

Determination of Myoglobin Stability by Circular Dichroism Spectroscopy: Classic and Modern Data Analysis

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In response to the current ACS guidelines for chemistry departments, many colleges and universities have begun to introduce biochemistry into their undergraduate programs throughout the curriculum. Because students are being exposed to biological structures (DNA and proteins in particular) in many science courses, there needs to be the simultaneous exposure to the various spectroscopic techniques currently employed to characterize these biological molecules. Circular dichroism spectroscopy (CD) is an important technique used to study globular protein structure and stability (for an excellent overview on CD see ref 1). Proteins with their asymmetric centers within the polypeptide backbone show unequal absorption of left- and right-handed circularly polarized light in the far-UV region (170–250 nm). The difference in absorbance is multiplied by a factor to relate the change in absorbance to an ellipticity value, θ , with units of degrees ($\theta = 33.98\Delta A$). Unique CD absorption bands are observed depending on the type of protein secondary structure present within the protein molecule: either α -helical type or β -strand type.

A previous article in this *Journal* (1) noted that there are only a few experimental descriptions using CD at the undergraduate level to investigate proteins. In fact, many biochemical technique manuals do not have any experiments or even a discussion of the theory and instrumentation for CD. To augment the number of applications using CD we describe an exercise involving the thermal denaturation of myoglobin that can be used in a biochemistry or physical chemistry teaching laboratory. Proteins, such as myoglobin, that have a high percentage of α -helical secondary structure will show a strong CD absorbance minimum at 208 and 222 nm. Upon protein unfolding there is a significant change in the absorbance at 208 and 222 nm and thus with incremental increases of temperature one can monitor the unfolding process. The temperature at which there is 50% folded and 50% unfolded protein is called the midpoint unfolding temperature, T_m , and gives an indication of stability. A laboratory session of 3 to 4 hours in length is needed for one complete denaturation experiment. Our approach was to have two groups of students run identical experiments and then compare and average the results.

Myoglobin was chosen for a number of reasons. First, it is a well-studied protein that is presented in practically all the leading biochemistry texts during discussion of protein structure and function (2–4) and consists entirely of α -helical type secondary structure. Second, myoglobin is readily available for purchase from several sources. Third, for comparison purposes, a laboratory exercise determining the myoglobin stability by visible spectroscopy has been reported in this *Journal* (5).

From a pedagogical perspective we also present a comparison of data analysis between a more traditional approach of constructing various linear plots to ascertain the thermodynamic quantities of enthalpy and Gibbs energy of unfolding versus a more modern approach of “fitting” the data using non-linear methods to a particular model describing the denaturation

process. All modern CD instruments have appropriate software that allows non-linear methods to be employed. The classical data analysis approach uses the traditional plots of ΔG° versus temperature and the van't Hoff plot of $\ln K_{eq}$ versus $1/T$ to determine the standard enthalpy and Gibbs energy change associated with the unfolding process. We feel that students need to learn both approaches followed by a discussion of advantages and disadvantages of each approach.

Experimental Procedure

A stock solution of 2 mg/mL myoglobin (horse skeletal muscle, Sigma #M-0630) was prepared using sodium phosphate buffer, 0.05 M, pH 7.0, in aqueous conditions (filtered and degassed). An aliquot (1.5 mL) of this stock was centrifuged at 14,000 rpm (16,000g) for 15 min at 20 °C to remove any particulate matter. Samples (1.5 mL) for CD analysis were prepared to a final concentration in the range of 0.4–0.2 mg/mL using the same phosphate buffer. A quartz cylindrical cell with a 1 mm path length was utilized and CD spectra were obtained using an Online Instruments Inc. (OLIS) RSM 1000 spectrometer configured with a CD module for dual beam CD. The instrument was fitted with a Peltier-type (OLIS and Quantum Northwest) cell for accurate temperature control. A complete CD absorbance spectrum (from 250 to 195 nm) was taken at each temperature (every 2 °C from 50 °C to 96 °C) with a delay time of 2 minutes to ensure equilibrium conditions. Non-linear fitting of the data was carried out with the OLIS GlobalWorks software.

Hazards

The sodium phosphate salts may cause irritation to the skin, eyes, and respiratory tract.

Results and Discussion

Typical student-generated CD spectra for myoglobin in the folded state at 52 °C and in the unfolded state at 96 °C are shown in Figure 1. The protein also exhibits complete reversibility upon lowering the temperature to 50 °C (Figure 1). In a more traditional approach to data analysis, the ellipticity at 222 nm is plotted versus temperature to obtain a thermal denaturation profile as shown in Figure 2. Pedagogically, students can appreciate that all data collection is carried out at equilibrium and thus an equilibrium constant (K_{eq} = unfolded/folded) can be calculated from the fraction of unfolded protein at each temperature near the transition point using a simple two-state model for the process (6, 7). The fraction of unfolded protein is calculated from knowing the ellipticity of the folded and the unfolded species. Since this is an equilibrium-based calculation the absolute amount or concentration of the sample is not needed for the calculations.

