PROTEIN STUDY I: Thermal Analysis of a Protein

INTRODUCTION

Most proteins are made from unique combination of 20 L-amino acids found in nature that define the protein sequence. Three of these essential amino acids absorb energy in the ultraviolet region of the electromagnetic spectrum and are largely responsible for the unique spectral properties of the protein.

![Amino acids phenylalanine (F), tryptophan (W), and tyrosine (Y)](image)

Fig. 1) Amino acids phenylalanine (F), tryptophan (W), and tyrosine (Y)

Based on the number of these amino acid residues (F, W, and Y) present and their location in a protein, these amino acids can provide information about protein structure.

In this experiment, we will determine the unique spectral properties of these pure amino acids in solution. We will use these spectral properties to characterize absorbance of protein in native and denatured state to monitor the stability of a protein as a function of temperature. The protein that will be used to characterize the thermodynamic properties as a function of temperature and pH is listed on the lab’s home page after the link for this experiment. This same protein will be used in Part II of the study, “Chemical Denaturation with Fluorescence Detection”.

BACKGROUND

Equation 1 shows a common depiction of the denaturation of a protein as a two-state model (Lumry et al., 1966).

\[ N \leftrightarrow D \quad (1) \]

This model can be used to define linear baselines for a hypothetical denaturation of a protein as shown in Figure 2.
Equations 2 and 3 define the linear baselines for the native and denatured states in Figure 2.

\[
y_{N,T} = m_N T + b_N \\
y_{D,T} = m_D T + b_D
\]

Where \( m_N \) and \( b_N \) are the slope and \( y \)-intercept of the native baseline. Likewise, \( m_D \) and \( b_D \) are the values for the denatured baseline. \( T \) is the absolute temperature.

Equation 4 shows the determination of the fraction of the protein denatured, \( \alpha_T \), by comparing the signal, \( A_T \), at any temperature to the native and denatured baselines.

\[
\alpha_T = \left( \frac{A_T - y_{N,T}}{y_{D,T} - y_{N,T}} \right)
\]

The equilibrium constant for the denaturation, \( K_{D,T} \), is obtained using Equation 5.

\[
K_{D,T} = \frac{[D]}{[N]} = \frac{\alpha_{D,T}}{1 - \alpha_{D,T}}
\]
Equation 6 shows the calculation of Gibb’s Free Energy, $\Delta G_{D,T}$ for the denaturation.

$$\Delta G_{D,T} = -RT \ln K_{D,T}$$  \hspace{1cm} (6)

Where R is the gas constant. Manipulation of the van’t Hoff equation can be used to determine the enthalpy, $\Delta H_D$ as shown in Equation 7.

$$\Delta H_D = -4RT^2_D \left( \frac{\delta \alpha}{\delta T} \right)_{TD}$$  \hspace{1cm} (7)

Where $(\delta \alpha / \delta T)_D$ is the slope of a line drawn through the steepest part of the transition and is used in conjunction with $T_D$.

**PRELIMINARY REPORT**

Complete and submit an individual preliminary report formatted as indicated on the lab web pages prior to the beginning of the 1st scheduled day of the experiment.

Include safety information on the handling of the following:

a) Phenylalanine, tryptophan, tyrosine  
b) Glycine  
c) Monosodium and disodium phosphate  
d) The protein (listed on lab home page, immediately following the link to this experiment).

**EXPERIMENTAL**

1. Obtain two 200mM buffers from your TA. Dilute the buffer to 20mM using deionized water. You should prepare approximately 100mL of each buffer. Store the buffers in a refrigerator at ~4°C.

2. Prepare ~10mL solutions of each of the 3 amino acids at the following concentrations, using each of the assigned buffers. Once the solutions are prepared, filter using a syringe filter.

   a) phenylalanine, 10mM  
   b) tryptophan, 0.5mM  
   c) tyrosine, 1mM

   Once prepared, the solutions not used within the lab period should be refrigerated at 4°C until use.
3. Set the Peltier Control at 25°C and obtain a UV-VIS spectrum of each of the amino acids using the Perkin Elmer Lambda 35 UV-VIS spectrometer in 408 from 220 to 400nm. Dilute with your filtered 20 mM buffer as necessary to obtain the maximum wavelength(s) with an absorbance approximately equal to 1. Save each of the spectra as ASCII files using the user directions that accompany the spectrometer.

4. Obtain a protein from the TA that has been diluted to 1mg/mL in the designated buffers. Handle the protein carefully.

5. Obtain a UV VIS Spectrum of the protein from 220 to 400nm at two temperatures, as follows:
   a) pH = 2.5 buffer (glycine): 18 and 90°C. Do not place the protein solution into the spectrometer until the temperature reaches 18°C. Allow it to equilibrate at least 2 minutes before obtaining the spectrum at this temperature. Next, raise the temperature to 90°C and allow it to equilibrate for at least 5 minutes before obtaining the spectrum at this temperature.
   b) pH = 4.0 buffer: 25 and 95°C. Allow the protein solution to equilibrate for at least 2 minutes before obtaining a spectrum at the lower temperature. Next, raise the temperature to 95°C and allow it to equilibrate for at least 5 minutes before obtaining the spectra at this temperature.

