Does Myoglobin Unfold in the Body?

A teaching experiment produced by the

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Does Myoglobin Unfold in the Body?[1]

Myoglobin is a protein found in muscles that binds oxygen when in the proper conformation. In the denatured or unfolded form, myoglobin cannot bind oxygen. Our body contains compounds called denaturants that cause denaturation or unfolding. Guanidine is a natural product of protein metabolism.[2] At physiological pH guanidine is protonated to form the guanidinium ion. Guanidinium ion is a strong protein denaturant.

In this experiment the thermodynamic parameters associated with the unfolding of myoglobin will be determined, providing information on the stability of the protein in the presence and absence of guanidinium and insight into the release and storage of oxygen in muscle cells.

Pre-experiment Questions Part One

1. Describe the structure of myoglobin and its role in the human body.
2. Why is it important to know the conformation of myoglobin?
3. What is guanidine-HCl (GuHCl), and what is the role of GuHCl in biochemistry?
4. What is the *soret band*?
5. Do the calculations to show how to make 1 L of 0.05 M potassium phosphate buffer (pH=7), 250 mL of 1 mg/mL myoglobin in the buffer; and 6 M GuHCl in the buffer from pure compound.
6. Do the calculations needed to determine how you would make 5.0 ml of 0.2 mg/mL myoglobin that is also 1.5 M in GuHCl using the stock solutions from Pre-experiment Question 5.
7. Explain why, when making the solution described in Pre-experiment Question 6, you must mix some buffer with GuHCl before adding myoglobin and diluting to the final concentration with buffer.

General Protocol

1. Obtain (or prepare) the buffer solution (0.05 M potassium phosphate, pH=7) and the myoglobin solution (1 mg/mL in the buffer).
2. Prepare the necessary solutions of myoglobin with the required concentration of GuHCl. The GuHCl stock solution is 6 M (prepared in buffer). The total volume of every solution made is 5.0 ml, and the concentration of myoglobin is constant at 0.2 mg/mL. All dilutions are made with buffer. Mix the GuHCl and some of the buffer before adding the myoglobin and the rest of the buffer. Mix well.
3. Equilibrate the solutions for a minimum of 20 minutes.

4. Prepare the UV-Vis spectrophotometer to record spectra:
    a. Spectra will be recorded from 350 to 600 nm.
    b. Record a reference spectrum of the buffer.
    c. Set up the software to overlay spectra on the same axes.
5. Record the spectra of the myoglobin solutions; label the spectra, noting the concentration of GuHCl for that solution. In your notebook record the λ_{max} values and corresponding intensities of any peaks. Export the spectra so that they may be opened in a spreadsheet program such as Excel.

Experiment Part One

How does the addition of the denaturant GuHCl affect the UV-Vis absorption spectrum of myoglobin? Each group will record the spectrum of three myoglobin solutions (0.2 mg/mL myoglobin) from each of the following ranges of concentrations of GuHCl:

- 0-1 M GuHCl
- 1-2 M GuHCl
- 2-3 M GuHCl

Before recording the spectra, predict how the absorbance of the myoglobin will change as you increase the concentration of the denaturant. In your notebook, sketch on the same axes the predicted spectra of the soret band for myoglobin at three different GuHCl concentrations: low, medium, and high. Discuss the predicted spectra with your group. Once the group has reached a consensus answer, sketch this on the board and prepare to explain your reasoning.

Thinking About the Data Part One

8. Create a spreadsheet combining the spectra collected for the three myoglobin solutions by each group; overlay the spectra on a single graph.
9. Create another spreadsheet combining the λ_{max} values and corresponding intensities of any peaks.
10. What are the λ_{max} values for the myoglobin soret band when myoglobin is in its native, folded conformation and when it is in its denatured, unfolded conformation?
11. Describe the effect of unfolding on the myoglobin soret band intensity.
12. Explain how you can use the soret band intensity to determine the concentration of native myoglobin. The class (group) must come to a consensus about which λ_{max} to use and why.

Part Two

The myoglobin conformational equilibrium can be modeled by assuming there are two states of the protein, native (N) and unfolded (U), according to $N \rightleftharpoons U$. A goal of physical chemistry is to determine the equilibrium constant, *K*, for this equilibrium, and the corresponding free energy of unfolding, $\Delta_u G°$.
The free energy change for this process, when it occurs in pure water (in the absence of denaturant), is $\Delta_{H_2O} G°$. The two free energies are related by

$$\Delta_u G° = \Delta_{H_2O} G° - mC \quad (1)$$

where m is the rate of change of the free energy of denaturation with respect to C, the concentration of denaturant ([GuHCl]). $\Delta_u G°$ can be determined experimentally from

$$\Delta_u G° = -RT ln K_{eq} \quad (2)$$

and K_{eq} can be determined experimentally by

$$K_{eq} = \frac{A_N - A}{A - A_u} \quad (3).$$

A_N is the absorbance of myoglobin for [GuHCl] = 0 M, A_U is the absorbance of myoglobin for large [GuHCl], and A is the absorbance of myoglobin for intermediate values of [GuHCl]. All absorbances are recorded at your chosen wavelength.

