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Laboratory Exercises

Labeling of Proteins with Fluorescent Probes

PHOTOPHYSICAL CHARACTERIZATION OF DANSYLATED BOVINE SERUM ALBUMIN*

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Fluorescence spectroscopy is a widely used technique in biophysical studies. One of the strategies frequently used consists of labeling biomolecules with fluorescent probes, which have distinctive photophysical properties. This methodology allows the study of a wide variety of structural features of the biomolecule. We describe a simple laboratory activity for undergraduate Biophysical Chemistry courses. The experimental work includes two activities: labeling BSA with dansyl chloride and analyzing the resulting absorption and fluorescence spectra. The discussion of these activities helps students to understand the basis of fluorescence spectroscopy with emphasis in the application to biological systems.

Keywords: BSA, fluorescent probes, fluorescence spectroscopy.

When a molecule absorbs light in the UV-visible range, it undergoes an electronic transition from the ground state to an excited state. This condition is unstable, and thus, the molecule spontaneously returns to the ground state (Scheme 1).

In the simplest relaxation mechanism, the molecule proceeds non-radiatively to the first singlet excited state (S_1)[1], in a process known as internal conversion. Then, it dissipates the excess energy by non-radiative mechanisms or through the emission of photons, in a process known as luminescence. Particularly, if the emission occurs without changing the electron spin multiplicity, the process is called fluorescence [1].

Electronic excitation can also occur if the molecule receives the energy from another molecule that was previously excited by the absorption of photons. This transfer can occur if the excess energy of the excited donor is similar to that involved in the excitation of the acceptor [1].

Absorption and emission of a photon by a molecule are virtually instantaneous processes during which neither intra- nor intermolecular processes can happen. Before emitting a photon, the molecule remains in the excited state for a few nanoseconds [2], the lifetime of this excited state, which is defined as the inverse of the rate constant for returning to the ground state. This period is long enough to allow either intramolecular rearrangements or interactions with other molecules [3]. Thus, the information obtained from absorption or fluorescence experiments usually differ. The former refers to the structural, static properties of the system; and the latter also includes information about changes occurring during the lifetime of the excited state. A very good discussion of fluorescence principles and applications can be found in a recent review of Croney *et al.* [4].

For some biophysical studies, the biomolecule requires labeling with a small foreign molecule that has distinctive photophysical properties and associates with the macromolecule by either covalent or noncovalent bonds [5]. This molecule, denoted as a spectroscopic probe, acts as a sensor of the changes occurring in the biomolecule and translates them into modifications of its photophysical properties.

Labeling biomolecules with fluorescent probes constitutes a commonly used technique in scientific research. This methodology allows the study of a wide variety of structural features, the dynamics of structural changes, and interactions among molecules [6, 7].

In this work, we present a simple procedure for labeling BSA with the fluorescent probe dansyl chloride. The labeled protein was examined by absorption and fluorescence spectroscopy. These activities were designed as an initial approach to fluorescence spectroscopy for Biophysical Chemistry undergraduate courses.

Dansyl chloride is a fluorescent probe widely used in protein structural studies since the earlier works of Gregorio Weber [8]. Protein amino groups, including the N terminus and the ϵ-amino of lysine (Lys) residues, react with this probe through the lone pair of electrons by a substitution reaction [9] (Scheme 2).

Because covalent labeling is a chemical modification procedure, it always affects, to some extent, the function of biomolecules. A suitable probe should produce only little changes on the structure and function of the macro-

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[1] The abbreviations used are: S, singlet state(s); MOPS, 3-(*N*-morpholino)propane-sulfonic acid; DMF, dimethylformamide; RET, radiationless resonance energy transfer.

molecule. This fact must be explored by using independent methods, such as electrophoresis, circular dichroism, and functional studies, to compare the properties of the labeled and unlabeled species before using it in spectroscopic studies [5]. It was demonstrated that neither denaturation nor changes in the biological activity occurred after coupling BSA with dansyl chloride [8]. These facts suggest that dansyl chloride is a suitable probe for biophysical studies of BSA.

