MEASUREMENT AND ANALYSIS OF RESULTS OBTAINED ON BIOLOGICAL SUBSTANCES WITH DIFFERENTIAL SCANNING CALORIMETRY

(IUPAC Technical Report)

Prepared for publication by
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Measurement and analysis of results obtained on biological substances with differential scanning calorimetry

(IUPAC Technical Report)

Abstract: Differential scanning calorimeters (DSCs) have been widely used to determine the thermodynamics of phase transitions and conformational changes in biological systems including proteins, nucleic acid sequences, and lipid assemblies. DSCs monitor the temperature difference between two vessels, one containing the biological solution and the other containing a reference solution, as a function of temperature at a given scan rate. Recommendations for DSC measurement procedures, calibration procedures, and procedures for testing the performance of the DSC are described. Analysis of the measurements should include a correction for the time response of the instrument and conversion of the power vs. time curve to a heat capacity vs. temperature plot. Thermodynamic transition models should only be applied to the analysis of the heat capacity curves if the model-derived transition temperatures and enthalpies are independent of the DSC scan rate. Otherwise, kinetic models should be applied to the analysis of the data. Application of thermodynamic transition models involving two states, two states and dissociation, and three states to the heat capacity vs. temperature data are described. To check the operating performance with standard DSCs, samples of 1 to 10 mg mL⁻¹ solutions of hen egg white lysozyme in 0.1 M HCl-glycine buffer at pH = 2.4 ± 0.1 were sent to six different DSC laboratories worldwide. The values obtained from proper measurements and application of a two-state transition model yielded an average unfolding transition temperature for lysozyme of 331.2 K with values ranging from 329.4 to 331.9 K, and an average transition enthalpy of 405 kJ mol⁻¹ with values ranging from 377 to 439 kJ mol⁻¹. It is recommended that the reporting of DSC results be specific with regard to the composition of the solution, the operating conditions and calibrations of the DSC, determination of base lines that may be model-dependent, and the model used in the analysis of the data.

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1. INTRODUCTION

Differential scanning calorimeters (DSCs) are widely used for the study of phase transitions and conformational changes in biological systems including proteins, nucleic acids, and lipid assemblies. From a single differential calorimetric scan, it is possible, in principle, to determine the following thermodynamic information for a reversible conformational transition: the transition temperature, the transition enthalpy, the heat capacity change accompanying the transition, the cooperativity, and the stoichiometry of the transition. Irreversible transitions in biological systems that are kinetically controlled have also been studied with DSCs. Rate constants and activation energies for the formation of the final irreversible state can be determined from these kinetically controlled changes. To optimize the scientific value of studies employing differential scanning calorimetry on biological substances, it is expeditious to develop a set of recommendations on the measurement procedures and on the analysis of results from DSC measurements.

A number of reviews on the application of DSC measurements to the study of biological systems have appeared over the past two decades. Privalov [1–2] has published several reviews on the analysis of the transition data obtained from DSC scans on simple and complex proteins in solution and on the design and operating characteristics of the DSC. The types of thermodynamic information obtainable from DSC measurements and the characteristics of commercially available DSCs have been also described in detail by Hinz [3], Chowdhry and Cole [4], and Benoist [5] and, more recently, by Pfeil [6], Leharne and Chowdhry [7], and Robertson and Murphy [8]. More highly sensitive DSCs that employ computer algorithms to correct for heat losses and changes in the scan rate during the DSC measurement and, thus, increase the precision of the DSC measurements have recently become commercially available [9,10]. Absolute heat capacity values of solutions as a function of temperature can be determined from these more highly sensitive DSCs [11]. Biltonen [12], Sturtevant [13], and Mabrey [14] have published descriptions on applications of DSC measurements to the study of various biological systems including lipid-phase transitions. DSC measurements have been used to determine the energetics of DNA transitions in solution [15]. Detailed DSC studies have appeared on the thermodynamics of ligand binding interactions [16,17], particularly ultratight interactions [18], intramolecular interactions in large proteins [19–21], and the effect of single mutations on the thermal stability of proteins [22]. DSC measurements have been made on the stability of proteins inserted in reverse micelles [23] and on the stability of proteins immobilized on solids [24]. DSC measurements have also been used to determine preferential solvation changes in biopolymers upon heat denaturation in mixed solvents [25]. In addition, DSC measurements have been employed in determining the rate constants and activation energy of irreversible transitions for proteins from the dependence of the transition properties on scan rate [26]. On a more applied level, DSC measurements have been employed in the determination of the amount of free water in brain tissue [27], of the stability of proteins in rabbit brain membrane fractions [28], of the effects of manufacturing operations on proteins in foods [29,30], and of new methods to improve freeze-drying of labile biological material [31].

