Intrinsic protein fluorescence

Fluorescence methods in life sciences

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Examples of fluorescent animals

What does really shine there?

Fluorophores around us

Fluorophores are divided into two general classes:

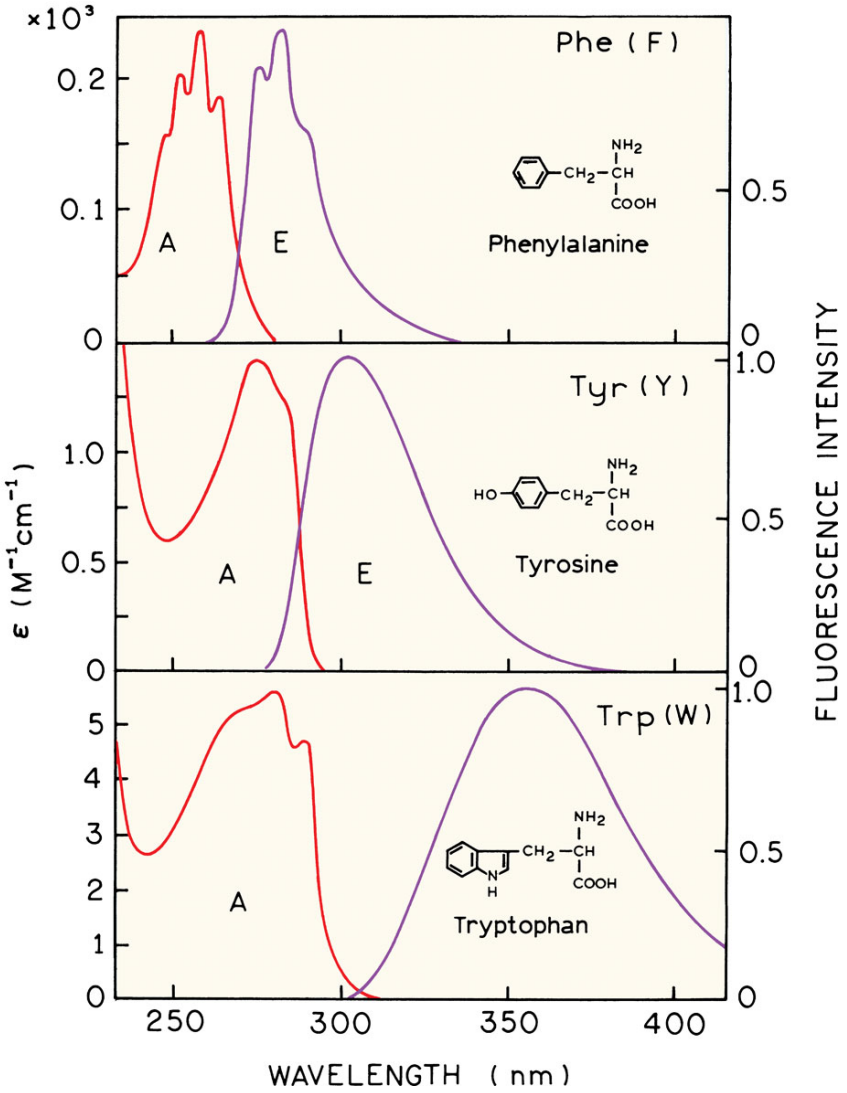
intrinsic – they occur naturally e.g. in proteins

extrinsic – they are added to samples which do not have suitable fluorescence properties

Protein fluorescence

Aromatic amino acids **tryptophan** (Trp), **tyrosine** (Tyr) a **phenylalanine** (Phe) are major fluorophores in proteins. Their absorption band lies between 240 and 300 nm, emissions is also in the ultraviolet region. Dominant fluorophore is tryptophan, respectively its indole group, because it has a much broader emission spectrum than tyrosine, whose fluorescence is due to its phenol ring. Phenylalanine practically does not contribute to the total fluorescence of proteins. Tryptophan fluorescence is very sensitive to the properties of its surroundings and therefore it can be used for the observation of protein conformational changes (e.g. in ligand binding or protein-protein interactions).

Absorption and emission spectrum of aromatic amino acids



- Proteins are fluorescent due to three aromatic amino acids:

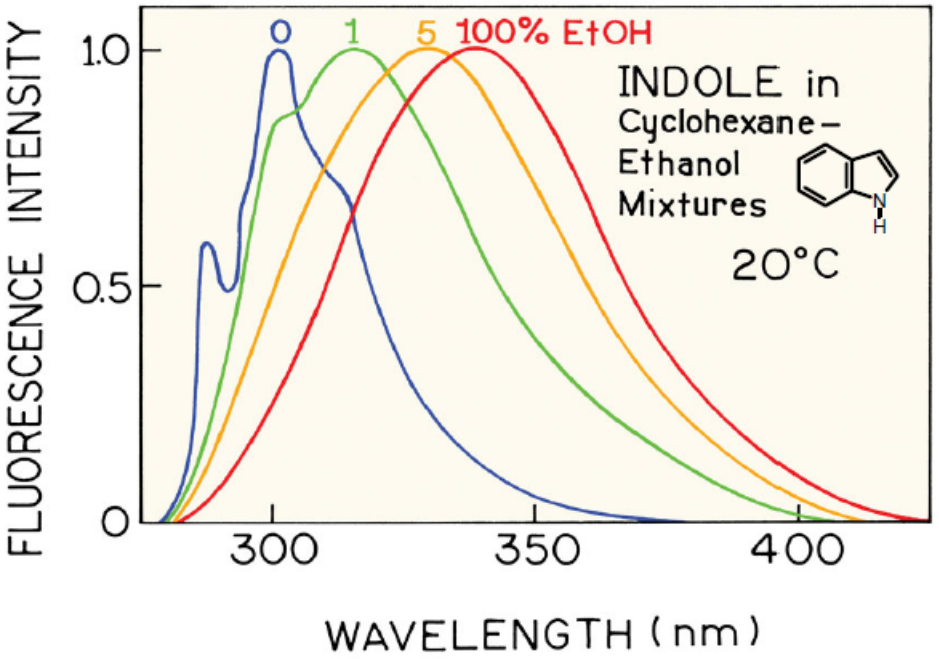
tryptophan, tyrosine and phenylalanine

- In the sequence they occur relatively little
- Tryptophan (Trp) occurs only in 1% of molecules, which is probably due to its relatively metabolically demanding synthesis
- Tryptophan is sensitive to the local environment

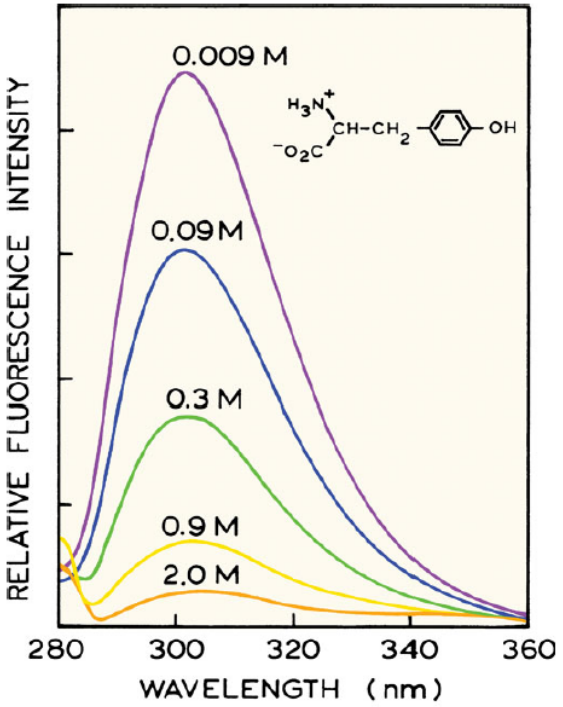
fluorophor	λ_{ex}^{max} (nm)	λ_{em}^{max} (nm)	quantum yield	Lifetime (ns)
tryptophan	295	353	0,13	3,1
tyrosine	275	304	0,14	3,6
phenylalanine	260	282	0,02	6,8

