Thermal Denaturation of Proteins and Chemical Equilibrium

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Abstract The thermal denaturation of proteins is considered as a process by means of which chemical equilibrium can be introduced to undergraduate students of Chemistry related curricula. In this approach chemical potential $\mu$, Gibbs energy $G$, degree of advancement $\xi$ and Le Châtelier principle are integrated with chemical equilibrium. With reference to $\alpha$-Chymotrypsinogen A as a test case, the process is discussed in terms of a simplified two-state model. The activity is addressed to physical chemistry students in combination with computer-aided work mainly involving storage/manipulation of large data sets and plot preparation.

Keywords: first-year undergraduate/physical chemistry; computer-aided learning; chemical equilibrium

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1. Introduction

Chemical equilibrium is a central topic in physical chemistry courses. Physical chemistry textbooks introduce chemical equilibrium in terms of Gibbs free energy $G$ vs. degree of the reaction advancement $\xi$. [1-7] Strictly connected to chemical equilibrium, Le Châtelier principle is a guide to predict qualitatively the response of the system at equilibrium to changes in the external conditions. [8] Over the years, several studies on protein denaturation have been reported [9-17] and some of them discussed in terms of equilibrium reactions. [10,13,14,15,16] While, in fact, for most proteins the denaturation occurs irreversibly, a number of proteins, including trypsin, are capable of reverting the process. [18,19] In particular, in the case of $\alpha$-Chymotrypsinogen A, whose structure in the active form has been reported [20] and is sketched in Figure 1, it has been found that, after increasing the temperature step by step from 20°C to 85°C, renaturation occurs cooling to 20°C. [14]

This allows us to discuss the thermal unfolding/folding of $\alpha$-Chymotrypsinogen A within the thermodynamic framework of chemical equilibrium. [3,5] Here we wish to report on our teaching experience in the classroom aimed at facilitating the discussion of results on the $\alpha$-Chymotrypsinogen A thermal denaturation. [14] In our opinion this activity greatly favors the student perception of chemical equilibrium and for students searching for a rigorous approach to the subject provides an excellent example of chemical equilibrium fully amenable to analytical treatment.

2. Materials and Methods

$\alpha$-Chymotrypsinogen A was purchased from Sigma and used without further purification. Following the experimental protocol [14], 2-5 mg of the protein were added to 1 mL of a buffer solution, 10 mM glycine and 10 mM NaCl, at pH = 3.0. The solution was diluted with buffer in order to have a protein concentration $\approx 0.3$ mg/mL. The absorption spectra of the $\alpha$-Chymotrypsinogen A solution were measured as a function of the temperature on a Cary 5 spectrophotometer in the spectral range 320 – 240 nm. The temperature of the absorption cell was controlled with a closed-circuit Haake L thermostat working between room temperature and 75°C.
3. Results

The spectral data acquired through absorption measurements in the range 20 °C - 75 °C have been collected and the equilibrium constants between 50 °C and 60 °C have been calculated. In Figure 2 the temperature dependence of UV spectra of α-Chymotrypsinogen A at selected temperatures is reported, showing the intensity decrease of the band maximum at ≈ 281 nm and the isosbestic point at ≈ 269 nm.

![Figure 2](image-url)

Figure 2. UV absorption spectra of α-Chymotrypsinogen A in aqueous dilute solution at selected temperatures between 20 °C and 75 °C. Optical path, 1 cm. The arrow indicates the spectral sequence with increasing temperature.

As reported [10,14], the equilibrium constants K are determined from the observed absorbance maxima A(T) and the corresponding absorbances A_N(T) and A_D(T) of the native and denatured protein, respectively, as \[ \frac{[A_N(T) - A(T)]}{[A(T) - A_D(T)]} \]. In the inset of Figure 3, the A_N(T) and A_D(T) values are obtained by linear regression extending the intensity behaviour with temperature of the native and denatured form in the temperature interval, ≈ 10 °C, where the thermal unfolding is completed. The equilibrium constants are plotted as a function of the temperature in Figure 3.

![Figure 3](image-url)

Figure 3. Equilibrium constants K calculated from the observed absorbance maxima A(T) and the corresponding absorbances A_N(T) and A_D(T) as explained in the text. In the inset A_N(T) and A_D(T) values are obtained in the 50 °C – 60 °C temperature range by linear regression.

