PROTEIN INTRINSIC FLUORESCENCE

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ABSTRACT

The analytical use of peptide and protein intrinsic fluorescence properties (tryptophan, tyrosine and phenylalanine fluorophores) combines the advantages of the non-invasive techniques, with a good sensitivity. By providing simultaneously structural and dynamic information, fluorescence spectroscopy is an extremely useful tool for the study of proteins and peptides. The structural information obtained by fluorescence spectroscopy is especially important in the study of systems for which crystallography data is not available, as it is the case of the large majority of membrane proteins. Furthermore, the dynamic information is hardly accessible by crystallography studies. Thus, for most of these applications, fluorescence spectroscopy is the only technique that can probe the time scale or the topological range of the molecular events. Examples of the different fluorescence spectroscopy methodologies used on membrane proteins and peptides studies (spectral characterization, time-resolved fluorescence, fluorescence anisotropy, energy transfer and fluorescence quenching) will be addressed.

INTRODUCTION

The aim of this work is to present the unique ability of the different fluorescence spectroscopy methodologies to assess the time scale and the topological range of the molecular events involving proteins or peptides. Tryp-

tophan (Trp), tyrosine (Tyr) and phenylalanine (Phe), the three aromatic amino acids are the only intrinsically fluorescent residues in peptides and proteins (Fig. 1), with the exception of some highly specific amino acids, not so common in Nature, such as kynurenine (1).

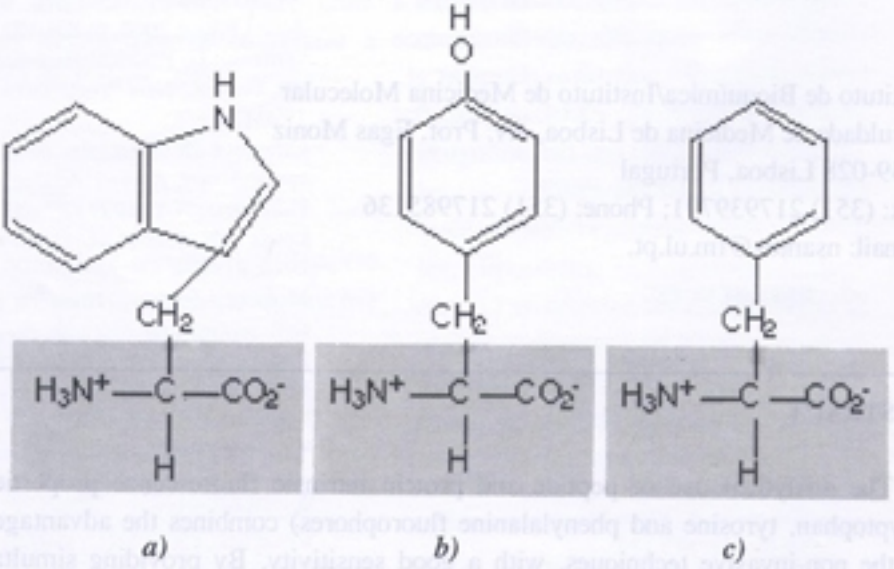

Fig. 1 – Molecular structure of the three aromatic acids: **a)** tryptophan (Trp), **b)** tyrosine (Tyr), and phenylalanine.

Each of the three aromatic amino acids have two absorption maxima at wavelengths (λ) between 200 and 300 nm. Based on their molar absorptivities (ε), it is possible to estimate the value of ε for a peptide or protein, as long as the aromatic amino acid residues of its primary structure are known (2). Many of the basic aspects of the intrinsic fluorescence of peptides and proteins were fully characterized in the first works published in this field (e.g., (3)); Namely, (i) at room temperature, peptides and proteins fluorescence emission lies on the UV; (ii) The fluorescence quantum yields (ϕ) of peptides and proteins are comparable to those of the free amino acids, although dependent on the amino acid residues micro-environment; (iii) Virtually all peptides and proteins with Trp, Tyr or Phe residues are fluorescent; (iv) Different peptides and proteins with identical aromatic amino acid residues composition can present substantially different quantum yields; (v) The quantum yields of peptides and proteins depend on the pH of the solution, decreasing in acid or alkaline solutions. The emission spectra of the three aromatic amino acids are presented in Fig. 2.

