Differential Scanning Calorimetry of the Irreversible Thermal Denaturation of Thermolysin†

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ABSTRACT: A differential scanning calorimetry study of the thermal denaturation of *Bacillus thermoproteolyticus rokko* thermolysin was carried out. The calorimetric traces were found to be irreversible and highly scan-rate dependent. The shape of the thermograms, as well as their scan-rate dependence, can be explained by assuming that the thermal denaturation takes place according to the kinetic scheme $N \xrightarrow{k} D$, where k is a first-order kinetic constant that changes with temperature, as given by the Arrhenius equation, N the native state, and D the unfolded state or, more probably, a final state, irreversibly arrived at from the unfolded one. On the basis of this model, the value of the rate constant as a function of temperature and the activation energy have been calculated. It is shown that the proposed model may be considered as being one particular case of that proposed by Lumry and Eyring [Lumry, R., & Eyring, H. (1954) *J. Phys. Chem. 58*, 110] $N \rightleftharpoons D \rightarrow I$, where N is the native state, D the unfolded one, and I a final state, irreversibly arrived at from D. Lastly, some comments are made on the use of the scan-rate effect on the calorimetric traces as an equilibrium criterion in differential scanning calorimetry.

The study of the in vitro unfolding process in proteins has been a subject of increasing interest during recent years. Thus, the transition from the native to the denatured state for many proteins has been followed by different techniques, using mainly the kinetic or the thermodynamic approach (Tanford, 1968, 1970; Lapanje, 1978; Ghelis & Yon, 1982). This transition has generally been achieved by denaturant agents, such as urea or Gdn·HCl,[1] by changes in the pH and/or ionic strength, or by temperature. In this latter case the development of high-sensitivity differental scanning microcalorimetry (DSC) (Privalov, 1980) has been very important, since this technique permits the direct calculation of all the unfolding thermodynamic functions and, furthermore, can also be used to check the validity of the two-state model for the process by calculating and comparing both the calorimetric and the van't Hoff enthalpies of denaturation (Sturtevant, 1974; Privalov, 1979; Pfeil, 1981; Mateo, 1984). In addition, a knowledge of the heat capacity value of the protein as a function of temperature permits the calculation of its molecular partition function and, through the appropriate algorithm (Freire & Biltonen, 1978; Privalov et al., 1981), the deconvolution of the unfolding thermal profile into two-state-type processes. This analysis has already been applied to several proteins of various sizes and complexity, leading to the definition and characterization of submolecular cooperative blocks as structural domains of the macromolecule (Privalov et al., 1981; Privalov, 1982; Potekhin & Privalov, 1982; Privalov & Medved, 1982; Novokhatny et al., 1984; Rigell et al., 1985; Tsalkova & Privalov, 1985).

An essential prerequisite for these thermal studies, however, is that there should be thermodynamic equilibrium in the sample throughout the temperature-induced unfolding process. The equilibrium criterion usually applied is the reproducibility of the trace in a second heating of the sample, the so-called calorimetric reversibility. In many cases, however, the second thermogram shows no thermal effect and, therefore, according to the above-mentioned criterion, thermodynamic functions for the process, such as changes in entropy and Gibbs function, cannot be extracted from the first trace.

It has recently been shown, nevertheless, that the DSC thermograms for the thermal denaturation of several proteins can be interpreted in terms of the van't Hoff equation, in spite of the calorimetric irreversibility (Manly et al., 1985; Edge et al., 1985; Hu & Sturtevant, 1987). This can be explained if we assume that an irreversible alteration of the unfolded state takes place, with little heat effect, at temperatures higher than those at which the calorimetric transition occurs. These results indicated that the lack of calorimetric reversibility does not necessarily preclude the derivation of thermodynamic information from the calorimetric trace. It is still conceivable, of course, to find DSC traces that do not follow equilibrium thermodynamics, that is, traces that are kinetically controlled and, therefore, scan-rate dependent.

In this paper we describe a simple model for this type of DSC thermogram, and we show that the traces for the thermal denaturation of thermolysin can be quantitatively interpreted according to this working scheme.

Experimental Procedures

Thermolysin (EC 3.4.24.4), as a crystallized and lyophilized powder (lot 95F-0551), FAGLA, and HEPES were obtained from Sigma, and NaCl was obtained from Merck. All chemicals used were of the highest available purity. Distilled, deionized water was used throughout.

