Calculating Thermodynamic Data for Transitions of any Molecularity from Equilibrium Melting Curves

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Synopsis

In this paper, we derive the general forms of the equations required to extract thermodynamic data from equilibrium transition curves on oligomeric and polymeric nucleic acids of *any molecularity*. Significantly, since the equations and protocols are general, they also can be used to characterize thermodynamically equilibrium processes in systems other than nucleic acids. We briefly review how the reduced forms of the general equations have been used by many investigators to evaluate mono- and bimolecular transitions, and then explain how these equations can be generalized to calculate thermodynamic parameters from common experimental observables for transitions of higher molecularities. We emphasize the strengths and weaknesses of each method of data analysis so that investigators can select the approach most appropriate for their experimental circumstances. We also describe how to analyze calorimetric heat capacity curves and noncalorimetric differentiated melting curves so as to extract both model-independent and model-dependent thermodynamic data for transitions of any molecularity. The general equations and methods of analysis described in this paper should be of particular interest to laboratories that currently are investigating association and dissociation processes in nucleic acids that exhibit molecularities greater than two.

INTRODUCTION

We have learned a great deal about the sequence-dependent conformational states present in naturally occurring DNA and RNA polymers from studies on specially designed and synthesized oligonucleotides.[1-5] Particular attention has been focused on thermodynamically characterizing the secondary structures formed by these oligomeric model systems through investigations of their temperature-dependent "melting" behavior. In fact, the results obtained from these model systems have provided databases from which thermodynamic libraries have been established that characterize all ten Watson–Crick nearest-neighbor interactions in both DNA[6] and RNA.[7-9] These thermodynamic data now provide an empirical basis for predicting the stability ($\Delta G°$) and temperature-dependent melting behavior ($\Delta H°$) of any DNA or RNA duplex region by inspection of its primary sequence.[6-9]

To date, the overwhelming majority of oligomer systems studied have modeled secondary structures formed by monomolecular (e.g., hairpins) and bimolecular (e.g., duplexes) associations of oligomer strands.[10-38] (Also see other work cited in Refs. 4 and 5). The equations required to extract thermodynamic data from melting studies on oligomer systems exhibiting these two molecularities are already published and are reasonably well known.[39-45] (See

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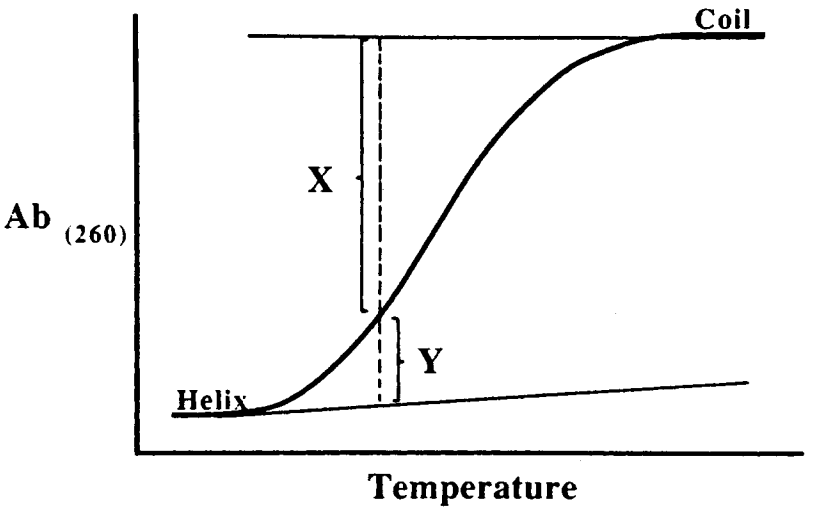
Ref. 39 for an excellent anthology and discussion of the original papers describing the early development of the theory of helix–coil transitions in biopolymers.) Undoubtedly, future modeling of biologically important structures will involve oligomer associations well beyond the bimolecular level. In fact, recent efforts to model cruciform formation by immobile junction structures have required oligomer association processes at the tetramolecular level.[46-51] Consequently, it would be useful to derive the general forms of the equations required to extract thermodynamic data from melting studies on oligomer systems *of any molecularity*. In this paper, we derive the relevant equations and describe how they can be used to calculate thermodynamic parameters from experimental data. We also describe a useful method for extracting complete thermodynamic profiles from calorimetric measurements in the absence of optical data. As part of an independent and concurrent effort, Privalov and Potekhin also have derived general equations that they use to analyze calorimetric heat capacity curves for protein transitions.[52] Although their and our formulations for analyzing calorimetric data differ significantly, the information content of the two sets of equations is similar. Significantly, however, in contrast to Privalov and Potekhin, we explicitly include a concentration-dependent term in several of our formulations. Such a term is important when using noncalorimetric techniques to characterize thermodynamically association or dissociation processes for relatively short oligomers.

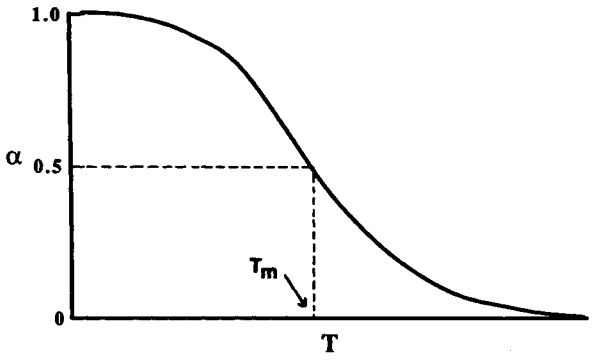
ANALYZING THE SHAPE OF AN EQUILIBRIUM MELTING CURVE TO CALCULATE ΔH_{VH}

A thermally induced order–disorder transition in any nucleic acid can be monitored by following, at an appropriate wavelength, the increase in uv absorption with increasing temperature. The resulting absorption vs temperature profile commonly is called a "uv melting curve." Figure 1(a) shows a typical experimental curve. The shape of this curve, as well as other equilibrium melting profiles (e.g., viscosity vs temperature, heat capacity vs temperature, etc.), can be analyzed to yield a value for the van't Hoff transition enthalpy. Below we describe how this analysis can be accomplished for a uv absorption vs temperature curve. However, keep in mind that the protocol is general and therefore can be used to analyze the temperature dependence of any equilibrium property that is directly related to the concentrations of the two equilibrating species.