The solvent can affect the fluorescence parameters of a molecule due to unspecific interactions, related with the polarity and polarizability of the solvent, or through specific solute--solvent interactions. This phenomenon has a great importance for tryptophan. The presence of a less polar en-

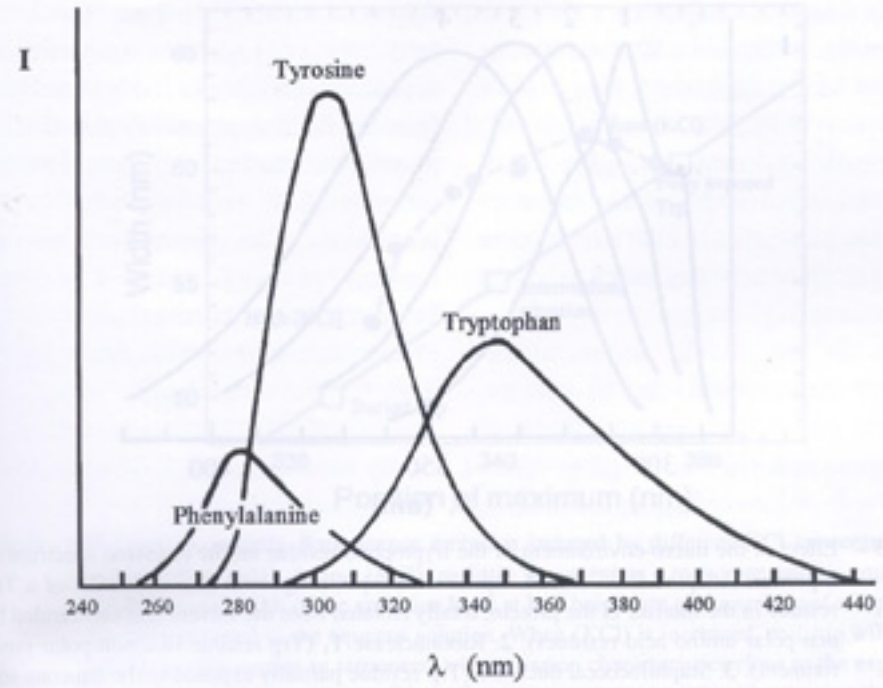

Fig. 2 – Emission spectra of tryptophan, tyrosine and phenylalanine in aqueous solution pH 7. The fluorescence intensities presented are proportional to the quantum yields (adapted from (4)).

vironment has a minimal effect on the absorption spectrum but leads to a significant blue-shift on Trp emission, to an increased quantum yield and to a decreased emission band width at half-height (5,6). On the contrary, these alterations are not significant for phenylalanine (7) and tyrosine (8).

The variability of the tryptophan fluorescence parameters induced by its micro-environment (viscosity, polarity and specific interactions of the solvent with the indole ring) is responsible for the large spectral shifts that occur in peptides and proteins containing tryptophan residues. The emission spectra of these proteins depend on the average micro-environment around the Trp residues, going from the blue-shifted emission, characteristic of a Trp residue in a non-polar environment, to the red-shifted emission of a Trp residue fully exposed to a polar environment (aqueous solution), and passing through the intermediate situations resultant of a partial exposure to the aqueous solvent or the formation of hydrogen bonds with adjacent amino acid residues (Fig. 3). This individual micro-environment enables the reaching of conclusions on its location (through the influence on the fluorescence properties of a specific residue) and, consequently, about the tertiary structure of the protein. Thus, the emission intensity maximum of a protein or polypeptide with Trp residues can go from the 324 nm of Ribonuclease T_1 to the 352 nm of glucagon and adrenocorticotropic hormone (ACTH), with the extreme situation of the emission maximum at 308 nm characteris-

