Emission Spectroscopy: Biophysics and FRET

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In performing this experiment, students should learn how to apply a variety of techniques to analyzing a chemical system. By using cytochrome *c* as a model system, students will learn how to determine the free energy associated with unfolding a protein. Students will also learn the principles of fluorescence quenching via Förster energy transfer and then they will calculate intramolecular distances for partially unfolded and fully folded protein structures.[1]

[1] Transcribed and adapted from Sanchez et al. [1].

Introduction

The study of biological macromolecular structure has become a major field in biochemistry and chemistry research laboratories. Numerous spectroscopic techniques, such as UV-Vis absorption, fluorescence, and circular dichroism, have been employed to investigate the thermodynamics and structural changes associated with protein folding and unfolding. In undergraduate laboratories, most experiments rely on a single technique to study a chemical problem. In this lab, a multifaceted approach is used to the study of the important biophysical problem of protein folding. Specifically, the combination of absorption, fluorescence, and Förster resonance energy transfer (FRET) techniques will provide complementary and in-depth information on structural changes and thermodynamics associated with these changes. The protein used in this experiment is a well-studied system, cytochrome *c* (cyt *c*). Cyt *c* will be unfolded chemically via a denaturant and monitored spectroscopically.

FRET is a spectroscopic technique that may be used to determine inter- or intramolecular distances. It has been applied to study a wide variety of systems to obtain structural information. FRET occurs via long-range dipole-dipole interactions between donor and acceptor molecules, and does not involve emission of a photon. Radiationless energy transfer from an excited-state donor to a ground-state acceptor is the fundamental principle behind FRET. A critical requirement for FRET is spectral overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor. The efficiency of FRET energy transfer is inversely proportional to the sixth power of the distance between the donor and acceptor. By utilizing this distance dependence, structural information about proteins can be obtained during denaturant-induced unfolding.

Cyt *c* is an electron transfer protein found in the inner membrane of mitochondria. This globular protein consists of a single polypeptide chain that contains one tryptophan residue at position 59 (Trp-59) and a covalently bound heme cofactor. The intrinsic fluorophore, tryptophan, serves

as the FRET donor while the heme cofactor serves as the acceptor. In the native structure of cyt *c*, Trp-59 is in close proximity to the heme group (fig. 1). When the protein is folded, energy is transferred from the excited tryptophan to the heme group, causing the tryptophan fluorescence to be quenched. Upon addition of a chemical denaturant to unfold the protein, the rise in fluorescence signal from Trp-59 indicates that the distance between donor and acceptor has increased; this change in distance as a function of folding state can be quantified via the equations that describe FRET.
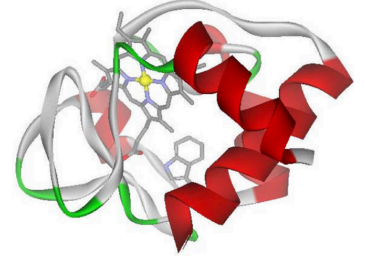
Theory

Förster Resonance Energy Transfer

According to Förster's theory on energy transfer, the rate of energy transfer k_T is related to the lifetime of the donor in the absence of acceptor (τ_D) and the distance between the donor and acceptor (r) via

$$k_T(r) = \frac{1}{\tau_D}\left(\frac{R_0}{r}\right)^6 . \tag{1}$$

The Förster distance, R_0, is the critical distance for energy transfer, and is defined as the distance at which the efficiency of energy transfer is 50 %. Förster distances typically range from 20 Å to 60 Å, and can be calculated using the relationship

$$R_0{}^6 = \left(8.79 \times 10^{23}\ \mathring{A}^6\mathrm{M/cm^3}\right)\left(\kappa^2 n^{-4}\Phi_D J_{DA}\right), \tag{2}$$

where κ^2 is the orientation factor between the transition dipoles of the donor and acceptor, n is the refractive index of the solvent, Φ_D is the quantum yield of the donor in the absence of acceptor, and J_{DA} is the overlap integral of the donor emission spectrum and the acceptor absorption spectrum. The numerical prefactor depends on the units of the overlap integral, which can be calculated as follows:

$$J_{DA} = \frac{\int F_D(\lambda)\,\varepsilon_A(\lambda)\,\lambda^4 d\lambda}{\int F_D(\lambda)\,d\lambda} . \tag{3}$$

