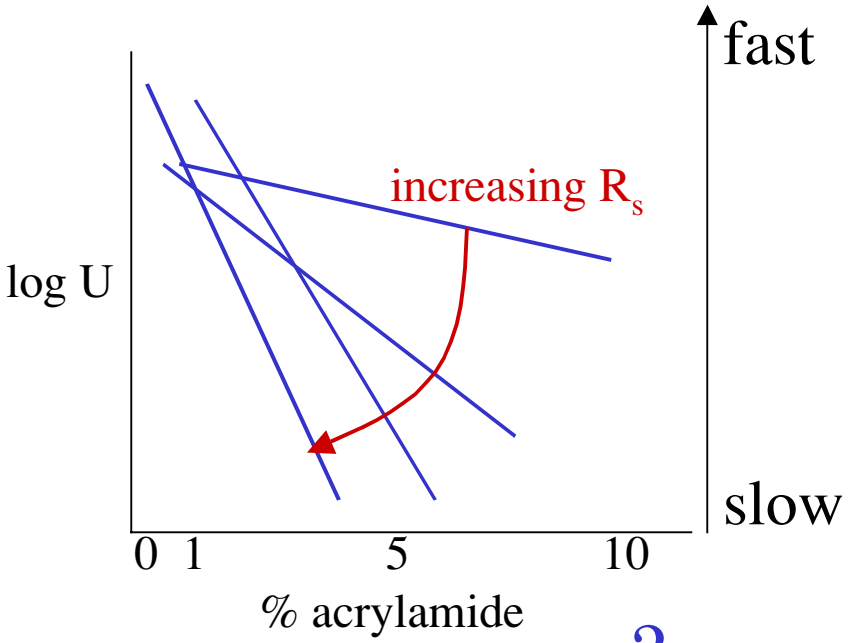
Ferguson Plot to determine the Stokes radius of non-denatured protein

$$\log U = \log U_o - K_R (\%\ T)$$

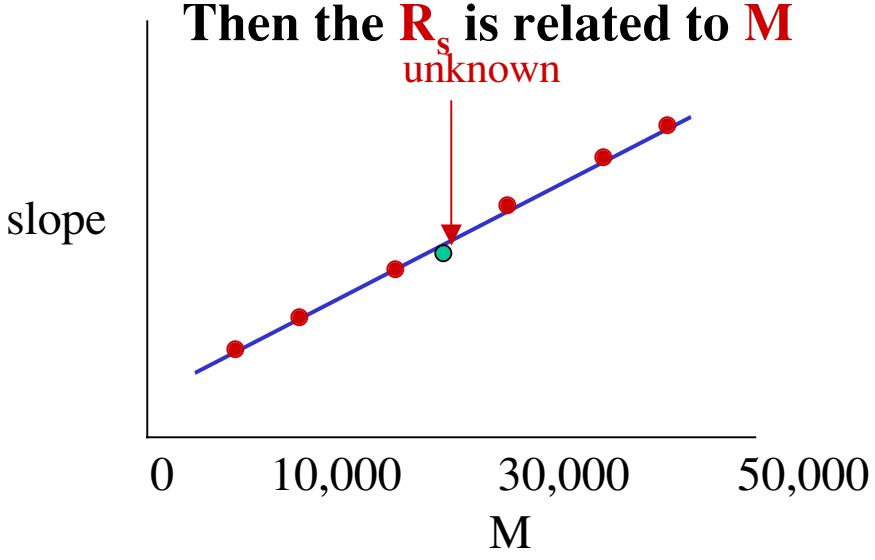
K_R = retardation coefficient
(%T) = % acrylamide

U_o - depends on Q and R_s
K_R - depends only on R_s

Ferguson Plot
non-denatured proteins



If standards and the unknown Protein have the same shape Then the R_s is related to M



slope $\Rightarrow R_s \overset{?}{\Rightarrow}$ M if standards and unknown have same shape

Example of the Application of the Ferguson Plot

GOAL: To determine the subunit composition of bacterial dioxygenases of potential use in environmental cleanup of polychlorinated biphenyls (PCPs)

purified enzymes contain two kinds of subunits, analyzed by SDS polyacrylamide gel electrophoresis.

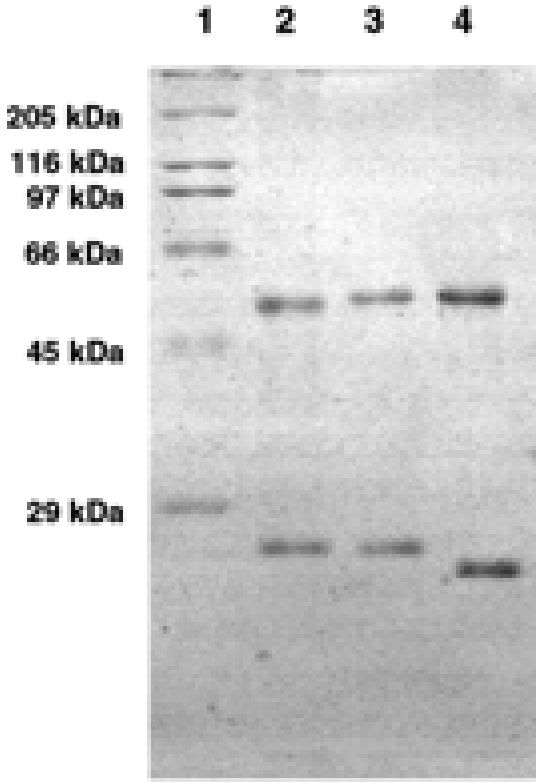
α subunit
β subunit

J. Biol Chem (2001)276,29833-38

Two enzymes were examined which have distinct substrate specificities. In addition, a hybrid enzyme was prepared and examined.

