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Laboratory Exercises

The Free Energy of Denaturation of Lysozyme

AN UNDERGRADUATE EXPERIMENT IN BIOPHYSICAL CHEMISTRY*

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An undergraduate laboratory experiment to measure the free energy of denaturation of the enzyme lysozyme is described. The fluorescence of the protein in solutions of varying concentrations of urea and guanidine hydrochloride is measured. The data are analyzed graphically assuming a two-state model for the denaturation.

Keywords: Laboratory instruction, biophysical chemistry, fluorescence, protein folding, thermodynamics.

The study of the structure and properties of proteins is an integral part of all basic courses in biochemistry. One of the most important concepts in this area of study is the existence of native and denatured states of the macromolecule, and consideration is often given to the thermodynamics of the conversion between these two states. Measurements of thermodynamic parameters for folding and unfolding are common in biophysical research. However, to date, no experiments on the thermodynamics of protein denaturation have been published for the instructional laboratory. The experiments described herein have been developed as part of an overall revision of advanced courses in chemistry and biochemistry. One of the main goals in the revision was to develop experiments that illustrate the importance of physical and analytical chemistry to the study of the structures and properties of biological macromolecules. The experiments reported here involve the use of fluorescence spectroscopy to determine the Gibbs free energy change for the unfolding of the protein lysozyme by the denaturants urea and guanidine hydrochloride.

MATERIALS

Denaturant Stock Solutions

Approximately 10 M urea (Ultra grade; Sigma) was prepared in 0.050 M citrate buffer, pH 3. The fluorescence background of this solution was measured, and, if necessary, the solution was purified with activated charcoal. Stock solutions of ~8 M guanidine hydrochloride (Sigma Ultra grade) were made in 0.1 M Tris buffer, pH 7.0, and used without further purification. The final concentrations of denaturant stock solutions were determined by measurement of the refractive index [1].

Lysozyme Stock Solution

A 0.12 mM solution of chicken egg white lysozyme (stock number L6876; Sigma) was prepared in citrate or Tris buffer, as appropriate.

METHODS

A series of solutions containing a fixed amount of lysozyme (3 μM) and varying concentrations of urea (4.0–9.0 M) or guanidine hydrochloride (1.0–7.0 M) were prepared from the stock solutions. The final volume of each sample was 4.0 ml. The solutions were allowed to sit for at least 1 h, and the fluorescence spectrum of each sample was then measured on a Photon Technology Inc. LS-100 spectrometer. The excitation wavelength was 280 nm, and the fluorescence emission spectrum was recorded over the wavelength range from 300 to 460 nm. All experiments were done at room temperature (~25 °C).

RESULTS AND DATA ANALYSIS

Typical fluorescence spectra for native and unfolded lysozyme in urea are shown in Fig. 1. For the analysis of the fluorescence data, both the fluorescence intensity at the maximum wavelength (340 nm) and the integrated fluorescence area were measured and used in subsequent data analysis. Both parameters yielded the same result within experimental error. Fig. 2 and Fig. 3 show typical data for the variation of the measured fluorescence intensity with denaturant concentration.

Experimental protein denaturation curves are known generally to exhibit a sigmoid dependence of the measured parameter on the denaturant concentration [3]. The fluorescence intensity data were therefore fit with an equation of the following type:

$$y = y_0 + \frac{ax^b}{c^b + x^b} \qquad \text{(Eq. 1)}$$

using the software SigmaPlot (SPSS, Inc.). This is a Hill equation in which y is the measured parameter (fluorescence intensity, I), y_0 is the baseline value of the parameter at low denaturant concentration, a is the difference between the maximum value of the parameter when the protein is completely unfolded and the baseline value ($y_{max} - y_0$), x is the denaturant concentration, b is the Hill coefficient necessary to fit the data, and c is an interaction constant, equal to the denaturant concentration where

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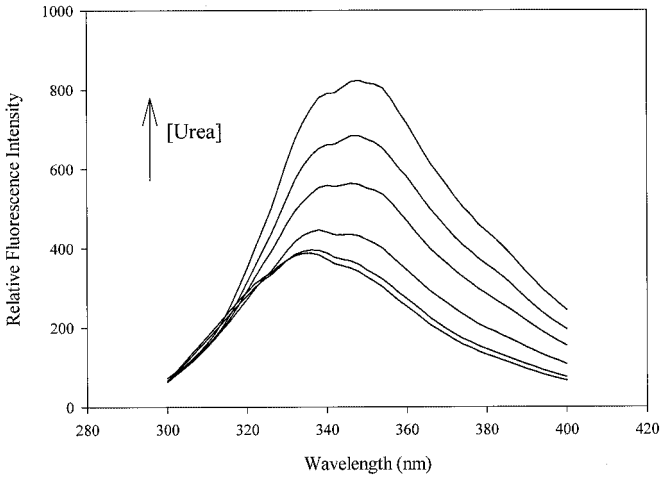

FIG. 1. **Fluorescence emission spectra of lysozyme (3 μM) in citrate buffer, pH 3, with increasing concentrations of urea (4–9 M).** The excitation wavelength was 280 nm.