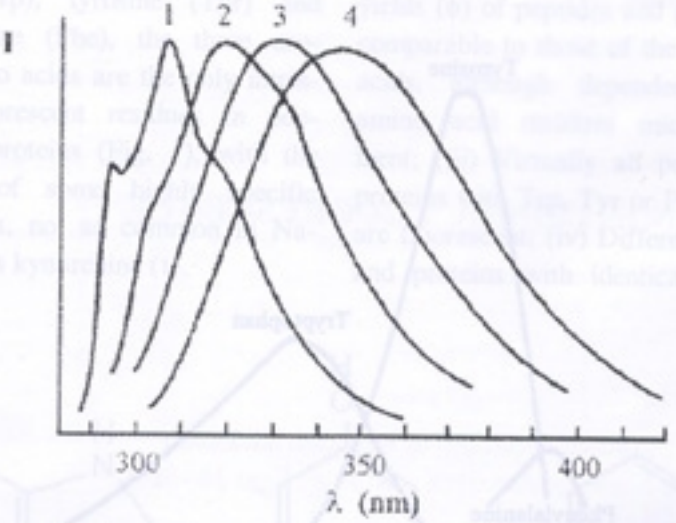

Fig. 3 – Effect of the micro-environment of the tryptophan residue on the emission spectrum of a protein (adapted from (9)). **1**. Apoazurine (special spectral characteristics of a Trp residue in the interior of the protein, totally isolated from the solvent and surrounded by non-polar amino acid residues). **2**. Ribonuclease T_1 (Trp residue in a non-polar environment). **3**. Staphilococcal nuclease (Trp residue partially exposed to the aqueous solvent). **4**. Glucagon (Trp residue fully exposed to the aqueous solution).

tic of the azurines (10). As expectable, the full denaturation of all these proteins results in a red-shift, leading to a spectrum identical to the emission of tryptophan in water, due to the exposure of all the residues to the aqueous environment (e.g., (11,12)). The special spectral characteristics of azurines can be explained by the location of their single Trp residue in the interior of the protein, totally isolated from the solvent and surrounded by non-polar amino acid residues (e.g., (13)).

The spectral shifts on the fluorescence of a tryptophan residue can also be used to identify modifications on its micro-environment resulting from a conformational alteration induced by a change on pH (14) or on the concentration of another ion (15,16), enzyme--substrate interaction (13), binding of small (non-fluorescent) molecules to receptor proteins (17), oligomerization (18,19), association of different proteins (20), or the incorporation of a peptide or protein in lipid membranes (21). As an example, Fig. 4 presents the changes on melittin fluorescence (emission maximum and width of the emission band at half height) induced by its KCl-dependent oligomerization process (22).

The insertion of an aromatic amino acid residue of a peptide or protein in a lipid membrane leads to an increase of its fluorescence quantum yield. This increase can be used for the determination of the partition coefficient (K_p) of the biomolecule between the aqueous and lipid phases (21).