4. Discussion

The denaturation process of α-Chymotrypsinogen A, assumed to occur in the solution volume V at temperature T and pressure p = 1 bar, may be discussed by means of the following two-state model [14]

\[ N \overset{K}{\rightleftharpoons} D \] (1)

where N and D are the native and denatured forms of the protein, respectively, with unit stoichiometric coefficients. At constant temperature and pressure the reaction solution goes spontaneously toward the equilibrium state, where reactant (N) and product (D) concentrations are unaltered in time. As the solution approaches equilibrium, the Gibbs free energy G decreases and at equilibrium is minimum or, in other words, the Gibbs free energy of the reactant, G_N, and of the product, G_D, are equal. The effect on G of the irreversible changes occurring in the solution due to denaturation is described by means of the chemical potentials of N and D. At mechanical and thermal equilibrium the free energy G is given for any composition of the two species by the expression [1,7,21]

\[ G = n_N \mu_N + n_D \mu_D \] (2)

where \( n_N, n_D \) are the number of moles and \( \mu_N, \mu_D \) are the chemical potentials. Having initially \( n_0 \) moles of the native species and being unitary the stoichiometric coefficients, \( n_N \) and \( n_D \) may be expressed in terms of the degree of process advancement \( \xi \) as

\[ n_N = n_0 - \xi \quad n_D = \xi \] (3)

where \( \xi \) units are mol. As the composition of the solution varies, the chemical potentials change accordingly, depending on the activity of the native and denatured forms, by

\[ \mu_i = \mu_i^0 + RT \ln a_i \quad i = N, D \] (4)

where \( \mu_i^0 \) is the standard potential at temperature T and pressure p = 1 bar and \( a_i \) is the activity of the i-th species. For a sufficiently dilute solution, \( a_i \) may be approximated [5] to the ratio of molar concentrations \( c/c^0 \), with \( c^0 = 1 \) M. Substituting eqs. (3) and (4) into eq. (2) and grouping the RT, the result is

\[ G = G_0 + \xi \Delta G^0 + RT \left\{ \left( n_0 - \xi \right) \ln \left( \frac{n_0 - \xi}{n_0} \right) + \xi \ln \left( \frac{\xi}{n_0} \right) \right\} \] (5)

where \( G_0 = n_0 \left[ \left( \mu_N^0 + RT \ln \left( n_0/V \right) \right)c^0 \right] \) is the free energy of \( n_0 \) moles of the native species in the volume V, \( \Delta G^0 = \mu_D^0 - \mu_N^0 \) is the so-called standard free energy of denaturation, i.e., the rate of change of G with respect to \( \xi \) [22,23,24], and \( RT \left( n_0 \xi \right) \ln \left( \frac{n_0 \xi}{n_0} \right) + \xi \ln \left( \frac{\xi}{n_0} \right) \) is the free energy of mixing of the two species. [21] Eq. (5) extends the expression valid for ideal gas mixtures [1,7,21] to the case of dissolved species in dilute solution. The G_0 term of this equation is independent of \( \xi \). The linear term, \( \xi \Delta G^0 \), would lead, in absence of the third term to the total conversion from the species of highest to that of lowest chemical potential. The equilibrium state, where both N and D are present, is reached since the third term further lowers G with respect to the linear profile. Alternatively,
the irreversible course of the reaction toward equilibrium may be followed considering \( \frac{\partial G}{\partial \xi} \)\( _{T,p} \), i.e., by differentiating \( G \) with respect to \( \xi \) at constant \( p \) and \( T \).

\[
\frac{(G - G_0)}{J} < T < T_d \\
\Delta G^0 > 0 \\
T = T_d \\
\Delta G^0 = 0 \\
T > T_d \\
\Delta G^0 < 0
\]

\[
\frac{\partial G}{\partial \xi} \bigg|_{p,T} / (J \text{ mol}^{-1})
\]

\[\xi/\text{mol}\]

