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Fluorescence Spectroscopy: A Powerful Tool for Biological Systems

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Introduction

Fluorescence spectroscopy is one of the most widely used spectroscopic techniques in the fields of biochemistry and molecular biophysics today. Although Fluorescence measurements do not provide detailed structural information, the technique has become quiet popular because of its acute sensitivity to changes in the structural and dynamic properties of the biomolecules and biomolecular complexes. Like most biophysical techniques, fluorescence spectroscopic studies can be carried out at many levels ranging from simple measurement of steady state emission intensity to quiet sophisticated time-resolved studies. The four basic rules of fluorescence are as follows

1.The Frank- Condon principle: the nuclei are stationary during electronic transitions, and so excitation occurs to vibrationally excited levels of the excited electronic state.

2.Emission occurs from the lowest vibrational level of the lowest excited singlet state because relaxation from the excited vibrational levels is much faster than emission.

3. The stokes shift: emission is always of lower energy than absorption due to nuclear relaxation in the excited state.

4.The mirror image rule: emission spectra are mirror images of the lowest energy absorption band.

Flourophores

Intrinsic or Natural fluorophores

A variety of biological molecules contain naturally occurring or intrinsic fluorophores.

Proteins: The intrinsic fluorescence of a protein is contributed by the aromatic amino acids tryptophan, tyrosine and phenylalanine. About 90% of total fluorescence arises from tryptophan residues of protein. When all three fluorophores are present in protein, pure emission from the tryptophan can be obtained by photoselective excitation around 295nm. Among the important characteristics of tryptophan fluorescence are the1) strong solvent dependence of its stokes's shift, 2) the pH dependence of its quantum yield, 3) the double exponential kinetics of its fluorescence decay at neutral pH and the 4) existence of two over lapping $S_0 \rightarrow S_1$ electronic transitions. Frequently, spectral shifts are observed as a result of ligand binding. protein-protein association , and denaturation. The emission maxima of proteins reflect the average exposure of their tryptophan residues to the aqueous phase. Proteins absorb light near 280nm and fluorescence emission maxima range from 320nm to 350nm. Fluorescence lifetimes of tryptophan residue range from 1 to 6 nsecs. Tyrosine is also highly fluorescent in solution, but its emission is generally weaker in proteins.

Nucleic acids

The common nucleic acid bases are generally nonfluorescent. However, Yeast $tRNA^{phe}$ contains highly fluorescent base known as Y-base, which has an emission maximum near 470nm.

Dihydronicotinamide

NADH and related coenzyme NMNH are highly fluorescent. They are often encountered as substrate /ligand for the dehydrogenase class of enzymes. The fluorescence of these coenzymes has been used to characterize the thermodynamics and kinetics of formation of these proteins-ligand complexes. Upon binding of NADH to proteins, the quantum yield of NADH generally increases several folds due to binding of NADH in an unstaked conformation. NADH shows absorption at 340nm and emission at around 450nm. The 340nm absorption of NADH provides for a large spectral overlap with tryptophan emission, so that resonance energy transfer occurs in protein-NADH complexes. The oxidized form of NADH, i.e., NAD + is nonfluorescent

Extrinsic Fluorophores

Frequently, the natural fluorescence properties of macromolecules are not adequate for desired experimentation, so fluorophores are chosen that bind at some or some specific sites covalently or noncovalently. If the probes are properly placed, a number of structural information can be obtained. Usually a probe is chosen so that it can be selectively excited. In this case the macromolecule is invisible and all information related to the probe molecule.

Cysteine groups are the prime choice for specific labeling of the proteins. Male ides are commonly used to label cysteine residues. The chromophoric and fluorescent derivatives of N-ethyl-malemide (NEM) are used for quantitation and analysis. Unlike Iodoacitamides , malimides do not react with tyrosine, histidine or methionine of proteins. Reactions with amides usually require higher pH than the reaction of malemides with thiols. Fluorescein , Eosin , Rhodamine , Coumarine and Pyrene malimides are useful fluorophores. Pyrene malimides are useful fluorophores. Pyrene malimide has several unique properties , its quantum yield markedly increases upon binding, its conjugates have a very long lifetime , and it shows excimer formation with a longer wavelength emission if two fluorophores are in close proximity.

Acryloyl derivatives generally react with thiols more slowly than the malimide. Acrylodan is an excellent sulfhydryl reactive probe, in that its adducts have strongly environment sensitive fluorescence that may be sensitive to ligand binding.

Amines are also common target for modification in peptides, proteins,polymers,ligands and synthetic oligonucleotides. Many proteins do not have free thiols but virtually all have lysine and most have free amino terminal group. Fluoroscein