SDS-PAGE: gives subunit molecular weight and approximate ratio

Relative staining intensities are consistent with 1:1 ratio of the two subunits



α (~50KDa)

β (~21 KDa)

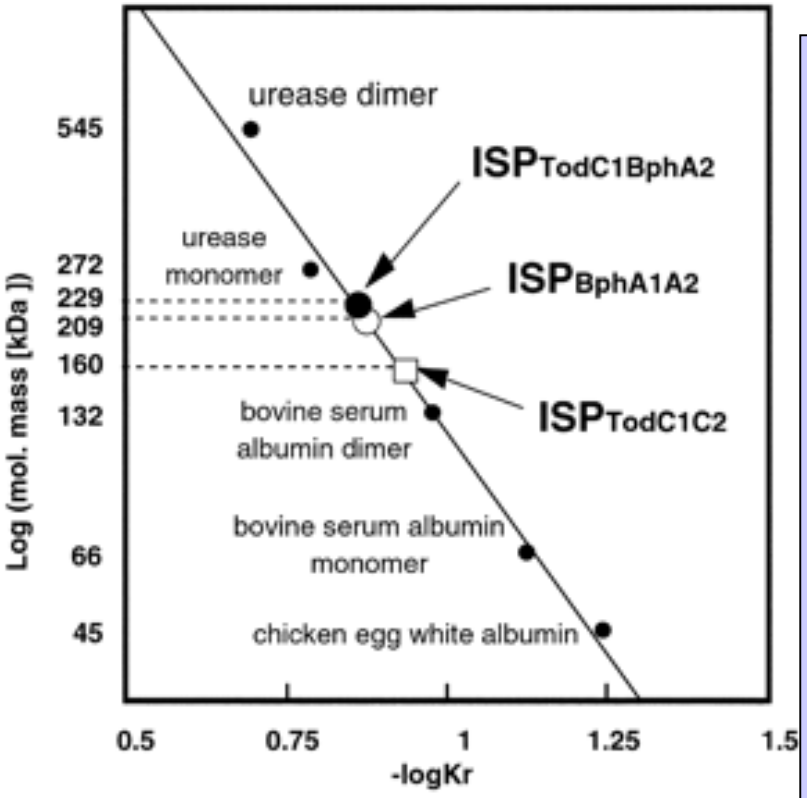
lane 2. biphenyl dioxygenase
A1 = α subunit
A2 = β subunit

lane 3. hybrid
C1 = α subunit
A2 = β subunit

lane 4. toluene dioxygenase
C1 = α subunit
C2 = β subunit

J. Biol Chem (2001)276,29833-38

Ferguson Plot Analysis of dioxygenases



ISPs	Mol. mass estimated by Ferguson plots	Mol. mass estimated by SDS-PAGE	
		α subunit	β subunit
$ISP_{BphA1A2}$	209	50.0	23.0
$ISP_{TodC1C2}$	160	52.5	21.5
$ISP_{TodC1BphA2}$	229	52.5	23.0

1. for each protein (called "ISPxx"), determine the mobility (R_f) as a function of percent acryamide (%T)

2. Determine the slope (K_r) of the plot of $\log R_f$ *vs* %T

3. Plot $\log K_r$ (slope) *vs* log (mol wt)

4. If standards and dioxygenases are the same shape, then molecular weight
can be determined. If the unknowns are highly asymmetric, the molecular weight will be incorrect.

Conclusions from Ferguson Plot anaylysis

1. biphenyl dioxygenase is a hexamer: $\alpha^3 \beta^3$

2. toluene dioxygenase is a tetramer: $\alpha^2 \beta^2$

3. hybrid enzyme is a hexamer: $\alpha^3 \beta^3$

ISPs	Mol. mass estimated by Ferguson plots	Mol. mass estimated by SDS-PAGE		Predicted mol. mass	
		α subunit	β subunit	$\alpha^3\beta^3$	$\alpha^2\beta^2$
$ISP_{BphA1A2}$	209	50.0	23.0	219.0	146.0
$ISP_{TodC1C2}$	160	52.5	21.5	222.0	148.0
$ISP_{TodC1BphA2}$	229	52.5	23.0	226.5	151.0

J. Biol Chem (2001)276,29833-38

Electrophoresis

of double stranded DNA

of denatured RNA (no secondary structure)

of SDS - protein complexes

$$\log U = \log U_o - K_R (\%T)$$

Within each set:

(1) shape is the same

(2) charge and size vary proportionally

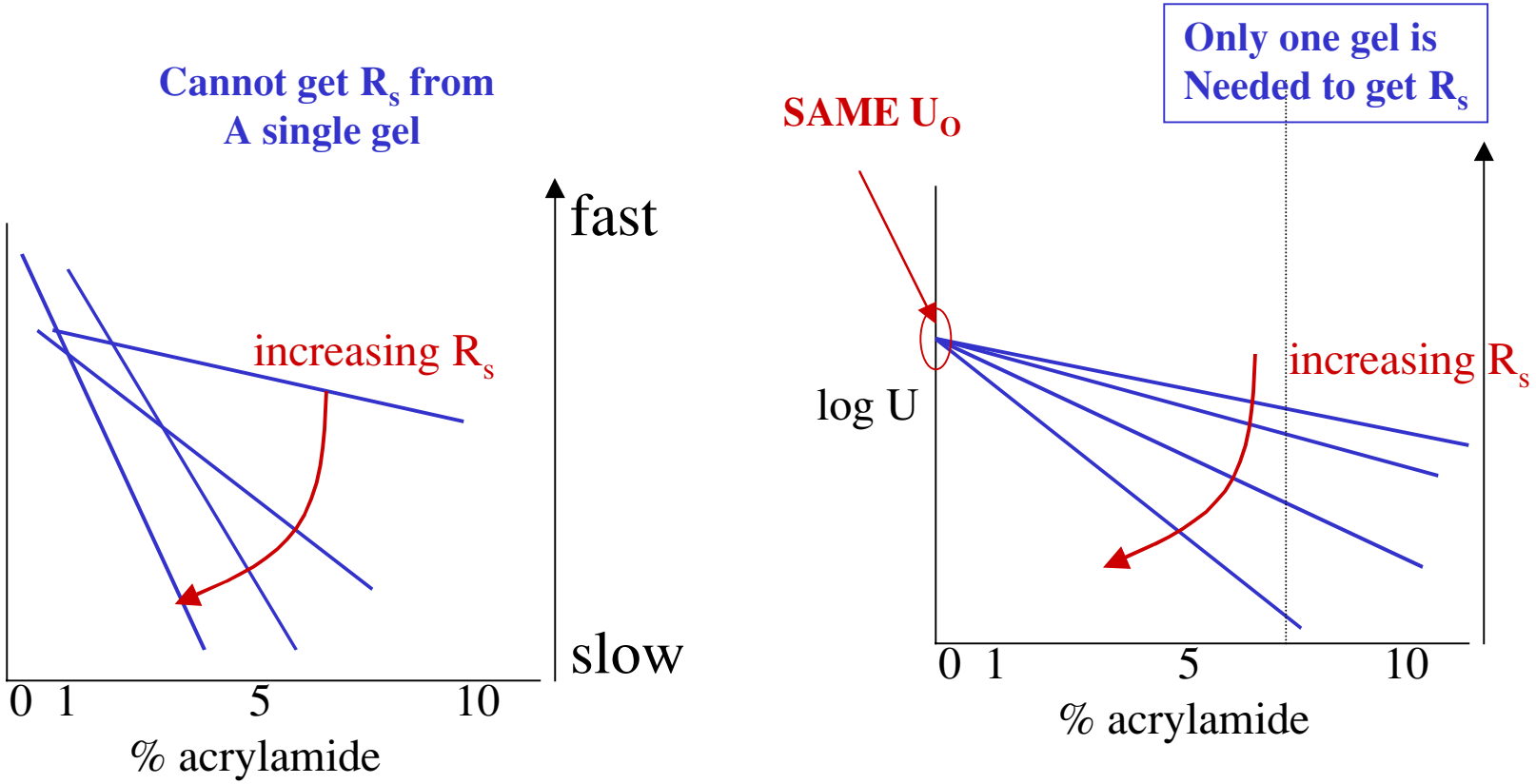
U_o does not vary with molecular weight

$$U_o = \frac{Q}{f} \quad \begin{matrix} \uparrow \text{ offsetting effects} \\ \uparrow \end{matrix}$$

Hence, in comparing (for example) DNA samples, **U** varies in proportion to Stokes radius

- No need to vary % acrylamide , so one gel is sufficient

Constant value of U_o for SDS-Protein Complexes allows the R_s to be determined by Determining the electrophoretic mobility on a single gel and comparing to standards

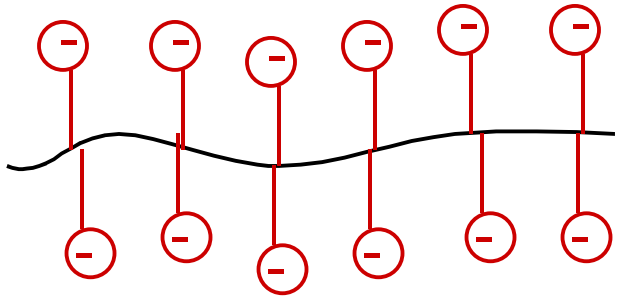


NATIVE PROTEINS

SDS-PROTEIN COMPLEXES

Since the SDS-Protein complexes all have the same shape:
R_s corrlates well with molecular weight (M)

Electrophoresis of Protein - SDS Complexes



-highly asymmetric

-as length of protein increases, so does Q

$$\log U = \log U_o - K_r (\%T)$$

U_o is the same for all complexes

relative mobility depends on R_s only
Since the shape is the same for all complexes
$R_s \Rightarrow$ Molecular Weight

Problems with SDS-PAGE one can get incorrect answers!

