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Laboratory Exercises

Protein Tryptophan Accessibility Studied by Fluorescence Quenching

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Matías Möller and Ana Denicola‡

From the Physical Biochemistry Laboratory, Facultad de Ciencias, Universidad de la República, Iguá 4225, 11400 Montevideo, Uruguay

A laboratory class is described to introduce the biochemistry major students to the basic concepts and various applications of fluorescence spectroscopy. Through simple and inexpensive experiments the students learn how to record excitation and emission spectra and measure intrinsic protein fluorescence and its quenching to elucidate the local tryptophan environment. Free tryptophan, ovalbumin, and bovine serum albumin are used for the experiments. The structural information that can be retrieved from emission spectra, as well as from quenching data, is discussed.

Keywords: Tryptophan fluorescence, tryptophan accessibility, fluorescence quenching, Stern-Volmer constant.

In fluorescence spectroscopy we have two processes going on [1]. First, there is the absorption of light, which promotes an electron to an excited state, and then there is the emission of light, which is when the excited electron returns to the ground state undergoing a radiative transition. Because some of the light energy initially absorbed is lost in transitions between vibrational energy levels (internal conversion), the light energy emitted is always of longer wavelength (see Scheme I). The efficiency of the emission is defined by the quantum yield (Φ), which is the ratio of the photons emitted over the total photons absorbed.

Many biological molecules display fluorescence, such as reduced nicotinamide dinucleotide (NADH), oxidized flavins (FAD, the adenine dinucleotide, and FMN, the mononucleotide), pyridoxal phosphate, chlorophyll, and proteins [1]. Intrinsic protein fluorescence is due to the aromatic amino acids, mainly tryptophan, considering that phenylalanine has a very low quantum yield, and emission by tyrosine in native proteins is often quenched. Fluorescence due only to tryptophan residues can be selectively measured by exciting at 295 nm, because at this wavelength there is no absorption by tyrosine. Tryptophan fluorescence is highly sensitive to the environment polarity, and shifts in its emission spectrum toward lower wavelengths (blue shift) can be seen as hydrophobicity increases. Changes in emission spectra from tryptophan can be seen in response to protein conformational transitions, subunit association, ligand binding, or denaturation, all of which can affect the local environment surrounding the indole ring.

The lifetime of a fluorophore (*i.e.* the time the fluorophore spends in the excited state) is very short, ranging from 0.5 to 100 ns, but sufficiently long for the excited fluorophore to react with external molecules to yield an excited product that decays by a nonradiative pathway and therefore diminishes the fluorescence quantum yield. This process is known as dynamic or collisional quenching, and the external molecule is known as the quencher [1]. The whole process can be summarized briefly by the following equations (Equations 1–3), where *X* represents the fluorophore, and *Q* represents the quencher.

$$X + h\nu \rightarrow X^* \text{ (excitation)} \quad \text{(Eq. 1)}$$

(*a*) Without quencher: $X^* \rightarrow X + h\nu'$ (emission) (Eq. 2)

(*b*) With quencher Q:

$$X^* + Q \xrightarrow{k} XQ^* \rightarrow XQ \text{ (no emission)} \quad \text{(Eq. 3)}$$

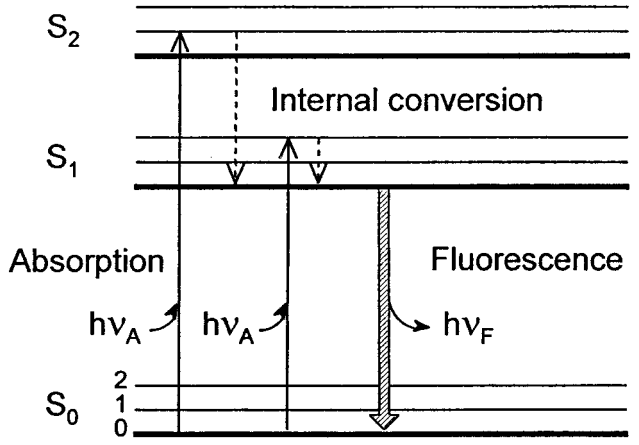
The change in fluorescence intensity due to the presence of the quencher is given by the Stern-Volmer relationship, shown in Equation 4,

$$I_0/I = 1 + k\,\tau\,[Q] \quad \text{(Eq. 4)}$$

where I_0 and I represent the fluorescence intensity in the absence and in the presence of a given concentration of *Q*, respectively. τ is the lifetime of the fluorophore, and k is the bimolecular quenching rate constant. The product $k\ \tau$ is known as the Stern-Volmer constant, K_{SV},[1] and is a parameter that senses the accessibility of the quencher to the fluorophore. In particular, tryptophan appears to be uniquely sensitive to collisional quenching, apparently because of a tendency of indole rings to donate electrons while in the excited state. Externally added quenchers like iodide, oxygen, and acrylamide can quench tryptophan fluorescence. Given the different size and charge of these molecules, quenching of intrinsic protein fluorescence can

‡ To whom correspondence should be addressed. Tel.: 5982-5258618; Fax: 5982-5258617; E-mail: denicola@fcien.edu.uy.