MATERIALS AND METHODS

Materials

BSA (catalog number A-7906) and dansyl chloride (catalog number D-2625) were obtained from Sigma Chemical Company. All other chemicals used in this work were of analytical grade.

Spectroscopic Measurements

Absorption measurements were performed in a Shimadzu UV-160A (Kyoto, Japan) spectrophotometer. Fluorescence spectra were registered using an SLM-AMINCO (SLM, Urbana, IL) BOWMAN Series 2 spectrofluorometer. The spectra were registered at 20 °C on 30 μM BSA or dansyl-BSA in 10 mM Tris-HCl (pH = 7.4).

BSA Labeling with Dansyl Chloride

To accomplish the labeling, students incubated the probe (300 μM in 10 mM 3-[N]-morpholinopropane-sulfonic acid [MOPS]-K [pH 8.4 at 25 °C]) with the protein (300 μM) during 1.5 h at 25 °C in the dark, with continuous stirring.

Given the pK_a values of the ϵ-amino of Lys residues and the N-terminal residue of proteins [10], these groups are protonated at a neutral pH, being unreactive for the dansylation. Thus, the media should be slightly basic to allow the reaction.

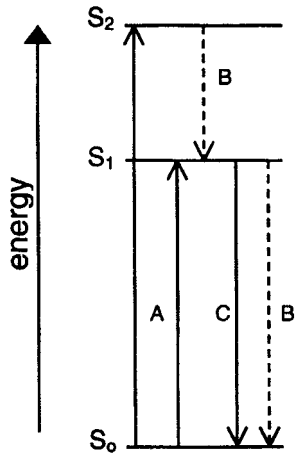
Because dansyl chloride is poorly soluble in water, it must be added to the sample dissolved in a nonaqueous solvent that is, additionally, nonreactive with the probe. Dimethylformamide (DMF) fits these conditions [9]. The concentration of dansyl chloride in the DMF solution was calculated spectrophotometrically after adding 10 μl of a solution consisting of nearly 100 mM dansyl chloride to 5 ml $CHCl_3$, considering that the extinction coefficient at 372 nm in this medium is 3,900 $M^{-1}cm^{-1}$ [9].

After labeling, 10 mM Tris was added. Because this buffer contains a primary amine group, it reacts with the free probe, stopping the reaction with BSA. The sample is then dialyzed against 10 mM Tris-HCl (pH 7.4 at 20 °C) to remove unreacted probe and secondary products such as that obtained from the hydrolysis of dansyl chloride [9].

The stoichiometry of labeling was determined by measuring the protein and probe concentrations in the labeled sample. The dye concentration was calculated using $\epsilon_{339\ nm}$ = 3,370 M^{-1} cm^{-1} [11]. This is possible because the unlabeled protein does not present significant absorption at this wavelength. BSA concentration after labeling was calculated according to Peterson [12]. By following this procedure, students determined a mole ratio of probe/protein = 0.95.

Structure of the Class Activities

The experimental work was organized in two time blocks of 3 h each. In the first time block, the students discussed the experimental design and performed the labeling reaction. In the second time block, they registered and analyzed the absorption and fluorescence spectra. Discussion was done in small groups of students and then extended to the whole class.



SCHEME 1. **Jablonski diagram.** The singlet ground state (S_o) and the first and second singlet excited states (S_1 and S_2) are represented. Absorption (*A*), internal conversion (*B*), and fluorescence transitions (*C*). *Continuous lines* show the processes that involve absorption or emission of photons. *Dashed lines* represent those processes occurring with heat dissipation. To simplify the scheme, vibrational levels associated with each electronic level and transitions involving triplet states are not represented.

