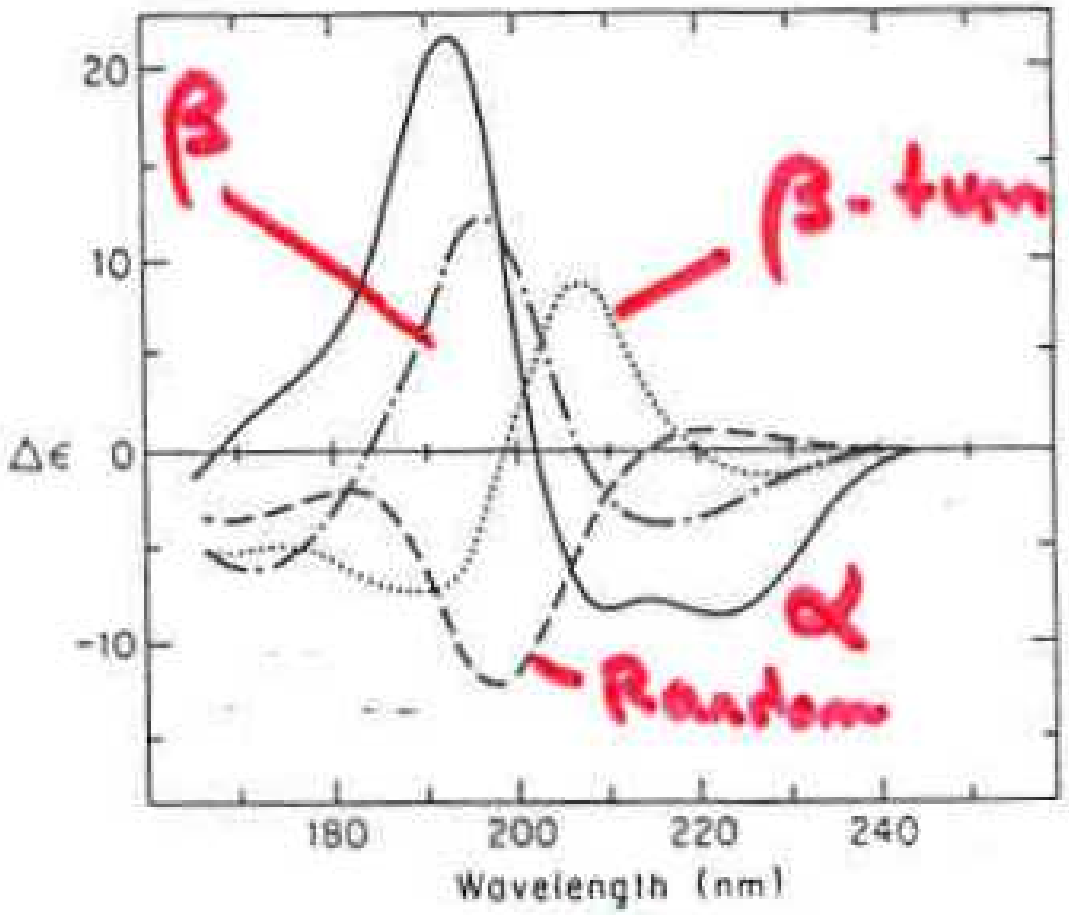
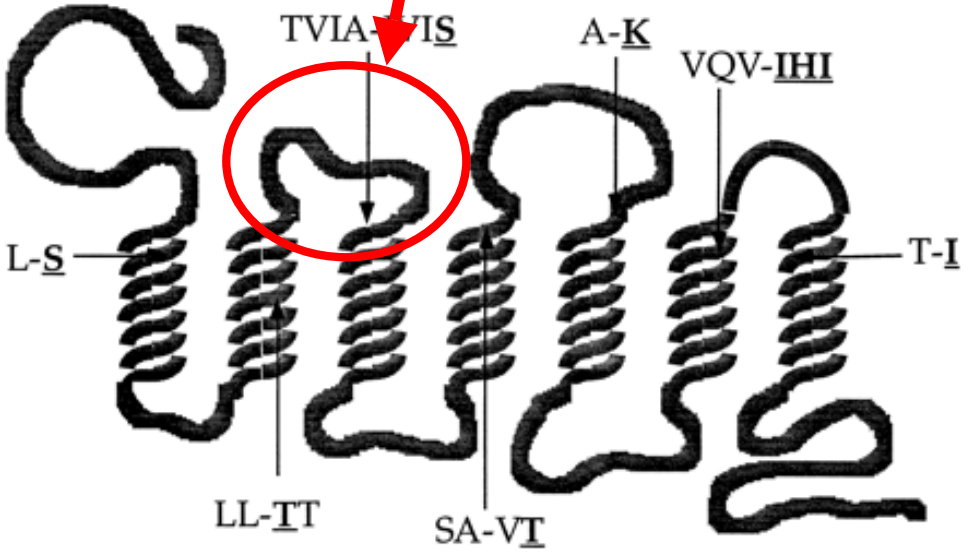
CD Basis Set of Spectra that is used is that derived from comparing the spectra of globular proteins whose secondary structures are known from X-ray crystallography



An example of the use of CD
Modeling an extracellular loop of the κ opioid receptor

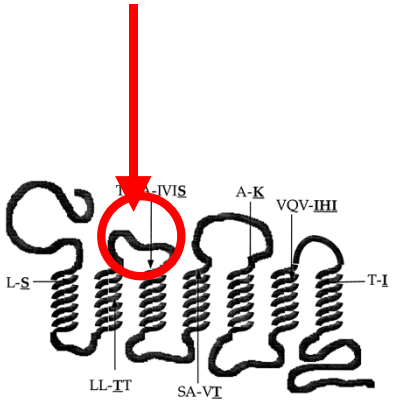
second extracellular "loop"
involved in binding to dynorphin

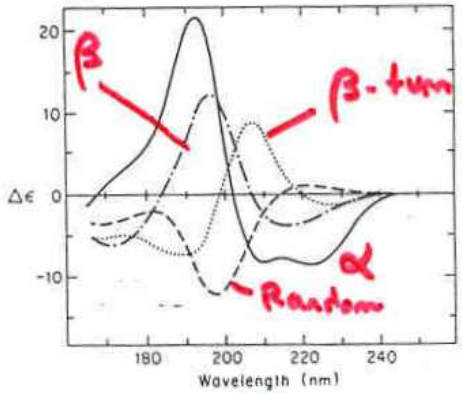


Biochemistry (2002) 41, 61-68

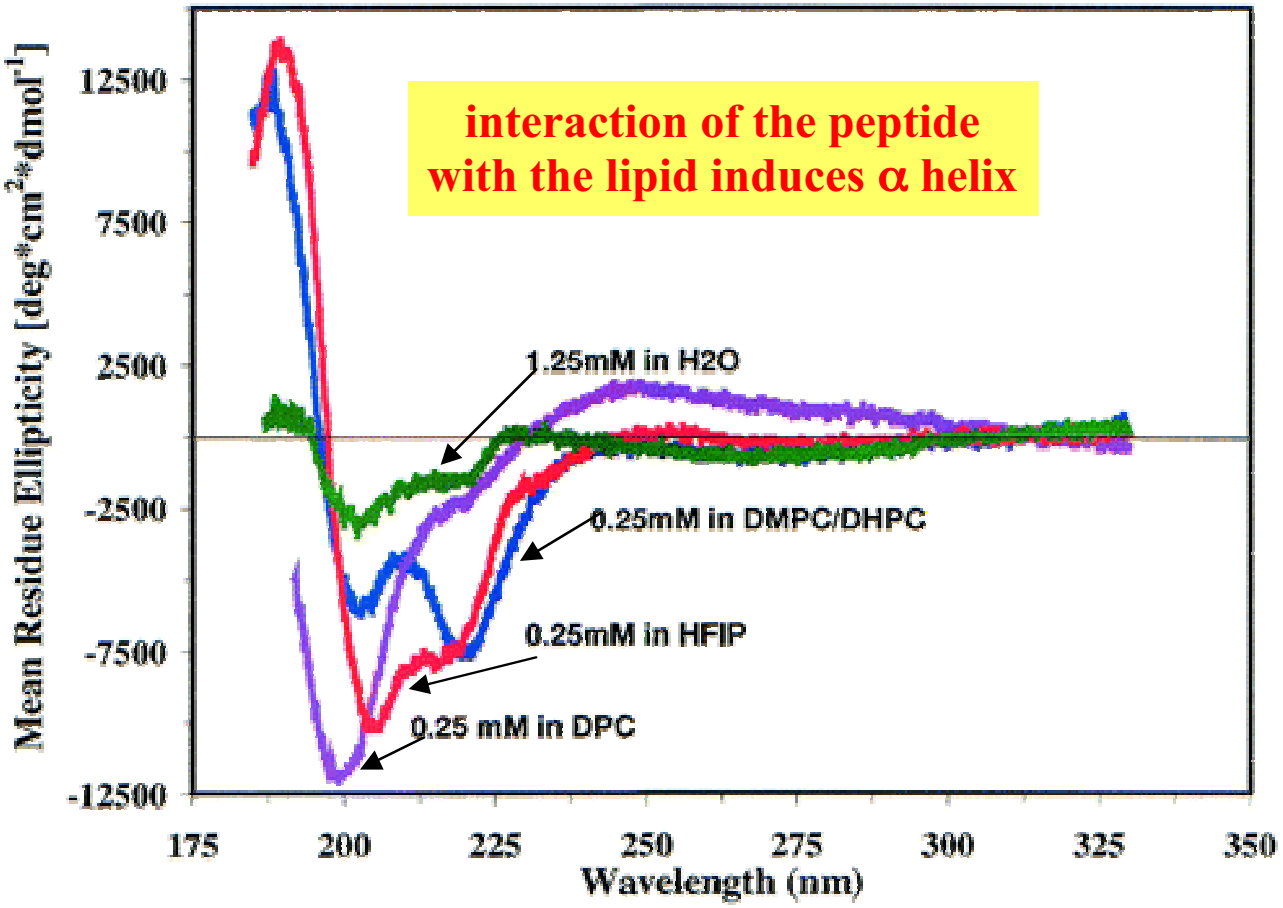
**No secondary structure of the peptide in water
but substantial α-helix in the presence of
phospholipids (DPC,DHPC,DMPC) or in the solvent hexafluoro-2-propanol**