Prior to use, the thermolysin was additionally purified by crystallization according to the method of Holmquist and Vallee (1974) and its concentration calculated from the absorbance at 280 nm by using an $E^{1\%}$ value of 17.65 (Ohta et al., 1966). The enzymatic assay of thermolysin was carried out by measuring the peptidase activity of the enzyme, using the chromophore FAGLA as substrate and following the de-

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[1] Abbreviations: DSC, differential scanning calorimetry; FAGLA, *N*-[3-(2-furyl)acryloyl]glycyl-L-leucinamide; Gdn·HCl, guanidine hydrochloride; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate.

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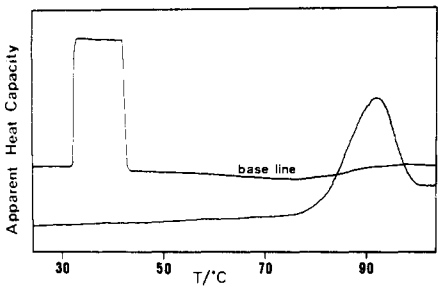

FIGURE 1: Original calorimetric recording of heat absorption of thermolysin solution at 1 K/min and pH 7.5, protein concentration 0.6 mg/mL. The endotherm on the base line corresponds to a 50-μW calibration mark.

crease in absorbance at 322 nm (Khan & Darnall, 1978).

All absorbance measurements were carried out in a Cary 210 spectrophotometer with the cells maintained at 25 °C. DSC experiments were performed in a DASM-1M calorimeter with cell volumes of 1 mL under an extrapressure of 1 atm to prevent any degassing during the heatings. Four different scan rates were used within the range of about 0.2–2 K/min. The calorimetric reversibility of the thermally induced transition was checked by reheating the protein solution in the calorimetric cell after the cooling from the first run. The calculation of the denaturation enthalpy was as has been described elsewhere (Mateo, 1984).

Protein solutions were prepared by dissolving dry samples in buffer solutions. Before the calorimetric experiments, the sample solutions were dialyzed for 24 h at 4 °C against three changes of a large volume of the dialysis buffer. The buffer used was 10 mM HEPES, 0.1 M NaCl, and 10 mM $CaCl_2$ at pH 7.5. For all the measurements the protein concentrations were in the range of 0.5–1.0 mg/mL. In the calculations of molar quantities the molecular weight used for the protein was 34 600, estimated from the known sequence of thermolysin (Matthews et al., 1974).

Results

Figure 1 shows an original DSC recording for the thermal denaturation of thermolysin at pH 7.5 and at a scan rate of 1 K/min. The traces were corrected for the calorimetric base line (see Figure 1) and for the difference in heat capacity between the initial and final states by using a linear chemical base line traced between the initial and final temperatures of the transition. The use of a sigmoidal base line, as described by Takahashi and Sturtevant (1981), leads to practically the same corrected traces. The corrected calorimetric traces, i.e., the excess heat capacity function versus temperature profiles, are given in Figure 2A at four different scan rates.

The reversibility of the process was checked as described under Experimental Procedures, and no transition was obtained in the second run of all the samples. As is evident in Figure 2A, the temperature corresponding to the maximum heat capacity, T_m, is highly dependent on the scan rate, which suggests that the denaturation process is kinetically determined.

In order to explain these results we have assumed that the process taking place in the calorimetric cell is a two-state irreversible process that can be represented as

$$N \xrightarrow{k} D$$

where N is the native state, D the unfolded state or, more probably, a final state, arrived at irreversibly from the unfolded one (see Discussion), and k a first-order kinetic constant, which changes with temperature according to the Arrhenius equation. The calorimetric experiment is assumed to start at a temperature low enough to make the reaction rate negligible and

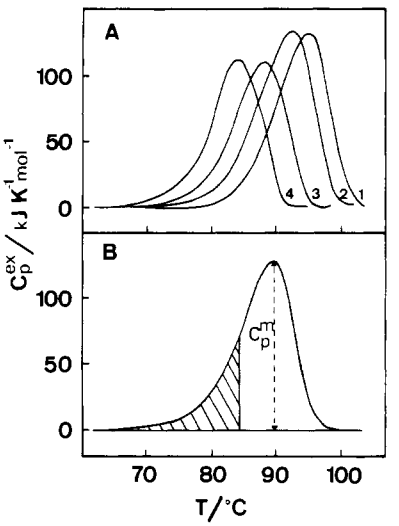

FIGURE 2: (A) Excess heat capacity of thermolysin at pH 7.5 versus temperature obtained at four different scan rates: (1) 1.9; (2) 1.0; (3) 0.5; and (4) 0.2 K/min. (B) Theoretical excess heat capacity curve versus temperature obtained according to eq A18; values used were T_m = 90 °C, specific denaturation enthalpy 39 J/g, and E = 282 kJ/mol.

hence the concentration of the native state equal to the total protein concentration.

