Published in final edited form as:

J Chem Educ. 2010 September 1; 87(9): 961–964. doi:10.1021/ed900029c.

Quenching of Tryptophan Fluorescence in Unfolded Cytochrome *c*: A Biophysics Experiment for Physical Chemistry Students

Diana E. Schlamadinger, Dina I. Kats, and Judy E. Kim*

Department of Chemistry and Biochemistry, University of California at San Diego, La Jolla, California 92093

Biophysics is a rapidly growing research field that draws scientists from diverse disciplines, including chemistry, biology, and physics. Despite the nationwide expansion of resources and programs that focus on biophysics, the typical undergraduate curriculum offers few, if any, opportunities for students to learn topics in this growing interdisciplinary field. In a previous article published in this *Journal* (1), we presented a biophysics module that described protein folding and Förster resonance energy transfer (FRET) experiments on the well-studied globular protein cytochrome *c* (cyt *c*), shown in Figure 1 (2). This prior work was designed to provide physical chemistry students an opportunity to explore topics in dipole–dipole energy transfer and protein thermodynamics, as well as guide them to measure Gibbs free energies and intramolecular distances associated with unfolding of cyt *c* in two denaturants. Here, we extend this previous experiment and introduce concepts in collisional quenching and solvent accessibility of proteins. Specifically, we describe the use of Stern–Volmer plots to determine the extent of solvent accessibility of the single tryptophan (trp-59) residue in different conformations of cyt *c*. Comparison of quenching efficiencies of free tryptophan in solution and buried in partially or fully unfolded cyt *c* highlights the persistence of residual structure in unfolded proteins and deepens students' understanding of protein structures and dynamics.

Fluorescence quenching via collisions differs from the dipole–dipole interactions that give rise to the FRET process, and the most important distinctions are the mechanisms and distance scales associated with these quenching processes. Whereas FRET occurs between spectrally overlapping fluorophore and quencher molecules that are typically ~50 Å apart, collisional quenching does not require spectral overlap and occurs only when the fluorophore and quencher are in molecular contact (within ~5 Å) (3). A key requirement in collisional quenching is that a quencher must diffuse to the fluorophore during the lifetime of the excited state of the fluorophore to cause a decrease in fluorescence intensity; this mechanism allows collisional quenching to be a valuable technique to measure the extent of fluorophore exposure to bulk solvent and permeation of quencher in the solution matrix (3, 4). When the natural fluorophore, tryptophan, is buried in the interior of a protein or membrane, quencher accessibility to the tryptophan residue is limited, and therefore

fluorescence emission is maximized. In contrast, a tryptophan residue that is fully exposed to the solvent results in quenching of its fluorescence by freely diffusing external quencher molecules. This phenomenon can be exploited to probe the effect of protein conformation on accessibility of the tryptophan residue to bulk solvent.

A number of experiments that focus on fluorescence quenching have been presented in this *Journal* (5–12); however, only one report describes collisional quenching in a biological system (13). The experiment presented here is advantageous because it may be performed in sequence with our prior report (1) to provide a comprehensive set of biophysics experiments. In addition, the current work investigates protein conformations at two points along the unfolding curve and presents discussion on essential correction factors based on screening effects that significantly affect data analysis.

This experiment is intended for a pair of students to complete within four three-hour lab periods of an upper-level physical chemistry laboratory course. The learning objectives of the experiment are the following: (i) understand principles of fluorescence collisional quenching and calculate quenching constants in Stern–Volmer plots; (ii) compare accessibility of solvent to trp-59 in partially and fully unfolded cyt *c* protein conformations; (iii) discuss different mechanisms of collisional quenching; and (iv) explore concepts in solvation and protein folding.