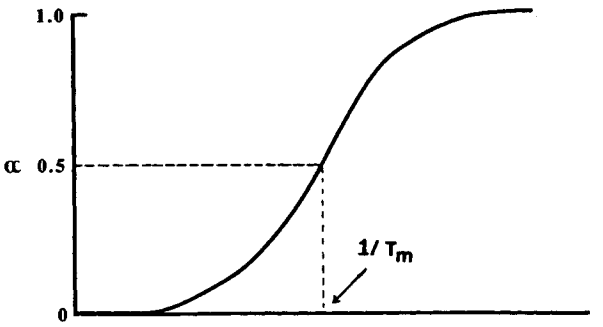
If we define α as equal to the fraction of single strands in the duplex state, then any experimental absorbance vs temperature curve can be converted into an α vs temperature profile by assuming that the fractional change in absorbance at any temperature monitors the extent of reaction. This conversion is accomplished graphically, as illustrated in Fig. 1, by taking the ratio at each temperature of the height between the upper baseline and the experimental curve (x) and the height between the lower and upper baselines ($x + y$). We now have constructed the curve in Fig. 1(b), which expresses how an equilibrium property α varies with temperature. Alternatively, we could have plotted α vs the reciprocal of the temperature $1/T$ to obtain the curve



(a)



(b)



(c)

Fig. 1. (a) A typical absorbance vs temperature melting curve. X corresponds to the distance between an upper baseline and the curve, while Y represents the distance between the lower baseline and the curve. (b) A plot of α vs T, where α equals the fraction of single strands in the duplex state. This curve is derived from (a) as described in the text. (c) A plot of α vs $1/T$ where α equals the fraction of single strands in the duplex state.

shown in Fig. 1(c). Since the equilibrium constant K for any transition can be expressed in terms of α, the curves shown in Fig. 1(b and c) reveal how K varies with T and $1/T$. This knowledge of the temperature dependence of K allows us to calculate the transition enthalpy using either form of the van't Hoff equation shown below:

$$\Delta H_{VH} = RT^2\left[\frac{d \ln K}{dT}\right] \quad \text{or} \quad \Delta H_{VH} = -R\left[\frac{d \ln K}{d(1/T)}\right] \tag{1}$$

Obviously, to complete the calculation of ΔH_{VH}, K must be expressed in terms of α. For this purpose, one generally assumes that the transition proceeds in a two-state (all-or-none) manner. When this condition prevails, the general expression for the equilibrium constant K, and its value at the melting temperature $K(T_m)$, will depend on the molecularity of the transition and the nature of the associating sequences (self-complementary vs non-self-complementary). In the sections that follow, we derive the general expressions for the equilibrium constant and explain how they can be used to calculate thermodynamic data.

Equilibria Involving Non-Self-Complementary Sequences

Consider the general equilibrium shown below for the association of *non-self-complementary* sequences to form an n-mer structure

$$A_1 + A_2 + A_3 + \cdots + A_n \leftrightarrow A_1A_2A_3 \cdots A_n$$

where n is the molecularity of the reaction, which equals the number of strands that associate to form the final n-mer complex. The general expression for the corresponding equilibrium constant K, in terms of α and n, is

$$K = \frac{[A_1A_2A_3A_n]}{[A_1][A_2][A_3]\cdots[A_n]} = \frac{\alpha(C_T/n)}{[(1-\alpha)C_T/n]^n}$$

$$= \frac{\alpha}{(C_T/n)^{n-1}(1-\alpha)^n} \quad \left(\begin{matrix}\text{for non-self-complementary} \\ \text{associations}\end{matrix}\right) \tag{2}$$

where C_T equals the total strand concentration and *each strand is present in equal concentration*; namely, C_T/n. This expression is applicable to the general case in which the associating strands all are non-self-complementary. The special case in which the associating sequences *all* are *self-complementary* is described in the next section.

We now have an expression for the equilibrium constant in terms of experimentally accessible parameters; namely, n, C_T, and α. If we define the melting temperature T_m, as the temperature at which $\alpha = 1/2$, then the general expression for the equilibrium constant shown in Eq. (2) reduces to

$$K_{T_m} = \frac{1/2}{(C_T/n)^{n-1}(1/2)^n} = \frac{1}{(C_T/n)^{n-1}(1/2)^{n-1}} = \frac{1}{(C_T/2n)^{n-1}} \tag{3}$$

This expression allows calculation of K at the T_m for an association reaction of any molecularity between non-self-complementary sequences.

We also can derive a general expression for calculating the transition enthalpy. To accomplish this, we substitute Eq. (2) into the first form of the van't Hoff expression (Eq. 1), differentiate, and solve for ΔH_{VH} at the T_m where $\alpha = 1/2$. These manipulations yield

$$\Delta H_{VH} = (2 + 2n)RT_m^2\left(\frac{\partial \alpha}{\partial T}\right)_{T=T_m} \tag{4a}$$

Since $\partial(1/T)/\partial T = -1/T^2$, Eq. (4a) also can be written as

$$\Delta H_{VH} = -(2 + 2n)R\left[\frac{\partial \alpha}{\partial(1/T)}\right]_{T=T_m} \tag{4b}$$

We could have obtained this expression directly by substituting Eq. (2) into the second form of the van't Hoff expression shown in Eq. (1). Many of the equations presented below can be reformulated in a similar manner.

Either general form of the van't Hoff expression derived above [Eq. 4(a) or 4(b)] allows calculation of ΔH_{VH} for an association reaction of any molecularity simply by evaluating the slope of an α vs T or an α vs $1/T$ melting curve at T_m. The resulting value of $(\delta\alpha/\delta T)_{T=T_m}$ or $[\partial\alpha/\partial(1/T)]_{T=T_m}$ and the known molecularity n of the reaction are then plugged into Eq. (4a) or (4b) to calculate ΔH_{VH}. [Equation (4a) or (4b) is derived for an *association* reaction. The same expression with the *opposite sign* can be used for *dissociation* processes.] These expressions [Eq. (4a) or (4b)] allow calculation of ΔH_{VH} for any equilibria involving *non-self-complementary* sequences. In the next section, we demonstrate that the same expressions [Eqs. (4a) or (4b)] also can be used to calculate ΔH_{VH} for equilibria that involve structures formed from *self-complementary* sequences.

