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The calorimetric criterion for a two-state process revisited

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Abstract

The "calorimetric criterion" is one of the important experimental approaches for determining whether protein folding is an "all-or-none" two-state transition (i.e., whether intermediates are present at equilibrium). The calorimetric criterion states that the equivalence of the "measured" calorimetric enthalpy change and the effective two-state van't Hoff enthalpy change demonstrates that there is a two-state transition. This paper addresses the essential question of whether the calorimetric criterion is a necessary and sufficient condition for a two-state process and shows that it is necessary but not sufficient by means of specific examples. Analysis of simple models indicates that the heat capacity curve, regardless of whether it originates from a two-state process or not, can always be decomposed in such a way that the calorimetric criterion is satisfied. Exact results for a three-state model and a homopolymer tetramer demonstrate that the deviation from the calorimetric criterion is not simply related to the population of intermediate states. Analysis of a three-helix bundle protein model, which has a two-state folding from a random coil to ordered (molten) globule, shows that the calorimetric criterion may not be satisfied if the standard linear interpolation of baselines (weighted or unweighted) is employed. A specific example also suggests that the more recently introduced deconvolution method is not necessarily better than the simple calorimetric criterion for distinguishing a two-state transition from a three-state transition. Although the calorimetric criterion is not a sufficient condition for a two-state process, it is likely to continue to be of practical utility, particularly when its results are shown to be consistent with those from other experimental methods.

Keywords: calorimetric criterion; free-energy barrier; protein folding; simple models; two-state transition

An important feature of proteins is the abrupt (cooperative) change upon heating in many of their properties, such as enzymatic activity and optical density (Johnson et al., 1954). Observation of these abrupt changes led Anson and Mirsky (1934) to introduce the two-state approximation to describe protein denaturation. The two-state approximation is a reference point for equilibrium and kinetic studies of protein folding (Chen & Schellman, 1989; Jackson & Fersht, 1991; Alexander et al., 1992; Chen & Matthews, 1994; Yi & Baker, 1996). In the two-state approximation, the protein folding/unfolding transition is described as a transition between a native state and a denatured state without intermediates that are populated at equilibrium. The native state is assumed to be the tightly-structured global minimum energy state, and the denatured state is thought to be a collection of many loosely structured states. Although the two-state approximation is widely used, the meaning of the term continues to be a subject of discussion (Lumry et al., 1966; Tanford, 1968; Privalov, 1979; Chan et al., 1995; Dill et al., 1995).

There are at least two ways to define a two-state transition for the protein folding reaction. If one defines the denatured state to consist of all protein conformations other than the native state, protein folding is automatically a two-state transition. The two-state definition based on measurements of enzymatic activity corresponds essentially to this definition. Also, many lattice model studies (Dill et al., 1995; Karplus & Šali, 1995; Shakhnovich, 1996) have been analyzed in this way. We refer to this definition as the "formal" definition and the corresponding two-state transition as the "formal" two-state transition. However, the formal definition is not used in most considerations of two-state behavior. Instead two-state behavior is associated with the requirement that intermediate states make a negligible contribution due to the existence of a single free energy barrier between the native and denatured states. If there is a single free energy barrier on the multidimensional free energy surface or "landscape," it provides a necessary and sufficient condition for ensuring that the population of intermediate species is negligible at the transition temperature.[4] It also makes the identification of two states possible because they

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[4] The intermediate here is the "transition" intermediate that should be distinguished from other equilibrium intermediates such as molten globules. A "transition" intermediate is the intermediate that is present during the transition while equilibrium intermediates are the products of transitions and are stable under certain thermodynamic conditions.

are associated with well-separated distributions when plotted in terms of appropriate order parameters. We refer to this definition as the "thermodynamic" definition and the corresponding two-state transition as the "thermodynamic" two-state transition. The thermodynamic definition contains information regarding the free-energy surface of the protein and the population of the intermediate states and provides a link with the macroscopic liquid-gas and liquid-solid two-state transitions. The latter are known to be induced by free-energy barriers between two states (Temperley, 1956), so that two populations exist in equilibrium at the transition temperature.