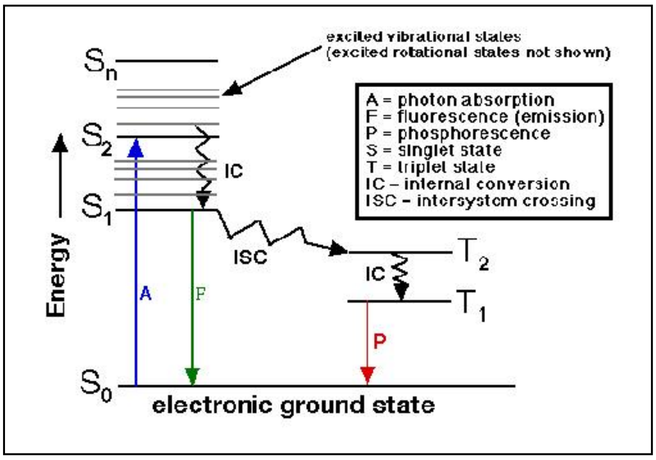
And Rhodamine Isocyanates and Isocyanates are widely used to label proteins . these dyes react primarily with lysine and α-amino groups on proteins. They have high quantum yield and are resistant to photobleaching. Again ,the long wavelengths of absorption and emission minimize the problem of background fluorescence from biological samples. The lifetime of these dyes are near 4nsec and their emission spectra are not significantly sensitive to solvent polarity. Dansyl Chloride is used to label proteins, especially where the polarization experiments are anticipated. It is in common use for protein amino terminus analysis and preparation of many fluorescent derivatives of oligonucleotides, amino acids, drugs and proteins. Its fluorescence has quite high environment sensitivity , and has one of the largest emission Stoke's shift known. The isomer of Dansyl Chloride has lower Stoke's shift but longer fluorescence lifetimes , which makes them useful for fluorescence polarization study.

1-Amino-8 naphthalene –sulfonic acid(1,8-ANS or ANS), Bis-ANS and similar derivatives are frequently used as common noncovalent labels for proteins and membranes. They are nonfluoroscent in water , but are highly fluorescent when dissolved in nonpolar solvents or when bound to macromolecules. The binding sites are likely to have both polar and nonpolar nature.

Fluorescence Quantum Yield

Conventionally the energy level diagram known as Jablonski diagram illustrates absorption and emission of light. The ground, first and second singlet electronic states are designated as S_0, S_1,S_2 respectively and first triplet state as T_1. At each of these electronic energy levels the fluorophores can exist in a number of

vibrational energy levels the fluorophores can exist in a number of vibrational energy levels. Absorption of light causes transition from S_0 to S_1 and S_2. It occurs in about 10^{-15} secs, obeying Frank-Condon principle. This is followed by internal conversion ($S_2 \rightarrow S_1$), and vibrational relaxation to the lowest vibrational level of S_1. From S_1several processes can occur to dissipate the excess energy, including radiative decay (k_F) to produce fluorescence, non-radiative interconversion to S_0 by various quenching mechanism (k_{IC}), and intersystem crossing to T_1 state (k_{ISC}). If quenchers (Q) are present, deactivation of S_1 will also occur with rate constants kq [Q]. All the above processes will compete directly to depopulate the excited singlet state.



The fluorescence quantum yield (ϕ_F) is defined as the fraction of excited singlets that decay by fluorescence.

$$[\phi_F] = k_F / [k_F + k_{IC} + k_{ISC} + kq[Q]]$$

Thus fluorescence quantum yield is equal to the ratio of photons ,emitted to photons absorbed by the system. The quantum yield can be close to one , if the rate of radiationless deactivation is much smaller than the rate of radiative decay. But the energy yield of fluorescence is always less than unity because of Stoke's losses. Absolute measurement of ϕ_F is quiet difficult. In practice one measures the relative quantum yields. A standard such as quinine sulfate in 1(N) H_2SO_4 (ϕ_F= 0.7) or fluorescein in 0.1(N) NaOH (ϕ_F= 0.93) is used to calibrate the instrument. Quantum yield can also be calculated from measurement of decay rates.

Fluorescence Intensity

The fluorescence intensity at a given wavelength , F can be expressed in the following way

$$F = I_A \Phi_F \text{..........} [1]$$

Where I_A is the initial population of the excited state and ϕ_F is the quantum yield. The above equation refers to the total fluorescence intensity emitted in all directions, but usually only a small amount is collected by the spectrophotometer. To account for this the equation must be multiplied by an instrument factor (Z). thus the above equation becomes

$$F = I_A \Phi_F Z \text{..........} [2]$$

Now, the population in the excited state depends on the amount of light absorbed , I_A, which can be calculated by Beer-Lambert law in the following manner.

$$I_A = I_1 - I_0 = I_0 [1 - e^{-2.3\varepsilon(\lambda)cl}] \text{..........} [3]$$

Where $\varepsilon(\lambda)$ is the extinction coefficient of the absorbing or exciting wavelength, C is the concentration of the absorbing molecules and l is the pathlength. For small absorbance ,

$e^{-2.3\varepsilon(\lambda)cl} << 1$, so by expanding and ignoring higher terms of equation (3) we get,

$$I_A = I_1 - I_0 = I_0 [1-(1-2.3\varepsilon(\lambda)cl\}]$$

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$= I_0(2.3\varepsilon(\lambda)cl)$ …….[4]

Replacing equation [4] in equation [2] we get the value of fluorescence intensity , as follows.