Equilibria Involving Self-Complementary Sequences

For an association reaction involving structures formed from self-complementary sequences, the general equilibrium can be written as

$$nA \leftrightarrow A_n$$

For this special case, the general expression for the equilibrium constant K, in terms of α and n, is

$$K = \frac{[A_n]}{[A]^n} = \frac{\alpha(C_T/n)}{[(1-\alpha)C_T]^n} = \frac{\alpha}{nC_T^{n-1}(1-\alpha)^n} \tag{5}$$

Note that this expression for the equilibrium constant for an association reaction between self-complementary sequences is *not* identical to the corresponding expression for non-self-complementary sequences. [Compare Eqs. (2) and (5).] This disparity reflects the statistical differences between these two classes of equilibria.

If we define the melting temperature T_m as the temperature at which $\alpha = 1/2$, the general expression for K shown above reduces to

$$K_{T_m} = \frac{1/2}{nC_T^{n-1}(1/2)^n} = \frac{1}{n(C_T/2)^{n-1}} \quad (6)$$

This expression allows calculation of K at the T_m for an association reaction of any molecularity between self-complementary sequences.

We also can derive a general expression for calculating the transition enthalpy for self-complementary associations. To accomplish this, we substitute Eq. (5) into the van't Hoff expression (Eq. 1), differentiate, and solve for ΔH_{VH} at the T_m where $\alpha = 1/2$. These manipulations yield equations for calculating ΔH_{VH}, which are identical to those derived above for the association of non-self-complementary sequences [Eqs. (4a) and (4b)]. This identity reflects the fact that the statistical differences between processes involving these two classes of sequences does not influence the expression used to calculate ΔH_{VH}.

Calculating ΔH_{VH} from Melting Curves

Equation (4a) can be used to calculate a van't Hoff transition enthalpy for a process of any molecularity. To date, bimolecular (e.g., duplex to single strands) and monomolecular (e.g., hairpin to single strand) processes represent the two most commonly studied classes of nucleic acid transitions. For a monomolecular process ($n = 1$), the leading coefficient in Eq. (4a) is 4 while for a bimolecular process ($n = 2$) the corresponding coefficient is 6. These two reduced forms of Eq. (4a) are well known and have been used by many investigators to extract van't Hoff transition enthalpies from the temperature dependence of various equilibrium properties for mono- and bimolecular processes. By contrast, the use of Eq. (4a) to extract van't Hoff transition enthalpies from processes exhibiting molecularities greater than 2 is much less common in the literature.

However, the recent use of multistrand oligomer systems to model more complex biological structures has resulted in studies on processes that exhibit molecularities greater than 2. Consequently, it is useful to examine the reduced forms of Eq. (4a) required to extract van't Hoff transition enthalpies from such studies. One example of a thermodynamic analysis on a higher molecularity process is described in the paper that follows. In this work, a cruciform is modeled by an immobile junction structure that forms through association between *four* 16-mer sequences.[51] To extract thermodynamic data from the melting curves associated with this tetramolecular process (where $n = 4$), the reduced form of Eq. (4a) shown below was used:

$$\Delta H_{VH} = 10RT_m^2\left(\frac{\delta\alpha}{\delta T}\right)_{T=T_m}$$

In the paper that follows, a critical appraisal of the ΔH_{VH} data obtained from this equation is provided by comparison with directly measured calorimetric data.

In summary, Eq. (4a) or (4b) allows one to calculate ΔH_{VH} for any association reaction for which the temperature dependence of an equilibrium property has been monitored. One simply needs to know the molecularity n of the reaction and then graphically determine the value of $(\delta\alpha/\delta T)_{T=T_m}$ from the experimental melting curve. Note that the same equation with the opposite sign can be used to evaluate dissociation or melting processes.

CALCULATING ΔH_{VH} FROM THE CONCENTRATION DEPENDENCE OF THE MELTING TEMPERATURE

The formation or dissociation of complexes of molecularity greater than one will result in a concentration-dependent equilibrium. Such equilibria therefore can be characterized by determining the concentration dependence of the melting temperature. This approach represents a second method for extracting thermodynamic data from experimental melting profiles. The relevant equations in general form are derived below.

Concentration-Dependent Equilibria Involving Non-Self-Complementary Sequences

Consider the general equilibrium reaction shown below for the association of *non-self-complementary* sequences to form an n-mer structure:

$$A_1 + A_2 + A_3 + \cdots + A_n \leftrightarrow A_1A_2A_3 \cdots A_n$$

Since, for any process at equilibrium, $\Delta G^\circ = -RT \ln K_{eq}$ and $\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$, we can derive an expression for K_{eq} in terms of ΔH° and ΔS° by equating these two expressions for ΔG° to yield

$$-RT \ln K = \Delta H^\circ - T\Delta S^\circ \tag{7}$$

Equation (3) provides us with an expression for K at T_m in terms of n and C_T when non-self-complementary strands associate. Plugging this expression into the equality given above yields

$$+RT_m \ln(C_T/2n)^{n-1} = \Delta H^\circ - T_m \Delta S^\circ$$

Upon rearrangement, this expression becomes

$$(n-1)RT_m \ln C_T - (n-1)RT_m \ln 2n = \Delta H^\circ - T_m \Delta S^\circ$$

Dividing by $T_m \Delta H^\circ$ and rearranging the terms yields

$$\underset{(y)}{\frac{1}{T_m}} = \underset{(m)}{\frac{(n-1)R}{\Delta H^\circ}} \underset{(x)}{\ln C_T} + \underset{(b)}{\frac{[\Delta S^\circ - (n-1)R \ln 2n]}{\Delta H^\circ}} \quad \left(\begin{array}{c}\text{for associations of}\\ \text{non-self-complementary}\\ \text{strands}\end{array}\right) \tag{8}$$

As emphasized by the symbols in parenthesis, this equation corresponds to a straight line when the reciprocal of the melting temperature $(1/T_m)$ is