The observation of sharp changes in the properties of a protein does not necessarily signal a thermodynamic two-state transition since sequential multistep transitions can show such behavior (Tsong et al., 1972; Chan et al., 1995). The most widely used criterion for a two-state transition is the calorimetric criterion (Tanford, 1968; Jackson & Brandts, 1970; Privalov, 1979; Schellman, 1987; Sturtevant, 1987; Jackson & Fersht, 1991), which requires the equivalence of the "measured" calorimetric enthalpy change (defined as the area enclosed by the heat capacity vs. temperature curve) and the calculated van't Hoff enthalpy change based on the two-state assumption. The same criterion is now being used in the analysis of homopolymer collapse transitions (Tiktopulo et al., 1994, 1995). The criterion is found to be satisfied for low molecular weight homopolymers but not for high molecular weight species that appear to have separate domains. For proteins, the calorimetric criterion has been complemented by other measurements. These include gel electrophoresis (Creighton, 1986), size exclusion chromatography (Uversky, 1993), hydrogen-deuterium exchange (Yi & Baker, 1996), as well as kinetic studies of the folding and unfolding transitions (Jackson & Fersht, 1991).

Although the use of multiple criteria has established that the folding transition of many proteins is two-state-like, a clear analysis of the calorimetric criterion is necessary to assess its scope and limitations. For the calorimetric method to be the criterion for a two-state process, it has to be both a necessary and a sufficient condition. A necessary condition means that if a process is two-state-like, the calorimetric criterion is satisfied while a sufficient condition states that if the calorimetric criterion is satisfied, the process is two-state-like. It is generally agreed that the calorimetric criterion is a necessary condition. However, whether or not the criterion is a sufficient condition is still a subject of discussion (Chan et al., 1995). Experimentally, deviation from the calorimetric criterion has been interpreted as being related to intermolecular transitions (Sturtevant, 1987), ligand-protein interactions (Shrake & Ross, 1990; Straume & Freire, 1992), weakly stable proteins (Haynie & Freire, 1994), and the existence of a non-negligible population of intermediate states including domain formation (Tanford, 1968; Privalov, 1979; Sturtevant, 1987).

In this paper, we provide an in-depth analysis of the calorimetric criterion by the use of simple well-defined models. We find that for any heat capacity curve, there always exist "two-state" solutions so that the calorimetric criterion is satisfied. An exact formula is obtained for the deviation from the calorimetric criterion in the case of a simple three-state model whose baselines are exactly known. It is found that the deviation from calorimetric criterion is not simply related to the population of intermediate states, as has been shown for a specific case by Freire (1995). That the calorimetric criterion is not a sufficient condition for a two-state transition is shown to be due to the fact that the two-state assumption is implicit in the approximation that the folding transition is taking place at a single temperature, rather than over a finite temperature range. The use of the deconvolution method for identifying a two-state transition (Freire & Biltonen, 1978; Freire, 1995) is also briefly discussed.

Results and discussion

The calorimetric criterion

In the calorimetric criterion, the standard enthalpy difference between state I and state II is calculated according to the van't Hoff equation and then compared to the value measured in a heat capacity vs. temperature experiment. A thermodynamic two-state transition is said to occur if the two values are equal at a temperature T_d (called the middle transition temperature) at which the populations of states I and II are equal.

The van't Hoff equation for the standard enthalpy change may be derived by assuming that a two-state transition exists and can be described as a chemical reaction between state I (the reactant) and state II (the product). The effective two-state "chemical" equilibrium constant K^{eff} associated with the transition from state I to state II is defined as (Privalov, 1979):

$$K^{\text{eff}}(T) = \frac{\langle\alpha(T)\rangle - \langle\alpha(T)\rangle_{\text{I}}}{\langle\alpha(T)\rangle_{\text{II}} - \langle\alpha(T)\rangle} = \frac{f_{\text{II}}}{1 - f_{\text{II}}}, \tag{1}$$

where $\langle\alpha(T)\rangle$ is the average value of some appropriate observable quantity, $\alpha(T)$, such as the enzymatic activity or optical density, $\langle\alpha(T)\rangle_{\text{I}}$ and $\langle\alpha(T)\rangle_{\text{II}}$ denote the average values of $\alpha(T)$ in states I and II, respectively, and f_{II} is the fraction of the system in state II ($\equiv (\langle\alpha(T)\rangle - \langle\alpha(T)\rangle_{\text{I}})/(\langle\alpha(T)\rangle_{\text{II}} - \langle\alpha(T)\rangle_{\text{I}})$). The two-state effective (van't Hoff) standard enthalpy change associated with the transition from state I to state II, $\Delta H^{\text{eff}}(T)$, can be obtained from the van't Hoff equation for the derivative of the effective equilibrium constant with respect to temperature T

$$\Delta H^{\text{eff}}(T) = k_B T^2 \frac{d \ln K^{\text{eff}}}{dT}, \tag{2}$$

where k_B is the Boltzmann constant.