$F = I_0(2.3\varepsilon(\lambda)cl)\ \Phi_F Z$ ……..[5]

From equation (5) , I_0 if the intensity of the exciting light is kept constant and assuming that the Φ_F and Z are independent of the wavelength of the exciting light , then for a given solution :

$F \propto \varepsilon(\lambda)$ …….[6]

Quenching of fluorescence

A process, which leads to the fluorescence intensity of a given substance to decrease , is defined as fluorescence quenching. There are varieties of processes,e.g., energy transfer, complex formation and collisional quenching , can result in quenching. There are two types of quenching , dynamic quenching and static quenching. The quenching resulting from collisional encounters between fluorophore and quencher is defined as collisional or dynamic quenching, where as static quenching arises due to complex formation between fluorophore and quencher. Both mode of quenching require molecular contact between fluorophore and quencher. In dynamic quenching , the quencher molecule must diffuses to the fluorophore during lifetime of the excited state. Upon contact the fluorophore returns to the ground state, without emission of a photon. In static quenching a complex is formed between fluorophore and quencher and this complex is non-fluorescent.

Dynamic quenching of fluorescence is described by the Stern-Volmer equation.

$$F_0/F = \Phi_0/\Phi = k_F + k_{nr} + kq[Q] \ / \ k_F + k_{nr}$$
$$= 1 + k_q\tau_0[Q]$$
$$= 1 + K_{SV}[Q]$$
$$= \tau_0 / \tau \qquad \text{where } K_{SV} = k_q\tau_0$$

In this equation F_0 and F are the fluorescence intensities in the absence and presence of quencher (Q) respectively, Φ_0 and Φ are the corresponding quantum yields, [Q] is the concentration of collisional quenchers, τ_0 and τ are the fluorescence life times in the absence and presence of Q, K_{SV} is the Stern-Volmer constant for the collisional quenching process, which is equal to $k_q\tau_0$ where k_q is the bimolecular rate constant for the quenching process. Both τ_0 and τ can be defined in terms of sum of rate constants

$\tau_0 = (k_F + k_{nr})^{-1}$ and $\tau = (k_F + k_{nr} + kq[Q])^{-1}$ respectively.

Where k_F is the rate of fluorescence in the absence of any interactions , k_{nr} is the sum for all the nonradiative rates in interactive system lacking the quencher . The above equation predicts a linear plot of F_0/F (or τ_0 / τ) vs. [Q] with an intercept of one on the y-axis and a slope equal to K_{SV} .A linear Stern-Volmer plot is generally indicative of a single class of fluorophore , all equally accessible to quencher.

In case of quenching in solution plots of F_0/F (or χ / τ) vs. [Q] have been found to be upward curving. To account for this curvature static quenching mechanism has been considered. Any quenching interaction requires that the chromophore and the quencher be within a certain distance of one another. In solution, this critical distance defines an interaction sphere of volume V. At the time of photon absorption , i.e., on excitation of chromophore and the quencher molecule may already be within this active volume and thus be able to quench instantaneously without the need for a diffusion –controlled collisional interaction. The probability of the quencher being within this volume at the time of excitation depends on the volume and on the quencher concentration. Assuming that the quencher is randomly distributed in solution , the probability of static quenching is given by a Poisson distribution, $e^{-V[Q]}$ and this factor can be introduced into the Stern-Volmer equation in order to describe both quenching modes.

$F_0/F = (1+ K_{SV}[Q])\ e^{-V[Q]}$

This equation represents a single species undergoing both static and dynamic quenching and sphere of action static quenching.

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Often the static quenching term is expressed as (1+ Ka [Q]), where Ka is the static constant, rather than exp(V[Q]) term . In such treatments the static quenching constants represents an association for the formation of a one –to-one dark complex chromophore and quencher.

$$A + Q = A\text{-}Q$$

This ground state complex is able to reach the initial Frank-Condon state, however, because the quencher is part of the complex, quenching occurs instantaneously and efficiently without the need for a diffusion controlled interaction.

In the absence of quencher, F_0 is proportional to the entire chromophore concentration, $[A_{total}].F$, however is proportional to the free chromophore concentration $[A_F]$,and that of quencher [Q]. Therefore

$$Ka = [A\text{-}Q] / [A_F].[Q]$$

Again, $$[A_{total}] = [A_F] [AQ]$$

Substituting fluorophore concentration with the fluorescence intensities

$$F_0/F = 1 + Ka [Q]$$

Therefore , by combining this type of static quenching mechanism with the dynamic one

$$F_0/F = (1 + K_{SV} [Q]) (1 + Ka [Q])$$

Above equation represents a single species undergoing both dynamic quenching and ground state complex static quenching. The process of both quenching modes results in upward curving plot of F_0/F (or τ_0 / τ) vs. [Q]. The lifetime or the temperature dependence of quenching and careful examination of absorption spectra of fluorophore can be used to distinguish between dynamic and static quenching. In case of dynamic quenching decrease in lifetime of fluorophore occurs because quenching is an additional rate process, which depopulates the excited state. Here decrease in yield occurs because the quenching rate depopulates the excited state without fluorescence emission. Whereas static quenching removes a fraction of fluorophores from observation , and the complex fluorophores are nonfluoroscent , hence the observed fluorescence arising from uncomplexed fluorophore and as it is unperturbed , its life time is τ_0 .Therefore , for static quenching $\tau_0 / \tau = 1$. In contrast , for dynamic quenching $F_0/F = \tau_0 / \tau$. Again, higher temperatures result in larger diffusion coefficient , hence bimolecular quenching constants increase with increase in temperature. In contrast , increased temperature is likely to result in decreased stability of complexes and thus lower value of static quenching constants. Dynamic quenching only affects the excited states of the fluorophores and thus no change in absorption spectra are observed. In contrast , ground state complex formation results in perturbation of the absorption spectra of the fluorophore.

