Förster Resonance Energy Transfer (FRET) in Cytochrome *c*

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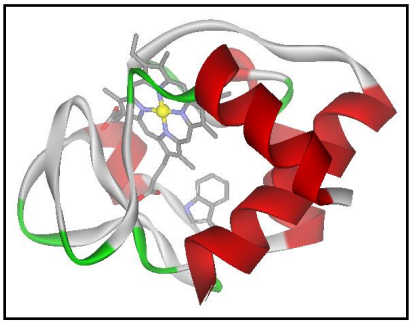
Goals: (1) Determine the free energy associated with unfolding a protein
(2) Understand principles of quenching via Förster energy transfer
(3) Calculate intramolecular distances for partially unfolded and fully folded structures

Introduction and Background

The study of biological macromolecular structure has become a major field in biochemistry and chemistry research laboratories. Different 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 one technique to study a chemical problem. Here, we describe a multifaceted approach towards 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 indepth 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.

Cytochrome *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 cytochrome *c*, Trp-59 is in close proximity to the heme group (shown on the right). 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.



FRET Theory

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

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

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 20-60 Å, and can be calculated using the relationship

$$R_0^6 = 8.79x10^{23}\left[\kappa^2 n^{-4}\Phi_D J_{DA}(\lambda)\right] \quad \text{in Å}^6 \quad (2)$$

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 overlap integral can be calculated as follows:

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

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

$$E = \frac{R_0^6}{R_0^6 + r^6} \quad (4)$$

Efficiency can be experimentally determined using the fluorescence intensities of the donor with and without the acceptor in the form of Equation 5:

$$E = 1 - \frac{F_{DA}}{F_D} \quad (5)$$

where F_{DA} is the fluorescence intensity of the donor in the presence of the acceptor, and F_D 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. A common chemical denaturant, urea, can 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 constant and free energy of unfolding are then given as

$$K_{eq} = \frac{[U]}{[F]} \quad \text{and} \quad \Delta G_U^o = -RT \ln K_{eq} = -RT \ln \frac{[U]}{[F]} \tag{7) and (8}$$

where ΔG_U^o is the free energy of unfolding in denaturant. Here, we are interested in the free energy of unfolding in the absence of denaturant. A theoretical treatment described by Pace (1984) and Jones (1997) 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, $\Delta G_{H_2O}^o$:

$$\Delta G_U^o = \Delta G_{H_2O}^o - mC \tag{9}$$

Here, m (kcal M^{-1} mol^{-1}) 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

$$\Delta G_{H_2O}^o = mC_m \tag{10}$$

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

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

it is relatively straightforward to obtain the following equation:

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

Experimental data points in an unfolding curve are fit to Equation 12, with f and C as dependent and independent variables, respectively, to yield values for m and C_m. Knowledge of the variables m and C_m then allows for determination of the free energy of unfolding in the absence of denaturant, $\Delta G^o_{H_2O}$, using Equation 10.

Lab 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 (F_D). The cyt *c* absorption spectrum is used to determine $\varepsilon_A(\lambda)$ of the acceptor. Finally, in part C, 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.

A. Measurement of unfolding curve

1. Prepare a stock solution (100 mL) of 20 mM pH 7.4 phosphate buffer. This buffer is called $\underline{KP_i}$. We have a concentrated phosphate buffer at pH 7.4 that you will need to dilute.
2. Prepare a stock solution (50 mL) of 10.0 M urea (CAS 57-13-6) in KP_i. You should add the urea to an empty volumetric flask and add buffer up to 50 mL. You probably will have to heat it gently to get everything to dissolved. This solution is called $\underline{urea/KP_i}$.
3. Prepare a stock solution (1.0 mL of about 500 μM) of cyt *c* (CAS 9007-43-6) in KP_i (ε_{410} for cyt *c* in this solution is approximately 105,000 $M^{-1}cm^{-1}$, ε_{530} is 11,200 $M^{-1}cm^{-1}$). Remove 0.1 mL of this stock solution, place in a vial and add 1.4 mL of the phosphate buffer. Take the UV-Vis spectrum of this solution in order to accurately determine the concentration of the stock solution. You will use the concentration of this stock solution to figure out the concentration of cytochrome c in all of your samples. You will also use the spectrum as described above to determine the $\varepsilon_A(\lambda)$ of the acceptor that you will need in equation 3.