33 amino acid peptide

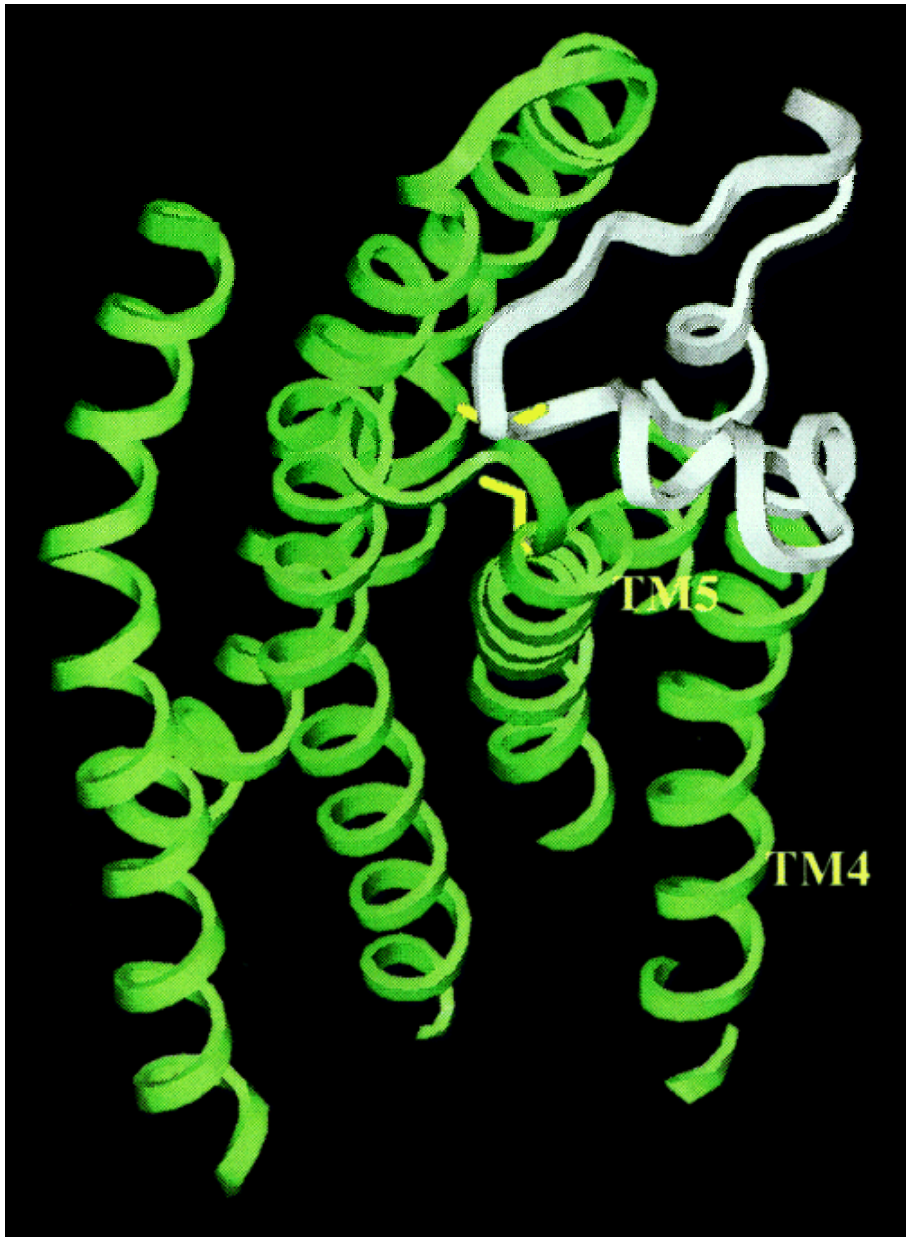




Biochemistry (2002) 41, 61-68



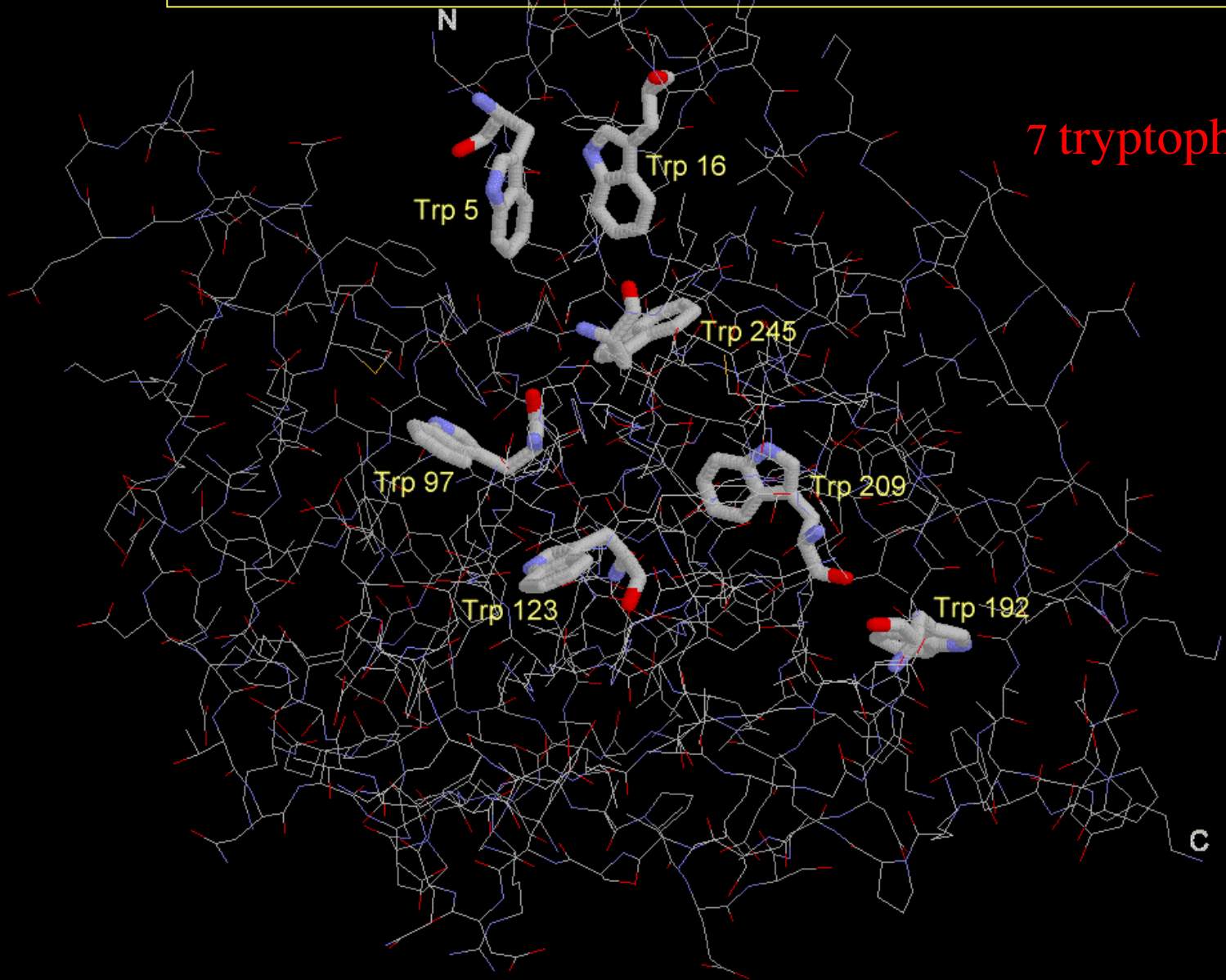
Modeling an extracellular loop of the κ receptor



combination of CD, fluorescence and NMR yields the model

Biochemistry (2002) 41, 61-68

Human Carbonic Anhydrase II: an example showing induced CD of tryptophans in a protein in the near UV



7 tryptophans

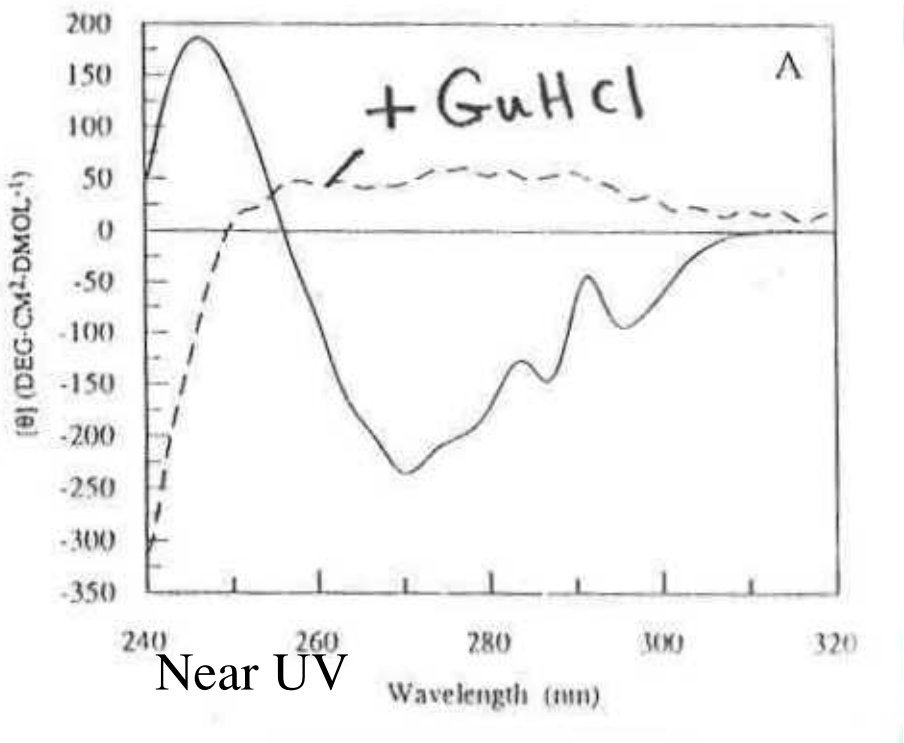
7% α

28% β

Biochem (1994) 33, 14281-88)

CD spectra of Human Carbonic Anhydrase II

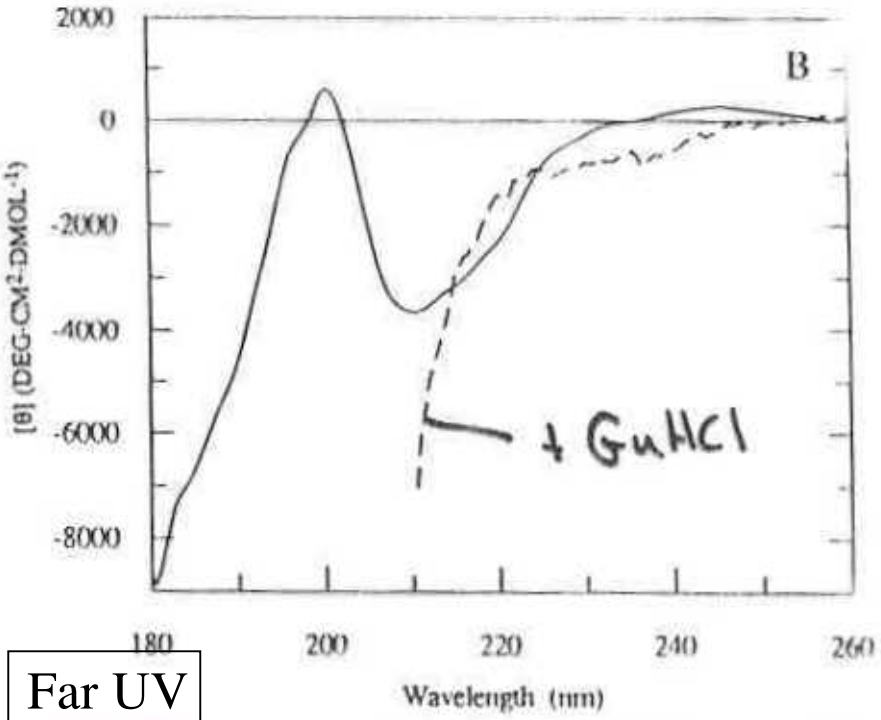
A: **near-UV** CD spectrum of $HCAII_{pwt}$ in 0 M GuHCL (solid) and 5 M GuHCL(dashed)



Near UV

from 7 tryptophans

B: The **Far-UV** CD spectrum of $HCAII_{pwt}$ in 0 M GuHCL (solid) and 5 M GuHCL (dashed)



Far UV

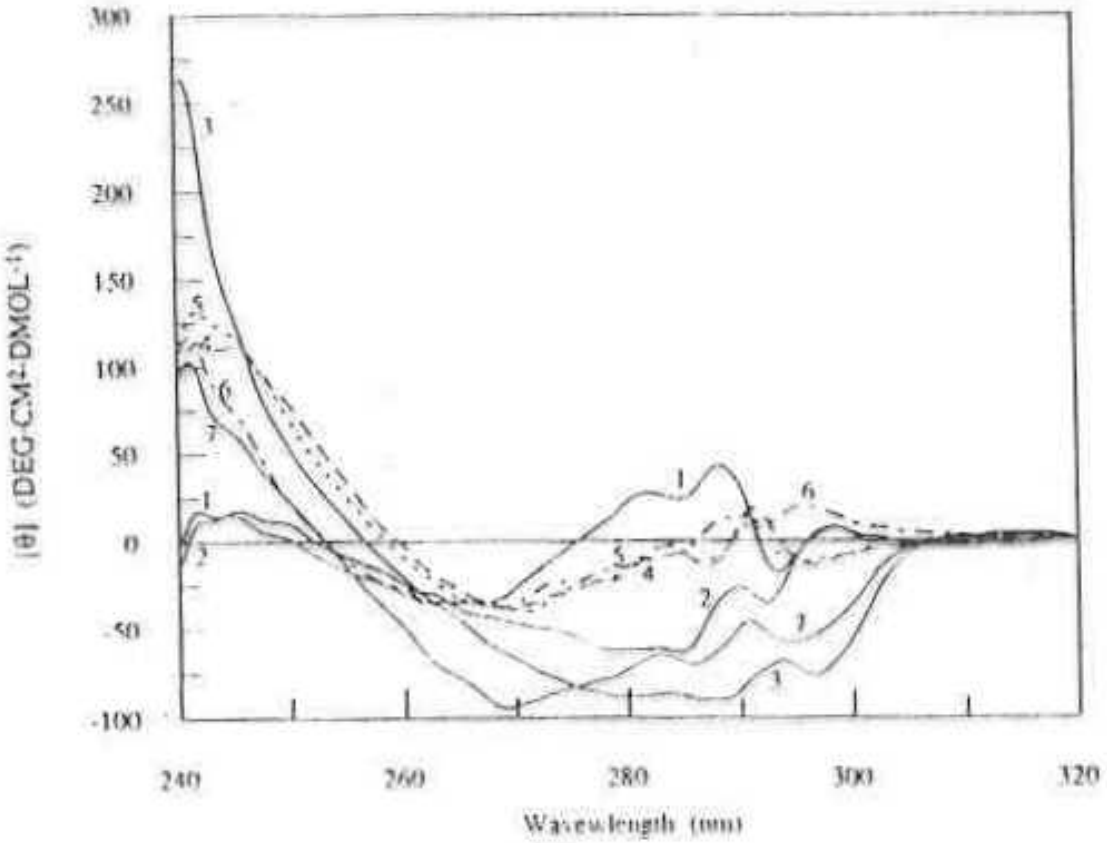
consistent with α (7%), β (28%)

$$[\theta] = \theta_{obs} \bullet \left[\frac{(\text{mol wt})}{C\ (\text{mg/ml})\ (10)(1\ \text{cm})} \right]$$

