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FOR THE RECORD

van't Hoff enthalpies without baselines

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Abstract: Analysis of thermal melting curves represents one important approach for evaluating protein stability and the consequences of amino acid substitution on protein structure. By use of the van't Hoff relationship, the *differential* melting curve can be robustly fit to only three parameters, two of which are the underlying physical constants of melting temperature (T_m) and van't Hoff enthalpy (ΔH_{vH}). Calculated T_m and ΔH_{vH} values are insensitive to the choice of pre- and post-transition baselines. Consequently, the method accurately computes T_m and ΔH_{vH} for extremely truncated data sets, in the complete absence of baseline information, and for proteins with low melting temperatures, where the traditional direct approach routinely fails. Moreover, agreement between ΔH_{vH} values obtained using points derived from pre- vs. post-transition data provide an independent method for detecting some classes of non-two-state transitions. Finally, fitting of the differential denaturation curve should prove useful for analysis of abbreviated data sets obtained from high throughput array analysis of protein stability.

Keywords: analysis of protein denaturation; differential melting curve; van't Hoff enthalpy

The thermodynamics underlying protein folding and the effects of amino acid substitution on protein stability are commonly analyzed by monitoring denaturation as a function of temperature (Kauzmann, 1959; Hermans, 1965; Freire, 1995). For proteins, denaturation is usually detected by circular dichroism (CD) or, less commonly, by absorbance and other methods. Thermodynamic information can be extracted from these thermal melting experiments using the van't Hoff relationship:

$$\frac{d \ln K}{d(1/T)} = \frac{-\Delta H_{vH}}{R}. \quad (1)$$

The shape of a denaturation curve is governed by two fundamental physical properties, the melting temperature (T_m) and transition enthalpy (ΔH_{vH}). T_m determines the transition midpoint, whereas the van't Hoff enthalpy ΔH_{vH} is inversely proportional to the width of the transition. The key to employing the van't Hoff relationship to extract thermodynamic information is to assume a relationship between the equilibrium constant K and the signal measured during denaturation.

Protein unfolding transitions, especially for small single domain proteins, are often two-state (Lumry et al., 1966; Fersht, 1999):

$$N \rightleftarrows D \quad (2)$$

where N and D are the native and denatured states, respectively. The equilibrium constant for this unimolecular reaction is given by

$$K = \frac{D}{N} = \frac{f}{1-f}. \quad (3)$$

The fraction denatured protein f can be obtained algebraically from the fractional signal change during thermal denaturation. The observed signals for the fully native and fully denatured states are also typically temperature dependent, resulting in sloping baselines that must be subtracted prior to calculating f. Thus, direct fitting of protein denaturation curves to obtain T_m and ΔH_{vH} involves optimization of six independently adjustable parameters: the two physical constants plus a slope and intercept for both upper and lower baselines.

The choice of baseline can lead to large variations in the calculated ΔH_{vH} (Allen & Pielak, 1998; Cooper, 1999). In many interesting cases, it is not possible to determine robustly both a good quality upper and lower baseline. These situations include measurements on proteins with very low or very high melting temperatures and on proteins that are not stable at temperatures removed from the transition midpoint. These experimental challenges are encountered routinely, for example, in the cases of protein variants containing destabilizing mutations (no lower baseline (Eriksson et al., 1992; Marmorino & Pielak, 1995; Marmorino et al., 1997)) or that aggregate at higher temperatures (no upper baseline (Elwell & Schellman, 1975; Hickey et al., 1991)).

Direct fitting of the *differential* melting curve provides an alternative method for analyzing thermal denaturation experiments. This approach has been widely employed for studying nucleic acid thermodynamics (Gralla & Crothers, 1973; Marky & Breslauer, 1987; John & Weeks, 2000) and has been shown to be relatively

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insensitive to baseline corrections (Gralla & Crothers, 1973). We explored whether this approach might be useful for studying protein stability and folding using van't Hoff enthalpies and melting temperatures.