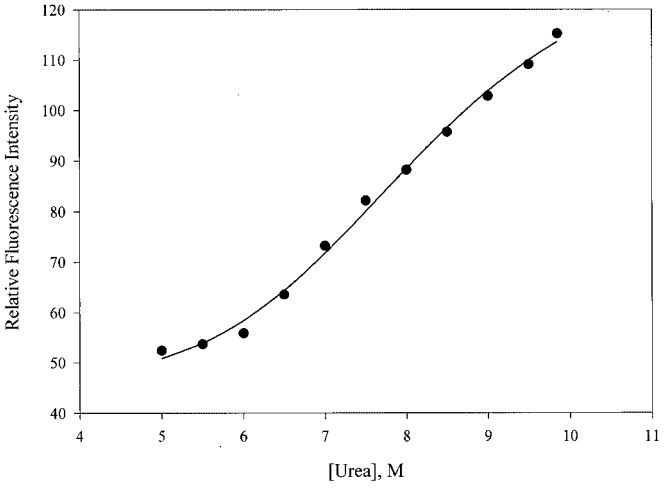

FIG. 2. **Variation of fluorescence intensity of lysozyme at 340 nm with concentration of urea in citrate buffer, pH 3, at room temperature (~25 °C).** *Closed circles* are actual data; *solid line* is the curve fit with Equation 1; $r^2 = 0.996$.

$\Delta G = 0$. The *solid lines* of Fig. 2 and Fig. 3 are the fitted curves, and the calculation returns the baseline (y_0) and maximum (y_{max}) values of the parameters. This was necessary, because the protein was not denatured completely even at the highest concentrations of denaturant that were used. This was particularly the case with urea and is consistent with the literature [3]. From the measured parameters and their calculated baseline and maximum values, the fraction of protein in the denatured state at any denaturant concentration may be calculated, assuming a two-state model [3], shown in Equation 2.

$$f_D = \frac{y - y_0}{y_{max} - y_0} \qquad \text{(Eq. 2)}$$

The apparent equilibrium constant, K_D, for the denaturation process may then be calculated as shown in Equation 3.

$$K_D = \frac{f_D}{1 - f_D} \qquad \text{(Eq. 3)}$$

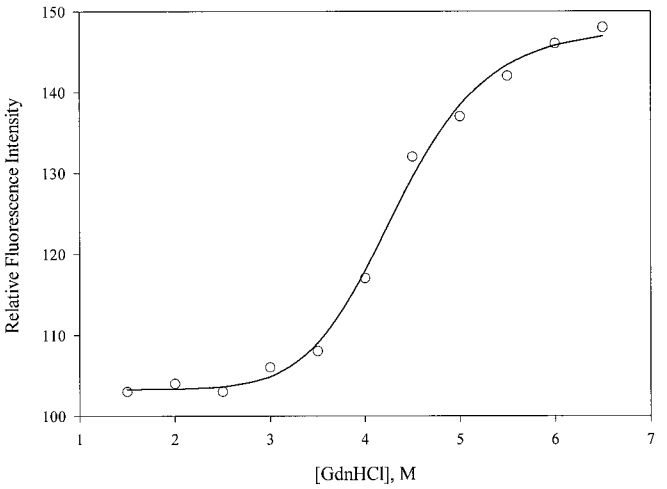

FIG. 3. **Variation of fluorescence intensity of lysozyme at 340 nm with concentration of guanidine hydrochloride in Tris buffer, pH 7, at room temperature (~25 °C).** *Open circles* are actual data; *solid line* is the curve fit with Equation 1; $r^2 = 0.995$

From K_D, the free energy change may be calculated from $\Delta G_D = -RT\ln K_D$. From these ΔG values, the ΔG value at zero denaturant concentration may be obtained using the linear extrapolation method [4]. Fig. 4 shows these results based on the data from Fig. 2 and Fig. 3. As can be seen, the experiments yield about the same extrapolated value (17–21 kJ mol^{-1}) for the free energy of denaturation of lysozyme in water. The mean value obtained by a group of 12–15 students in the biochemistry laboratory was 19.4 $\pm$ 2.3 kJ mol^{-1}.

DISCUSSION

An assumption made in this experiment is that at any concentration of the denaturant, lysozyme will exist in only two states, the native (*N*) and completely unfolded or denatured (*D*) states. These two states are assumed to be in equilibrium, as shown in Scheme I.

$$N \underset{}{\overset{K_D}{\rightleftharpoons}} D$$

SCHEME I

As the concentration of denaturant increases, the relative concentration of the denatured state should increase. Although the two-state model for lysozyme has not been proven unequivocally, strong evidence suggests that it is valid [5, 6].