-U_o might be different for unknown and for the standards

1. Membrane proteins (run fast)

2. Glycoproteins (run slow)

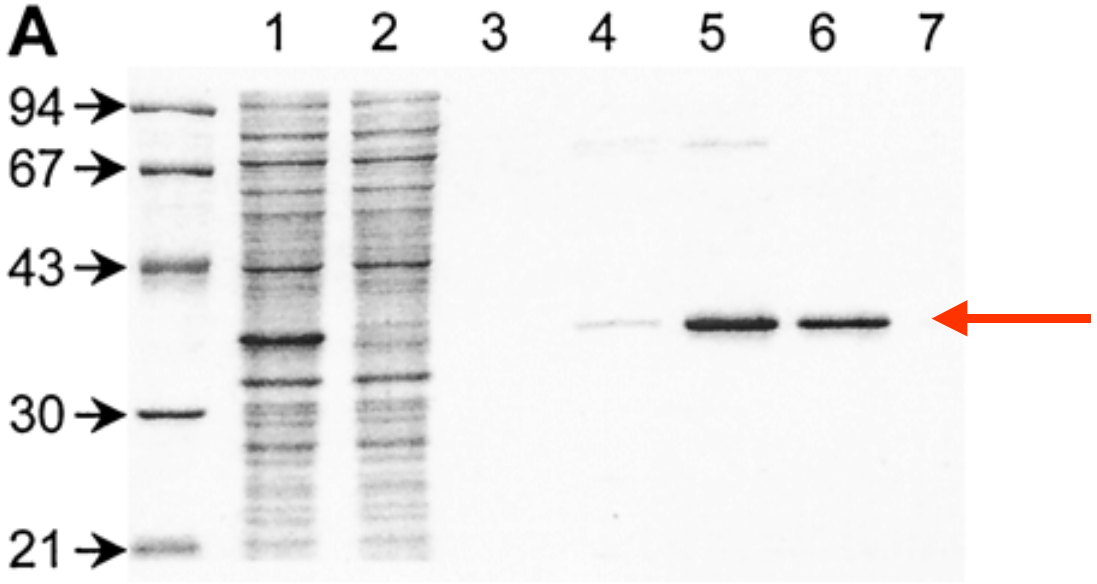
-more or less SDS bound: so (Q/f) is not the same
(usually 1.4 g SDS / g protein for a typical soluble protein)

-glycoproteins can have substantial carbohydrate component that does not bind SDS

-shape may not be same due to incomplete unfolding (membrane proteins)

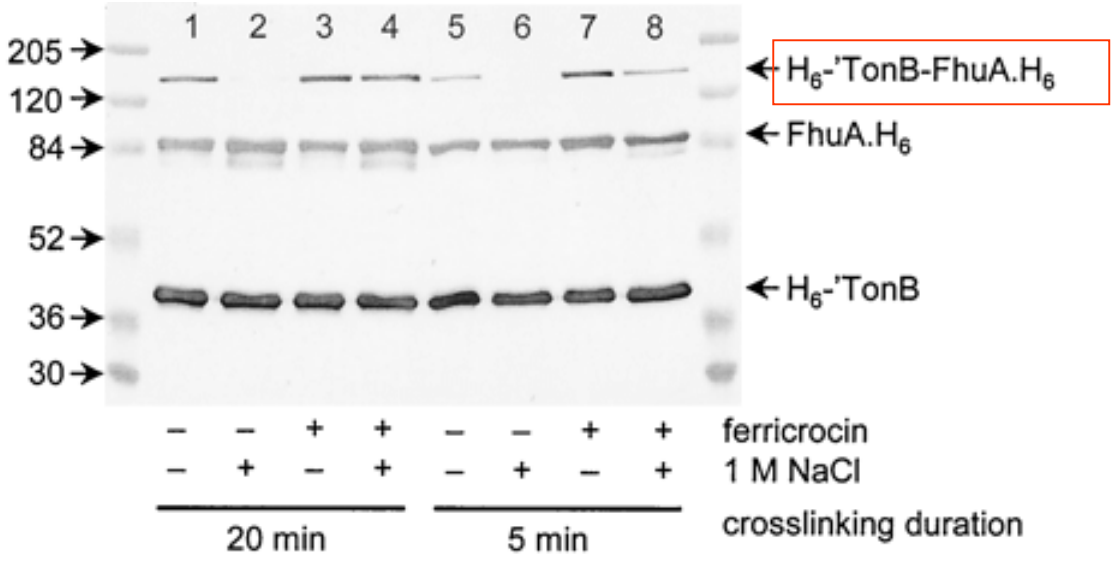
SDS-PAGE of Purified H_6-TonB Gives an incorrect value of the molecular weight

Expected molecular weight: 24.9 kDa
Estimated M_r from SDS-PAGE Mobility: 35 kDa
same as wild type TonB
reason is not known



Journal of Bacteriology, May 2001, p. 2755-2764, Vol. 183, No. 9

Chemical Crosslinking of FhuA and His_6-TonB Shows the presence of a 1:1 Complex Enhanced by ferricrocin and inhibited by 1 M NaCl



Journal of Bacteriology, May 2001, p. 2755-2764, Vol. 183, No. 9

Electrophoresis of double-strand DNA

$$\log U = \underbrace{\log U_o}_{\text{constant}} - K_R (\%T)$$

Relative mobility in gel $\Rightarrow$ R_s $\Rightarrow$ # basepairs

U_o constant

Same shape for unknown and standards

→compare with standards←

Whenever you have secondary structure this approach fails

1. U_o need not be the same for unknown and standard set

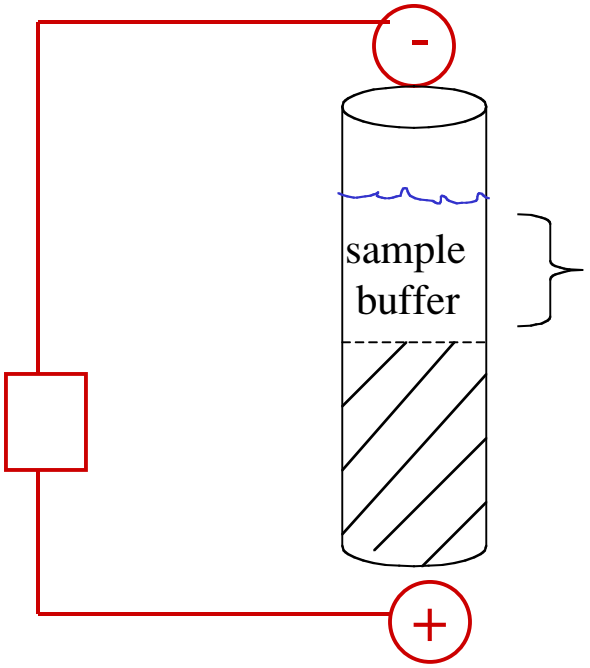
2. R_s will not simply be related to # basepairs (or mol wt) if the shape of the unknown is not the same as the shape of the standard set

-Hence, one needs to denature RNA to obtain the correct molecular weight from a single gel.