4.1. Classroom Activity

\((G - G_0)\) and \(\frac{\partial G}{\partial \xi} \)\( _{T,p} \) vs. \( \xi \) graphs are generated in Figure 4 according to eqs. (5) and (6), respectively, starting with \( n_0 = 1 \) mol of native protein in the solution volume \( V \), at three (or more) temperatures; one is the denaturation temperature \( T_d \) and the remaining are distributed below and above \( T_d \). The denaturation temperature \( T_d \) is defined \([14]\) as the temperature at which protein at equilibrium is half native and half denatured, i.e., \( \xi_{eq} = n_0 - \xi_{eq} \) and \( c_{eq,N} = c_{D,eq} \). At this temperature \( \Delta G^0 = 0 \) and \( K = 1 \). The volume \( V \) is assumed sufficiently large so that activities may be approximated to concentrations for both protein forms. Typically, data are stored on a few columns of a worksheet:

1. in the first column, the X-axis, the \( \xi \) values between 0 and 1 mol are given with 0.05 step;
2. in the second and third column the \((G - G_0)\) and \(\frac{\partial G}{\partial \xi} \)\( _{T,p} \) values are tabulated according to eqs. (5) and (6), respectively.

Alternatively, the total \((G - G_0)\) result is resolved into two contributions, the linear term in \( \xi \) and the nonlinear (logarithmic) term. Then the \((G - G_0)\) values fill the fourth column using the second column for the linear term in \( \xi \) and the third the logarithmic term. Splitting \((G - G_0)\) into two contributions clarifies a point, not often mentioned in textbooks, i.e., the purely chemical term \( \xi \Delta G^0 \) would lead to the complete conversion from one protein form to the other and only mixing is responsible for the occurrence of the two-component equilibrium solution. \([3]\). The three graphs of Figure 4 result from the equilibrium constants of Figure 3 on \( \alpha \)-Chymotrypsinogen A, i.e., \( T_d = (273+55) \) K, \( K = 1; T_{below} = (T_d - 2) \) K, \( K = 1/3; T_{upper} = (T_d + 2) \) K, \( K = 3 \). Since denaturation is known to be an endothermic \((\Delta H^o > 0)\) and disordering \((\Delta S^o > 0)\) process \([3, 5]\), at \( T < T_d \) \( \Delta G^o > 0 \) (K < 1) and at \( T > T_d \) \( \Delta G^o < 0 \) (K > 1). As the temperature increases, the composition at equilibrium shifts from native-rich to denatured-rich, crossing the \( c_{N,eq} = 1 \) mol equality at the denaturation temperature. This can be seen from Figure 4 looking at the \( \xi \) value of the \((G - G_0)\) minimum (on the left) or of the zero value of the \(\frac{\partial G}{\partial \xi} \)\( _{T,p} \) derivative (on the right).

4.2. Le Châtelier Principle Exemplified

For the two-state model of eq. (1), it is rewarding also to view the equilibrium constant \( K = c_{D,eq}/c_{N,eq} \) as the proportionality factor relating the concentrations of the native and denatured species at equilibrium, i.e., \( c_{D,eq} = K \cdot c_{N,eq} \). Therefore, in a plot \( c_D \) vs. \( c_N \) the equilibrium states lie on a straight line graph of slope \( K \), which in Figure 5 is 1 for the particular case presented here at the protein’s denaturation temperature.

\[\text{C}_D \text{ vs. } \text{C}_N\]

**Figure 4.** Gibbs free-energy of protein denaturation (G-G0, left) and derivative \(\frac{\partial G}{\partial \xi} \)\( _{T,p} \), (right) as a function of the degree of advancement \( \xi \) at the denaturation temperature \( T_d \) (middle), below (upper) and above (lower) \( T_d \) according to eqs. (5) and (6), respectively. The calculation refers to the \( \alpha \)-Chymotrypsinogen A denaturation with \( T_d = (273+55) \) K, \( K = 1; T_{below} = (T_d - 2) \) K, \( K = 1/3; T_{upper} = (T_d + 2) \) K, \( K = 3 \). Since denaturation is known to be an endothermic \((\Delta H^o > 0)\) and disordering \((\Delta S^o > 0)\) process \([3, 5]\), at \( T < T_d \) \( \Delta G^o > 0 \) (K < 1) and at \( T > T_d \) \( \Delta G^o < 0 \) (K > 1). As the temperature increases, the composition at equilibrium shifts from native-rich to denatured-rich, crossing the \( c_{N,eq} = 1 \) mol equality at the denaturation temperature. This can be seen from Figure 4 looking at the \( \xi \) value of the \((G - G_0)\) minimum (on the left) or of the zero value of the \(\frac{\partial G}{\partial \xi} \)\( _{T,p} \) derivative (on the right).