A mathematical elaboration of this model (see Appendix) allows us to calculate the activation energy of the kinetic process in several different ways.

(A) The rate constant of the reaction at a given temperature, T, can be obtained by using

$$k = vC_p/(Q_t - Q) \quad (1)$$

where v (K/min) stands for the scan rate, C_p for the excess heat capacity, Q_t for the total heat of the process, and Q for the heat evolved at a given temperature, T (proportional to the hatched area in Figure 2B). The energy of activation, E, can then be obtained from the values of k at several temperatures by using the Arrhenius equation $k = A \exp(-E/RT)$. The corresponding Arrhenius plot, ln k versus $1/T$, including data from the four scan rates used, is given in Figure 3A. There is excellent agreement between the rate constants calculated from the traces obtained at four different scan rates. A value of 275 ± 5 kJ/mol for the energy of activation can be calculated from the slope of the Arrhenius plot in Figure 3A.

(B) The proposed kinetic model predicts that the temperature value corresponding to the maximum of the heat capacity curve, T_m, should change with the scan rate according to

$$\frac{v}{T_m^2} = \frac{AR}{E} e^{-E/RT_m} \quad (2)$$

Therefore, a plot of ln (v/T_m^2) versus $1/T_m$ should result in a straight line with a slope equal to $-E/R$. This plot is shown in Figure 3B for the four scan rates involved; the four data points fit a straight line very well, and the calculated activation energy is 269 ± 5 kJ/mol.

(C) The dependence of the heat evolved with temperature can be expressed as

$$\ln\left(\ln\frac{Q_t}{Q_t - Q}\right) = \frac{E}{R}\left(\frac{1}{T_m} - \frac{1}{T}\right) \quad (3)$$

Thus, a plot of ln [ln $[Q_t/(Q_t - Q)]$] versus $1/T$ should give rise to straight lines, the slope of each one being $-E/R$. These plots are given in Figure 3C, and the average activation energy

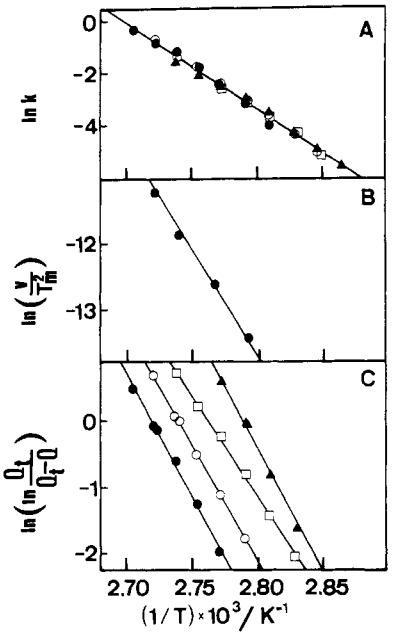

FIGURE 3: (A) Arrhenius plot including k data from the four scan rates used (Figure 2A): (●) 1.9; (○) 1.0; (□) 0.5; and (▲) 0.2 K/min. At the four scan rates only k values corresponding to the thermal effects higher than 5% or lower than 95% of the total unfolding heat were used to avoid the relative higher uncertainty at the beginning and the end of the transition. (B) Plot of ln (v/T_m^2) versus $1/T_m$. Here the $1/T$ coordinate actually represents $1/T_m$. Each data point corresponds to one of the four scan rates used. (C) Values of ln [ln $[Q_t/(Q_t - Q)]$] plotted versus $1/T$. Symbols are the same as those used in (A).

thus obtained is 296 ± 14 kJ/mol.

In addition, the values of T_m can be calculated from the X-axis intercepts in these plots. The values obtained were 367.6, 365.0, 362.0, and 358.6 K for the scan rates 1.9, 1.0, 0.4, and 0.2 K/min, respectively. These T_m values compare well with the corresponding ones obtained directly from the calorimetric traces: 367.2, 364.8, 361.5, and 358.1 K.

It is interesting to note that, according to eq 3, when $T = T_m$, the value of ln [ln$(Q_t - Q)$]] must be equal to zero, and therefore $Q_t/(Q_t - Q_m) = e$, where Q_m is the heat evolved at T_m. The average value of $Q_t/(Q_t - Q_m)$ for the four scan rates is 2.59 ± 0.1, with a percentage deviation from the e value of lower than 5%.

