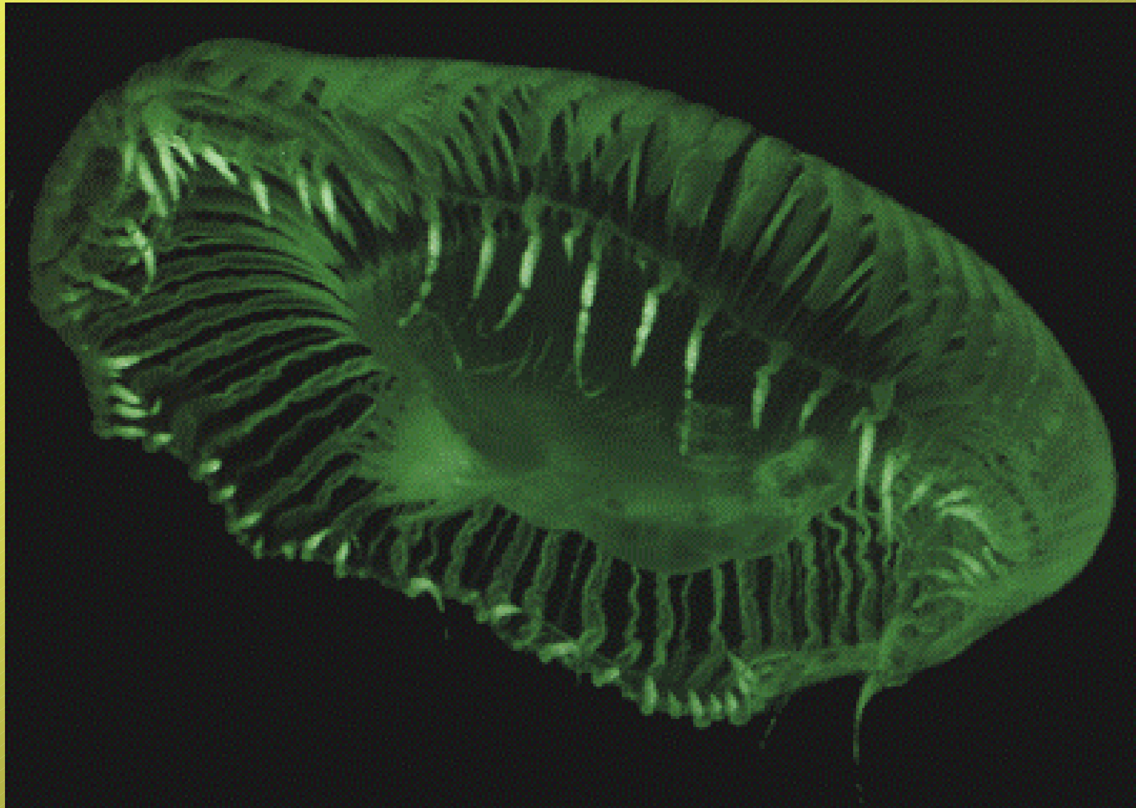
The Green Fluorescent Protein

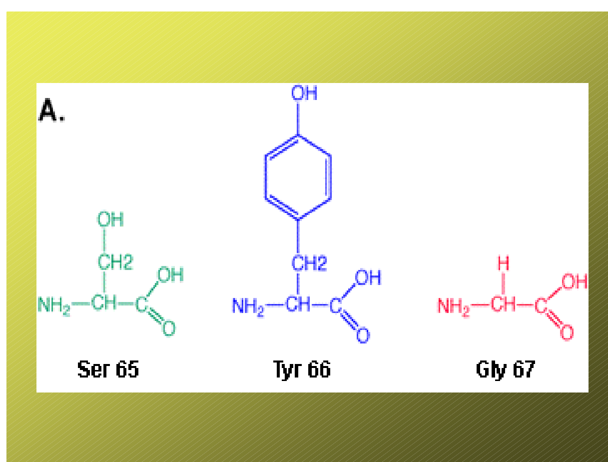
v.chem.uwec.edu/Chem412_S99/ppt/green.ppt

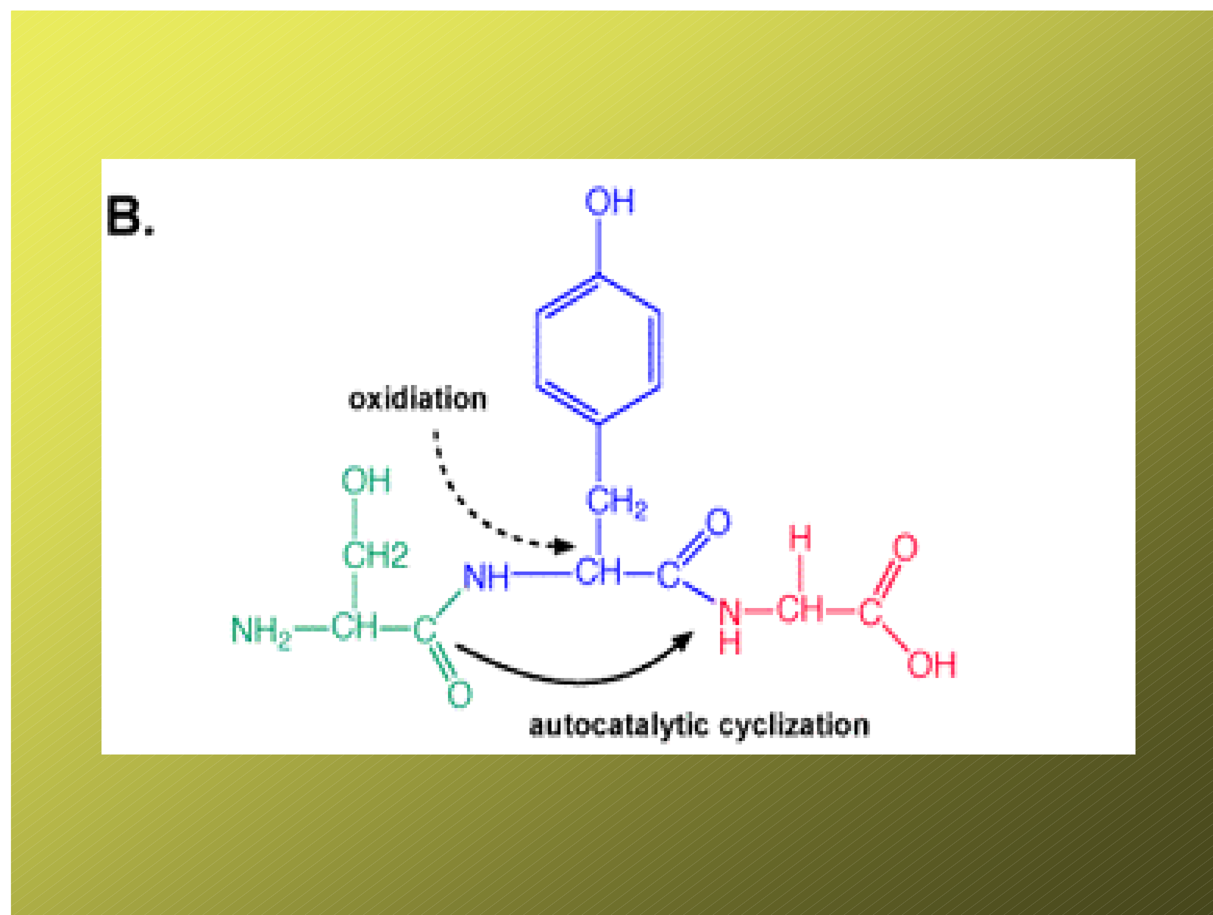
Protein (gene) is from a jellyfish:
Aequorea victoria