4. Prepare several ~ 10 μM cyt *c* (1.5 mL each) samples by mixing the appropriate amounts of the stock solutions to achieve final urea concentrations of ranging from 0M to10M.

5. Record fluorescence and absorption spectra of KP_i, urea/KP_i, and each of the samples prepared in part above. The fluorescence spectra should be recorded with 290 nm excitation, and scanned from 305 nm to 500 nm. Use a relatively 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 changes of Trp-59 as the probe.

B. Determination of the Förster distance

1. Prepare 5 mL of a ~20 μM solution of the tryptophan model compound, N-acetyl-tryptophanamide (NATA) (CAS 2382-79-8) in KP_i. Use this stock solution to make two more NATA solutions of ~10 μM and ~5 μM

2. Measure absorption and fluorescence spectra of the three NATA solutions prepared above. Determine the actual concentration of NATA in each based on the value for the extinction coefficient at 280 nm (ε_{280} for NATA is 5630 $M^{-1}cm^{-1}$). These are going to be pretty small absorbance signals so make sure you either blank the instrument with a sample of pure buffer or acquire the spectrum of pure buffer so you can subtract it later. When doing the fluorescence, make sure that you are not saturating the fluorometer detector– you will need to dilute the sample (and redo the UV-Vis) if this is happening.

We are going to do this experiment in two (or three) weeks

Week 1 **• Make buffer, urea stock solution and cytochrome c stock solution**
• UV-Vis and Fluorescence of cyt c/urea samples 0.0M, 2.0M, 4.0M, 6.0M, 8.0M, 10.0M

Week 2: **•UV-Vis and Fluorescence of more cyt c/urea samples pick 10-14 concentrations of urea based on the previous week's results that make sense (check with instructor)**
• UV-Vis and Fluorescence of NATA samples

Week 3: Redo anything that needs to be redone

C. Calculations/Data Analysis

1. To obtain an unfolding curve, subtract background fluorescence spectra of the buffers KP_i or urea/KP_i from corresponding fluorescence spectra of protein to obtain a corrected fluorescence spectrum.

   This corrected fluorescence intensity should be plotted as a function of urea concentration. The y-axis should be scaled from 0 to 1 (max). From the analysis of this data (Eqs. 10 and 12) determine the free energy of the unfolding process.

2. Also use the absorbance spectrum to generate an unfolding curve. Plot the shift in Soret absorption near 410 nm as a function of denaturant concentration to generate an unfolding curve and determine the free energy of unfolding for cyt *c* (if possible). How do these results compare with those from the fluorescence experiments?

3. Use the fluorescence spectrum collected in part B2 along with the absorption spectrum of ~10 μM cyt *c* in the absence of urea to calculate the Förster distance of the trp-heme pair. You will need Equations 2 and 3. Other pertinent information: $\kappa^2=2/3$, $n=1.4$, $\Phi_D=0.13$.

4. Using Equation 4 and your calculated R_0, make a graph 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 your donor-acceptor pair.

5. Use Equations 4 and 5 and the corrected fluorescence spectra to determine the distance between Trp-59 and the heme group as a function of denaturant concentration. For F_D, you should use the fluorescence intensity of NATA at the same concentration as the cyt c samples. You may have to interpolate from the data you acquired in B2 in order to get a good value for F_D. Make a graph to display the how the distance between heme and Trp-59 changes as a function of denaturant concentration. 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?

D. Lab Report

This lab report will be a journal-style report (abstract, introduction, experimental results, discussion, conclusions, references). You and your partners can choose to each hand in your own reports or to hand in a joint report. If you do hand in a joint report, I will assume that you each contributed equally.

E. References

(1) Lakowicz, J. R. *Principles of Fluorescence Spectroscopy*, 3rd ed.; Springer: New York, 2006.
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(4) Shirley, B. A. Urea and guanidine hydrochloride denaturation curves. In *Protein Stability and Folding*; Shirley, B. A., Ed.; Human Press Inc.: Totowa, 1995; Vol. 40; pp 177-190.