Differential scanning calorimetry consists of measuring the temperature difference between a sample solution and a reference solution as a function of temperature. This temperature difference, which is expressed in units of a power difference, is converted to a difference in the heat capacity between the sample solution and the reference solution as a function of temperature. First-order transitions in the biological sample solution are observed as large discrete changes in this heat capacity dif-
ference over a small temperature range, i.e., a transition peak. The following quantities, which are specified here for proteins, but can, accordingly, be applied to other systems such as polynucleotides and lipid assemblies, can be determined from a DSC scan.

- The transition or calorimetric enthalpy ($\Delta_{trs}H$), which is the heat capacity difference between the sample and baseline integrated over the transitional temperature range per amount of protein in g or mol undergoing the transition.
- In general, three characteristic temperatures can be determined [32]: (i) $T_{1/2}$, which is the temperature where 50% of the protein population is unfolded and, thus, this temperature is observed by definition at $\alpha_u = 0.5$; (ii) $T_m$, the temperature of the maximum of the transition peak; and (iii) $T_G$, the temperature where the standard molar Gibbs energy change of the transition $\Delta_{trs}G^o$ is equal to zero. It is important to note that only for a two-state transition with a stoichiometry of 1:1, i.e., $N = U$, do these three temperatures coincide. For all other stoichiometries, $T_{1/2} < T_m < T_G$.
- The difference between the extrapolated pre- and posttransitional baselines at the transition temperature, i.e., the difference between the heat capacities of the protein in its native state and unfolded state ($\Delta_{trs}C_p$).

Additional valuable information can be obtained from the analysis of the shape of the transition peak, provided that the shape is not determined by kinetic effects. For example, application of the appropriate thermodynamic model to the baseline-corrected transition profile yields a van’t Hoff enthalpy ($\Delta_{trs}H_{vH}$) for the transition and the stoichiometry for the transition. For transitions, which exhibit a dependence of $T_m$ on scan rate after correcting for the time response of the DSC, information can be obtained only in terms of a kinetic model for the transition. With the recent, more highly sensitive DSCs an important advancement has been made: it is now feasible to obtain absolute partial heat capacities of biological molecules over a large temperature range. These theoretically significant values can be determined by subtraction of the heat capacity data for the buffer vs. buffer scan from the heat capacity data for the solution vs. buffer scan. The absolute partial heat capacities yield information not only on conformational changes of the biological substance as a function of temperature in solution but also on hydration effects accompanying these changes [11]. In case of cold denaturation, where the denatured state occurs at a lower temperature than the native state, it is recommended to use primed symbols for the parameters, for example $T_G'$ for the transition temperature, $\Delta_{trs}H'$ for the transition enthalpy, etc.

2. RECOMMENDATIONS FOR DSC MEASUREMENTS

2.1. DSC measurement procedures

A sample solution should be well characterized as to its pH at the transition temperature, its ionic strength, and the concentrations of chemical substances. It is recommended that DSC vessels which are built into the DSC be completely filled to the known volume of the vessel while removable DSC vessels be filled with identical masses of the sample solution and reference solution. In general, for the built-in DSC vessels, the sample and reference solutions should be de-gassed by stirring under vacuum prior to filling the DSC vessels to minimize the formation of air bubbles in the sample. It is advisable to make sure that no concentration changes result from this procedure.

It is recommended that prior to the scan, the DSC vessels be equilibrated at a starting temperature sufficiently below the onset of the transition so as to allow for a sufficient pretransitional baseline to accurately extrapolate this baseline to $T_G$ under the transition peak. The sample should be scanned to a temperature above the temperature where the heat capacity returns to the baseline value so as to allow for a sufficient posttransitional baseline to accurately extrapolate this baseline to $T_G$ under the transition peak. The samples should be rapidly cooled after the transition and then rescanned after thorough equilibration at low temperature to determine if the transition reappears and, thus, may be con-
considered repeatable. Alternatively, controlled cooling scans could be performed, if the particular DSC permits this mode of operation. However, the usually required equilibration time at elevated temperature before the start of the cooling scan may be potentially harmful to the sample since prolonged high temperature exposure of the unfolded state of a biological substance may enhance aggregation and degradation, thereby reducing repeatability of the transition.

It is recommended that a baseline scan consisting of the reference solution in both the sample and reference vessels be performed and then subtracted from the sample solution vs. reference solution scan to obtain the dependence of the heat capacity of the biological substance on temperature. In many DSCs, a baseline of buffer vs. buffer or water vs. water is scanned first, stored in a computer file, and then used to automatically subtract the baseline scan from the DSC scan of a sample vs. the buffer. When initiating a new series of DSC scans, it is standard procedure to run first the buffer vs. buffer scan several times before running the sample vs. buffer scans. The second or latter buffer vs. buffer scan is usually used for the baseline scan, since, by experience, the first scan frequently deviates from all subsequent scans.

It is recommended that DSC scans be performed on identical samples at different scan rates to determine any dependence of the shape of the transition peak on scan rate arising from the response time of the DSC or the irreversible nature of the transition. Samples of the solution should be scanned at different concentrations to determine any dependence of $T_{1/2}$, $T_m$, and $T_G$ on concentration. Other methods such as ultracentrifugation, light-scattering measurements, or mass spectrometry should be employed to characterize the oligomeric state of the initial, low-temperature form of the biological substance [33].

