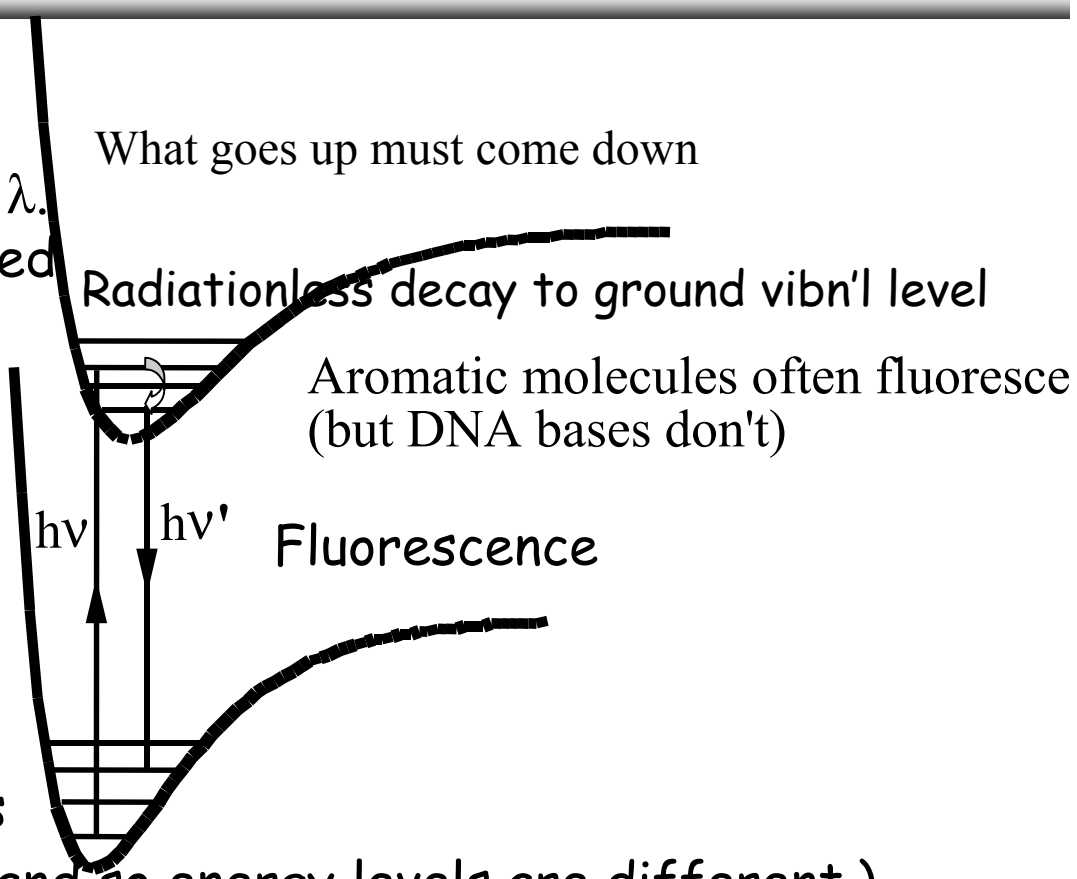
Fluorescense

Two types of spectra:
Excitation: Detection λ fixed and scan excitation λ.
Emission: Excitation λ fixed (usually at A_{max}) & scan detection λ.

Excitation ~ absorbance
(Differences due to:
poor λ calibration
or
solvent structure changes
around excited molecule and so energy levels are different.)