Change of emission spectrum depending on the environment

Polarity



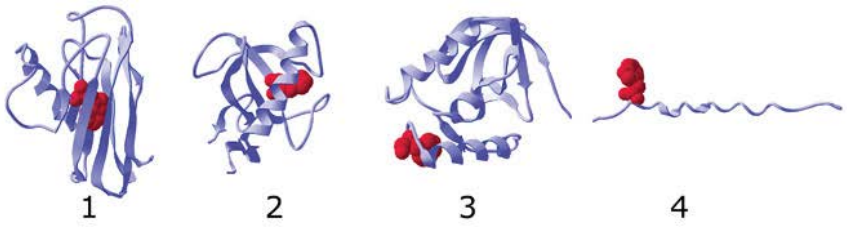
ionic strength

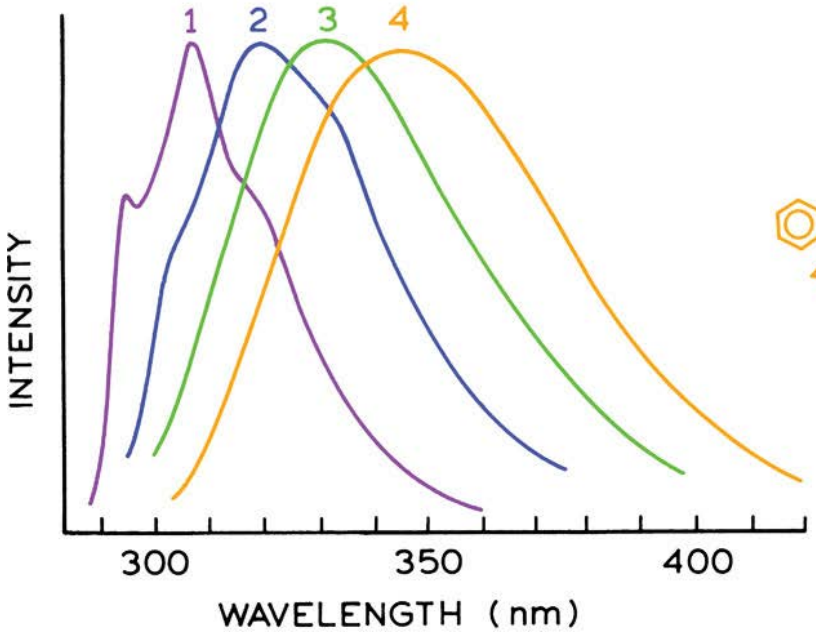


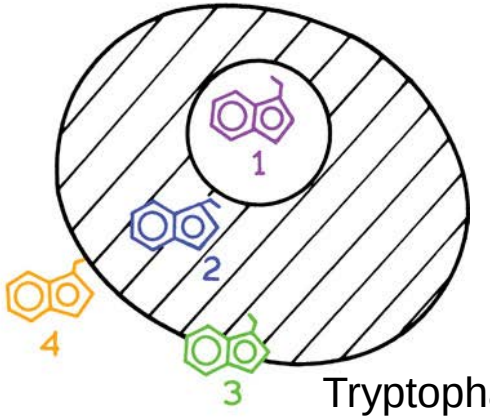
Tryptophan emission is influenced by the surrounding environment – by the local electric field of protein, polarity of solvent and ionic strength of solution

http://micro.magnet.fsu.edu/primer/java/jablonski/solventeffects/index.html

Influence of the protein environment on the emission spectrum of tryptophan







1 apoazurin PFl

2 ribonuclease T_1

3 Staphylococcus nuclease

4 glucagon

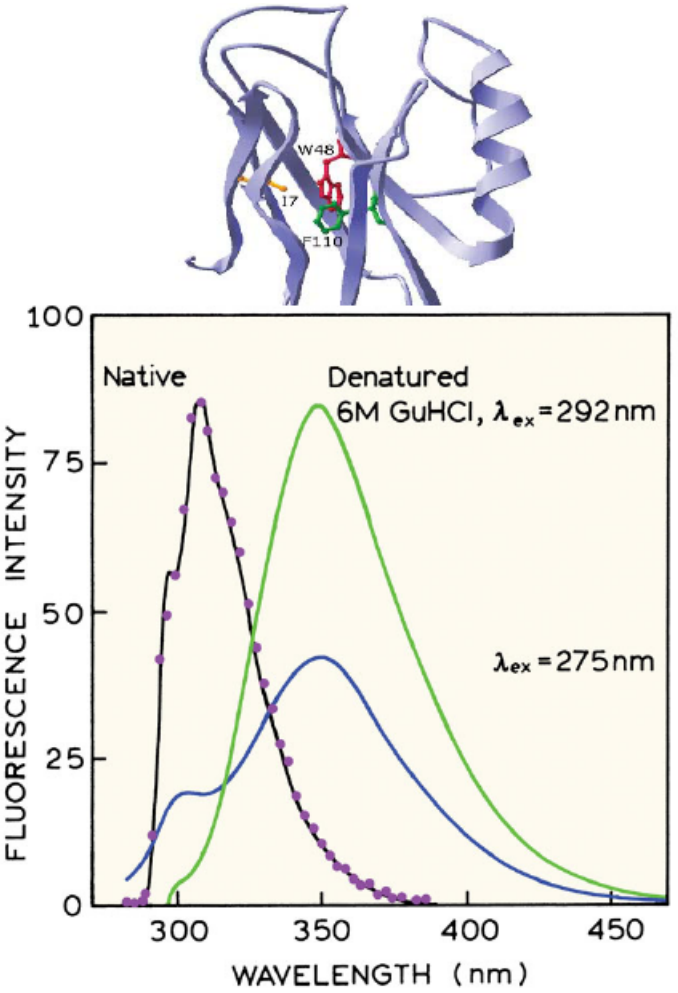
Tryptophan in a nonpolar environment has emission maximum shifted to the shortest wavelength (apoazurin)

Tryptophan exposed to the polar solution has a maximum at the longest wavelength (glucagon)

Factors influencing the tryptophan emission

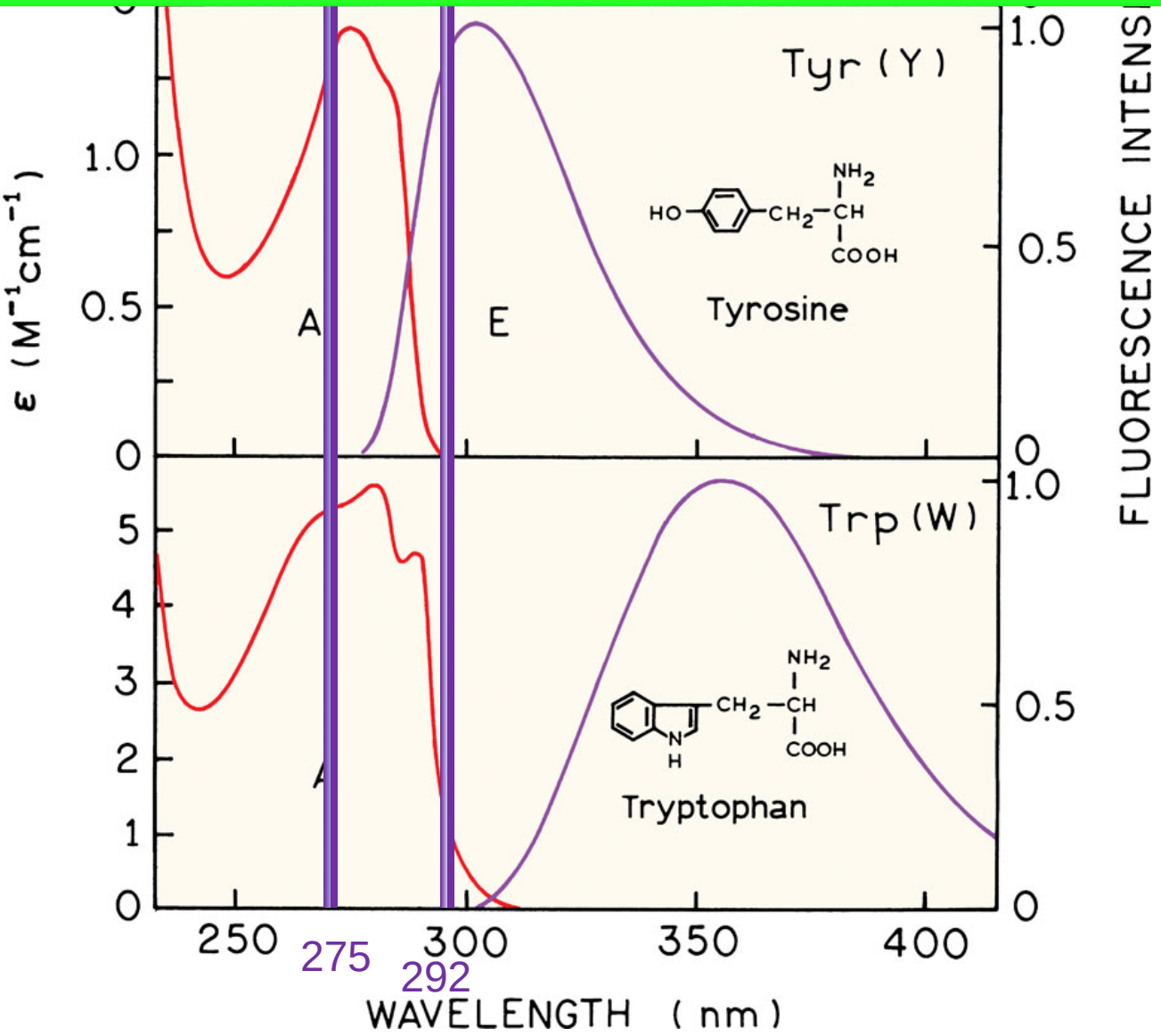
- Quenching by proton transfer from close amino groups
- Quenching by electron acceptors (COO^-)
- Quenching by electron transfer from disulfides and amides
- Quenching by electron transfer from peptide bonds of protein backbone
- FRET between different molecules of tryptophan