2.2. DSC calibration and test procedures

Both the differential power and temperature calibrations should be performed on a regular basis since the calibration constants may change with time. Preferentially, the manufacturer’s procedures should be used to perform the power and temperature calibrations of the DSC. It is recommended that test solutions be also scanned to further evaluate the operating performance of the DSC. A test solution of a very pure dialkylphosphatidylcholine suspension in phosphate buffer can be used to check the temperature calibration of the DSC since these suspensions exhibit extremely narrow gel to liquid phase transition peaks at well-known temperatures [34]. However, it is important to follow established preparation procedures for multilamellar vesicles in order to obtain consistent results for transition temperatures. The phase transitions occur at temperatures from 287 K for 1,2-ditridecanoyl-sn-glycero-3-phosphocholine to 354 K for 1,2-ditetrascanoyl-sn-glycero-3-phosphocholine in sodium phosphate buffer at pH = 7.0. Since the transition peak half-widths are <0.2 K, $T_m$ is recorded as the transition temperature. If the DSC has a slow response time, then the DSC scan should be performed at different scan rates and the lipid results extrapolated to zero scan rate to obtain the correct transition temperature. The functional dependence of $T_m$ of the lipid-phase transition on scan rate might be used to determine the time response of the instrument which may be needed to correct for response time distortion of the DSC signal as described below. However, recording of the response of the DSC instrument to an instantaneous electrical calibration power signal is probably a better basis for the evaluation of the response time of the instrument.

Commercially available DSCs have internal calibration heaters proximal to the DSC vessels, which supply known quantities of heat to the DSC vessel for calibration. The calibration heater provides a power pulse of known output in terms of voltage and current. The power difference between the solution and reference vessels is recorded before and after the power pulse to obtain an average baseline reading so that the power reading corresponding to the power pulse can be subtracted from this average to obtain the instrument power reading $\Delta P(T)$ due to the power pulse, $\Delta P_p(T)$. Since the response of the thermoelectric sensors is temperature-dependent, this calibration should be performed
as a function of temperature to obtain an analytical expression \((F(T))\) for the conversion of the instrument power reading into the true power difference as a function of temperature, so that

\[
\Delta P(T) = F(T) \Delta P_p(T)
\] (1)

Usually, this conversion is performed automatically by the DSC software so that the “raw” data are actually in the form of \(\Delta P(T)\) vs. temperature.

It is also recommended that test solutions consisting of protein solutions that exhibit known transition enthalpies such as simple globular proteins like ribonuclease A [35], lysozyme [36], and lactoglobulin [37] be scanned to check the operating performance of the DSC. To evaluate the applicability of a protein test solution in checking the operating performance of different DSCs in different laboratories, samples of hen egg white lysozyme solutions were sent to six different international laboratories. The lysozyme was 95 mass % pure and the samples, which ranged from 1 to 10 mg mL\(^{-1}\) in concentration, were extensively dialyzed in 0.1 M HCl-glycine buffer at pH = (2.4 ± 0.1). The results of these independent measurements are summarized in Table 1. The average value for the transition temperature \(T_G\) was 331.2 K with values ranging from 329.4 to 331.9 K. The average transition enthalpy \((\Delta_{\text{trs}} H)\) was 405 kJ mol\(^{-1}\) with values ranging from 377 to 439 kJ mol\(^{-1}\) as shown in Table 1. Some of the variation in \(\Delta_{\text{trs}} H\) might be attributed to transportation of the lysozyme samples for different amounts of time and under different temperature conditions. In view of these possible problems, the agreement between the results is very good. Calculations of the van’t Hoff enthalpy \(\Delta_{\text{vH}} H\) determined from the application of a \(N = U\) two-state thermodynamic transition model to the heat capacity difference data yield an average value of 395 kJ mol\(^{-1}\) with a range of 363 to 418 kJ mol\(^{-1}\) as shown in Table 1. Values of \(\Delta_{\text{vH}} H\) depend on the determination of the amount of lysozyme in the sample whereas values of the van’t Hoff enthalpy are independent of this determination. The test solution was scanned from 293 to 363 K at a scan rate of 60 K h\(^{-1}\) since some aggregation may occur at slower scan rates. The concentration of the lysozyme solutions was determined from UV absorption measurements at 280 nm where a 1 mg mL\(^{-1}\) solution in a 1-cm cell has an optical density of 2.65 [38]. The molar mass of lysozyme used in the calculation was 14 321 g. The lysozyme can be obtained commercially at high enough purity so that it is only necessary to dialyze the lysozyme solution in the glycine buffer to dilute any salts present in the samples prior to the DSC scan. The test solutions should be freshly prepared since upon storage in a refrigerator, lysozyme tends to aggregate with time.