Advantages of fluoresence

More sensitive than absorbance — require $A < 0.1$ units
else get problems

Very sensitive to environment — changes in fluorecence
used to probe protein folding / unfolding,
DNA/drug interactions (if drug is fuorescent),
solvent exposure
effects of pH, temperature, ionic strength *etc.*

Fluorescence signal is proportional to concentration of
particular fluorophore in given environment
→ determine $L_f + S_f \leftrightarrow L_b$ equilibrium binding
constants

$$K = \frac{[L_b]}{[L_f][S_f]}$$

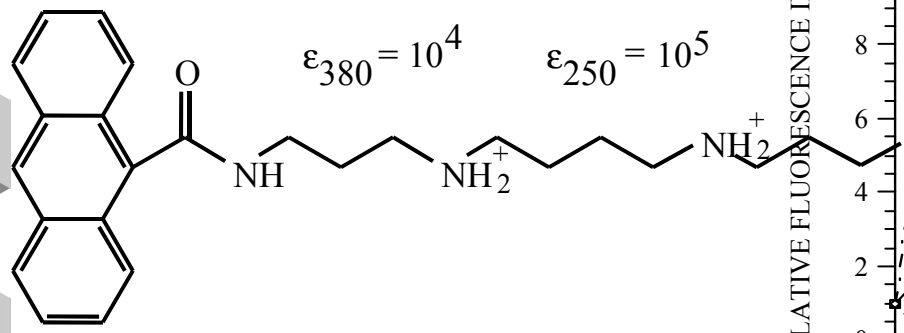
Fluorescence quenching/enhancement

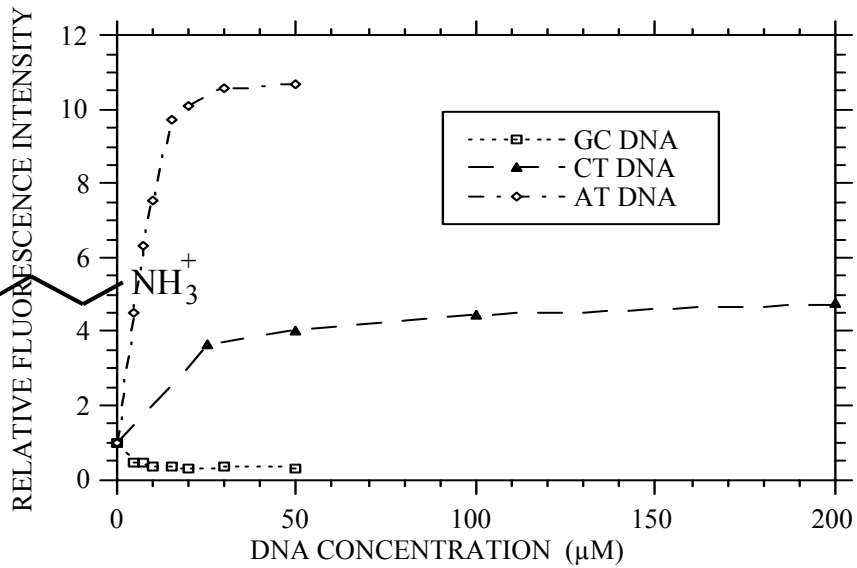
Some molecules provide an alternative route for energy loss — they quench the fluorescence.

Other molecules inhibit quenchers — they enhance it

Quenchers include: oxygen (O_2), $[Fe(CN)_6]^{4-}$, I_2, I^-

DNA binder:





Fluorescence polarisation anisotropy

Excite molecule with linearly polarized light

If the molecule hasn't moved then the emitted photon will have the same polarization

Any amount of depolarization means the molecule has rotated before emitting

IF you know the excited state lifetime then you can calculate the mobility of the fluorophore — determine rotation rate of macrmolecules, motion of drugs on DNA *etc.*

Fluorescence lifetime & quantum yield

Depends on fluorophore environment

Measure lifetimes to determine number of different species in solution

$$\phi = \frac{\text{no. emitted photons}}{\text{no. absorbed photons}} = \frac{\text{rate of photon emission}}{\text{rate of photon absorption}}$$

ϕ = 1 if an absorbed photon leads to emitted photon

Fluorescence resonance enery transfer

Energy transfer experiments if distance < 10-18 Å
Some trp → tyr transfer
Alt fluorescence label and measure transfer
Acceptor must be a fluorophore. Donor need not be.
E.g. DNA

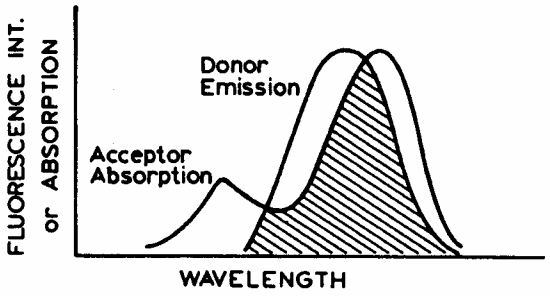


Figure 1.15. Spectral overlap for fluorescence resonance energy transfer (RET).

