Ribonuclease T_1 and Alcohol Dehydrogenase Fluorescence Quenching by Acrylamide

A Laboratory Experiment for Undergraduate Students

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The proposed experiment consists of an introduction to the fluorescence technique with a concomitant illustration of relevant aspects of protein structure. Two convenient aspects of this work are its use of easily available instrumentation and its fast experimental execution.

Tryptophan (Trp) is a sensitive fluorescent probe of protein structure and dynamics due to the dependence of its photophysical parameters on both its molecular environment and its intrinsic mobility in the macromolecule (*1*). The ability of some solutes to decrease the protein quantum yield by collisional quenching can be used to determine the degree of exposure to the medium of the protein's emitting amino acid residues (*2*). If they are deeply buried in the protein core, the quencher molecule can hardly reach them, and low protein fluorescence quenching by the solute is expected. Upon significant conformational transition of the protein chain, as in denaturation, a large increase in the efficiency of quenching of the protein should be observed.

In the present experiment, information about Trp exposure in

- ribonuclease T_1 (RNase T_1) (E.C. 3.1.27.3)
- alcohol dehydrogenase (LADH) (E.C. 1.1.1.1)

is obtained from the determination of their accessibility to a neutral quencher solute, acrylamide. These well-known proteins (*3*, *4*) have only one and two Trp residues per polypeptide chain, respectively, thus facilitating a detailed steady-state fluorescence quenching kinetic study.

An initial characterization of acrylamide as a protein quencher is carried out in aqueous medium using Trp as a model compound. The fluorescence quenching study of these enzymes both in their native and denatured (8 M urea) states is then used to demonstrate the importance of protein tertiary structure in determining the fluorescence properties of Trp and its solvent exposure.

Theoretical Background

Dynamic Quenching of a Single Species

The process of collisional fluorescence quenching is described by the Stern–Volmer relationship (*5*):

$$\frac{I_o}{I} = 1 + K_{SV}[Q] \tag{1}$$

where I_o and I are the fluorescence intensities in the absence and presence of a concentration of quencher [Q], respectively; and K_{SV} is the Stern–Volmer quenching constant. The derivation of this equation has already been presented in *this Journal* (*6*).

The Stern–Volmer constant can be expressed as

$$K_{SV} = k_q\tau_o \tag{2}$$

where k_q is the bimolecular quenching rate constant and τ_o is the fluorophore decay lifetime in the absence of quencher.

From a steady-state fluorescence quenching experiment, k_q values can be obtained from the slope of a plot of I_o/I vs. [Q] once τ_o is known. Sometimes, however, Stern–Volmer plots show a positive deviation from linearity for the highest concentrations of quencher used, thus requiring alternative quenching models.

Dynamic and Static Quenching of a Single Species

These deviations, at an elementary level, are interpreted according to the sphere-of-action static quenching model (*5*): Within a volume V' around the fluorophore, a quenching reaction occurs instantaneously after excitation with unit efficiency. Assuming that the quenchers are distributed among these volumes according to a Poisson distribution, the following expression for the combined existence of dynamic and static quenching is obtained,

$$\frac{I_o}{I} = (1 + K_{SV}[Q])e^{V[Q]} \tag{3}$$

where V is the static quenching constant. ($V = V'N_A/1000$ where N_A is the Avogadro number.)

Dynamic Quenching of Multiple Species

Nonlinear Stern–Volmer plots can also be observed when there are multiple classes of fluorophores in solution, each with its own K_{SV} (*2*). The simplest case to consider is a mixture of two noninteracting fluorophores, A and B, in solution. One is totally protected from contact with the quencher solute (A), whereas the other is exclusively quenched in a dynamic fashion (B). In this way, the initial total fluorescence intensity

$$I_o = I_o^A + I_o^B$$

is reduced to

$$I = I_o^A + I^B$$

upon addition of quencher, where I^B is related to I_o^B by eq 1.

On these assumptions, eq 4 is derived.

$$\frac{I_o}{I} = \frac{1 + K_{SV}^B[Q]}{(1 + K_{SV}^B[Q])(1 - f_B) + f_B} \tag{4}$$

where

$$f_B = \frac{I_o^B}{I_o}$$

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is the fraction of initial fluorescence intensity emitted by the species B; and K_{SV}^{B} is its Stern–Volmer constant. Equation 4 is usually presented in a linearized form known as the Lehrer plot (*7*).

Experimental

Reagents

Trp (Merck, No. 8374) and RNase T_1 from *Aspergillus oryzae* (Sigma, No. R-8251) were used as received.

LADH from equine liver (Boehringer Mannheim, No. 102741) was extensively dialyzed against 20 mM phosphate buffer (pH 7.4) with 0.1 M NaCl at 4 °C for 24 h. The undissolved material was removed by centrifugation.