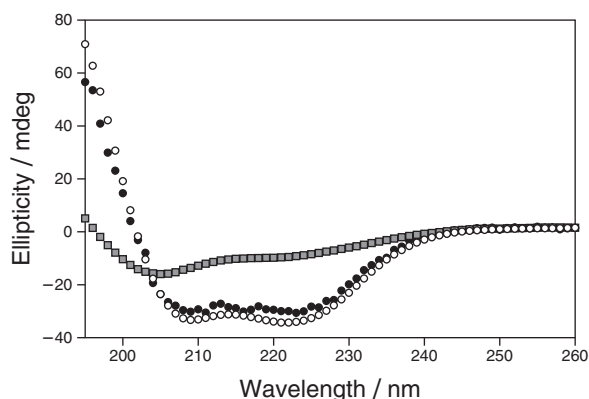


Figure 1. Far-UV CD spectra for myoglobin at 52 °C (open circles), at 96 °C (filled squares), and the return in temperature to 50 °C (filled circles). Myoglobin concentration was 0.4 mg/mL. Digital filtering (9 pt) was applied to each spectrum.

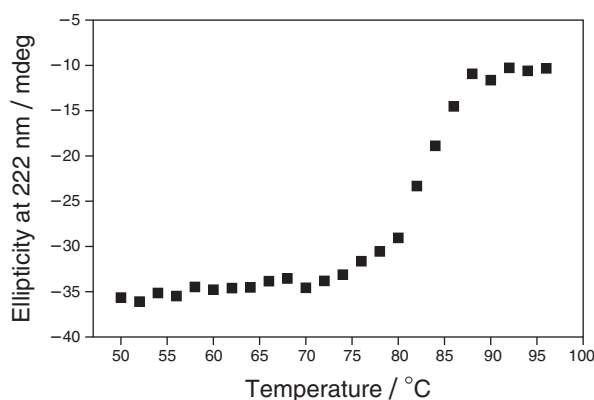


Figure 2. Thermal denaturation profile of myoglobin. At each temperature (50 °C to 96 °C, every 2 °C) the full CD spectrum was obtained from 260 to 195 nm. The individual values at 222 nm were used to generate this figure.

Next the standard Gibbs energy ($\Delta_u G^\circ$) for unfolding of myoglobin at each temperature is calculated using

$$\Delta_u G^\circ = -RT \ln K_{eq} \quad (1)$$

where R is the ideal gas constant and T is the specific temperature. The standard Gibbs energy is then plotted versus temperature; Figure 3 shows this plot using the data obtained in Figure 2. Using the slope of the plot of $\Delta_u G^\circ$ versus temperature one can calculate the transition temperature, T_m , because at the T_m the ratio of unfolded to folded species is 1 and $\Delta_u G^\circ = 0$. From the slope of the plot (Figure 4) of $\ln K_{eq}$ versus $1/T$ one can employ the van't Hoff equation

$$\frac{\partial(\ln K_{eq})}{\partial(1/T)} = \frac{-\Delta_u H^\circ}{R} \quad (2)$$

to obtain $\Delta_u H^\circ$ for unfolding process. Note that a change in entropy ($\Delta_u S^\circ$) can be determined from the y intercept in the van't Hoff plot, but a more reliable calculation would be from $\Delta_u H^\circ$ and $\Delta_u G^\circ$ (see the online material). For comparison purposes to literature values an estimate of the $\Delta_u G^\circ$ at 25 °C

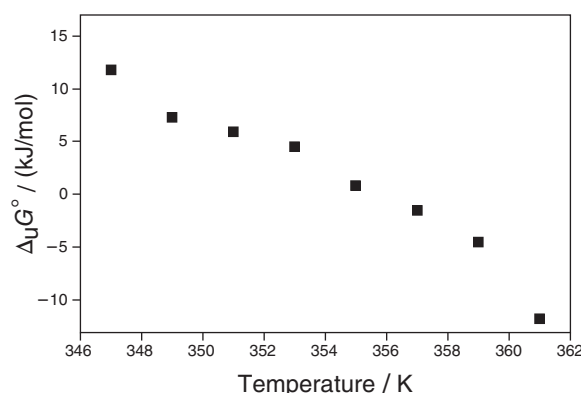


Figure 3. Plot of $\Delta_u G^\circ$ versus temperature for myoglobin.