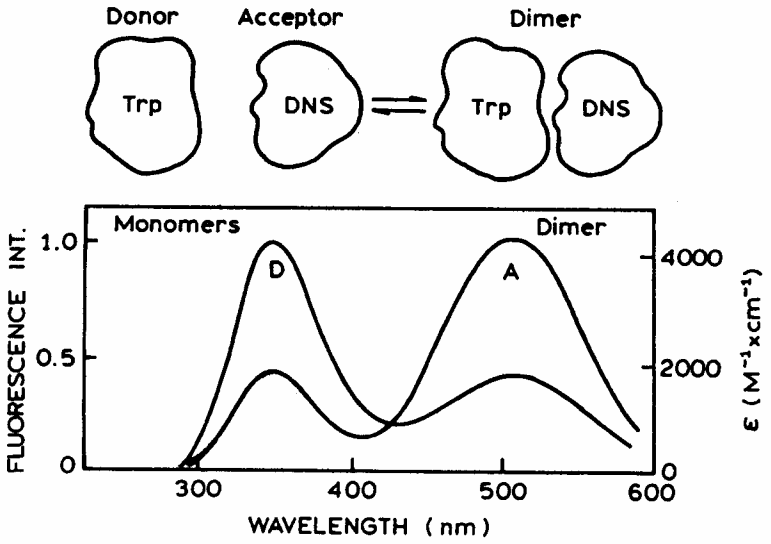


Figure 1.23. Energy transfer between donor (D) and acceptor (A)-labeled monomers, which associate to form a dimer. In this case the donor is tryptophan and the acceptor is a dansyl group (DNS).

The experiment

Excitation light comes in to one face of cuvette and usually measure fluorescence coming out at 90°

Need 4 clear sides on cuvette

Don't usually measure fluorescence at 180° as signal swamped by unabsorbed light

Don't (usually) measure fluorescence at 0° as would block excitation beam

Usually report relative intensities as measure depends on instrument. lab temperature, *etc.*

Proteins and aromatic amino acids

Tyrosine, tryptophan and phenylalanine all fluoresce
For proteins usually excite at 280 nm (so miss Phe)
Can selectively excite trp at 295 – 305 nm
Tyr emission ~ indep. of solvent polarity
Trp emission very solvent dependent
Tyr > trp in solution but
native proteins excited at 280 nm: fluorescence dominated by trp
Blue shift of fluorescence in native vs in H_2O
Tyr fluorescence in native proteins SMALL — easily quenched by neighbouring groups
Trp Hydrophobic: Fl max ~330 nm;
Hydrophilic: Fl max ~350 nm

Protein fluorescence

Proteins are usually excited at 280 nm and their fluorescence is dominated by tryptophans.

If excite at 295 nm only get tryptophan fluorescence.

Due to hydropobic/hydrophilic shift, fluorescence indicates folding or unfolding of proteins