Enzyme concentrations were determined spectrophotometrically using

- $\varepsilon_{278nm} = 2.1 \times 10^4\ M^{-1}\ cm^{-1}$ (*8*) for RNase T_1
- $\varepsilon_{280nm} = 3.5 \times 10^4\ M^{-1}\ cm^{-1}$ (*9*) for LADH

Acrylamide and urea were both purchased from Sigma (electrophoresis reagents, purity > 99%) and were recrystallized from ethyl acetate and methanol, respectively, when found necessary. All other reagents were *pro analysis* grade.

Procedure

- ***Caution***: Acrylamide is *neurotoxic*. Urea is an *irritating reagent*. Handle both with care.

The fluorescence quenching experiments were carried out in

- 10 mM Mes buffer (pH 7.0) with 0.1 M NaCl for Trp
- 20 mM Tris–HCl buffer (pH 7.0) for RNase T_1
- 20 mM phosphate buffer (pH 7.4) with NaCl 0.1 M for LADH

The absorbance of each solution at the exciting wavelength (λ = 295 nm) was measured and was always less than 0.1/cm optical pathlength. Small aliquots (5–10 μL) of an 8 M acrylamide stock solution were then sequentially added to 1.1 mL of each fluorophore solution under study, using a 50-μL syringe. Final quencher concentrations ranged from 0 to 0.6 M. An identical procedure was repeated in the absence of fluorophore in order to obtain fluorescence background intensities.

Protein unfolding was achieved by incubating each enzyme in 8 M urea for at least 2 h at 25 °C. The denaturant solution was prepared by diluting 9 M urea and protein stock solutions with the buffer used in each assay.

Apparatus

Any conventional spectrofluorimeter with right-angle geometry and monochromatic excitation can be used to selectively excite the protein Trp residues at 295 nm. To minimize acrylamide absorption at this wavelength (ε_{295nm} = 0.25 $M^{-1}cm^{-1}$ (*2*)) fluorescence cells with a reduced exciting optical pathlength l should be used. In our study, most measurements of fluorescence quenching were carried out using 0.4-cm × 1.0-cm cells, but similar results were also obtained with l = 1.0 cm.

Results and Discussion

Correction of Quenching Data

This experiment describes how to take into account the use of a quencher solute that also absorbs at the exciting wavelength. When the quencher concentration is increased, two effects are observed:

An increasing fraction of the exciting light is absorbed by the quencher.

The overall increase of the sample absorbance leads to less penetration of the exciting light into the solution, implying a decrease in the detected fluorescence intensity for a right-angle geometry (*10*).

Thus, it is necessary to correct the measured fluorescence intensities by multiplying their values by the correction factor C, which takes into account both effects.

$$C = \frac{A_t}{A_f}\left(\frac{1 - 10^{-A_f}}{1 - 10^{-A_t}}\right) \qquad (5)$$

where A_f and A_t are the absorbances at the exciting wavelength for the fluorophore and total solution, respectively.

This expression may be readily derived considering the definition of quantum yield, the Beer–Lambert law, and the absorbance of a mixture of two noninteracting chromophores. Before considering the described artifacts, the fluorescence data should be corrected for the background intensities as well as for the progressive dilution of the sample by the added quencher.

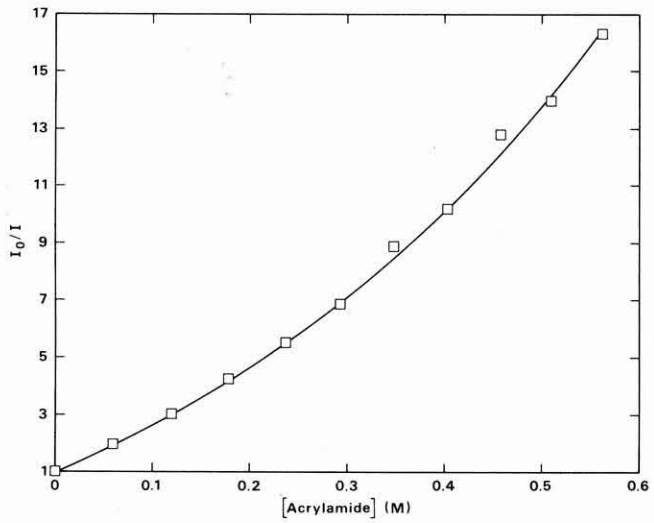

Figure 1. Stern–Volmer plot of Trp fluorescence quenching by acrylamide (λ_{em} = 340 nm). Fit of eq 3 to experimental data (—) (K_{SV} = 13.7 M^{-1} and V = 1.14 M^{-1}).