Finally, obtain a difference spectra by subtracting the absorption versus wavelength of the high temperature spectra from the low temperature spectra for each buffer. Plot as a function of wavelength and determine the wavelength at which the maximum difference occurs.

TIP: Check the cuvet for the presence of bubbles at temperatures greater than ~40°C. These bubbles could reduce the measured absorbance. If present, remove the cuvet from the spectrometer, then carefully use the end of a Pasteur pipet to remove them.

6. Using the wavelength determined from the difference spectra, obtain UV VIS spectra of a fresh sample of the protein at a peak wavelength (obtained from difference spectra) by varying the temperature at 2°C intervals (1°C intervals at maximum absorbance changes) from the minimum to maximum temperature range specified for your buffer. Always remember to incubate your protein for 2 min at the indicated temperature for stabilization of the absorbance reading before acquiring the spectra. Remember to check the cuvet for the presence of bubbles.

FINAL REPORT INFORMATION

Follow the normal Physical Chemistry Laboratory report guidelines for a final report. Reports should be submitted individually. Please include the following for both pH/buffer solutions:

1. Absorbance spectra of each of the amino acids (in both pH/buffer solutions). Indicate the final concentration, the buffer, and the pH on each, as well as the A vs. λ for each.

2. Spectra of the protein at the high and low temperatures.
3. A graph showing the difference spectra of the protein solution as a function of the wavelength.

4. A graph showing the normalized absorbance vs. temperature of the protein. To normalize the absorbances to values between 0 and 1, use Equation 8.

\[ x_n = \frac{x - A}{B - A} \]  

(8)

Where \( x_n \) is the normalized data (absorbance), \( x \) is the data you wish to normalize, \( A \) is the data set’s minimum value, and \( B \) is the data set’s maximum value.

5. Notice the graph of \( A_n \) vs. \( T \) has three distinct areas. Fit each of these areas with an equation that depicts the best-fit equation for a line:
   
   a) the protein in its natural state (c.f. Equation 2)
   b) the protein in its denatured state (c.f. Equation 3)
   c) the protein undergoing a transition (from a to b)

6. Determine the temperature where \( \alpha = 0.5 \) (c.f. Equation 4). The temperature value, \( T_D \), must be located on the best-fit line in the transition area of the graph.

7. Report the thermodynamic properties: \( K_{D,T} \), \( \Delta G_{D,T} \), and \( \Delta H_D \) for the protein at each pH/buffer. Include sample calculations.

8. Discuss hydrogen bond interactions i.e., differences in the protein and protein data which are caused by pH.

REFERENCES


ACKNOWLEDGEMENTS

The following people assisted in the development of this experiment: Dr. Ashutosh Tiwari, Kelley M. Smith, M.S., and Katlyn C. Mehne (B.S., 2011). Michigan Technological University, Houghton, MI 49931 (2010).
A. Setting up the Method

2. Open the software icon. Username & password: “analyst” & <enter> (no password).
3. Select “New” then “Method” from the “File” menu.
4. Select “Next” when the New Method Wizard opens, then “Next” again (Lambda 35).
5. Next, choose either scan or a wavelength program (not the quantitative wavelength program), then “Next”.
6. Select the “Peltier” box, then “Next”.
7. Select “Save/Finish” and save in a CH3541 folder.
8. Set wavelength(s)
   * Scans must be from 200-800nm (The Amino Acids absorb only in the 200-300nm region)
   * For a wavelength method, remove the default wavelengths and add $\lambda_{\text{max}}$ (obtained from the “difference spectrum”).

B. Setting up the Task

1. Select the “TASK” menu from the side menu.
2. Set up the maximum number of samples (25?).
3. Edit the samples. The samples will be processed from the most recently edited to the least recently edited, so use caution when adding details. (It might be better to record details in a notebook for each sample number and use good naming practices to save the files.)
4. Press the blue arrow in the upper menu.
5. You will soon be prompted to remove the sample(s). Place both of the matched cuvets containing the buffer into the instrument and press “OK”.
6. Once the calibration is complete, you will be prompted to begin running your samples. The cuvet in the reference holder should remain in the spectrometer unless your buffer changes.

C. Adjusting the Temperature

1. Set the cooler to about 15°C.
2. Use the Peltier’s control panel to control the temperature. Both the reference and sample should be changed. The $\lambda$-35’s software will not control the Peltier, so it should be ignored.

D. Processing the Data

1. To save a scan (i.e., wavelength data as a function of temperature should be recorded in a notebook), right click directly on one of the absorbances of the spectra you are viewing and select, “Save as an ascii file.” Specify a file on a USB drive or other media separate from the hard drive.
2. To open a scan in Excel, first go to the “Data” menu (upper menu bar selection), then choose “From Text”. Find the file (remember it will be a *.* file!) and open it using a space as the delimiter. The data will be imported as non-numerical (i.e., unusable) if you don’t use the “delimeter” option!