Stacking and Discontinuous Gels

Zonal analysis depends on having sharp, well defined bands

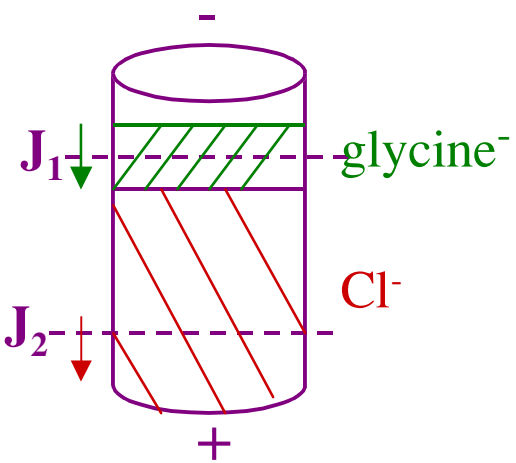
1 create a large electric field (voltage drop) in the sample buffer to concentrate the protein prior to separation by the gel



want large Electric Field to rapidly pile up protein at the surface of the gel.

Stacking

current (ion flux) must be constant throughout the gel



$NH_2CH_2CO_2^-$ (charged)

pH-dependent mobility

$NH_3^+CH_2CO_2^-$ (neutral)

$J_1 = J_2$

$C_1V_1 = C_2V_2$

C = concentration

V = net velocity

$$U = \text{Mobility} = \left(\frac{V}{E}\right)$$

$$J_1 = C_1U_1E_1 = C_2U_2E_2 = J_2$$

glycine — Cl$^-$

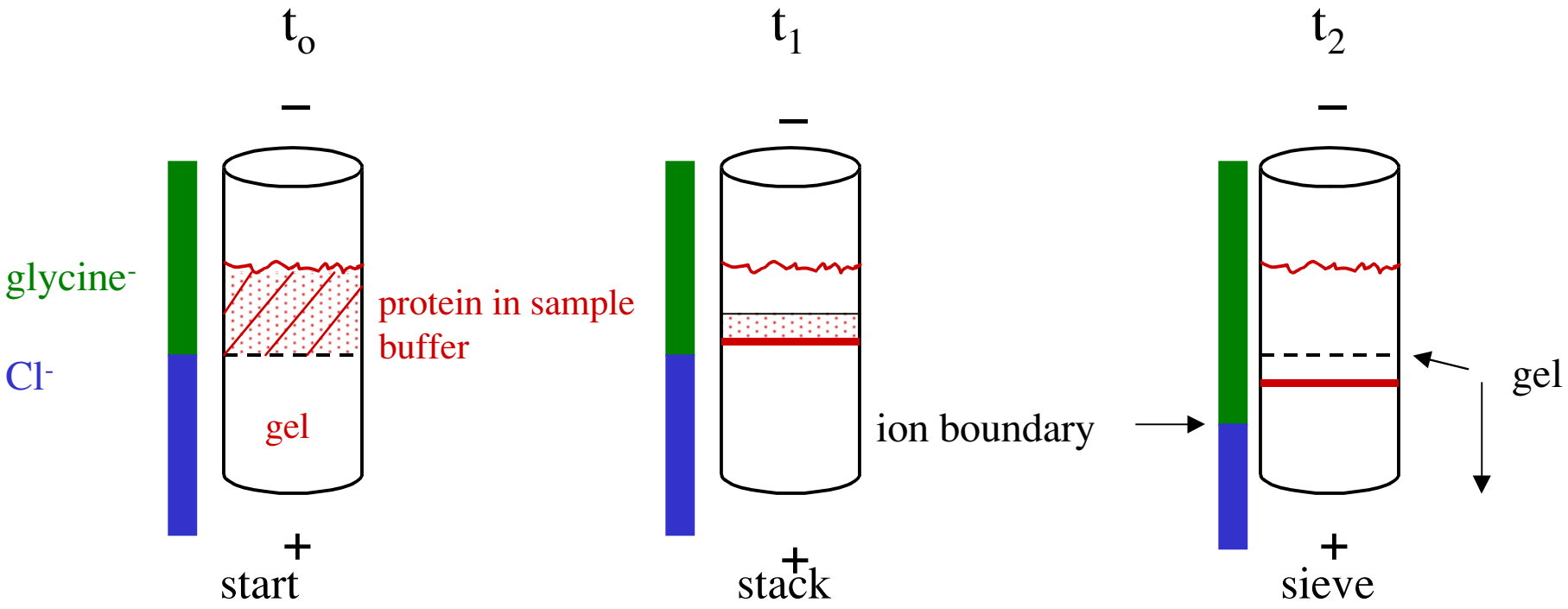
if $(C_1U_1) \ll (C_2U_2) \Rightarrow E_1 \gg E_2$

Protein in the glycine buffer experiences a high electric field because of the low electrophoretic mobility of glycine at the pH used

-the protein moves to the ion boundary if

$U_{gly} < U_{prot} < U_{Cl-}$

Stacking



$U_{glycine^-} < U_{ce}$

(depends on pH)

