Emission Spectroscopy:

Biophysics and FRET

Carina Sirochinsky

Lab Partner: Philip To

March 10, 2016

Introduction and Objective

Proteins exist in an equilibrium between their folded and unfolded states. This equilibrium is dictated by environmental conditions such as the polarity of the solvent, temperature, pH, and the presence of chemical denaturants. In this laboratory experiment, we are interested in how the chemical denaturant, urea, causes the protein ferric cytochrome-c (cyt-c) to unfold. To study the relationship between urea concentration and the cyt-c equilibrium, we utilize the concept of Förster resonance energy transfer (FRET). The protein of interest contains a fluorophore, a tryptophan residue at position 59 (Trp-59). In the folded state, the fluorescence of Trp-59 is quenched by the nearby presence of a heme group. The Trp therefore acts as the FRET donor and the heme as the FRET acceptor. As the protein is unfolded, due to increasing concentrations of urea, the acceptor and donor are moved away from each other, decreasing the efficiency with which heme absorbs the energy released by Trp, and fluorescence intensity increases. The distance between the FRET donor and acceptor is referred to as the Förster distance. Thus, by studying how the observed cyt-c fluorescence changes with urea concentration, it is possible to correlate the folded-unfolded protein equilibrium to the denaturant concentration. In part A, we correlate fluorescence data with urea concentration to generate an unfolding curve for cyt-c. In part B, we determine the characteristic Förster distance between tryptophan and heme using a model tryptophan compound, N-acetyl-tryptophanamide, NATA. In part C, we calculate the actual distance between try-59 and heme in the cytochrome-c protein, using the fluorescence data from A in conjunction with the Förster distance determined in B to gain insight on the structural changes that occur upon denaturing of cytochrome-c with urea.

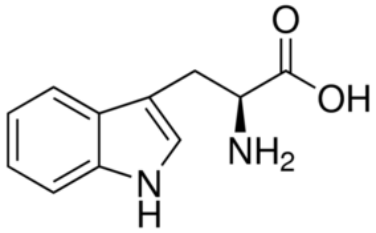


Figure 1: Tryptophan amino acid found at position 59 in the cytochrome-c protein

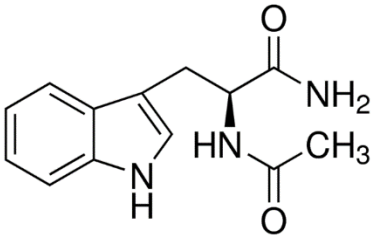


Figure 2: Tryptophan model compound, N-acetyl-tryptophanamide, NATA

Experimental Procedure

Stock solutions of 20 mM pH 7.4 phosphate buffer (KP_i), 10.0 M urea in KP_i (urea/KP_i), and 500 µM cyt-c in KP_i, were pre-prepared. KP_i and urea/KP_i were combined in various volumes to create 21 samples ranging from 0 M urea concentration to 10.0 M [urea] in 0.5 M increments. Pipettes were used to transfer volumes of the stock solutions to Eppendorf tubes. The mixed urea/buffer solution was transferred from the Eppendorf tube to a glass cuvette starting with the 0 M [urea] sample. The absorbance and fluorescence spectra were then obtained without any protein present in solution. The fluorometer used in the lab was The samples were exposed to an excitation wavelength of 290 nm and the emission spectrum was collected from 305 nm to 500 nm; the "high" sensitivity and 5 nm bandpass settings were used for both excitation and emission. The UV-Vis Spectrometer used in lab was The absorption spectra for each sample were collected from 350 nm to 650 nm. After both spectra were collected for the urea/buffer mixture, enough cyt-c was added to create 10 µM solution. Then the absorbance and fluorescence spectra were obtained once again. This process was then repeated with the 0.5 M [urea] sample, first collecting the spectra without cyt-c and then collecting the spectra once the

protein was added. This was repeated for all 21 samples. The protein was kept on ice at all times and exposure to light was limited to prevent photo-bleaching of the fluorophore. A 10 µM solution of NATA was created from a stock solution of 0.10 M NATA in KP_i. The fluorescence and absorbance spectra were collected for this sample using the same parameters described above.

