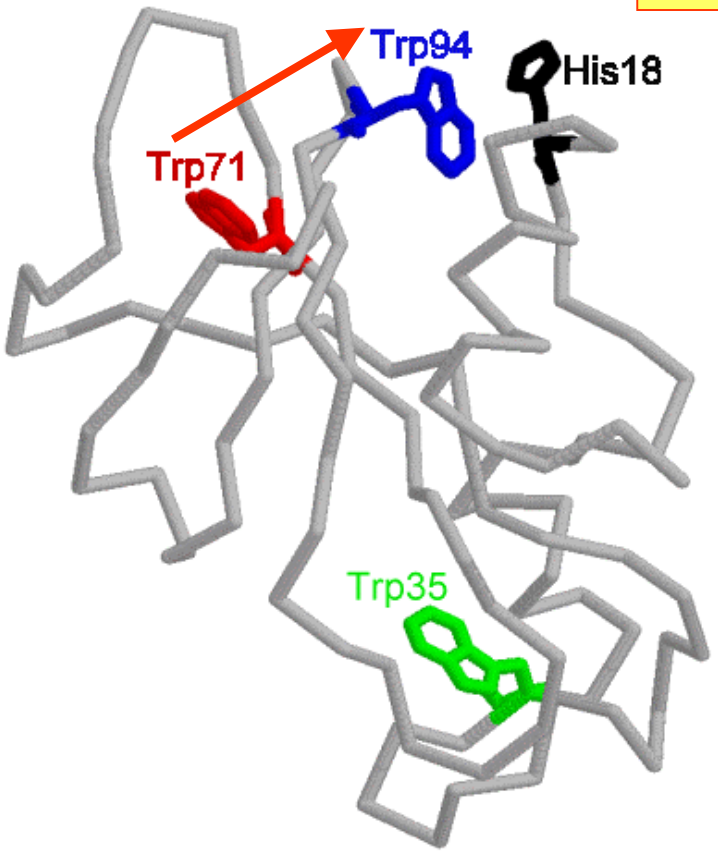
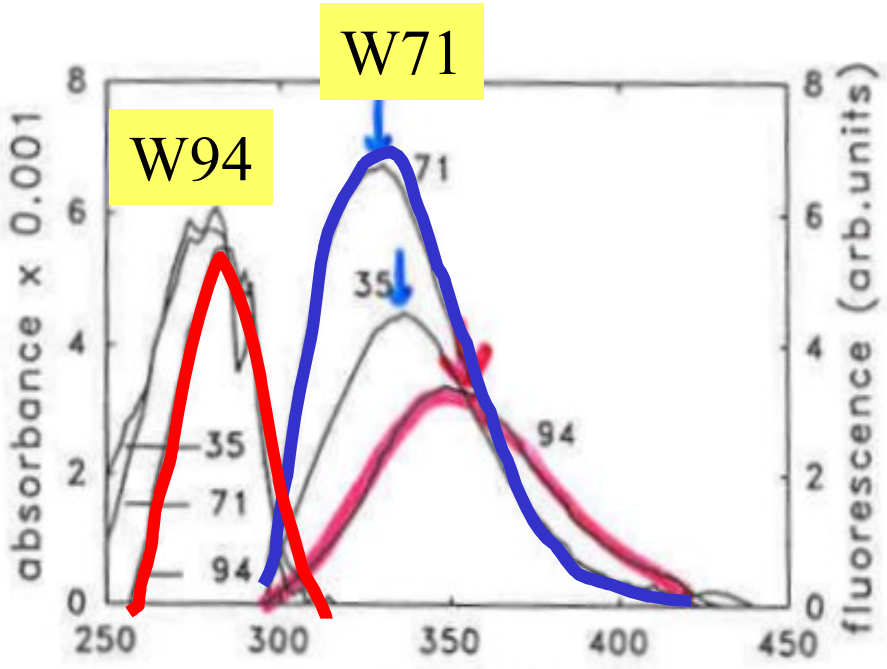
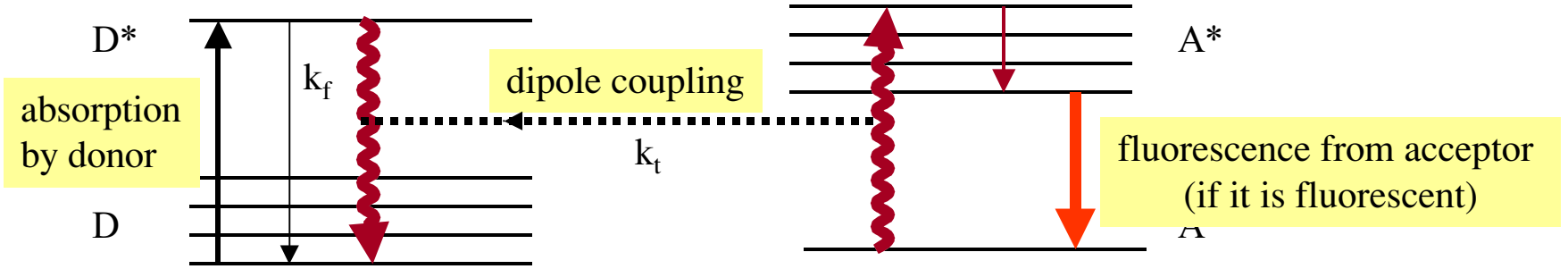
FRET: Fluorescence Resonance Energy Transfer

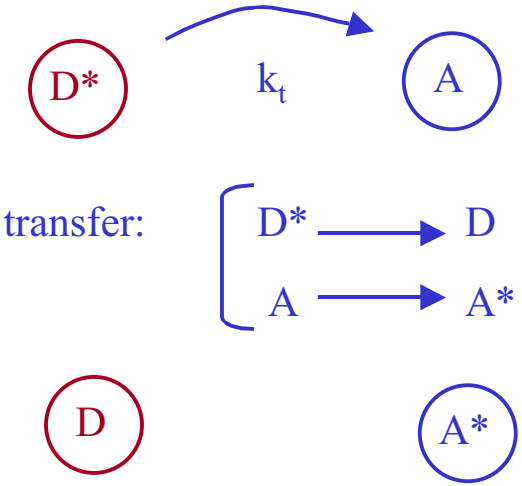
example: Barnase
Donor is W71 Acceptor is W94

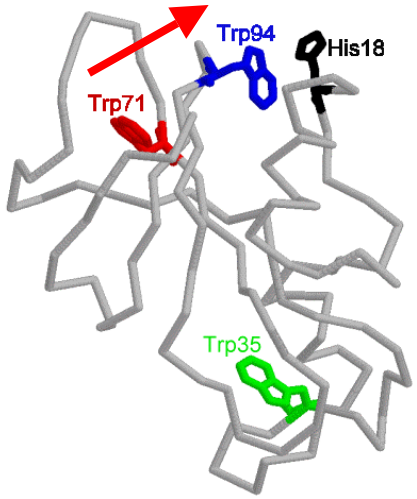




"trap" of transfered excitation by internal conversion





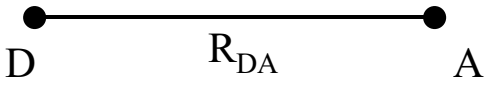


Can measure:

(1) Fluorescence quenching of Donor due to Acceptor => k_t

(2) If Acceptor also fluoresces, measure induced (enhanced) fluorescence of the Acceptor => k_t

From k_t , get R_{DA} , distance



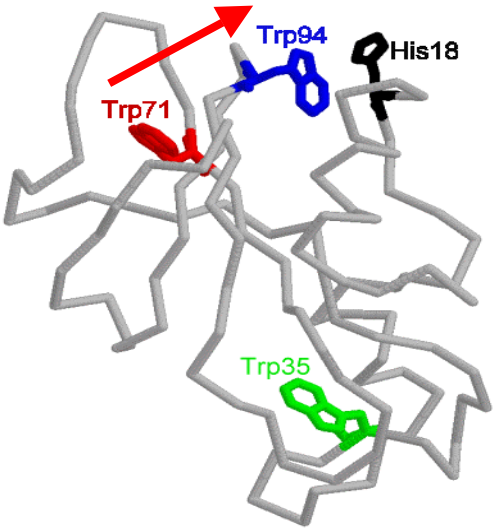
Transfer rate depends on:

(1) Distance

(2) Orientation of $\underset{\sim}{\mu}_D$, $\underset{\sim}{\mu}_A$

(3) Probability of donor de-excitation and acceptor excitation
- transition probabilities ($\underset{\sim}{\mu}_D^2$, $\underset{\sim}{\mu}_A^2$)

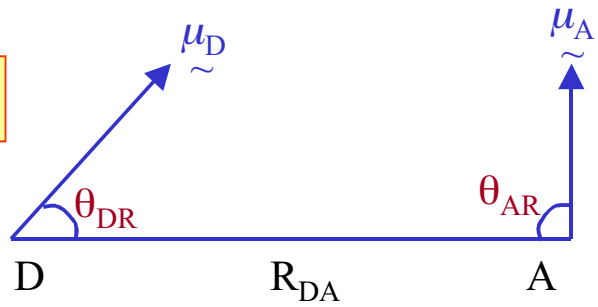
(4) Energy matching



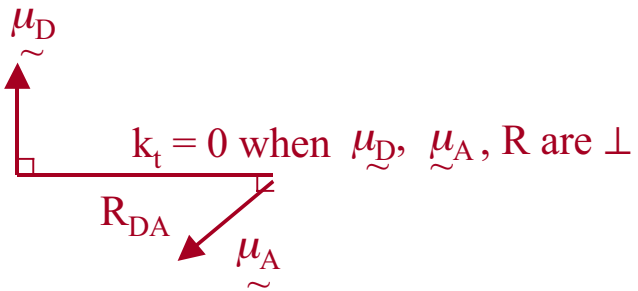
Interaction energy given by dipole-dipole term

interaction energy of two dipoles →

$$U = \frac{1}{n^2 R^3}\left[\underset{\sim}{\mu}_D \cdot \underset{\sim}{\mu}_A - 3(\underset{\sim}{\mu}_D \cdot \hat{R})(\underset{\sim}{\mu}_A \cdot \hat{R})\right]$$



$$k_t \propto U^2 \propto 1 / R^6$$



Rate constant for energy transfer:

Donor transition probability

Acceptor transition probability

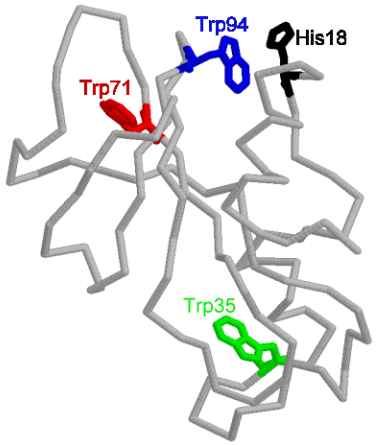
Orientation factor

$$k_t \propto \frac{|\tilde{\mu}_D|^2 \, |\tilde{\mu}_A|^2 \, \kappa^2}{n^4 R^6} J$$