Lysozyme, like most globular proteins, contains aromatic amino acids. These amino acids are known to be fluorescent, but tryptophan fluorescence dominates usually the fluorescence of the macromolecule. Furthermore, when lysozyme is denatured, the exposure of tryptophan residues in the hydrophobic core of the molecule results in a relative fluorescence increase (Fig. 1). The relative fluorescence intensity may then be used as a measure of the extent of unfolding of the protein. The fluorescence spectrum of the protein actually shifts somewhat to longer wavelengths as the concentration of the denaturant increases. However, the fluorescence band is relatively broad, and our selection of an analysis wavelength that is

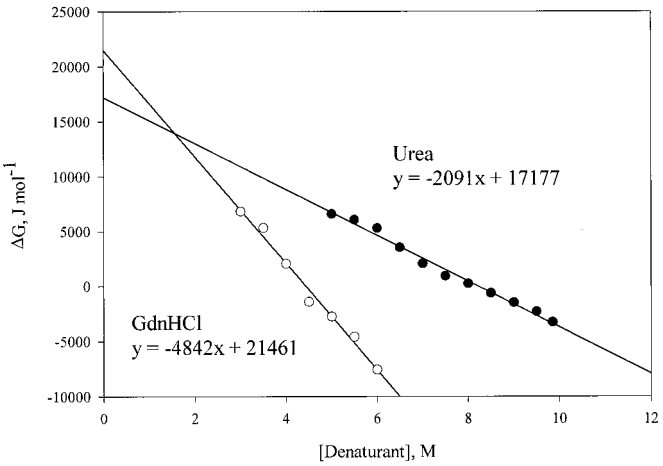

FIG. 4. **Variation of ΔG of denaturation of lysozyme with denaturant concentration.** Conditions are those used for Fig. 2 and Fig. 3.

centered in the spectrum yielded satisfactory results. The values of ΔG obtained using the two different denaturants are in reasonable agreement and are similar to literature values for lysozyme obtained using other methods [3, 7].

It is essential that the samples reach equilibrium prior to the acquisition of data. If these experiments are to be done in one laboratory period, then the best procedure is for the students to prepare the samples for fluorescence measurements at the beginning of the period. Then while these samples are equilibrating the instructor can provide information and discussion on the theory and important practical considerations for the lab. We have found that the samples reach equilibrium after approximately 1 h, but 1.5–2 h is better. The students could also determine this for themselves by following the increase in fluorescence as a function of time, but equipment limitations will preclude this for a large class. For a class of 15–20 students, we divide them into groups of two and assign one of the two denaturants to each group.

The data points used in the linear extrapolation plot normally are only those from the transition zone of the plot of fluorescence intensity *versus* denaturant concentration, *i.e.* data near 0 and 100% denaturation are usually excluded. Because of the curve fitting procedure that is used, the use of the calculated baseline fluorescence of the native protein will result in negative and/or undefined values of subsequent parameters for data points with intensities below the baseline value. Research has shown that these baselines are not always flat, and a more involved extrapolation procedure has been used to obtain the baseline values [5]. However, we have found that the curve fitting analysis we have used is much simpler and results in reasonable values for ΔG.

Ideally, the extrapolated values for ΔG of denaturation in water using the two different denaturants should be the same. However, rather large errors (± 15%) in these values should be expected because of the extrapolation method used and because the values are very sensitive to the curve fitting results [3]. Even with these caveats, however, we have found that reasonable values for ΔG are obtained using as few as six-eight data points in the transition zone. The main purpose of the laboratory is to demonstrate how the thermodynamics of this important process may be studied. For discussion of theoretical and experimental aspects of protein denaturation, the reader is referred to the review by Pace [3].

Results on the thermodynamics of denaturation of lysozyme are known to be sensitive to pH [8]. The two buffers that we chose were those that provided the most consistently reproducible results for this experiment.

This experiment provides the opportunity for discussion and reinforcement of important concepts concerning protein folding. The students determine experimentally that the unfolding process is endergonic and thus may conclude that the reverse process of folding is thermodynamically favorable. In the lab report the students should be asked to discuss the various entropic and enthalpic factors that contribute to the free energy of folding. Information on these factors may be obtained conveniently from most comprehensive textbooks of biochemistry [9–11].

CONCLUSIONS

A simple experiment to measure the free energy of denaturation of lysozyme is described. The experiment provides the opportunity for students to use the important physical method of fluorescence spectroscopy to study an important concept in protein biochemistry. The experiment provides for reinforcement and expansion of important concepts from the lecture course. The experiment is appropriate for advanced laboratory courses in chemistry, biochemistry, and biophysics.

NOTES

It is not necessary that the protein concentration be the same as that used in our experiments, because in the unfolding process the number of molecules does not change, and thus the free energy of denaturation should be approximately independent of the protein concentration. The 3 μM lysozyme concentration that we used provided good spectra using our instrument and parameters.

The concentrations of the stock solutions of urea and guanidine hydrochloride are near the solubility limits of these denaturants, and because of practical problems that sometimes occur in the preparation of such concentrated solutions, we have determined the actual concentration of the stock solutions by measuring the refractive index. However, we have found that the error in the concentration was never more than 5% and would suggest that the use of the nominal concentration of the stock solution is satisfactory for the purposes of instruction. The error in concentration affects only the parameter *c* in the curve fit. This has the result of changing the slope of the linear extrapolation but not the intercept.

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