www.chem.uwec.edu/Chem412_S99/ppt/green.ppt

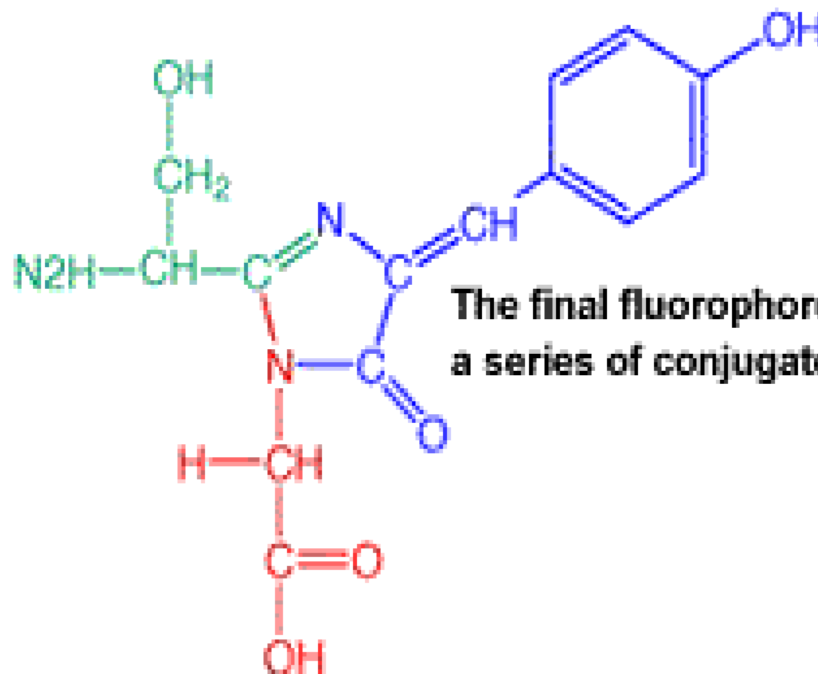
Autocatalytic reaction of three amino acids in the protein: cyclization and Oxidation by O_2





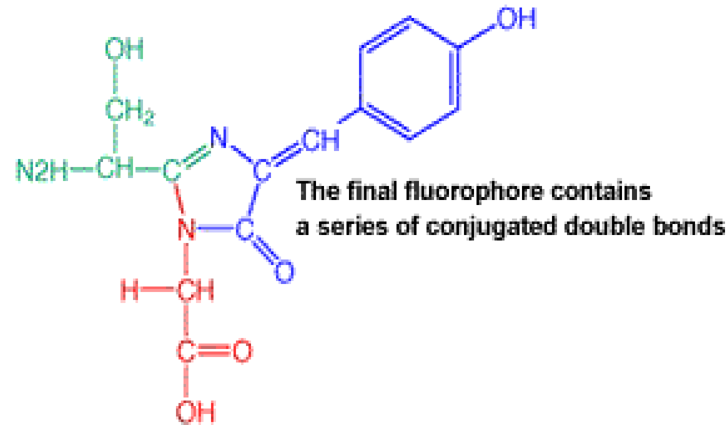
www.chem.uwec.edu/Chem412_S99/ppt/green.ppt

Rigid Protein “solvent” Enhances the Fluorescence Quantum Yield



www.chem.uwec.edu/Chem412_S99/ppt/green.ppt

Spectral Tuning By Protein Environment by mutagenesis



T203Y: π-stacking of the tyrosine to the aromatic chromophore stabilizes the excited state and results in a "red shift" **Yellow Fluorescence**

yellow emission maximum is **529 nm**

Also there are **blue (448 nm)** and **cyan (485 nm)**

Protein Fusions with Green/Yellow/Cyan Fluorescent Proteins Used to Localize Proteins within Cells *in vivo*

examples: **Gα protein** and **Ca^{2+}- binding protein** (Calnuc)

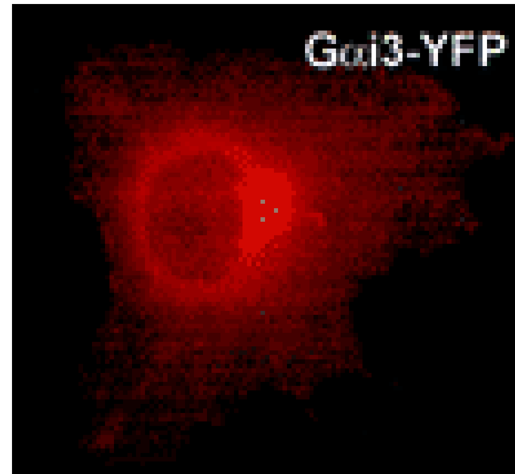
fusion of Yellow fluorescent protein to G protein

fusion of Cyan fluorescent protein to Ca^{2+} binding protein

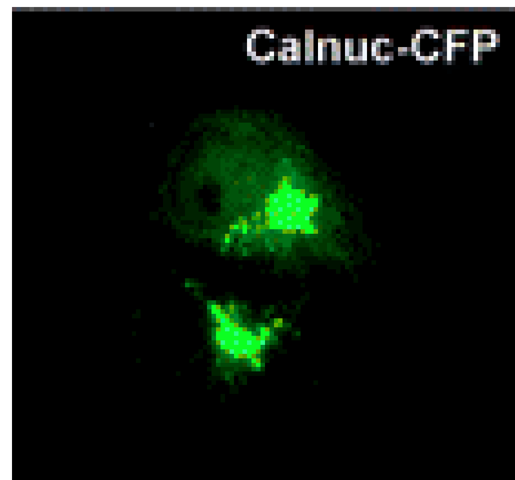
PNAS (2001) 98 14961-66

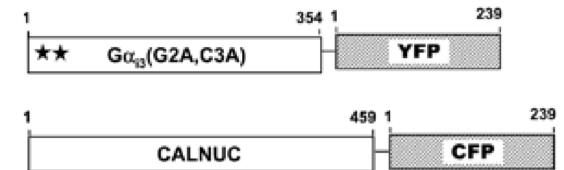
Gαi3 and calnuc fusion proteins expressed in COS-7 cells