Emissions of tryptophan in protein nonpolar environment - azurin



- Azurin from *Pseudomonas aeruginasa* is a protein that contains a copper ion and is involved in electron transfer of denitrification bacteria
- Structure is composed of α-helix and 8 β-sheets, which form β-barel structure with a hydrophobic core
- In the native state, the emission spectrum is the same as in the case of a nonpolar environment (e.g. cyclohexane)
- In the denatured state, the emission spectrum is shifted, which correspond to the movement of tryptophan into the polar environment
- The emission spectrum is different at different excitation wavelength, because at 275 nm **tyrosine** is also excited (peak ~ 300 nm), while at 292 nm only **tryptophan** is excited

Absorption and emission spectrum of aromatic amino acids



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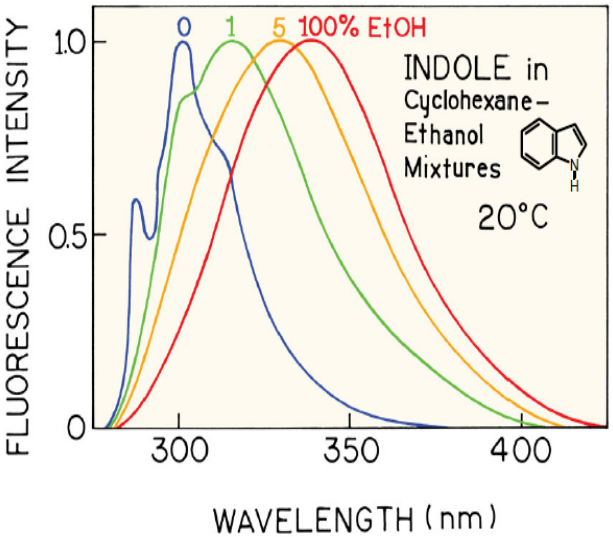
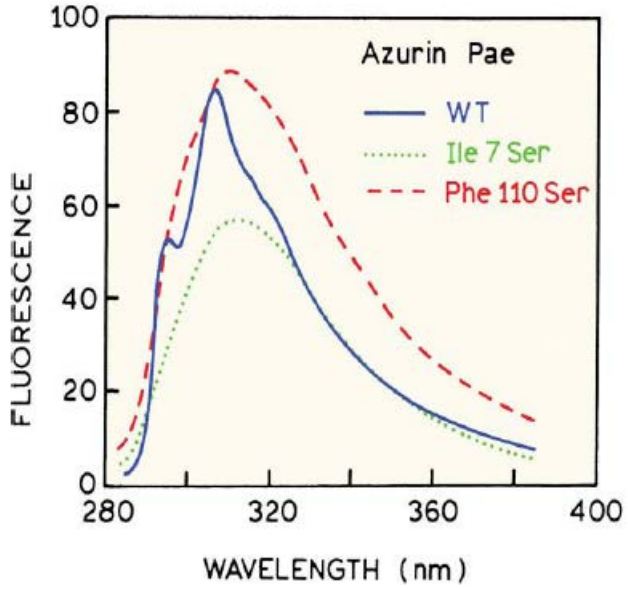
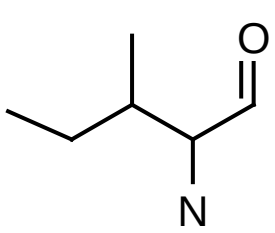
Emissions of tryptophan when ambient amino acids are exchanged

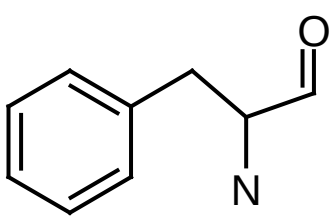




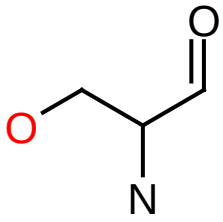
- When nonpolar isoleucine and phenylalanine were replaced by directed mutagenesis of the azurin for polar serin, there was a shift of the spectrum to longer wavelengths
- Substitution at serine with OH group induces a similar effect as if Trp is in ethanol
- Just a small change nearby Trp caused a significant shift of spectrum



isoleucine



phenylalanine



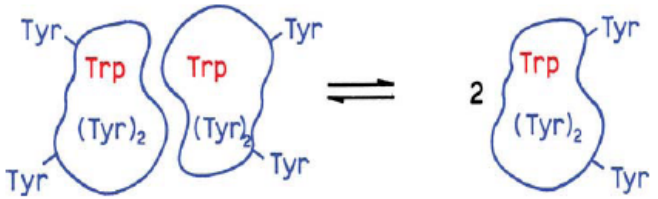
serine

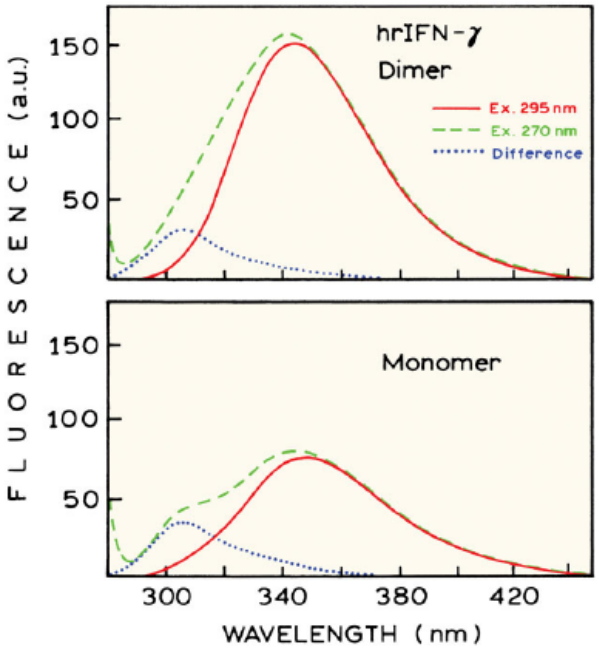
FRET between amino acids

- Between different aromatic amino acids, a resonant energy transfer is found
- FRET between tyrosine and tryptophan is most common because they most often occur in protein sequence and they both are excited at 275 nm
- The rate of energy transfer is given by the emission spectrum and quantum yield of amino acid which serves as a donor
- The rate of energy transfer is dependent on the surrounding environments of individual amino acids
- The resonant transfer may also occur between two molecules of Trp in the case that one is in a nonpolar environments and the second is exposed to the external environment (polar solution)

Donor	Akceptor	R_0 (A)
Phe	Tyr	11-13
Tyr	Tyr	9-16
Tyr	Trp	9-18
Trp	Trp	4-16

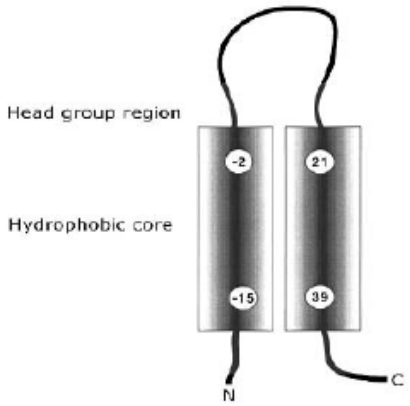
FRET between tyrosine and tryptophan for interferon γ