(D) The activation energy can also be calculated from the heat capacity at the maximum of the trace, C_p^m, according to

$$E = eRC_p^m T_m^2/Q_t \qquad (4)$$

When this expression is applied to the four traces of Figure 3C, an average activation energy of 287 ± 9 kJ/mol is obtained.

We must point out that the four methods employed to obtain the activation energy involve different approximations (see Appendix) and use different experimental information. Thus, method D uses parameters from the maximum of the excess heat capacity curve, while method C is based on the effect of the scan rate on the temperature of the maximum and method B tests the shape of the traces. These three methods assume that the Arrhenius equation holds true. Method A, on the other hand, checks that the rate constants calculated from the traces obtained at different scan rates agree and would continue to be valid even if the Arrhenius equation did not hold good or if the activation energy were strongly temperature dependent (nonlinear Arrhenius plot). It is clear that the excellent agreement between the results obtained by using all of these four methods supports the validity of the proposed kinetic model for the thermal denaturation of thermolysin.

The overall average value for the activation energy (at pH 7.5, including all the results obtained, by using the four methods) is 282 ± 8 kJ/mol, which compares well with the values reported by Fujita et al. (1979), 330 kJ/mol at pH 8.2, and Voordouw et al. (1976), 275 kJ/mol at pH 6, using noncalorimetric techniques. The slight differences observed can be attributed to a certain small pH dependence of the energy of activation. In fact, we have carried out DSC experiments on the thermolysin thermal unfolding under the same conditions as those of Fujita et al. (1979) and found an E value of 329 ± 5 kJ/mol, which agrees exactly with their value (results not reported). There is also agreement between the rate constants given by these authors and those calculated from the calorimetric traces by using our method A.

Finally, the denaturation calorimetric enthalpy of thermolysin increases slightly with the scan rate, that is, with the T_m value. The average specific enthalpy obtained was 38.6 ± 1.5 J/g (1336 ± 50 kJ/mol), a value that compares well with those published for compact globular proteins of similar denaturation temperatures (Privalov, 1979).

Discussion

We have shown that the DSC thermograms for the thermal denaturation of thermolysin can be interpreted in terms of a kinetic process

$$N \xrightarrow{k} D$$

where k is a first-order kinetic constant that changes with temperature as described by the Arrhenius equation.

This model predicts that the calorimetric traces should be highly dependent on the scan rate, as is the case for thermolysin. It should be borne in mind, however, that the time response of the calorimeter may also produce a scan-rate dependence of the traces, especially when one is dealing with sharp transitions such as, for instance, the main lipid transition. Lechuga (1985) has shown how the effect of the finite time response of the instrument can be corrected for. When his procedure is applied to thermolysin calorimetric traces, it is found that the corrected and noncorrected traces are virtually identical, which is to be expected given the broadness of thermolysin transitions. It is clear, therefore, that the observed scan-rate dependence can be attributed to chemical kinetic factors. The analysis carried out under Results demonstrates that the proposed kinetic model is able consistently to explain such a dependence.

This model would appear to suggest that the unfolded state is thermodynamically more stable than the native one throughout the temperature range of the calorimetric experiment. We must emphasize, however, that while the proposed model is the simplest one, other more complex models may come back to a first-order kinetic process. For instance, a more realistic representation of the irreversible thermal denaturation of a protein might be (Lumry & Eyring, 1954)

$$N \underset{k_2}{\overset{k_1}{\rightleftharpoons}} D \xrightarrow{k_3} I$$

where I is a final state irreversibly arrived at from the unfolded state, D. If we assume that all three processes are first-order ones and that $k_3 \gg k_2$ at any moment, most of the D molecules will be converted to I instead of returning to N through the process D → N. As a result the equilibrium between N and D will not be established, the concentration of D always being very low, and thus the denaturation may be considered as being an irreversible process, N → I, kinetically controlled by the

relatively slow conversion from N to D. That is

$$N \xrightarrow{k_1} I$$

which would lead to the same kinetic equations as those resulting from our original model, N → D. It is of interest to note that if the D → I process is much faster than the D → N one, the kinetic order of the former is immaterial.

