Fitting Thermal and Chemical Denaturation Data

Here we derive a general equation for fitting sigmoidal two-state denaturation curves to a Boltzmann distribution and then modify that expression to included specific parameters for the fitting of thermal and chemical denaturation data.

Consider a two-state denaturation equilibrium, where only the native (N) and denatured (D) states of a protein are significantly populated:

$$N \rightleftharpoons D$$

The equilibrium constant for this reaction is defined as:

$$K_{eq} = \frac{[D]}{[N]} \tag{1}$$

A standard thermodynamic identity shows that the free energy under standard conditions may be calculated from the equilibrium constant according to Equation 2, which may be rearranged to give Equation 3. Note that, for convenience, the prime indicating standard conditions is dropped from ΔG and replaced by a subscript $D-N$ to indicate that the standard free energy is that for unfolding.

$$\Delta G^{\circ} = -RT.lnK_{eq} \tag{2}$$

$$K_{eq} = exp\left(\frac{-\Delta G_{D-N}}{RT}\right) \tag{3}$$

Unfolding is studied by introduction of a perturbant (e.g. a temperature change, a pH change or an increasing concentration of denaturant) which incrementally decreases the stability of the native state, or increases the stability of the denatured state. Any observed spectroscopic signal that changes upon increasing perturbation may be expressed as a sum of contributions from the native and denatured states. For a given degree of perturbation, the observed signal is given by Equation 4, where fX is the fractional occupation of state X and sX is the signal from state X when $fX = 1$:

$$Observed\,Signal = fN.sN + fD.sD \tag{4}$$

Expressing fD in terms of concentration of D and N (square brackets omitted for clarity), rearranging, and substituting according to Equation 1 gives Equation 5 for fD:

$$fD = \frac{D}{D+N} \quad \Rightarrow fD = \frac{\frac{D}{N}}{\frac{D}{N}+\frac{N}{N}} \quad \Rightarrow fD = \frac{K_{eq}}{K_{eq}+1} \tag{5}$$

A similar process gives Equation 6 for fN

$$fN = \frac{1}{K_{eq} + 1} \tag{6}$$

Substituting Equations 5 and 6 into Equation 4 gives:

$$Observed\ Signal = \frac{sN + sD.K_{eq}}{1 + K_{eq}} \tag{7}$$

Expressing K_{eq} according to Equation 3 gives:

$$Observed\ Signal = \frac{sN + sD.e^{-(\frac{\Delta G}{RT})}}{1 + e^{-(\frac{\Delta G}{RT})}} \tag{8}$$

In practice, the native and denatured states tend to have sloping baselines since there are intrinsic signal changes for native and denatured states upon increasing perturbation. Expressing Equation 8 with terms for sloping baselines gives (Santoro and Bolen 1988; Clarke and Fersht 1993):

$$Observed\ Signal = \frac{(\alpha_N + \beta_N.P) + (\alpha_D + \beta_D.P).e^{-(\frac{\Delta G}{RT})}}{1 + e^{-(\frac{\Delta G}{RT})}} \tag{9}$$

Where P is the degree of perturbation, α_X is the signal from state X when $P = 0$ and β_X is the rate of change of the signal from state X with increasing P. For example, in thermal denaturation the perturbation is achieved by increasing temperature so the fitting equation becomes:

$$Observed\ Signal = \frac{(\alpha_N + \beta_N.T) + (\alpha_D + \beta_D.T).e^{-(\frac{\Delta G}{RT})}}{1 + e^{-(\frac{\Delta G}{RT})}} \tag{10}$$

Where αN is the native state signal at 0 K, βN is the slope of the native state baseline, αD is the denatured state signal at 0 K, βD is the slope of the denatured state baseline, T is the temperature in Kelvin (Centigrade + 273.15), R is the ideal gas constant ($1.987 cal.mol^{-1}.K^{-1}$) and ΔG_{D-N} is the free energy of unfolding.

To fit thermal denaturations, ΔG_{D-N} in Equation 10 can be substituted by the Equation 11, a rearrangement of the Gibbs Helmholtz relationship (Jackson and Fersht 1991; Nicholson and Scholtz 1996). This formalism expresses ΔG_{D-N} in terms of temperature, midpoint of the thermal denaturation T_m (which may be accurately determined from the data), the enthalpy of denaturation at the transition midpoint ΔH_m, and the change of heat capacity of denaturation ΔC_p.

$$\Delta G_{D-N} = \Delta H_m \left(1 - \frac{T}{T_m}\right) + \Delta C_p \left[T - T_m - \left(T.ln\frac{T}{T_m}\right)\right] \tag{11}$$

ΔC_p can be experimentally determined by calorimetry, or by measuring denaturation curves at multiple pH or concentrations of denaturant where the T_m shows some variation. The variation of ΔH_m in such experiments is dominated by the effect of ΔC_p, which causes ΔH_m to change with the change in T_m ($\Delta C_p = \frac{\delta \Delta H}{\delta T}$).

ΔC_p can also be estimated from empirical correlations based on protein size. In the absence of an experimental value or estimate for ΔC_p, using a value of zero will yield an acceptable fit and essentially correct parameters for most thermal denaturation data. This is because Equation 11 has a parabolic form with non-zero ΔC_p, but that function is closely approximated by a linear form (ΔC_p set to zero) at temperatures close to T_m, where most of the experimentally observable change in the population of N and D states occurs.

To fit chemical denaturation curves ΔG_{D-N} in Equation 9 was substituted by Equation 12 (Fersht 1998):

$$\Delta G_{D-N} = m\left([Denaturant]_{50\%} - [Denaturant]\right) \tag{12}$$

Where $[Denaturant]_{50\%}$ is the concentration of denaturant at the midpoint of the unfolding transition (50 % population of the unfolded state) and m is the slope of the transition: $m = \frac{\delta \Delta G_{D-N}}{\delta [denaturant]}$. In addition, the two baseline slopes in equation 9, $\beta_X P$ were replaced by $\beta_X [Denaturant]$