Results and Discussion

Part A

First, it was necessary to find the fluorescence of just cyt-c, correcting for the fluorescence of the urea and KP_i also present in solution. This was done by subtracting the 350 nm fluorescence of just the urea/buffer solution from the 350 nm fluorescence of the urea/buffer/cyt-c solution. This was done for each of the 21 pairs of data. Next, the concentration of cyt-c was determined in each of the 21 samples by first correcting the absorbance data in a similar manner as the fluorescence correction described above to find absorbance of pure cyt-c; the absorbance data was corrected at 410 nm. Then, rearranging the Beer-Lambert Law, $A = \varepsilon lc$ (where *A* is absorbance of a compound at a given wavelength, ε is the extinction coefficient of the compound at that wavelength, *l* is pathlength, and *c* is the concentration of the compound in solution) it was possible to determine the concentration of cyt-c in each of the 21 samples; $\varepsilon_{410} = 105{,}000\ M^{-1}cm^{-1}$ was used as the extinction coefficient. Next, the fluorescence of each sample (with different urea concentrations) was divided by the corresponding concentration of cyt-c in determined to be in that sample to find the fluorescence per concentration of protein in each sample.

To create an unfolding curve of the protein, it was necessary to determine the f of fraction unfolded molecules at each concentration of urea, *C*. To do this it was assumed that at *C* = 0 M, 100% of the protein was in its folded state and the corresponding fluorescence, F_0, represented the fluorescence of the folded protein and that at *C* = 10 M, the protein was fully unfolded and the corresponding fluorescence, F_∞, represented the fluorescence of the unfolded protein. Thus the following relationship was established, the fraction of unfolded protein, *f*, is equal to

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

(where *[U]* is the concentration of unfolded protein and *[F]* is the concentration of folded protein, and thus *[U]+[F]* is the total protein concentration in solution) and could be correlated to the concentration of urea as shown below:

$$f = \frac{F(C) - F_0}{F_\infty - F_0}$$

where *F(C)* is the corrected and normalized fluorescence of the cyt-c in solution as a function of urea concentration. This result, *f*, was plotted against urea concentration, *C*, as shown in Graph 1 below.

A theoretical way to determine the fraction of unfolded molecules can be derived from the laws of thermodynamics as shown in the Appendix. The equation below represents the result of the derivation, with the fraction of unfolded molecules as a function of *C*, where *m* (kcal $M^{-1}mol^{-1}$) is the rate

of change of the free energy of denaturant with respect to denaturant and C_m (M) is the midpoint concentration of denaturant at which *[U]=[F]*.