3. RECOMMENDATIONS FOR THE PRESENTATION OF DSC DATA

3.1. Introduction

Since the interpretation of DSC data may be dependent on scan rate, it is necessary to correct for any time-dependent distortion of the signal. The more recent DSCs exhibit minimal time distortions of the signal while older models may exhibit large time distortions that can be corrected as described below. Determining the dependence of the transition parameters on scan rate after correcting for instrument response time is crucial in determining whether a thermodynamic or kinetic model should be applied to the analysis of the data. In addition, the large variety of models that can be applied to the interpretation of DSC data necessitates a complete description of the model employed in the analysis. Application of the models to the data is also dependent on the extrapolation of the pre- and posttransitional baselines under the transition peak and, thus, this extrapolation should be adequately described.

3.2. Correction for the time response of the DSC

The design of the DSC results in a time response or “thermal lag” between the time an “instantaneous” change in heat transfer occurs in the solution vessel and the time this change is monitored. This thermal lag results from the rate of heat transfer to the thermoelectric sensor and the electronic processing
circuitry, which is designed with high- and low-band pass filters to minimize electronic noise. Any thermal lag can result in a distortion of the shape of a DSC power pulse that would affect the DSC quantities determined from the profile shape of the transition, as for example, the van’t Hoff enthalpy \( \Delta_{\mathrm{trs}} H_{\nu \mathrm{H}} \). To determine the effect of any thermal lag on the DSC measurements, the DSC scans should be performed at different scan rates with a change in scan rate of at least an order of magnitude. Any dependence of the DSC thermodynamic quantities on scan rate due to the slow response time of the DSC can be corrected by the Tian equation which relates the observed power difference \( \Delta P_o(t) \) to the instantaneous power change, \( \Delta P \) as follows [39].

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Table 1 DSC results on test solutions of lysozyme in 0.1 M HCl-glycine buffer.

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<th>( T_G )</th>
<th>( \Delta_{\mathrm{trs}} H )</th>
<th>( \Delta_{\mathrm{vH}} H_{\nu \mathrm{H}} )</th>
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\(^a\)Letters A through F include the NanoDSC, VPDSC, MC-2, SCAL 1, DASM-4, and DASM-1.

\(^b\)Laboratory 5 redialyzed these samples for a second scan. \(^c\)Laboratory 5 made up this sample.
\[ \Delta P = \Delta P_0(t) + \tau \frac{d\Delta P_0(t)}{dt} \] (2)

where \( \tau \) is the “thermal lag” time or response time of the calorimeter. The response time can be obtained from monitoring the time response of an “instantaneous” power pulse, for example, from turning off the DSC calibration heater or from a dialkylphosphatidylcholine gel-liquid phase transition which occurs over a narrow temperature range of about 0.2 K in dionized water.

It is recommended to apply the Tian equation correction to the initial power difference vs. temperature data to determine if this correction makes the DSC quantities independent of scan rate. A similar dynamic correction has been developed by Freire et al. [40] and, more recently, by Kirchhoff [41]. The response time of the DSC should be reported, if a correction has been applied.

### 3.3. Conversion of DSC data to heat capacity vs. temperature data

It is recommended that the DSC results be presented in terms of specific heat capacity difference in J K\(^{-1}\) g\(^{-1}\) (\(C_p(T)\) g\(^{-1}\)) or molar heat capacity J K\(^{-1}\) mol\(^{-1}\) (\(C_p(T)\) mol\(^{-1}\)). If molar values are reported, the molar mass used for conversion of the specific heat capacities should be reported. DSC data are initially obtained in power differences \(\Delta P(T)\) as a function of temperature as just described. They are converted into heat capacity differences by dividing the power differences at each temperature by the scan rate at that temperature. Since the scan rate may not be constant with temperature in older DSCs, it is recommended that an appropriate analytical expression be determined for the scan rate. For example, if temperature \(T\) changes with time \(t\) according to the equation,

\[ T = at + bt^2 + ct^3 \] (3)

where \(a\), \(b\), and \(c\) are constants, then taking the first derivative:

\[ \frac{dT}{dt} = a + 2bt + 3ct^2 \] (4)

yields the scan rate. Therefore at a given temperature,

\[ C_p = \frac{\Delta P}{(dT/dt)}. \] (5)

### 4. RECOMMENDATIONS FOR THE ANALYSIS OF DSC DATA

#### 4.1. Transition baseline extrapolations

Essential to the analysis of the transition peak data is the extrapolation of the baseline. It is recommended that the pre- and posttransitional baselines be determined first by least square fits. The pretransitional baseline can usually be approximated very well by a straight line. The posttransitional baseline is in principle not linear [42]. However, for short extrapolations down to the transition temperature, a linear extrapolation is frequently acceptable. The difference between the extrapolated baselines at the transition temperature is a good approximation of the transitional heat capacity change, \(\Delta_{\text{trs}}C_p(T)\). This extrapolation may be further refined into a sigmoidal baseline where the pretransitional baseline and the posttransitional baseline extrapolations mimic the normalized progress of the unfolding transition as a function of temperature, as first described by Takahashi and Sturtevant [43] and later by Kirchhoff [41]. The type of extrapolated baseline used in the analysis should be specified.