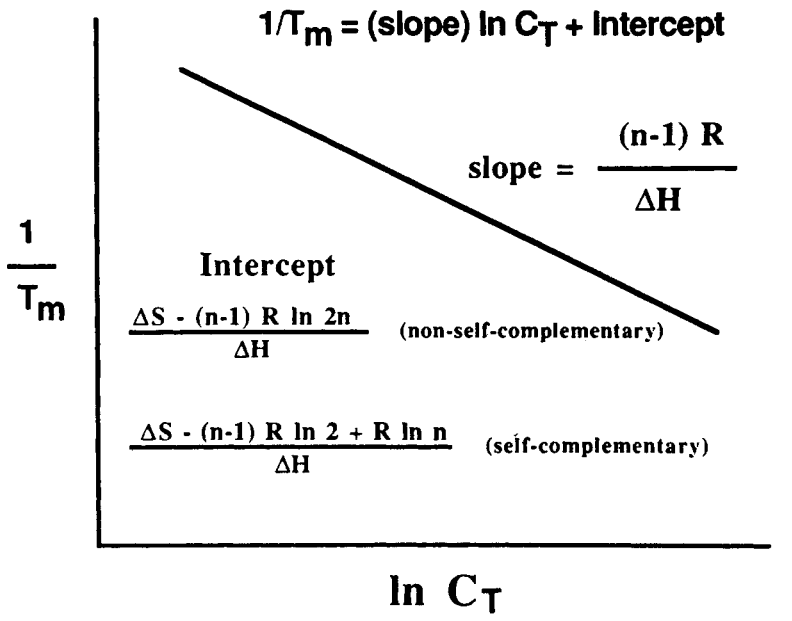


Fig. 2. A plot of $1/T_m$ vs $\ln C_T$. This plot reveals the concentration dependence of the melting temperature. The slope is inversely proportional to the transition enthalpy while the X-axis intercept is proportional to the transition entropy. The equations describing the specific relationships are shown in the plot as well as described in the text.

plotted against the natural logarithm of the strand concentration ($\ln C_T$). The slope (m) of such a plot is equal to $[(n-1)R]/\Delta H°$ and the intercept (b) is equal to $[(1/\Delta H°)/(\Delta S° - (n-1)R \ln 2n]$. Figure 2 shows a typical $1/T_m$ vs $\ln C_T$ plot in which these features are emphasized.

Concentration-Dependent Equilibria Involving Self-Complementary Sequences

For the case in which the associating sequences all are self-complementary rather than non-self-complementary, the equilibrium constant expression shown in Eq. (5) rather than Eq. (2) is used to derive the appropriate relationship between T_m and $\ln C_T$. Paralleling the procedure outlined above, one obtains the expression shown below, which is similar to but different from Eq. (8):

$$\frac{1}{T_m} = \frac{(n-1)R}{\Delta H°} \ln C_T + \frac{[\Delta S° - (n-1)R \ln 2 + R \ln n]}{\Delta H°} \quad \left(\begin{array}{c}\text{for associations of}\\ \text{self-complementary strands}\end{array}\right) \quad (9)$$

Note that Eqs. (8) and (9) have the same expression for their slopes, but different expressions for their intercepts. This disparity reflects the statistical difference between these two classes of equilibria. Significantly, it should be noted that this statistical factor only influences the intercept and not the

slope. Thus, the statistical differences between self-complementary and non-self-complementary equilibria do not influence the enthalpy term. As with Eq. (4), our derivation of Eq. (8) or (9) assumes a two-state transition and a temperature-independent enthalpy. However, this method for determining ΔH_{VH} is much less sensitive to the choice of baselines when analyzing experimental melting curves. This feature results from the fact that the slope of a $1/T_m$ vs $\ln C_T$ line results from *differences* in the T_m values obtained from several melting curves. Consequently, as long as the baselines for a family of concentration-dependent melting curves are selected in a *consistent* manner, the slope of the resulting $1/T_m$ vs $\ln C_T$ line provides a good measure of the van't Hoff enthalpy for transitions that proceed in a two-state manner. Any deviations of the selected baselines relative to the "true" baselines simply produce a parallel line that has a different intercept but the same slope, thereby altering $\Delta S°$ but not ΔH_{VH}.

The method of data analysis described above is uniquely applicable to relatively short oligomer transitions where the concentration dependence of the melting temperature can be observed. For long oligomers and polymers, this approach cannot be applied, since the monomolecular helix growth steps dominate the bimolecular helix initiation step, thereby producing an artificially reduced concentration dependence or a pseudo-first-order equilibrium for which the melting temperature is concentration independent.

Calculating ΔH_{VH} from the Concentration Dependence of T_m for Some Common Transitions

Clearly, the melting temperature of a monomolecular process (e.g., hairpin formation) will not exhibit a concentration dependence. This feature is illustrated by the fact that when $n = 1$, Eq. (8) or (9) reduces to $1/T_m = \Delta S°/\Delta H°$. This simplified form of Eq. (8) or (9) for the $n = 1$ case shows that for a monomolecular process the melting temperature T_m, depends only on $\Delta S°$ and $\Delta H°$, and is independent of C_T. Consequently, to obtain a value for ΔH_{VH} from a melting curve of a monomolecular process requires application of Eq. (4).

Equation (8) or (9) can be used to evaluate any process that exhibits a molecularity of 2 or above. For example, for the bimolecular duplex formed between two strands of a *self-complementary* sequence, Eq. (9) reduces to the well-known expression

$$\frac{1}{T_m} = \frac{R}{\Delta H°} \ln C_T + \frac{\Delta S°}{\Delta H°} \quad \left(\begin{array}{c}\text{for a bimolecular association of two}\\ \text{self-complementary strands}\end{array}\right)$$

However, if the two strands that associate are *non-self-complementary* rather than self-complementary, then the *intercept* (b term) includes an additional factor to account for the entropic differences between these two association reactions. Consequently, when analyzing a bimolecular association between non-self-complementary rather than self-complementary strands, Eq. (8) reduces to the expression

$$\frac{1}{T_m} = \frac{R}{\Delta H°} \ln C_T + \frac{\Delta S° - R \ln 4}{\Delta H°} \quad \left(\begin{array}{c}\text{for a bimolecular association of two}\\ \text{non-self-complementary strands}\end{array}\right)$$