Energy overlap

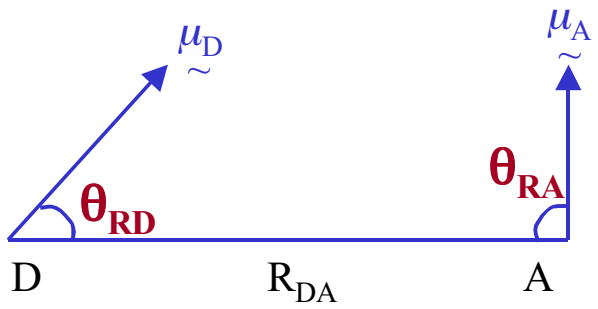
Index of refraction

Distance



Orientation factor

$$\kappa = \cos\theta_{DA} - 3\cos\theta_{RD} \cdot \cos\theta_{RA}$$



For random orientation: $\kappa^2 = 2/3$: this is the value that is used in most estimations

Donor transition probability

Acceptor transition probability

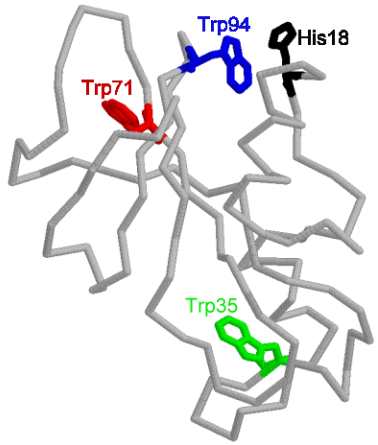
Orientation factor

$$k_t \propto \frac{|\tilde{\mu}_D|^2 \; |\tilde{\mu}_A|^2 \; \kappa^2}{n^4 \, R^6} \; J$$

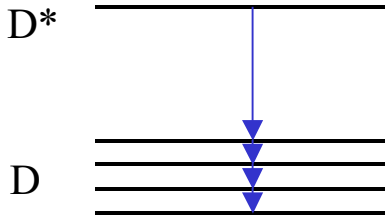
Index of refraction

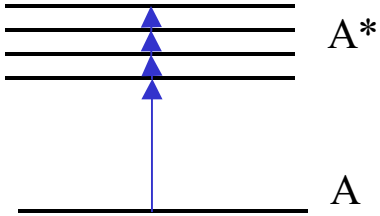
Distance

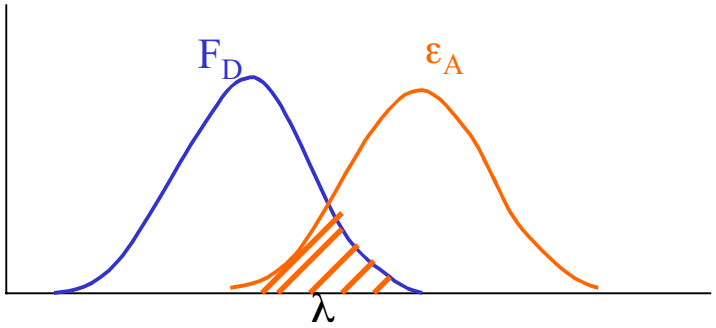
Energy overlap



Energy overlap Factor: J

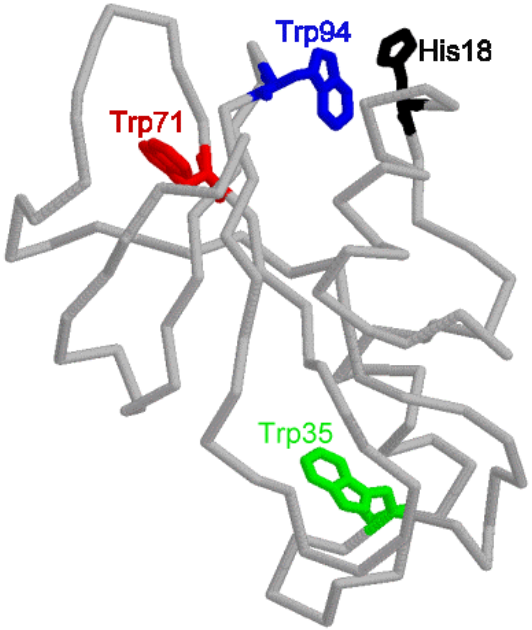


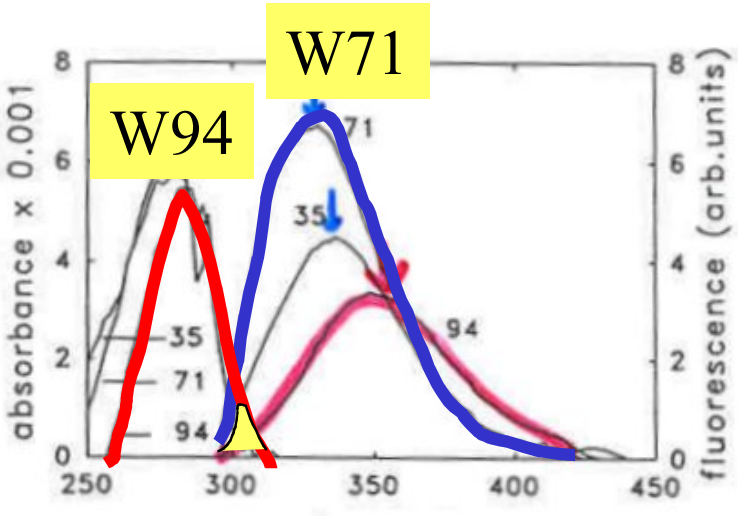




$$\frac{\int F_D(\bar{\nu}) \cdot \varepsilon_A(\bar{\nu}) \frac{d\bar{\nu}}{\bar{\nu}^4}}{\int F_D(\bar{\nu}) d\bar{\nu} \cdot \underbrace{\int \varepsilon_A(\bar{\nu}) d\bar{\nu}}_{|\mu_A|^2}} = J$$

Barnase: donor is W71 acceptor is W94





Transition probability of donor absorption $(\mu_D)^2$ is related to the rate constant for fluorescence from the donor (k_f)

$$k_t \propto \frac{|\tilde{\mu}_D|^2 \; |\tilde{\mu}_A|^2 \, \kappa^2}{n^4 R^6} J$$

$$|\mu_D|^2 \propto k_f = (Q_f^D / \tau_D)$$

$$\frac{\text{Quantum yield}}{\text{lifetime}}$$

$$k_t = \text{constant} \times \frac{\kappa^2 Q_f^D J}{n^4 \tau_d R^6}$$

Defining R_0 for a donor/acceptor pair

$$k_t = \text{constant} \times \frac{\kappa^2 Q_f^D J}{n^4 \tau_d R^6}$$

orientation factor: often assigned value of 2/3

quantum yield of donor in the absence of acceptor

Define: $R_0 = \left[\frac{\text{constant} \times \kappa^2 Q_f^D J}{n^4} \right]^{1/6}$

spectral overlap factor

index of refraction between donor/acceptor: usually guessed for hydrophobic interior of protein

$$k_t = \frac{1}{\tau_d} \left[\frac{R_0}{R^6} \right]^6$$

R_0 depends on the properties of the Donor and Acceptor and their relative orientation (usually $\kappa^2 = 2/3$)

R_0 has units of distance (Å) and can be calculated in advance based on the spectral properties of the donor and acceptor pair if the orientation factor is guessed (2/3)

Experimental: Measure the efficiency of energy transfer
What fraction of the excited donors transfer their energy to the acceptor?

Quantum yield of donor - no Acceptor

$$Q_{f,D} = \frac{k_f}{k_f + k_I}$$

Quantum yield of donor - plus Acceptor

$$Q_{f,D}^{+A} = \frac{k_f}{k_f + k_I + \mathbf{k_t}}$$

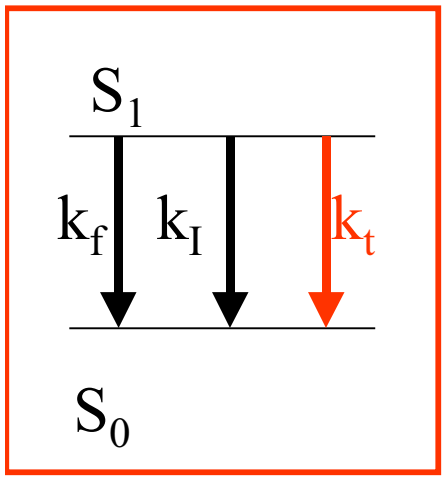
added route for returning to the ground state

$$E = \frac{Q_{f,D} - Q_{f,D}^{+A}}{Q_{f,D}} \equiv \frac{k_t}{k_f + k_I + k_t}$$

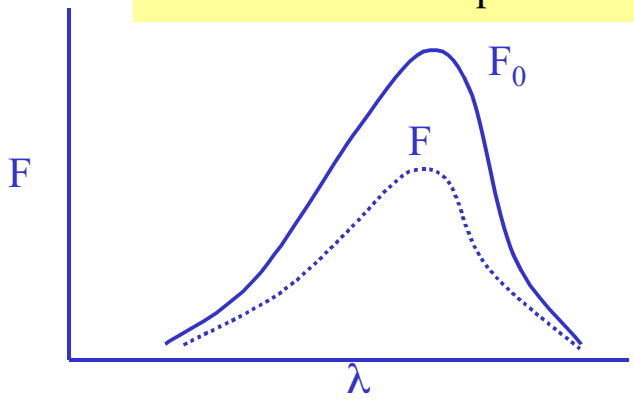
$$E = \left[1 - \frac{Q_{f,D}^{+A}}{Q_{f,D}}\right]$$

$$E = \left[1 - \frac{F}{F_0}\right]$$

$$E = \left[1 - \frac{\tau_D^{+A}}{\tau_D}\right]$$



Measuring quenching of the donor fluorescence due to energy transfer to the acceptor



Once the efficiency of energy transfer is determined, one can determine the distance between donor and acceptor if one knows R_0

E = Efficiency of energy transfer

$$E = \frac{k_t}{k_f + k_I + k_t} = \frac{\frac{1}{\tau_D}\left(\frac{R_0}{R}\right)^6}{\frac{1}{\tau_D} + \frac{1}{\tau_D}\left(\frac{R_0}{R}\right)^6}$$

$$E = \frac{1}{1 + (R/R_0)^6}$$

or

$$\boxed{R = \frac{(1-E)^6}{E} \cdot R_0}$$

1) Calculate R_0
2) Measure E
determine R

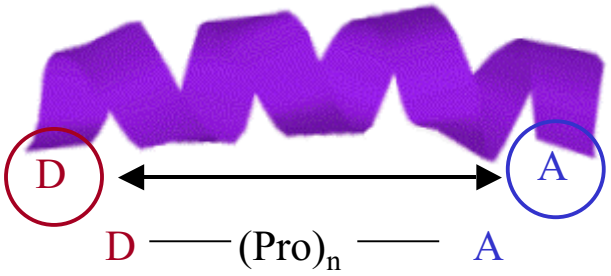
Note that when $R = R_0$, E = 50%

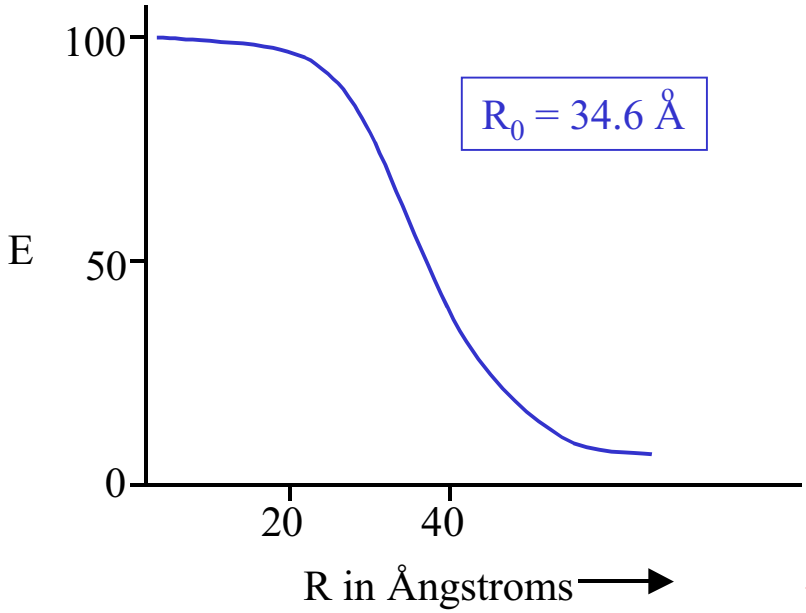
R_0 is the distance at which 50% of the excited state donor molecules transfer their energy to the acceptor.