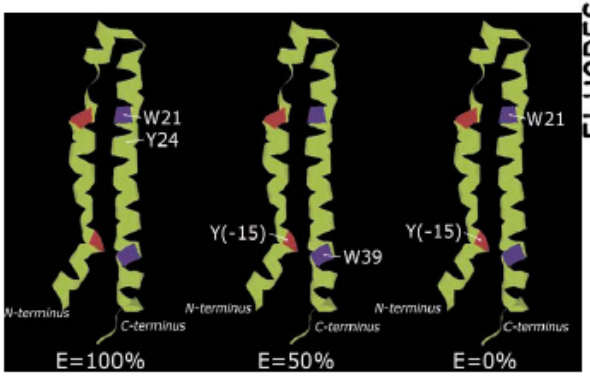


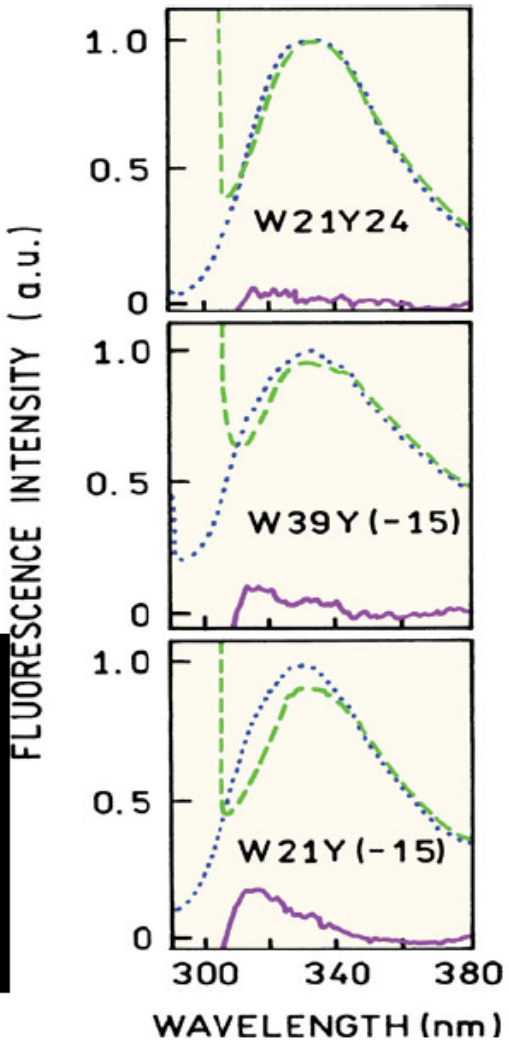


- Interferon γ is produced by activated lymphocytes and has antiviral and immunoregulatory effects
- Interferon activity is dependent on a proportional amounts of dimers
- Intrinsic fluorescence of interferon which is given by 4 tyrosines and + tryptophan was used for observation of dissociation of dimers to monomers
- Emission spectrum of dimer at the excitation of 270 nm shows that tryptophan and tyrosine contributes to fluorescence
- Only tryptophan emission is observed at the excitation of 295 nm
- The difference in the emission spectra at excitation of 270 nm and 295 nm indicates the spectrum of tyrosine
- In the case of dimer, the relative emission intensity of tyrosine is 20 % compared with the emission of tryptophan
- In the case of monomer, the relative emission intensity of tyrosine is 50 % compared with the emission of tryptophan
- Increase of tyrosine relative intensity after dissociation is given by tryptophans moving away (FRET acceptors) which were in dimer in close proximity to tyrosines
- Reduction of energy transfer between tryptophan and tyrosine showed that during dissociation four "external" tyrosine residues are moved away from tryptophans

Utilization of FRET between amino acids to determine the spatial arrangement







- Membrane protein M13 is nested into the membrane of *E. coli* when connecting phage particles
- It was suggested that two α helices of this protein are in the membrane in close proximity
- Protein variants were created by directed mutagenesis, these variants include tyrosine and tryptophan (donor -akceptor FRET) in different positions
- Only one pair tyrosine - tryptophan was always kept
- Emission of fluorescence at excitation of 280 (---) a 295 nm (…) was observed and from the difference tyrosine emission was determined
- If the distance of tyrosine and tryptophan is larger, the emission intensity of tyrosine is higher
- If tyrosine and tryptophan are in close proximity, FRET occurs, that significantly reduces the emission of tyrosine
- Reduction of tyrosine emission intensity in case of W39Y(-15) demonstrated that the α helices are in close proximity

Fluorescence quenching

- **Fluorescence quenching** can be defined as bimolecular process that reduces the quantum yield of fluorescence (ie. fluorescence intensity) without changing of fluorescence emission spectrum.
- **Collision (dynamic) quenching** occurs when the fluorophore in excited state is deactivated (i.e. it returns to the ground state without radiation) in collision with molecule of quencher. In this process the molecules are not chemically modified in contrast to
- **static quenching** when non-fluorescent complex is formed after contact the fluorophore and the quencher
- **Self-quenching** means quenching of the fluorophore by itself; it occurs with high concentrations of fluorophore or with high density of labeling.

Dynamic quenching

Reduction of fluorescence intensity by dynamic quenching is described by Stern-Volmer equation:

$$F_0/F = \tau_0/\tau = 1 + k_q\ \tau_0\ C_q$$

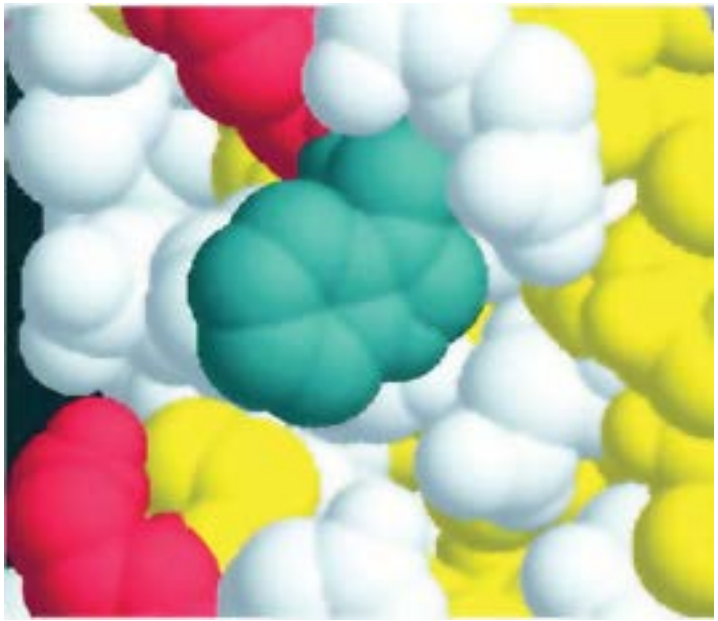
F_0 – fluorescence intensity in the absence of quencher,

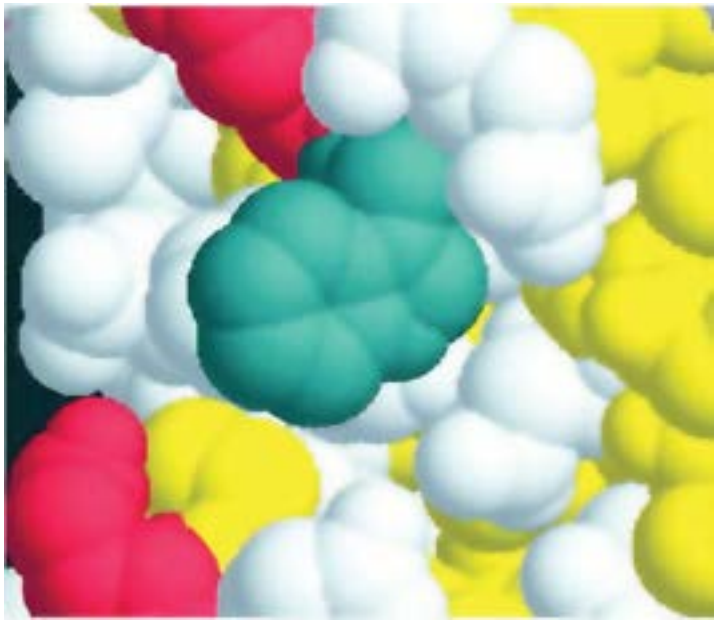
F - the same in the presence of quencher with concentration C_q, τ_0 – fluorescence lifetime without quencher, τ - lifetime in the presence of quencher, k_q – bimolecular quenching constant (= bimolecular rate constant determined by diffusion multiplied by efficiency of quenching).

k_q value indicates the concentration of quencher at which the fluorescence intensity is reduced to half.

The most effective dynamic quencher of fluorescence and phosphorescence is molecular **oxygen** (O_2). Furthermore, halogen atoms such as **bromine** and **iodine** quench the fluorescence (as a result of inter-system conversion). **Acrylamide** is also frequently used quencher.

How is it possible, that oxygen gets into internal regions of the protein?





- It is small and proteins „breathe"

Static quenching

- Complex of fluorophore and quencher is formed which already does not emit fluorescence
- The Stern-Volmer equation is also valid for it:

$$F_0/F = 1 + K_a \, \tau_0 \, C_q$$

K_a is the association constant of the fluorophore and quencher

No change of life time! (on/off state of fluorophore)

Typical static quenchers are:

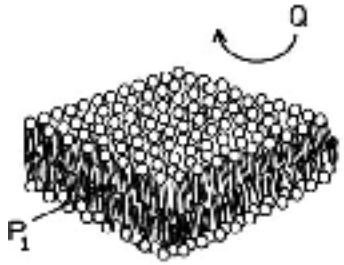
Bases of nucleic acids

mainly Guanine

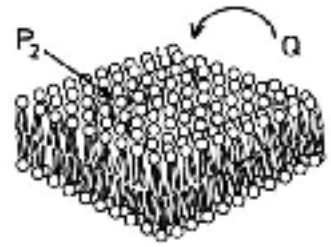
Nicotinamide

heavy metals

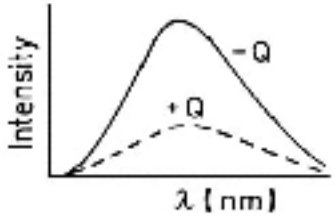
Use of quenching in fluorophore localization