Theory

Collisional quenching is also called dynamic quenching. The decrease in fluorescence intensity because of quenching is described by

$$\frac{F_0}{F} = 1 + K_{SV} [Q] \quad (1)$$

where F is the fluorescence intensity in the presence of quencher, F_0 is the intensity in the absence of quencher, $[Q]$ is quencher concentration, and K_{SV} is the Stern–Volmer quenching constant. While K_{SV} is a general term that may refer to either dynamic or static quenching processes, in this report we focus on K_{SV} in eq 1 as a dynamic quenching constant. K_{SV} values are the slopes of a linear regression fit of a plot of F_0/F versus quencher concentration. This plot is called a Stern–Volmer plot, and fluorescence quenching via collisions results in a linear dependence of F_0/F on quencher concentration. In many instances, the Stern–Volmer plot is not linear. The presence of an upward-curving Stern–Volmer plot indicates the presence of a static quenching mechanism in which the fluorophore and quencher form a nonfluorescent complex. Alternatively, the quencher and fluorophore may not necessarily form a complex, but are in such close proximity that fluorescence is quenched immediately upon excitation. This latter mechanism is called sphere-of-action quenching and affects the fluorescence according to

$$\frac{F_0}{F} = (1 + K_D [Q]) \exp([Q]V) \quad (2)$$

Here, V is the volume of the sphere around the fluorophore and quencher and is interpreted as a static quenching constant and K_D is the dynamic component of the quenching

mechanism. As V approaches zero, the sphere-of-action mechanism is interpreted to be dominated by dynamic quenching. A relatively large dynamic quenching constant in cases of nonlinear or linear Stern–Volmer plots indicates that the fluorophore is accessible to bulk solvent, whereas a small quenching constant suggests that the fluorophore is relatively inaccessible to solvent, or buried. Downward-curving Stern–Volmer plots may also be observed, and this curvature may be interpreted as two populations, one that is accessible to quenchers and another that is inaccessible to quenchers.

To obtain the proper fluorescence intensity values, fluorescence data must be corrected for the inner filter effect caused by attenuation of the excitation beam and emission signal because of absorption by quencher and fluorophore. These absorption events lead to artificial decreases in the fluorescence intensities; this effect is corrected with knowledge of the absorbance (or optical density, OD) values from the corresponding absorption spectra,

$$F_{\text{corr}} = F_{\text{obs}} \log^{-1} \frac{\text{OD}_{\text{ex}} + \text{OD}_{\text{em}}}{2} \quad (3)$$

where F_{corr} and F_{obs} are the corrected and observed fluorescence intensities, respectively. OD_{ex} is the absorbance value at the excitation wavelength (290 nm), and OD_{em} is the absorbance value at the emission wavelength (350 nm) (3). A final correction associated with changes in solvent properties, such as viscosity, is required and is discussed below.

Experimental Procedures

The experiment is divided into four sections: (i) preparation of primary and secondary stock solutions; (ii) preparation of 5 sets of 6 samples each; (iii) collection of absorbance and fluorescence spectra for all 30 samples; (iv) analysis of data. A flowchart accompanies the methods described here and is found in the supporting information. Each of these sections can be accomplished in one three-hour lab period. Alternatively, the primary and secondary stocks can be prepared ahead of time so that the students can complete the entire experiment in two or three lab periods. Stock solutions are described and labeled according to the flowchart found in supporting information. Four primary stock solutions are (i) 80 mL of 20 mM potassium phosphate solution (109 mg of KH_2PO_4 , 139 mg of K_2HPO_4); (ii) 80 mL of 7 M urea/20 mM potassium phosphate solution (109 mg of KH_2PO_4 , 139 mg of K_2HPO_4 , and 33.6 g of urea); (iii) 80 mL of 10 M urea/20 mM potassium phosphate solution (109 mg of KH_2PO_4 , 139 mg of K_2HPO_4 and 48.1 g of urea); and (iv) 5 mL of 1 mM NATA (*N*-acetyl-L-tryptophanamide, Fisher BioReagents, NJ) solution (1.2 mg of NATA, and 5 mL of potassium phosphate buffer). Secondary stocks are also described in the flowchart. The stock solutions are used to make 5 sets of 6 samples; each set corresponds to NATA or cyt *c* (Acros Organics, NJ) in 0.0, 7.0, or 10.0 M urea (MP Biomedicals, OH) with increasing acrylamide (Promega Corp., WI) concentrations of 0, 50, 100, 150, 200, or 250 mM. NATA is an uncharged model compound of tryptophan. NATA experiments are included here to measure the inherent extent of fluorescence quenching in the absence of protein environment.