TIME-SOLVED FLUORESCENCE SPECTROSCOPY

At variance with steady-state fluorescence spectroscopy, time-resolved

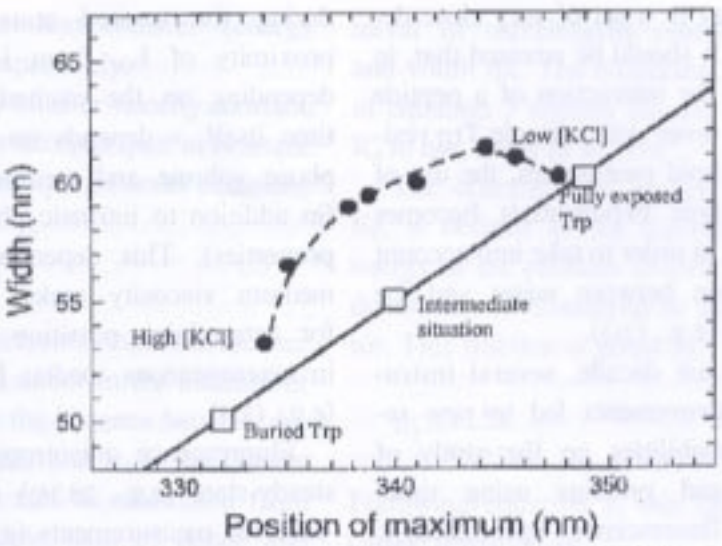

Fig. 4 – Changes on melittin fluorescence emission induced by different KCl concentrations (adapted from (22)). At low (KCl), melittin is present as a monomer, and its emission maximum and width of the emission band at half height are characteristic of a Trp residue fully exposed to the aqueous solution. When (KCl) is increased, melittin will progressively oligomerize in tetramers, with emission characteristics close to the expectable for Trp residues not accessible to the aqueous environment, without going through the intermediate situation of a partially exposed residue. The experimental position--width points above the straight line are characteristic of a mixture of the two emitting species (monomers and tetramers).

fluorescence spectroscopy enables the identification of inter and intramolecular events occurred during the lifetime of the excited state of the fluorophore. In protein studies, this time scale includes, eventually, the rotation of the whole macromolecule, domains of the peptide chain, or amino acid residue side-chains (for a specific review see, e.g., (9,23-25)).

The analysis of studies carried out with different single Trp proteins indicates that their fluorescence decays must be described by a sum of two exponentials (except for apoazurine (26)). In the majority of the peptides and proteins with more than one tryptophan residue, more than two exponentials are required for the fit to the experimental fluorescence decay. The proteins with identical fluorescent residues, but in different micro--environments, behave as a mixture of different fluorophores. In some situations, it is possible to identify the contribution of each one of the residues to the fluorescence decay (23). However, the association of different microenvironments to the intrinsically biexponential decay of Trp, makes it impossible (or, at least, doubtful) to establish a correspondence between each residue and a pair of components. Usually, in these proteins it is possible to group the residues in classes of fluorophores with similar properties (e.g., residues exposed to the solvent and internal residues). In each class, the emitting species must have the same spectral distribution and average fluorescence lifetime, in order to enable their contributions to be analyzed as a

single decay or a set of very close decays (27). It should be stressed that, in studies of the interaction of a peptide or protein (even with a single Trp residue) with lipid membranes, the use of at least three exponentials becomes necessary, in order to take into account the partition between water and the membrane (e.g., (21)).

In the last decade, several instrumental improvements led to new research possibilities on the study of peptides and proteins using time-resolved fluorescence spectroscopy; Namely, some studies of protein conformational changes (28), enzyme dimerization (29), relation between the differences (30) or similarities (31) among some proteins with their fluorescence decays, temperature effect on protein structure (32,33) and the use of a long component of tryptophan fluorescence lifetime as a probe for slow orientation fluctuations in proteins (34).

FLUORESCENCE ANISOTROPY

Fluorescence anisotropy studies are based on fluorescence intensity measurements carried out with vertically polarized excitation light. The fluorescence emission is measured with a second polarizer placed parallel (I_{VV}) or perpendicular (I_{VH}) to the excitation polarizer. The dimensionless parameter anisotropy, r, can be calculated using the equation,

$$r = \frac{I_{VV} - I_{VH}}{I_{VV} + 2I_{VH}} \qquad (1)$$

and it provides information on rotational freedom of the fluorophore during the excited state (i.e., the proximity of I_{VH} from I_{VV}). Beside depending on the excited state lifetime itself, r depends on the fluorophore volume and medium viscosity (in addition to intrinsic photophysical properties). This dependence on the medium viscosity makes it suitable for determining partition coefficients in heterogeneous media, for instance (e.g., (35)).