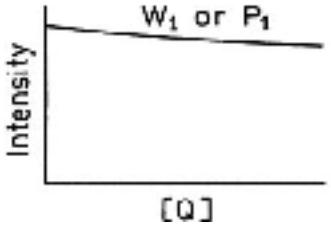


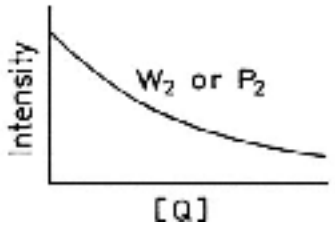
Burried in the membrane



Exposed on the surface







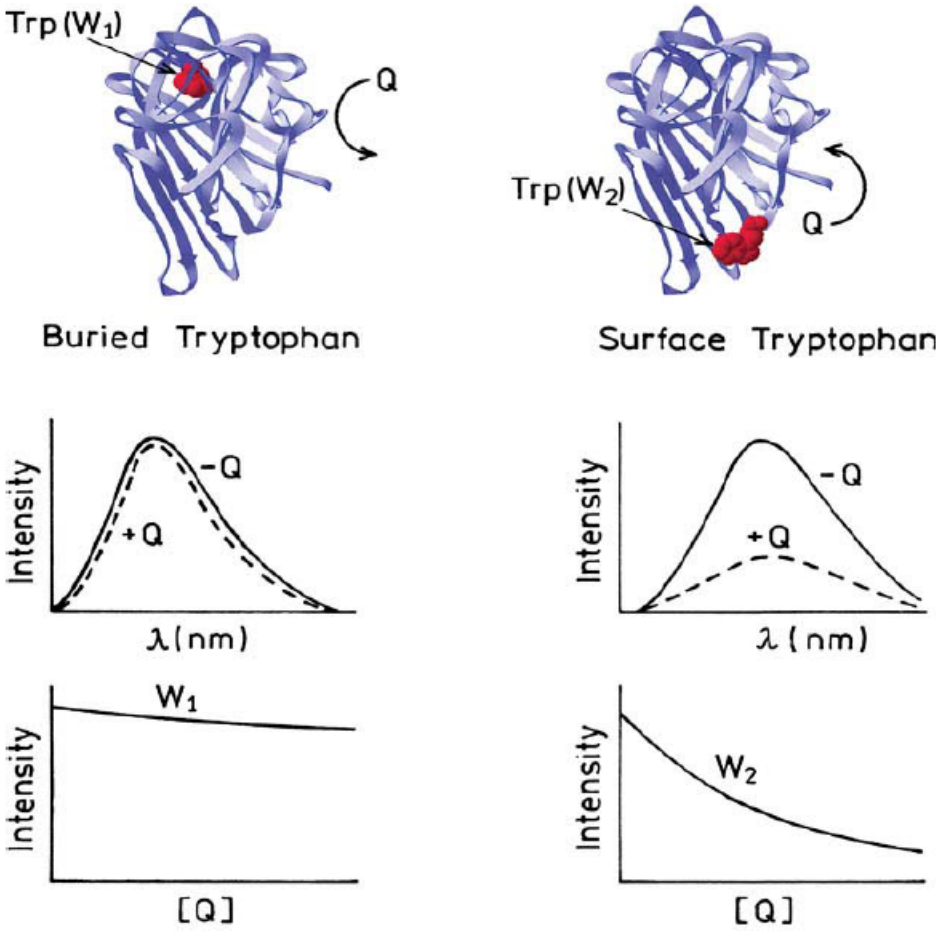
In membrane

- If fluorophore P1 is buried into membrane, it is unavailable for quencher Q and there is almost no quenching.
- With increasing concentration of quencher the fluorescence intensity is almost unchanged.

On the surface

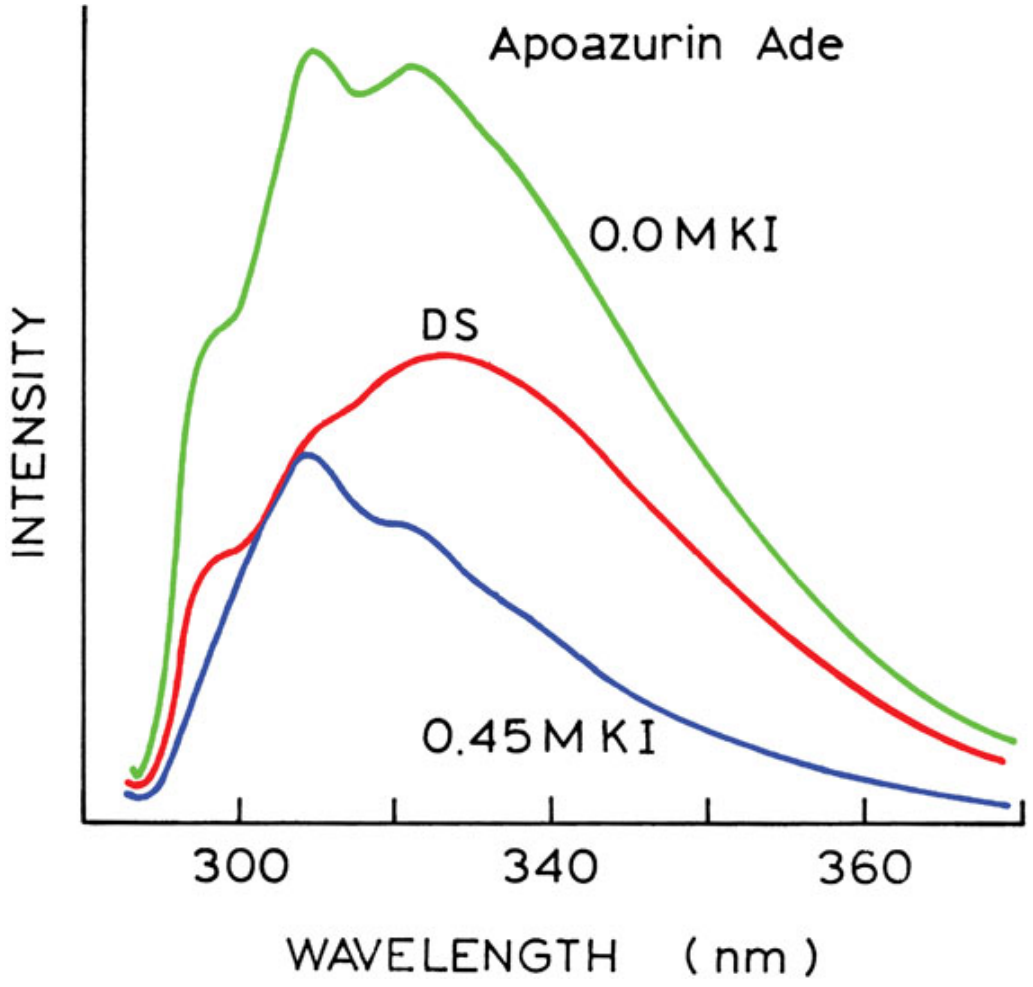
- If fluorophore P2 is on the surface the efficient quenching occurs.
- With increasing concentration of quencher the fluorescence intensity decreases dramatically.

Quenching in the study of protein structure
or
Is tryptophan on the surface of protein?



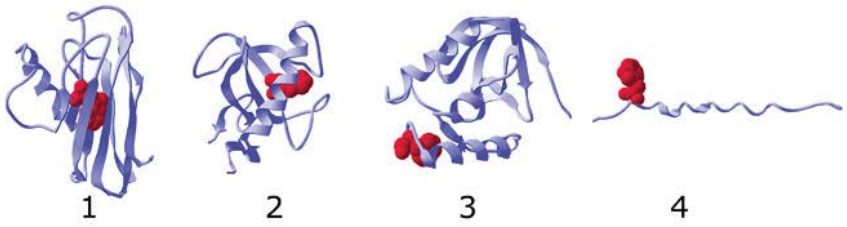
- Dynamic quenching requires direct contact of the fluorophore and quencher
- When tryptophan is located „inside“ protein, its quenching does not occur (W1)
- When tryptophan is located on the surface of protein, quenching occurs (W2)

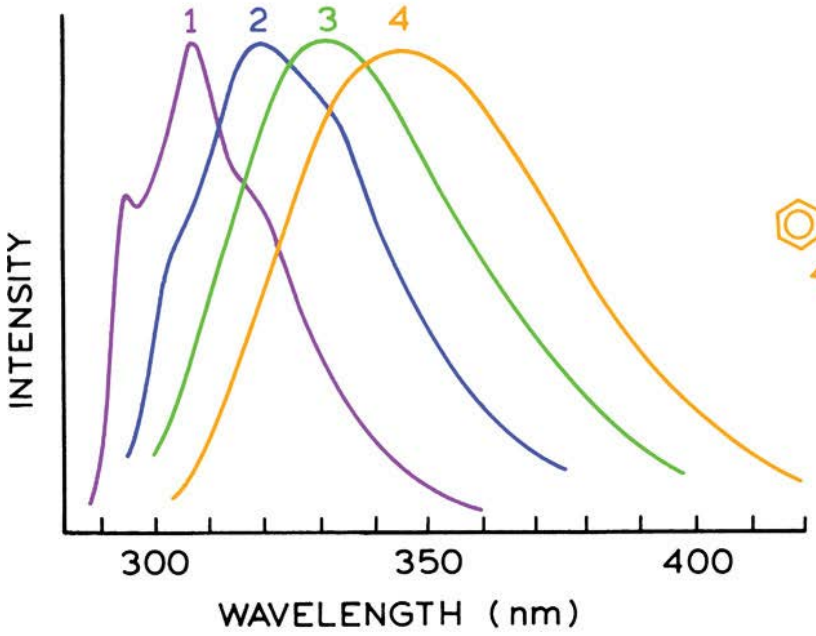
Quenching in the study of apoazurin

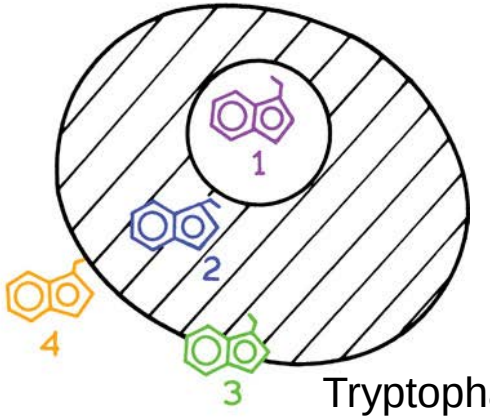


- Apoazurin has two tryptophans in the sequence
- One tryptophan is on the surface, the second is buried
- After adding quencher (KI) to the solution, quenching of tryptophan on the surface occurs
- DS is a differential spectrum