Any aggregation observed in the sample after the transition is completed may induce shifts in the posttransitional baseline because of heat contributions from the aggregation process [44]. The presence of aggregates in the sample may be ascertained by another method, e.g., light-scattering measurements on the sample, after the DSC scan. The DSC scanning conditions can sometimes be modified by using a faster scan rate or minimizing the temperature range of the posttransitional scan to reduce the effect of aggregation on the baseline.
4.2. Analysis of transitions that can be described by equilibrium thermodynamic models

It is recommended that a thermodynamic model be used to analyze the transition if the transition is repeatable, and if \( T_m \) is independent of scan rate. Although repeatability implies that the transition can be reversed within the cooling time of the instrument, there may be cases where a repeatable transition may still exhibit a scan rate dependence because equilibration between the two states proceeds slowly compared to the scan rate of the instrument [33]. In this special case, the results should be extrapolated down to zero scan rate. A detailed description of fitting these thermodynamic models to DSC data has been described by Kirchhoff [41]. The model may be a simple two-state model in going from a native conformation to an “unfolded” state (N = U), involving dissociation (N\(_n\) = nU), or more complex models involving intermediate states. The following criteria are useful in the simplest cases; i.e., for the N = U and N\(_2\) = 2U models to choose the appropriate analysis procedure:

- The state of dimerization of the sample in its initial and final states: If it exists as a dimer only in the initial state, then the N\(_2\) = 2U model may be applicable.
- The shape of the transition profile: A transition profile that is unsymmetrical about the transition temperature may result from the presence of additional transitions occurring at slightly different temperatures or from dissociation-association processes [45] occurring during the transition.
- The dependence of \( T_{1/2} \), \( T_m \), and \( T_G \) on sample concentration: If these temperatures increase with sample concentration, then apparently dissociation accompanies the transition and the N\(_2\) = 2U model is applicable.

For the simplest case of a two-state transition, the temperature dependence of the enthalpy (H) relative to the native state (H\(_N\)), the van’t Hoff enthalpy, \( \Delta_{\text{trs}}H_{\text{vH}} \), is given by the equation:

\[
\Delta_{\text{trs}}H_{\text{vH}} = H - H_N = \frac{K_{\text{trs}}}{1 + K_{\text{trs}}} \Delta_{\text{trs}}H(T) = \alpha_U \Delta_{\text{trs}}H(T)
\]

where \( K_{\text{trs}} = [U]/[N] \) is the equilibrium constant of the two-state N = U transition and \( \alpha_U \) is the degree of the transition that has gone to completion at temperature T. A simplified form of this equation derived by Sturtevant is as follows:

\[
\Delta_{\text{trs}}H_{\text{vH}} = 4RT_m^2 \frac{C_p(\text{max})}{(\text{peak area})},
\]

where \( C_p(\text{max}) \) is the maximum peak height of the transition in J K\(^{-1}\) g\(^{-1}\) and (peak area) is the area of the transition peak in J g\(^{-1}\). The corresponding variation of the heat capacity, \( C_p \), is given by the following equation where \( \bar{N}C_p \) is the heat capacity of the initial state:

\[
C_p = \bar{N}C_p + \Delta_{\text{trs}}C_p \frac{K_{\text{trs}}}{1 + K_{\text{trs}}} + \left( \frac{\Delta_{\text{trs}}H(T)}{RT} \right)^2 \frac{K_{\text{trs}}}{1 + K_{\text{trs}}} \right)
\]

The relation of the equilibrium constant to the standard Gibbs energy change for the transition (\( \Delta_{\text{trs}}G^0 \)) is \( K_{\text{trs}} = \exp(-\Delta_{\text{trs}}G^0/RT) \). Both \( C_p \) and the van’t Hoff enthalpy are model dependent.

The following equations can be used to fit a two-state transition of the type, N\(_2\) = 2U where \( K_{\text{trs}} = [U]/[N_2] \), to the data [32, 46]:

\[
\Delta_{\text{trs}}H_{\text{vH}} = H - H_N = \frac{\alpha_U}{1 + \alpha_N} \Delta_{\text{trs}}H(T)
\]

and

\[
C_p = \bar{N}C_p + \Delta_{\text{trs}}C_p \frac{\alpha_U}{1 + \alpha_N} + \frac{(\Delta_{\text{trs}}H(T))^2}{RT^2} \frac{2\alpha_N \alpha_U}{(1 + \alpha_N)^3}.
\]

where \( \alpha_U = [U]/(2[N_2] + [U]) \) and \( \alpha_N = 2[N_2]/(2[N_2] + [U]) \).