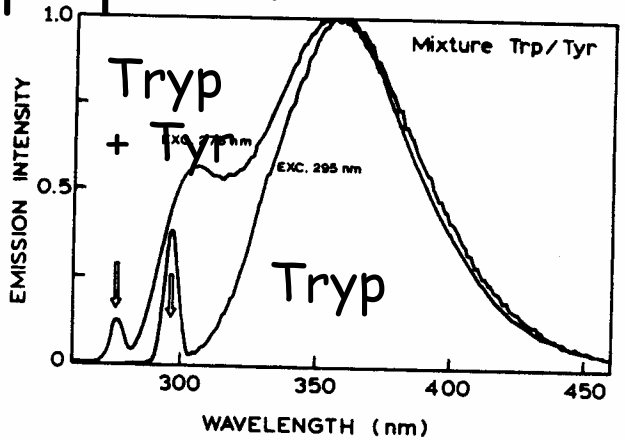
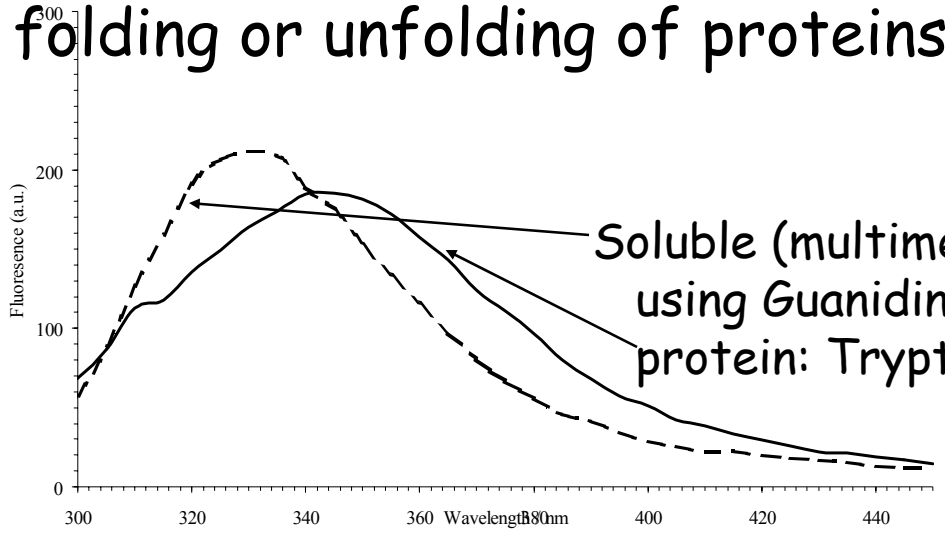


Figure 2.22. Emission spectra of tryptophan with a trace impurity of tyrosine, obtained with excitation at 275 and 295 nm. From Ref. 8.



Soluble (multimer of) Pre-PSbW and Unfolded using Guanidinium Chloride to denature protein: Tryptophan fluorescence

Mutants

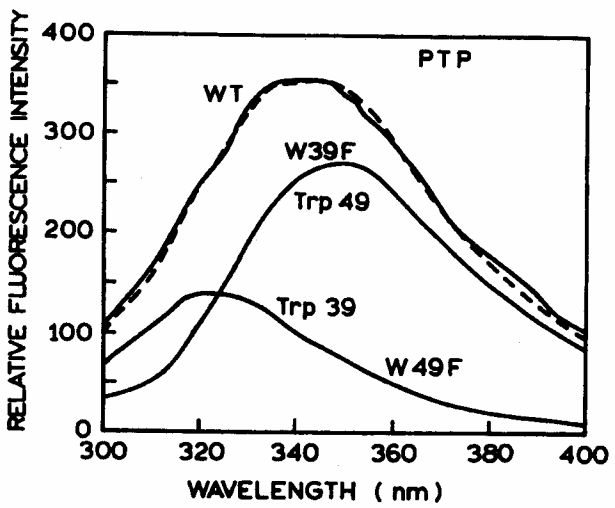


Figure 16.42. Emission spectra of wild-type (WT) protein tyrosyl phosphatase (PTP) and the single-tryptophan mutant proteins W39F and W49F at 20 °C. Excitation was at 295 nm. The composite spectrum from the sum of the contributions due to W39F and W49F is indicated by the dashed curve. Revised from Ref. 113.