Influence of protein environment on tryptophan emission spectrum







1 apoazurin PFl

2 ribonuclease T_1

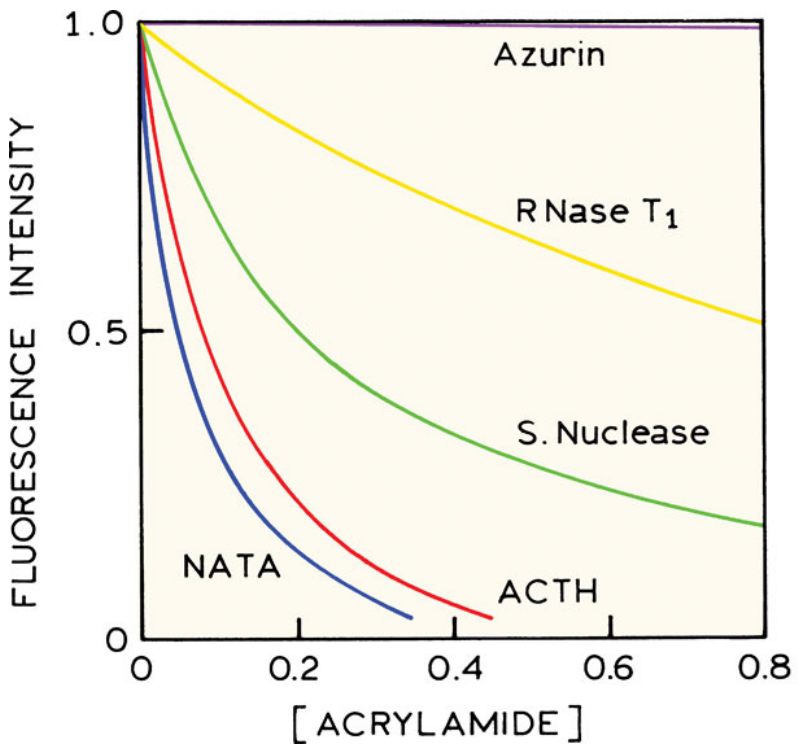
3 Staphylococcus nuclease

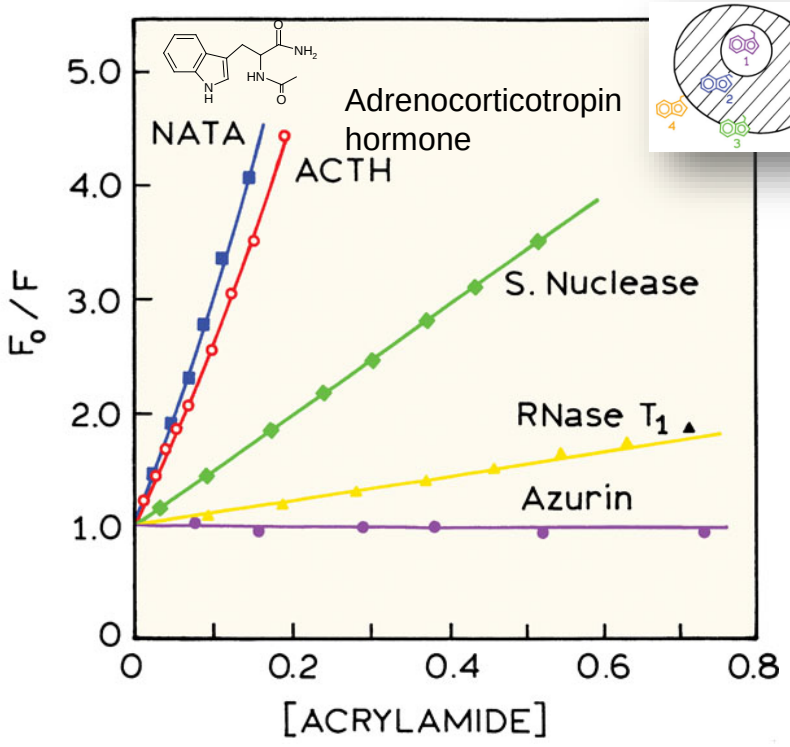
4 glucagon

Tryptophan in a nonpolar environment has emission maximum shifted to the shortest wavelength (apoazurin)

Tryptophan exposed to the polar solution has a maximum at the longest wavelength (glucagon)

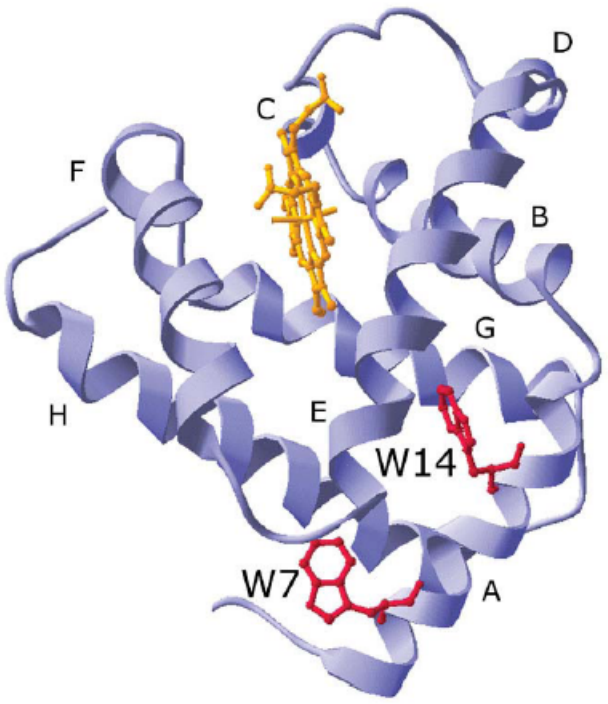
Quenching of protein with one tryptophan in acrylamide solution

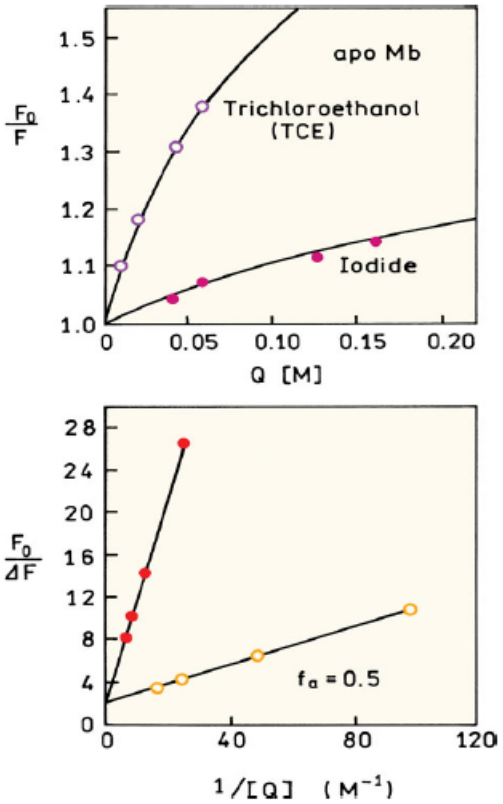


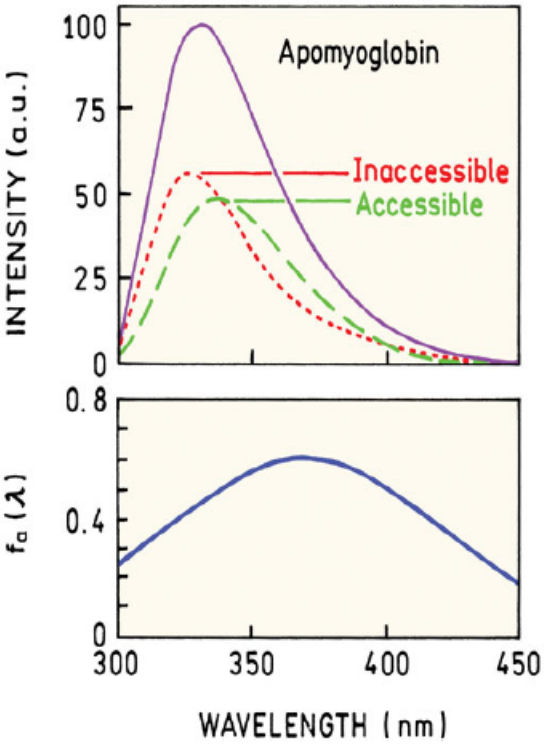


- The higher slope of the Stern-Volmer graph, the greater degree of quenching
- Tryptophan which is free in solution is the best quenched (represented by NATA- N-acetyl-L-tryptophanamide)
- Tryptophan in azurin is the least accessible for quencher