**Figure 5.** Effect of concentration change on the protein denaturation equilibrium at \( T_d \). Lines AA’ and AA”, additions of native and denatured protein, respectively; lines A’B and A”B, paths to reach the new equilibrium state. The temperature is kept constant during the two additions.

This diagram offers a vivid application of the Le Châtelier principle. \([8]\) All the equilibrium states of the
two-component solution are points on the straight line; any other point does not correspond to an equilibrium state. What happens when \(2 \times 10^5\) moles of native protein are added to the dilute equilibrium solution at point A (\(c_{\text{NA}} = c_{\text{DA}} = 1 \times 10^{-5}\) M, see Figure 5)? Soon after the addition, the system reaches point A’ which, not being on the straight line, corresponds to a non-equilibrium state. The system shifts spontaneously toward equilibrium minimizing, according to the Le Châtelier principle, the effect of the disturbance. Since for each mole of native protein that unfolds at the denaturation temperature \(T_d\), one mole of denatured protein appears at equilibrium, and the concentration of N decreases by the same amount that the concentration of D increases (i.e., \(-\Delta c_N = +\Delta c_D\)), the path moving toward equilibrium is a straight line with slope \((-1)\) equal to \(+\Delta c_D/-\Delta c_N\), intersecting the equilibrium line at point B. It is graphically evident from Figure 5 that the final concentration of native protein, \((c_{\text{NA}} + c_{\text{NA}'})/2\), is greater than the initial concentration, \(c_{\text{NA}} = 1 \times 10^{-5}\) M, but less than \(c_{\text{NA}} = 3 \times 10^{-5}\) M, the concentration that would have been if no response from the system had occurred. It is equally clear from Figure 5 that, given the same addition of denatured protein (point A’), the system will move to the same equilibrium state, point B, reached before and that the changes in concentration for native and denatured forms are the same but of opposite signs. In the second case the denatured protein is assumed to undergo renaturation to form more native protein, which may not be necessarily true for a large part of actual proteins. It is left to the students as a geometrical exercise to discuss the more general case, \(K \neq 1\).

The effect of a temperature change on the denaturation equilibrium can be also visualized on the graphical plot of Figure 6.

![Figure 6](image)

Figure 6. Effect of temperature change on the protein denaturation equilibrium at \(T_d\). The equilibrium states at \(T_d\) and \(T'\) (with \(T' > T_d\)) are represented on the two straight lines, respectively. For the denaturation process (\(\Delta H^\circ > 0\)) the temperature increase from \(T_d\) to \(T'\) corresponds to the counterclockwise rotation

Each straight line starting from the origin represents the whole group of equilibrium states sharing the same equilibrium constant at a given temperature and the set of such straight lines in the first quadrant is in a one-to-one correspondence with the set of equilibrium constants. For an endothermic reaction the temperature increase is graphically associated with the counterclockwise rotation around the origin from the original to the final position of the straight line. Going from any point of the equilibrium line at temperature \(T_d\) to a point of the equilibrium line at temperature \(T'\) (\(T' > T_d\)) along the trajectory with \((-1)\) slope, the concentration of the denatured form increases and that of the native form decreases by the same amount, as it is visualized in Figure 6.

5. Conclusions

In summary, in this study the thermal denaturation of proteins has been considered as an example of chemical equilibrium. Chemical potential \(\mu\), Gibbs energy \(G\), degree of advancement \(\xi\), two-state model constitute basic requisites to understand the chemical equilibrium of the denaturation reaction. The equilibrium state is specified looking at the minimum of the Gibbs free energy or alternatively, using the van’t Hoff equation, at the zero value of the \((\partial G/\partial \xi)_{T,P}\) derivative. Results obtained combining the analytical treatment of the process with spectroscopic data are visualized with the aid of currently used program packages. External perturbations on the denaturation equilibrium, due to variation of N and D concentrations and of temperature, have been discussed on the basis of the Le Châtelier principle. Making recourse to x-y graphs the path of the reacting solution from the original to the new equilibrium state is followed so as to minimize the effect of the disturbance.

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