[1] The abbreviations used are: K_{SV}, Stern-Volmer constant; BSA, bovine serum albumin; OVA, ovalbumin; λ_{em}, emission wavelength; λ_{ex}, excitation wavelength.



SCHEME 1. **Jablonski diagram.** At each electronic energy level (S_0, S_1, S_2), the fluorophore can exist in a number of vibrational energy levels (*0*, *1*, *2*, etc.). The fluorophore is usually excited to some higher vibrational level of either S_1 or S_2 but rapidly relaxes to the lowest vibrational level of S_1 (*dotted arrows*), a process called internal conversion, that occurs in less than 10^{-12} s. Because the lifetime of the fluorophore is near 10^{-8} s, internal conversion is complete prior to emission, which results from deactivation from the lowest vibrational state of S_1 to ground level S_0.

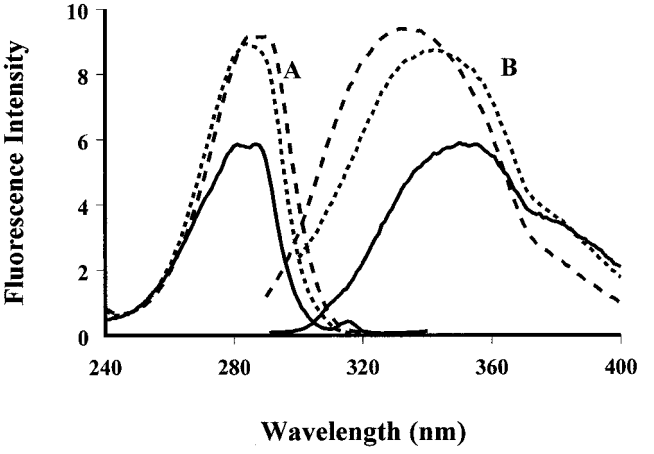


FIG. 1. **Excitation (λ_{em} = 350) (*A*) and emission (λ_{ex} = 280) (*B*) spectra of tryptophan in solution (*solid line*), BSA (*dotted line*), and OVA (*dashed line*).**

be used to differentiate the location of tryptophan residues and also to follow protein conformational changes [2]. The emission intensity of a tryptophan residue located on the protein surface will be strongly decreased in the presence of a charged water-soluble quencher like iodide. On the other hand, the emission of a buried tryptophan residue will be less affected by the presence of the quencher.

The aim of this class is to present to the student some standard applications of intrinsic protein fluorescence, determination of λ_{max} of emission and quenching by iodide of tryptophan in solution, as well as part of a protein, which enables the study of differential accessibility of tryptophan residues [3].

EXPERIMENTAL PROCEDURES

Solutions—The solutions needed for these experiments are as follows: 100 mM sodium phosphate buffer, pH 7.4, 3 M sodium chloride, 50 μM tryptophan dissolved in distilled water, stock solution of 1 M potassium iodide also dissolved in distilled water (this solution should be prepared the same day of the experiment, because it easily becomes oxidized, and small amounts of sodium dithionite should be added), 5 mg/ml bovine serum albumin (BSA), and 9 mg/ml ovalbumin (OVA). Pure OVA is not really needed, and a solution containing a high percentage of OVA can be obtained directly from hen egg white by dilution of 1:10 in phosphate buffer and filtration through filter paper. Protein concentration is determined by Biuret assay [4].

Tryptophan Emission and Excitation Spectra—Prepare 5 μM tryptophan by diluting the tryptophan stock solution with phosphate buffer. Aromatic amino acids are known to absorb light at the near ultraviolet region, around 280 nm. This is the excitation wavelength (λ_{ex}) we will use. After setting the excitation wavelength to 280 nm, proceed to acquire the emission spectrum recording between 300 and 400 nm. From this spectrum, the maximum emission wavelength (λ_{em}) can be obtained, and this wavelength can be used to obtain the excitation spectrum, *i.e.* fix λ_{em}, and sweep the λ_{ex} between 240 and 340 nm. Repeat the procedure with 0.1 mg/ml OVA and 0.2 mg/ml BSA.