Present on Golgi and Plasma membrane



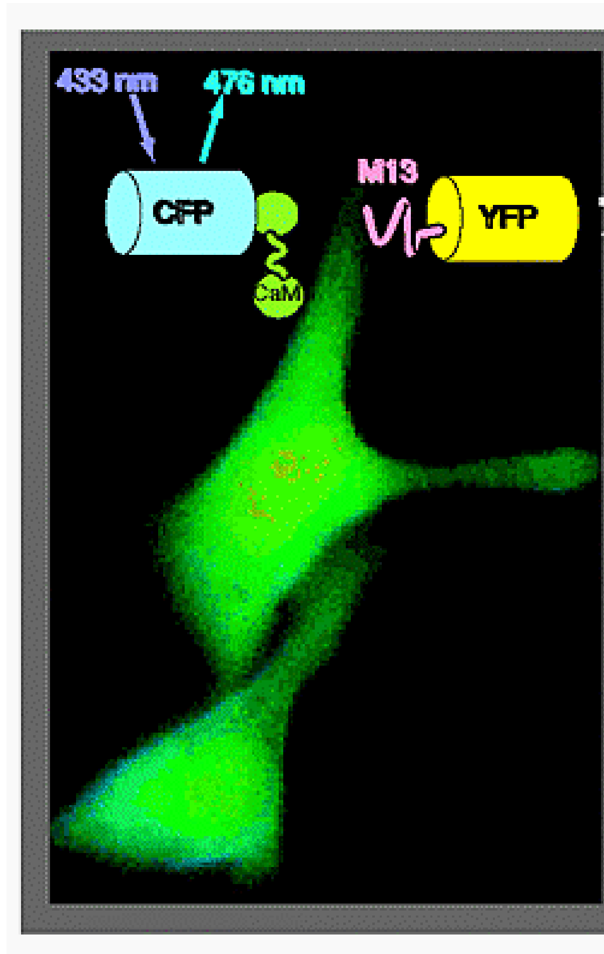
Present on Golgi





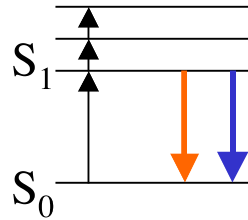
PNAS (2001) 98 14961-66

Calmodulin (CaM) in cytoplasm labeled with CFP
(and Calmodulin Binding Protein)



1. Tsien, R.Y. & Miyawaki, A. *Science* **280**, 1954-1955 (1998).

Quantum Yield and Lifetime



$N^*(t) = [S_1]_t$

rate up: $(S_o \rightarrow S_1) = I_o$, # photons absorbed/sec

rate down: $(S_1 \rightarrow S_o) = (k_f + k_I) \cdot N^*(t)$

$N^*(t)$ = concentration of excited state molecules at any time, t

k_I = sum of all rate constants other than that for fluorescence for de-excitation

rate up rate down

steady state

$$0 = \frac{dN^*(t)}{dt} = I_o - (k_f + k_I)N^*(t)$$

$$I_o = (k_f + k_I)N^*(t)$$

Q_f = fluorescence quantum yield

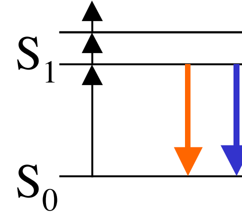
Fraction of excited state molecules that relax to the ground state by emitting a photon

photons/sec emitted

$$Q_f = \frac{k_f \bullet N^*}{I_o} = \frac{k_f N^*}{(k_f + k_I) \bullet N^*}$$

photons/sec absorbed

since $I_o = (k_f + k_I)N^*(t)$



$$Q_f = \frac{k_f}{k_f + k_I}$$

Fluorescence lifetime

a measure of how long the molecule remains in the excited state

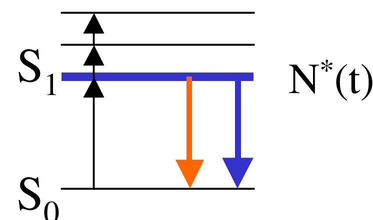
Excite some molecules to S_1 with a brief pulse of light

At $t = 0$, N_o^* excited state molecules

Decay of the excited state population is exponential

$$\frac{dN^*(t)}{dt} = -(k_f + k_I)N^*(t)$$

So $$N^*(t) = N_o^* e^{-(k_f + k_I)t}$$



Define: lifetime $\tau = \dfrac{1}{k_f + k_I}$

then

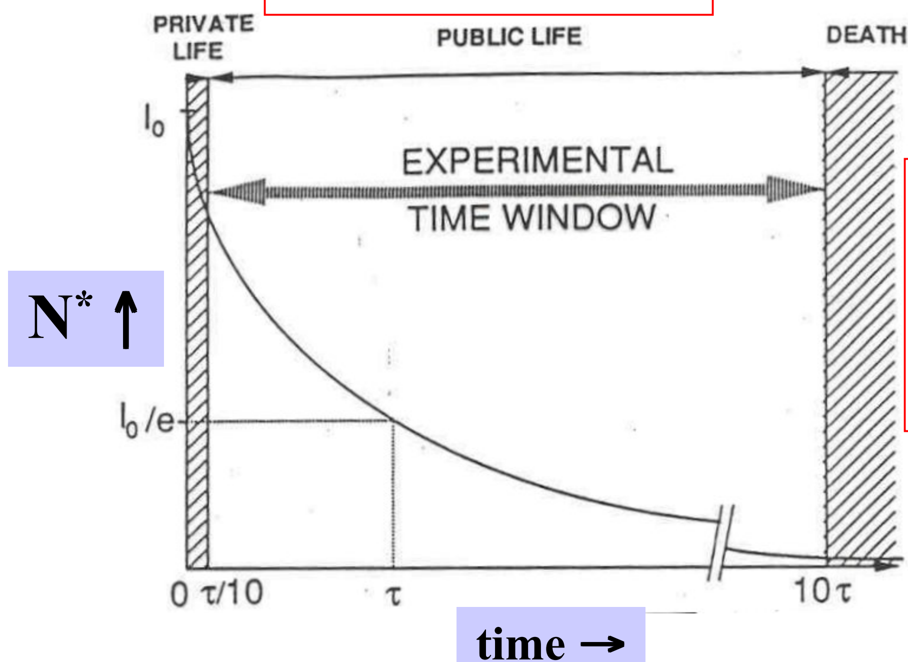
$$N^*(t) = N_o^* e^{-t/\tau}$$

$$Q_f = k_f \bullet \tau$$

Since $Q_f = \dfrac{k_f N^*}{I_o} = \dfrac{k_f N^*}{(k_f + k_I)N^*}$

Events that influence the excited state from τ/10 to 10τ can be probed by fluorescence spectroscopy

$N^*(t) = N_o{}^*e^{-t/\tau}$



N* ↑

time →

Note: τ has units of time (seconds)