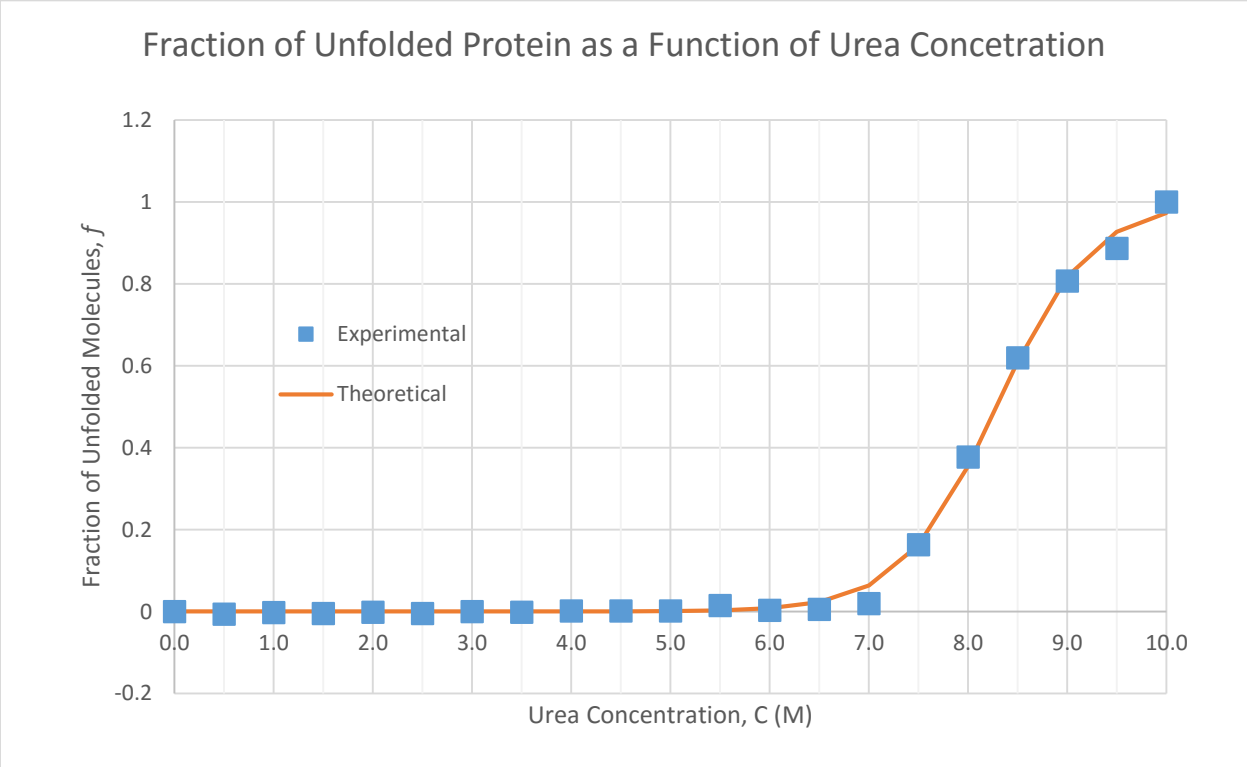
$$f = \frac{e^{-m\left(\frac{C_m - C}{RT}\right)}}{1 + e^{-m\left(\frac{C_m - C}{RT}\right)}}$$

Arbitrary values were chosen for *m* and C_m and *f* was plotted against *C*. The *Solver* tool on Excel was used to adjust the values of *m* and C_m in the theoretical equation in order to minimize the square of the differences between the experimental and theoretical values for *f* and determine the equation of the curve that would describe the experimental results. Using these optimized values for *m* and C_m, it is possible to determine the free energy of unfolding in absence of denaturant, using the equation

$$\Delta G^{\circ}_{H_2O} = mC_m$$

The values determined by the *Solver* program were

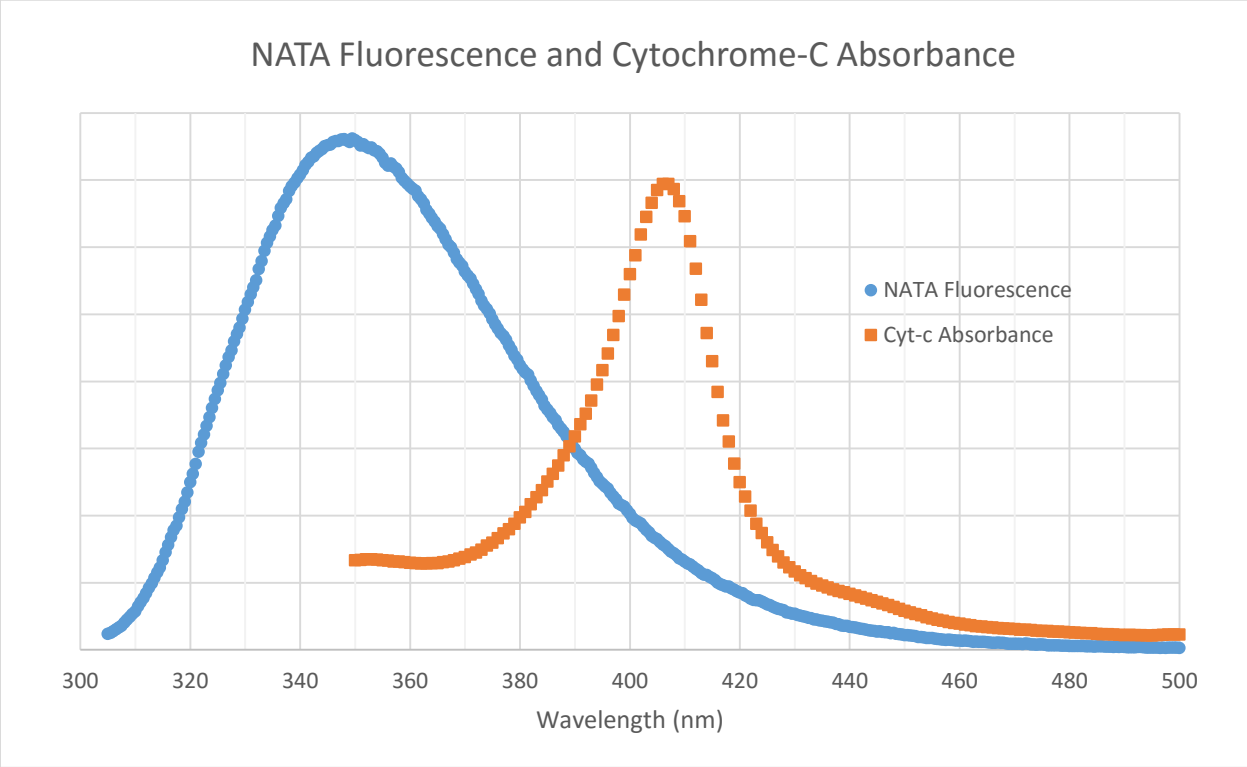
m (kcal M^{-1} mol^{-1})	1.240356464	1.2
C_m (M)	8.284904	8.3
$\Delta G°_{H2O}$ (kcal mol^{-1})	10.27623423	10