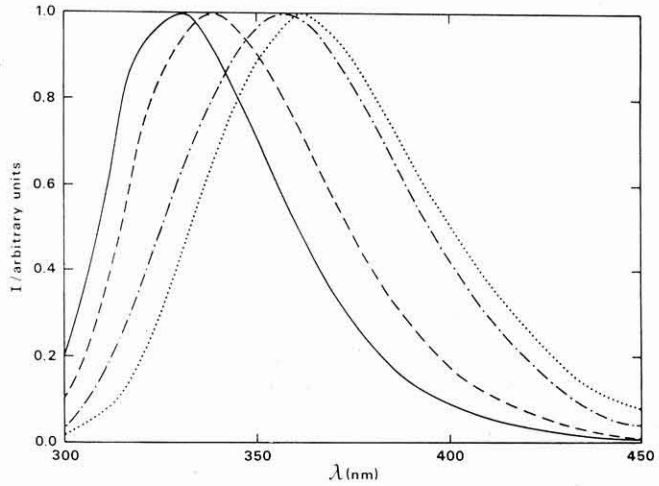

Figure 2. RNase T_1 and LADH emission spectra (λ_{exc} = 295 nm): native (—) and denatured (8 M urea) (— · —) RNase T_1 emission spectra in 20 mM Tris–HCl buffer (pH 7.0); native (– – –) and denatured (8 M urea) (·····) LADH emission spectra in 20 mM phosphate buffer (pH 7.4) with NaCl 0.1 M.

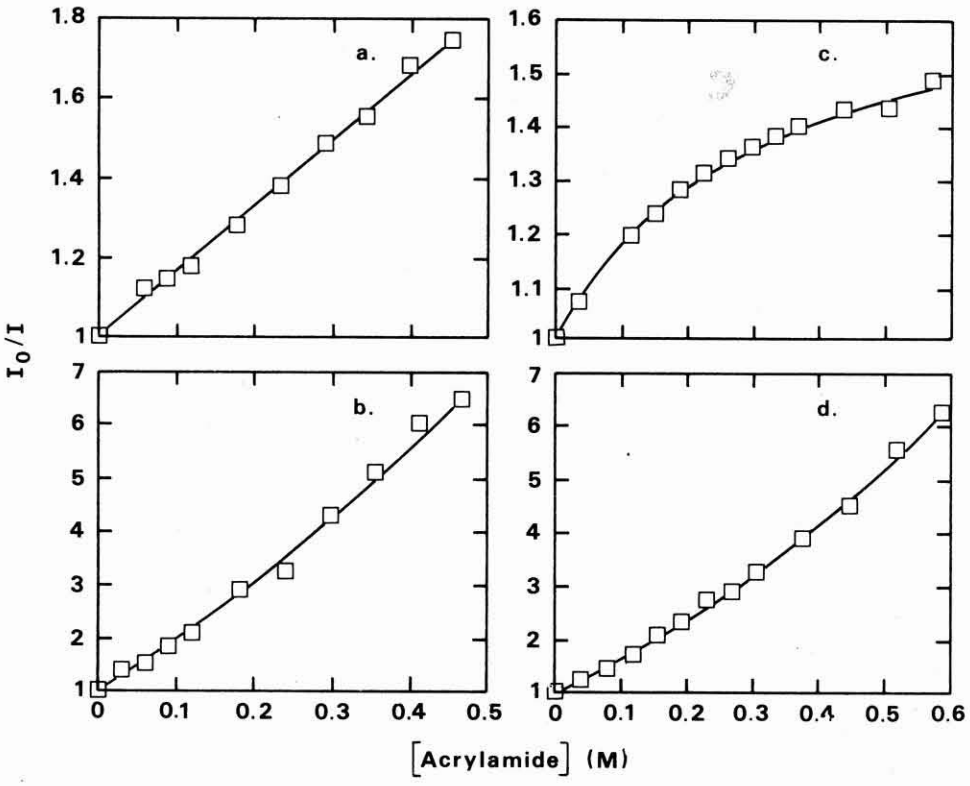

Figure 3. Stern–Volmer plots of RNase T_1 (a, b) and LADH (c, d) fluorescence quenching by acrylamide: a) native state (λ_{em} = 340 nm); Fit of eq 1 to experimental data (—) (K_{SV} = 1.63 M^{-1}). b) denatured state (λ_{em} = 360 nm); Fit of eq 3 to experimental data (—) (K_{SV} = 9.24 M^{-1} and V = 0.48 M^{-1}). c) native state (λ_{em} = 340 nm); Fit of eq 4 to experimental data (—) (K_{SV} = 5.52 M^{-1} and f_B = 0.42). d) denatured state (λ_{em} = 360 nm); Fit of eq 3 to experimental data (—) (K_{SV} = 6.30 M^{-1} and V = 0.71 M^{-1}).