Pre-experiment Questions

13. Use your data collected in Part One to determine A_N, A_U, and A for each of the three myoglobin solutions for which you recorded spectra. Go on to determine K_{eq} for these three solutions.
14. Identify the independent and dependent variables and the slope and intercept in equation (1); what must be varied to determine the free energy of unfolding in the absence of denaturant?
15. Complete the table showing the solutions necessary to clarify how myoglobin unfolds in the presence of GuHCl. Indicate the concentration and volume GuHCl and the volume of buffer needed in each 5mL flask. The volume and concentration of myoglobin is the same for each sample.

	GuHCl concentration (0-3M)	GuHCl volume (mL)	Buffer volume (mL)
Sample 1	0.0	0	4.0
…			
…			
Sample ##	3.0	2.5	1.5

Experiment Part Two

How does the addition of the denaturant GuHCl affect the UV-Vis absorption spectrum of myoglobin? Each group will repeat Experiment One for all the solutions listed in your table. Predict *more specifically* the effect of the concentration of GuHCl on the extent of unfolding of myoglobin by sketching a graph of % unfolded myoglobin (y-axis) versus concentration of GuHCl (x-axis). Each group should draw their consensus graph on the board and explain their reasoning for this predicted graph.

Thinking about the Data Part Two

16. Sketch a rough graph in your notebook to illustrate the interdependence of the GuHCl concentration and absorbance (intensity) at your chosen wavelength. Describe the general appearance of this figure. Compare your graph to that of at least two other groups.
17. Relate the three distinct "regions" in this graph to what is happening to the myoglobin as the concentration of GuHCl increases. Write your answer using complete sentences. The whole class should come to a consensus answer.
18. We use Equation (1) to model the "transition region" of the graph where there is an appreciable concentration of both native and unfolded myoglobin. Examine your graph to decide how many points you have obtained in this region.
19. Do you need additional data points? If so, prepare additional solutions and record the needed spectra.
20. In your spreadsheet,
    a. determine A_N and A_U and
    b. set up columns for [GuHCl], A, K, and $\Delta_u G°$.
21. Construct the appropriate graph to determine $\Delta_{H_2O} G$ and *m* by fitting Equation (1) to your data.
22. Is the unfolding of myoglobin in water thermodynamically spontaneous? Justify your answer.
23. The concentration of GuHCl where [N]=[U] is labeled C_m. If [N]=[U] and $C=C_m$, then (complete each equality)
    a. K =___
    b. $\Delta_u G°$ =___
    c. $\Delta_{H_2O} G°$ = ______
24. Determine C_m using your results from Question 23. Compare your result to that of at least two other groups.

Thinking About the Data Part Three:

Although physical chemists often use linear models like that in Equation (1) to model data, nonlinear models can also be used. The whole dataset relating myoglobin absorbance to [GuHCl] can be modeled by the equation

$$f = \frac{e^{\left[-m\frac{C_m - C}{RT}\right]}}{1+e^{\left[-m\frac{C_m - C}{RT}\right]}} \qquad (4)$$

where f, the fraction of unfolded protein, is given by

$$f = \frac{[U]}{[U]+[N]} = \frac{K_{eq}}{1+K_{eq}} = \frac{A_N - A}{A_N - A_U} \qquad (5)$$

25. Use your spreadsheet to determine the experimental values of f for your results; graph f versus the concentration of GuHCl. Should the experimental values of f be plotted as points or a line? Describe and explain the appearance of your graph in words.
26. Using the values of m and C_m from the Thermodynamics I analysis, make a column in your spreadsheet for *calculated* values of f from Equation 4. In your equation make sure that m and C_m are values that may be varied (i.e., point to another cell in the spreadsheet). Add these calculated values of f to your graph (second series) of f versus [GuHCl]. Should the calculated values of f be plotted as points or a line?
27. Add a column to your spreadsheet in which you square the difference between the experimental and calculated values of f. In a cell near your values of m and C_m, add a cell in which you sum all the values in this new column. This value is called "chi-squared", χ^2, or the "sum of the square of the residuals."
28. Use the "Solver" add-in in Excel to improve the values of m and C_m by minimizing χ^2. Solver is located under the Data tab. Set the "Target Cell" to the cell containing χ^2; select "Min" for "Equal to" by "Changing Cells" with the values of m and C_m. Then click on "Solve."
29. Calculate a value for $\Delta_{H_2O}G°$ based on m and C_m determined from the nonlinear fit of Equation 4 to your data. Tabulate in your notebook values of $\Delta_{H_2O}G°$, m, and C_m obtained by fitting either Equation (1) or (4) to the data.

Post Experiment Questions

30. Show the algebraic steps needed to recast $f = \frac{[U]}{[U]+[N]}$ as $f = \frac{K_{eq}}{1+K_{eq}}$.
31. Use $\Delta_u G° = -RT ln K_{eq}$ and $\Delta_u G° = \Delta_{H_2O}G° - mC$ to derive the two-state model expression for *f*. Hint: How is $\Delta_{H_2O}G°$ related to m•C_m?
32. Compare your results to the published results[1] for myoglobin.
33. At what concentrations of GuHCl is myoglobin folded or unfolded in the body?

34. What keeps a native protein folded (enthalpy or entropy)? Justify your answer based on thermodynamics and the structure of myoglobin. What experiment would you do to test that idea?

35. What could cause a false reading of denaturation?

For further reading see references 3 and 4.

References

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