The absorption spectrum of each sample was acquired using an Agilent 8453A absorption spectrophotometer. Absorption spectra were used to determine cyt *c* concentrations ($\epsilon_{530} =$

11,200 mol⁻¹ L cm⁻¹) (14) and to correct fluorescence spectra for the inner filter effect (eq 3). The fluorescence spectra were measured on a Horiba Jobin Yvon fluorometer, model FL 3-11, using an excitation wavelength of 290 nm and emission range of 305–500 nm. Spectra of acrylamide secondary stock solutions were also collected and subtracted from NATA and cyt *c* spectra to remove Raman scattering and background arising from water, phosphate, and urea. Resulting background-corrected fluorescence spectra were then corrected for variations in cyt *c* concentrations. Fluorescence intensities for each quencher concentration were tabulated and finally adjusted for the inner filter effect (eq 3). F_0/F values are plotted as a function of quencher concentration to generate Stern–Volmer plots. For all plots, the data are fit to eqs 1 and 2. Quenching constants are obtained based on the results of these fits and then adjusted for screening effects.

Hazards

Extreme care is recommended when using acrylamide, which is a potential carcinogen, toxin, and skin sensitizer and can cause skin and eye irritations upon contact; it can also sublime leading to an inhalation hazard. Urea can cause skin irritations upon contact. Cytochrome *c* and the phosphate buffer are only slightly hazardous in case of skin and eye contact or ingestion and inhalation. The toxicology of NATA has not been fully investigated; it may be harmful or act as an irritant.

Results and Discussion

Fluorescence spectra of the free, unbound model compound NATA (top panel) and trp-59 of cyt *c* (bottom panel) in 10.0 M urea and varying concentrations of acrylamide are presented in Figure 2. Stern–Volmer plots for NATA in 0, 7.0, and 10.0 M urea and for cyt *c* in 7.0 and 10.0 M urea are shown as insets. At these urea concentrations, cyt *c* is partially unfolded (7.0 M) or fully unfolded (10.0 M) (1). Quenching experiments on folded cyt *c* in 0.0 M urea were not performed because of the inherently weak fluorescence signal in folded protein; the close proximity of trp-59 to the heme group in folded cyt *c* results in efficient quenching via the FRET mechanism. The Stern–Volmer plots of NATA (Figure 2) were fit to eq 2 because of the apparent upward curvature and because this model system has been shown to exhibit sphere-of-action quenching (3). The plots of cyt *c*, however, were fit to eq 1 because of the clear linearity of the plots. The values determined here are consistent with other reports (13, 15).

Typical quenching constants obtained with both sphere-of-action (K_D) and pure dynamic (K_{SV}) collisional mechanisms are summarized in Table 1 for one set of experiments. Multiple trials from different students gave results that varied less than 15%. The decrease in K_D and K_{SV} for NATA in 7.0 and 10.0 M urea relative to in 0.0 M urea arises because of changes in viscosity and refractive index as a function of urea concentration; this dependence of the diffusion coefficient on viscosity as well as dependence of fluorescence quantum yield on refractive index are well documented (3) and are collectively referred to as a general screening effect. These general trends are also apparent in cyt *c*, though to a much smaller extent because cyt *c* Stern–Volmer plots additionally reflect changes in solvent accessibility of fluorophore due to protein conformation. To isolate the effect of protein

conformation on quenching, cyt *c* quenching constants were corrected for screening effects by multiplying measured cyt *c* quenching constants by the appropriate scalar by which NATA quenching constants changed. Here, we utilized K_{SV} values for NATA fit to eq 1 instead of K_D values fit to eq 2 for simplicity; to obtain accurate K_D values, a greater number of data points, roughly 15–20, are required to adequately reflect the upward curvature, and such an experiment may be time-prohibitive in an undergraduate lab. An example of the correction is as follows: The 7.0 M urea cyt *c* K_{SV} value of 3.6 obtained directly from measurement was multiplied by 1.13. This factor of 1.13 was determined by dividing the K_{SV} value of NATA in 0.0 M urea (22.1) by the K_{SV} value of NATA in 7.0 M urea (19.6). The resultant value of 4.0 reflects the corrected K_{SV} ($K_{SV,corr}$) value for cyt *c* in 7.0 M urea without contribution from general screening effects. The correction factor for 10.0 M urea is 1.33 (22.1/16.6). These corrected quenching constants, $K_{SV,corr}$, are tabulated in Table 1.