Analyzing the Shape of a Differentiated Equilibrium Melting Curve

An approach very similar to the one just described can be used to obtain a van't Hoff transition enthalpy from a *differentiated* melting curve. Figure 3 shows a tracing of a typical differentiated melting curve where $\partial\alpha/\partial(1/T)$ is plotted against T. Such a curve can be derived from the temperature dependence of any equilibrium property or obtained directly from techniques such as temperature-jump measurements. As originally shown by Gralla and Crothers for mono- and bimolecular transitions,[16] the full width or the half-width of a differentiated melting curve at the half-height is inversely proportional to the van't Hoff transition enthalpy. For a general equilibrium of the form

$$nA \leftrightarrow A_n$$

the relevant general forms of the van't Hoff equation are

$$\Delta H_{VH} = \frac{B}{(1/T_1) - (1/T_2)} \quad \left(\begin{array}{c}\text{for the full width}\\ \text{at the half-height}\end{array}\right) \tag{10}$$

$$\Delta H_{VH} = \frac{B'}{(1/T_{max}) - (1/T_2)} \quad \left(\begin{array}{c}\text{for the upper half width}\\ \text{at the half-height}\end{array}\right) \tag{11}$$

where T_{max} is the temperature at the maximum, and T_1 and T_2 correspond to the lower and upper temperatures, respectively, at which the change in the observable with temperature is equal to one-half of $[\partial\alpha/\partial(1/T)]_{max}$ (see Fig. 3). Both B and B' are constants that depend on the molecularity of the

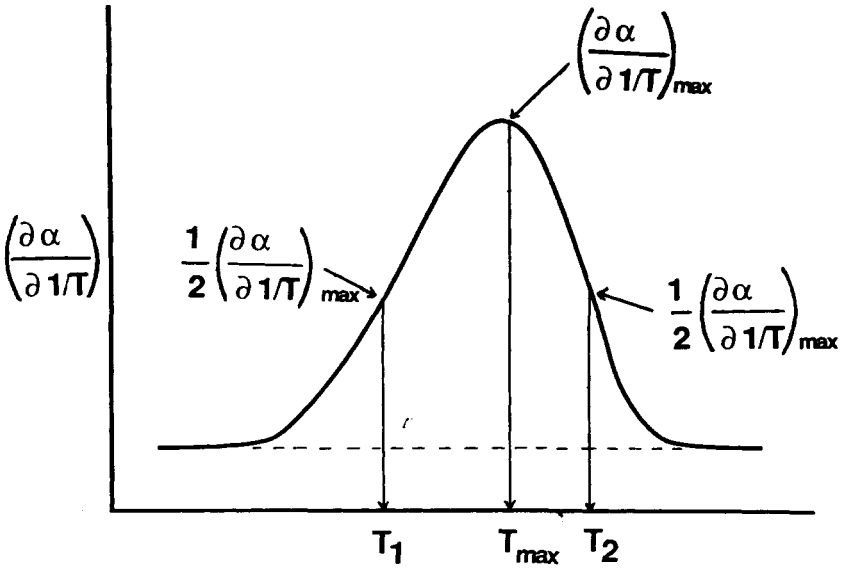


Fig. 3. A typical differentiated melting curve. Several reference temperature points are specifically defined in the figure and described in the text.

TABLE I
Values of the Constants B and B' in Eqs. (10) and (11) for Association Reactions Exhibiting Molecularities (n) Between 1 and 5. B and B' in cal/K-mol.

n	$-B$	$-B'$
1	7.00	3.50
2	10.14	4.38
3	12.88	5.06
4	15.40	5.63
5	17.79	6.14

process under investigation. Specific values of B and B' are given in Table I for processes involving several different molecularities. The detailed derivations of Eqs. (10) and (11) are given in the appendix. Examination of these derivations reveals that this method of analyzing differentiated melting curves still incorporates the assumption of a two-state transition and a temperature-independent enthalpy. However, when Eq. (11) is employed, only the high-temperature half of the transition need be obtained experimentally (between T_{max} and T_2). Consequently, this approach provides a means of circumventing the *lower* baseline problem that one frequently encounters in the analysis of the *overall shapes* of integral absorbance vs temperature curves. Specifically, for transitions with low T_ms, where it is difficult to define the lower baseline (or even to obtain the lower half of the melting curve), this method of data analysis permits a transition enthalpy to be calculated from just the upper half of a melting profile (see Fig. 3).

When a differentiated melting curve is obtained directly from temperature-jump experiments, the problem of baseline selection may be reduced further. If the molecular events that give rise to the sloping baselines correspond to processes that are very fast, then the temperature-jump experiment provides a means of kinetically resolving the relatively slow, cooperative helix melting from these fast baseline effects.[16,53] In such a case, one obtains a differentiated melting curve that is kinetically filtered of the baseline problems encountered with integral melting curves.

CALORIMETRIC DETERMINATION OF TRANSITION ENTHALPIES

Differential scanning calorimetry (DSC) also can be used to detect and follow thermally induced order–disorder transitions in oligonucleotides and other thermally labile molecules. However, with DSC the excess heat capacity (ΔC_p) rather than the change in absorbance of the solution is monitored. Experimentally, one obtains a ΔC_p vs T melting profile, as illustrated in Fig. 4(a). Since $\Delta H^\circ = \int \Delta C_p \, dT$, the area under such a calorimetric transition curve is equal to the transition enthalpy.

In contrast to the model-dependent (usually two-state) ΔH_{VH} values indirectly derived from the temperature dependence of an equilibrium property, the calorimetrically determined transition enthalpy does *not* depend on the nature of the transition. As with optical studies, with calorimetry one first must define an experimental baseline to analyze for the transition of interest.

