

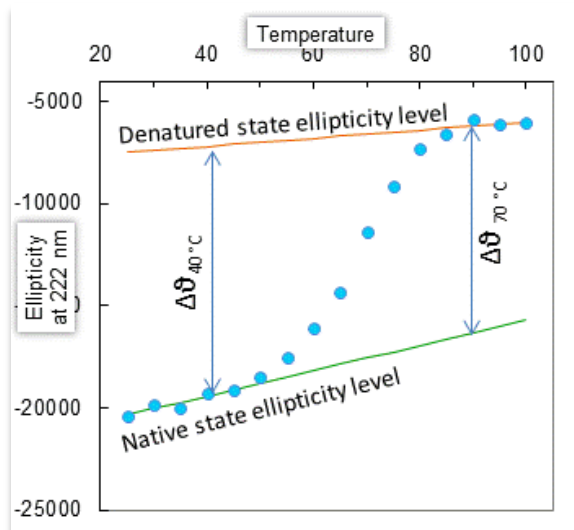
# Using optical methods for assessing thermodynamics of protein stability

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This approach can be applied to any observable, sensitive to the changes in protein conformation. It can be fluorescence intensity (or maximum), CD signal, size of the molecule or anything else.

## Determination of the Gibbs free energy

Suppose you have a helical protein with the typical spectral minimum at 222 nm. You want to follow the changes in helicity upon heating and you obtain the following experimental data:



A visual observation tells you that the native state, N, characterized by more negative ellipticity values, is populated at 20 to about 40 °C. The denaturation transition appears to be completed at 90 °C, where the signal intensity is less negative and thus represents the denatured state of the protein, D. Assuming that ellipticity of both native and denatured states is a linear function of temperature we can draw two baselines (the pre-transitional baseline is green and the post-transitional one is orange). The difference between these lines, shown by the arrows, reflects the change in ellipticity at 222 nm upon transition from N to D and its value is  $\Delta\theta = \theta_D - \theta_N$ . Notice that the value of  $\Delta\theta$  is not the same at different temperatures, since the pre- and post-transitional baselines are not parallel.

The denaturation process can be expressed as an elementary chemical reaction:



with an equilibrium constant K:

$$K = \frac{[D]}{[N]}$$

The above equation can be expressed through populations (or fraction) of the native and denatured molecules,  $P_N$  and  $P_D$ :

$$P_N = \frac{[N]}{C}; P_D = \frac{[D]}{C}$$

Where C is the total concentration of the protein:

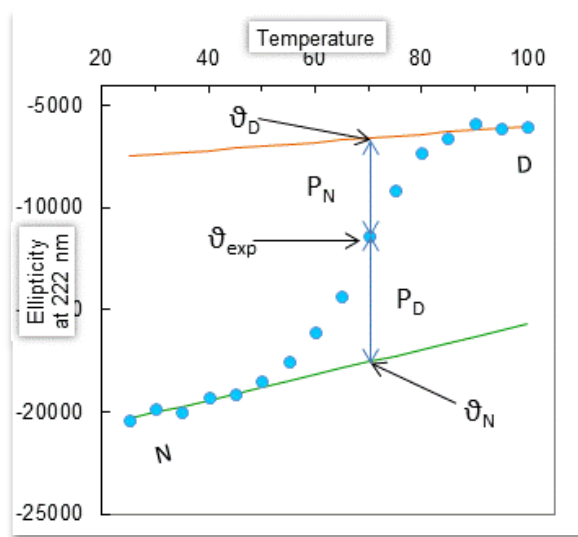
$$C = [N] + [D]$$

Then the equilibrium constant becomes:

$$K = \frac{P_D}{P_N}$$

The values for  $P_N$  and  $P_D$  at each temperature can be obtained from the experimental data. For example, at 70 °C, the experimental ellipticity value is  $\vartheta_{exp}$  and the fraction of the molecules in native and denatured states are:

$$P_N = \frac{\vartheta_{exp} - \vartheta_D}{\vartheta_N - \vartheta_D}; P_D = 1 - P_N = \frac{\vartheta_N - \vartheta_{exp}}{\vartheta_N - \vartheta_D}$$



Now we can calculate K at 70 °C

$$K = \frac{\vartheta_N - \vartheta_{exp}}{\vartheta_{exp} - \vartheta_D}$$

and at all other temperatures, where experimental data are available. When this is done, we can calculate the Gibbs free energy at all experimental temperatures using the formula:

$$\Delta G = -RT \cdot \ln K$$

where  $R = 1.987 \text{ cal}/(\text{K}\cdot\text{mol})$  is the gas constant and  $T$  is the absolute temperature (*i.e.* expressed in Kelvin). For example at  $70 \text{ }^\circ\text{C}$ ,<sup>1</sup> the ratio of  $P_D$  to  $P_N$  is 1.28 and the Gibbs free energy is  $-0.18 \text{ cal/mol}$ , the negative sign indicating that transition to D state is favorable at this temperature. At  $40 \text{ }^\circ\text{C}$  the  $\Delta G = 3.2 \text{ kcal/mol}$  and the N state is more stable than the D.