RESULTS AND DISCUSSION

Absorption Spectra Analysis

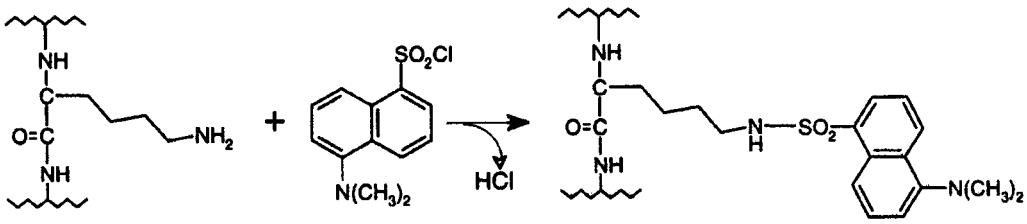
Figure 1 shows the absorption spectrum of unlabeled BSA and dansyl-BSA. Both spectra present a maximum at 280 nm, which is characteristic of tryptophan (Trp) and tyrosine (Tyr) residues in proteins [13]. The labeled albumin also presents a second maximum at 340 nm that corresponds to dansyl absorption after conjugation with proteins [9].

Fluorescence Emission Spectra Analysis

Students registered the fluorescence spectra of BSA and dansyl-BSA on excitation at those wavelengths corresponding to the maxima detected in the absorption spectra (Fig. 2).

On excitation at 340 nm, only the labeled protein fluoresces, as can be expected considering that aromatic residues of BSA do not absorb light at this wavelength. The fluorescence spectrum presents a maximum at 470 nm, corresponding to dansyl emission [9].

After excitation at 280 nm, the fluorescence spectrum of



SCHEME 2. **Reaction of dansyl chloride with a Lys residue of a protein.**

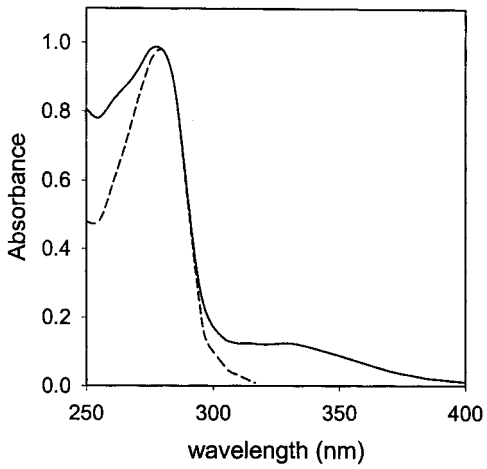


FIG. 1. **Absorption spectra of BSA (*dashed line*) and dansyl-BSA (*continuous line*).**

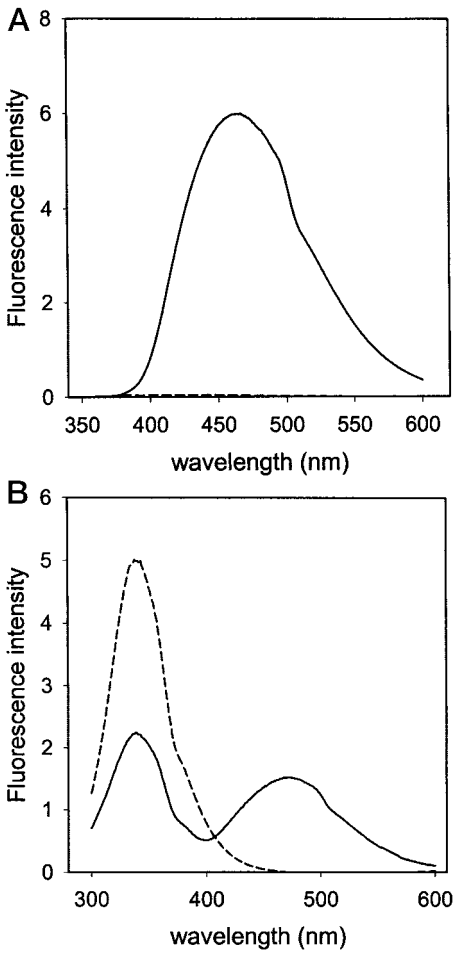
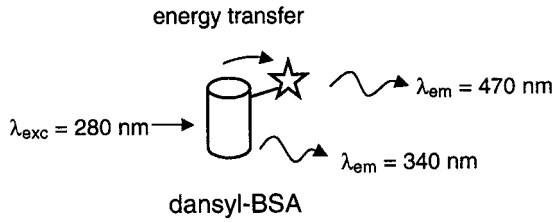