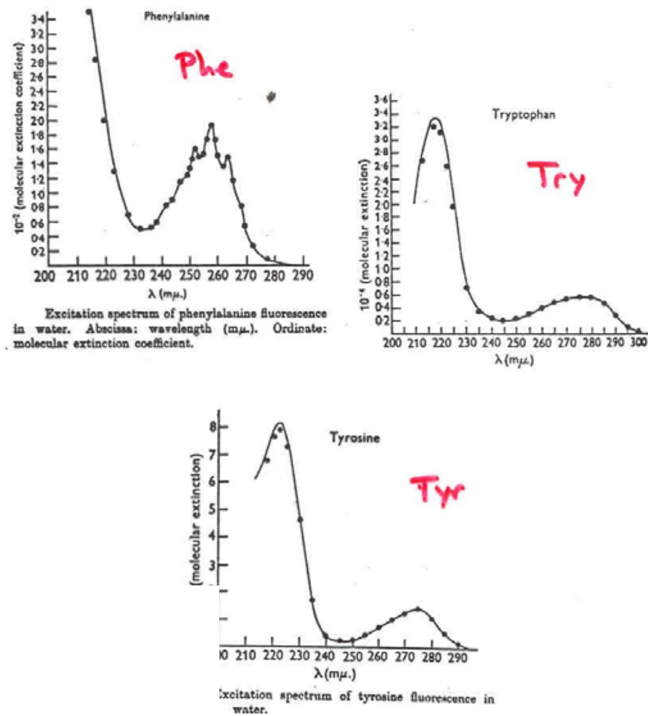
$(k_I + k_f)$ has units of $seconds^{-1}$

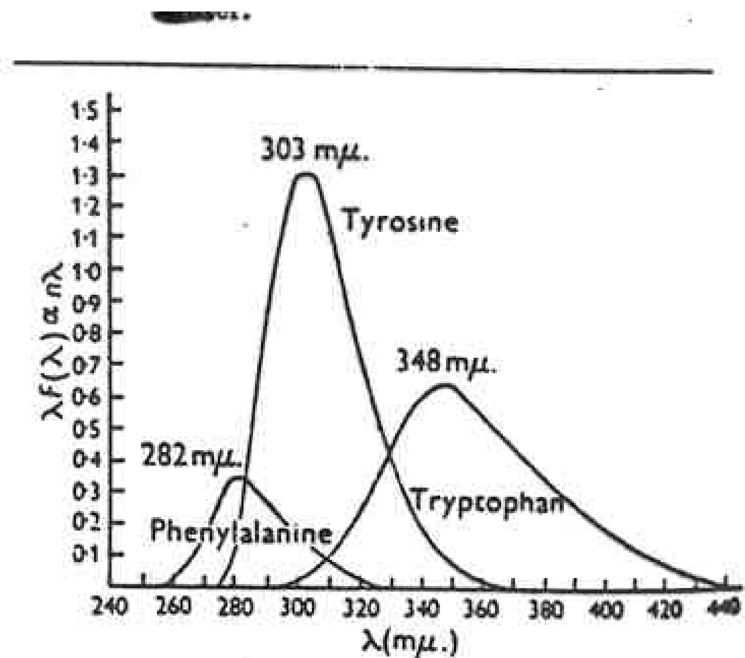
$\tau = 1/(k_f + k_I)$

Example: tryptophan: $\tau \approx 4$ nsec

"window": 0.4 - 40 nsec

Intrinsic Fluorescence of Proteins



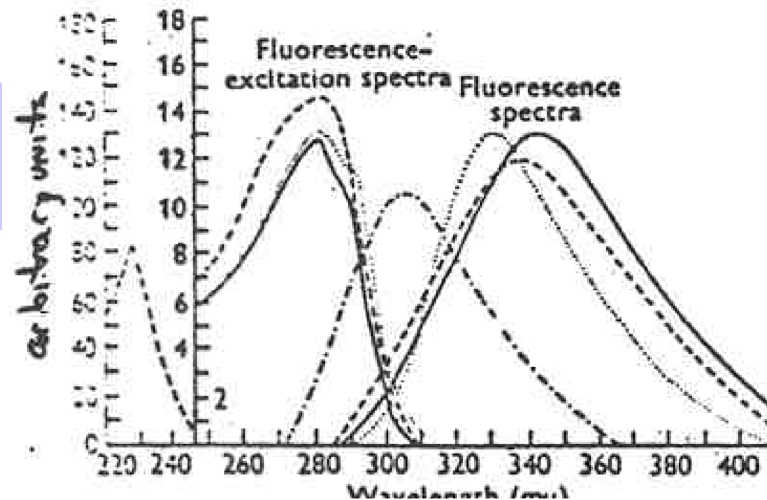


Excitation spectra: same as the absorption spectra

Fluorescence spectra of amino acids in water

Spectra of several proteins

Mostly due to tryptophan



Fluorescence-excitation spectra and fluorescence spectra of proteins in aqueous solutions. Chymotrypsin (…), insulin (-.-), human-serum albumin (---), pepsin (-). In the excitation spectrum of human-serum albumin between 220nm and 247nm, the ordinate of extinction is increased tenfold

Quantum yields of free amino acids and of amino acids in BSA proteins.

	Q_f (%)	
	free	in BSA
tyrosine	21%	2% ← quenched
tryptophan	20%	47%
phenylalanine	4%	0% ← quenched

In most proteins, the fluorescence is dominated by tryptophan

For example, in bovine serum albumin, tyrosine and phenylalanine fluorescence is mostly quenched by interactions with surrounding protein residues

	Q_f (%)	
	free	in BSA
tyrosine	21%	2%
tryptophan	20%	47%
phenylalanine	4%	0%

An example of the use of fluorescence spectroscopy:

Fluorescence from **Barnase**

Extracellular ribonuclease from Bacillus amyloliquefaciens

Biochem (1992) 31, 711-

Biochem (1991) 30, 6775-

-similar to Ribonuclease T1

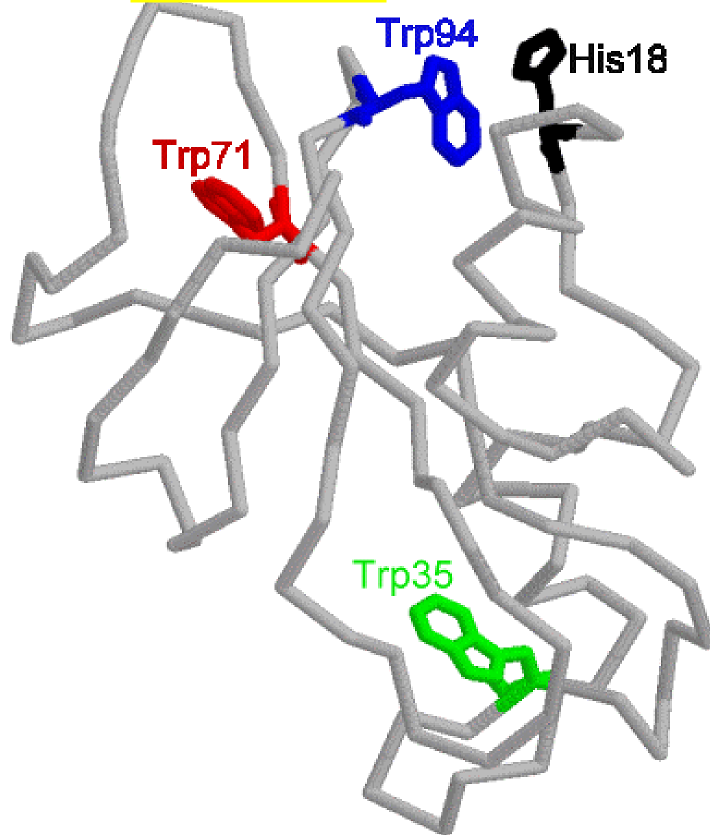
-focus of many studies to examine protein structure, folding, etc by mutagenesis