A nonlinear F_0/F plot also arises a system containing multiple chromophores, e.g., a protein molecule with more than tryptophan residues having different K_{SV} term due to either a different k_q, τ_0 or both. In most of the cases the excitation and/or emission energies of each species overlap. The measured F_0 and F values, therefore consists of weighted sums of the F_0 and F values for each species. Hence fluorescence intensity ratio is a function of Σ (1+ K_{SV} (i)[Q]) , where i denotes the specific quenching constant/species. Assuming that the individual species do not interact with one another , the complete expression for the dynamic quenching of multiple species must weight each element of this summation by the fractional intensity, f(i), of each emitting species. These f(i) are a function of the concentration, extinction, quantum yield and emission spectrum of each species; by definition, $\Sigma f(i)=1$. The complete expression for multiple (n) species undergoing only dynamic quenching is given by

$$F_0/F = [\Sigma_{i=1}{}^{n} f(i)/\{1 + K_{SV} (i)[Q]\}]^{-1}$$

Now, let us consider each species also undergoes static quenching by either the sphere of action or the ground state complex model. The resulting expression for the multiple species/ sphere of action model is given by,

$$F_0/F = [\Sigma_{i=1}{}^{n} f(i)/\{1 + K_{SV} (i)[Q]\} e^{-V(i)[Q]}]^{-1}$$

Where V(i) is the interaction sphere of volume for ith species.

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Considering ground state static quenching model, the expression for the ratio of fluorescence intensities is given by,

$$F_0/F=[\Sigma_{i=1}{}^{n}f(i)/\{1+ K_{SV}(i)[Q]\}\{ 1+ Ka)(i)[Q]\}]^{-1}$$

To analyze the quenching in heterogeneous systems, particularly those proteins showing downward curving plots of F_0/F vs. [Q] , a modified Stern-Volmer equation, originally proposed by Lehrer, is used. Considering two tryptophan popularities, one of which is accessible (a) to quencher and the other is inaccessible or buried (b), the modified Stern-Volmer equation is given by

$$F_0/\Delta F = (1/f_a K_{SV}[Q]) +(1/f_a)$$

Where f_a is the fraction of initial fluorescence, which is accessible to quencher.

$$f_a = F_{oa}/ (F_{oa} + F_{ob})$$

The o subscript refers to fluorescence intensity in absence of quencher. A plot of $F_0/\Delta F$ vs. 1/[Q] is linear, giving information concerning the fraction of fluorescence arising from quenchable fluorophores (from intercept, f_a^{-1}) and the effective quenching constant for the accessible fluorophores (from the slope of the plot ,($f_a K_{SV}$) ^{1}). The value of $F_0/\Delta F$ at infinite quencher concentration (1/[Q]=0) represents the reciprocal of the fluorescence, which is quenched. At this concentration only the inaccessible residues are present.

Another consideration regarding quenching reaction is that the quencher may or may not be efficient. If the efficiency , γ, of quenching is unity , then every encounter between quencher and the excited fluorophore results in the deactivation . $\gamma<1$ indicates that the quenching reaction is inefficient. Three quenchers of indole fluorescence which have $\gamma=1$ (nearby) are molecular oxygen, iodide and acrylamide. When fluorophore is other than tryptophan , it should be noted that acrylamide may be inefficient. For tryptophan in proteins , these three quenchers provide a useful, complementary set for studying the topography of the tryptophan residue. Oxygen is a small and apolar quencher and has greatest ability to diffuse into interior of globular proteins. Acrylamide is a larger, polar quencher molecule. It has greater selectivity in that it preferentially quenches solvent exposed tryptophan residues. Iodide, which is negatively charged and hydrated, has an even greater selectivity in being able to quench only exposed residues. The steric factor plays an important role in quenching. The planar fluorophore ethidium bromide (EB) intercalates between the base pair of double helical DNA. Upon binding to DNA the fluorescence lifetime of EB increases by 10-fold and bi-molecular quenching constant decreases by 30-fold. This is interpreted as the steric protection of EB provided by the DNA. The base pairs of DNA are staked on both sides of the fluorophore and prevent collisions with oxygen.

Sensitivity of fluorescence to the environment

Fluorescence is generally much more sensitive to the environment of the chromophore than light absorption. Hence it is an effective technique for following the binding of ligand or conformational changes. The sensitivity of fluorescence is a consequence of the relatively long time a molecule stays in an excited singlet state before deexcitation. During this time period (10^{-9} to 10^{-8}secs) a number of processes can occur, including protonation or deprotonation can occur, local conformational changes, and any processes coupled to translational or rotational motion.

Emission spectra are the most readily measured spectral parameter. The solvent sensitivity of emission spectra of many fluorophores is used to detect its binding to macromolecules or to infer the polarity of binding site on the macromolecule. The polarity dependent spectral shifts result from both the interactions of the dipolemoment of the fluorophores with reactive fields induced in the surrounding solvent, and from specific interaction between the fluorophore with solvent molecules. A variety of emission maxima are found for native proteins, which are found to be red shifted on denaturation. Thus shifts in emission maxima of proteins are considered to reflect the average degree of exposure to tryptophan residues to the aqueous phase. The emission maxima of 1,8-ANS bound to apomyoglobin is found to be strongly blue shifted which indicates ANS is bound to a site, which is not accessible to the external aqueous phase.