An early experimental test from 1976:

Haugland + Stryer

Oligo prolines



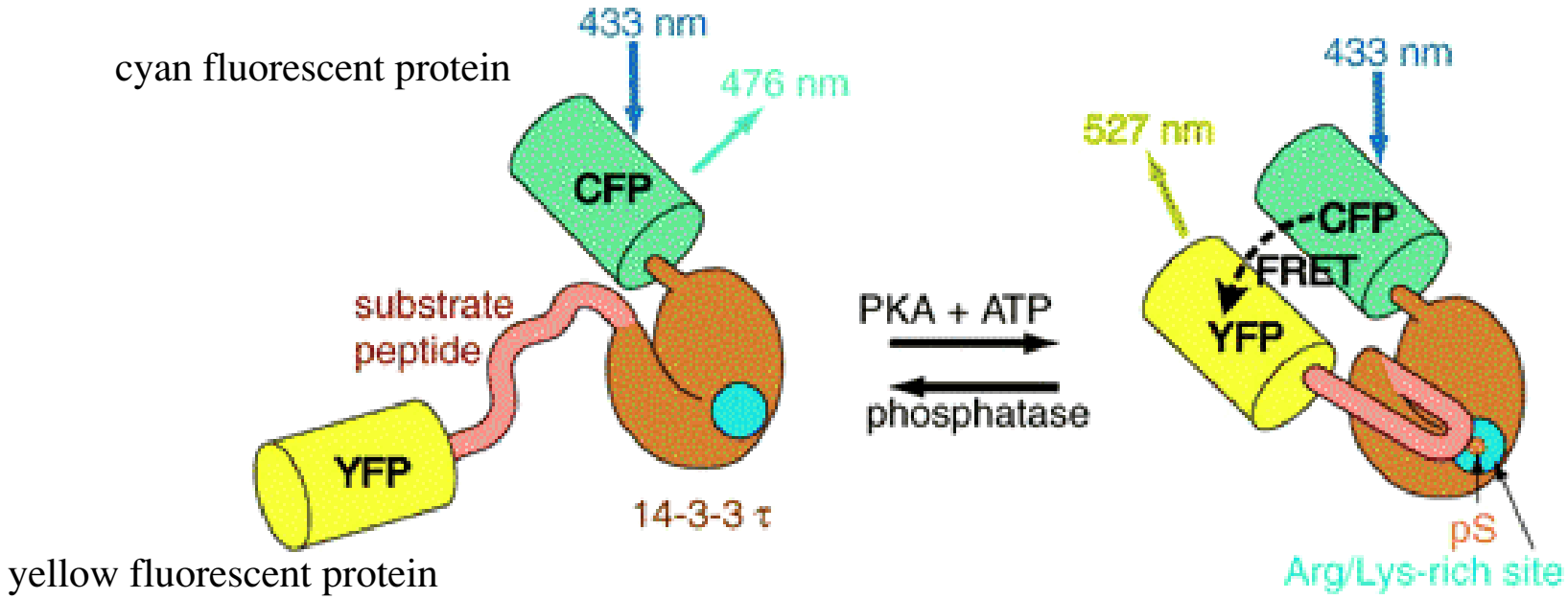


Biochemistry (1976) **15**, 2097 -

Method is useful to distinguish close from far

R_0 values range from 15 to 50 Å or more

Using Two GFPs as an Assay for Protein Kinase A Activity

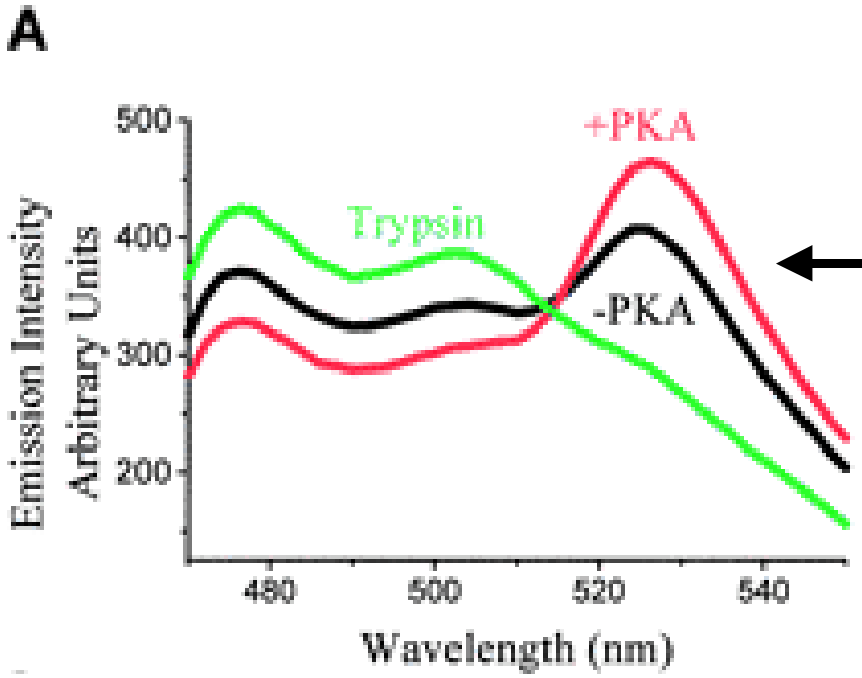


Phosphorylation results in binding of the substrate peptide to an phosphorylation recognition domain engineered into the same protein

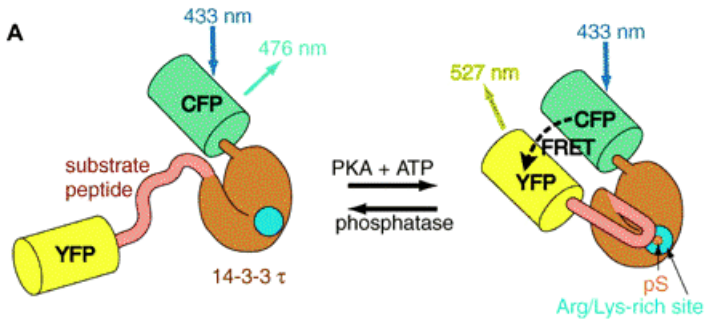
The conformational change brings the YFP and CFP close to each other and gives a FRET signal

PNAS (2001) 98, 14997-15002

FRET Assay of Phosphokinase A Activity



increased fluorescence at 527 nm from YFP due to energy transfer



PNAS (2001) 98, 14997-15002

FRET Showing Conformational Change in Motor Protein Myosin Associated with ATP Binding and Hydrolysis