Fluorescence anisotropy, either in steady-state (e.g., 20,36)) or in time-resolved measurements (e.g., (32,37)), has been of great utility on the study of diverge processes involving peptides and proteins, as well as their interaction with other macromolecules and membrane model systems. Some of these applications are also associated with energy transfer processes.

ENERGY TRANSFER

The term energy transfer is here used for the process in which a molecule in the excited state (donor) returns to the ground state by transfering its energy to another molecule (acceptor) without the emission of a photon or the contact between the molecules (38). The other types of energy transfer, involving the emission and subsequent re-absorption of a photon, the contact between two molecules or the formation of complexes, are quite specific and, thus, were left out of the scope of this work (for specific reviews see, e.g., (9,13,39-44)). Depending on the occurrence of the energy transfer process between two different or equal fluorophores, it is named hetero-transfer (the majority of the studied

situations) or homotransfer (energy migration), respectively.

The energy transfer velocity constant, k_T, for a donor-acceptor pair in isotropic medium is given by the Förster equation,

$$k_T = \frac{1}{\tau_D}\left(\frac{R_o}{R}\right)^6 \quad (2)$$

where τ_D represents the fluorescence lifetime of the donor in the absence of acceptor, R is the distance between donor and acceptor and R_o is the Förster radius, which can be calculated from the spectroscopic data of donor and acceptor, using the expression (45):

$$R_o = 0.02108\sqrt[6]{\frac{J\kappa^2\phi_D}{n^4}} \quad (3)$$

where κ^2 is a factor that depends on the relative orientation of donors and acceptors, ϕ_D is the fluorescence quantum yield of the donor in the absence of acceptor, and n is the refractive index of the solvent. The determination of κ^2 is complex. Usually, a value of 2/3 is assumed for this parameter, corresponding to the situations in which the transition momentum of donor and acceptor sweep all the possible orientations in a short time (e.g., (42,46)). The parameter J represents the spectral overlap integral between the emission spectrum of the donor and the absorption spectrum of the acceptor:

$$J = \int \lambda^4 \varepsilon_A(\lambda) f_D(\lambda) d\lambda \quad (4)$$

where $\varepsilon_A(\lambda)$ and $f_D(\lambda)$ represent the molar absorptivity of the acceptor and the fraction of the fluorescence emitted by the donor, respectively, in the interval of wavelengths centered in λ and width $d\lambda$. The numerical constant of equation 3 implies the use of λ and R_o in nm, and ε in $M^{-1} cm^{-1}$.

The efficiency of energy transfer, E_T, is defined as the fraction of the energy of the photons absorbed by the donor that is transfered to the acceptor. This fraction is given by,

$$E_T = \frac{k_T}{k_T + \tau_D^{-1}} \quad (5)$$

Following equation 2, this expression can be rewritten as,

$$E_T = \frac{R_o^6}{R_o^6 + R^6} \quad (6)$$

Looking into the last equation, it can easily be concluded that the Förster radius, R_o, represents the distance between donor and acceptor for which the efficiency of energy transfer is 0.5. It can also be verified that, in an energy transfer process, the fluorescence lifetime and quantum yield of the donor in the presence of the acceptor, $\tau_{D,A}$ and $\phi_{D,A}$, respectively, decrease according to the equations:

$$\tau_{D,A}^{-1} = \tau_D^{-1} + k_T \quad (7)$$

$$\frac{\phi_{D,A}}{\phi_D} = 1 - E_T = \frac{R^6}{R_o^6 + R^6} \quad (8)$$

At variance with the heterotransfer, in a homotransfer process the fluorescence decay law is not modified. In these situations the best way to follow the energy transfer is using fluorescence anisotropy measurements. In this formalism r_f and r_{et} represent the fluorescence anisotropies of the fluorophore directly excited and of the

fluorophore excited by energy transfer, respectively (47):

$$r = r_1 \frac{1+\left(\frac{R_0}{R}\right)^6}{1+2\left(\frac{R_0}{R}\right)^6} + r_{et} \frac{\left(\frac{R_0}{R}\right)^6}{1+2\left(\frac{R_0}{R}\right)^6} \quad (9)$$