Graph 1: The fraction of unfolded molecules in solution as a function of denaturant (urea) concentration. With experimental results shown as points on the graph. The theoretical model was fit to the experimental data to produce values for m *and* C_m.

Part B

Below the fluorescence of the model tryptophan compound, NATA, is shown alongside the absorption spectrum of cyt-c in a solution of 10.0 M urea. The NATA curve is assumed to represent the full fluorescence of tryptophan-59 (the FRET donor) and the cyt-c curve is meant to represent the absorbance the heme group (the FRET acceptor). In accordance with FRET theory, we see that the spectra of the donor and acceptor do in fact overlap.



Graph 2: The NATA fluorescence approximates the fluorescence of the trp-59 residue in cytochrome-c. The absorbance spectrum of cytochrome-c in 10.0 M urea, in which the protein is assumed to be fully denatured and therefore, the trp-59 and heme are as far apart as possible, approximates the absorbance of heme in ferric cytochrome-c.

To determine the Förster distance between trp-59 and heme, it was first necessary to calculate the overlap integral between, J_{DA}, the fluorescence and absorbance demonstrated in Graph 2 above. The integrals of the curves in Graph 2 are approximated as Riemann sums. The equation below demonstrates how the overlap integral can be calculated:

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

where $F_D(\lambda)$ is the fluorescence of NATA, $\varepsilon_A(\lambda)$ is the extinction coefficient of cty-c at any given wavelength. Once J_{DA} is known, it is possible to calculate R_0, the Förster distance, using Equation 2 from the manual shown below:

$$R_0^6 = (8.79 \times 10^{-5}) \left(\frac{\kappa^2 \Phi_D}{n^4} \right) J_{DA}$$

where $\kappa^2 = ⅔$, n = 1.4, and $\Phi_D = 0.13$. The values for J_{DA} and R_0 are shown below