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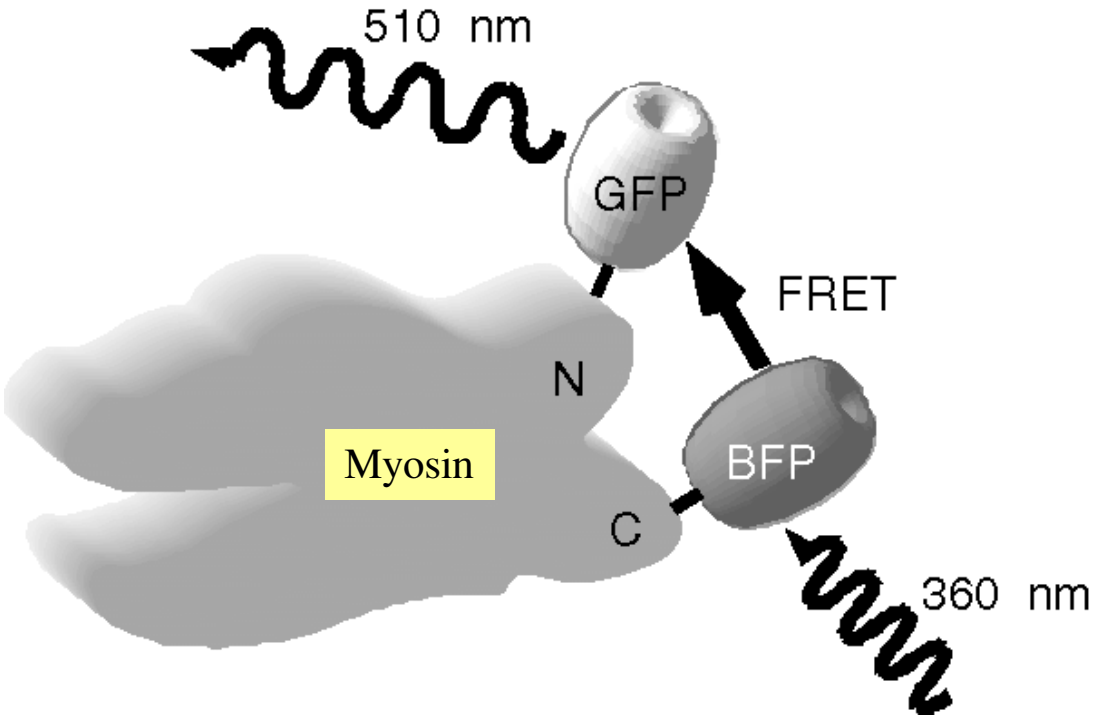
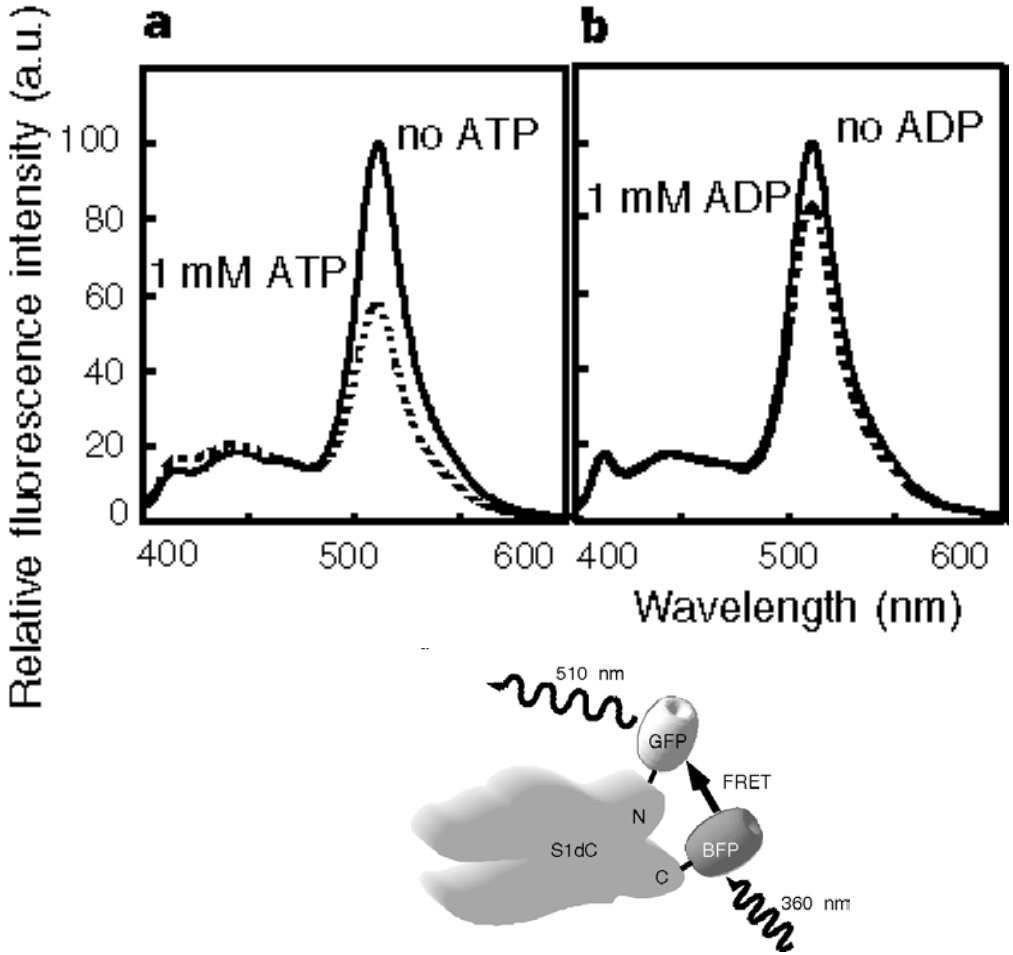
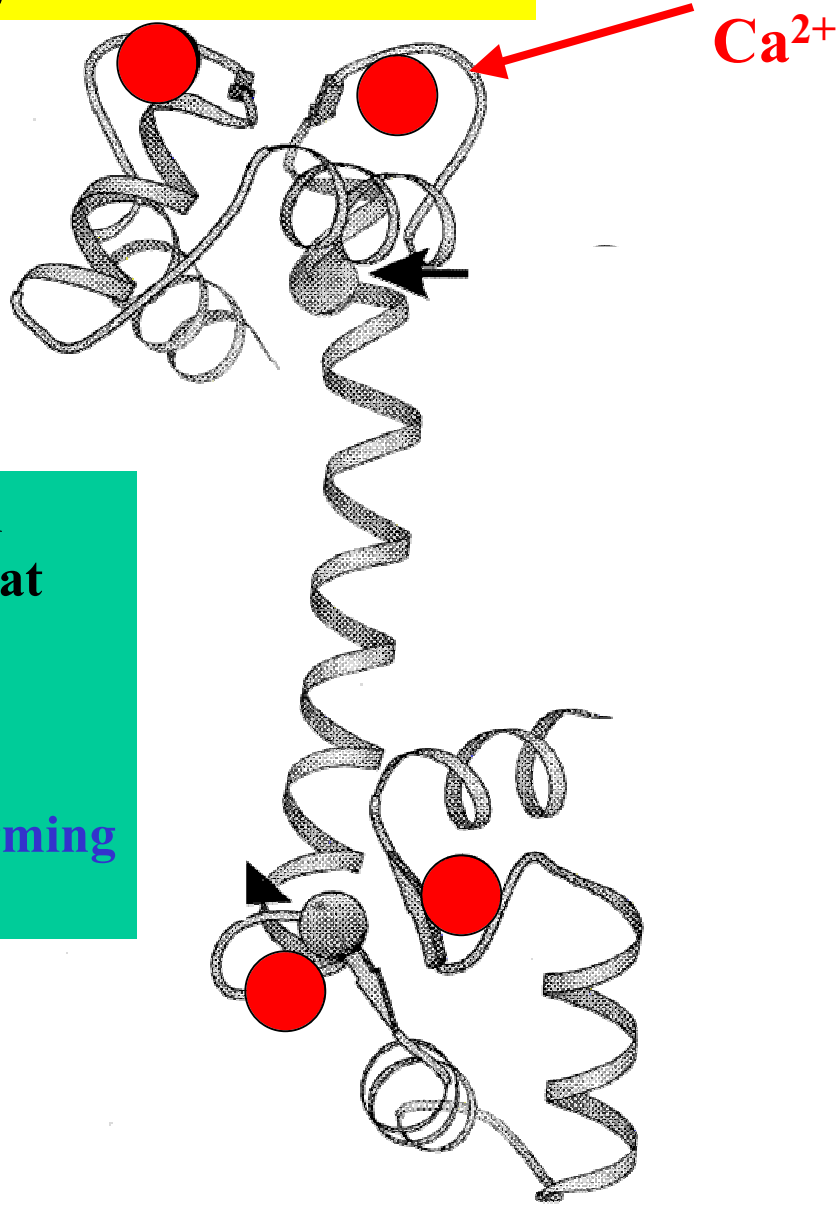


N- and C- terminal Domains Move Apart Upon ATP binding/hydrolysis



Nature **396**, 380 - 383 (1998)

Calmodulin: two globular domains and a long central helix



Ca^{2+} binding causes conformational changes in each globular domain that expose residues that bind to a wide variety of target proteins

The central helix collapses upon forming a complex with the target protein

Biochem (1999) 38, 12266-12279

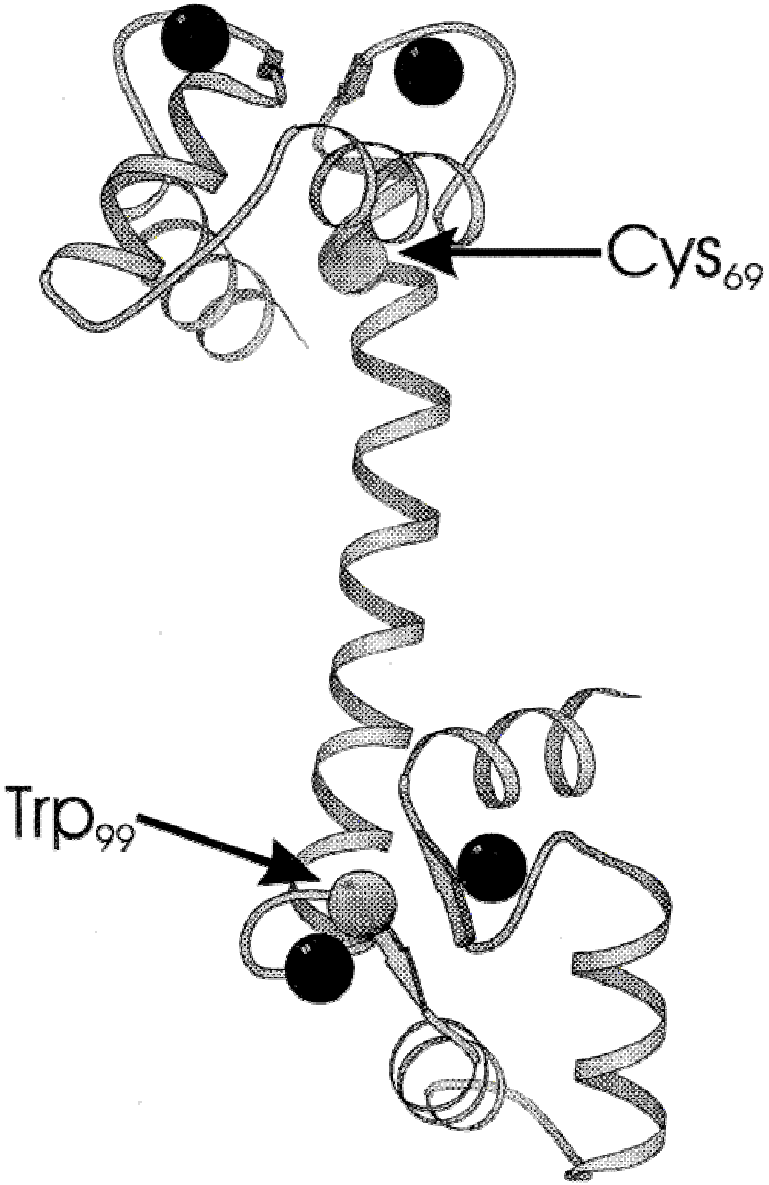
Biochem (2001) 40, 9605-9617

Calmodulin

Calmodulin complexed to
CaMKinase II

Calmodulin

Question: Does Ca^{2+} binding itself cause a change in the central helix that alters the conformational relationship of the two globular domains?



Use FRET to address this.

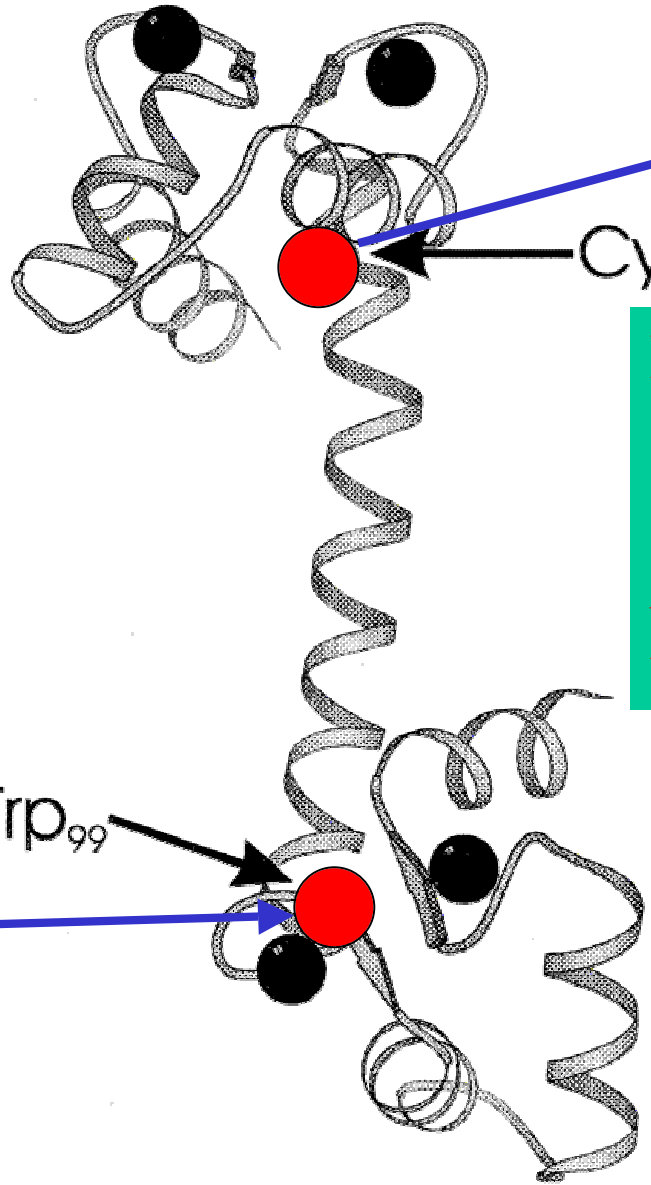
Construct a double mutant:

Tyr99→Trp

Leu69→Cys

Biochem (1999) 38, 12266-12279
Biochem (2001) 40, 9605-9617

Calmodulin



hν 500 nm

FRET Acceptor

use a covalent probe AEDANS that binds to Cys -SH

FRET Donor

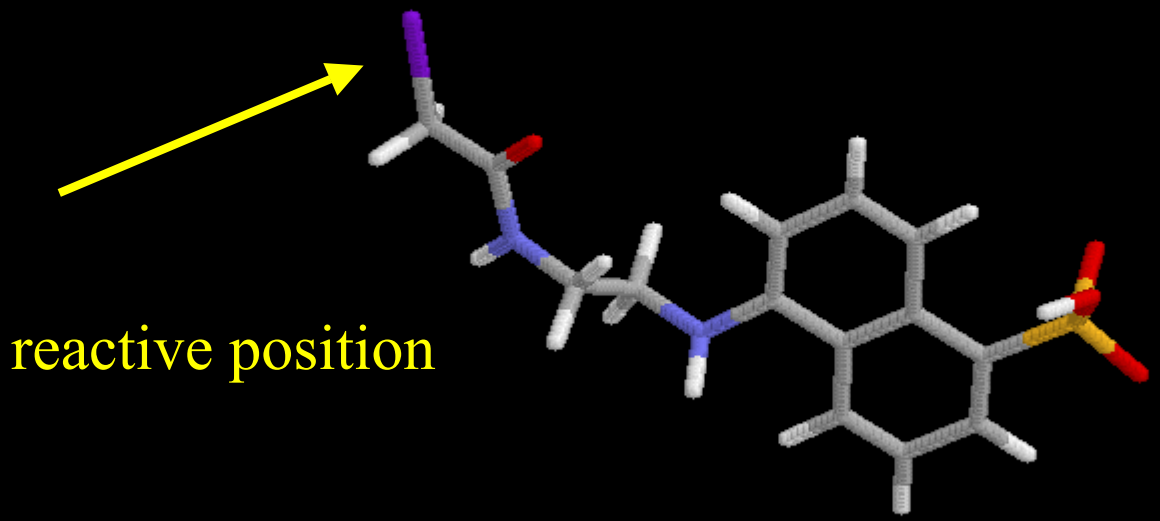
only Trp in the protein

hν 297 nm

Biochem (1999) 38, 12266-12279
Biochem (2001) 40, 9605-9617

IAEDANS

FLUORESCENT LABEL FOR
COVALENT ATTACHEMENT TO CYSTEINES



MDL

5-((((2-Iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid

Calculating R_0 between Tryptophan-99 and AEDANS

overlap integral
calculated from spectral overlap
numerical integration

$9 \times 10^{-15}\ M^{-1}cm^3$

$$R_0 = (9.79 \times 10^{-5})\ (n^{-4}\ \kappa^2 Q_d J)^{1/6}$$

1.40

estimated index
of refraction
like non-polar solvent

2/3

orientation factor:
assumes random
orientations of donor and
acceptor--rapid isotropic
motion

measured: 0.098 ($+Ca^{2+}$)

quantum yield of donor:
compare area under
the emission spectrum to
a standard: L-tryptophan,
which has a known
quantum yield of 0.14)

$R_0 = 22.6$ Å

FRET Results: measure fluorescence from Trp99 in the presence and absence of AEDANS

fluorescence of Trp99
in the absence of AEDANS

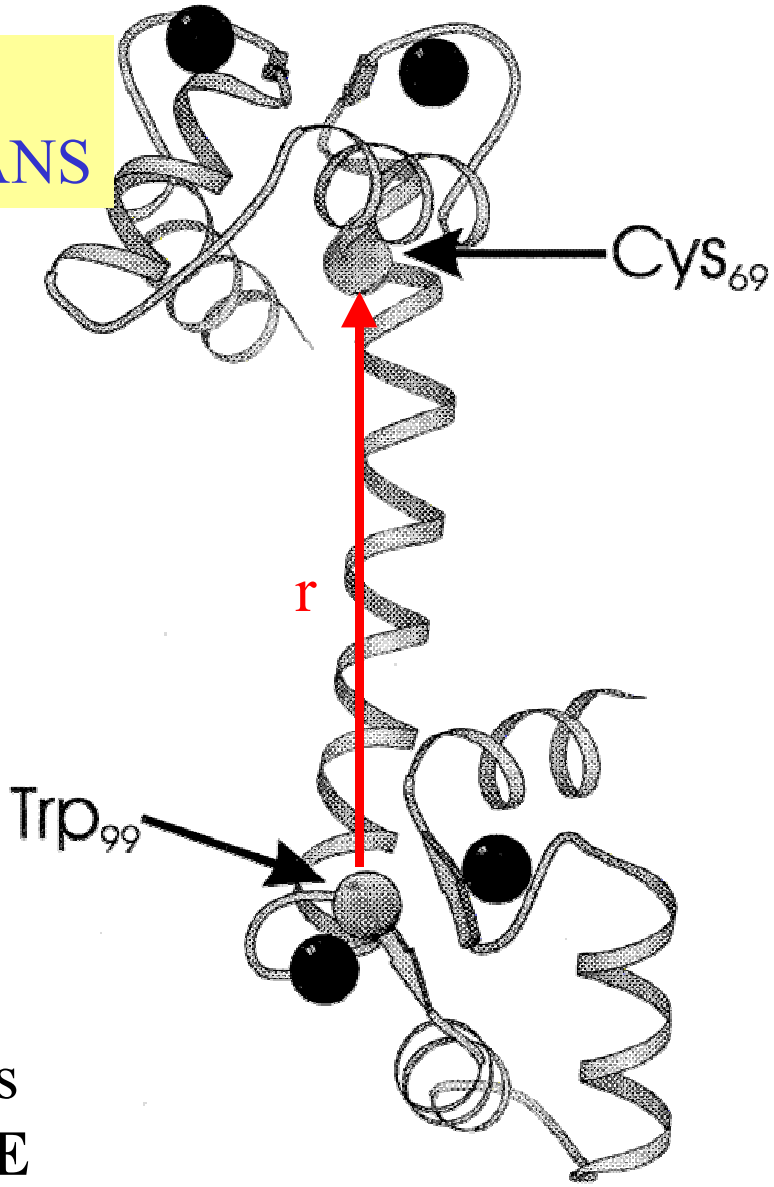
$E = 1 - F_{da}/F_d$ experimental relationship

fluorescence from Trp99
in the presence of AEDANS

$E = R_0^6/(R_0^6 + R^6)$

theoretical reltionship

want to get this
by measuring **E**

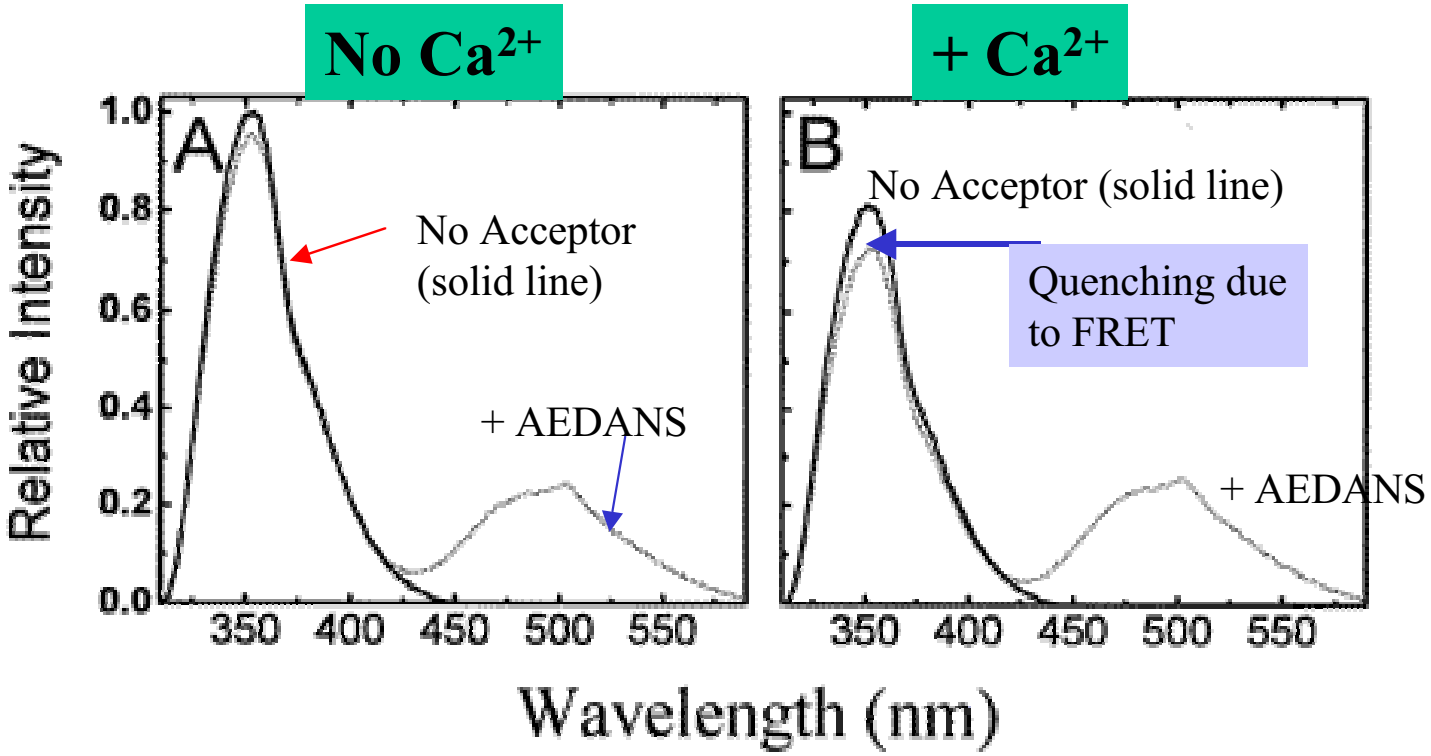


Measuring FRET from Trp99 to AEDANS by steady state fluorescence spectroscopy

What is the distance between the two domains?