Here, J_{DA} is in $\mathrm{cm^3/M}$, λ is in units of cm, $F_D(\lambda)$ is the fluorescence of the donor in the absence of the acceptor, and $\varepsilon_A(\lambda)$ is the extinction coefficient of the acceptor in units of $1/(\mathrm{M \cdot cm})$ at wavelength λ. The energy transfer efficiency, E, is defined by the following relationship:

$$E = \frac{R_0{}^6}{R_0{}^6 + r^6} . \tag{4}$$

Efficiency can be experimentally determined using the fluorescence intensities of the donor with and without the acceptor in the form of

$$E = 1 - \frac{F_{DA}(\lambda)}{F_D(\lambda)}, \tag{5}$$

Figure 1: Crystal structure of cyt *c* (PDB: 1HRC). Trp-59 and the heme group are shown. Image reproduced from Sanchez et al. [1].

where $F_{DA}(\lambda)$ is the fluorescence intensity of the donor in the presence of the acceptor and $F_D(\lambda)$ is the fluorescence intensity of the donor in the absence of the acceptor.

Free Energy of Protein Unfolding

Native tertiary structures of biomolecules can be disrupted by a variety of methods, including changes in temperature and pH, as well as addition of chemical denaturants. Two common chemical denaturants, guanidinium hydrochloride and urea, are used to disrupt native protein structures easily. The mechanisms by which these denaturants unfold proteins is an active area of research, and hypotheses regarding their modes of action involve direct solvation of peptide bonds and other hydrophobic regions as well as significant modification of solvent structures. By measuring the relative concentrations of folded and unfolded proteins as a function of denaturant concentration, one generates an unfolding curve which can be further analyzed to determine protein stability.

Spectroscopic techniques are commonly used to determine relative concentrations of folded and unfolded proteins for a given denaturant concentration. A critical requirement is that the folded and unfolded species exhibit unique spectroscopic signatures. Typical optical methods that may distinguish between native and denatured structures include circular dichroism, UV-Vis absorption spectroscopy, and fluorescence spectroscopy. Shifts in absorption/emission maxima as well as changes in intensities often reflect changes in global and local protein structures. By utilizing these spectroscopic shifts to monitor changes in population, unfolding curves can be generated to yield free energies of unfolding in the absence of denaturant.

The simplest model of protein folding/unfolding describes a two-state system of folded (F) and unfolded (U) species:

$$F \rightleftharpoons U \tag{6}$$

The equilibrium consant and free energy of unfolding are then given as

$$K_{eq} = \frac{[U]}{[F]} \qquad \text{and} \tag{7}$$

$$\Delta G_U^{\leftrightarrow} = -RT \ln K_{eq} = -RT \ln \frac{[U]}{[F]}, \tag{8}$$

where $\Delta G_U^{\leftrightarrow}$ is the free energy of unfolding in the presence of the denaturant. Here, we are interested in the free energy of unfolding in the absence of denturant. A theoretical treatment described by Pace [2] and Jones [3] approximates a linear perturbation of free energy as a function of denaturant concentration wherein extrapolation of this relationship to zero denaturant concentration gives rise to the free energy of unfolding in

the absence of denaturant, $\Delta G^{\ominus}_{\mathrm{H_2O}}$:

$$\Delta G^{\ominus}_{\mathrm{U}} = \Delta G^{\ominus}_{\mathrm{H_2O}} - mC, \tag{9}$$

where m reflects the rate of change of the Gibbs energy with respect to denaturant concentration and C is the molar concentration of denaturant. The denaturant concentration that gives rise to equal populations of folded and unfolded proteins is referred to as the midpoint concentration, C_{m}.

Here, m (kcal/(M·mol)) describes the rate of change of the free energy of denaturant with respect to denaturant concentration and C (M) is the molar concentration of denaturant.