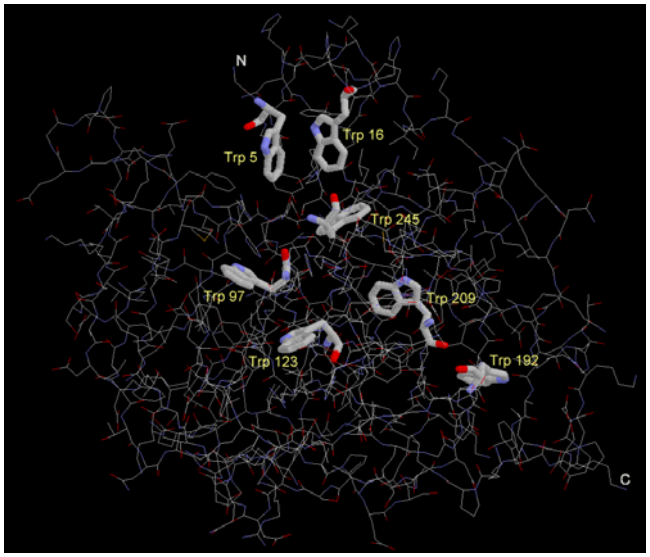
$$\frac{\text{deg} \bullet \text{cm}^2}{\text{dmol}}$$ Units of molar ellipticity

Biochem 33, 14281-

Contribution to CD from each tryptophan is different: monitor of tertiary structure.



determine CD of site-directed mutants eliminating each tryptophan and determining the difference spectrum



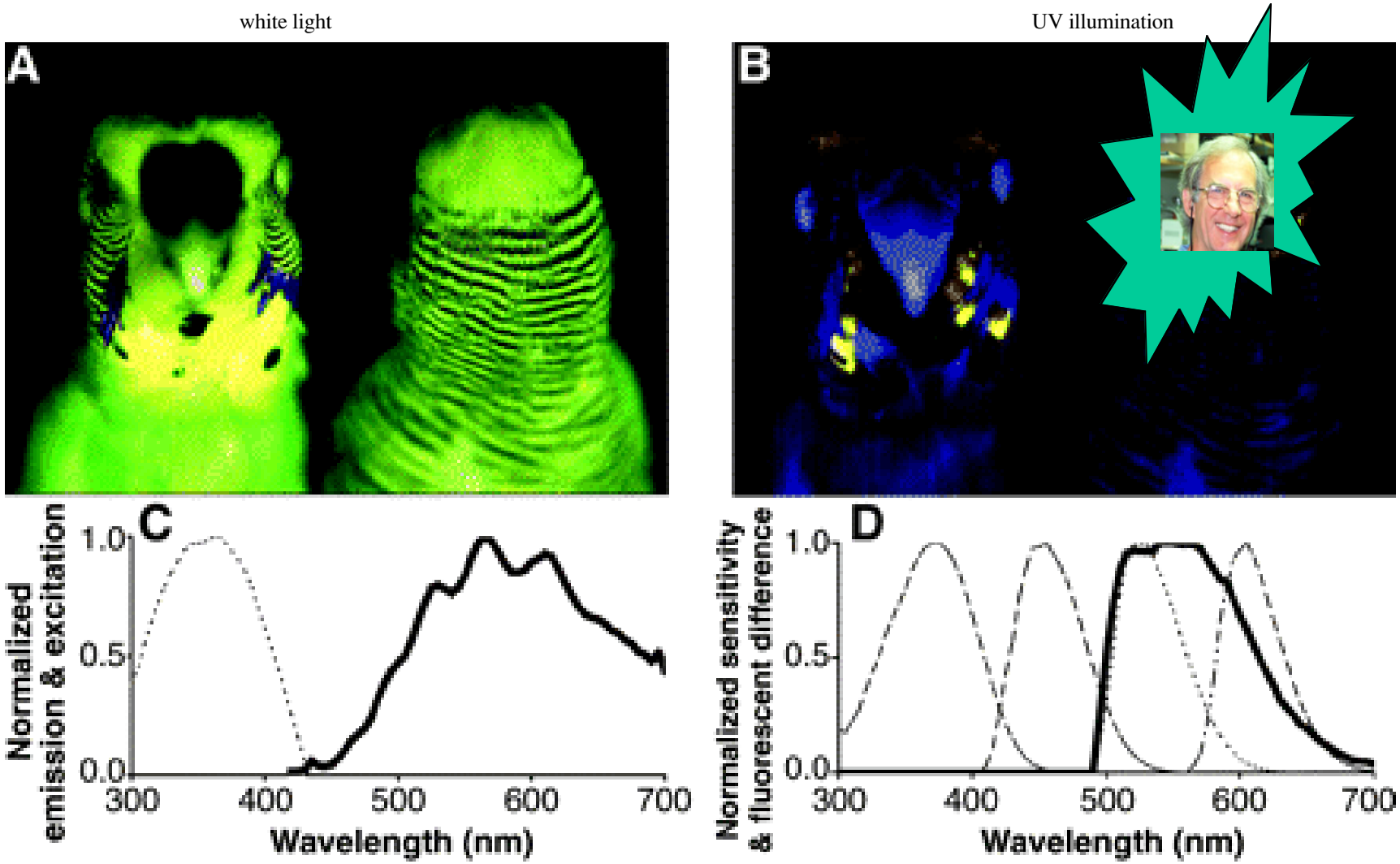
Biochem 33, 14281-

Fluorescence Spectroscopy and SEX

Fluorescent Plumage of Parrots is a Sexual Signal!

white light

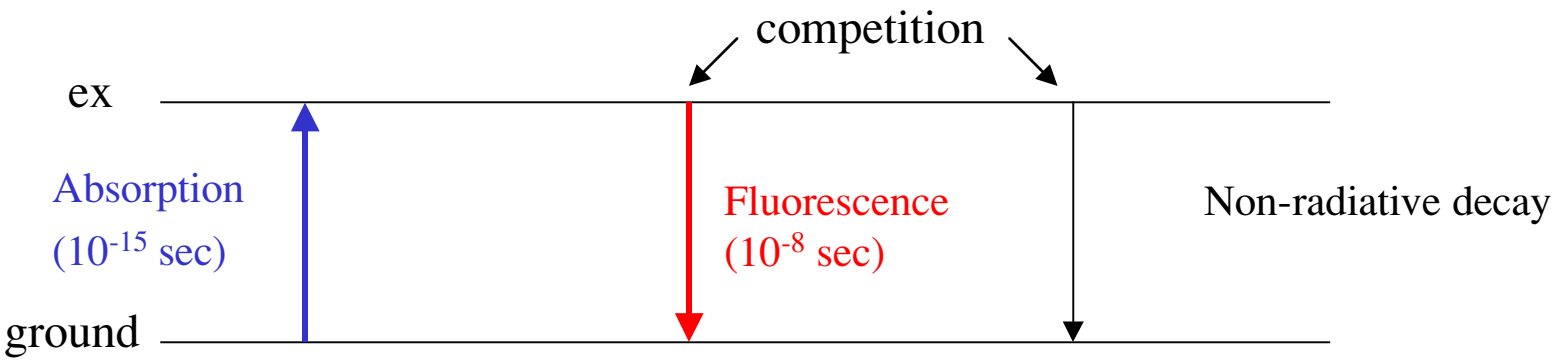
UV illumination



Science (2002) 295, 92

Fluorescence Spectroscopy

Decay from excited state back to ground state with emission of a photon



Fluorescence properties depend on what happens to the molecule during the ~10^{-8} sec during which it is excited

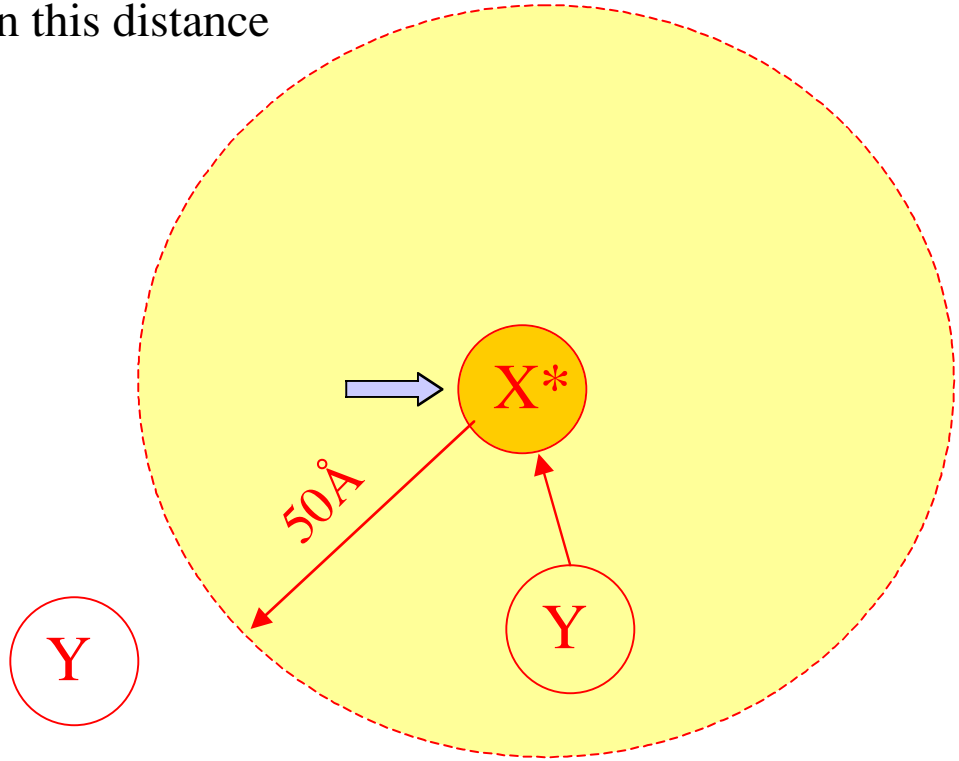
-dynamic properties of biomolecules are probed by fluorescence

Example of what can happen to the excited state: collision with another molecule

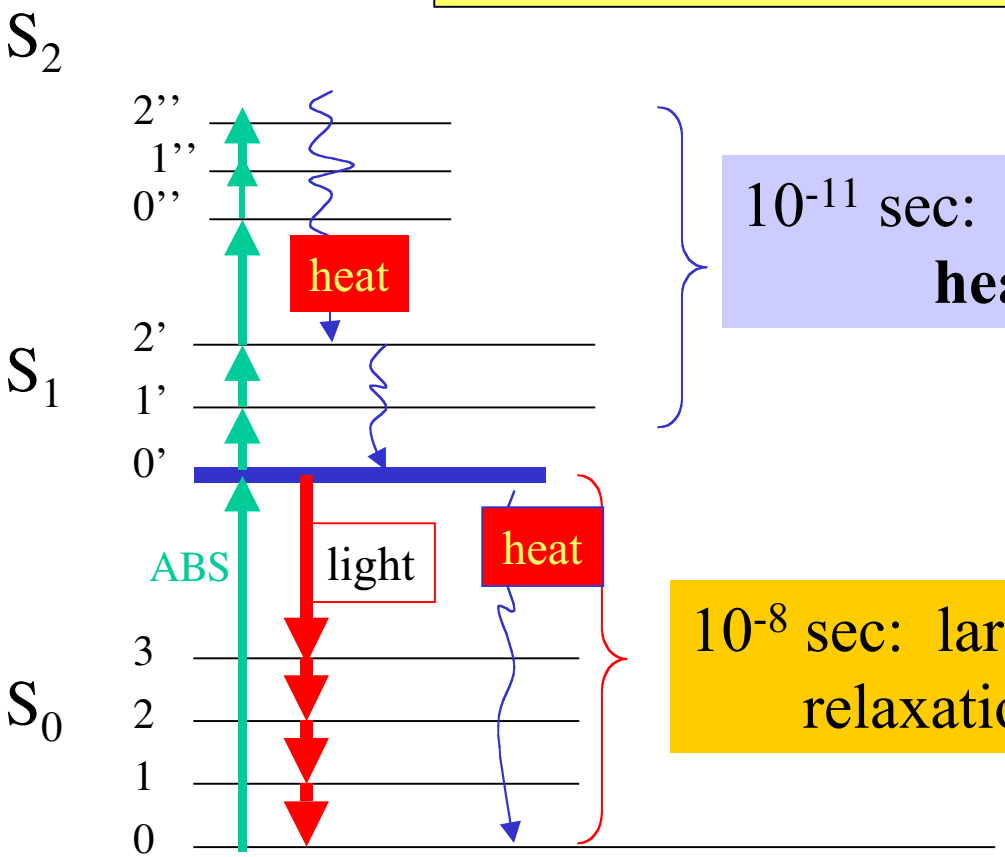
$$X^* + Y \xrightarrow[k]{} XY$$

In 10^{-8} sec, a small molecule can diffuse ≈ 50Å (water at 25°C)