Alternative: Dilute running buffer (1/10) to make sample buffer ($U_1 = U_2$)

$$J_1 = c_1 U_1 E_1 = C_2 U_1 E_2 = J_2$$

(low) (high) in sample region

Two variants of gel electrophoresis

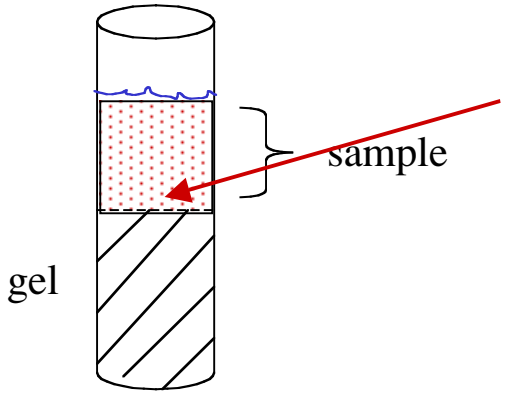
1. **Gel mobility-shift assay for protein-DNA interactions**

2. **Pulsed field gel electrophoresis for separating very large DNA (chromosomes)**

Gel Mobility - Shift Assay

for quantitative and qualitative characterization of DNA-protein interactions

DNA protein (eg lac repressor) DNA-protein complex

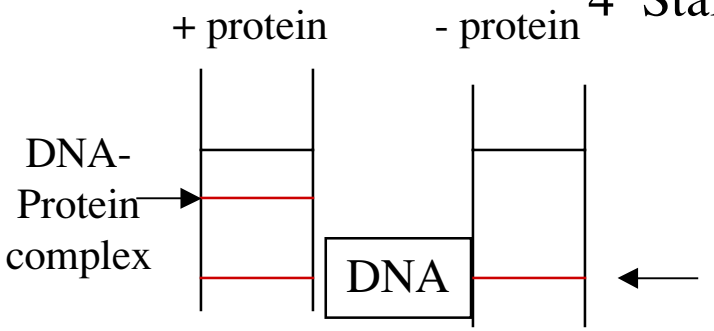


1 Equilibrate Reaction Mixture

2 Load onto gel (e.g., 7.5% Polyacrylamide Tris-Borate-EDTA buffer)

3 Run gel

4 Stain with ethidium bromide for DNA (or detect using radioactive labels)

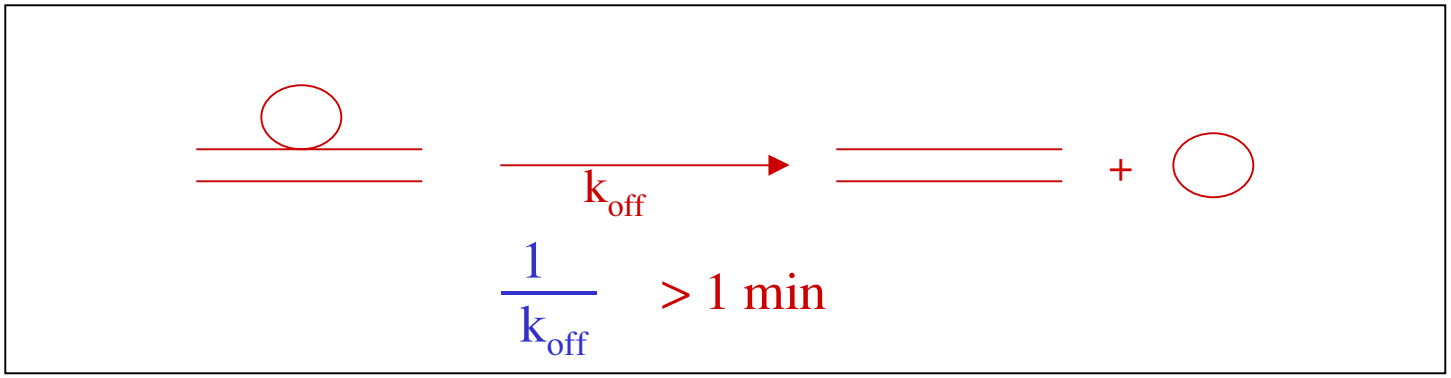


Gel Mobility shift assay

Why does this work?

1. Free DNA and **Bound DNA** species (along with free protein) are stacked and moved into the gel **before there is any time for protein dissociation**

Takes ~ 1 min to complete this process



2 Once in the gel, the DNA-protein complexes are virtually **locked together**

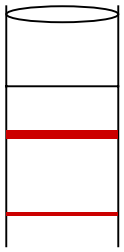
WHY?

- low salt
- excluded volume effect of gel
- "cage" effect of the gel

(not fully understood)

Gel Mobility Shift Assay

can provide all the information for a binding isotherm



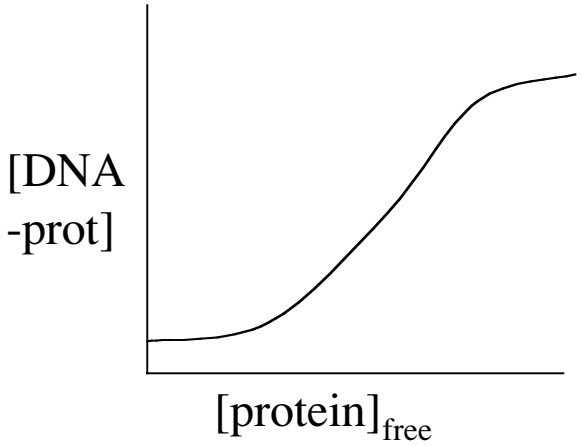
After running the gel: visualize the DNA: protein-DNA complex is retarded