The irreversible step in the denaturation pathway of many proteins has been put down to a variety of mechanisms, including protein aggregation, deamination of Asn/Gln residues, isomerization of proline residues, chain separation in oligomeric proteins, loss of cofactor, etc. [see Privalov (1982), Ahern and Klibanov (1985), and Zale and Klibanov (1986)]. As far as the irreversible thermal denaturation of thermolysin is concerned, autolysis might be playing a part, and this would appear to be borne out by SDS gel electrophoresis of the scanned samples. It is worth pointing out, however, that the thermal denaturation of thermolysin does not seem to be a unique case, and, in fact, results obtained in the authors' laboratory indicate that the DSC traces for other proteins, such as procarboxypeptidase A, in which no evidence for autolysis has been found, may also be interpreted according to the proposed kinetic model (manuscript in preparation).

In order to apply equilibrium thermodynamics to the analysis of DSC transitions, the prime necessity is for chemical equilibrium to exist throughout the temperature range of the transition. It is usually assumed that this condition is fulfilled in those cases in which calorimetric reversibility is found. Furthermore, it has recently been reported (Edge et al., 1985; Manly et al., 1985; Hu & Sturtevant, 1987) that some calorimetric transitions corresponding to thermal denaturation of proteins can be adequately analyzed in terms of equilibrium thermodynamics, in spite of calorimetric irreversibility. In these cases the equilibrium criterion applied was the agreement between the van't Hoff enthalpy values calculated from the shape of the trace and those calculated from the effect of the ligands and from the protein concentration on the transition temperature. Within the context of the model $N \rightleftharpoons D \rightarrow I$, these results can be easily understood if we assume that $k_3 \ll k_2$ within the temperature range at which the transition occurs; the irreversible step may, nevertheless, be faster at higher temperatures, thus originating the calorimetric irreversibility.

A conclusion that can be drawn from this work, as well as from general principles, is that if the process giving rise to the calorimetric transition is kinetically controlled, the calorimetric traces should be dependent on the scan rate. Accordingly, we would like to suggest that the effect of scan rate on the thermograms (after correcting, if necessary, for the effect of the finite time response of the instrument) may be used as an alternative equilibrium criterion in differential scanning calorimetry. Thus, the detection of a clear scan-rate dependence of the traces should preclude their interpretation in terms of equilibrium thermodynamics. This idea has been advanced before by several authors, but rarely heeded. For instance, Jackson and Sturtevant (1978) proposed that equilibrium thermodynamics could be applied to the 80 °C transition of *Halobacterium halobium* purple membranes on the basis of its scan-rate independence.

Acknowledgments

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Appendix

We will consider here a two-state endothermic transition, from the native, N, to the denatured, D, state of a protein, which takes place upon heating at a scan rate of v K/min and which is determined by a first-order kinetic process

$$N \xrightarrow{k} D$$

where k is a first-order kinetic constant that changes with temperature according to the Arrhenius equation.

A simple irreversible DSC melting profile of the form found for several proteins is shown in Figure 2B. For the sake of simplicity we will consider that there is no net heat capacity change on denaturation, that is, $\Delta_d C_p = C_p(D) - C_p(N) = 0$.

(A) For a given temperature, T, the heat evolved, Q, will be proportional to the hatched area in Figure 2B. This value, as well as the total heat of the process, Q_t, can be expressed by

$$Q = [D]\Delta HV \qquad Q_t = c\Delta HV \tag{A1}$$

where [D] stands for the molar concentration of the denatured state at the temperature T, c for the total molar concentration of protein in the sample cell, V for the cell colume, and ΔH for the total heat (enthalpy) of the process per mole of protein. The Q_t value is thus proportional to the total area under the transition curve.

For a first-order kinetic process

$$d[N]/dt = -k[N]$$

and since the scan rate is $v = dT/dt$

$$k = -\frac{v}{[N]}\frac{d[N]}{dT} \tag{A2}$$

where [N] is the concentration of the native state at the temperature T. This value can also be expressed as

$$[N] = c - [D] = (Q_t - Q)/\Delta HV \tag{A3}$$

and thence

$$\frac{d[N]}{dT} = \frac{-1}{\Delta HV}\frac{dQ}{dT} = \frac{-C_p}{\Delta HV} \tag{A4}$$

and by substitution in (A2)

$$k = vC_p/(Q_t - Q) \tag{A5}$$

which is eq 1 used in method A under Results.