So an excited state (X*) can be perturbed by a collision with molecule (Y) that is within this distance



Within about 10^{-11} sec, all excited state molecules lose heat to decay to the lowest vibronic level of the first excited state



10^{-11} sec: internal conversion **heat** loss to solvent

10^{-8} sec: larger energy gap results in slower relaxation to ground electronic state

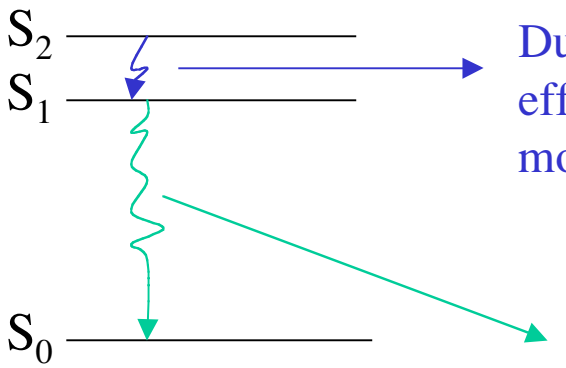
Collisions with solvent molecules

The rate of internal conversion within the excited state manifold is due to loss of energy to the solvent via collisions and is dependent on the rate of collisions with the solvent

collision rate = k_{coll} [solvent]

$\sim 10^{10}$ M^{-1} sec^{-1} 55M for water

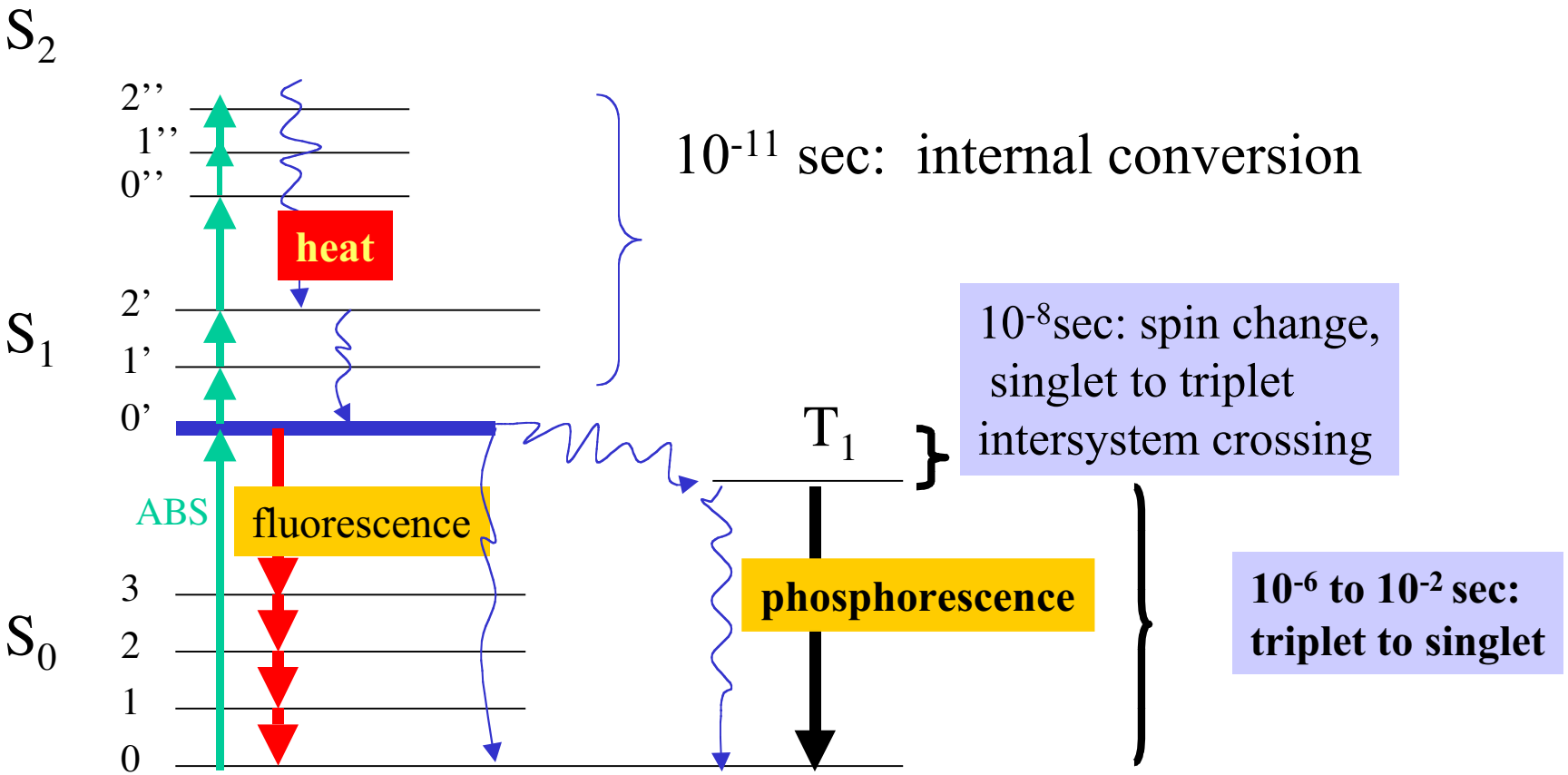
In 55 M solvent, the rate of collisions of a single molecule is $\approx 10^{11}$ -10^{12} sec^{-1}



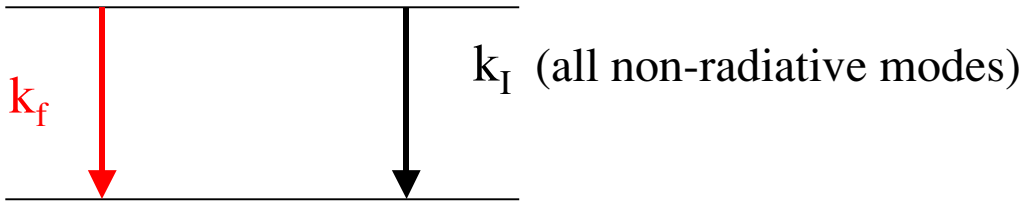
Due to small energy gaps, collisions are very effective so that within 10^{-11} sec all excited state molecules are in the lowest vibrational state of S_1

However, the non-radiative loss of energy to the ground state, Internal Conversion to S_0, is <u>much slower</u> due to the large energy gap

Phosphorescence results from emission of a photon from the lowest triplet state (electron spins aligned)



What can you monitor by fluorescence?



Competition between rate of fluorescence and rates of non-radiative modes

Faster non-radiative de-excitation

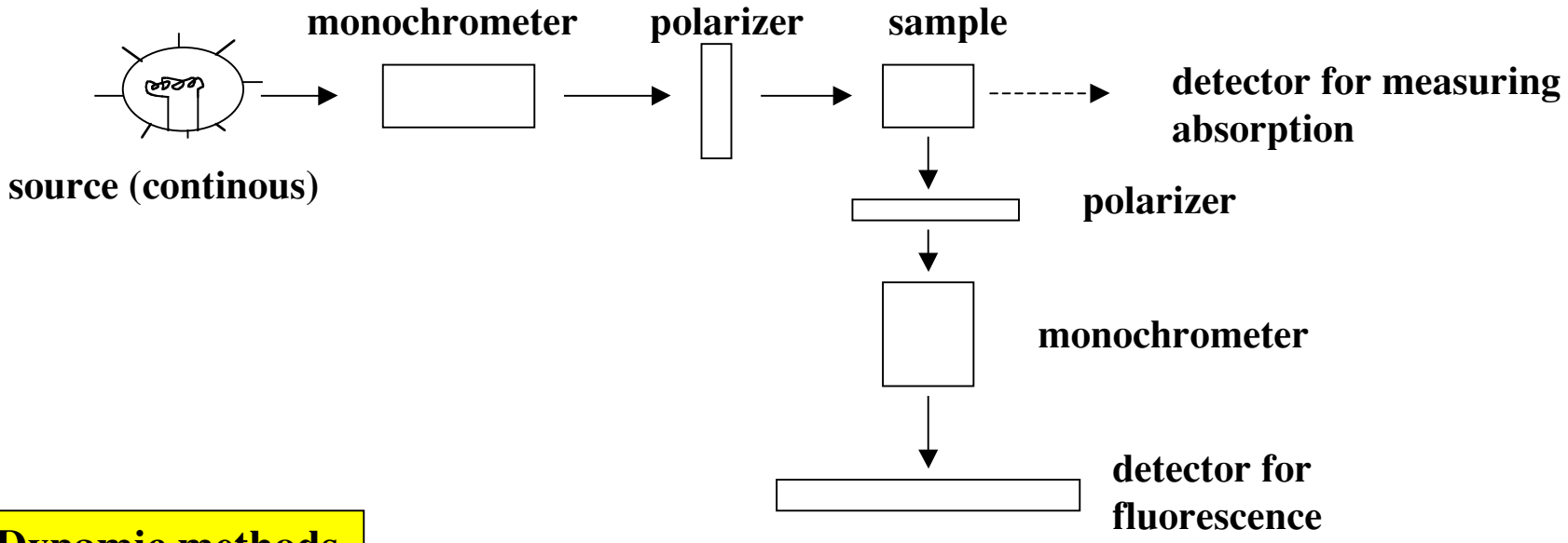
⇒ less fluorescence

Many processes occur to a molecule during its excited state lifetime that influence the fluorescence:

(1) collisions (quenching) ⇒ accessibility
(2) energy transfer ⇒ distance
(3) solvent relaxation ⇒ solvent polarity
(4) chromophore rotation ⇒ molecular size/viscosity

Most common methods of measuring fluorescence

Steady state measurement



Dynamic methods

Pulse spectroscopy: Measure the intensity of emitted light after a very brief pulse of light (nsec duration)

-measure fluorescence lifetime

Also: **Phase and Modulation Spectroscopy**

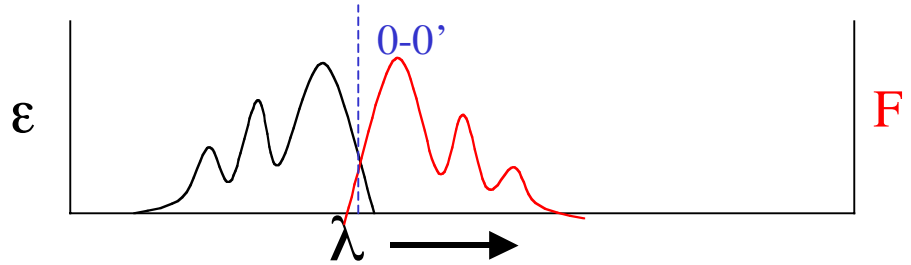
See Ann. Rev. Biophys Bioeng. (1984) 13, 105-124

Excitation Spectrum

Dependence of the emission intensity on the excitation wavelength, measured at a constant emission wavelength

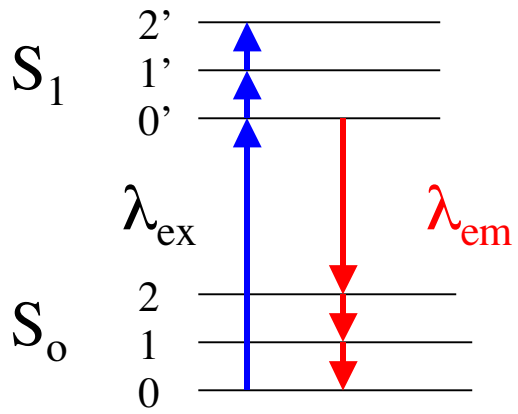
Emission Spectrum

Wavelength dependence of the emission intensity, measured with a constant excitation wavelength



What parameters are measured?