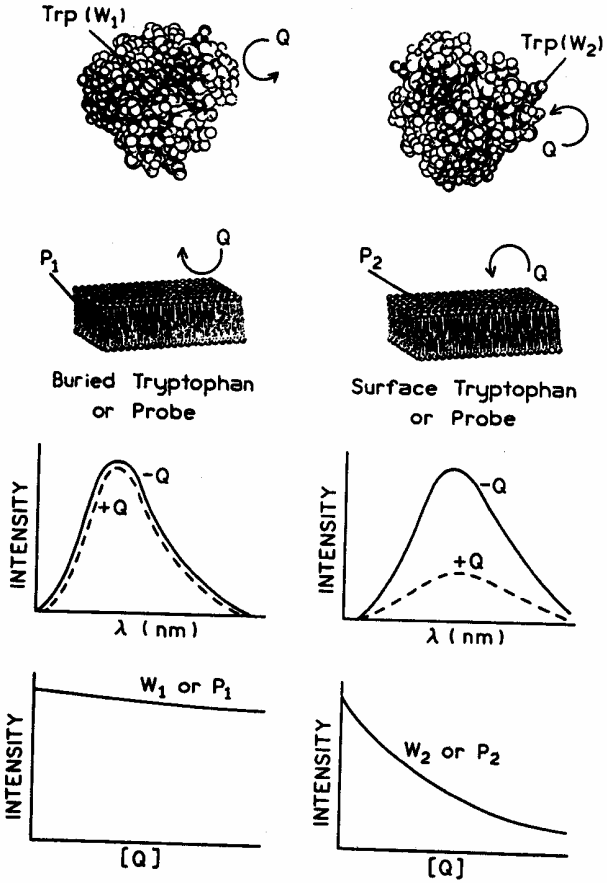
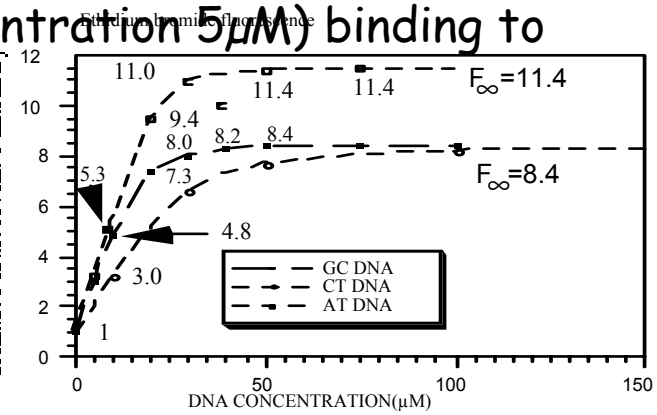


Figure 1.21. Accessibility of fluorophores to the quencher (Q^-). Reprinted, with permission from Wiley-VCH, STM, from Ref. 20.

Binding constant from fluorescence

Determine K for ethidium bromide (EB, concentration 5μM) binding to poly(dG-dC)$_2$ from the fluorescence data.



Assume each EB requires 4 DNA bases to bind.

$$\frac{L_{total}F}{F_{free}} = \frac{L_bF_b}{F_{free}} + \frac{L_fF_{free}}{F_{free}} = \frac{L_bF_b}{F_{free}} + \frac{(L_{total} - L_b)F_{free}}{F_{free}}$$

$$L_b = L_{total}\left(\frac{F - F_{free}}{F_b - F_{free}}\right)$$

$$K = \frac{L_b}{L_fS_f}$$

$$\frac{KS_f}{[DNA]} = \frac{L_b / [DNA]}{L_f} = \frac{r}{L_f}$$

$$\frac{r}{L_f} = \frac{K([DNA]/n - L_b)}{[DNA]}$$

GC data summary:

[DNA]/μM	*10*	*20*	*30*	*40*	*50*
F	*4.8*	*7.3*	*8.0*	*8.2*	*8.4*

Scatchard plots for binding constants: EB + AT example

$$\frac{r}{L_f} = \frac{KS_f}{C_M} = \frac{K}{n} - rK$$

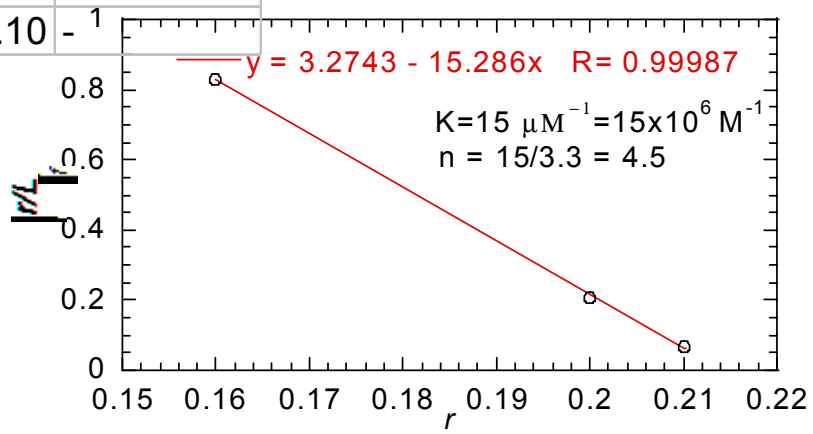
$r = L_b/[\text{DNA}] = L_b/C_M$

n = number of bases per ligand

$K = L_b/(L_f S_f)$ $S_f = [\text{DNA}]/n - L_b$

EB conc	DNA conc	F	Ff	Fb	Lb	r=Lb/[DNA]	r/Lf=r/(l-Lb)
5	10	5.3	1	11.4	2.07	0.21	0.07
5	20	9.4	1	11.4	4.04	0.20	0.21
5	30	11	1	11.4	4.81	0.16	0.83
5	40	11.3	1	11.4	4.95	0.12	2.57
5	50	11.4	1	11.4	5.00	0.10	-

Didn't use 40 μM data: big error



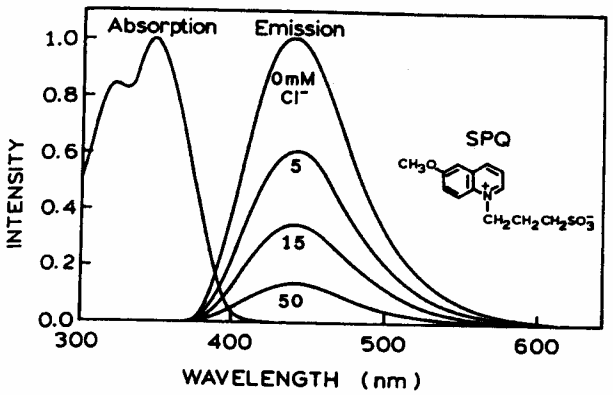


Figure 3.48. Absorption and emission spectra of 6-methoxy-*N*-(3-sulfopropyl)quinolinium (SPQ) in water with increasing amounts of chloride. From Ref. 74.

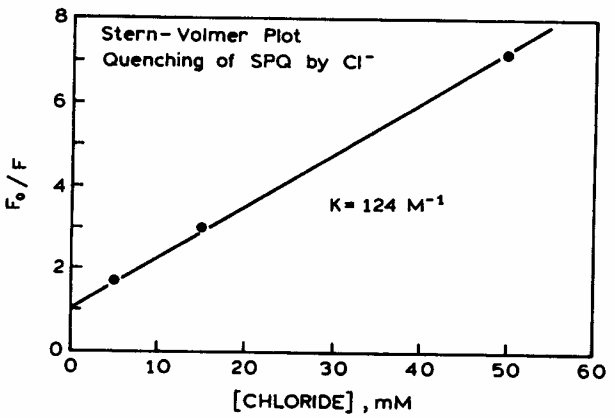


Figure 3.49. Stern–Volmer plot for the quenching of SPQ by chloride.