(B) From eq A2 and the Arrhenius relationship, we have

$$\frac{d \ln [N]}{dT} = -\frac{k}{v} = -\frac{A}{v}\exp(-E/RT) \tag{A6}$$

The value of $d^2 \ln [N]/dT^2$ will, then, be

$$\frac{d^2 \ln [N]}{dT^2} = -\frac{A}{v}\frac{E}{RT^2}\exp(-E/RT) = \frac{E}{RT^2}\frac{d \ln [N]}{dT} \tag{A7}$$

but also

$$\frac{d^2 \ln [N]}{dT^2} = \frac{1}{[N]}\frac{d^2[N]}{dT^2} - \frac{1}{[N]^2}\left(\frac{d[N]}{dT}\right)^2 \tag{A8}$$

Now from eq A4 it follows that

$$C_p = -V\Delta H\frac{d[N]}{dT} \tag{A9}$$

where, for $T = T_m$, we have that $dC_p/dT = 0$ and therefore $|d^2[N]/dT^2|_{T=T_m} = 0$.

Comparing (A7) and (A8), for $T = T_m$, we arrive at

$$\frac{E}{RT_m^2}\frac{1}{[N]}\left(\frac{d[N]}{dT}\right)_{T_m} = -\frac{1}{[N]^2}\left(\frac{d[N]}{dT}\right)^2_{T_m} \tag{A10}$$

or

$$\frac{E}{RT_m^2} = -\left(\frac{d \ln [N]}{dT}\right)_{T_m} \quad (A11)$$

Taking eq A6 into account and rearranging, we have

$$\frac{v}{T_m^2} = \frac{AR}{E} \exp(-E/RT_m) \quad (A12)$$

which is eq 2 in the text used in method B to calculate the energy of activation.

(C) The derivation, from (A6), of an explicit equation giving the concentration of the native state as a function of temperature requires the evaluation of the so-called Arrhenius integral; this must be done numerically (Norris, 1981) or by carrying out some approximation.

We can relate T_m to any temperature by $T = T_m + \Delta T$ and apply the Taylor expansion of $1/T$ in the neighborhood of $1/T_m$ using only the first expansion term, given that within the range of the calorimetric transition $\Delta T \ll T_m$

$$1/T = 1/T_m + (d(1/T)/dT)_{T_m}(T - T_m) = 1/T_m - \Delta T/T_m^2 \quad (A13)$$

Substituting (A13) in (A6) and using (A12), we arrive at

$$d \ln [N]/dT = -A/v \exp(-E/RT_m) \exp[(E/RT_m^2)\Delta T] = -W \exp(W\Delta T) \quad (A14)$$

where $W = A/v \exp(-E/RT_m) = E/RT_m^2$ (see eq A12).

Upon integration from $\Delta T_0 = T_0 - T_m$, where T_0 is a temperature low enough to make the reaction rate negligible, we obtain

$$\ln [N]/[N]_0 = \exp(W\Delta T_0) - \exp(W\Delta T) \quad (A15)$$

If T_0 is low enough, and for the temperature range of the calorimetric transition, $\exp(W\Delta T) \gg \exp(W\Delta T_0)$ (note that $\Delta T_0 < 0$). Therefore

$$\ln \frac{[N]_0}{[N]} = \exp(W\Delta T) = \exp\left[\frac{E}{R}\left(\frac{T - T_m}{T_m^2}\right)\right] \quad (A16)$$

Now, from eq A1 we have

$$\frac{[N]_0}{[N]} = \frac{Q_t}{Q_t - Q} \quad (A17)$$

and taking into account that, within the relatively narrow range of the transition $(T - T_m)/T_m^2 = 1/T_m - 1/T$ (eq A13), we finally obtain

$$\ln\left(\ln \frac{Q_t}{Q_t - Q}\right) = \frac{E}{R}\left(\frac{1}{T_m} - \frac{1}{T}\right) \quad (A18)$$

which is eq 3 in the text used in method C. Clearly, we could have used an equation similar to (A18) with $(T - T_m)/T_m^2$ instead of $1/T_m - 1/T$ in the right-side term. Both equations, in fact, lead to the same results; we have used (A18) because it allows us to use the same X axis for the three plots in Figure 3.

Note that for $T = T_m$ eq A18 leads to

$$\ln \frac{Q_t}{Q_t - Q_m} = 1 \qquad \frac{Q_t}{Q_t - Q_m} = e \quad (A19)$$

(D) Finally, at $T = T_m$ and by use of eq A6 and A12, eq A5 becomes

$$\frac{v\, C_p^m}{Q_t - Q_m} = \frac{Ev}{RT_m^2} \quad (A20)$$

from whence, and by application of eq A19, we obtain

$$E = \frac{RT_m^2 C_p^m}{Q_t - Q_m} = \frac{eRT_m^2 C_p^m}{Q_t} \quad (A21)$$

which is eq 4 in the text used in method D.

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