Measurement of Lysozyme Gibbs Free Energy via Chemical Denaturation

Summary

- The Gibbs Free Energy of unfolding ($\Delta G°$) and midpoint of inflection denaturation concentration (C_m) are measured for the globular protein lysozyme (acetate buffer, pH 5.2)
- Chemical denaturation was used to unfold the protein and the change in fluorescence spectrum was measured with the SUPR-CM fluorescence plate reader
- The ratio of intensities at 354 nm and 338 nm was used to chart the unfolding process as the denaturant concentration was increased
- Fitting of a two-state function produced a Gibbs Free Energy and midpoint of inflection values with low errors and that align with literature values.

Applied Innovations in Protein Characterisation

Introduction

The change in Gibbs energy ΔG indicates whether a chemical reaction/process will occur spontaneously or not, with values equal to zero indicating that the system has reached equilibrium.

The unfolding of a protein will typically have a positive ΔG as energy needs to be applied to unfold the protein. However, the value of ΔG varies for each protein and the solution environment that protein is in. Comparing the ΔG values between different proteins and the solvent solutions provides a means to assess which proteins are more stable.

This is particularly useful within Antibody Engineering and Formulations as ΔG values can be compared between different antibody constructs or formulations so that only the most stable proceed to the next stage of development.

A common approach to assessing protein stability is thermal denaturation, where the protein is heated, and the unfolding process monitored via calorimetric (like Differential Scanning Calorimetry) or optical techniques like fluorescence (as with Differential Scanning Fluorimetry). However, thermal measurements are often irreversible, non-equilibrium measurements limiting the accuracy of Gibbs free energy measurements.[1]

An alternative approach is isothermal chemical denaturation (ICD) which uses chaotropic agents (like Urea and Guanidine Hydrochloride) to unfold the protein. The advantage with ICD is that measurements can be done at equilibrium and with new systems like the SUPR-CM, measurements can be performed rapidly and with low sample volumes.

In this technical note, the fundamentals of ICD will be illustrated via measurement of the Gibbs Free Energy of lysozyme, using the SUPR-CM fluorescence plate reader.

Method

Chemicals and Stock Solutions

Sodium acetate and acetic acid (glacial) where acquired form Sigma Aldrich. Guanidine hydrochloride (GuHCl) was supplied from Carl Roth and lysozyme from egg white was acquired from Roche.

Acetate buffer was prepared at 0.1 M concentration by mixing a 0.07 M stock of sodium acetate with a 0.03 M stock of acetic acid. The pH was measured and adjusted to 5.2 with sodium hydroxide. A 7 M stock solution of GuHCl was prepared by dissolving the GuHCl in acetate buffer, dropwise, until the crystals where completely dissolved. The pH was adjusted back to 5.2 before topping off the volume with acetate buffer. The lysozyme

[1] Svilenov H, Markoja U, Winter G. Isothermal chemical denaturation as a complementary tool to overcome limitations of thermal differential scanning fluorimetry in predicting physical stability of protein formulations. European Journal of Pharmaceutics and Biopharmaceutics. 125, 106–113. 2018.

stock was prepared at 1.0 $^{mg}/_{ml}$ concentration in acetate buffer and mixed using a vortex mixer.

Preparation of Microplate

A 384-well microplate was prepared using the Mantis® Liquid Handler (Formulatrix®). 24 denaturant concentrations from 0 M to 6 M were used such that the concentration increased across the row of the microplate. Final well volume was maintained at 50 µl and the protein concentration after dispensing was 140 $^{µg}/_{ml}$. Samples were prepared in triplicate and after dispensing, the microplate was incubated for 24 hours at 20°C.

Measurement and Analysis

The 384-well microplate was measured using the SUPR-CM fluorescence plate reader. The well measurement time was set to 250 ms which resulted in all 72 sample wells being measured within 20 secs. Full fluorescence spectra (**figure 1**) where used to calculate the ratio of intensities at 354 nm and 338 nm. A bandpass value of 5 nm was used to average the intensity values at 338 nm and 354 nm to account for instrumental noise. The ratio of intensities values were plotted against denaturant concentration to produces denaturation curves (**figure 2**).

The denaturation curve data was fitted to a two-state function to determine the Gibbs Free Energy of unfolding value. The midpoint of inflection was calculated via the linear interpolation method. Initial parameter values were provided in order to ensure the correct global minimum was reached during the fitting process.

Results

Fluorescence Intensity Spectra

The introduction of the denaturant GuHCl has resulted in a change of the fluorescence spectra of lysozyme. This change (seen in **figure 1**) is the result of the protein unfolding and exposing the internal tryptophan molecules to a hydrophilic microenvironment.

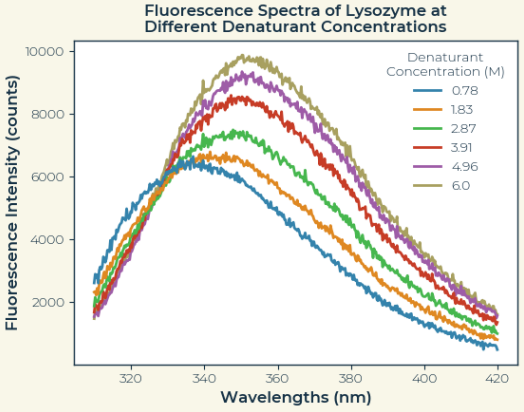

Figure 1 – Fluorescence spectra of Lysozyme at different GuHCl concentrations. The redshift in the spectrum can be used to chart the unfolding of the protein.