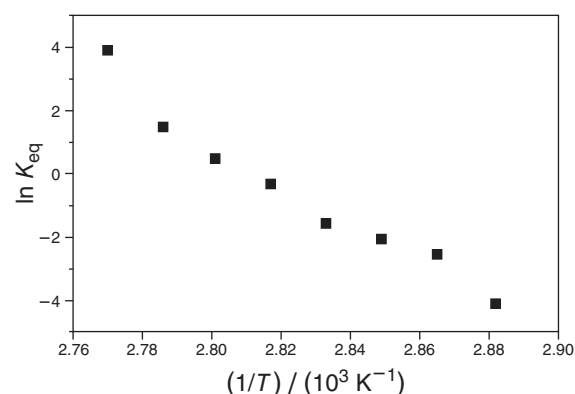


Figure 4. Van't Hoff plot of $\ln K_{eq}$ versus $1/T$ for myoglobin.

is needed and $\Delta_u C_p$ is required since there is a change in heat capacity that accompanies protein unfolding (the heat capacity of a folded protein is different from its unfolded form). To determine a rough estimate for $\Delta_u C_p$, the number of amino acids in myoglobin (153) is multiplied by 50.2 J/(mol K) (7). The $\Delta_u G^\circ$ at 25 °C can be calculated using (7)

$$\Delta_u G^\circ(T) = \Delta_u H^\circ \left(1 - \frac{T}{T_m}\right) - \Delta_u C_p \left[(T_m - T) + T \ln \frac{T}{T_m} \right] \quad (3)$$

The values determined using the classic linear approach described above are summarized in Table 1.

Students would also have the opportunity to compare the values obtained in the classic approach with a more modern, non-linear approach, of allowing computer software programs to "fit" the data to a given model of protein unfolding. Manufacturers of CD instruments will provide appropriate software for the non-linear method. The results of

Table 1. Summary of Thermodynamic Data for the Unfolding of Myoglobin

Analysis	Reference	Method	T_m (°C)	$\Delta_u H^\circ$ / (kJ/mol)	$\Delta_u C_p$ / [kJ/(mol K)]	$\Delta_u G^\circ$ (25 °C) / (kJ/mol)
Classic ^a	This work	CD (Thermal)	82.0	509 ± 40	7.7	44.4 ± 6
Modern ^a	This work	CD (Thermal)	82.4	416 ± 28	4.3	46.0 ± 5
Literature Values	9	DSC	81.6	548 ± 20	7.8	50.0 ± 3
	6	Fluorescence (GuHCl) ^b	ND ^c	ND	ND	38.5
	5	Visible (GuHCl)	ND	ND	ND	45.7 ± .7
	10	CD (GuHCl)	ND	ND	ND	46.0
	11	CD (Thermal)	81.3	ND	ND	ND

^aValues represent the average of two unfolding experiments. ^bGuanidine hydrochloride as a denaturant. ^cNot determined.

fitting the thermal unfolding data to a simple two-state model (native \rightleftharpoons unfolded) with the enthalpy varying with temperature are shown in Figure 5. The GlobalWorks (8) software allows a singular value decomposition (SVD) factor analysis on the entire data set using all the points from each scan (250 nm to 195 nm) at each temperature: thus, using a three-dimensional data matrix. The values obtained using the GlobalWorks software are summarized in Table 1.

Both approaches to data analysis, the classic (linear) and modern (non-linear), yield values close to those previously published in the literature (Table 1). Is one approach better than the other? Clearly in the non-linear approach more data points are being utilized and it is a much faster analysis. We feel that for the best understanding of the process of protein unfolding and the thermodynamic entities involved, students should follow through the classic approach first and then compare values with those from a more modern non-linear fitting program.

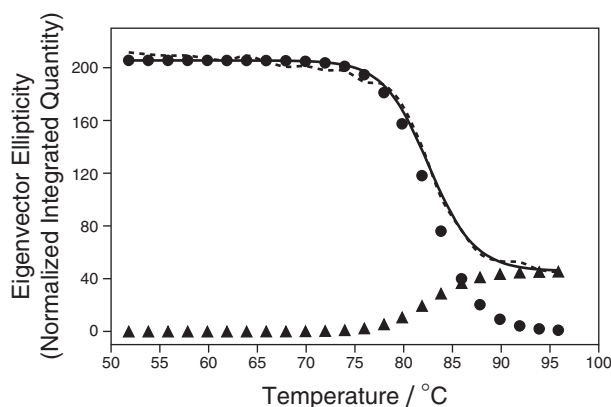


Figure 5. Fitting results from the GlobalWorks software. The relative quantity of unfolded species (filled triangles). The relative quantity of folded species (filled circles). The overall fit results (solid line) and the actual data (dashed line).

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