(1) **Excitation Spectrum**
(2) Emission Spectrum
(3) Quantum Yield (Q_f)
(4) Lifetime (τ)
(5) Polarization (Anisotropy)



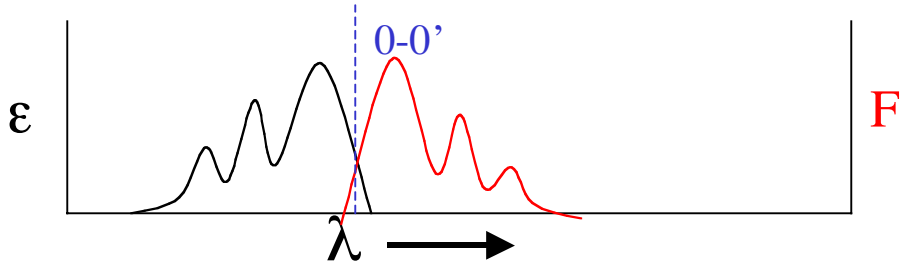
Excitation spectrum

Measure fluorescence as a function of excitation wavelength at fixed λ_{em}

Identical to the absorption spectrum

Since all emission comes from the same energy level ($S_{\upsilon'=0}$), the only thing that changes with λ_{ex} is the probability of absorption

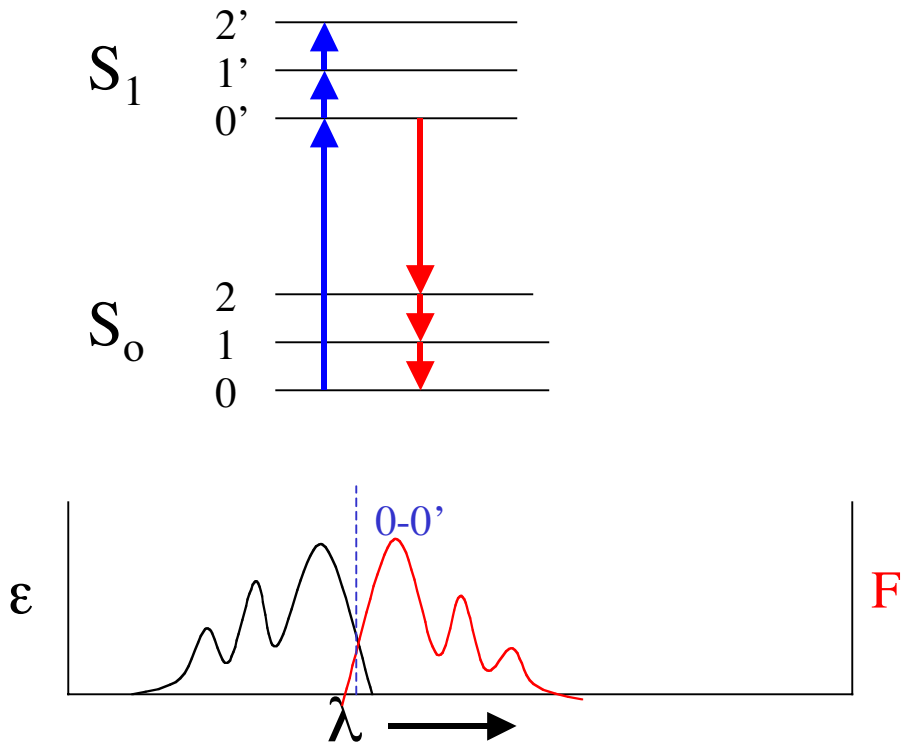
$$F(\lambda_{ex}) \propto \varepsilon(\lambda_{ex})$$



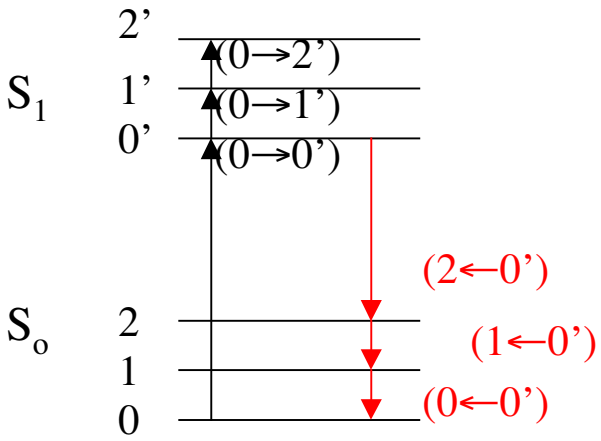
Emission spectrum

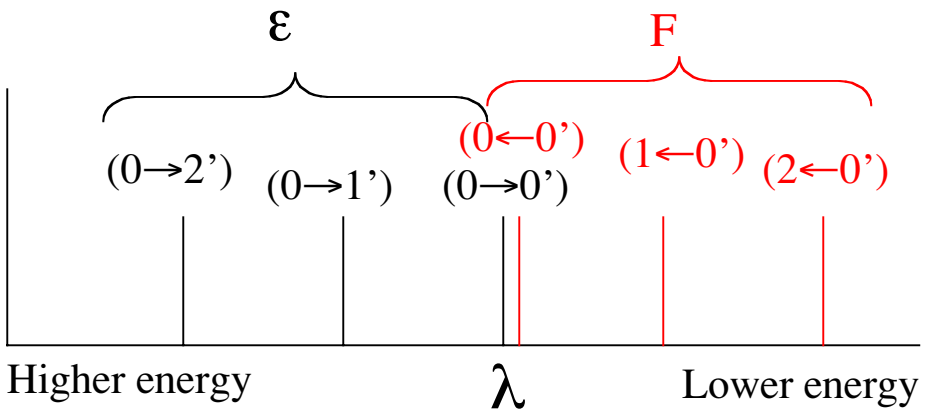
Measure fluorescence at fixed λ_{ex} as a function of λ_{em}

Emission spectrum is always red-shifted (lower energy) compared to the absorption spectrum

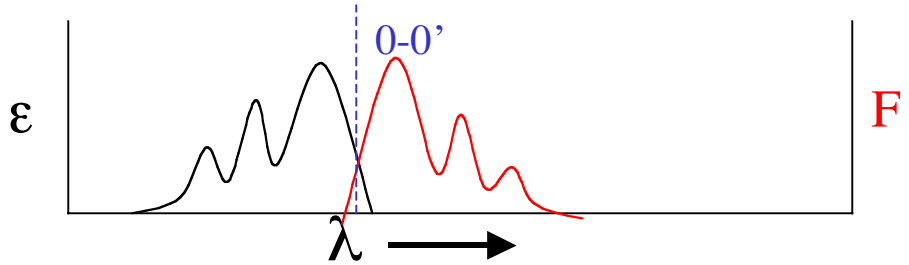


Fluorescence: Mirror-Image Rule

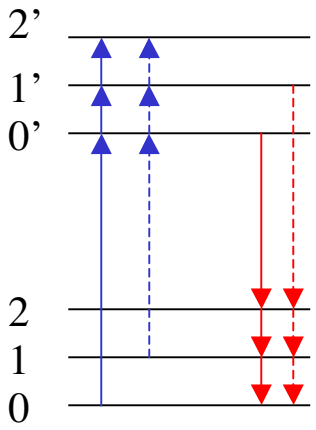




Franck-Condon Overlap Factors: Prob (0'→2') ≅ Prob (2'←0') etc



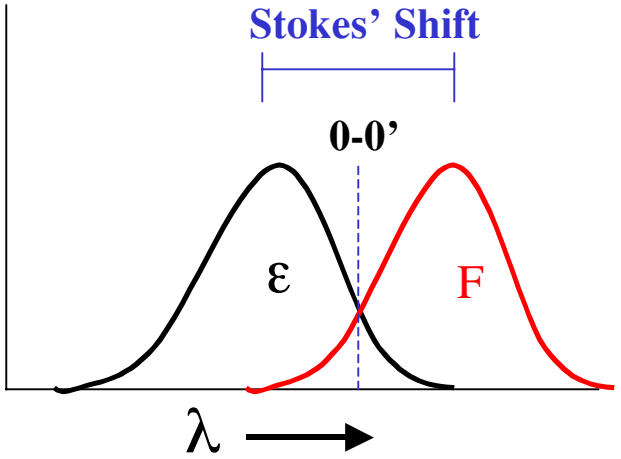
Fluorescence: Self-Overlap



Thermal population of υ’ = 1 state results in some molecules having blue-shifted emission

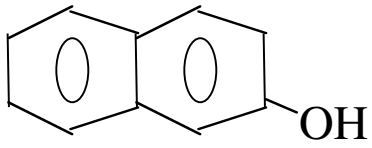
Solvent-induced heterogeneity also results in self-overlap

Thermal population of υ = 1 state results in some molecules with red-shifted absorbance



The ground state and excited state of molecules can have very different properties

Example 1: β-naphthol

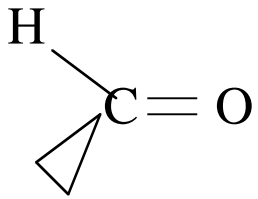


pK_a (S_o) = 10.8

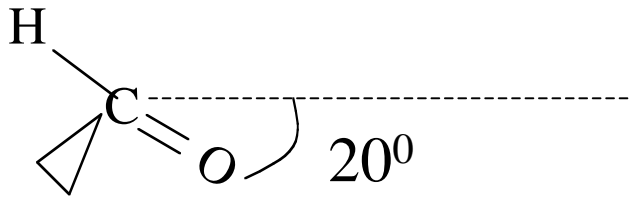
pK_a (S_1) = 3.5

Deprotonates upon excitation at pH 7

Example 2: Formaldehyde changes geometry and permanent dipole upon excitation



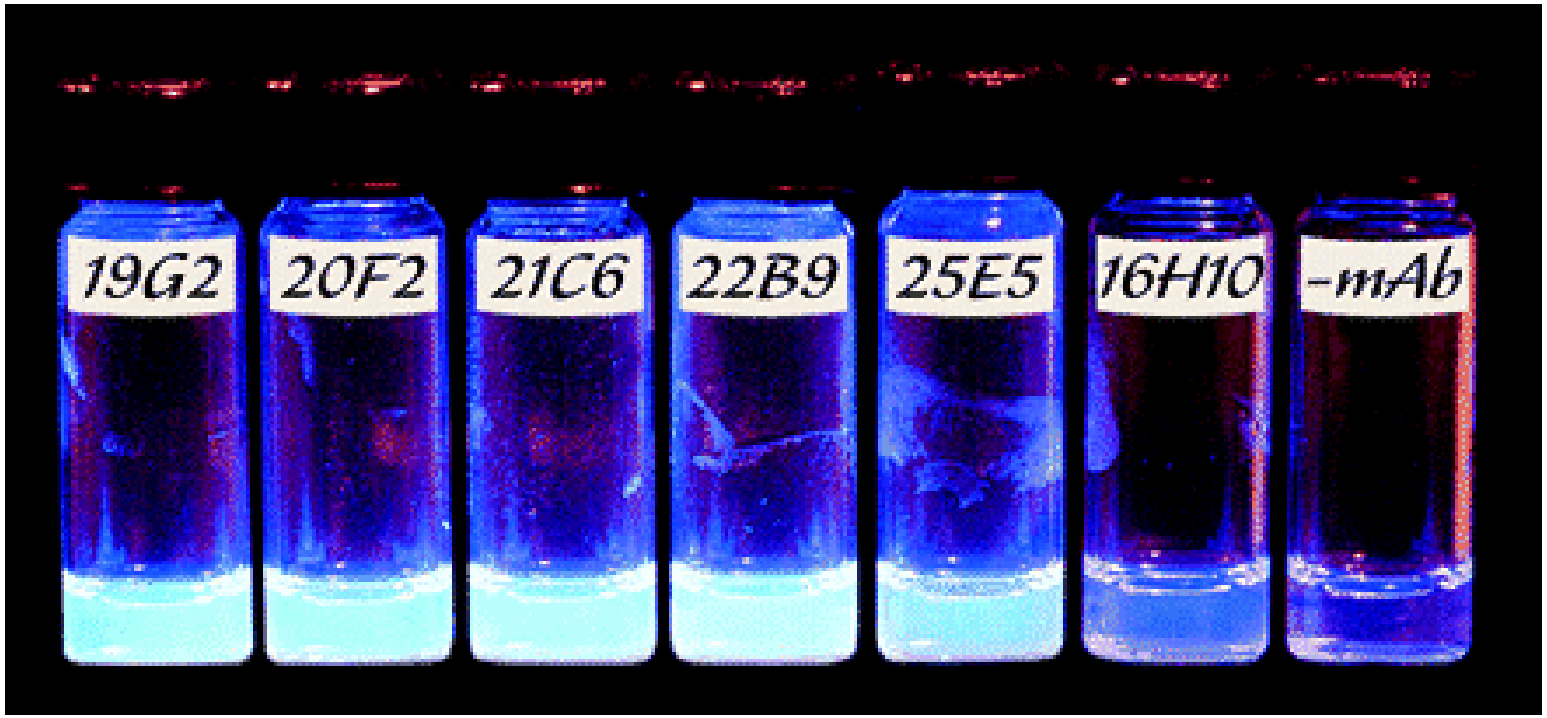
S_0, planar

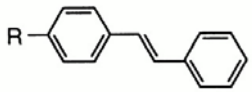


S_1, non-planar

An example where the excited state has different properties than the ground state