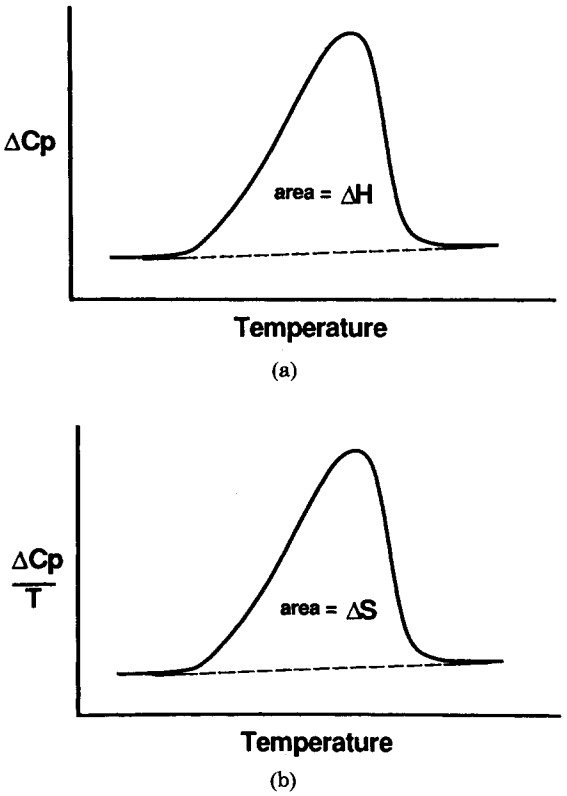


Fig. 4. (a) A typical calorimetric transition curve, which shows how the heat capacity ΔC_p, changes with temperature. The area under this curve is equal to the transition enthalpy. (b) A $\Delta C_p/T$ vs temperature curve, which can be derived from the experimental calorimetric transition curve shown in (a). The area under this curve is equal to the transition entropy.

One then measures the total energy required to go from the initial to the final state from the area under the curve and above the baseline. The shape of the curve, which does depend on the nature of the transition, need not be analyzed as is done with optical and other noncalorimetric data. Thus, the calorimetric measurement provides a direct, model-independent determination of the transition enthalpy. For this reason, a comparison of the model-dependent van't Hoff transition enthalpy and the model-independent calorimetric transition enthalpy provides insight into the nature of the transition, as explained in the next section. Calorimetry also provides a direct measure of the heat capacity change ΔC_p° accompanying the transition. Consequently, one need not assume that ΔC_p° is zero, as usually is done when analyzing optical data. Interestingly, in contrast to proteins, our DSC studies do not detect significant heat capacity changes accompanying thermally induced transitions in DNA molecules. In an independent and concurrent effort, Privalov and Potekhin also recently have derived equations that express the dependence of calorimetric enthalpies on n, the reaction molecularity.[52] However, their formulation is quite different from the one presented here.

It is of interest to note that a van't Hoff transition enthalpy also can be calculated from the calorimetric data. In a manner paralleling the analysis of

a differentiated melting curve, the shape of a calorimetric heat capacity curve can be analyzed using Eq. (10) or (11) to yield a van't Hoff transition enthalpy that we designate as ΔH_{VH}^{cal}. Values obtained in this manner do not always agree with the corresponding optical ΔH_{VH} derived from Eqs. (4), (8), or (9). For bimolecular transitions we have observed the largest differences between ΔH_{VH}^{cal} and ΔH_{VH} for longer oligomers.[5] This result suggests that the thermal and the optical windows may not always equivalently monitor the extent of reaction. For the tetramolecular transition of an immobile junction, we also observe disparities between the ΔH_{VH} data derived by the different methods described above. A critical discussion of the potential origins of these disparities is presented in the paper that follows.

NATURE OF THE TRANSITION

The methods described above for extracting thermodynamic data from the temperature dependence of an equilibrium property only can be applied rigorously to transitions that proceed in a two-state (all-or-none) manner. For transitions in which intermediate states are significantly populated, any integral or differentiated equilibrium melting curve will be broadened. According to Eqs. (4), (10), (11), this broadening will lead to a reduced ΔH_{VH} relative to the true calorimetric value. By contrast, as noted earlier, the calorimetrically determined transition enthalpy is derived directly from the area under (rather than the shape of) a heat capacity curve. Thus, ΔH_{cal} is independent of the nature of the transition, as one would expect for a state function.

Comparison of the model-dependent ΔH_{VH} and the model-independent ΔH_{cal} allows one to conclude if the transition proceeds in an all-or-none fashion, thereby providing a test for the applicability of the two-state model to a given transition. If $\Delta H_{VH} < \Delta H_{cal}$, then the transition involves a significant population of intermediate states. However, if $\Delta H_{VH} = \Delta H_{cal}$, then the transition proceeds in a two-state manner and meaningful thermodynamic data can be obtained by monitoring the temperature dependence of an equilibrium and by using the equations presented in this paper.

A quantitative comparison of the van't Hoff and calorimetric transition enthalpies provides further insight into the nature of a transition. Specifically, the ratio $\Delta H_{VH}/\Delta H_{cal}$ provides a measure of the fraction of the structure that melts cooperatively; in other words, the size of the cooperative unit. This ability to define the size of the cooperative unit represents an important and unique advantage of the calorimetric measurement. In the paper that follows, we employ this approach and discuss its potential limitations when applied to complex structures such as immobile junctions.

CALCULATING $\Delta G°$ AND $\Delta S°$ FROM MELTING CURVE DATA

As described above, a van't Hoff transition enthalpy (ΔH_{VH}) can be determined by analyzing the shape of an integral or differentiated melting curve using either Eq. (4), (10), or (11) which each assume a two-state model. By contrast, a model-independent calorimetric transition enthalpy (ΔH_{cal}°) can be determined directly by evaluating the area under an experimental heat capacity curve. The free energy and entropy changes ($\Delta G°$ and $\Delta S°$) that

correspond to these transition enthalpies (ΔH_{VH} and ΔH°_{cal}) then can be determined by one of the methods described below.