Small, monomeric, single domain

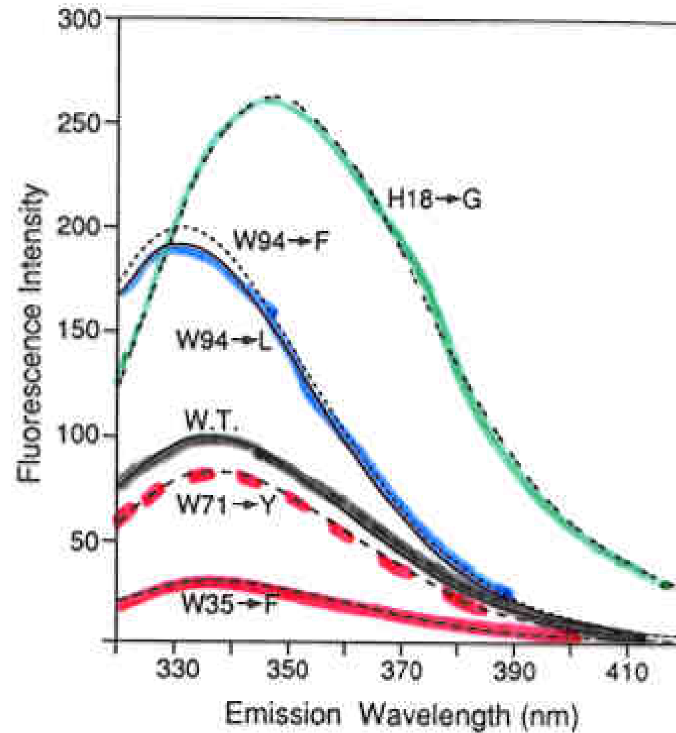
5 antiparallel β strands, 2 major α helices

3 Tryptophans

-use mutagenesis to understand contributions to intrinsic fluorescence and how it can be used



340 nm emission **pH 5.5**



Fluorescence emission spectra of the different mutants in Bis-Tris buffer (ionic strength = 10mM) at pH 5.5. All spectra were recorded at the same protein concentration of 4 υM. The maximal value of the intensity of emission of the wild-type protein has been arbitrarily taken at 100

(1) fluorescence dominated by Trp35:

(2) small contribution from Trp71

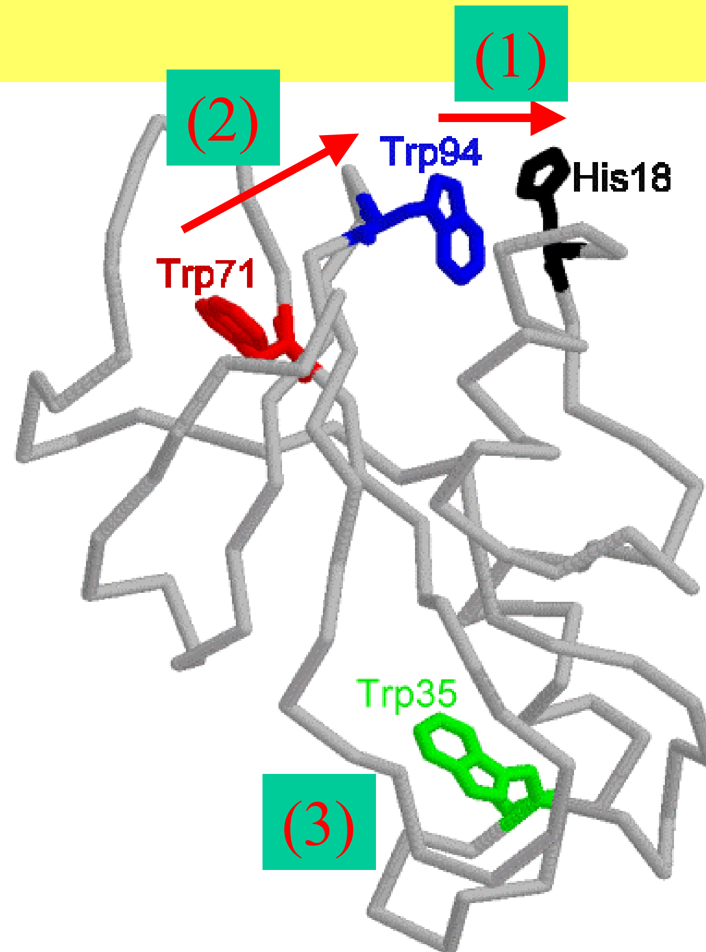
(3) enhanced fluorescence when Trp94 is removed (—)

(4) enhanced fluorescence and red-shift when His18 is replaced (—)

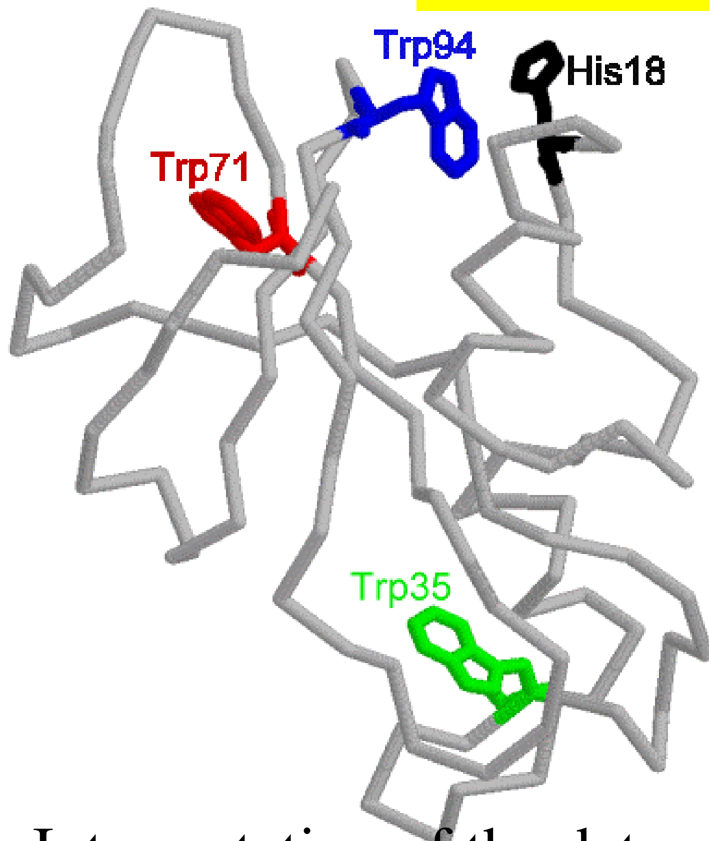
Barnase fluorescence comes from 3 tryptophans:

strong effects from

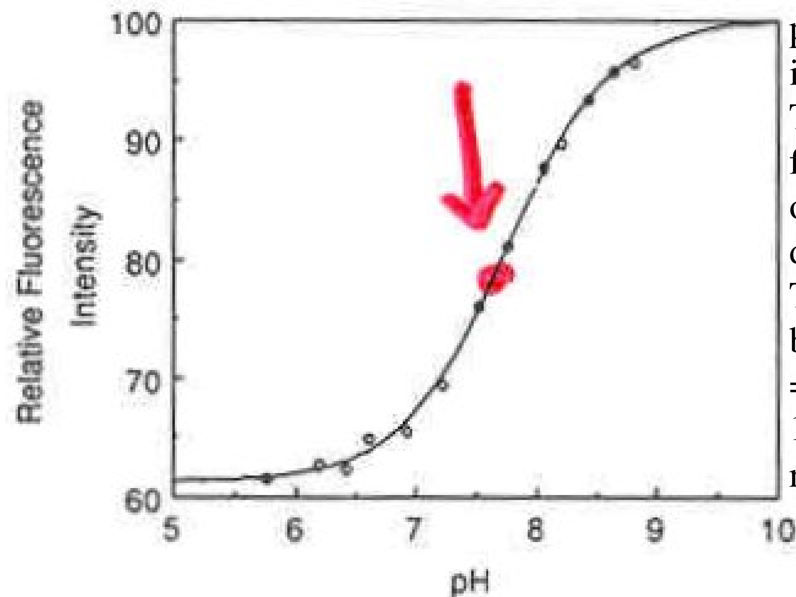
1) static quenching
2) Fluorescence energy transfer
3) Stokes shift from solvent relaxation



Barnase: Fluorescence is pH-dependent



pK corresponds to His18 protonation (known from previous NMR studies)



pH titration of His-18 in wild-type barnase. The intensity of fluorescence emission of solutions of different pH in either Tris or Bis-Tris buffers (ionic strength = 10 mM) containing 1 μM barnase was recorded at 340 nm.

Interpretation of the data:

(1) Protonation of **His18** (pK_a = 7.75) quenches the fluorescence from **Trp94**

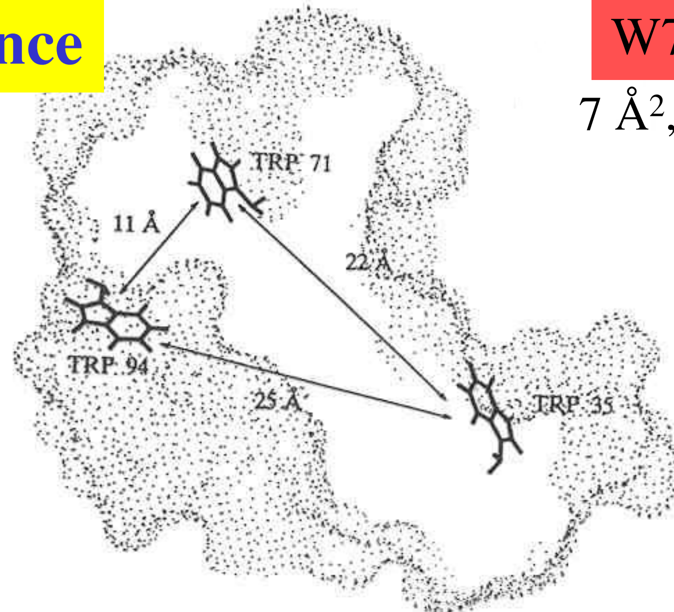
(2) Efficient energy transfer from **Trp71** to **Trp94** eliminates the fluorescence from **Trp71**.

(3) Replacement of **Trp94** enhances emission from **Trp71**

Barnase fluorescence

W94

58 Å², solvent accessible surface



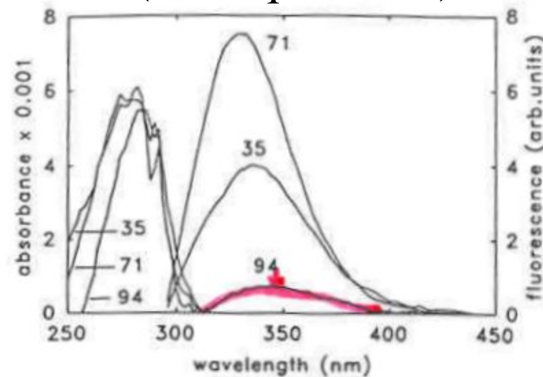
W71

7 Å², solvent accessible area

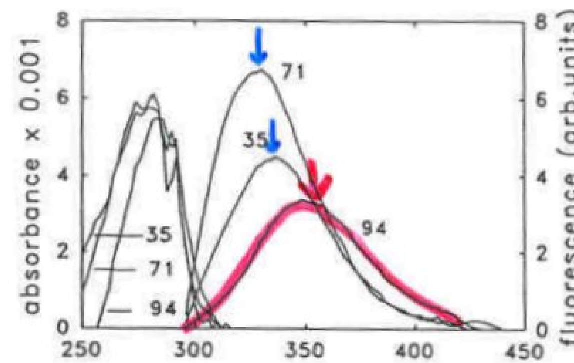
W35

10 Å², solvent accessible area

pH 5.5
(W94 quenched)



pH 9.4



W94 is quenched by protonated His18

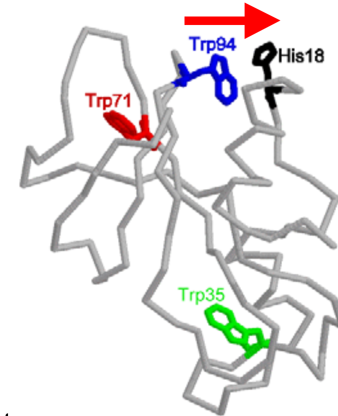
Abosrption and emission spectra calculated for the individual tryptophan residues, in the absence of energy transfer, at low pH (left) and high pH (right). The spectra are calculated by subtraction of different mutant spectra.