The case of the Blue-Fluorescent Antibodies raised against *trans*-stilbene

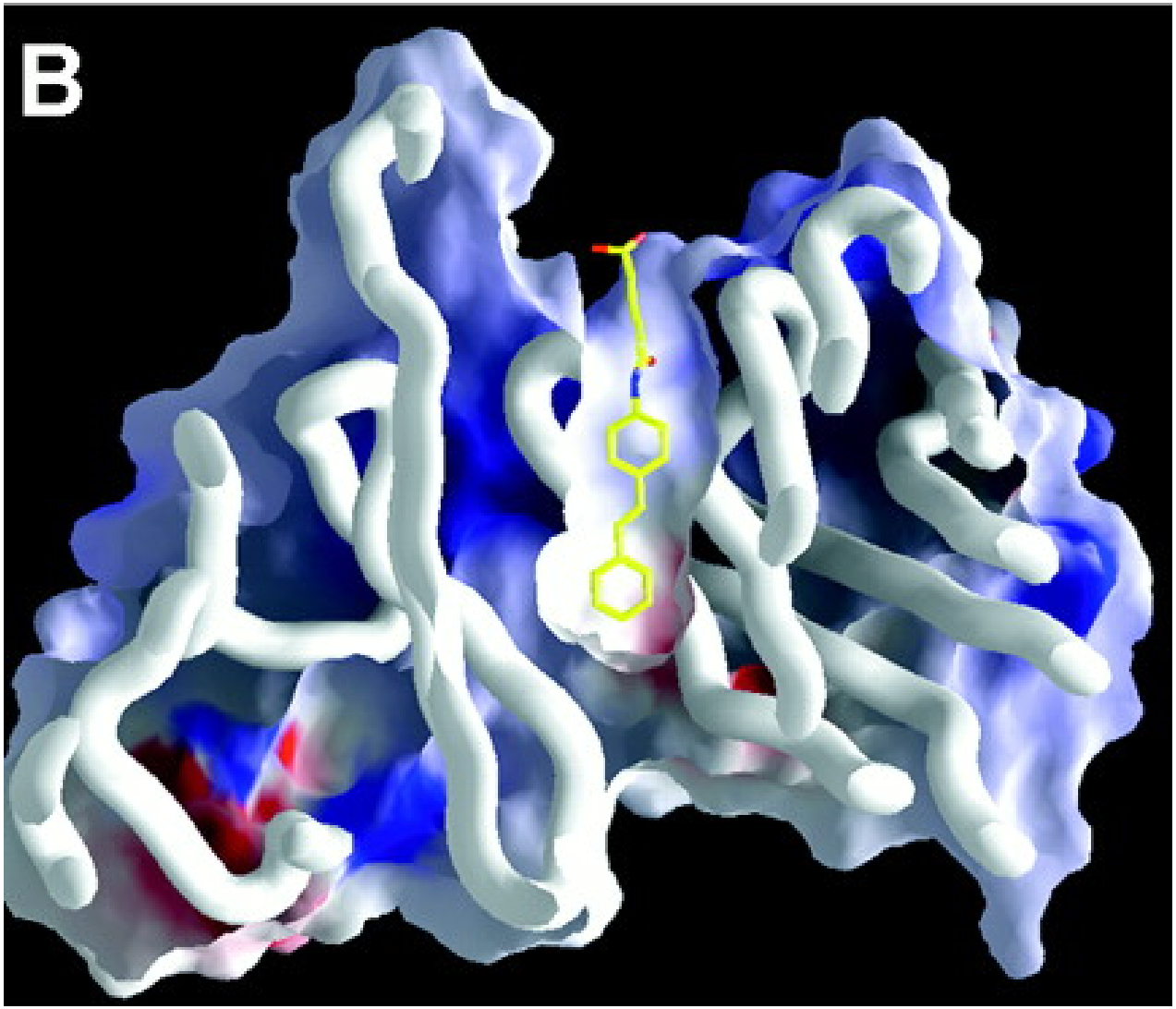




trans-stilbenes

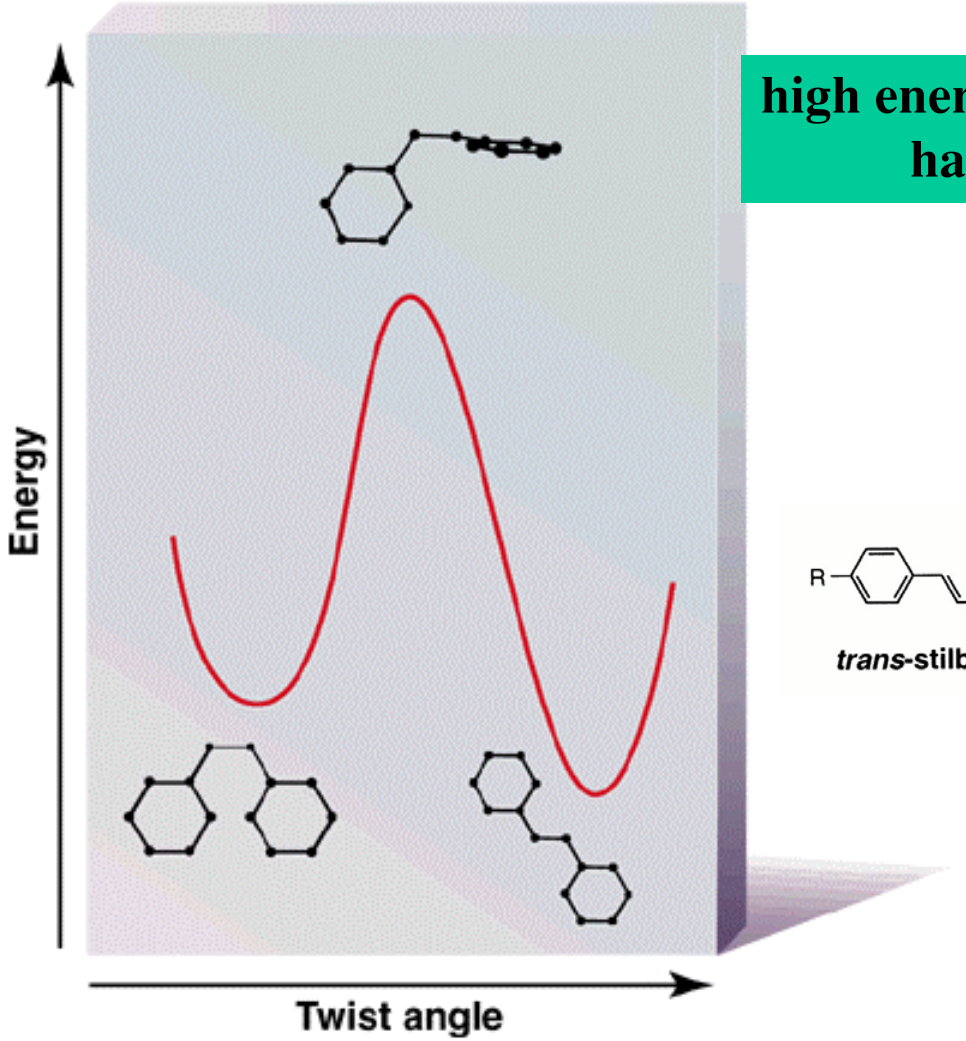
Science (2000) 290, 307-313

Antibodies binds to *trans*-stilbene:
The protein inhibits thermal relaxation from the excited state



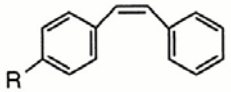
Science (2000) 290, 307-313

Ground State of stilbene: 45 kcal/mol energy barrier to twist from the *trans* to the *cis* conformation



high energy transition state has a 90° twist

***trans*-stilbenes**

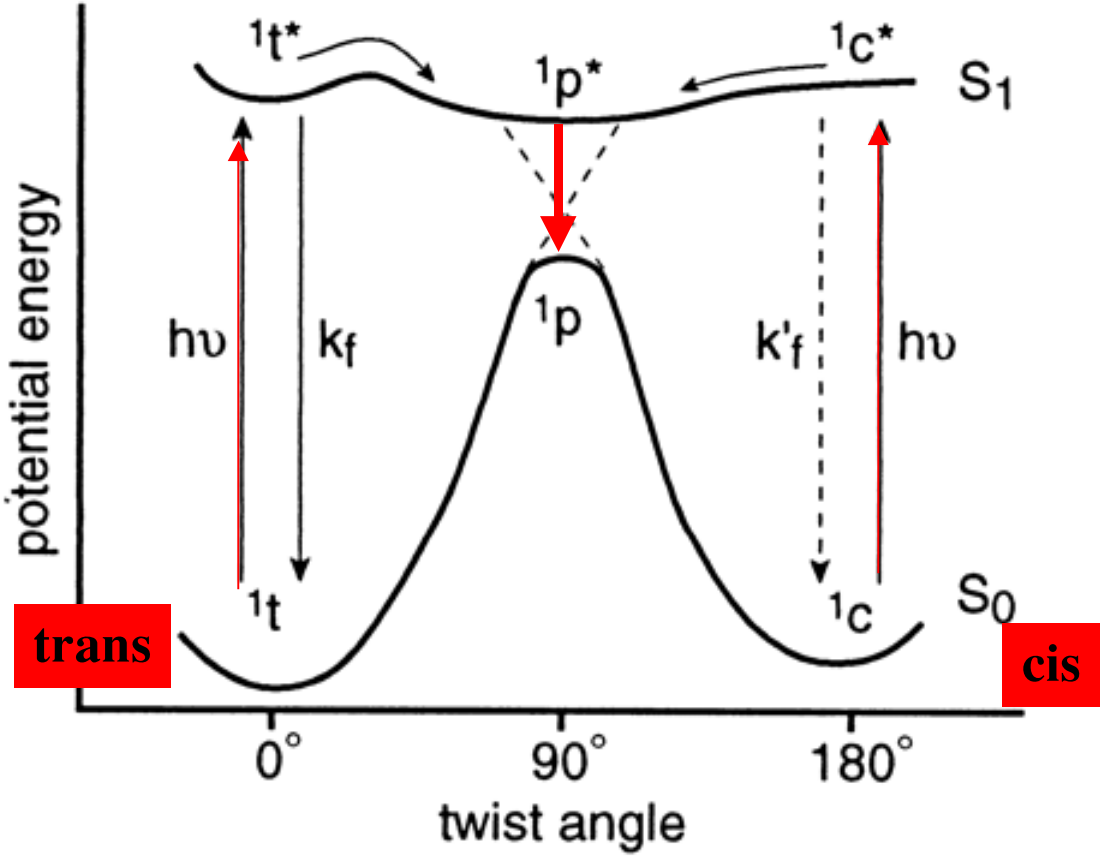


***cis*-stilbenes**

Science (2000) 290, 307-313

Excited state isomerizes very easily
low energy barrier to 90° twist
(electron is in antibonding π^* orbital)

But the 90° twisted state is the main pathway for thermal relaxation, so most of the energy is lost as heat after absorption from either *cis* or *trans* stilbene