These corrected cyt *c* Stern–Volmer plots are presented in Figure 3 along with the plot for NATA in 0.0 M urea. The plots in Figure 3 reflect the corrected K_{SV} values of partially and fully unfolded cyt *c*. These corrected Stern–Volmer constants reflect the local environments of trp-59 in the absence of urea and can therefore be appropriately compared to each other as well as to the K_{SV} value of free NATA in 0.0 M urea. These corrected quenching constants indicate that trp-59 is more accessible to bulk solvent in the fully denatured form than in the partially unfolded state. This result is consistent with the expectation that partially unfolded cyt *c* maintains some native structure and, therefore, trp-59 remains at least partially buried. The corrected quenching constants for cyt *c* are lower than the values obtained for NATA at all urea concentrations, and this finding suggests that unfolded cyt *c* remains collapsed instead of fully extended, as evidenced by partial burial of trp-59. The quenching constants found here for fully denatured cyt *c* are lower than those found for other denatured proteins (13), suggesting a greater degree of collapsed structure even in 10.0 M urea. This finding is supported by previous results found for cyt *c* at high concentrations of urea (16, 17).

Several sources of error contribute to the accuracy and precision of this experiment. The trends shown here are obtained relatively easily, and therefore, this experiment is successful from an educational perspective. However, the specific experimentally measured values of K_{SV} may vary and depend on the actual concentration of urea. The reason for this dependence is because the unfolding curve for cyt *c* in urea is steep near the midpoint (~7.2 M), and as a result, small changes in urea concentration result in large variation in protein conformation and, hence, solvent accessibility of trp-59. An additional challenge is that urea is hygroscopic, so if the urea sample is old, its concentration may be ~10% lower than intended. If time permits, students are encouraged to measure the urea concentration with a refractometer (reference provided in supporting information). Scatter in the data points in the Stern–Volmer plots of cyt *c* (Figure 3) can be attributed to a variety of sources of error. For example, the quencher concentration is not independently verified in this experiment, and deviation of the quencher concentration from the intended concentration will cause errors. Protein concentrations are another source of error. Whereas absorption spectra are acquired to determine protein concentrations, shifts in absorption baseline and slight variation in extinction coefficient for folded and unfolded protein will affect this

measurement. Finally, the presence of other quenchers is an important consideration. The heme group, peptide backbone, and side chains, such as cysteine, may also quench tryptophan fluorescence (1, 18), and the efficiency of quenching is highly dependent on the dynamics of the protein under the specific conditions. Both inter- and intramolecular quenching of tryptophan by these groups can cause variations in the fluorescence intensity especially under denatured conditions.

The experimental results provide students with the opportunity to discuss significant topics in fluorescence quenching and protein folding. Students should be encouraged to discuss general mechanisms of FRET and collisional quenching, as well as devise experiments that may discern dynamic versus static quenching processes (e.g., temperature dependence measurements). Other important topics in physical chemistry, such as refractive index, viscosity, diffusion, and excited-state lifetimes, may be discussed. Important experimental considerations and sources of systematic error, such as the inner filter effect, may also be explored. Finally, results from this experiment illustrate the complex nature of denatured and partially unfolded states of proteins and encourage discussions of, for example, collapsed and extended structures, the role of solvent in protein stability, and alternative models for cyt *c* unfolding (e.g., two-state vs continuous). The downward-curving Stern–Volmer plot of the partially folded state in 7.0 M urea in Figure 3 may also be analyzed in terms of two quenching populations for folded and unfolded states (3). Analogous quenching experiments may be pursued using different unfolding methods, such as guanidinium hydrochloride or changes in temperature or pH (17), to highlight the dependence of unfolded conformation on denaturing mechanism. Collectively, these experiments provide sufficient background and experience for undergraduate students to appreciate topics in the active research field of protein folding as well as numerous other problems in biophysics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment

We thank Guipeun Kang and Jennifer Pomponio for feedback on this experiment. This work was supported by an NSF CAREER award to J.E.K.