Calculating ΔG° and ΔS° from Noncalorimetric Melting Curves

Method I

We have shown by Eqs. (2) and (5) that the equilibrium constant K for any two-state association reaction can be expressed in terms of the molecularity of the process n and the extent of association α (which corresponds to the fraction of single-stranded molecules in the complexed state). Consequently, we can evaluate K for a process of any molecularity at any value of α. Usually, a value of K is determined at the melting temperature T_m, where $\alpha = 0.5$ [see Eqs. (3) and (6)]. This $K(T_m)$ value then is extrapolated to some reference temperature T (usually 298 K) using the experimentally measured melting temperature T_m, the calculated van't Hoff transition enthalpy ΔH_{VH} (assumed to be temperature independent), and the integrated form of the van't Hoff equation shown below:

$$\ln[K(T_m)/K(T)] = \frac{\Delta H^{\circ}}{R}\left(\frac{1}{T} - \frac{1}{T_m}\right) \tag{12}$$

Using the calculated value of $K(T)$, one can determine the Gibbs free energy change for the transition using the standard thermodynamic relationship $\Delta G^{\circ} = -RT \ln K(T)$. The corresponding entropy change then can be calculated from the standard equation

$$\Delta G^{\circ} = \Delta H^{\circ} - T\Delta S^{\circ} \tag{13}$$

It should be emphasized that if ΔH° is not known exactly and $(T_m - T)$ is large, the required temperature extrapolation can introduce serious errors.

For a monomolecular process such as hairpin formation, $\ln K(T_m) = 0$ since $K = 1$ at the melting temperature. Consequently, for monomolecular associations, the integrated form of the van't Hoff equation reduces to

$$-\ln K(T) = \frac{\Delta H^{\circ}}{R}\left(\frac{1}{T} - \frac{1}{T_m}\right)$$

Multiplying both sides by RT yields

$$-RT \ln K(T) = \Delta H^{\circ}\left(1 - \frac{T}{T_m}\right) = \Delta G^{\circ} \tag{14}$$

This simplified expression for monomolecular processes can be used to calculate the transition free energy ΔG° at any temperature of interest T from the experimentally measured values of T_m and ΔH_{VH}. The corresponding ΔS° value then can be calculated using Eq. (13). Significantly, this simplified expression for monomolecular processes also can be used to evaluate melting curves of polymer complexes that formally have molecularities greater than

one. This possibility exists because polymer complexes melt in a pseudomonomolecular manner since the growth steps that are monomolecular dominate the bimolecular initiation steps. This pseudomonomolecular behavior is reflected by the fact that the melting temperatures of polymer complexes are concentration independent.

Method II

If a van't Hoff transition enthalpy is determined from concentration-dependent melting studies, a more direct procedure can be employed to determine $\Delta G°$ and $\Delta S°$. Inspection of Eq. (8) or (9) and Fig. 2 reveals that, while the slope of a $1/T_m$ vs $\ln C_T$ plot yields ΔH_{VH}, the intercept permits calculation of $\Delta S°$. These two thermodynamic parameters then can be used to calculate $\Delta G°$ by application of the standard thermodynamic relationship shown in Eq. (13). In principle, for a two-state transition this method should be equivalent to Method I described above. However, since transition enthalpies calculated by Method I are more sensitive to baseline assignments, the thermodynamic profiles calculated by Method II probably are more reliable.

For a monomolecular association or dissociation process $n = 1$. Thus, at the melting temperature, Eq. (8) or (9) reduces to

$$\Delta S° = \frac{\Delta H°}{T_m}$$

Thus, the free energy change $\Delta G°$ at the T_m can be calculated from $\Delta S°$ and $\Delta H°$, and extrapolated to any temperature of interest using Eq. (14).

Significantly, all of the methods outlined above for calculating thermodynamic parameters from equilibrium melting curves are applicable only to transitions that proceed in a two-state manner. Such a characterization of the nature of a transition is not possible based exclusively on the temperature dependence of an equilibrium property. Consequently, additional independent information concerning the nature of a transition is required before the approaches described above can be applied to a given oligomer.

Calculating $\Delta G°$ and $\Delta S°$ from Calorimetric Melting Curves

Method III

As noted earlier, a single calorimetric transition curve gives a direct, model-independent measure of both the heat capacity and enthalpy changes accompanying a thermally induced conformational change. Frequently, these calorimetric data have been used in conjunction with independent equilibrium measurements of $\Delta G°$ to calculate complete thermodynamic profiles. Significantly, however, as described below, this dependence on noncalorimetric data is not necessary.

In a given DSC experiment, one directly obtains a heat capacity (ΔC_p) vs temperature (T) curve [see Fig. 4(a)]. This ΔC_p vs T curve can be converted into a $\Delta C_p/T$ vs T curve [see Fig. 4(b)]. Since $\Delta S° = \int(\Delta C_p/T)\,dT$), the area under such a curve provides a "direct" measure of the entropy change. Thus,

from a single calorimetric transition curve one can obtain ΔC_p, $\Delta H°$, and $\Delta S°$. Using these data, the corresponding value of $\Delta G°$ can be calculated at any temperature. This calorimetric approach has two significant advantages relative to optical methods. First, one obtains a *model-independent* measure of the transition enthalpy and entropy rather than the "two-state values" obtained from analysis of optical data. Second, the calorimetric experiment provides a direct measure of ΔC_p so that the temperature-dependent stability can be assessed. We have used this calorimetric method to characterize thermodynamically the transitions of many oligomers and have compared these results with the corresponding data obtained indirectly from optical melting curves. Significantly, we find that, for transitions that proceed through multiple states, the optically derived values can seriously deviate from the calorimetrically determined values. Only for two-state transitions do the optical methods of analysis described above yield meaningful thermodynamic data. By contrast, the calorimetric method yields meaningful thermodynamic data regardless of the nature of the transition.

Calculating Melting Temperatures

One of the primary practical applications of thermodynamic data is its use in calculating melting temperatures. This generalization particularly applies to molecular biologists who frequently need to predict the thermal stabilities (T_m) of probe-gene complexes or other local DNA duplex domains. A melting temperature can be calculated for any transition by application of Eq. (8) or (9). One simply needs to know the molecularity of the transition n, the total strand concentration C_T (when $n > 1$), and the transition enthalpy and entropy. The latter two thermodynamic parameters can be obtained experimentally or calculated using published nearest-neighbor data.[6,9]

CONCLUDING REMARKS

In this paper we have derived the general forms of equations that can be used to extract thermodynamic data from experimental results obtained by either calorimetric or noncalorimetric techniques. We believe these equations will be of widespread interest considering the large increase in efforts designed to characterize thermodynamically the molecular forces that dictate and control the structural preferences of nucleic acids in solution.