If an intermediate is involved in the transition to the unfolded state, the transition mechanism can be described by the following equations,
N = I = U with $^1K = [I]/[N]$ and $^2K = [U]/[I]$, (11)

For the enthalpy change, 
\[
\Delta_{\text{trs}}H_{\text{vH}} = H - H_N = \Delta_{\text{trs}}^1H (\alpha_I + \alpha_U) + \Delta_{\text{trs}}^2H\alpha_U,
\]
and the corresponding equation for fitting the heat capacity curve is, 
\[
C_p = NC_p + \Delta_{\text{trs}}^1C_p (\alpha_I + \alpha_U) + \Delta_{\text{trs}}^2C_p\alpha_U + \left(\frac{(\Delta_{\text{trs}}^1H)^2}{RT^2}\right)\alpha_N (\alpha_I + \alpha_U) + \\
\left\{\frac{(\Delta_{\text{trs}}^2H)^2}{RT^2}\right\}\alpha_N \alpha_U.
\]

The $\alpha$ values refer to the respective degree of transition of the species N, I, and U. The superscript 1 refers to the transition from the folded state to the intermediate state, and the superscript 2 refers to the transition from the intermediate state to the unfolded state. In general, when numbering the transition parameters, the transition referred to by the number should be clearly specified. The formula for more complex transition models can be found in ref. 47. However, the requirements for very precise data become in these cases so high that under normal circumstances, the unambiguous discrimination between the different types of transition models may become very difficult.

A qualitative indicator of whether a transition involves intermediates is the cooperativity of the transition, which can be calculated from the ratio of the van’t Hoff enthalpy to the transition enthalpy, $\Delta_{\text{trs}}H_{\text{vH}}/\Delta_{\text{trs}}H$. For proteins, a cooperativity $<1.00$ implies a sequence of intermediate transitions in the observed transition profile. This can be analyzed in terms of the intermediate state transition model as just described. These transitions may be identified from structural information as transitions of different physical components of the sample, which occur within the observed transition profile. A cooperativity $>1.00$ may indicate that an aggregate of the sample is unfolding at the same temperature. It should then be ascertained as to whether any of the previous models could be applied to the analysis of the DSC data.

If the transition is not reversible and independent of scan rate, it may still be analyzed in terms of a thermodynamic model provided certain kinetic conditions hold. The basis for this is the fact that the transition itself is reversible between the N and U states but that the U state converts to an irreversible X state on a time scale much slower than that of the transition [48]:

\[
N = U \rightarrow X \text{ (irreversible)}
\]

In such a case, if it can be shown experimentally that the transition temperature shift with scan rate approaches a limiting value at high scan rates, an equilibrium analysis using any of the formula given above may be valid. Further details can be found in ref. 47. It is recommended that these tests be stated clearly if such a thermodynamic model is applied to a transition that does not reappear upon rescanning of the solution.

A sample may exhibit transition profiles at different temperatures, and each of the transition peaks may be analyzed by the models just described if they are repeatable or exhibit transition quantities that are scan rate-independent. These multiple transitions may be conceptualized in terms of parts of the system termed (thermodynamic) domains, e.g., substructures of the sample, undergoing a conformational change. A (thermodynamic) domain may be identified with one or several structural domains of the sample from X-ray crystallography or NMR information. Additional information can be obtained by comparing the transition properties of the system to the transition properties of the domains if the domains can be isolated as separate entities [19,21]. For example, for proteins, this can be done by limited proteolysis, by synthesizing the domain via molecular biological methods, and/or by peptide synthesis. Differences between the transition quantities determined from the domain transition of the sample and the transition of the isolated domain can be identified as the thermodynamic quantities that characterize the domain–domain interactions in the sample [19]. It is recommended that multiple transitions in a DSC scan be identified by the transition temperature of each transition peak and by the corresponding transition enthalpies and, if possible, by the transition heat capacity changes.

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4.3. Phase diagrams of the stability of the initial state of the sample

DSC scans are usually employed to determine the thermal stability of a biological substance as a function of temperature. This information is usually conveyed in the form of phase diagrams where the standard Gibbs energy of a transition or one of its components, the transition enthalpy or/and transition entropy, is plotted as a function of the temperature. The phase diagram can be determined using the following thermodynamic equation,

$$\Delta_{\text{trs}} G^0(T) = \Delta_{\text{trs}} H^0(T) - T \Delta_{\text{trs}} S^0(T)$$  \hspace{1cm} (15)

and the heat capacity change, $\Delta_{\text{trs}} C_p$. For a reversible two-state transition at $T_G$, $\Delta_{\text{trs}} G^0 = 0$ yielding $\Delta_{\text{trs}} H^0/T_G = \Delta_{\text{trs}} S^0$, then

$$\Delta_{\text{trs}} G^0(T) = \Delta_{\text{trs}} H^0(T_G) - T \Delta_{\text{trs}} S^0(T_G) + \Delta_{\text{trs}} C_p(T - T_G - T \ln(T/T_G))$$  \hspace{1cm} (16)

Values for $\Delta_{\text{trs}} C_p$ should be obtained from the difference between the extrapolated pre- and posttransitional baselines extrapolations. Determination of $\Delta_{\text{trs}} C_p$ from $d(\Delta_{\text{trs}} H)/dT = \Delta_{\text{trs}} C_p$ assumes that $\Delta_{\text{trs}} C_p$ is temperature-independent, which is not always the case. However, from a pragmatic point of view, this procedure often provides an acceptable value since determination of $\Delta_{\text{trs}} C_p$ from individual transition peaks can yield strongly scattering values unless extreme care is taken to perform the measurement. A more detailed description of the fundamental relationships between $\Delta_{\text{trs}} H$, $\Delta_{\text{trs}} S$, and $\Delta_{\text{trs}} C_p$ can be found in ref. 49.