Does calcium binding change the distance?

excite at 297 nm: only the tryptophan is excited at this wavelength.
The AEDANS fluorescence is entirely due to FRET

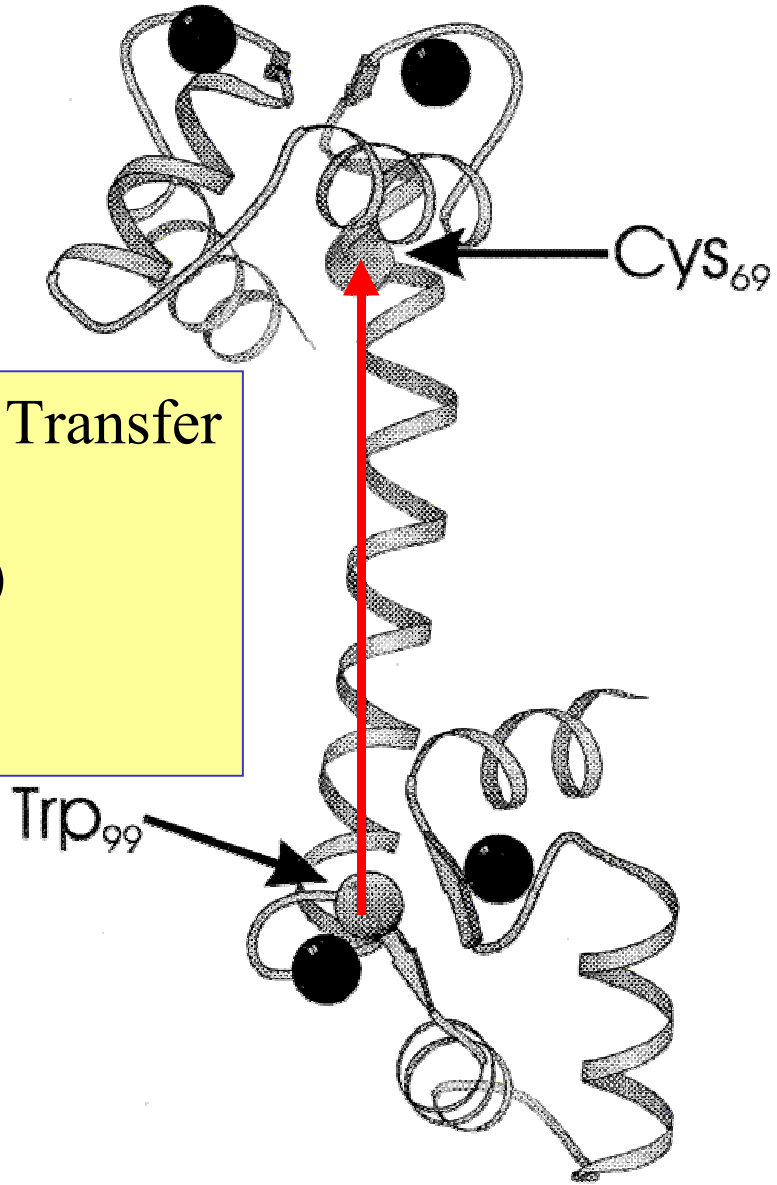


FRET Results: increase in energy transfer upon calcium binding

Efficiency of Energy Transfer

15% (+ Ca^{2+})

7% (no Ca^{2+})



FRET Results

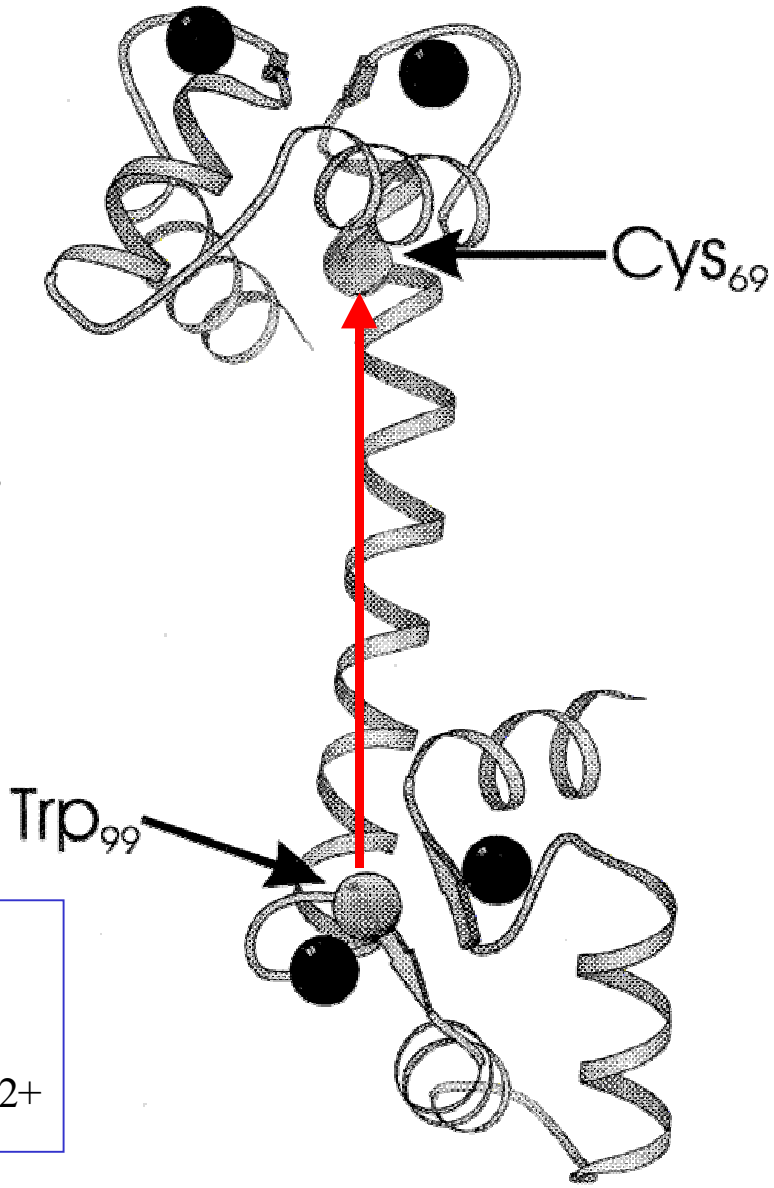
$$E = R_0^6/(R_0^6 + R^6)$$

want to get this

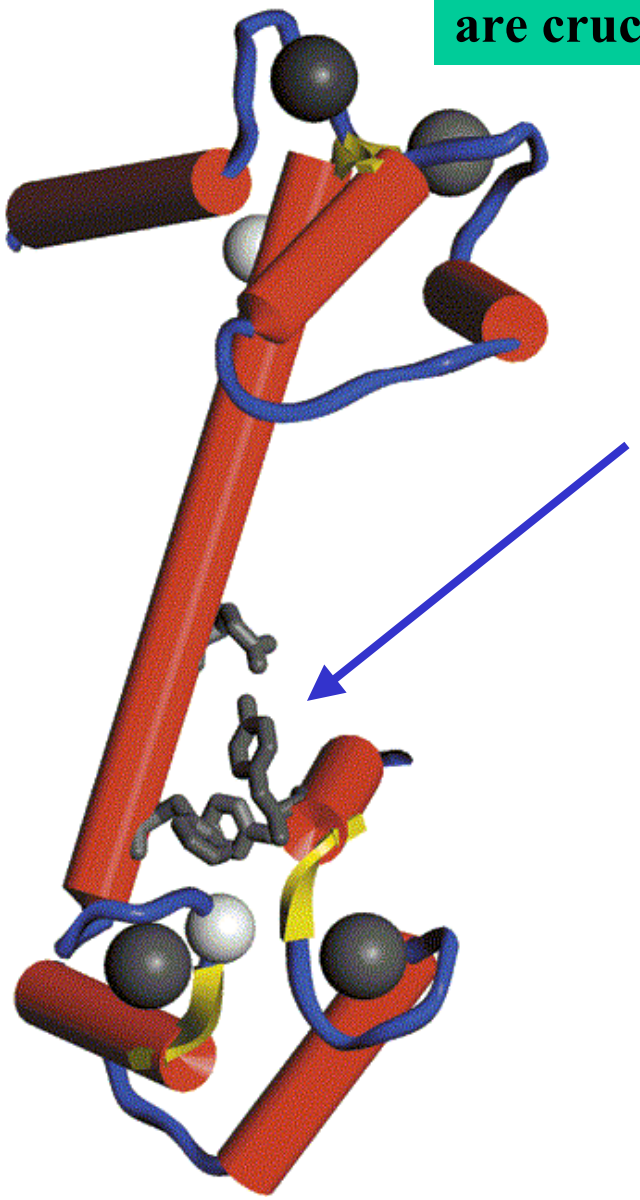
r = 31 Å (+ Ca^{2+})

r = 38 Å (no Ca^{2+})

conclude: there are two distinct conformations of the central helix in the presence and absence of Ca^{2+}



Interactions with tyrosine 138
are crucial to maintain the extended structure of calmodulin

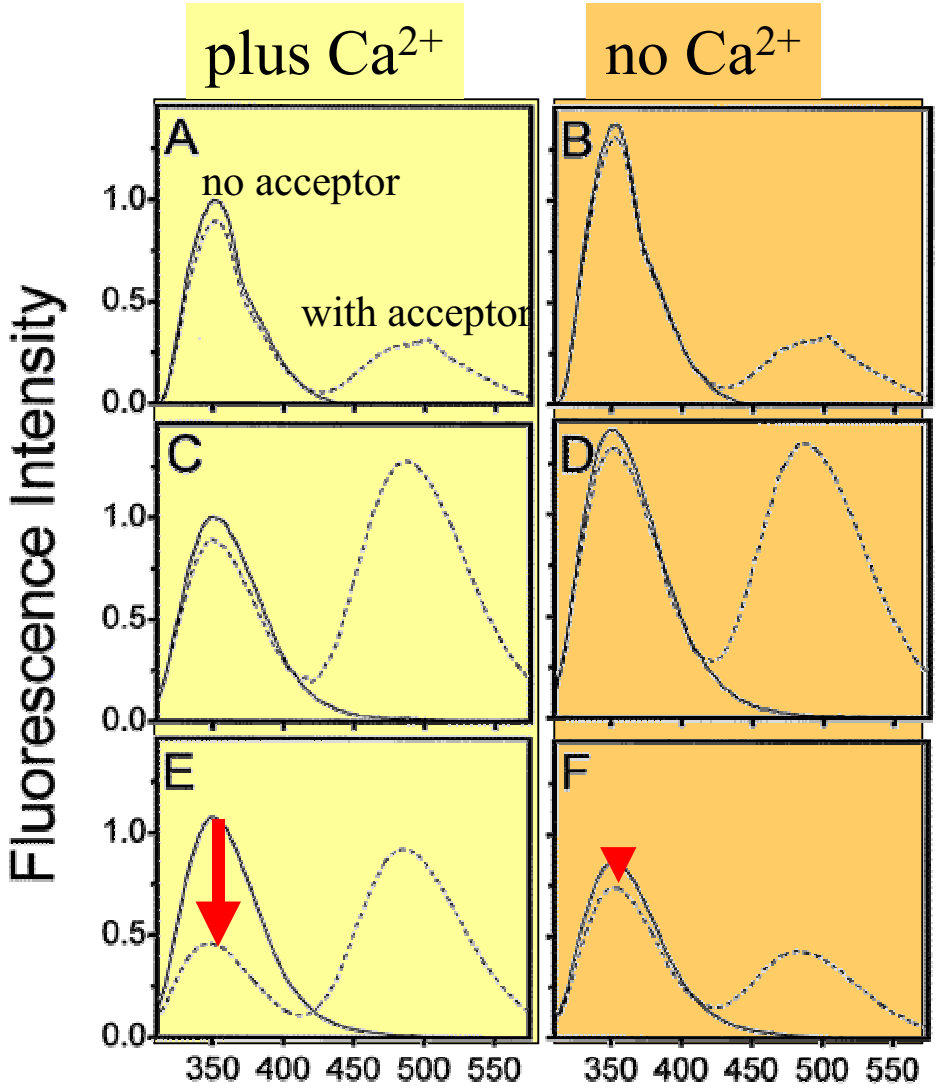


tyrosine 138 mutants disrupt the structural coupling between the two globular domains

Tyr138 interacts with residues in the central helix to stabilize the structure.