Note: red shift of W94 (water exposed)

Fluorescence Quenching

Static quenching: formation of a "dark" ground state complex

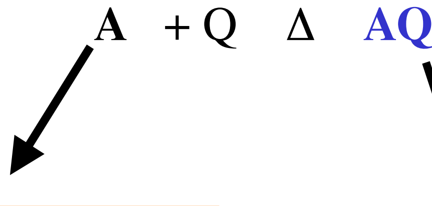
In the Barnase example
A=W94
AQ= W94- (H^+)His18



Both A and AQ complex absorb light but only A is fluorescent

A + Q Δ AQ

K_{eq} equilibrium constant

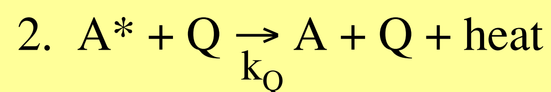


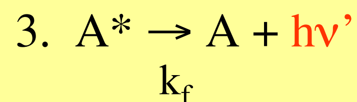
More [Q] results in lower [A], so there is less fluorescence

Q_f is decreased BUT τ_o is unchanged

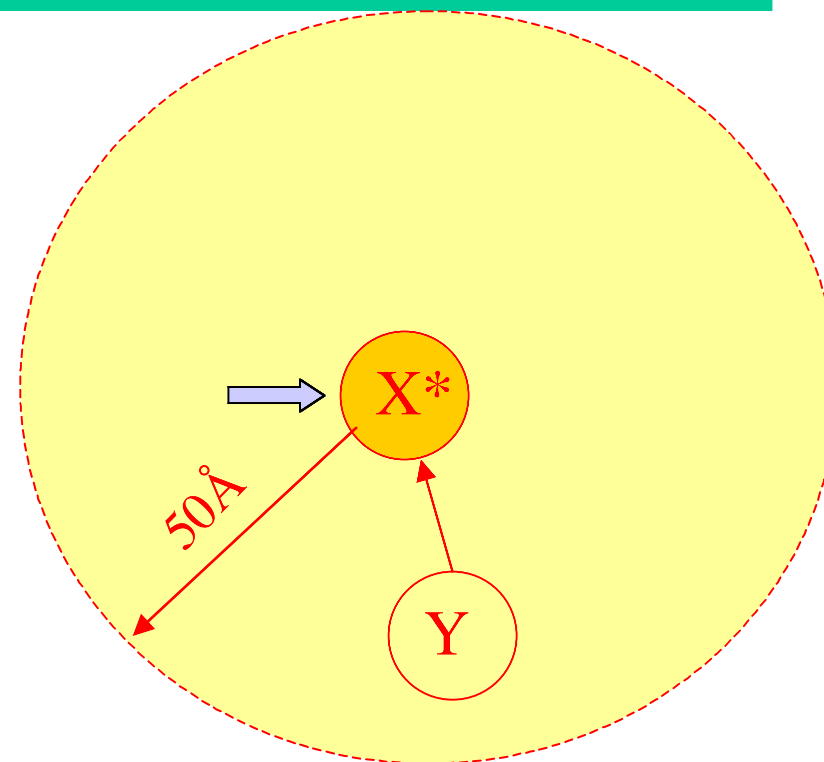
Fluorescence Quenching

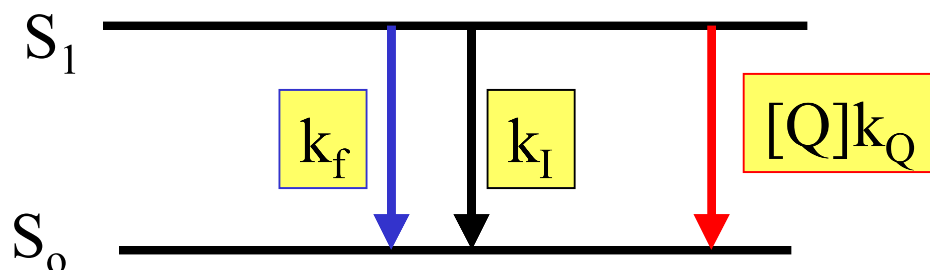
Dynamic quenching: collision with the excited state



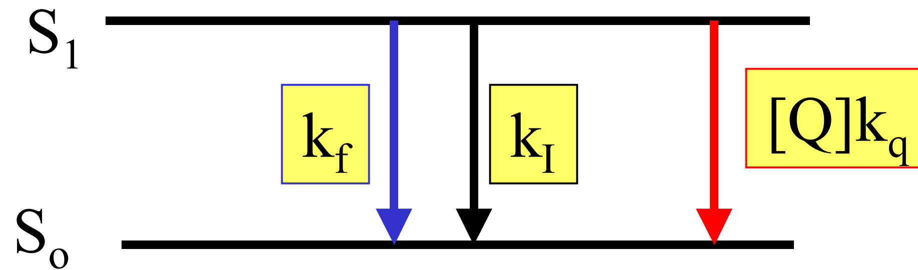


k_q = second order rate constant for collisional quenching





Dynamic Quenching



No Quencher: $Q_f^o = \dfrac{k_f}{k_f + k_I}$

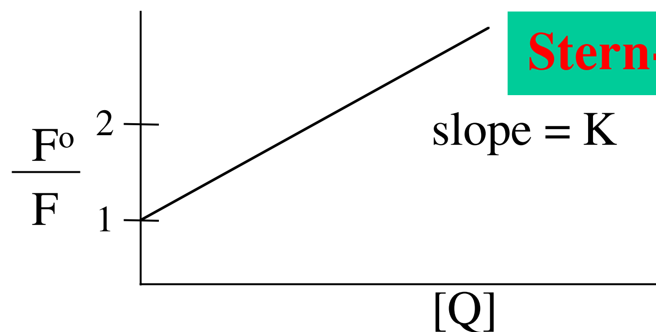
+Quencher: $Q_f = \dfrac{k_f}{k_f + k_I + k_Q[Q]}$

$$\frac{Q_f^o}{Q_f} \approx \frac{F^o}{F} = \frac{k_f}{k_f + k_I} \bullet \frac{k_f + k_I + k_Q[Q]}{k_f} = 1 + \frac{k_Q[Q]}{k_f + k_I}$$

$$\frac{F^o}{F} = 1 + k_Q\tau_o[Q] = 1 + K[Q]$$

K = Stern-Volmer constant

k_Q = quenching constant

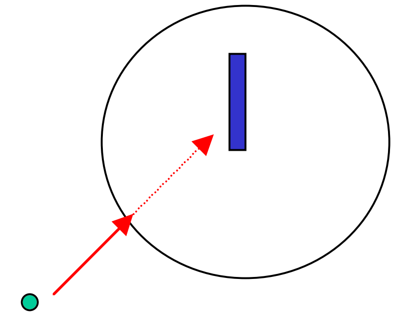


Stern-Volmer Plot

Example:

Quenching of tryptophan in proteins-

An early indication of protein dynamics



Experiment:

-Observe proteins with single buried tryptophans (known from X-ray)

-Quench with (a) Iodide, (I^-)
(b) O_2 (use high pressures to increase $[O_2]$)

Result: (1) Tryptophans are inaccessible to I^- but accessible to O_2

(2) However, no room in X-ray structures for O_2!