4.4. Extraneous effects on thermodynamic transition quantities

Not only pH, but also the presence of a cosolvent may affect the transition temperature if the cosolvent promotes the stabilization of the N or U state relative to the other state. If the N state is further stabilized by the cosolvent, then an increase in $T_G$ would be observed, whereas if the U state is stabilized, a decrease in $T_G$ would be observed. For example, simple globular proteins with a relative large number of hydrophobic residues, unfold at lower transition temperatures as the amount of ethylene glycol is increased in the solution since the interior hydrophobic residues are exposed to the more favorable hydrophobic solvent in the U state [50].

In the presence of a ligand that binds tightly only to the N state, the transition peak may broaden or appear as two transition peaks at subsaturate ligand concentrations and then appear as a single transition at saturating ligand concentrations [16,18]. In the simplest case where ligand binding only enhances the stabilization of the N state, the broadening and doubling of the transition is maximal at one half saturation of the ligand binding sites and depends on the product of the binding constant ($K_b$) and the site concentration ($K_b[\text{Site}]$). Such broadening is evident at $K_b[\text{Site}] = 10$ for simple globular proteins [18]. For weaker binding ligands, the broadening of the peak is barely detectable, and super saturated concentrations of ligand have to be employed to observe shifts in $T_G$. For both cases and using a two-state transition model, the binding constant and enthalpy of binding can be obtained at the transition temperature. In fact, for ultra-tight ligand-protein binding reactions, DSC is a well-suited technique since the double peak separation increases with the binding constant. At super saturation concentrations, the transition temperature shift may be reduced if the ligand binds to additional sites exposed in the U state. In more complex proteins, ligand binding may also have additional affects, e.g., inducing additional conformational changes in the protein [44], influencing domain–domain interactions [51], which affect the DSC results. Again, independent methods should be employed to determine the effects of ligand binding on the transition between the native and unfolded state.

4.5. Analysis of transitions that depend significantly on scan rate

If, after correcting for the time response of the DSC, the transition quantities still exhibit a scan rate dependence, then thermodynamic models are not applicable. The information content of these scans is
thermodynamically not very useful since it will depend on scan rate and, thus, on the design of the DSC. However, kinetic models have been developed for analysis of these scan dependent transitions such as those described in references [26,32,52–54].

5. RECOMMENDATIONS ON THE REPORTING OF RESULTS

1. SI units and their internationally recognized symbols as listed in the Glossary should be employed. Functional expressions should be used for specifying the pH, ionic strength, buffer, and temperature where the transition occurs. For example, for the calorimetric transition enthalpy of the unfolding of a protein at pH = 3 the transition temperature of \( T_G = 330 \) K and the ionic strength \( I = 0.1 \) mol dm\(^{-3}\) information should be provided in the following manner;

\[
\Delta_{trs} H(pH = 3.00, I = 0.10 \text{ mol dm}^{-3}, T_G = 330 \text{ K})
\]  

(17)

Since the pH of the buffer may be temperature-dependent, the pH value given should be preferentially the pH at the transition temperature. For practical reasons, it may be difficult to determine the pH at high temperature. In such cases, the pH at 298 K should be specified with the implication that the heat of ionization of the buffer will determine the temperature dependence of pH. The composition of the solution, the molecular mass, and the purity of the sample (protein, DNA, lipid assembly, etc.) should also be stated.

2. When the determination of protein and DNA concentration involves UV absorption measurements, the absorption coefficient together with the wavelength used for the particular mass of protein or DNA should be stated. It is recommended that the method of preparation should be described for lipid assemblies.

3. The experimental results should be reported as specific heat capacities, \( c_p \), or molar heat capacities, \( C_p \), as a function of the thermodynamic temperature. Power is not recommended since its value depends on the scan rate of the DSC. It is recommended that the calorimetric enthalpy, \( \Delta_{trs} H \), for a particular transition be in terms of mole of sample of the N state and that \( \Delta_{trs} C_p \) obtained from the extrapolation of the baselines be reported.

4. The scan rate, \( dT/dt \), and the amount of sample undergoing the thermal transition in moles should be reported.

5. The model and the analytical expressions used to determine the reported calculated van’t Hoff enthalpy, \( \Delta_{trs} H_{vH} \), should be reported since the calculated van’t Hoff enthalpy depends on the thermodynamic model or kinetic model used to analyze the transition.