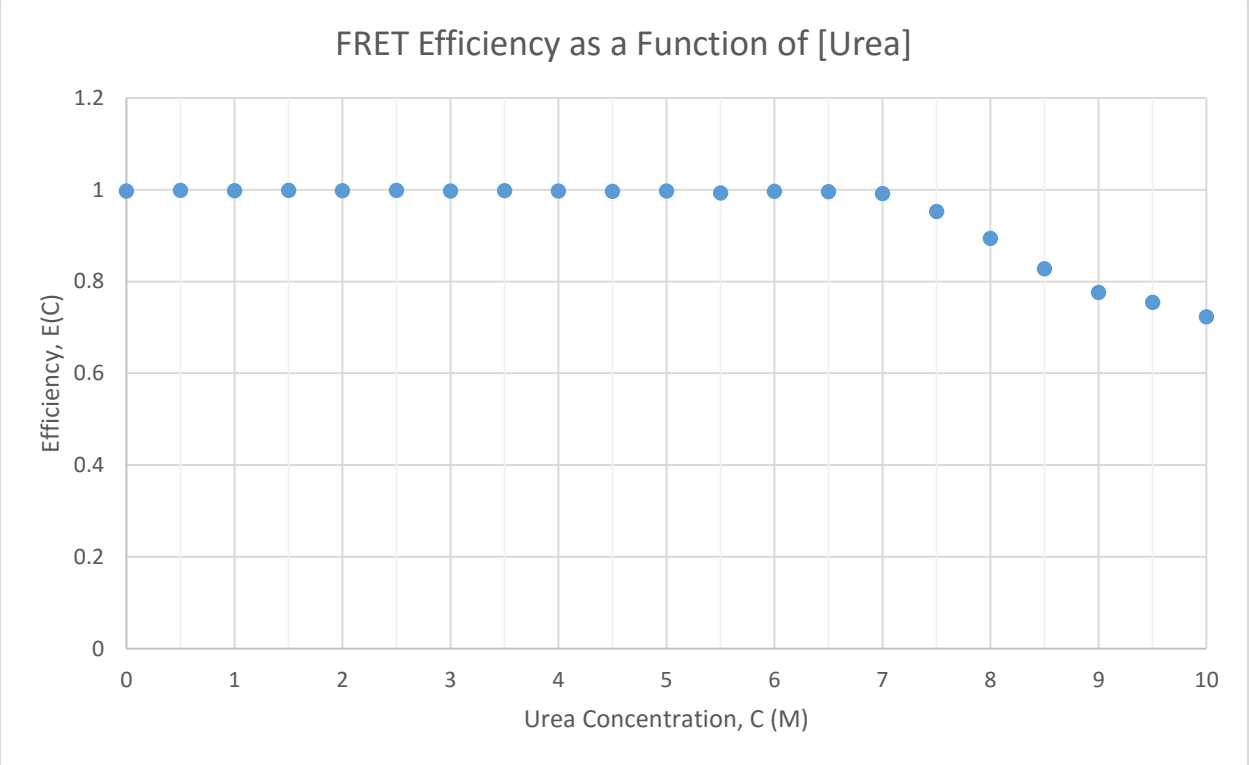
J_{DA} ($M^{-1} cm^3$)	7.76833×10^{14}	7.8×10^{14}
R_0 (Å)	33.98412781	34

Part C

The Förster distance indicates the distance at which FRET efficiency is 50%. Using the fluorescence data from A and this new insight from B, changes in FRET efficiency can be related to the denaturing of the protein. It is assumed that in a solution of 0 M urea FRET efficiency, *E*, is 1 and as the urea concentration, *C*, is increased, *E* falls off to 0 as the FRET donor and acceptor are separated beyond FRET range. The relationship between observed fluorescence and FRET efficiency is expressed by the equation below:

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

When cyt-c is fully denatured, the apparent fluorescence of trp-59, F_{DA}, can be approximated as the fluorescence of NATA, this value is F_D, and the efficiency would be 0, as expected; and when the fluorescence of trp-59 is being fully quenched by the heme, efficiency would be 1. F_{DA} is the fluorescence of cyt-c in varying concentrations of urea that has been corrected and normalized as described in Part A. F_D is the fluorescence of NATA at 350 nm, normalized for concentration (0.1 M). Efficiency was plotted against urea concentration as shown in Graph 3 below:



Graph 3: Efficiency is calculated as a function of concentration based on the fluorescence of cytochrome-c in various concentrations of urea compared to the full fluorescence of a model tryptophan compound, NATA.