Trp99→AEDANS Energy Transfer

Structural difference due to Tyr138Gln is again evident



Tyr138

Tyr138Phe

Tyr138Gln

Note large difference due to Ca^{2+} binding

Trp99 → AEDANS Energy Transfer

Tyr138Phe eliminates the Ca^{2+} effect on the conformation of the central helix
The distance between domains does not get shorter upon calcium binding

Tyr138Gln causes the structure of the central helix
to partially collapse, resulting in the two globular domains
getting closer together

	Energy Transfer Efficiency	Distance
Tyr138/+ Ca^{2+}	15%	30Å
Tyr138/- Ca^{2+}	7%	37Å
Tyr138Phe/+ Ca^{2+}	9%	35Å
Tyr138Phe/- Ca^{2+}	7%	37Å
Tyr138Gln/+ Ca^{2+}	**51%**	**23Å**
Tyr138Gln/- Ca^{2+}	16%	29Å

Interaction of Ca-calmodulin with its target proteins
also results in collapse of the central helix structure
likely by altering key interactions, such as that of Tyr138

Sample problem 1: fluorescence and absorption

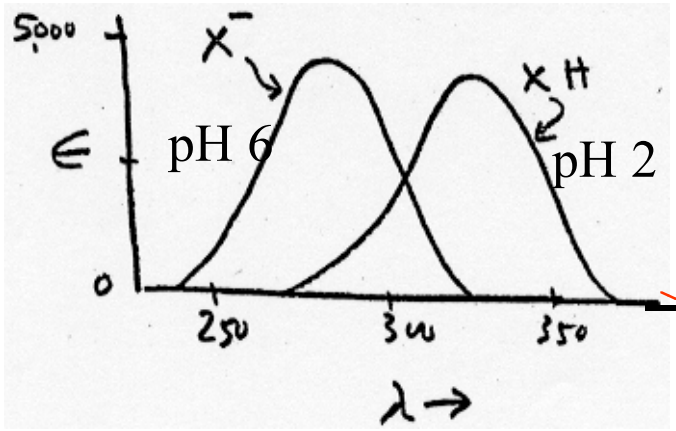
A fluorescent molecule has a single dissociating proton:

$$XH \quad X^- + H^+$$

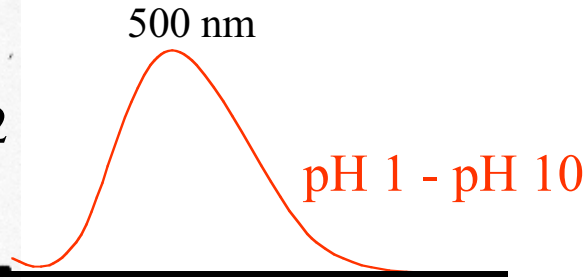
XH and X^- have distinct absorption spectra, with a $pK_a = 3.5$.

Part 1: Over the pH range 1 - 10 the emission spectrum does not change at all. **Explain.**

absorption spectra

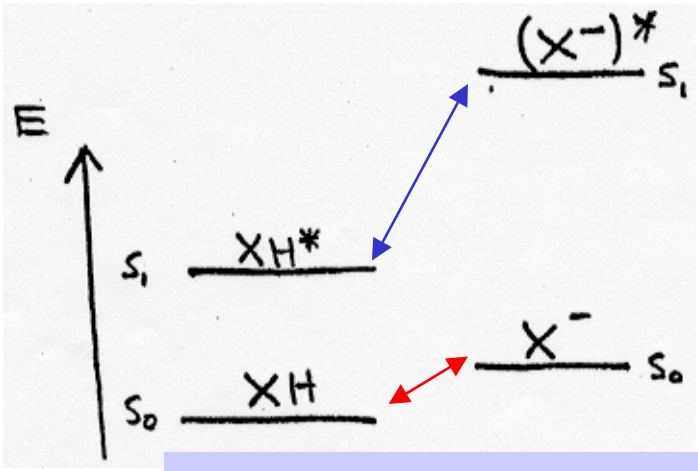


fluorescence spectrum of both XH and X^-



1) **The equilibrium state of protonation of the ground state evidently does not influence the emission spectra.**

2) The excited state must reach a rapid equilibrium with the protons within the excited state lifetime (about 10^{-8} sec).

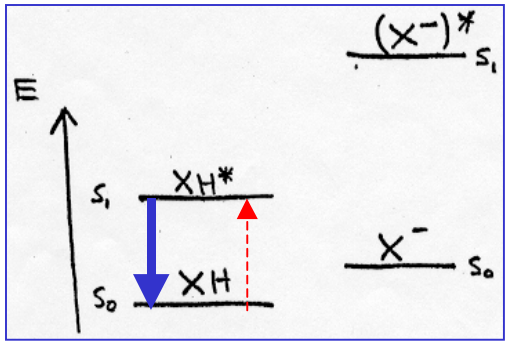


ground state pK = 3.5

The pK of the excited state must be outside the range examined (pH 1 to 10) Most likely, the pK > 10.

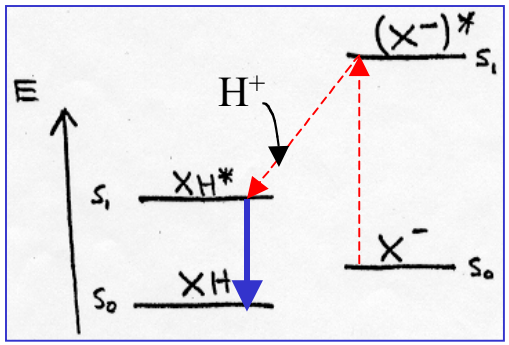
The proton equilibrates before emission of the photon.

Ground state is deprotonated at pH 6 but there is rapid proton equilibration during the excited state lifetime so the excited state can protonate before emission of a photon



pH 2

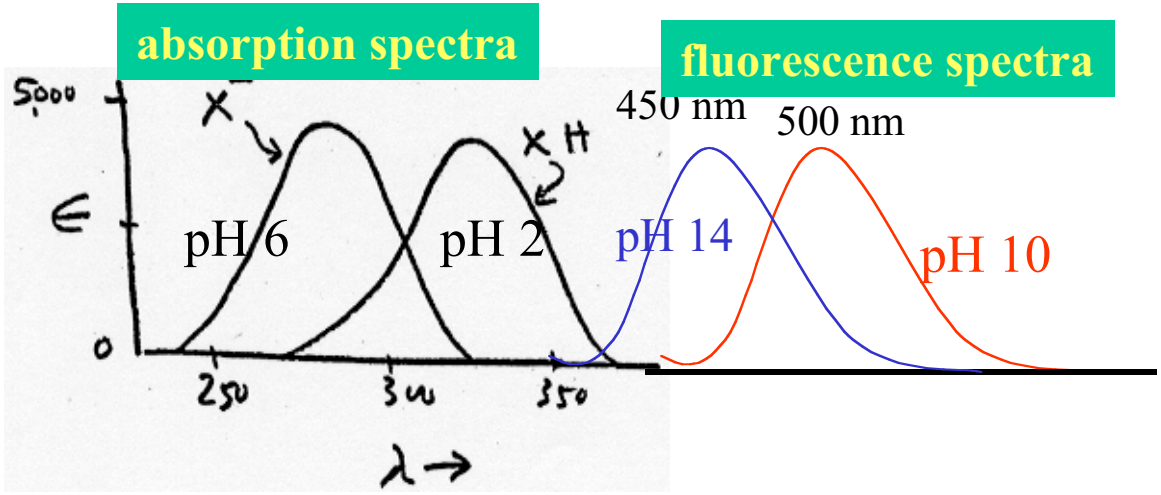
450 nm emission



pH 6

450 nm emission

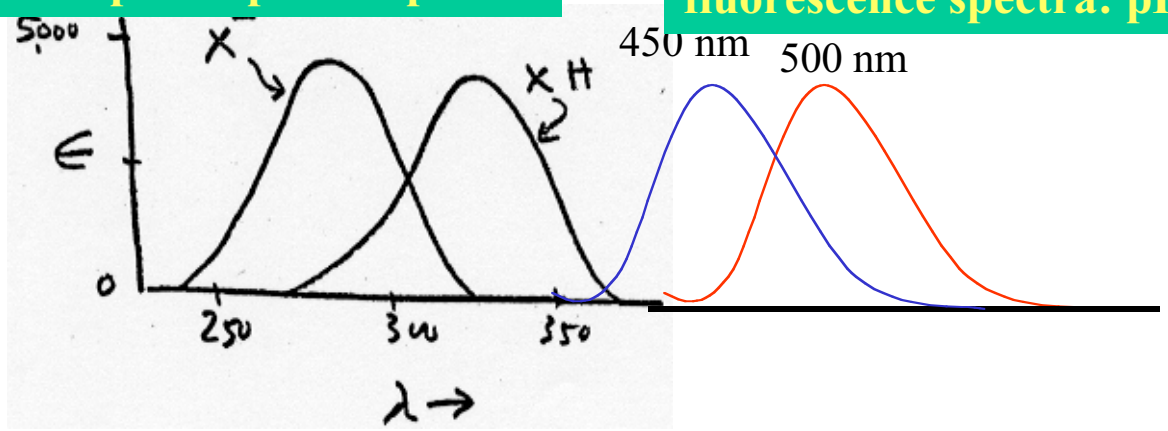
Part 2: As the pH is changed from pH 10 to 14, the emission peak shifts to the red from 500 nm to 450 nm. Explain what is occurring.

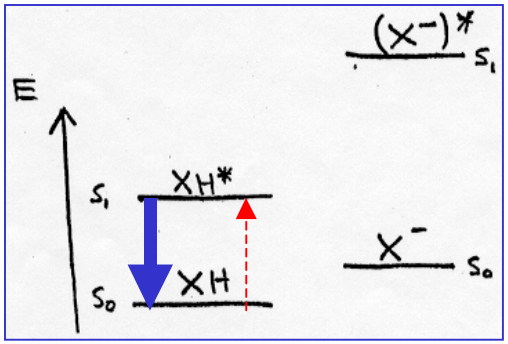


The blue shift denotes a change in the emitting species from XH* to (X^-)* as the pH is increased above pH 10. The excited state pKa is apparently about pH 12.