By taking the natural logarithm of both sides of Equation 3 and differentiating with respect to $(1/T)$ we obtain:

$$\frac{d\ln K}{d(1/T)} = \frac{1}{f}\left(\frac{df}{d(1/T)}\right) - \frac{1}{1-f}\left(\frac{-df}{d(1/T)}\right) \tag{4}$$

substituting Equation 1 and collecting terms gives

$$\frac{df}{d(1/T)} = \frac{-\Delta H_{vH}}{R} f(1-f) \tag{5}$$

where f is a function of both T_m and ΔH_{vH}. In practice, we achieve good results by fitting differential denaturation curves to

$$\frac{d(\text{signal})}{dT} = Af(1-f)(T^2) \tag{6}$$

where $d(\text{signal})/dT$ can be the algebraic derivative of any denaturation-sensitive signal in arbitrary units and A is a scaling factor. We take advantage of the relationship $d(1/T) = dT/T^2$. T_m and ΔH_{vH} are obtained by substituting into Equation 6, first,

$$f = \frac{K}{K+1} \tag{7}$$

and, second, the integrated form of the van't Hoff equation for unimolecular reactions.

$$K = \exp\left[\frac{\Delta H_{vH}}{R}\left(\frac{1}{T_m} - \frac{1}{T}\right)\right]. \tag{8}$$

Thermal denaturation curves are fit to Equations 6–8 using only three adjustable parameters, two of which are the physical constants T_m and ΔH_{vH}. Our implementation of this approach for personal computers, written in Kaleidagraph (Synergy Software, Reading, Pennsylvania), is available upon request.

We tested this method using thermal denaturation curves monitored by CD for yeast iso-1-ferricytochrome *c* mutants. The full melting profile (Allen & Pielak, 1998; Hostetter et al., 1999) for yeast iso-1-ferricytochrome *c* at pH 4.6 is shown in Figure 1A. These data represent a complete data set for which both upper and lower baselines can be reliably determined. The data were subsequently smoothed over a three degree window and algebraically differentiated with respect to temperature to yield the corresponding differential melting curve shown in Figure 1E. The thermodynamic parameters obtained by a nonlinear best fit to Equations 6–8 yields $T_m = 326.8 \pm 0.1$ K and $\Delta H_{vH} = -81 \pm 3$ kcal/mol. T_m and ΔH_{vH} values obtained by fitting the differential denaturation curve are the same within error as those determined by directly fitting the thermal melting curve to six parameters, 328.0 ± 0.1 K and -84 ± 2 kcal/mol, respectively (data not shown). Thus, where complete baselines can be obtained, direct fitting of the unmanipulated curve and of the differential denaturation curve yields thermodynamic parameters of comparable precision.