This equation is valid only in the absence of rotation on the same time-scale as the migration, i.e., when all the depolarization is due to the homotransfer process

The fluorescence spectroscopy studies of energy transfer between aromatic amino acid residues have shown a large applicability in the determination of intra- and intermolecular distances. This energy transfer is possible due to the overlapping between the absorption and emission spectra of phenylalanine, tyrosine and tryptophan. As the absorption spectra are almost not influenced by the location of the amino acid residue in the protein, there is a large R_o variability, mainly due to the different emission spectra and quantum yields (induced by the different micro-environments of the donor) and to changes on pH.

The energy transfer from tyrosine to tryptophan residues is the most used in studies involving the intrinsic fluorescence of peptides and proteins. The quantification of the efficiency of energy transfer can be carried out using the relative absorption of aromatic amino acids in the ratio under evaluation (48). To measure the energy transfer efficiency from tyrosine to tryptophan residues in a molecule it will be enough to measure the fluorescence quantum yield of the peptide or protein, ideally at several excitation wavelengths (λ_{exc}), choosing an emission wavelength above 350 nm (in order to prevent the emission from tyrosine). The E_T value obtained is used to calculate the distance between the two aromatic amino acid residues.

In our work, equation 9 was used to study the homotransfer between two Trp residues upon the dimerization of a synthetic peptide with the same sequence as the major epitope of HIV-1 membrane glycoprotein gp41 (21). The change of the fluorescence anisotropy between monomer and dimer, due to the energy migration, was used to calculate a distance between the two residues of ≈ 6 Å.

Beside the studies of energy transfer between aromatic amino acid residues, other studies have focused on the energy transfer from these residues to other fluorophores, such as the energy transfer from tryptophan residues to a heme group in proteins (49), or to fluorescent probes incorporated in lipid membranes (50). It should be stressed that many of the energy transfer studies use the labeling of the peptide or protein with fluorescent probes chosen in order to have a R_o value close to the distance that is intended to be measured (e.g., (47,51,52)).

FLUORESCENCE QUENCHING

Although, in its broad sense, the term fluorescence quenching can be generically used for any process involving the decrease of the fluorescence intensity of a sample (thus, including phenomena such as energy transfer), in a stricter sense it is only used to describe phenomena of fluo-

rescence quantum yield decrease due to direct molecular interactions by static and/or dynamic mechanisms. Both processes require the contact of the fluorescence quencher (the molecule or functional group responsible for the phenomenon) with the fluorophore (for specific reviews see, e.g., (9,13,53,54)).

The dynamic process of fluorescence quenching is described by the Stem-Volmer equation,

$$\frac{I_o}{I}=\frac{\phi_o}{\phi}=\frac{\tau_o}{\tau}=1+K_{SV}[Q]=1+k_q\tau_o[Q] \quad (10)$$

where I_o, ϕ_o, τ_o, I, ϕ and τ represent the fluorescence intensity, quantum yield and lifetime in the absence and presence of a quencher concentration, [Q]. K_{SV} is the Stern-Volmer constant and k_q the bimolecular velocity constant. This last parameter can be estimated using the Smoluchowski and Stokes-Einstein equations (e.g., (9)).

In some situations, the Stern-Volmer plot presents positive divergences from linearity. These divergences can be explained using the quenching sphere-of-action model where a sphere of volume V and centered on the fluorophore, in which the fluorescence quenching process occurs with efficiency γ, is considered. In steady-state, the quantification is made according to the equation,

$$\frac{I_o}{I}=(1+K_{SV}[Q])e^{V[Q]N_A\gamma} \quad (11)$$

(N_A is the Avogadro number). In other situations, the Stern-Volmer representation presents negative divergences from linearity, following a hyperbolic behavior. In general, these situations result from the existence of more than one class of fluorophores, with different K_{SV} values. The simplest situation, with a great applicability on the study of peptides and proteins (12,21,55), considers the existence of a population of fluorophores protected from the contact with the quencher, and a population (B) accessible to it. In this case, the fluorescence data can be analyzed using the Lehrer equation (55),