Quenching of Fluorescence by Iodide—Prepare several tubes containing 5 μM tryptophan and increasing concentrations of iodide (0 to 50 mM) in buffer. Set the excitation and emission λ at the maximum values you found previously. Measure the fluorescence of all your tubes. Repeat this procedure with 0.1 mg/ml OVA and 0.2 mg/ml BSA instead of tryptophan (similar tryptophan molar concentrations). Repeat the quenching assays now using 0.2 mg/ml BSA in the presence of 2 M sodium chloride. Display the data as a Stern-Volmer plot, I_0/I *versus* [iodide]. Use the modified Stern-Volmer equation (see "Results and Discussion") to calculate fractional tryptophan accessibility.

RESULTS AND DISCUSSION

Fig. 1 shows the excitation and emission spectra of tryptophan in solution and as part of protein structures. The λ_{max} of absorption and emission can be determined, and it can be seen that fluorescence occurs at a higher λ than that of the incident light. Tryptophan in solution displays an excitation maximum near 280 nm and emits near 350 nm. The emission spectrum of tryptophan is sensitive to solvent polarity. If the tryptophan residue is buried in a hydrophobic environment within a protein, the emission may be blue-shifted. In fact, we can observe that emission λ_{max} from BSA tryptophans is near 340 nm and that from OVA tryptophans is close to 330 nm, 20 nm lower than for free tryptophan (Fig. 1).

Fig. 2 shows the Stern-Volmer plots for the quenching by iodide of tryptophan in solution, as well as part of the two proteins studied, OVA and BSA. From the slopes of Fig. 2, the K_{SV} values can be determined and are summarized in Table I.

It was expected that the highest K_{SV} value corresponded to tryptophan in solution, because there is no barrier to the encounters between fluorophore (tryptophan) and quencher (iodide). The value obtained is high indeed ($K_{SV} \sim 8\ M^{-1}$), though not the highest, and this will be discussed later.

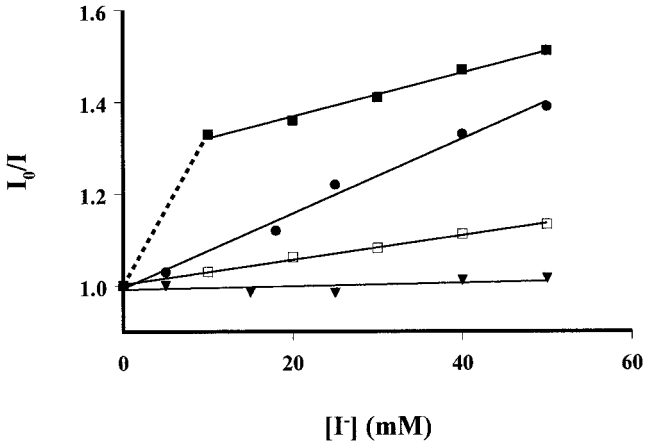


FIG. 2. **Stern-Volmer plots for the quenching by iodide of tryptophan in solution (●), OVA (▼), and BSA at low ionic strength (■) and BSA at ionic strength = 2 M (□).**

TABLE I
Quenching by iodide

	K_{SV} (M^{-1})
Trp (solution)	8.1 ± 0.4
OVA	0.33 ± 0.02
BSA_1[a]	26 ± 0.6
BSA_2[a]	4.7 ± 0.3
BSA[b]	2.1 ± 0.2

[a] K_{SV} determined at low ionic strength.
[b] K_{SV} determined at ionic strength = 2 M.

The K_{SV} obtained for the native OVA is low (K_{SV} = 0.33 M^{-1}) indicating that the three tryptophan residues of OVA are essentially unquenched by iodide. This result supports a buried rather than a surface site for the OVA tryptophans, which is in agreement with the observed blue shift in emission λ_{max} (Fig. 1).

BSA quenching needs a more extensive analysis. BSA quenching is clearly biphasic ($K_{SV1} \sim 26\ M^{-1}$ and $K_{SV2} \sim 4.7\ M^{-1}$). The K_{SV} calculated for the first phase is 3-fold higher than the K_{SV} determined for free tryptophan. Because free tryptophan has no barrier to the encounter with iodide it can be said that its K_{SV} should correspond to the maximum expected because of simple collisions. Therefore it can be hypothesized that there is some kind of interaction, so far unknown, that considerably increases fluorescence quenching. The second phase has a more reasonable K_{SV} value, nearly half of that determined for free tryptophan, which can easily be attributed to collisional quenching by free iodide to BSA.

Given the charged nature of iodide, quenching will depend not only on how exposed tryptophan residues are but also on its surrounding charges. Negative charges will result in decreased quenching whereas positive charges will have the opposite effect [5, 6]. It is expected that in the presence of increasing medium ionic strength, such effect should decrease because of diminished electrostatic attraction and because of anions (chloride) occupying iodide binding sites. It can be seen in Fig. 2 (*open squares*) that the biphasic quenching pattern indeed disappears at high ionic strength (ionic strength = 2 M). Another possibility that could be considered is that given that BSA has two tryptophan residues, the observed biphasic quenching would be due to differential accessibility of the two tryptophans.