$[DNA]_{total} = [DNA]_{free} + [DNA\text{-}protein]$

know this measure relative amounts from gel

$[protein]_{total} = [protein]_{free} + [DNA\text{-}protein]$

know this calculate this measure from gel

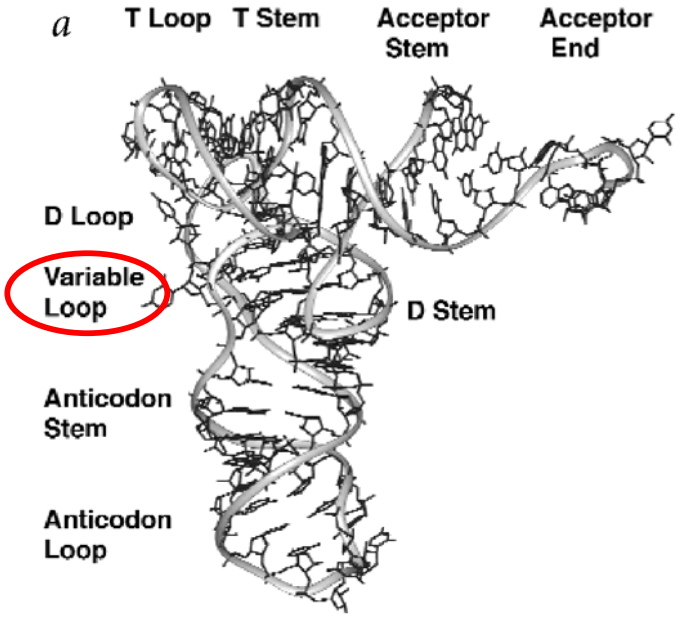


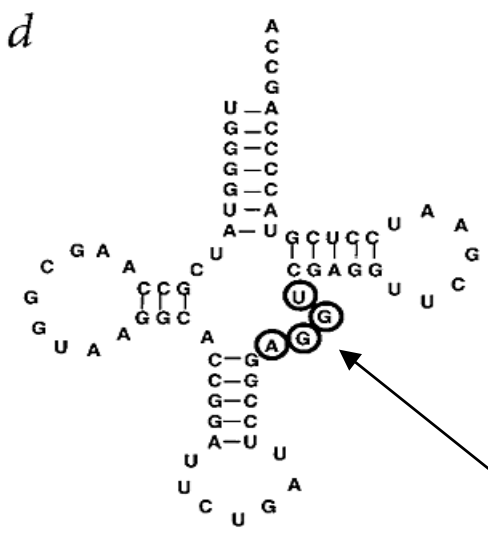
can measure K_d ~10^{-8} to 10^{-12} M

(see JBC (1991) 266 13661-)

Gel Mobility Shift Assay of a Protein-RNA Complex

Goal is to measure the K_d of the complex formed between a t-RNA^{gln} mutant and the glutaminyl-tRNA synthetase

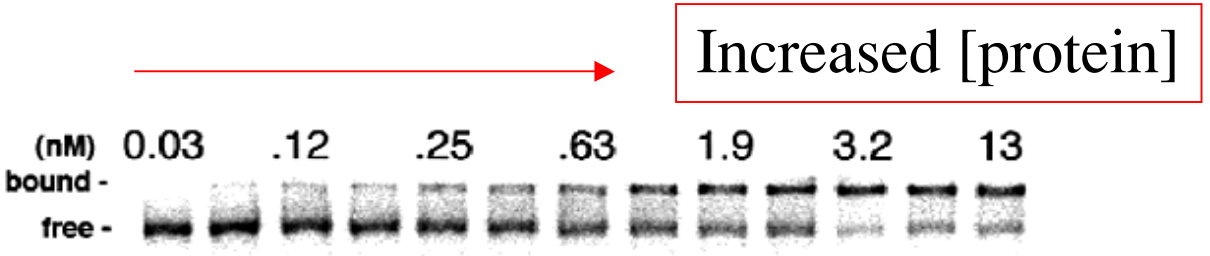




Residues altered in high affinity mutant

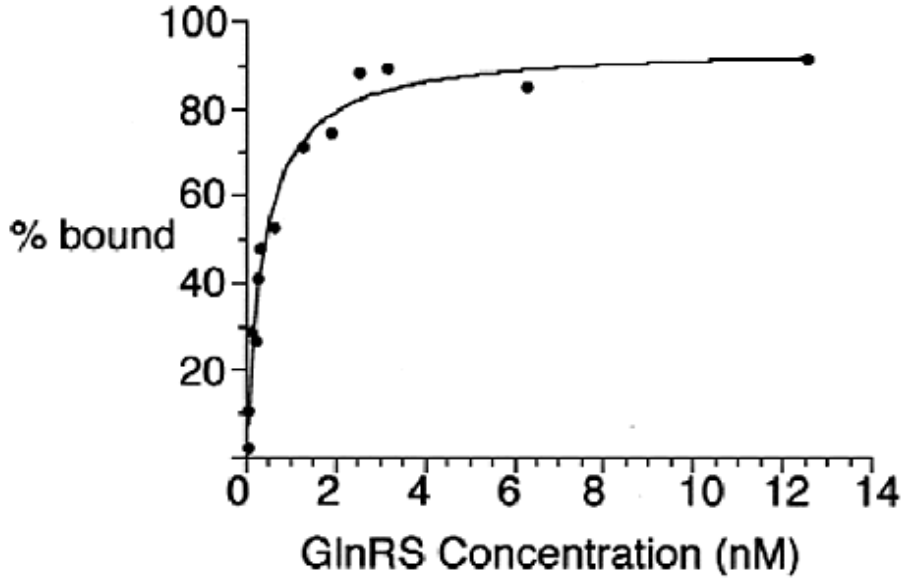
Gel shift assay

1. Use radioactive label on tRNA to detect on the gel
2. Incubate tRNA/protein mixture for 15 min
   - 200 pM tRNA
   - 6.6 nM to 66 pM Gln-tRNA Synthase (GlnRS)
3. Load onto 20% polyacrylamide gel and run for 5 h at 4° C
4. Autoradiography to determine bound and free tRNA
5. Fit to binding isotherm for 1:1 complex formation