A number of fluorescent molecules have a very interesting property; in aqueous solution their fluorescence is very strongly quenched, but in nonpolar or rigid environment an enormous enhancement, by more than a

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factor of twenty is observed. If the probe binds to a rigid or nonpolar site on a protein or nucleic acid, the fluorescence spectrum will be dominated by the bound species. A chromophore free in aqueous solution is quite susceptible to such quenching. When incorporated into a macro-molecular structure, it may be considerably shielded from the solvent. This shielding can show up as a protection against quenching. Fluorescence, measured as a function of quencher concentration, often can help to discriminate residues on the surface of a protein from those deeply buried. 1,8-ANS, Bis-ANS, PRODAN, ethidium-bromide etc. are such environment sensitive probe. Since these probes are highly sensitive to solvent polarity, they can potentially reveal the polarity of their immediate environment. Quantum yield of 1,8-ANS is very small in aqueous solution but it increases considerably when 1,8-ANS makes complex with the proteins. The emission maxima also shift towards shorter wavelength. These two factors indicate that 1,8-ANS binds to hydrophobic pocket in proteins. Quantum yield of PRODAN also increases upon binding to hydrophobic region of a protein. Ethidium-bromide in aqueous solution has very weak fluorescence, but when it intercalates into double – helical region of a nucleic acid, the fluorescence is very intense.

A particular property of the excited state of some aromatic fluorophore is the ability to from an excited state dimmer, excimer. It can be used to obtain information about proximity at close distances. Pyrene and its derivatives etc. are most useful fluorophores, which show excimer fluorescence. Phenylalanine and tyrosine also exhibit intrinsic excimer fluorescence in proteins if pairs of these groups can approximately interact. The efficient excimer fluorescence can be used to probe conformational changes associated with a variety of protein interactions.

Applications of fluorescence spectroscopy to studies of proteins

Fluorescence spectroscopy has evolved as one of the most important tools in the study of protein structure and function. Though it cannot provide detail structure like X-ray crystallography or Nuclear Magnetic Resonance (NMR), the sensitivity of the technique allows one to work at or near intracellular concentrations of macromolecules and ligand.

õ ***Study of ligand binding***

The biological function of almost all proteins is related to the ability to bind ligand. Quantitative analysis, e.g., the strength, stoichiometry and specificity of ligand binding reactions contribute crucially in the study of protein structure, function as well as engineering. To study ligand binding one only requires that a change in quantum yield occurs upon ligand binding and it can be measured either by observing ligand fluorescence or intrinsic protein fluorescence or fluorescence of a covalently or noncovalently bound fluorescent probe which is sensitive to ligand binding. Changes in quantum yield upon ligand binding are extremely common, being caused by a variety of different mechanism such as sensitivity of fluorescent ligand to the environment, energy transfer from protein to ligand causing quenching of protein fluorescence, a conformational change in ligand on binding to the protein, or a ligand induced conformational change of protein.

Many environment sensitive fluorescent probes have been synthesized, e.g., ANS, PRODAN and Bis-ANS. The quantum yields and/or emission maxima of these probes change dramatically upon binding to hydrophobic sites and have been used to detect conformational changes.

Binding isotherms of these compounds can be derived by tritametric method. In case of environment insensitive fluorescent ligand, where ligand binding to protein does not lead to fluorescent enhancement or quenching, fluorescence polarization may be used to obtain binding constants. Binding of a ligand to a protein often leads to quenching or enhancement of tryptophan fluorescence. Binding constants can be calculated by double reciprocal plot when the protein concentrations are much below the dissociation constant. Stoichiometry may be obtained from titration with protein at a concentration at or above the dissociation constant.

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Binding of tRNA (L) to aaRS (P) has been determined from quenching of tryptophan fluorescence of synthetase enzyme. Considering the situation, where synthetase has a single site for binding of tRNA, the appropriate equilibrium for this interaction being described by the following:

$$P + L = PL$$

The binding constant is

$$K = [PL]_{eq} / ([P].[L]_{eq})$$

Where $[A_i]_{eq}$ represents equilibrium molar concentration of ith species. At low concentrations the fluorescence intensity is directly proportional to fluorescent solute concentration according to $F = 2.303\ \varepsilon(\lambda)C.l.I_0.\phi_F.\ f(\lambda).d$, where F is the fluorescence intensity, I_0 is the intensity at excitation source, $\varepsilon(\lambda)$ is the molar absorptivity at excitation wavelength (λ), C is the molar concentration , I is the path length , ϕ_F is the fluorescent quantum yield., $f(\chi)$ is the fraction of total emission that occur at wavelength and λ and d is the fraction of radiation emitted at λ actually collected by the detector. Because of this direct proportionality , the quantitative treatment of fluorescent binding data starts with the premise that the intensity is a sum of contributions. Considering ligand (L) is nonfluoroscent , we can write

$$F_0 = K_p[P]_0$$

$$F = K_p[P]_{eq} + K_{comp}[PL]_{eq}$$

$$F_\infty = K_{comp}[PL]_T = K_{comp}[P]_0$$

Where the Ki represent proportionality constants connecting the intensities and concentrations of the species. F_0 , F and F_∞ are the initial fluorescence intensity, i.e., in absence of ligand (L), fluorescence under equilibrium condition and that at infinite ligand concentration when the substrate (P) is completely converted to the bound form. $[P]_0$ and $[P]_{eq}$ are initial and equilibrium substrate concentrations respectively. $[PL]_{eq}$ and $[PL]_T$ are equilibrium complex concentrations and that at saturating conditions respectively.