Quenching of 2 tryptophans in myoglobin



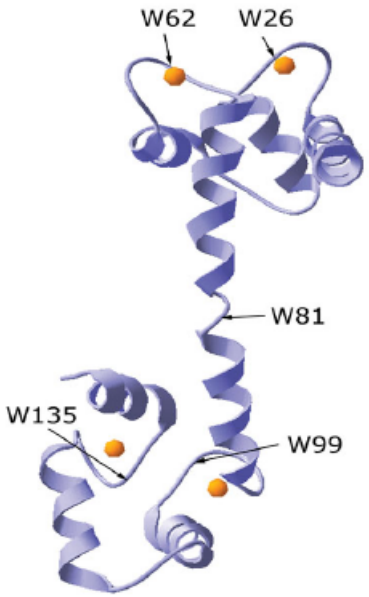


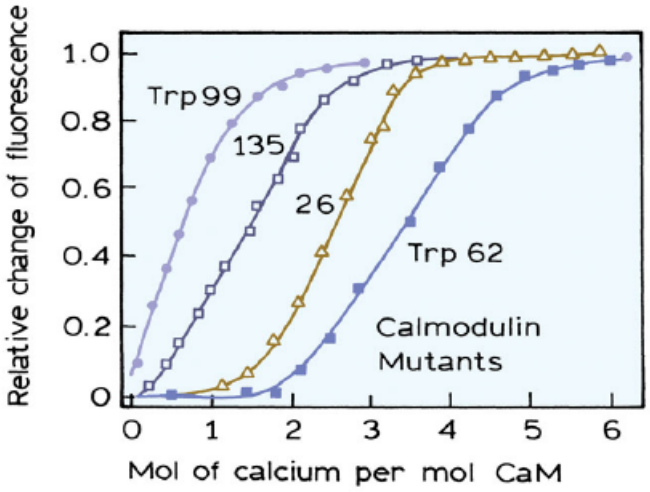


During quenching of tryptophans from horse myoglobin (contains 2 Trp) it was found that the proportional half of intensity is quenched.

This led to confirmation that tryptophan W14 is located in the inner part of the structure between α helices, while W7 is on the protein surface

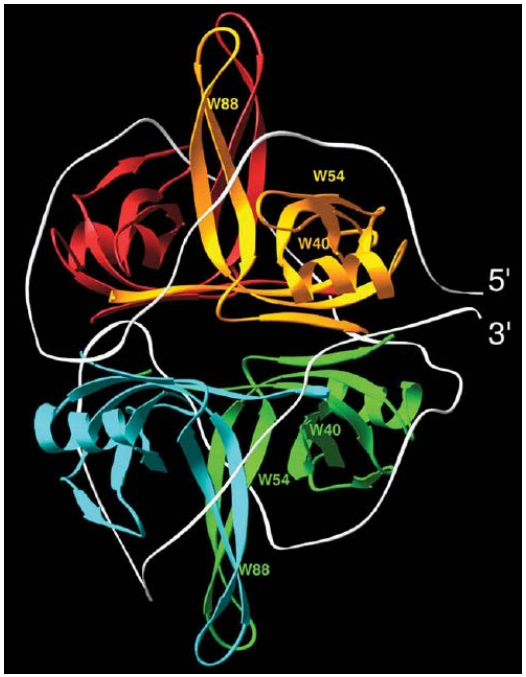
Order determination of occupancy of binding sites

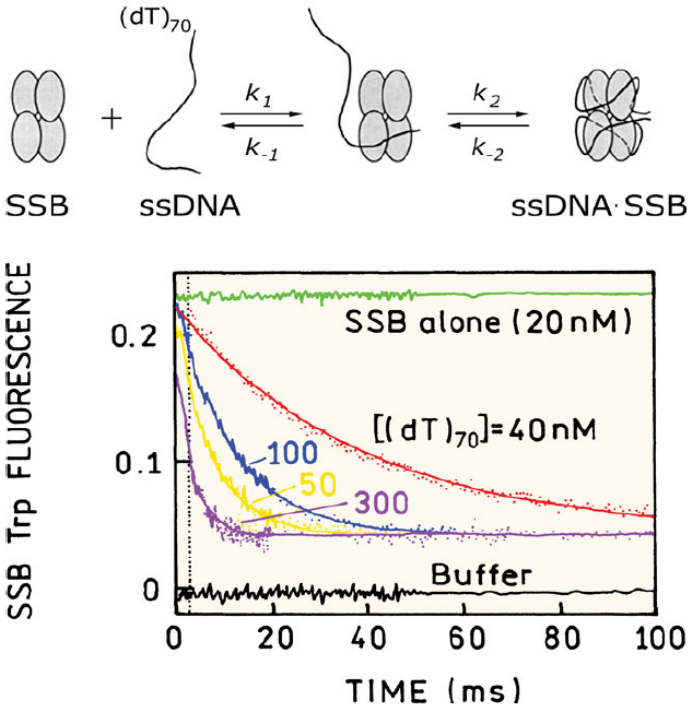




- **Calmodulin** binds to calcium ions, its structural change occurs, resulting in increased ability of binding to receptors and proteins, which metabolism regulates.
- One tryptophan was introduced always close to one of the four calmodulin binding sites and the change in fluorescence was observed with increasing concentrations of calcium ions
- Comparison of dependencies showed order of occupancy of individual binding sites by calcium cations

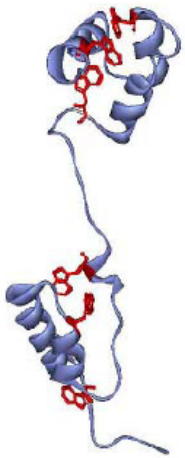
Protein-DNA interaction

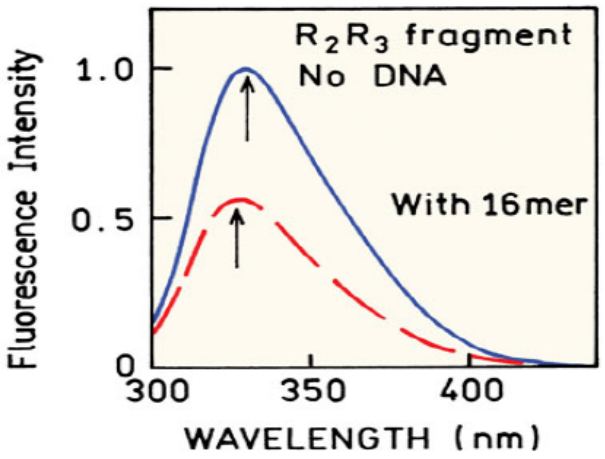


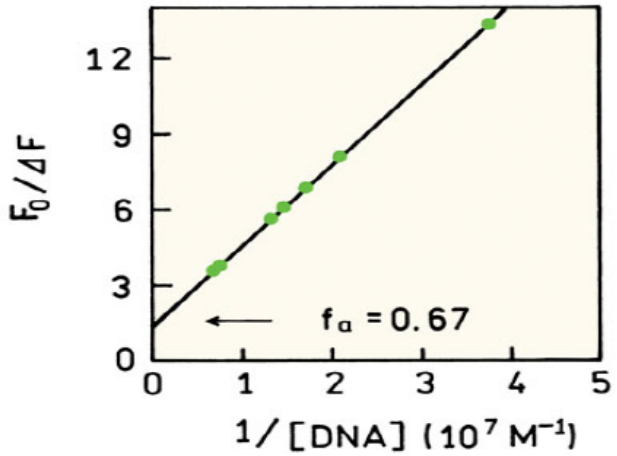


- During interaction of the protein with DNA, quenching of tryptophan by DNA bases occurs
- Protein SSB (single-stranded DNA binding) binds DNA with high affinity but low specifity
- Homotetramer is formed, that binds to DNA with a length of about 70 nucleotides
- Reduction of tryptophan emission after association of DNA and protein showed that there is a DNA strand wrapping around tetramer
- Labelling of DNA at one end by donor and at the other end by acceptor showed DNA wraping around protein complex