FRET efficiency can also be illustrated as a function of distance between the donor and acceptor, *r* as illustrated by the equation below:

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

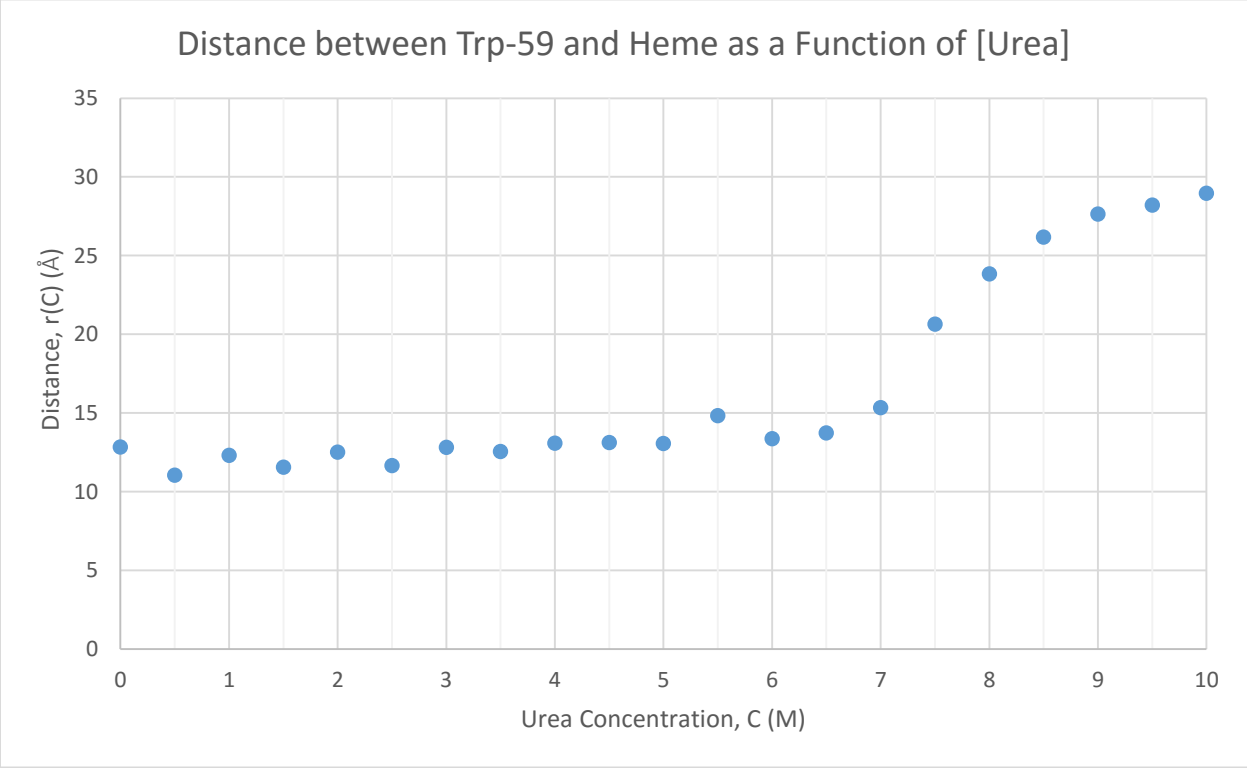
This equation can be rearranged for *r*:

$$r = \sqrt[6]{\frac{{R_0}^6}{E} - {R_0}^6}$$

If *E* is replaced with the equation relating efficiency to fluorescence, the equation for *r* becomes:

$$r = \sqrt[6]{\frac{{R_0}^6}{1 - \frac{F_{DA}(C)}{F_D}} - {R_0}^6}$$

And the distance between trp-59 and heme can be plotted as a function of urea concentration as shown in Graph 4 below:

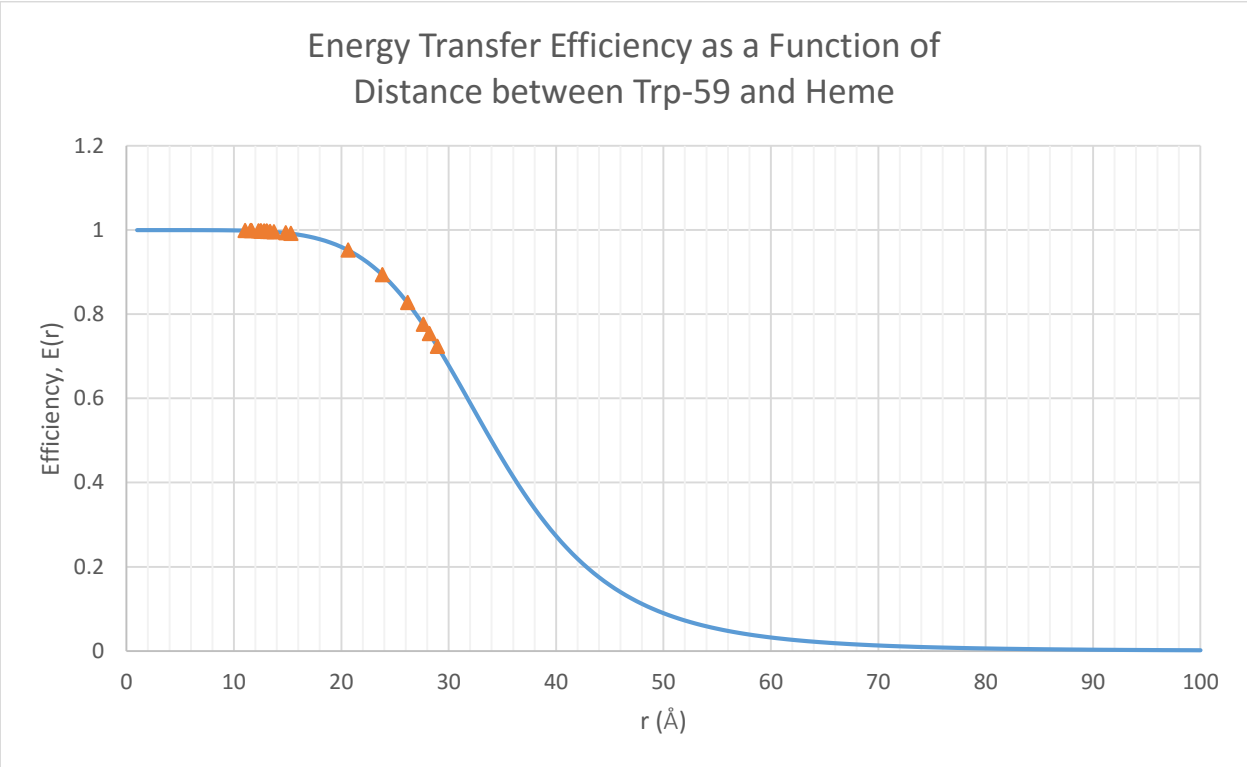


Graph 4: The separation of trp-59 and heme as the concentration of the denaturant, urea, is increased.

Using this equation

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

and choosing a range of values of *r*, here 0 Å to 100 Å, near the determined Förster distance, we can create a standard curve for FRET efficiency in a tryptophan-heme system, shown as the solid line in Graph 5 below. Then correlating the *r(C)* and *E(C)* for each concentration of urea from the data manipulations above, 21 data points are generated that lie on or near the standard curve as seen below.



Graph 5: Comparison of the standard curve for FRET efficiency in the tryptophan(NATA)-heme system and the experimentally determined dependence of efficiency on distance between the trp-59 and heme in cytochrome-c.

In Part A, it was assumed that the observed fluorescence of cytochrome-c in the 10.0 M urea sample was the full fluorescence of cytochrome-c and that none of the energy was being quenched by the heme group, meaning that in a solution of 10.0 M urea, the cytochrome-c was completely denatured and the fraction of unfolded molecules was 1. This is reflected in the calculation for fraction of unfolded molecules in which F_∞ was equated to *F(10.0 M).* Graph 1 shows that the protein does not start denaturing until the solution reaches a urea concentration of about 6.0 M. The graph also shows that the C_m, the concentration of urea at which [U]=[F], is 8.3 M. This suggests that the experiment should have been conducted with urea concentrations higher than 10.0 M, perhaps as high as 20.0 M, to fully denature the protein. F_∞ for cytochrome-c should be equal to the fluorescence of NATA. If this was true, then in Part C, when calculating efficiency as $E(C) = 1 - \frac{F_{DA}(C)}{F_D}$, plugging in the fluorescence at 10.0 M urea should have yielded an efficiency of 0. As seen in Graph 3, however, *E*(10.0 M) = 0.72. This incorrect assumption is also revealed in Graph 4, where the point at 10.0 M corresponds to *r* = 28.96 Å. In Part B

the Forster distance was determined to be approximately 34 Å. Thus at 10.0 M urea, the donor-acceptor FRET pair are closer together than R_0, and FRET dominates over fluorescence, indicating that the F_∞ should definitely not have been approximated as the fluorescence of cytochrome-c at 10.0 M urea. If Graph 3 is extrapolated to $E = 0$ and Graph 4 is extrapolated to $r = 34$ Å, then the concentration of urea required to fully denature cytochrome-c can be approximated to be 16.0 M.