Again, $[P]_0 = [P]_{eq} + [PL]_{eq}$

And $[L]_T = [L]_{eq} + [PL]_{eq}$

Where $[L]_T$ and $[L]_{eq}$ are total ligand concentration and that at equilibrium respectively.

Using above equations:

$$[PL]_{eq} = \frac{([P]_0 + [L]_T + 1/K_{eq}) \pm \sqrt{\{([P]_0 + [L]_T + 1/K_{eq})^2 - 4[P]_0[L]_T\}}}{2}$$

Therefore ,

$$\frac{F}{F_0} = 1 + \frac{\{(F/F0)-1\}\{[P]_0 + [L]_T + 1/K_{eq}) \pm \sqrt{\{([P]_0 + [L]_T + 1/K_{eq})^2 - 4[P]_0[L]_T}}{2[P_0]}$$

$$\frac{F}{F_0} = 1 + \frac{\{(F/F0)-1\}\{[P]_0 + [L]_T + K_d) \pm \sqrt{\{([P]_0 + [L]_T + 1/K_d)^2 - 4[P]_0[L]_T}}{2[P_0]}$$

Where $K_d = 1/K_{eq}$; K_d termed as dissociation constant of complex. The fluorescence quenching data are fitted to the above single site binding equation by a nonlinear least-square fit procedure, the initial fluorescence F_0 , F_∞ and K_d are systematically varied with in a given range. The set of values that gave the lowest χ^2 is chosen at the best-fit curve.

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õ ***Protein association –dissociation***

Heterologous and homologous protein association governs many important biological processes. Regulation of gene expression is found to bed controlled by protein-protein contact. Association plays a major role in the mechanism of regulation. Subunit association of multi-meric proteins plays a vital role in many catalytic and regulatory processes. Fluorescence spectroscopic technique can be used to study protein association-dissociation at equilibrium and at submicromolar concentrations. There are two principle fluorescence methods that are used to study protein-protein association: fluorescence anisotropy and fluorescence energy transfer.

Fluorescence anisotropy method is based on the fact that protein association results an increase in molecular weight, thereby increase in rotational correlation time. Therefore fluorophore rigidly attached to protein would also experience an increased rotational correlation time and hence an increased anisotropy value. Here lifetime of fluorophore should not very much smaller than the correlation value. As steady state anisotropy does not measure molecular weight, independent molecular weight estimates are needed at both ends of association-dissociation equilibrium. Then anisotropy values can be fitted to legitimate model using nonlinear least square fit procedure.

The energy transfer method is based labeling of one subunit of protein with fluorescence donor and another subunit with fluorescence acceptor and maintaining a large R0 for donor-acceptor pair. The dissociation of the hybrid dimmer causes average distance between the subunits to increase beyond the range of energy transfer, resulting loss of energy transfer that existed in the hybrid dimmer. The method is suitable for dimmer-monomer dissociation but is inapplicable for tetramer or higher order structures because of subunit reshuffling.

õ ***Conformational change***

Conformational changes in proteins can be monitored through fluorescence spectroscopy. Quantum yield and emission maxima of fluorophores are found to be very much sensitive to the microenvironment. Any change in conformation, which causes change in micro environment, results change in fluorescence properties of fluorophore. Tryptophans are most commonly used as probes for conformational change study. Single tryptophan proteins are very much useful for such purpose since the localization of the conformational change possible. Site-directed mutagenesis technique can also be used to construct single tryptophan proteins within the restrictions imposed by the protein structure. Phase modulated spectrometers and pico-second laser system allow tryptophan signal in protein to be resolved in the time domain resulting detailed residue level analysis of conformational change in multi-tryptophan protein. External environment sensitive fluorescent probes are also used to detect conformational change. For anilinio-napthalene sulfonic acid and its derivatives particularly 1,8-ANS and Bis-ANS, the quantum yield and to a moderate extent emission maxima are found to be very much sensitive to environment polarity. Any conformational change, causing change in polarity of microenvironment, leads to enhancement or quenching of fluorescence. The emission maxima of PRODAN and its sulfhydryl reactive analogue acrylodan is extraordinarily sensitive to polarity, and hence are sensitive to conformational changes. Quantum yield of di-cyano-vinyl-julolidfine is found to be sensitive to the polarity, and hence it can be used to detect conformational changes that increase or reduce mobility restrictions.

õ ***Folding and unfolding of proteins***

A major change in microenvironment of side chains arises during denaturation and renaturation of proteins. Hence environment sensitive external fluorescent probes as well as tryptophan can be used to probe structural changes during denaturation and renaturation of proteins. Several fluorescent techniques are useful in studying protein folding –unfolding reaction and characterization of intermediate states. Molten globule state, an intermediate state in protein folding pathway is often characterized by the accessible hydrophobic areas of proteins and bind dyes such as 1,8 ANS to a great extent. To estimate the compactness of the intermediate fluorescence energy transfer and polarization measurements are often used.