The denaturant concentration that gives rise to equal populations of folded and unfolded proteins is referred to as the midpoint concentration, C_{m}. At C_{m}, the free energy of unfolding is zero so that

$$\Delta G^{\ominus}_{\mathrm{H_2O}} = mC_{\mathrm{m}}. \tag{10}$$

Using the definition of fraction of unfolded molecules, f, given by:

$$f = \frac{[\mathrm{U}]}{[\mathrm{U}] + [\mathrm{F}]}, \tag{11}$$

it is relatively straighforward to obtain the following equation:

$$f = \frac{\exp\left[-m\left(\frac{C_{\mathrm{m}} - C}{RT}\right)\right]}{1 + \exp\left[-m\left(\frac{C_{\mathrm{m}} - C}{RT}\right)\right]}. \tag{12}$$

Experimental data points in an unfolding curve are fit to eq. (12), with f and C as dependent and independed variables, respectively, to yield values for m and C_{m}. Knowledge of the variables m and C_{m} then allows for determination of the Gibbs energy of unfolding in the absence of denaturant, $\Delta G^{\ominus}_{\mathrm{H_2O}}$ using eq. (10).

Safety Precautions

- Urea is a skin irritant, be sure to use gloves when handling urea and urea-containing solutions. If denaturant comes in contact with the skin, eyes, or respiratory tract, wash the affected area(s) with copious amounts of water.

- Standard PPE should be worn at all times during the lab.

- While cyt *c* and phosphate buffer are relatively harmless, care should be taken while handling all proteins and buffers. Other proteins and buffers can be potentially hazardous.

Procedure

In this experiment, you will determine the free energy of unfolding ferric cyt *c* using the chemical denaturant urea. You will monitor changes in fluorescence from Trp-59 as a function of urea concentration to generate an unfolding curve for cyt *c* (part A). In part B, you will determine the Förster distance of the Trp–heme pair using a tryptophan model compound, *N*-acetyl-tryptophanamide, to approximate the donor in absence of acceptor (FD). The cyt *c* absorption spectrum is used to determine $\varepsilon_A(\lambda)$ of the acceptor. Finally, in the analysis, you will combine the knowledge gained in parts A and B to determine changes in distance between Trp-59 and the heme moiety as a function of denaturant.

Part A: Measurement of the Unfolding Curve

1. Turn on the instrumentation (fluorescence and absorption spectrometers) immediately upon arriving in the laboratory to allow the electronics to warm up and stabilize.

2. Prepare a stock solution (50 mL) of 20 mM pH 7.4 phosphate buffer. This buffer is called KP_i. Both components of the conjugate acid–base pair should be weighed out separately to obtain the desired ratio and then dissolved in water. Use potassium phosphate monobasic (CAS 7778-77-0) and potassium phosphate dibasic (CAS 7758-11-4).

3. Prepare a stock solution (50 mL) of 10.0 M urea (CAS 75-13-6) in 20 mM KP_i. You should add the urea and phosphates to an empty volumetric flask and add water up to the mark. This solution is called urea/KP_i.

4. Prepare a stock solution (0.8 mL of ∼500 μM) of cyt *c* (CAS 9007-43-6) in KP_i (ε_{410} for cyt *c* in this solution is ∼105 000/(M·cm), ε_{530} is 11 200/(M·cm)).

5. Prepare several samples of ∼10 μM cyt *c* (1.5 mL each) by mixing the appropriate amounts of stock solution from item 4 with stock solutions made in items 2 and 3 to achieve final urea concentrations of 0.0 M to 10.0 M in 1.0 M increments. You should have 11 samples in 11 plastic Eppendorf tubes.

6. Record fluorescence and absorption spectra of KP_i, urea/KP_i, and each of the 11 samples prepared in item 5. The fluorescence spectra should be recorded with λ_{ex} set to 290 nm and λ_{det} should be scanned from 305 nm to 500 nm. Use "high" sensitivity and 5 nm slits for both excitation and emission. These spectra will be used to determine the free energy of unfolding of cyt *c* using fluorescence shanges of Trp-59 as the probe. You will see a large peak at ∼323 nm in the spectra of

the buffers (KP_i and urea/KP_i) as well as in some of your cyt c samples. Discuss the origin of this peak with your lab instructor.