Trp Fluorescence Quenching

Trp fluorescence intensity decreases as the acrylamide concentration increases. From the fit of eq 3 to the experimental data (Fig. 1) the values of

$$K_{SV} = 13.7\ M^{-1} \text{ and } V = 1.14\ M^{-1}$$

are recovered (see the table) leading to

$$k_q = 5.5 \times 10^9\ M^{-1}s^{-1}$$

when considering τ_o = 2.5 ns (*11*).

This value is close to the one obtained for the diffusion-limited rate constant of indole fluorescence quenching by acrylamide, $k_d = 7.4 \times 10^9\ M^{-1}\ s^{-1}$ (*12*), using the Smoluchowski equation, showing that acrylamide is an efficient quencher of Trp.

On the other hand, from the active-sphere volume, a radius of 7.7 Å is obtained. This value is in agreement with the sum of van der Waals radii of indole and acrylamide (6–7 Å (*13*)), supporting the existence of a contact interaction at the instant of Trp excitation.

RNase T_1 Fluorescence Quenching

The emission of RNase T_1 underwent a large red shift upon its denaturation in 8 M urea, as shown in Figure 2. In addition, the fluorescence-quenching curves of RNase T_1 by acrylamide are completely distinct for the native (Fig. 3a) and denatured (Fig. 3b) enzyme. In the first case, a linear Stern–Volmer plot is obtained, whereas for the denatured protein a strong positive deviation from linearity is apparent. The models applied to the quenching data and the recovered parameters are presented in the table.

RNase T_1 is a globular monomeric protein (M_r: 11,085) with only one Trp residue in its primary structure (*3*). This emitting residue is thought to be partially accessible to the solvent, although highly immobilized within its protein matrix (*17*).

Upon protein denaturation the macromolecule switches from a well-defined tertiary structure to a ramdomly coiled conformation, and Trp rotational freedom increases, as does its exposure to the medium. The appearance of a static quenching component and the larger k_q value obtained for the unfolded state of the enzyme, with the bathochromic shift observed in its emission spectra, clearly reflect this major structural alteration.

LADH Fluorescence Quenching

At variance with RNase T_1, the fluorescence quenching of native LADH by acrylamide yields a Stern–Volmer plot with a negative deviation from linearity (Fig. 3c). From the fit of eq 4 to the experimental data, the values

$$K_{SV} = 5.52\ M^{-1} \text{ and } f_B = 0.42$$

are obtained (see the table), showing that a large fraction of protein fluorescence is not accessible to the quencher.

In fact, X-ray crystallographic studies (*4*) show that the two Trp residues of each polypeptide chain of this homodimeric protein (M_r: 80,000)

$$Trp_{15} \text{ and } Trp_{314}$$

Acrylamide Quenching Data for Trp, RNase T_1, and LADH[a]

System	Eq[b] Fitted	K_{SV} (M^{-1})	V (M^{-1})	f_B	τ_o (ns)	$k_q \times 10^{-9}$ ($M^{-1}s^{-1}$)	r (Å)	λ_{max}[c] (nm)
Trp	3	13.7	1.14	—	2.5[d]	5.5	7.7	365
RNase T1 (Native)	1	1.63	—	—	3.5[d]	0.5	—	332
RNase T1 (Denatured)	3	9.24	0.48	—	3.6[e]	4.3[h]	5.8	356
LADH (Native)	4	5.52	—	0.42	6.9[f]	0.8	—	339
LADH (Denatured)	3	6.30	0.71	—	3.4[g]	3.1[h]	6.5	362

[a]Temperature, 25 °C; λ_{exc} = 295 nm.

[b]Equations were fitted to experimental data using a nonlinear regression program.

[c]Uncorrected fluorescence spectra.

[d]Lifetime value from ref *11*.

[e]Mean lifetime obtained from ref *14* (RNase T_1 in acetate buffer (pH 5.5) with 7 M guanidinium–HCl).

[f]Lifetime of the exposed Trp from ref *15*.

[g]Mean lifetime (Coutinho, A.; Prieto, M., unpublished results).

[h]Value corrected for the increase in the solution viscosity due to 8 M urea (*16*).

have distinct locations in the macromolecule. Trp_{15} lies near the surface, whereas Trp_{314} is buried near the subunit interface region.

The alteration in LADH fluorescence quenching upon its denaturation with 8 M urea follows the trend observed for RNase T_1. In other words, there is a large increase in its fluorescence quenching (Fig. 3d) with a concomitant red shift of the emission spectrum (Fig. 2). This result could be anticipated: Stern–Volmer plots should be similar for both enzymes considering that denaturation "normalizes" the environment and exposure of Trp residues in proteins.

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