As mentioned above, the results in Part A are systematically skewed by the assumption that cytochrome-c is fully denatured in 10.0 M urea. This means that the values of m, C_m, and $\Delta G°_{H2O}$ are incorrect. For example, $C_m = 8.3$ M, which suggests the experiment should be extended with urea concentrations surpassing 10.0 M, but even this value does not truly represent the equilibrium when [U] = [F], but the value when $[\mathrm{U}] = \frac{[\mathrm{U}]_{10.0M}}{2}$, when the number of unfolded proteins is one-half the number of unfolded proteins in the 10.0 M urea solution. In Part B, the fluorescence of NATA was not properly normalized for concentration because absorbance data was not collected, and even if it was, a reliable extinction coefficient for NATA at a wavelength the spectrophotomer could detect was not available in literature. In Part C, J_D at 10.0 M urea and J_{DA} were assumed to be equivalent when discussing FRET efficiency. It was assumed that in a 10.0 M urea solution, cytochrome-c would be fully denatured ~~and the fluorescence intensity would be equivalent to that of free tryptophan (simulated by NATA) which is not quenched by a FRET accepter~~. Clearly, the results in Part C, show this assumption to be correct, as FRET efficiency is well above 50% even in a 10.0 M urea solution. ~~This entire discussion, however, hinges on the assumption that in cytochrome-c's denatured form, trp-59 and heme are far enough apart from each other that FRET efficiency does indeed equal 0.~~ Perhaps 10.0 M of urea is enough to fully denature the protein, but in its denatured form the FRET pair remains in close enough in proximity that the FRET efficiency is always relatively high.

Conclusion

In a paper by Sanchez, Schlamadinger, Gable, Kim, a similar experiment of FRET efficiency in cytochrome-c was conducted with urea and guanidinium hydrochloride. For the trials with urea, the m was determined to be 1.0 kcal M^{-1} mol^{-1} and C_m was 7.2 M; the resulting $\Delta G°_{H2O}$ was 7.2 ± 0.3 kcal/mol. These values are similar to those calculated in our experiment.

Variable	Experimental	Literature	Percent Difference
m (kcal M^{-1} mol^{-1})	1.2	1.0	24
C_m (M)	8.8	7.2	15
$\Delta G°_{H2O}$ (kcal mol^{-1})	10	7.2	43
R_0 (Å)	33.98	34	0.059

The r value for cyt-c in its native form was determined in our experiment to be approximately 13 Å, while the paper reported a value closer to 20 Å. For the r value in the denatured form we calculated 30 Å, while the paper reported ~33 Å. Although the absolute numbers are different the change in r, the ranges between the native and denatured forms are similar. The article states "Consistent with a previous study, the fully unfolded protein exhibits ~50% fluorescence signal relative to the free model compound NATA, indicating that Trp-59 and the heme group remain in sufficient proximity for energy transfer under

denaturing conditions." This coincides with the suggestion made in the Results section above – the fully denatured form of cyt-c does not correspond to a FRET efficiency of 0. However, it is still unclear what the necessary urea concentration is to fully denature the cyt-c and whether correcting for this variable will provide better results. Saturating the protein solution with an excess of urea in future experiments may provide insight to this problem. In addition, other techniques of protein structure analysis, like x-ray crystallography may be needed to determine the distance between Trp-59 and heme in the native and denatured states.

References

Sanchez KM, Schlamadinger DE, Gable JE, Kim JE. Förster Resonance Energy Transfer and Conformational Stability of Proteins: An Advanced Biophysical Module for Physical Chemistry Students. *Journal of chemical education*. 2008;85(9):1253-1256.