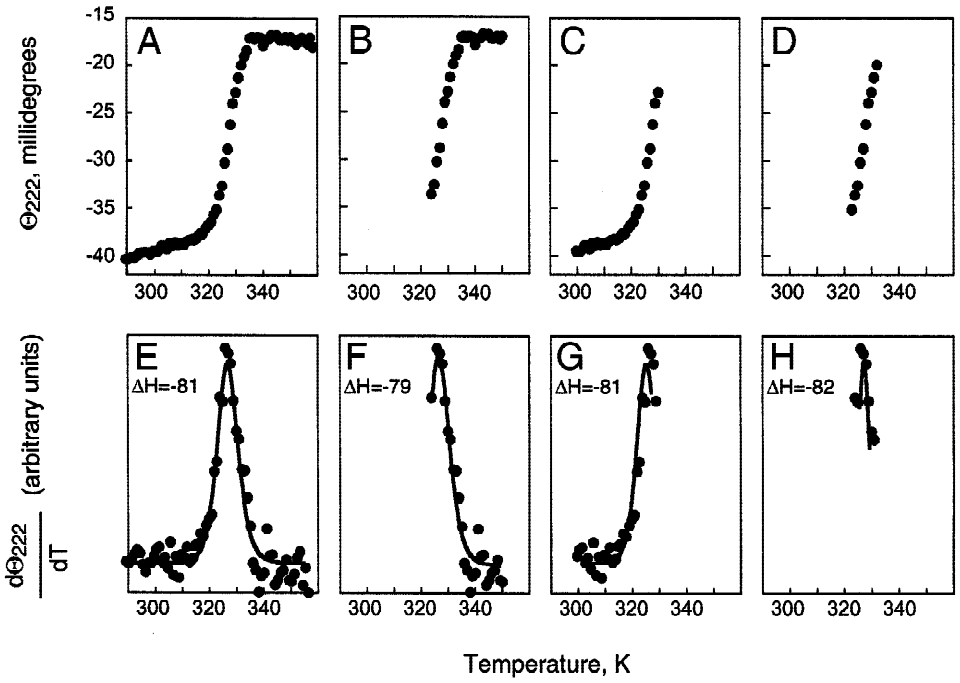

Fig. 1. Thermal denaturation of yeast iso-1-ferricytochrome *c* monitored by CD. (**A**) The complete denaturation profile or (**B, C, D**) representative abbreviated data sets were smoothed over a three degree window and algebraically differentiated to yield the curves **E, F, G, H**. Solid lines indicate best fits to Equations 6–8. Calculated van't Hoff transition enthalpies (in kcal/mol) are shown explicitly; in all cases T_m is 326.8 ± 0.3 K. Data are from Allen and Pielak (1998); denaturation was monitored at 222 nm using the C102T variant of cytochrome *c* at 30 μM in 50 mM sodium acetate, pH 4.6.

We then tested the importance of baselines for fitting the differential melting curve by deleting the entire lower baseline from our test data (Fig. 1B). The traditional six parameter method cannot fit this truncated data set (Marmorino & Pielak, 1995). The corresponding differential denaturation curve is shown in Figure 1F. The calculated T_m and ΔH_{vH} values obtained from these abbreviated data are 326.5 ± 0.3 K and −79 ± 6 kcal/mol, respectively. These values are identical within error to those obtained for the complete curve (compare Figs. 1E and 1F). We also deleted the upper baseline from the test data as illustrated in Figures 1C and 1G. Fitting the differential melting curve yields T_m and ΔH_{vH} of 326.8 ± 0.3 K and −81 ± 5 kcal/mol, respectively; these values are again identical, within error, to those obtained for both the complete and lower baseline-truncated curves.

We pushed this approach to its logical extreme and determined the fewest data points required to obtain robustly the melting temperature and transition enthalpy. Only 10 data points spanning the inflection point of the thermal melting curve (Fig. 1D) are required to give precise values for T_m and ΔH_{vH}. This region translates to the top of the differential denaturation curve (Fig. 1H).

Given that severely truncated data sets are robustly fit by the differential method, we applied this approach to an example where the six parameter method clearly has difficulty. We calculated T_m and ΔH_{vH} values for the A-state (pH 2.1) of the L94T mutant of yeast iso-1-ferricytochrome *c* (Marmorino & Pielak, 1995) whose profile has no native baseline and is missing a significant portion of the pre-melting transition region. Moreover, the transition is very broad making it difficult to distinguish the observed upper baseline from the denaturation transition (see Fig. 2A). For our analysis, the relatively noisy data were smoothed over a three degree window twice. Smoothing the complete data analyzed in Figure 1A twice had no significant effect on the calculated T_m and ΔH_{vH} values, emphasizing that parameters obtained using the differential curve fitting approach are insensitive to these manipulations. T_m and ΔH_{vH} values for the L94T mutant were calculated to be 280.1 ± 5.1 K and −17 ± 5 kcal/mol, respectively (Fig. 2B). Fitting the differential denaturation curve is thus useful even in this very unfavorable case of a broad transition (small ΔH_{vH}) and no lower baseline (due to low T_m).

Differential fitting of thermal denaturation data is independent of baseline corrections because the method essentially ignores the baseline slopes. Ignoring the baseline translates the entire differential melting curve up or down on the *y*-axis by the amount of the slope. To explore the potential systematic error due to neglecting baseline slopes, we added or subtracted reasonable values from the test differential denaturation curve (Fig. 1E) and recalculated T_m and ΔH_{vH} as shown in Table 1. The slopes of the lower and upper baselines in Figure 1A are +0.08 and −0.02, respectively. Therefore, a realistic test is to introduce 50% error, ±0.04, to the differential curve. Calculated ΔH_{vH} values are 80 ± 3 or −84 ± 3 kcal/mol, respectively; both values are within fitting error of the original data (Table 1). Addition or subtraction of 0.08 (100% error in the lower baseline) to the differential curve yields calculated ΔH_{vH} values that differ by <8% (Table 1); T_m values are identical in all cases.