Part B: Determination of the Förster Distance

1. Prepare 5 mL of a ~10 µM solution of the tryptophan model compound, N-acetyl-tryptophanamide (NATA, CAS 2382-79-8) in KP_i. The actual concentration is not important, as long as you are not saturating the fluorometer detector. You may need to start with a concentrated stock solution of NATA and dilute it to the desired ~10 µM. Regardless of how you prepare the sample, you must determine the actual concentration based on the value for the extinction coefficient at 280 nm (ε_{280} for NATA is 5630/(M·cm)).

2. Measure absorption and fluorescence spectra of the solution prepared in item 1.

Part C: Data Analysis

1. To obtain an unfolding curve, subtract the background fluorescence spectra of the buffer (KP_i or urea/KP_i) from the corresponding protein fluorescence spectra to obtain a corrected fluorescence spectrum. These corrected spectra should be made using the following general procedure:

   Spectrum A: raw fluorescence from cyt c in buffer/denaturant

   Spectrum B: raw fluorescence from buffer/denaturant

   Corrected Spectrum: Spectrum A − Spectrum B

   The fluorescence intensity of the corrected spectrum at 350 nm should then be normalized for protein concentration (via the absorption value at 530 nm) and plotted as a function of urea concentration. The y-axis should be scaled from 0 to 1. What is the free energy of unfolding? You will need eqs. (10) and (11). Fitting your data to eq. (12) is best accomplished with advanced analytical software; the fitting routines in the SciPy library are well-suited to this task.

2. Plot the change in the absorption spectra as a function of denaturant concentration to generate an unfolding curve and determine the free energy of unfolding for cyt c.

3. Use the fluorescence spectrum collected in item 2 along with the absorption spectrum of ~10 µM cyt c (0.0 M urea) from item 1 to calculate the Förster distance of the Trp–heme pair. You will need eqs. (2) and (3). Determination of the integrated areas can be done numerically as a sum or using the integration functions built in to SciPy.[2]

[2] Additional pertinent information for this system:

$$\kappa^2 = 2/3 \,, \tag{13}$$
$$n = 1.4 \,, \tag{14}$$
$$\Phi_D = 0.13 \tag{15}$$

4. Using eq. (4) and your calculated R_0 from item 3, make a plot of energy transfer efficiency, E, as a function of distance, r, between donor and acceptor. This is the theoretical distance dependence of energy transfer for the donor–acceptor pair.

5. Use eqs. (4) and (5) and the corrected and (concentration) normalized spectra from item 6 to determine the distance between Trp-59 and the heme group as a function of denaturant concentration. For F_D, use the fluorescence intensity of NATA at the same concentration as the cyt c samples.

Questions and Further Thoughts

1. What is the source of the large peak at $\sim$323 nm in the spectra of the buffers (KP_i and urea/KP_i) as well as in some of the cyt c samples? What could you do to verify (experimentally) the source of this peak?

2. How do the results of unfolding with item 2 differ from those obtained in item 1 and what are some possible origins of the differences (if any)?

3. Compare the distance between Trp-59 and the heme group for the fully folded protein (0.0 M urea) with distances obtained from the known crystal structure (PDB: 1HRC). How do these compare?

4. What assumptions are made to determine $\Delta G_{H_2O}^{\ominus}$ from part A and intramolecular distances from part C? Do these assumptions conflict?

References

(1)   Sanchez, K. M.; Schlamadinger, D. E.; Gable, J. E.; Kim, J. E. *Journal of Chemical Education* **2008**, *85*, PMID: 19756254, 1253.

(2)   Pace, C. In *Enzyme Structure Part L*, Hirs, C. H. W., Timasheff, S. N., Eds.; Methods in Enzymology, Vol. 131; Academic Press: 1986; Chapter 14, pp 266–280.

(3)   Jones, C. M. *Journal of Chemical Education* **1997**, *74*, 1306.

Further Reading

•   Lakowicz, J. R., *Principles of Fluorescence Spectroscopy*; Springer Science+Business Media: 2006.

•   Shirley, B. A. In *Protein Stability and Folding: Theory and Practice*, Shirley, B. A., Ed.; Methods in Molecular Biology, Vol. 40; Humana Press: Totowa, NJ, 1995, pp 177–190.