absorption spectra: pK 3.5

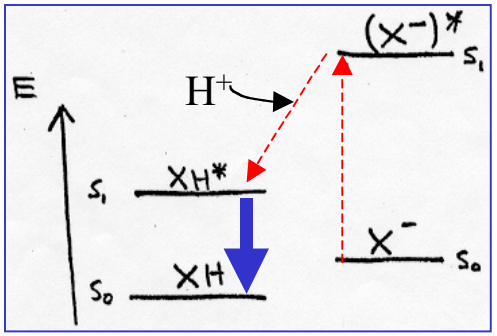
fluorescence spectra: pK 12





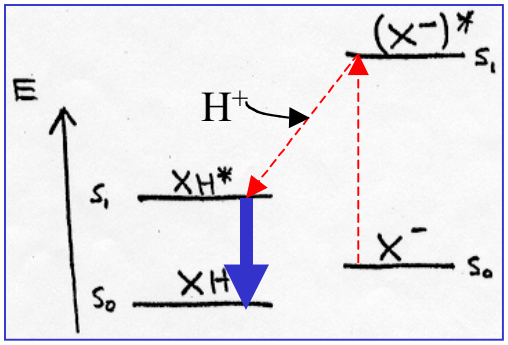
pH 2

500 nm



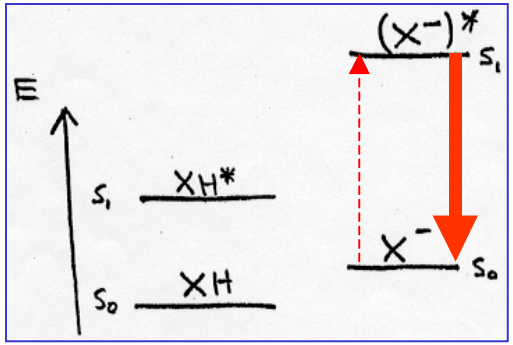
pH 10

500 nm



pH 6

500 nm

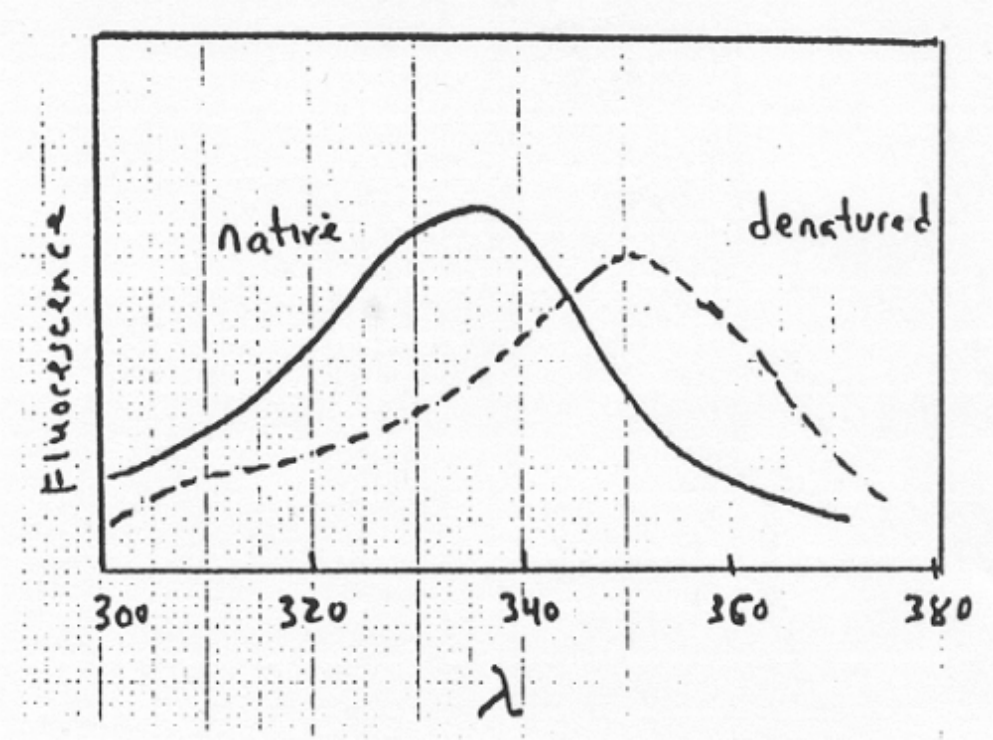


pH 14

450 nm

Sample problem:

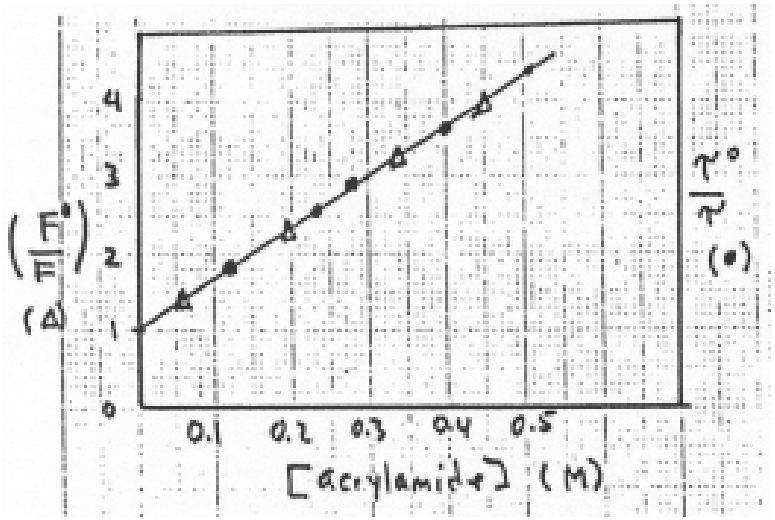
A protein contains two tryptophan residues which each fluoresce with equal quantum yields. Upon urea denaturation of the protein, the fluorescence changes as shown. **Explain.**



solution: the tryptophans are fully exposed to water when the protein is denatured, but are buried in the protein in the native form. Solvent relaxation results in the Stokes shift to the red.

b. Acrylamide was used to quench the tryptophan fluorescence. A linear Stern-Volmer plot was obtained which could be measured by either steady state fluorescence or by lifetime measurements

What is the mechanism of quenching? Explain.



$$F^o/F = 1 + k_Q\tau_o[Q]$$

Solution: Dynamic quenching: the lifetime changes in parallel with the fluorescence intensity

A modified "reciprocal" form of the Stern-Volmer plot is shown below

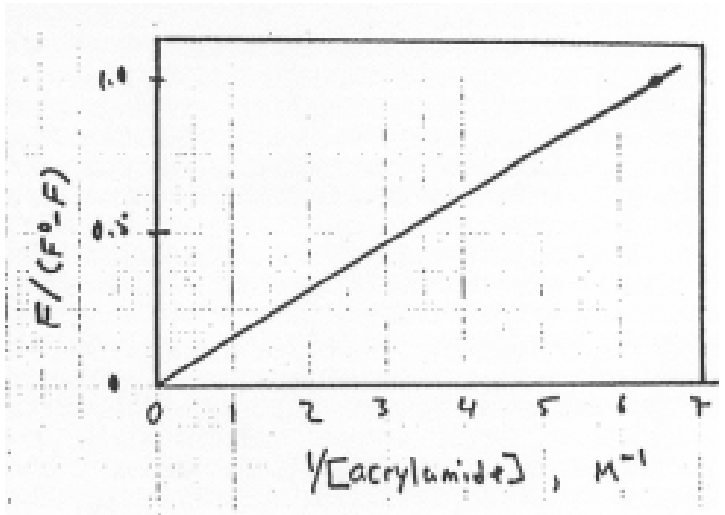
Are all the tryptophan residues buried?

reciprocal "modified form of the Stern-Volmer equation

$F^o/F = 1 + k_Q\tau_o[Q]$

$(F^0/F) - 1 = k_Q\tau_o[Q]$

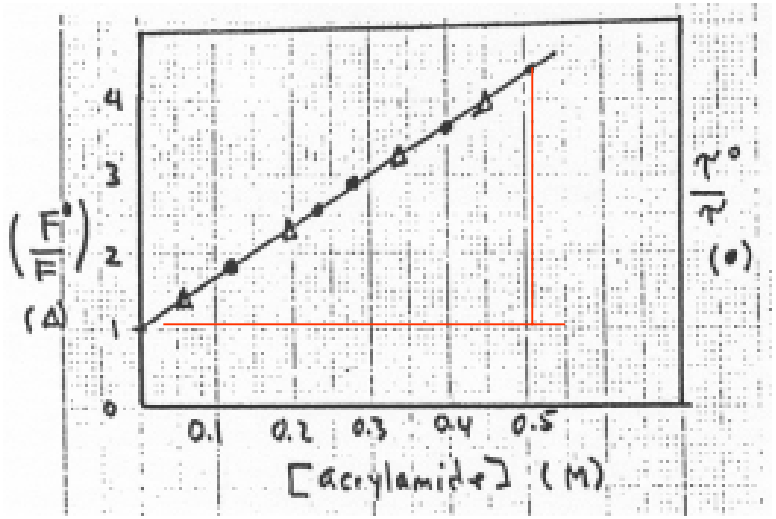
$F/(F^0 - F) = 1/(k_Q\tau_o[Q])$



Solution: as [acrylamide] is increased to infinite concentration the fluorescence, F, goes to zero.

This implies complete exposure to acrylamide to collisional (dynamic) quenching. Acrylamide is small and hydrophobic and can penetrate proteins to quench even buried tryptophans

What is the value of the collisional rate constant?
How does this value compare with the maximum possible?



$F^o/F = 1 + k_Q\tau_o[Q]$

Slope = 6.5 $M^{-1} = k_Q\tau_o$

Since τ_o = 3.6 nsec, we can calculate that $k_Q = 1.8 \times 10^9\ M^{-1}\ s^{-1}$

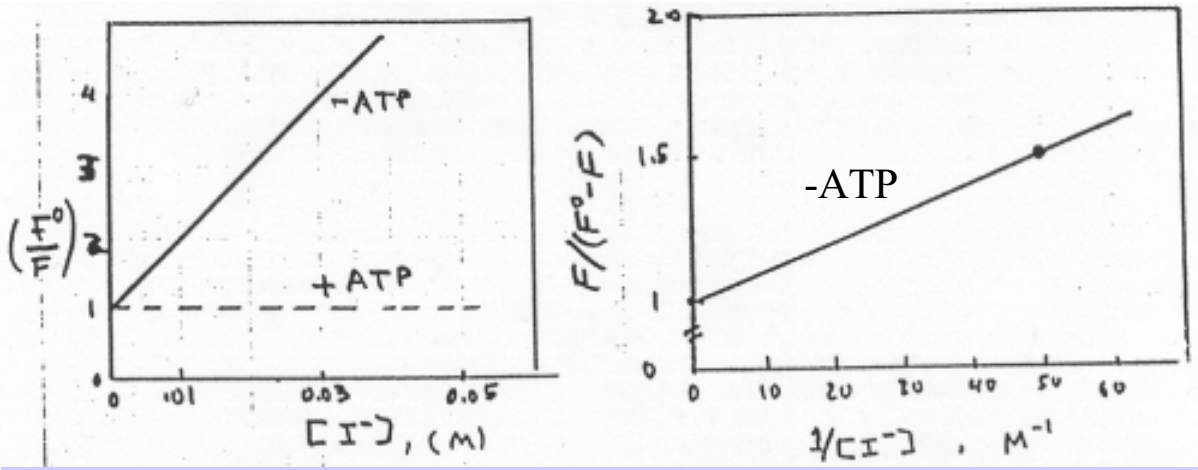
This is close to the diffusion limit of $10^{10}\ M^{-1}\ s^{-1}$

Iodide (I^-) was used as a quencher with the same protein. **In this case, the fluorescence lifetime did not change.** The quenching pattern was dramatically altered in the presence of ATP, known to bind to the proein.

1. What kind of quenching is caused by iodide in this case?

2. Are all the tryptophans accessible to iodide?

$F^o/F = 1 + k_Q\tau_o[Q]$ $\quad$ $F/(F^0 - F) = 1/(k_Q\tau_o[Q])$



Since the lifetime does not change, this is **static quenching**, probably due to iodide binding at some site near a tryptophan residue.

The intercept of the modified Stern-Volmer plot is 1, indicating that half the fluorescence is not quenched even at infinite iodide ($F = 0.5\ F^o$). One tryptophan residue is not accessible to iodide.