$$\frac{I_o}{I}=\frac{1+K_{SV}[Q]}{(1+K_{SV}[Q])(1-f_B)+f_B} \quad (12)$$

where f_B is the ratio between $I_{o,B}$ (the fluorescence intensity from the fluorophore sub-population accessible to the fluorescence quencher, in its absence) and I_o. If both sub-populations (accessible and non-accessible) have identical quantum yields, f_B is the mole fraction of the accessible population.

The application of this method to studies with proteins has the main objective of determining how many (or which) fluorescent amino acid residues are exposed to the solvent, using an aqueous quencher (e.g., acrylamide, iodide, lead). It can also be used to identify the transmembrane residues in a membrane peptide or protein, using a fluorescence quencher incorporated in the lipid bilayer. In this last situation, there are some formalisms to calculate the position or depth of the fluorescent residue in the membrane (Figs. 5 and 6), by comparing the fluorescence quenching efficiencies obtained with phospholipids or fatty acids derivatized with a quencher group (e.g., bromide, doxyl) at different positions (22,56-61).

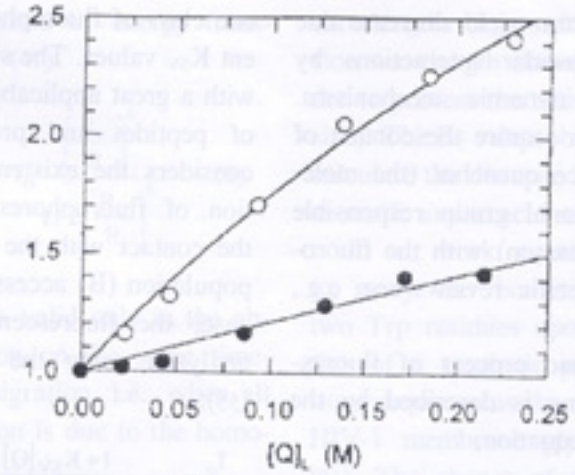

Fig. 5 – Fluorescence quenching of a synthetic peptide identical to a segment of the HIV membrane protein gp41, incorporated in the membrane of lipid vesicles, by fatty acids derivatized with a doxyl group at the carbon 5 (○) or 16 (●), and fitting line obtained using equation 12 (adapted from (21)).

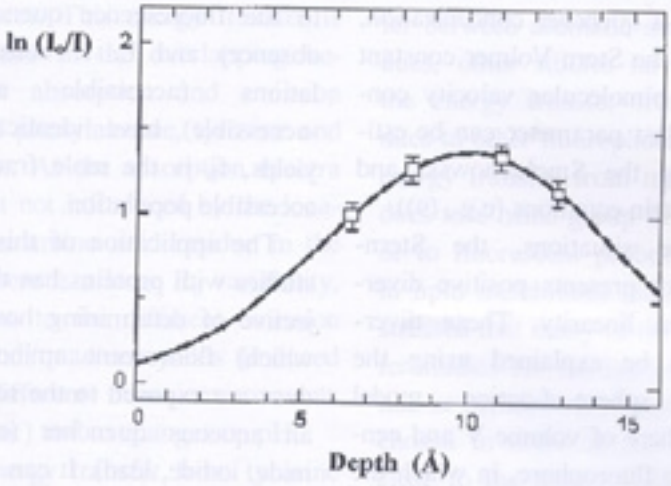

Fig. 6 – Fluorescence quenching study of a cytochrome b_5 mutant, using four bromolipids derivatized at different positions of the fatty acid chains (adapted from (22)). Data analysis indicates that the distance from the fluorophore to the center of the bilayer is (10.1±4.4) Å.

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