Literature Cited

1. Sanchez KM, Schlamadinger DE, Gable JE, Kim JE. *J. Chem. Educ.* 2008; 85:1253–1256. [PubMed: 19756254]
2. Englander SW, Sosnick TR, Mayne LC, Shtilerman M, Qi PX, Bai Y. *Acc. Chem. Res.* 1998; 31:737–744.
3. Lakowicz, JR. *Principles of Fluorescence Spectroscopy*. 3rd ed.. New York: Springer; 2006.
4. Eftink MR, Ghiron CA. *Anal. Biochem.* 1981; 114:199–227. [PubMed: 7030122]
5. Chatellier DS, White HB III. *Chem. Educ.* 1988; 65:814–815.
6. Cumberbatch T, Hanley QS. *J. Chem. Educ.* 2007; 84:1319–1322.
7. Ebeid E-ZM. *J. Chem. Educ.* 1985; 62:165–166.
8. Legenza MW, Marzzacco CJ. *J. Chem. Educ.* 1977; 54:183–184.

9. Marciniak B. *J. Chem. Educ.* 1986; 63:998–1000.
10. Fraji LK, Hayes DM, Werner TC. *J. Chem. Educ.* 1992; 69:424–427.
11. Tucker SA, Acree WE Jr. *J. Chem. Educ.* 1995; 72:A31–A33.
12. Bigger SW, Watkins PJ, Verity B. *J. Chem. Educ.* 2003; 80:1191–1193.
13. Coutinho A, Prieto M. *J. Chem. Educ.* 1993; 70:425–428.
14. Eaton WA, Hochstrasser RM. *J. Chem. Phys.* 1967; 46:2533–2539. [PubMed: 6039380]
15. Eftink MR, Hagaman KA. *Biophys. Chem.* 1986; 25:277–282. [PubMed: 3103704]
16. Tsong TY. *Biochemistry.* 1975; 14:1542–1547. [PubMed: 235957]
17. Shiu Y-J, Jeng U-S, Huang Y-S, Lai Y-H, Lu H-F, Liang C-T, Hsu I-J, Su C-H, Su C, Chao I, Su A-C, Lin S-H. *Biophys. J.* 2008; 94:4828–4836. [PubMed: 18326641]
18. Chen Y, Liu B, Yu H-T, Barkley MD. *J. Am. Chem. Soc.* 1996; 118:9271–9278.

Figure 1.
Structure of folded cyt *c* (PDB 1HRC).

Figure 2.

Fluorescence spectra and Stern–Volmer plots of NATA and cyt *c*. (top) NATA in 10.0 M urea with 0–250 mM acrylamide quencher. The inset shows Stern–Volmer plots for NATA in 0.0 (circles), 7.0 (squares), and 10.0 M (triangles) urea with fits using eq 2. (bottom) Cyt *c* in 10.0 M urea with increasing acrylamide concentration. The inset shows Stern–Volmer plots for cyt *c* in 7.0 (squares) and 10.0 M (triangles) urea with fits obtained using eq 1.

Figure 3.

Typical Stern–Volmer plot of NATA in 0.0 M urea (circles) and corrected Stern–Volmer plots of cyt *c* in 7.0 M (squares) and 10.0 M (triangles) urea from one experiment. The plots for cyt *c* have been corrected for general screening effects. An alternative fit to the downward-curving plot of cyt *c* in 7.0 M urea is discussed in the text.

Table 1

Typical Quenching Constants for NATA and Cyt *c* in Different Urea Concentrations

molecule	[urea]/M	sphere-of-action ^d		dynamic ^d	
		<i>V</i> /(L mol ⁻¹)	<i>K_D</i> /(L mol ⁻¹)	<i>K_{SV}</i> /(L mol ⁻¹)	<i>K_{SV,corr}</i> /(L mol ⁻¹)
NATA	0.0	1.4	15.2	22.1	NA
NATA	7.0	1.4	13.4	19.6	NA
NATA	10.0	1.7	10.3	16.6	NA
Cyt <i>c</i>	7.0	ND	ND	3.6	4.0
Cyt <i>c</i>	10.0	0.8	3.4	3.7	4.9

^dData obtained using eq 1 (dynamic) or eq 2 (sphere of action).

NA: not applicable. ND: not determined. Sphere-of-action analysis was not performed for cyt *c* in 7.0 M urea based on the downward-curving shape of the Stern–Volmer plot.