The data show that precise thermodynamic parameters are returned, largely independent of baseline selection. However, if desired, a small improvement in the calculated ΔH_{vH} could be achieved by either manually subtracting an average upper and lower baseline or by explicitly fitting the average baseline (b) by adding a "+ *b*" term to Equation 6.

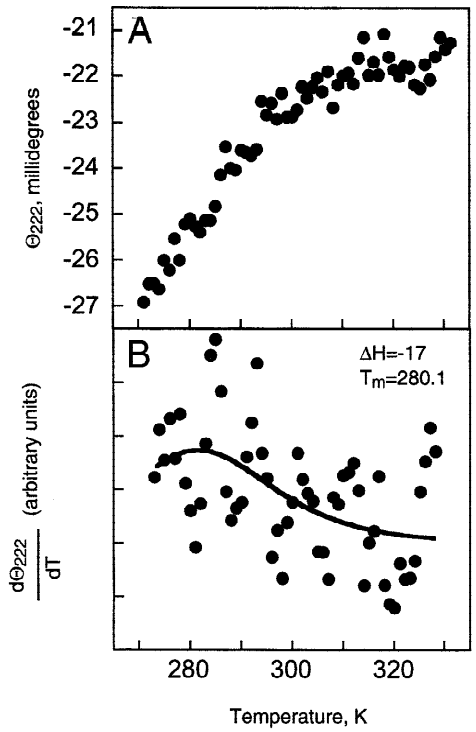

Fig. 2. Thermal denaturation of the A-state of the L94T mutant of yeast iso-1-ferricytochrome *c*. Raw data collected by (**A**) CD is missing the lower baseline and pretransition regions. Data were smoothed over a three degree window twice, algebraically differentiated, and fit to Equations 6–8 to yield the (**B**) best-fit curve. Fitting error for the van't Hoff transition enthalpy and melting temperature are ±5 kcal/mol and ±5 K, respectively. Data are from Marmorino and Pielak (1995); denaturation of the mutant protein was monitored at 222 nm at a concentration of 30 μM in 0.33 M sodium sulfate, pH 2.1.

This approach for determining T_m and ΔH_{vH} requires that we assume the underlying transition is two state. Although a two-state assumption can be an oversimplification, this analysis can still be useful. Intermediates often accumulate preferentially under conditions far from the T_m. Such intermediates include pre-melting "fraying" transitions and aggregation. In favorable cases, the denaturation

Table 1. *Effect of baseline correction on calculated* ΔH_{vH} *and* T_m[a]

y-Axis translation	ΔH_{vH} (kcal/mol)	Difference (%)
0.0	−81 ± 3	—
+0.04	−80 ± 3	1.2
+0.08	−75 ± 3	7.5
−0.04	−84 ± 3	3.7
−0.08	−87 ± 4	7.5

[a] T_m = 326.8 ± 0.5 K in all cases. *y*-Axis translation was performed using the data shown in Figure 1E. T_m and ΔH_{vH} were obtained from best fits to Equations 6–8.

transition closely approaches a two-state process at the midpoint of folding (Fersht, 1999). Moreover, differential transition profiles are approximately symmetrical about T_m. Therefore, agreement between fits using primarily pre- vs. post-transition data (compare Figs. 1F and 1G) provides an independent method for identifying some classes of non-two-state transitions. For example, early fraying transitions prior to cooperative denaturation of the protein core should be readily detected.

Conclusion: Fitting differential protein denaturation curves is superior to and much more robust than the traditional direct fitting method. The differential method takes advantage of the large information content of the data at the inflection point and deemphasizes the importance of selecting accurate upper and lower baselines. The differential method employs only three adjustable parameters, two of which are the fundamental physical constants T_m and ΔH_{vH}. That the upper and lower baselines can be completely eliminated and still give reliable values for the van't Hoff enthalpy and T_m (Fig. 1) will be especially useful for studying proteins with low or high melting temperatures and for proteins that exhibit nonideal behavior at temperatures removed from T_m. Finally, the method may prove especially useful for high throughput analysis of protein stability in array formats.

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