In **figure 1** the fluorescence spectra change in two significant ways, as the concentration of denaturant increases. The first change is the increase in fluorescence intensity which is evidence that more of the excitation energy has not been transferred internally or to another amino acid, within the lysozyme structure. This is expected for the unfolded state as the tryptophan molecules will be spaced out and no longer within the internal structure of the protein.[1]

The second change is the transition in fluorescence maximum from ~ 330 nm to ~ 350 nm, commonly referred to as a redshift in fluorescence maximum. This behaviour is expected of an unfolding protein as the microenvironment of the internal tryptophan molecules changes from hydrophobic to hydrophilic as the tryptophan molecules are exposed to more of the solvent environment.[1]

Denaturation Curve and Two-State Fitting

The unfolding process present in **figure 1** was quantified by taking the ratio of intensities at 354 nm and 338 nm and plotting the values against denaturant concentration as shown in **figure 2**. The

[1] Kurtin W E, Lee J M. The free energy of denaturation of lysozyme: An undergraduate experiment in biophysical chemistry. Biochemistry and Molecular Biology Education. 30(4), 244–247. 2002.

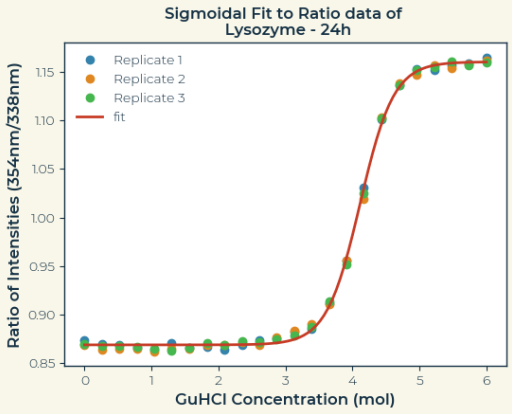

Figure 2 – The ratio of fluorescence intensities against denaturant concentration. The trendline for a two-state fit is plotted over the ratio data and shows good agreement.

wavelengths 354 nm and 338 nm were chosen as this is where the fluorescence maxima where for the folded and unfolded states (0 M and 6 M). Alternative methods for quantifying the spectral shift included within the software are the barycentric mean and the intensity value at a single wavelength.

The ratio of intensities data was fitted to the two-state function:

$$y = \frac{(F + m_F x) + e^{-\frac{\Delta G^0}{RT}}(U + m_U x)}{1 + e^{-\frac{\Delta G^0}{RT}}}$$

where ***y*** is the ratio of intensities value, ***x*** is the denaturant concentration, ***R*** is the molar gas constant, ***T*** is the incubation temperature, ***F*** and ***U*** are the *y*-intercepts for the folded and unfolded plateaus respectfully. The gradients for the folded and unfolded plateaus are m_F and m_U respectfully. Finally, ΔG° is the Gibbs Free Energy of unfolding which is equal to:

$$\Delta G^0 = m_g(C_m - x)$$

Where C_m is the denaturant concentration at the midpoint of inflection (where half the proteins are in the unfolded state) and m_g is the change in Free Energy of unfolding with respect to the denaturant concentration (the gradient at the midpoint of inflection).

Table 1 – Fitted parameters and derived errors from fitting a two-state function to the ratio of intensity data of **figure 2**.

Fitting Parameter	Fitted Value	Error
F	0.87	0.001
U	1.16	0.001
m_F (M^{-1})	0	0 (fixed)
m_U (M^{-1})	0	0 (fixed)
C_m (M)	4.12	0.006
m_g (kJ mol^{-1} M^{-1})	9.71	0.20
ΔG° (kJ mol^{-1})	39.95	0.83

The trendline (from fitting **equation 1**) is plotted with the ratio of intensities data in **figure 2** and show good agreement with the measured data. The fitted parameter values are shown in **table 1** along with their derived error value.

The pre and post transition baseline gradients of figure 2 show no significant change with concentration and where therefore fix at 0 M^{-1}. The rest of the variables where left free.

The results from fitting a two-state function visually show good agreement with the ratio of intensities data and the derived errors are low with the most significant being the Gibbs Free Energy error percentage at ~ 2%.

Comparing the fitted Gibbs Free Energy value of table 1 with literature values shows good agreement with Ahmad et. al. who reported Gibbs Free Energy values for lysozyme of ~ (37 – 38) kJ mol^{-1}.[1,2] Differences in exact pH value, buffer type and lysozyme batch can account for the (1-2) kJ mol^{-1} difference between the values reported by Ahmad et al. and the measured value of **table 1**.

[1] Ahmad F, Bigelow CC. Estimation of the Free Energy of Stabilization of Ribonuclease A, Lysozyme, a-Lactalbumin, and Myoglobin. The Journal of Biological Chemistry. 257 (21), 2935–2938. 1982.

[2] Ahmad F, Contaxis CC, Bigelow CC. Free Energy Changes in Lysozyme Denaturation. Journal of Biological Chemistry. 258 (13), 7960–7963. 1983.

Conclusion

Fluorescence measurements of lysozyme at different concentrations of the denaturant GuHCl showed spectral changes that are consistent with the unfolding of the protein. The spectral changes were quantified by calculating the ratio of intensities at 354 nm and 338 nm and plotting them against denaturant concentration. A two-state function was fitted to the denaturation curve data and the Gibbs Free Energy values determined. Fitting shows good agreement with experimental data and the derived errors from fitting a two-state function are below 3%. Comparing the determined Gibbs Free Energy value with the literature values shows close agreement given variance in solution conditions like pH and buffer selection. In addition, because the SUPR-CM can fit to the full dataset, the C_m error is significantly lower (~0.15%), allowing for a high sensitivity when rank ordering related conditions or proteins for stability.