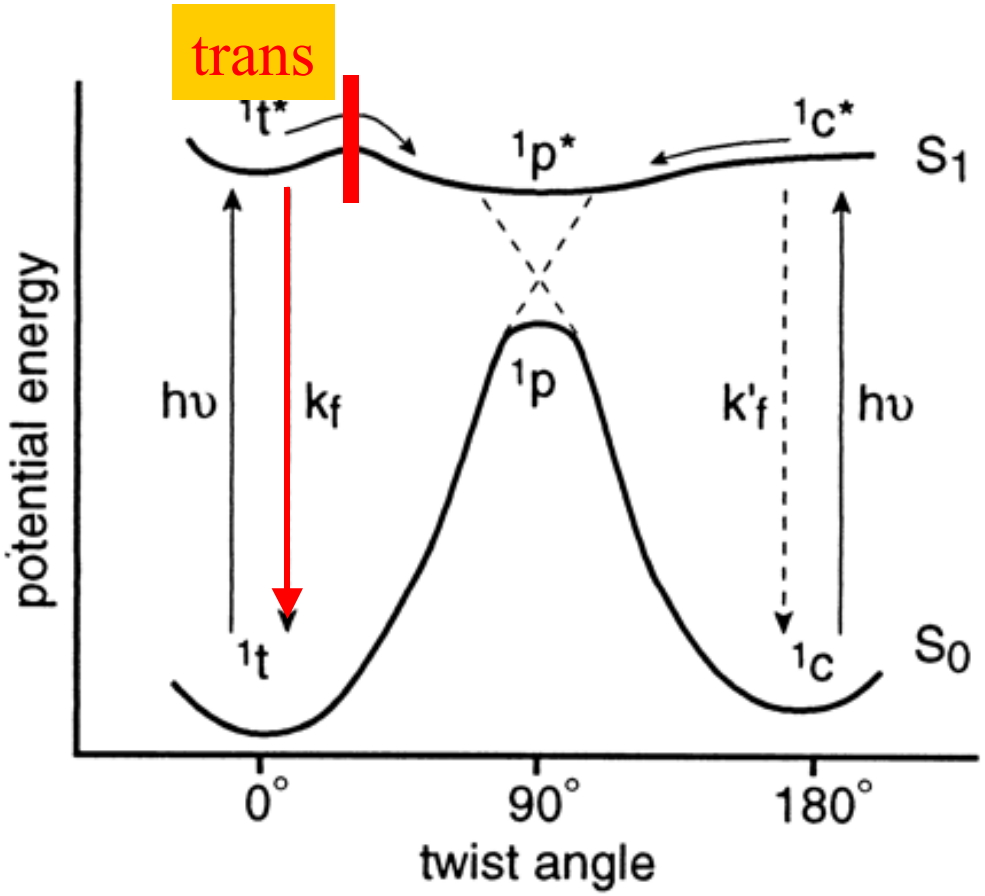
only 2% of molecules fluoresce

Science (2000) 290, 307-313

Antibody binding to *trans*-stilbene:

Locks the stilbene in the trans-configuration

70% of excited molecules that absorb a photon fluoresce
(only 2% for stilbene in solution)

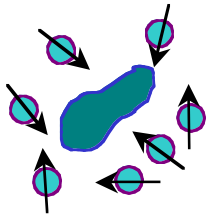


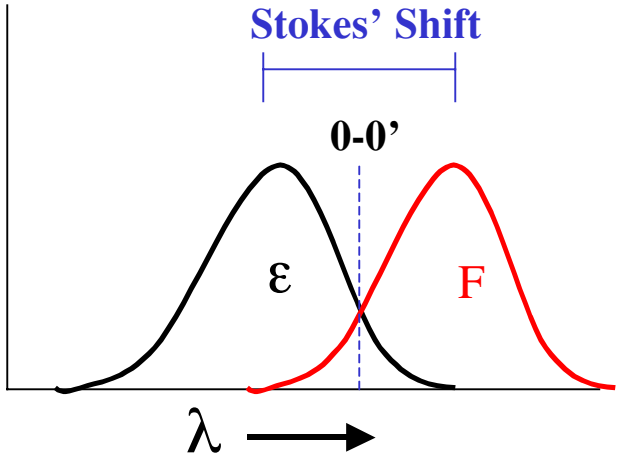
Science (2000) 290, 307-313

Stokes shift

Difference in wavelengths of the peaks of the excitation and emission spectra

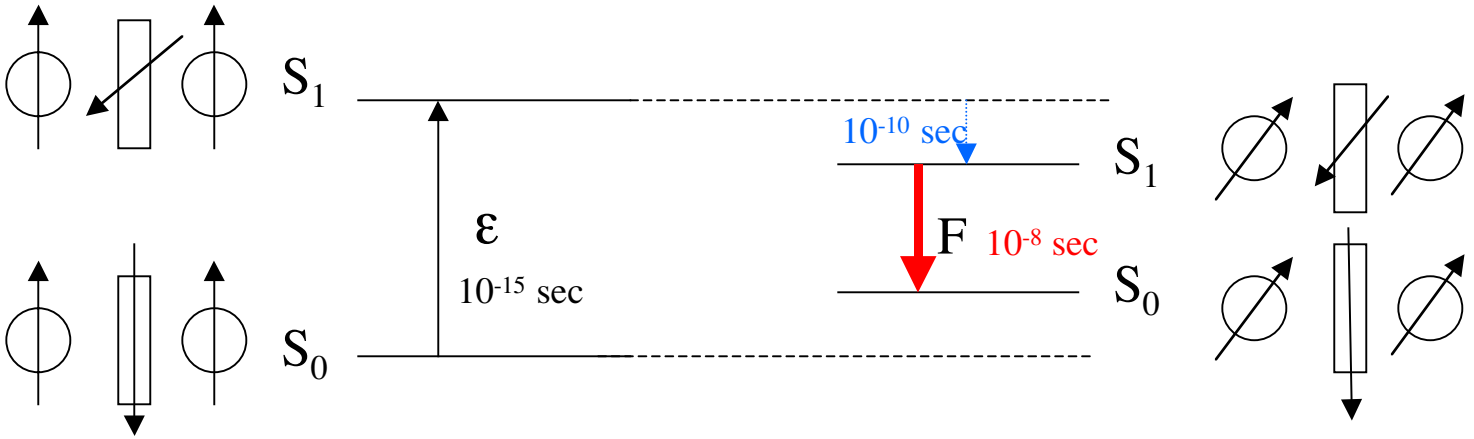
Due to solvent relaxation around the alteration in the electronic redistribution in the excited state.





The Stokes shift is a measurement of the environment around the fluorophore

Solvent relaxation and the Stokes Shift



Absorption ~ 10^{-15} sec

Solvent relaxation ~ 10^{-10} sec

Fluorescence ~ 10^{-8} sec

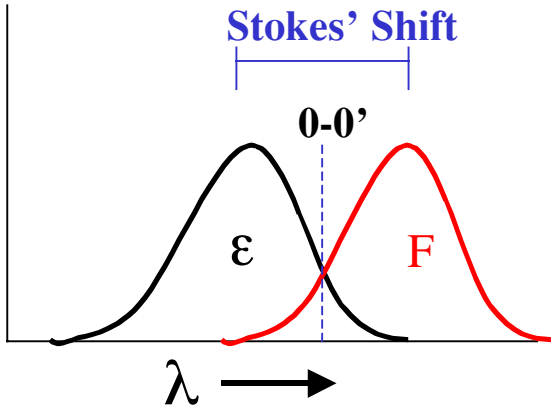
Permanent Dipoles of solvent re-orient to adjust to altered dipole of fluorophore upon excitation

dipole-dipole interaction

Requires:

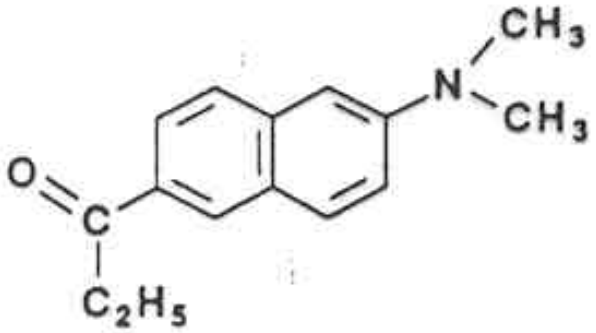
(1) solvent polarity (dielectric, ε)

(2) mobility of solvent (reorientation of solvent dipoles)

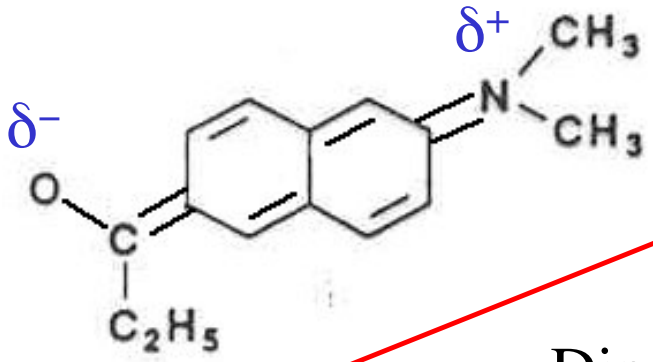


PRODAN

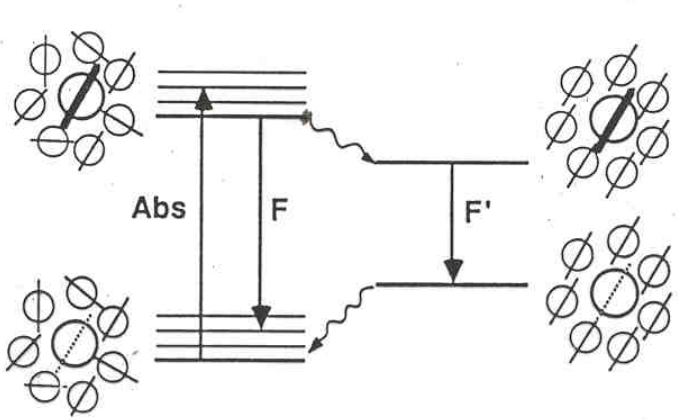
μ



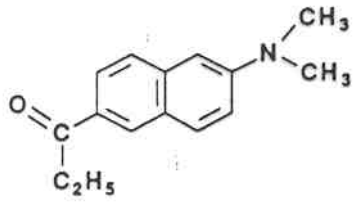
μ^*



Dipole moment of excited state

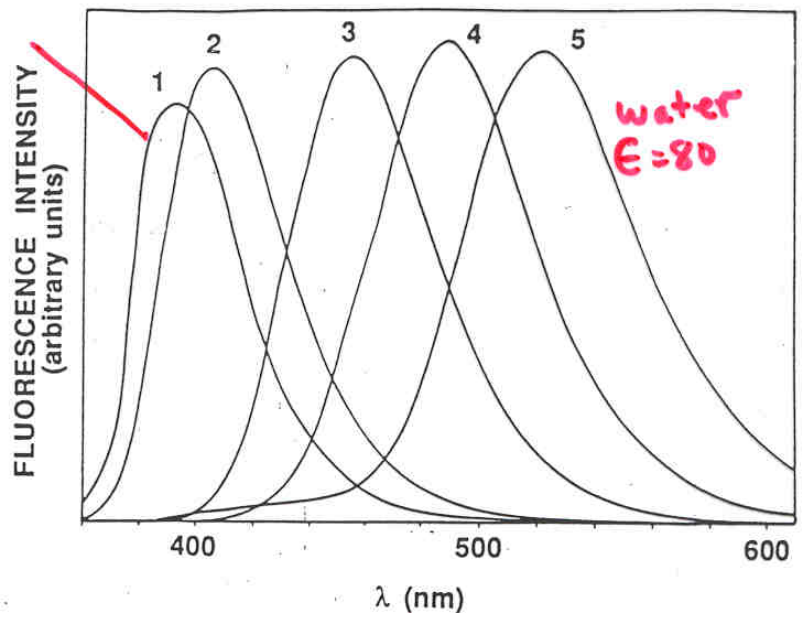


Direction of the permanent dipole is the same, but magnitude is greater in the excited state



PRODAN

In cyclohexane
$\varepsilon = 2$



PRODAN in cyclohexane (1), chlorobenzene (2), dimethylformamide (3), ethanol (4) and water (5).