FIG. 2. **Emission spectra.** The spectra of BSA (*dashed line*) and dansyl-BSA (*continuous line*) were registered under the conditions indicated (see "Materials and Methods"), setting the excitation wavelength at 340 nm (*A*) or 280 nm (*B*).

unlabeled BSA presents a maximum at 340 nm, corresponding to Trp and Tyr residues emission [14]. Conversely, dansyl-BSA presents two maxima located at 340 nm and 470 nm. This last corresponds to dansyl emission, as was verified in the previous experiment (Fig. 2*A*). This is a surprising result, because the dansyl probe should not be excited at 280 nm. Also, the fluorescence intensity of



SCHEME 3. **Intramolecular RET in dansyl-BSA.** The cylinder represents the BSA molecule, and the star represents the dansyl probe attached to it.

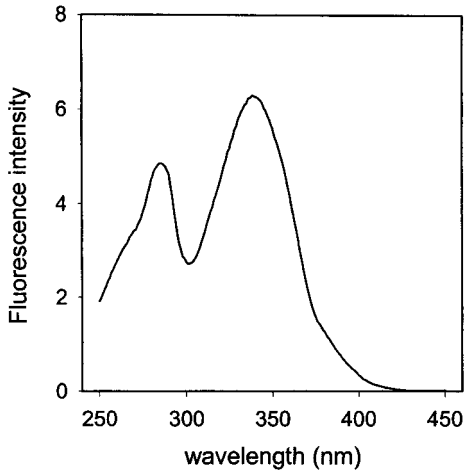


FIG. 3. **Excitation spectrum of dansyl-BSA.** The spectrum was registered under the conditions indicated (see "Materials and Methods"), setting the emission wavelength at 470 nm.

the band at 340 nm, corresponding to dansyl-BSA, is ~60% lower than that of unlabeled BSA, although the protein concentration was the same.

Comparing Figs. 1 and 2*B*, it can be observed that the emission band of BSA and the absorption band of dansyl are both centered at 340 nm. This fact determines that some Trp and Tyr residues of the protein in the excited state can transfer the excess energy to the fluorescent probe.

This phenomenon does not occur with the emission and reabsorption of a photon, it arises from the coupling of the transition dipoles of the donor and acceptor [1], and it is known as radiationless resonance energy transfer (RET). It can be demonstrated that the efficiency of the coupling is inversely related to the sixth power of the donor-acceptor distance [15]. Thus, RET is only possible if both fluorophores are separated by 1–10 nm [16]. This distance is on the order of the dimension of the BSA molecule [17, 18].

Intramolecular energy transfer in dansyl-BSA produces a decrease in the fluorescence of the protein as a consequence of the appearance of a new non-radiative way of de-excitation. This is just the observation students have made about the emission spectra of Fig. 2*B*. Scheme 3) represents the sources of the two emission bands observed when excited at 280 nm.

Fluorescence Excitation Spectra Analysis

Finally, students were asked about the expected characteristics of the excitation spectrum of dansyl-BSA. After discussion, they registered this spectrum by setting the

emission wavelength at 470 nm (*i.e.* where dansyl emits) (Fig. 3). The spectrum shows two maxima at 280 and 340 nm. The first maximum corresponds to indirect excitation of the probe through energy transfer, and the latter corresponds to direct excitation of the dansyl probe.

CONCLUSIONS

This laboratory class has been successfully implemented during 3 years in undergraduate courses of Biophysical Chemistry. Students found it simple and useful to understand the basis of spectral analysis in biophysics.

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