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Distance Information

Fluorescence techniques can be used to calculate distance between two fluorophores. Forster energy transfer between two fluorophores occurs in the range of 10-80 A^0 and the magnitude of energy transfer is inversely proportional to r^6, where r is the distance of separation. The distance calculations become difficult in multiple –donor single acceptor systems, which often arise in multiple-tryptophan proteins. If the energy donors differ in accessibility toward a collisional quencher and the acceptor fluorescence is in accessible to quenchers, then donor quenching can be used to resolve contribution to net energy transfer from a particular donor in favorable cases. In these cases, a collisional quencher is selected in such a way that it quenches donor fluorescence, without affecting acceptor fluorescence. Quenching of energy transfer can thus be attributed to accessible donors and Lehrer-type plot can be used to estimate the fraction of energy transfer from accessible and inaccessible donors.

Fluorescence anisotropy decay

Upon excitation with polarized light, the emission from fluorescent sample is also polarized. The molecules oriented with transition dipoles symmetrically around the Z-axis will be preferentially excited, when the polarized light along z-axis is applied. The principle is known as photoselection. Because the distribution of the excited molecule is anisotropic, the resulting fluorescence will be anisotropic. The polarization or anisotropy measurement reveals the average angular displacement of the fluorophore, which occurs between the absorption and subsequent emission of photon. This angular displacement is dependent on the rate and extent of rotational diffusion, which in turn depend on the viscosity of the solvent, and the size and the shape of the diffusive species. As a rule, degree of emission polarization is measured at a direct angle with the direction of excitation. By monitoring the emission that occurs parallel, $I_{||}$ and vertically, $I_{\perp}$, to a vertically polarized beam, the anisotropy of the emission is,

$$A = \frac{I_{||} - I_{\perp}}{I_{||} + 2 I_{\perp}}$$

And the polarization of the emission is

$$P = \frac{I_{||} - I_{\perp}}{I_{||} + I_{\perp}}$$

For a mixture of fluorophores, the average anisotropy is given by , $A=\Sigma f_i A_i$, where A_i indicates the anisotropy of the individual species and the f_i is the fractional fluorescence intensity.

The polarization of the light is defined as the fraction of the light , which is linearly polarized. The anisotropy , A of the light source is defined as the ratio of polarized component to the total intensity. The polarization is more useful and appropriate parameter for describing a light source, when the light is directed along a particular axis. But the radiation emitted by a fluorophore is symmetrical about Z-axis (when the excited light is Z axis is polarized.) Hence the total intensity is not given by $I_{||} + I_{\perp}$ but rather by

$I_{||} + 2 I_{\perp}$. This is because; the light radiated by a dipole is equally distributed along the X-axis and Y-axis. So it perhaps more appropriate to use the anisotropy parameter to describe fluorescent emission. The steady state anisotropy of the emission of the molecule depends on the average angle,θ, in the space between a vector representing the absorption transition and a vector representing emission transition orientation for an ensemble of chromophore,

$$A = \frac{3\cos^2\theta - 1}{5}$$

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If the absorption and emission oscillators remains parallel, i.e., $\theta=0$, no orientation occurs during the excited lifetime, then an anisotropy of 0.4 is observed. This value is referred to the limiting anisotropy, A_0. Several processes however can cause, θ, not be zero.

1.Motional effects, which lead to reorientation of the molecule during the excited state lifetime.

2. Energy transfer effects, in which there is difference in the orientation of the donor and the acceptor.

The individual depolarizing factors, d_1, are related to the average angle, θ, of reorientation by a given process, through the following relationship:

$$d_i = \frac{3\cos^2\theta i - 1}{2}$$

rotational diffusion is the dominating cause of fluorescence depolarization. For an isotropically rotating sphere, having a rigidly affixed chromophore , the steady state anisotropy is related by the Perrin equation, to the life time , τ_1, and the rotational correlation time, τ_c, as follows.

$$A = \frac{A_0}{1+ \tau_F / \tau_c}$$

The value of τ_c is an inverse measure of the speed of rotation of the sphere, and can be related to the rotational diffusion coefficient, D_R, and the effective hydrodynamic molar volume, V of the sphere by, τ_c -1/6 $D_R= V\eta/RT$, where $\eta$is the viscosity of the solution.

$$A^{-1}= A_0^{-1} (1+ \tau_F /\tau_c)= A_0^{-1} [1+(\tau_F RT)/V\eta]$$

A plot of A^{-1} versus $1/\eta$ should be linear at a constant temperature , with intercept equal to A_0^{-1} and with a slope /intercept that is directly proportional to τ . A nonlinear plot indicates more than one rotational mode.

Measurement of anisotropy

L-format or single channel method : This is the most frequently used method for anisotropy measurement. In this method, the excitation monochromator partially polarizes the incident light. As a result of which rotation of the excitation polarizer to the horizontal(H) and vertical (V) position yields different intensities of incident light, even if light source generates unpolarized light. Similarly, emission monochromator has a different transmission efficiencies for H and V. Hence the measured intensities are not actual $I_{||}$ and $I_{\perp}$.