Binding isotherm for tRNA/protein Complex

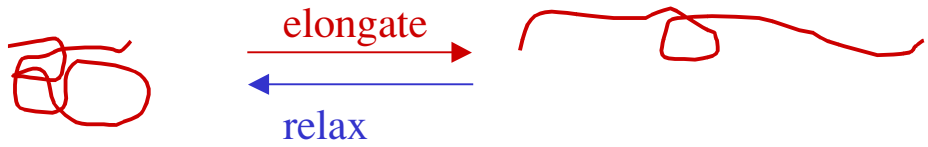
$K_d = 0.27$ nM



Pulsed Field Gel Electrophoresis

In normal electrophoresis - electrophoretic mobility is **independent** of molecular weight for large DNA (> 50 kbp)

because it becomes elongated in the electric field



Pulsed field gel electrophoresis is designed for separating very large DNA fragments (>100 kbp) and takes advantage of this relaxation/elongation property

(PNAS (1987) 84, 8011 - 8015;PNAS (1991) 88, 11071 - 11075)

In pulsed field gel electrophoresis, the DNA is allowed to "relax" after a brief pulse of electric field and then the direction of the electric field is changed

- this results in a strong length-dependence of electrophoretic behavior

Several variations of technique using different field direction changes and pulse times

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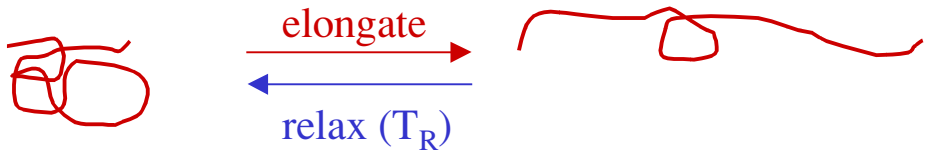
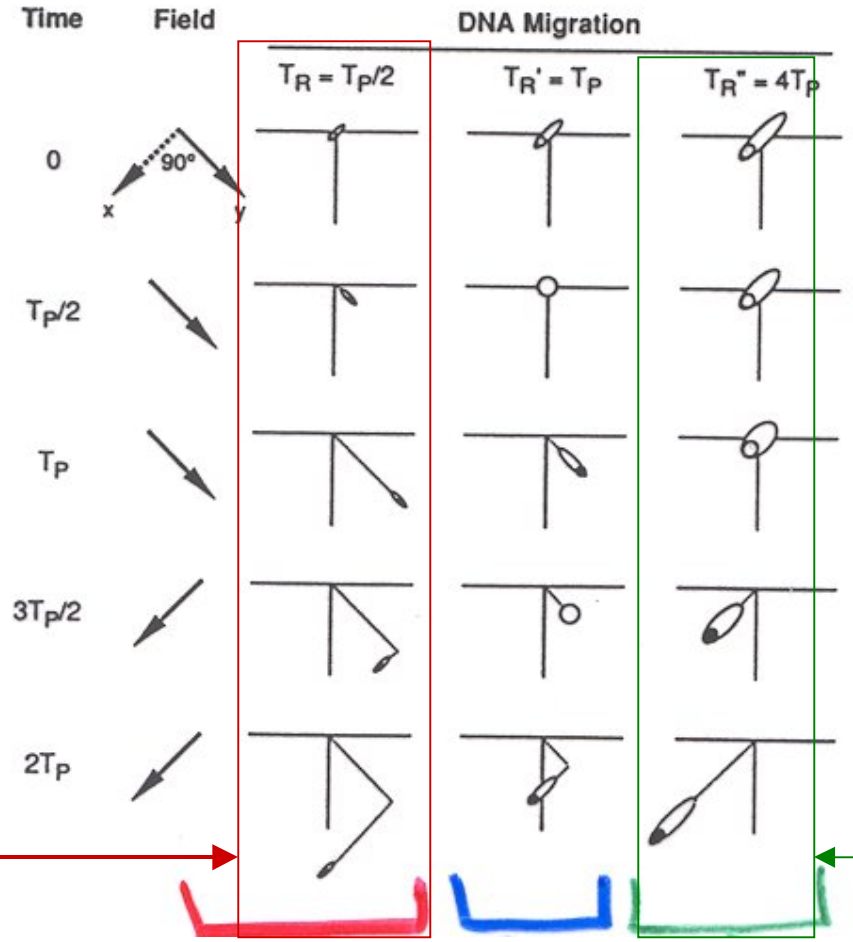
critical parameters

- DNA relaxation time (T_R) vs electrophoresis pulse time (T_P)



Alternate field direction by 90º

pulse time: T_p



small DNA
responds rapidly
to changing field

Very large DNA
After continuous field along $\hat{x}$, no adjustment along $\hat{y}$, so it resumes motion in $\hat{x}$ direction
-faster mobility

larger DNA
$T_R \approx T_p$
slow migration: field changes just as DNA changes shape
(minimum mobility)

PNAS 88 11071