6. Uncertainties in the reported quantities should be stated. The uncertainties should be based on a valid statistical method for treating data as described in ref. 55. Examples are the standard deviation of a mean value of \( T_G \) from several scans of identical samples, standard deviations of parameters from least squares fits of the models to the data, etc. The statistical uncertainties often do not reflect all possible errors, and it is often necessary to report estimated uncertainties based on estimates of possible systematic errors in the final result.

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## GLOSSARY

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Name</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_p$</td>
<td>molar heat capacity of a sample solution relative to a reference solution in a DSC scan at constant pressure</td>
<td>J K$^{-1}$ mol$^{-1}$</td>
</tr>
<tr>
<td>$c_p$</td>
<td>specific heat capacity</td>
<td>J K$^{-1}$ g$^{-1}$</td>
</tr>
<tr>
<td>$\Delta_{trs}C_p$</td>
<td>heat capacity change at $T_G$ in the solution associated with a transition</td>
<td>J K$^{-1}$ mol$^{-1}$</td>
</tr>
<tr>
<td>$T_{1/2}$</td>
<td>temperature at which 50% of the sample has been converted to the unfolded state (i.e., $\alpha_U = 0.5$)</td>
<td>K</td>
</tr>
<tr>
<td>$T_m$</td>
<td>temperature at which the transition peak exhibits the maximum heat capacity</td>
<td>K</td>
</tr>
<tr>
<td>$T_G$</td>
<td>temperature at which $\Delta_{trs}G^o = 0$</td>
<td>K</td>
</tr>
<tr>
<td>$K_{trs}$</td>
<td>equilibrium constant for a two-state transition</td>
<td>dimensionless</td>
</tr>
<tr>
<td>$\Delta_{trs}G^o$</td>
<td>standard Gibbs energy of a transition</td>
<td>J mol$^{-1}$</td>
</tr>
<tr>
<td>$\Delta_{trs}H$</td>
<td>transition enthalpy</td>
<td>J mol$^{-1}$</td>
</tr>
<tr>
<td>$\Delta_{trs}S$</td>
<td>van’t Hoff enthalpy for a transition</td>
<td>J mol$^{-1}$</td>
</tr>
<tr>
<td>$\Delta_{trs}H^{vH}$</td>
<td>transition entropy</td>
<td>J K$^{-1}$ mol$^{-1}$</td>
</tr>
<tr>
<td>$\Delta_{trs}H^{vH}/\Delta_{trs}H$</td>
<td>cooperativity ratio of a transition</td>
<td>dimensionless</td>
</tr>
<tr>
<td>$\Delta P$</td>
<td>the instantaneous power difference between the sample and reference solution in a DSC scan</td>
<td>W</td>
</tr>
<tr>
<td>$\alpha_N$</td>
<td>the degree of the transition that has not gone to completion</td>
<td>dimensionless</td>
</tr>
<tr>
<td>$\alpha_U$</td>
<td>the degree of the transition that has gone to completion</td>
<td>dimensionless</td>
</tr>
<tr>
<td>$\alpha_I$</td>
<td>the degree of the transition that has gone to the intermediate state</td>
<td>dimensionless</td>
</tr>
<tr>
<td>$\tau$</td>
<td>time constant</td>
<td>s</td>
</tr>
<tr>
<td>$\Delta_{trs}C_p^i$</td>
<td>heat capacity change in going from state $i-1$ to state $i$; $i = 1, 2, \ldots$ The process referred to by a number must be unambiguously defined in the text.</td>
<td>J K$^{-1}$ mol$^{-1}$</td>
</tr>
<tr>
<td>$\Delta_{trs}H^i$</td>
<td>transition enthalpy in going from state $i-1$ to state $i$; $i = 1, 2, \ldots$ The process referred to by a number must be unambiguously defined in the text.</td>
<td>J mol$^{-1}$</td>
</tr>
<tr>
<td>pH</td>
<td>$pH = -\lg{a(H^+)}$</td>
<td>dimensionless</td>
</tr>
<tr>
<td>R</td>
<td>gas constant $(8.314 \ 472 \ J \ K^{-1} \ mol^{-1})$</td>
<td>J K$^{-1}$ mol$^{-1}$</td>
</tr>
<tr>
<td>T</td>
<td>temperature</td>
<td>K</td>
</tr>
<tr>
<td>c</td>
<td>concentration</td>
<td>mg mL$^{-1}$</td>
</tr>
<tr>
<td>$I_m$</td>
<td>ionic strength (molality basis)</td>
<td>mol kg$^{-1}$</td>
</tr>
</tbody>
</table>

### Abbreviations

- **DSC**: differential scanning calorimeter
- **I**: denotes an intermediate state in a transition
- **N**: denotes the native state of a biological substance
- **N$_2$**: denotes the native state of a biological substance consisting of two subunits
- **U**: denotes the unfolded or non-native state of a biological substance in a reversible transition
- **X**: denotes the unfolded state of a biological substance in an irreversible transition

REFERENCES