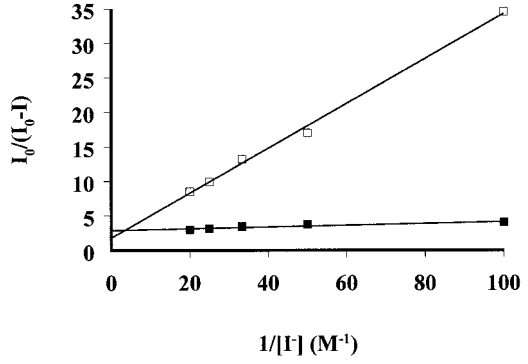


FIG. 3. **Modified Stern-Volmer plots to estimate fractional tryptophan accessibility to the quencher for BSA at low ionic strength (■) and BSA at ionic strength = 2 M (□).**

In the case of a protein containing one fluorophore, the ratio of the fluorescence intensities with and without quencher is given by the Stern-Volmer relationship (Equation 4). In the case of a protein containing two nonidentical tryptophan residues, the quenching is described as follows in Equation 5 [3, 5],

$$I = \frac{I'_0}{1 + K'_{SV}[Q]} + \frac{I''_0}{1 + K''_{SV}[Q]} \qquad \text{(Eq. 5)}$$

where I'_0 and I''_0 are the fluorescence intensities of each tryptophan residue in the absence of quencher, K'_{SV} and K''_{SV} are the corresponding quenching constants, and I is the observed fluorescence in the presence of a concentration of the quencher $[Q]$. If one of the tryptophans is unavailable to the quencher, then the equation reduces to the form represented by Equation 6,

$$I = \frac{I'_0}{1 + K'_{SV}[Q]} + I''_0 = \frac{I'_0}{1 + K'_{SV}[Q]} + I^t_0 - I'_0 \qquad \text{(Eq. 6)}$$

where I^t_0 is the total fluorescence intensity (because of the tryptophans) in the absence of quencher. A plot of I *versus* $[Q]$ would not be linear. However a plot of $I^t_0/\Delta I$ *versus* $(1/[Q])$ should be linear where $\Delta I = I^t_0 - I$. This is shown in Equation 7.

$$\frac{I^t_0}{I^t_0 - I} = \frac{I^t_0}{I'_0} \cdot \frac{1}{K_{SV}} \cdot \frac{1}{[Q]} + \frac{I^t_0}{I'_0} \qquad \text{(Eq. 7)}$$

The reciprocal of the ordinate value at $(1/[Q]) = 0$ represents the fraction of I^t_0 due to the quenchable tryptophan residue, and the ratio of intercept to slope gives K'_{SV}.

Applying Equation 7 to the data obtained for BSA, both at low and high ionic strength, straight lines were obtained (Fig. 3) that intersect the *y* axis at a value of 2, meaning that half of the fluorescence is due to quenchable tryptophan. This data therefore conforms to the assumptions of Equation 7 that only one of two nonidentical tryptophan residues of BSA are quenchable whereas the other is buried within the protein, unavailable to the quencher.

The biphasic iodide quenching observed for BSA was originally observed in human serum albumin, which has only one tryptophan residue. The first phase (*dotted line* in Fig. 2) was ascribed to quenching by an iodide molecule

that binds near the tryptophan, whereas the second phase is due to quenching by free diffusing iodide. In both cases the quenching is collisional, but the binding considerably increases the probability that iodide reacts with the excited tryptophan [3, 6]. BSA has two tryptophan residues, tryptophan 134 and 214. Noel and Hunter [3] showed that only tryptophan 214 can be quenched by iodide, whereas tryptophan 134 is normally protected.

As being exemplified for OVA and BSA, quenching of tryptophan fluorescence provides information on the location of this amino acid residue in the protein. The accessibility, as defined by K_{SV}, in general will depend upon "steric exposure," and in the case of charged quenchers it will depend additionally on local charge. The use of charged quenchers minimizes the problem of the partitioning or the diffusion of the quencher within the hydrophobic core of proteins. Local charge effects can be studied by varying ionic strength as we did in class. Quenching experiments can be used to calculate fractional fluorophore accessibility and determine the number of accessible tryptophans.

In addition, the quenching reaction can be used not only to probe topological features of the macromolecular structure but also to follow any protein conformation change that affects tryptophan accessibility. Indeed, any treatment of the native protein that involves a change in the tryptophan environment can be followed by fluorescence quenching. The tryptophan residue can act as a reporter group for local conformation change.

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