### Calculation of Enthalpy

Unlike the Gibbs free energy, the values for the enthalpy cannot be directly obtained from this type of experimental data. However, it is easily done by a primitive fitting analysis, for example in Excel. The required fitting parameters are the mid-temperature of the denaturation transition,  $T_m$ , the enthalpy of denaturation,  $\Delta H$ , and the heat-capacity change upon denaturation,  $\Delta C_p$ . Here, we assume that the unfolding reaction is a simple two-state process, *i.e.* the protein is either in the native, N, or in the denatured, D, states:



Since

$$\Delta G = \Delta H - T\Delta S$$

and

$$\Delta H = \Delta H_m + \Delta C_p \cdot (T - T_m)$$

$$\Delta S = \Delta S_m + \Delta C_p \cdot \ln\left(\frac{T}{T_m}\right)$$

then the Gibbs free energy can be expressed as

$$\Delta G = \Delta H_m + \Delta C_p \cdot (T - T_m) - T \left( \Delta S_m + \Delta C_p \cdot \ln\left(\frac{T}{T_m}\right) \right)$$

where the subscript “m” designates the values of thermodynamic parameters at the melting temperature. Choosing  $T_m$  as a reference is convenient because for a two-state process this is the temperature, where  $\Delta G = 0$ .<sup>2</sup> In addition, at this temperature  $\Delta H_m = T\Delta S_m$ , which allows elimination of the entropy from the above equation. Thus,

$$\Delta G = \Delta H_m + \Delta C_p \cdot (T - T_m) - T \left( \frac{\Delta H_m}{T_m} + \Delta C_p \cdot \ln\left(\frac{T}{T_m}\right) \right)$$

Note that we apply this formula to calculate theoretical values of the Gibbs free energy, using the fitting parameters  $T_m$ ,  $\Delta H$ , and  $\Delta C_p$ . To compare the calculated values with the experimental data we

<sup>1</sup> The data for this example are given in the Appendix.

<sup>2</sup> Follows from the notion that at  $T_m$ , *i.e.* at the mid-point of the reaction,  $[N]=[D]$ , hence  $K=1$ .

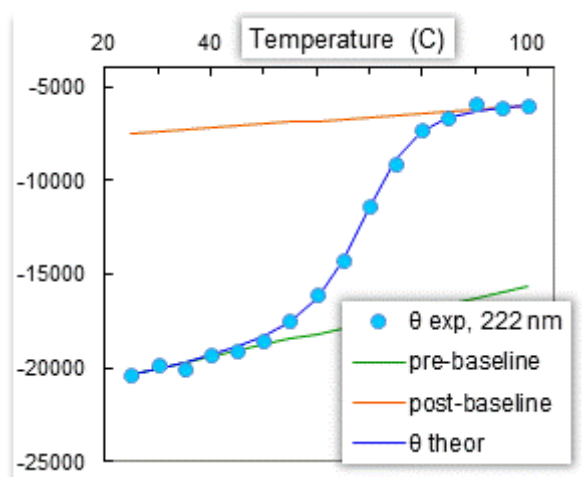
need to obtain the theoretical values for the ellipticity. This is done by rearranging the formulas, used at the beginning of this note. First we calculate the equilibrium constant,  $K_{theor}$ , where the subscript “theor” is used to distinguish the values of  $K$  calculated on the basis of the fitting parameters from those obtained directly from the data:

$$K_{theor} = e^{-\Delta G/RT}$$

Then we calculate the ellipticity values:

$$\vartheta_{theor} = \frac{\vartheta_N + K_{theor}\vartheta_D}{K_{theor} + 1}$$

Now, it’s time to use Excel’s Solver to fit the experimental values of ellipticity to the theoretical ones. Using the data from the example (see the Appendix) we obtain  $T_m = 69$  °C,  $\Delta H = 44.8$  kcal/mol, and  $\Delta C_p = 0.7$  kcal/mol and fit is shown in the graph below.



## Appendix

An example of experimental CD data, obtained during the heat denaturation of bovine insulin. The baselines are calculated to fit the first 5 and the last 3 experimental points.

T, C	$\theta$ exp, 222 nm	pre-baseline	post-baseline
25	-20400	-20340	-7500
30	-19800	-20030	-7400
35	-20000	-19720	-7300
40	-19300	-19410	-7200
45	-19101	-19101	-7100
50	-18500	-18791	-7000
55	-17500	-18481	-6900
60	-16100	-18171	-6800
65	-14300	-17861	-6700
70	-11400	-17552	-6600
75	-9100	-17242	-6500
80	-7300	-16932	-6400
85	-6600	-16622	-6300
90	-5900	-16312	-6200
95	-6100	-16003	-6100
100	-6000	-15693	-6000