Let S_V and S_H be the sensitivities of the emission channel for vertically and horizontally polarized components. I_{HV} be the horizontally polarized excitation and vertically polarized emission. For vertically polarized excitation , the observed polarized intensities are,

$$I_{VV} = K S_V I_{||}$$

$$I_{VH} = K S_H I_{\perp}$$

K is the proportionality constant which include the quantum yield of fluorophore and other instrumental factor.

$$I_{VV} / I_{VH} = S_V I_{||} / S_H I_{\perp} = G I_{||} / I_{\perp}$$

G is the ratio of sensitivities of detection system for vertically and horizontally polarized light. It depends upon the emission wavelength and bandpass of the monochromator. G can be easily measured using horizontally polarized excitation. When this is performed, both the horizontally and vertically polarized components are proportional to $I_{\perp}$. This is because both this orientations are perpendicular to the polarization of the excitation. Hence , any measured difference in I_{VV} and I_{VH} must be due to properties of the detection system.

$$I_{HV} / I_{HH} = S_V I_{\perp} / S_H I_{\perp} = S_V / S_H = G$$

Therefore, $I_{||} / I_{\perp} = I_{VV} / I_{HH} = 1/G$

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The anisotropy is given by,

$A= (I_{||}/ I_{\perp})-1/(I_{||}/ I_{\perp})+2 = (I_{VV} -G I_{VH}) / (I_{VV} +2G I_{VH})$

Fluorescence anisotropy decay

Fluorescence anisotropy measures the average angular displacement of a fluorophore that occurs between the absorption and the subsequent emission of a photon. The excitation of isotropically distributed fluorophores in a medium with linearly polarized light produces nonuniformity in the orientational distribution of the fluorophore in the ground and excited state. This induces anisotropy in the orientational distribution of the fluorophore, which in turn gives rise to orientational or rotational relaxation due to the time varying torque experienced by the fluorophore in collision with the surrounding molecules. The randomization of the rotational motion of the fluorophore leads to a depolarization of the fluorescence and a decay of the induced dichroism.

Theory of time-resolved anisotropy decay in an organized assembly

The decay of time-resolved fluorescence anisotropy provides direct information on orientational motion of a fluorophore in an organized assembly. The time dependent fluorescence anisotropy, r(t) is given by

$$r(t) = \frac{I_{||}(t) - GI_{\perp}(t)}{I_{||}(t) + 2GI_{\perp}(t)}$$

where G is a correction factor for instrumental anisotropy.

Recently , fluorescence anisotropy decay has been studied in many organized assembly such as micelles, reverse micelles, cyclodextrins, polymer, and in biomolecules such as DNA, protein, and lipid. Although in a homogeneous solution, rotational diffusion coefficient is inversely related to viscosity, it has been pointed out that rotational dynamics of a probe in an organized assembly is much more complex and involves more than one kind of motion. According to the "wobbling-in-cone" model, the anisotropy decay r(t), as a result of three independent motions-I) wobbling motion, $r_w(t)$ of the dye molecule in a cone ii) translational motion , $r_T(t)$ of the dye along the surface of the macromolecular assembly and iii) overall rotation , $r_M(t)$ of the macromolecule. Thus,

$$r(t)= r_w(t)\, r_T(t)\, r_M(t)$$

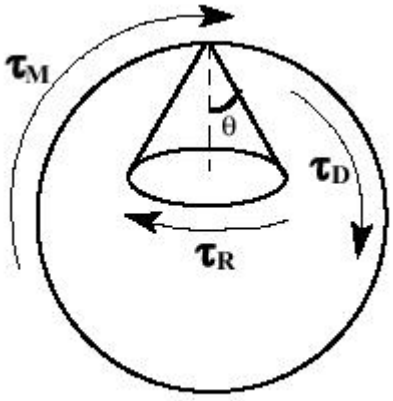

Fig. 2-4. "Wobbling-in-cone" Model

According to this model , the decay of r(t) is biexponential ,

$$r(t)= r_o[\beta \exp(-t / \tau_{slow})] + (1-\beta) \exp (-t / \tau_{fast})]$$

If denotes the initial anisotropy (at t = 0), and τ_R, τ_D, τ_M are the time constants respectively for wobbling, translational and overall rotation,

$$r(t) = r_o [S^2 + (1-S^2)\exp(-t/\tau_R)]\exp\{-t(1/\tau_D + 1/\tau_M)$$

where S is the order parameter related to the semicone angle θ in the wobbling –in-cone model as,

$$S = 0.5 \cos\theta (1+\cos\theta)$$

Comparing equations one immediately gets

$$\beta = S^2$$

$$1/\tau_{fast} = 1/\tau_R + 1/\tau_D + 1/\tau_M$$

$$1/\tau_{slow} = 1/\tau_D + 1/\tau_M$$

When the probe is attached to a spherical particle, τ_M is given by

$$\tau_M = 4\pi\eta r_h^3/3KT$$

where η=viscosity of water and r_h = hydrodynamic radii of the spherical macromolecule. Since τ_M is obtained from precious equation, Thus time constants of all three kinds of motions may be obtained. From the time constants the diffusion coefficients for the different kinds of motions are obtained.

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