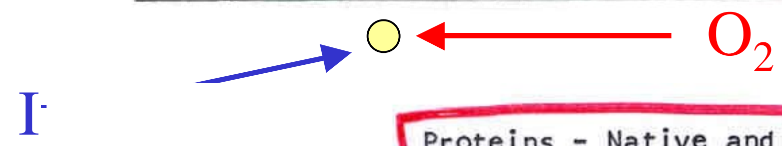
Conclude: X-ray structure is only an average. The protein must open and close on a time scale sufficient to allow O_2 inside

Lakowicz + Weber. Biochem (1973) 12, 4161 - 4171

Data from O_2 and I^- quenching of small molecules and buried tryptophan

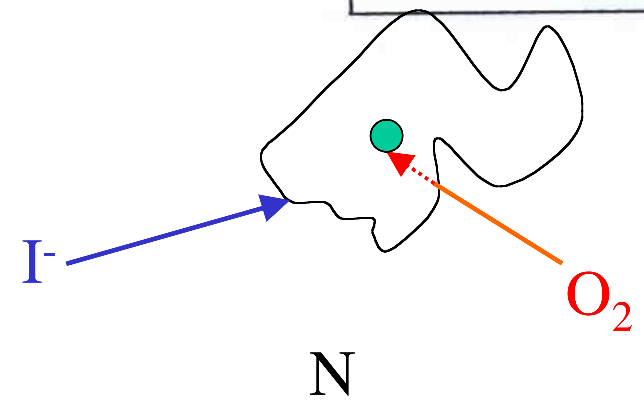
: Small Molecules in Water

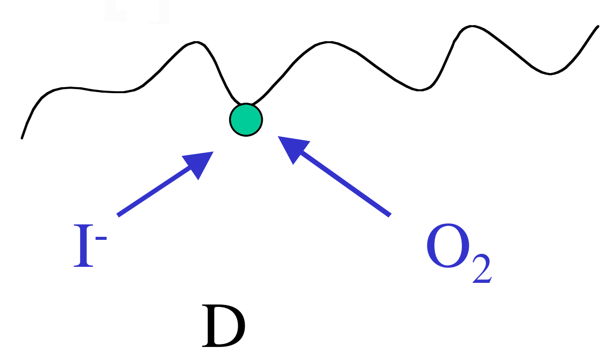
Cpd	Quencher	K(M^{-1})	τ_0 (nsec)	k_Q ($M^{-1}sec^{-1}$)
indol	O_2	50.4	4.1	1.23×10^{10}
tryptophan	O_2	32.5	2.7	1.2×10^{10}
tryptophan	I^-	10.5	2.7	0.39×10^{10}
tyrosine	O_2	39.0	3.2	1.2×10^{10}



Proteins - Native and Denatured (Random Coil)

Protein	Form	k_Q ($M^{-1}sec^{-1}$) O_2	k_Q ($M^{-1}sec^{-1}$) I^-
Trypsinogen	native	0.43×10^{10}	0.034×10^{10}
	denatured	1.17×10^{10}	0.24×10^{10}
Carboxypeptidase A	native	0.38×10^{10}	0.032×10^{10}
	denatured	0.78×10^{10}	0.21×10^{10}

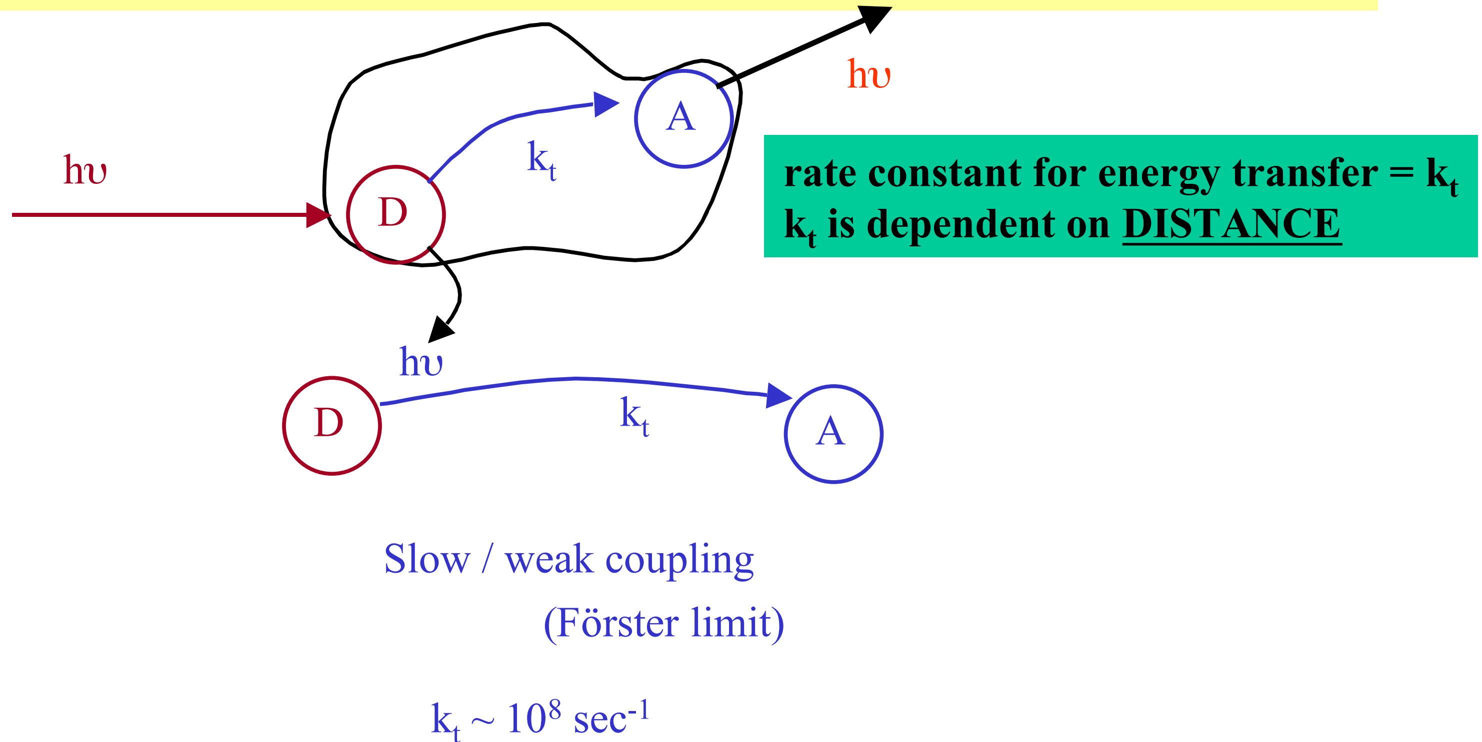




Fluorescence Resonance Energy Transfer (FRET)

"Förster" energy transfer
"singlet - singlet" energy transfer
"radiationless" energy transfer

Quenching of "Donor" Fluorescence due to the presence of an "Acceptor"
and
Stimulation of Fluorescence of the Acceptor upon Excitation of the Donor



(for review see) Nature Structural Biology (2000) 7, 730

Fluorescence Resonance Energy Transfer (FRET)

In the Barnase example, energy transfer is observed from W71 to W94