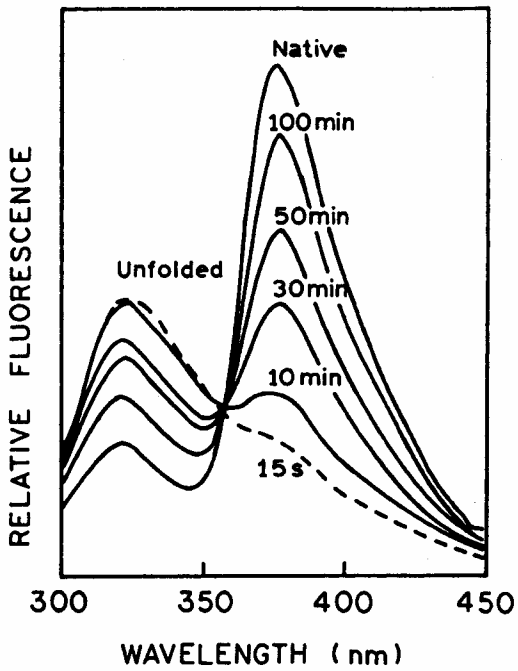


Figure 13.10. Emission spectra of trp-183 in PyP-5′-serine hydroxymethyltransferase during refolding. Excitation at 290 nm. Revised from Ref. 29.

It should be noted that the use of quenching-resolved spectra may not always be successful. One possible reason for failure would be that one of the tryptophan residues is not in a unique environment. In this case, each tryptophan residue may display more than one emission spectrum, each of which would be quenched to a different extent. In fact, quenching-resolved spectra have already been reported for proteins which contain a single tryptophan residue.[93,95] These results have been interpreted in terms of the protein being present in more than a single conformational state. Also, since a single tryptophan residue can display a multiexponential decay, there is no *a priori* reason to assume that the residue is quenched with the same quenching constant at all emission wavelengths.

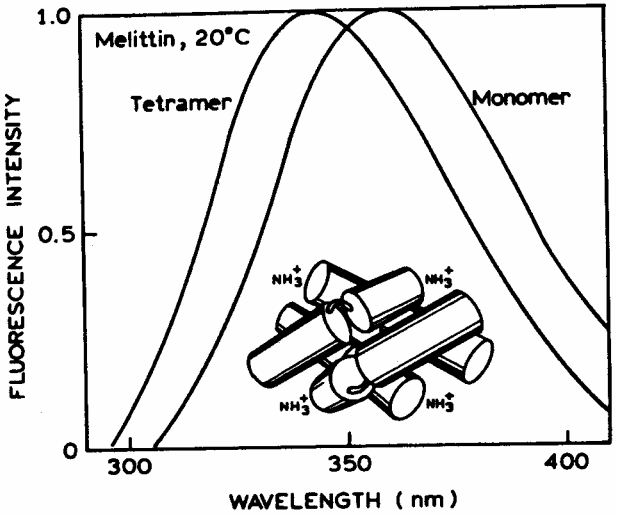


Figure 16.34. Emission spectra of melittin monomer and tetramer.[96] Excitation was at 295 nm. In the schematic structure, the tryptophans are located in the center between the four helices.

16.6. ASSOCIATION REACTIONS OF PROTEINS

16.6.A. Self-Association of Melittin and Binding to Calmodulin

A valuable use of fluorescence is to study the association of proteins with other proteins, lipids, or nucleic acids. Such studies take advantage of the high sensitivity of fluorescence for measurements in dilute solutions. Also, the sensitivity of tryptophan emission to the local environment typically results in spectral changes upon association. Among examples of protein self-association, one of the most studied by fluorescence is the association of melittin monomers to tetramers. When self-association occurs, the random-coil monomers adopt an α-helical conformation. In the α-helical state, one side of the helix is covered with nonpolar amino acid side chains. These nonpolar surfaces come together to form the center of the melittin tetramer. is covered with nonpolar amino acids. This side of melittin also contains the tryptophan residue. Binding of melittin to calmodulin results in a blue shift of the melittin emission and an increase in intensity (Figure 16.35). The association of melittin and calmodulin also results in an increase in the anisotropy. From such data, one can determine the binding constants and the stoichiometry of the association. It appears that the increase in anisotropy saturates at a calmodulin/melittin ratio of 1, indicating a 1:1 complex.

16.6.B. Ligand Binding to Proteins

Intrinsic protein fluorescence has also been widely utilized to study binding of ligands to proteins. In many cases the ligands are also fluorescent, and their fluorescence