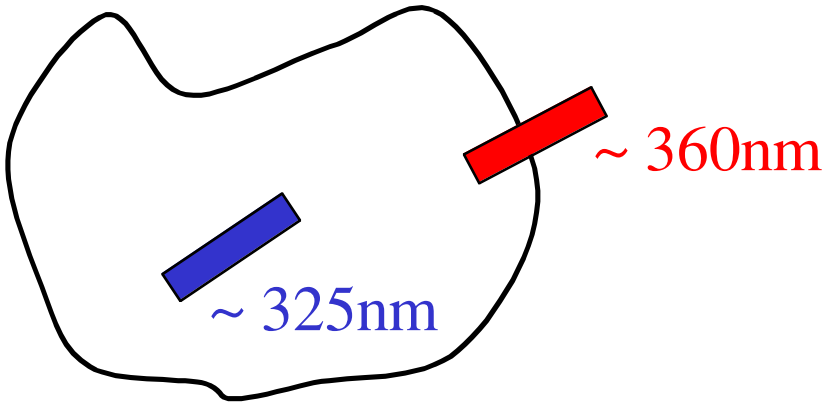
Stokes shift: example of tryptophan fluorescence

tryptophan

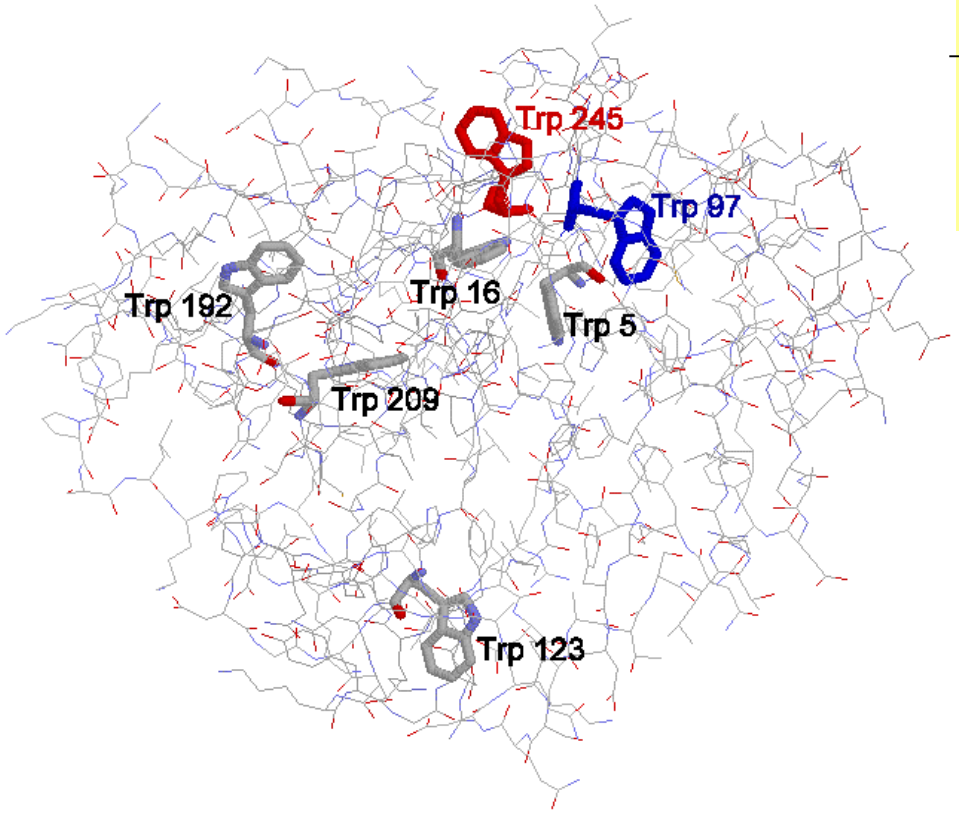
In non-polar solvents	λ_{em} ~ 325nm
In polar solvents	λ_{em} ~ 360nm

(1) Protein denaturation ⇨ tryptophan emission often is red-shifted

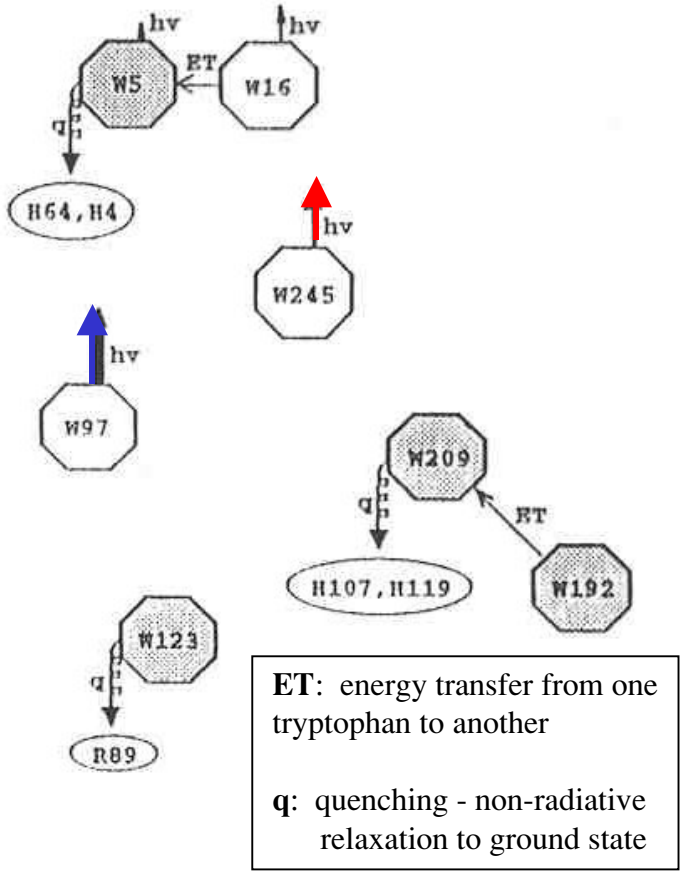
(2) buried vs exposed tryptophans in native proteins can be distinguished



Fluorescence from the 7 Tryptophan Residues of Human Carbonic Anhydrase II



% total fluorescence
W97 : 52%
W245 : 38%



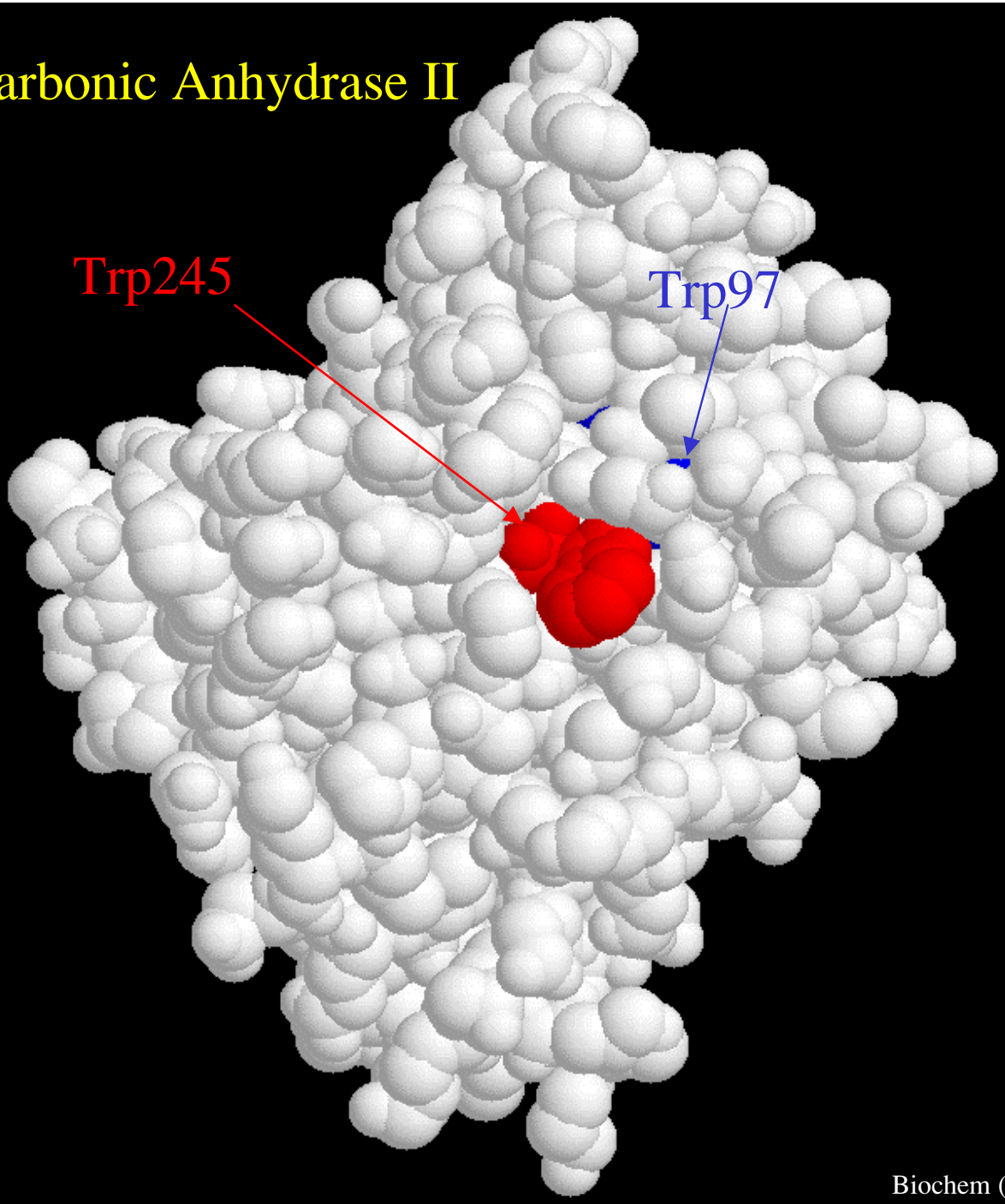
ET: energy transfer from one tryptophan to another

q: quenching - non-radiative relaxation to ground state

5 of the 7 tryptophans have little fluorescence due to enhanced thermal relaxation (quenching) from interactions with other amino acids such as histidines

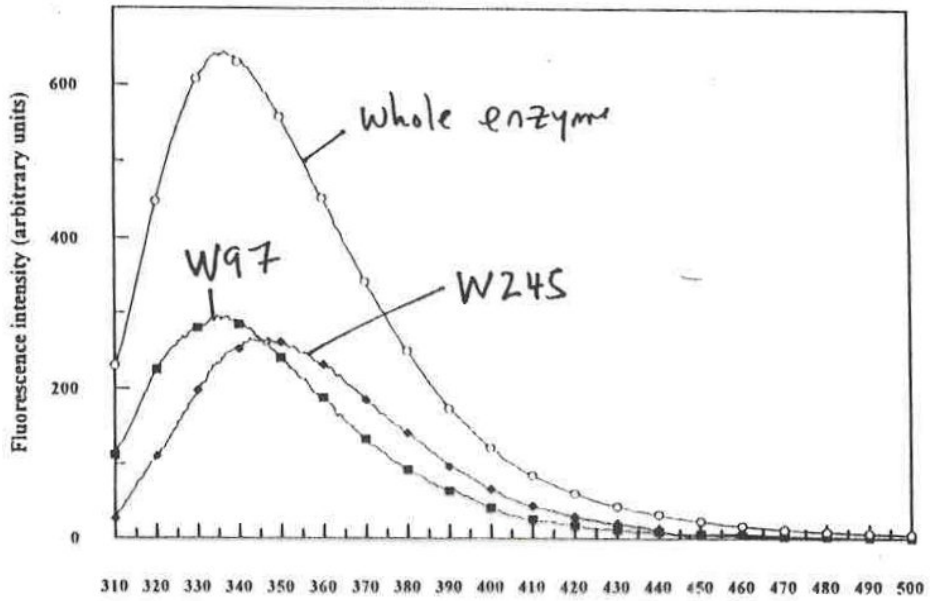
Biochem (1995) 34, 1011-1021

Human Carbonic Anhydrase II



Biochem (1994) 33, 14281-88)

W97 and W245 Fluorescence



W97 : buried : blue shifted spectrum

W245 : exposed to surface : red shifted spectrum

Table 3: Solvent Accessible Surface Area for Individual Tryptophan Side Chains of HCA II

Trp residue	surface area (Å²)[a]	fraction R[b]	fraction T[c]
5	22	0.10	0.23
16	4	0.02	0.04
97	0	0	0
123	6	0.03	0.06
192	13	0.06	0.14
209	4	0.02	0.04
245	47	0.22	0.49

[a] Solvent accessible surface area in angstroms for the specific Trp residue. [b] Fraction of solvent accessible surface area to side chain atom of a Trp residue. [c] Fraction of solvent accessible surface area to total solvent accessible surface area of the sum of Trp residues in HCA II. Accessible surface area of Trp in a Gly-Trp-Gly tripeptide in an extended conformation has been used in this calculation. The total solvent accessible surface area for the Trp side chain in this tripeptide is 217 Å² (Miller et al., 1987).

Biochem (1995) 34, 1011-1021