Significantly, the protocols described in this work can be used to evaluate association and dissociation reactions of any molecularity. This feature is particularly important since the modeling of more complex biological structures will involve equilibria well beyond the simple bimolecular level. An example of more complex modeling is illustrated in the paper that follows, where a tetramolecular association reaction is employed to form an immobile junction structure designed to model cruciform formation.

APPENDIX

In this appendix, we present the derivation of the general equation that can be used to calculate ΔH_{VH} for any equilibrium process by evaluation of the width of a *differentiated* melting curve.

We begin by considering the general equilibrium in which n single strands associate to form a n-mer complex:

$$\underset{(n \text{ single strands})}{A_1 + A_2 + A_3 + \cdots + A_n} \leftrightarrow \underset{(\text{a } n\text{-mer complex})}{A_n}$$

As described in the text, if the equilibrium occurs in a two-state manner, we can derive two general expressions for the equilibrium constant. If the associating strands are self-complementary (e.g., identical), the appropriate expression is given in the text as Eq. (5). By contrast, if the associating strands are non-self-complementary (e.g., nonidentical) then the relevant expression for the equilibrium constant is given in the text as Eq. (2). In these general expressions [Eqs. (2) and (5)], α is the fraction of single strands in the helical state and C_T is the total concentration of single strands. Consequently, we can write a general expression for α as

$$\alpha = \frac{n[n\text{-mer complex}]}{C_T}$$

Equations (2) and (5) in the text express how the equilibrium constant varies with C_T and α. The variation of the equilibrium constant with temperature is given by the van't Hoff expression [Eq. (1)]. Integration of this equation between two arbitrary temperatures T_1 and T_2 yields the form of the van't Hoff equation shown in the text as Eq. (12). Solving Eq. (12) for ΔH_{VH} yields

$$\Delta H_{VH} = \frac{-R \ln[K_{T_2}/K_{T_1}]}{[(1/T_2) - (1/T_1)]} \tag{A1}$$

Since the expression in the numerator is a constant for any given values of T_1 and T_2, we can set it equal to B and write

$$\Delta H_{VH} = -\frac{B}{[(1/T_2) - (1/T_1)]} \tag{A2}$$

Thus, ΔH_{VH} can be calculated by dividing the appropriate value of B by the difference between the reciprocals of any two reference temperatures T_1 and T_2. For a process of any molecularity, the specific value of the constant B can be calculated as described below.

We begin by differentiating Eq. (2) or (5) of the text with respect to $(1/T)$ to obtain an expression for $\partial \ln K/\partial(1/T)$. Note that the differential obtained is the same for either Eq. (A1) or (A2). The resulting expression then can be incorporated into the van't Hoff expression [Eq. (1)] to yield

$$\frac{\partial \alpha}{\partial(1/T)} = \frac{\alpha(1-\alpha)}{1 + (n-1)\alpha}\left(\frac{-\Delta H_{VH}}{R}\right) \tag{A3}$$

This expression describes how the temperature dependence of any observable $[\partial\alpha/\partial(1/T)]$ is related to the van't Hoff transition enthalpy. In other

words, Eq. (A3) relates the shape of a differentiated melting curve with ΔH_{VH}. Clearly, if α is known at any two temperatures (T_1 and T_2) a value for B can be defined and ΔH_{VH} can be calculated using Eq. (A2). Thus, we describe below how values of α can be determined at two experimentally convenient temperatures to yield specific values of B.

The temperature corresponding to the maximum of a differentiated melting curve (T_{max}) provides a convenient temperature at which to calculate α. The value of α at T_{max} can be determined by recognizing that, at the maximum of a differentiated melting curve, $[\partial^2\alpha/\partial(1/T)^2] = 0$. Thus, by taking the second derivative of Eq. (A3) and setting it equal to zero we obtain

$$\left[\frac{\partial\alpha}{\partial(1/T)}\right]_{max} = \frac{1}{(\sqrt{n}+1)^2}\left(\frac{-\Delta H_{VH}}{R}\right) \tag{A4}$$

Thus, at $T = T_{max}$, $\alpha_{max} = 1/(\sqrt{n}+1)$.

By selecting two temperatures, T_1 and T_2 at which $\partial\alpha/\partial(1/T)$ is equal to one half of $[\partial\alpha/\partial(1/T)]_{max}$, we have two additional convenient points at which to calculate α. Thus we can write

$$\left[\frac{\partial\alpha}{\partial(1/T)}\right]_{T=T_1 \text{ or } T_2} = \frac{\alpha(1-\alpha)}{1+(n-1)\alpha}\left(\frac{-\Delta H_{VH}}{R}\right) = \left(\frac{1}{2}\right)\frac{1}{(\sqrt{n}+1)^2}\left(\frac{-\Delta H_{VH}}{R}\right) \tag{A5}$$

This Eq. (A5) reduces to

$$2(1+\sqrt{n})\alpha^2 - (3+\sqrt{n})\alpha + \frac{1}{\sqrt{n}+1} = 0 \tag{A6}$$

Solving for α, we obtain

$$\alpha_{\pm} = \frac{(3+\sqrt{n}) \pm \sqrt{(n+6\sqrt{n}+1)}}{4(1+\sqrt{n})} \tag{A7}$$

where $\alpha_+ = \alpha_1$ at T_1 and $\alpha_- = \alpha_2$ at T_2.

Substituting these values of α into Eq. (2) or (5), and then into Eq. (A1) yields

$$\Delta H_{VH} = -R \ln\left[\frac{\alpha_2(1-\alpha_1)^n}{\alpha_1(1-\alpha_2)^n}\right] \Big/ \left[\frac{1}{T_1} - \frac{1}{T_2}\right] \tag{A8}$$

Comparison of Eqs. (A8) and (A2) allows us to conclude that

$$B = R \ln\left[\frac{\alpha_2(1-\alpha_1)^n}{\alpha_1(1-\alpha_2)^n}\right] \tag{A9}$$

This general expression can be used to calculate a specific value of B for a

reaction of any molecularity n. We have listed some of these values in Table I. B values should be used when the full width of the curve at the half-height is evaluated between T_1 and T_2, while B' values should be used when only the width of the upper half of the curve is evaluated at the half-height between T_{max} and T_2.

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