Quenching of tryptophan fluorescence in Myb oncoprotein during DNA binding



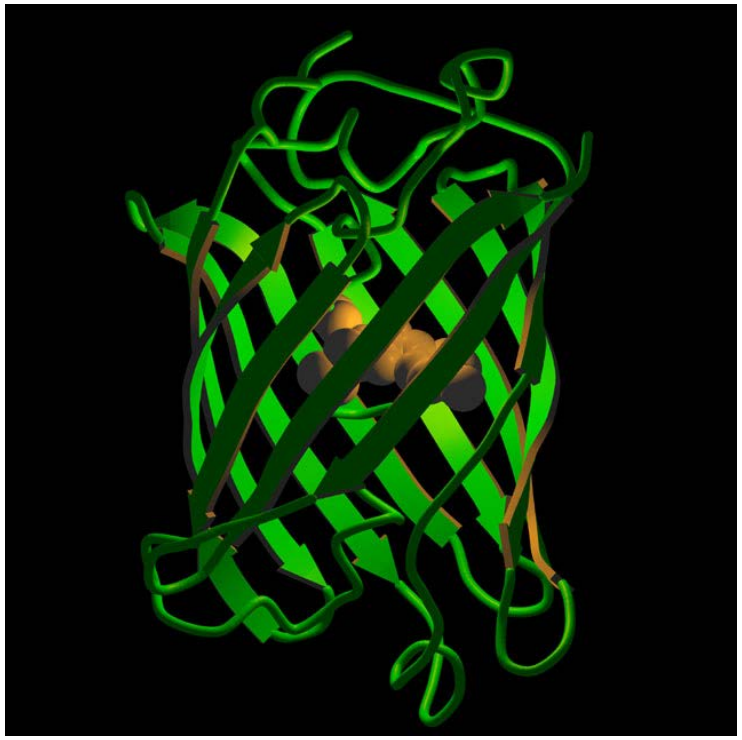




- Myb oncoprotein is associated with chromatin and regulates gene expression
- N-terminal part of the protein contains R1, R2 a R3 domains
- Each domain contains three tryptophans that are highly conserved in sequence, suggesting that they are involved in DNA binding
- Fluorescence of R2R3 fragment of Myb oncoprotein is partially quenched after binding to DNA
- After plotting of fluorescence dependence on the concentration of DNA, it was found that there was a intensity quenching of 67%
- It follows that 2 of 3 tryptophans in each domain are involved in the interaction with DNA

Protein with fluorescence in the name

Green Fluorescent Protein



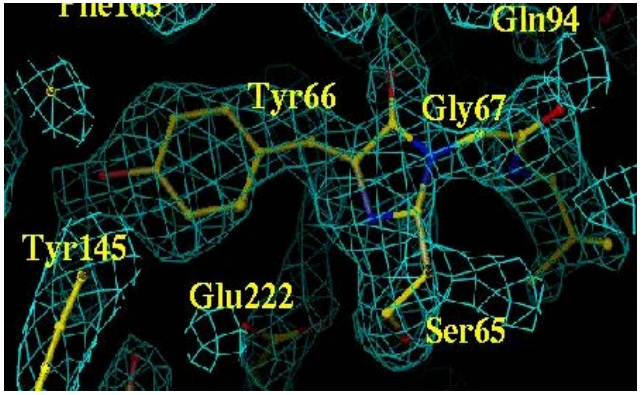
λ_{ex}= 488 nm, λ_{em}=507nm

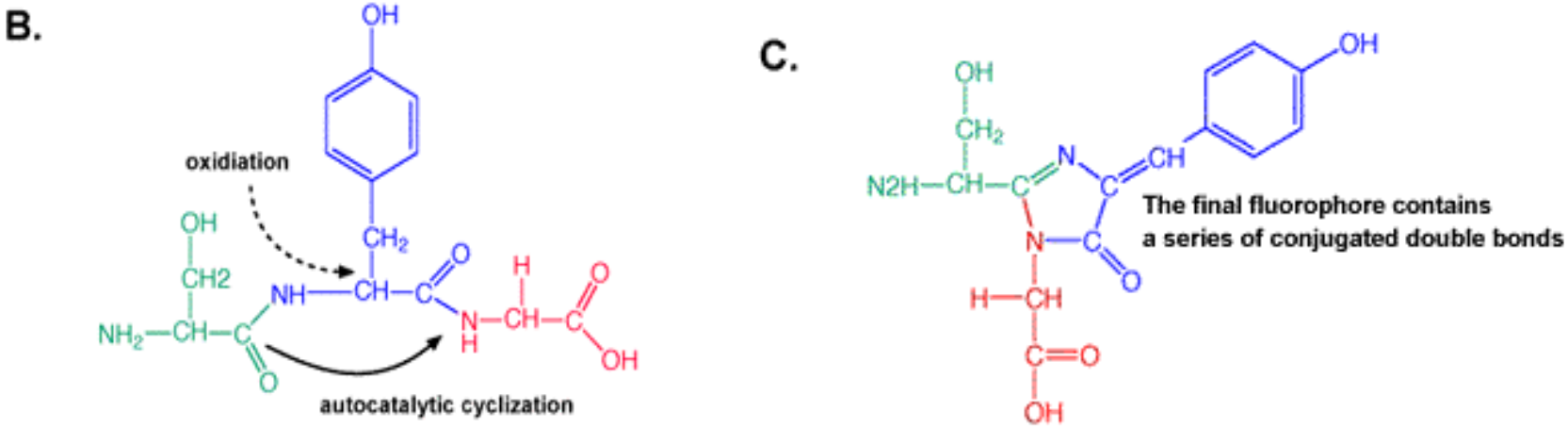
- Composed of 238 amino acids
- “Paint in a can”
- Monomer is composed of a central α helix surrounded by 11 antiparallel β chain in the shape of cylinder
- Diameter of the structure is 30A and length is 40A
- Fluorophore is located on the central α helix

http://www.cytographica.com/animations/FRET2.html

Fluorophore in GFP

- Ser65-Tyr66-Gly67
- Fluorophore is formed autocatalyticaly during protein folding (2-4 hours)
- Tyr66 is a source of fluorescence
- The fluorophore was created by oxidation of Tyr66 and subsequent spontaneous cyclization
- The resulting fluorophore contains conjugated double bonds

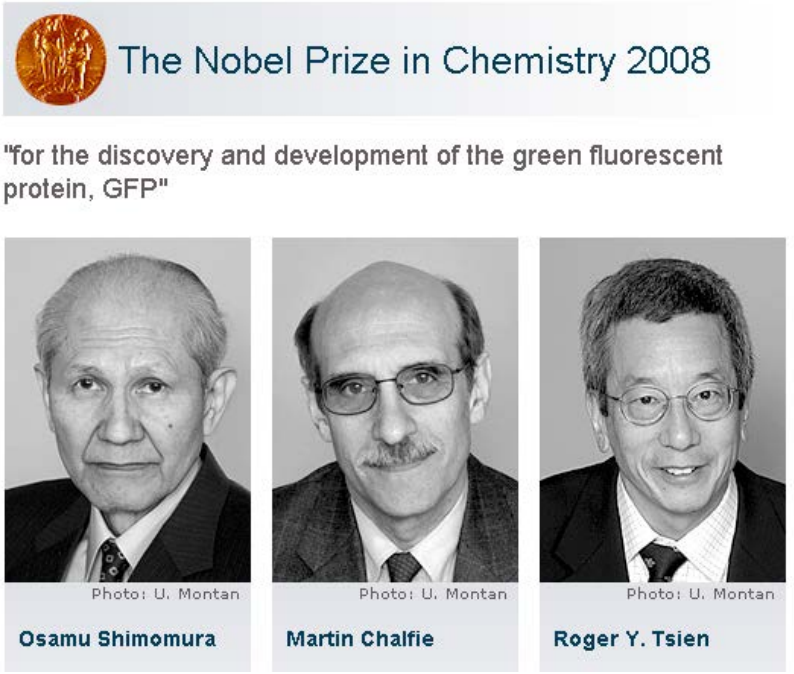




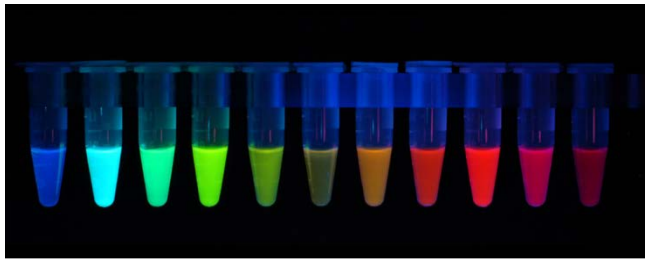
http://www.olympusconfocal.com/java/fpfluorophores/gfpfluorophore/index.html

Utilization of GFP

- Protein localization
- Dynamics of proteins and organelles *in vivo*
- Transport between organelles
- Reporter of gene expression
- Flow cytometry
- Protein purification
- Movement of cells

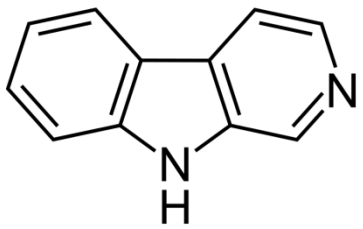


GFP can be used to label proteins in living cells of various organisms due to its inherent fluorescence (only gene coding GFP is needed – c-DNA 1992, Prasher)



Why does scorpion emit fluorescence?

Superstitionia donensis



β carboline

- Cuticula of scorpion contains **beta-Carboline** (9*H*-pyrido[3,4-b]indole)
- It is a derivative of tryptophan, which is formed after hardening of the breastplate

Stachel, Shawn J; Scott A Stockwell and David L Van Vranken (August 1999). "The fluorescence of scorpions and cataractogenesis". *Chemistry & Biology* (Cell Press) **6**: 531–539.

Literature

- Lakowicz J.R.: Principles of Fluorescence Spectroscopy. Third Edition, Springer + Business Media, New York, 2006.

- Fišar Z.: FLUORESCENČNÍ SPEKTROSKOPIE V NEUROVĚDÁCH http://www1.lf1.cuni.cz/~zfisar/fluorescence/Default.htm

Acknowledgment

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