In the calorimetric criterion, $\Delta H^{\text{eff}}(T)$ is compared with the experimentally "measured" value of the standard enthalpy change, $\Delta H^{\text{expt}}(T)$, associated with the transition. By definition, $\Delta H^{\text{expt}}(T)$ is the excess heat released during the transition from the initial state to the final state at *constant* temperature T. However, unlike liquid-solid and liquid-gas transitions in infinite systems, protein denaturation by heating (or cooling) does not occur at a single temperature but instead occurs over a finite temperature range. Thus, the measurement of $\Delta H^{\text{expt}}(T)$ from heat denaturation experiments on proteins is not direct. Instead, one measures the system heat capacity $C_p(T)$ over a range of temperatures that extend from T_{I}, the temperature at which the system is assumed to be entirely in state I, to T_{II}, the temperature at which the system is assumed to be entirely in state II. The "measured" enthalpy change, $\Delta H^{\text{expt}}(T)$, is then taken to be the area under the peak enclosed by the $C_p(T)$ curve and a baseline curve, which is chosen to represent the hypothetical situation in which the system transforms directly from pure state I to pure state II at temperature T (Jackson & Brandts, 1970) or gradually from pure state I to pure state II over a temperature range (Ackermann & Ruterjans, 1964; Beck et al., 1965; Privalov, 1970; Takahashi & Sturtevant, 1981; Sturtevant, 1987). A schematic illustration of such a measurement is given in Figure 1. The area of the heat capacity peak is assumed to represent the excess heat released during the transition. However, since the excess heat is released over a finite temperature range, it cannot be identified directly as ΔH at a specific temperature such as T_d (the middle transition temperature) or T_c (the heat capacity maximum temperature) unless the transition is approximated as a two-state transition occurring at that temperature; i.e., it is assumed implicitly that no intermediate states contribute to the transition. Thus, the common experimental method used to measure ΔH^{expt} implicitly assumes that a two-state transition exists. In other words, similar to ΔH^{eff}, the so-called ΔH^{expt} is an approximate way to evaluate the enthalpy change $H_{\text{II}}(T) - H_{\text{I}}(T)$ even if intermediates exist.

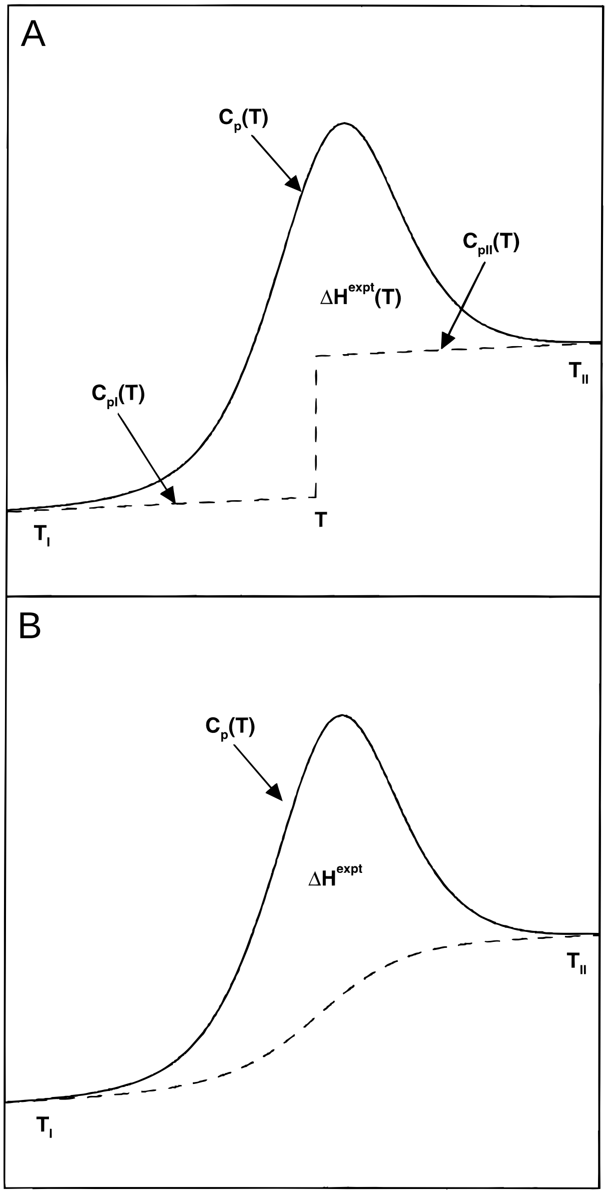

Fig. 1. Schematic plot of temperature dependence of heat capacities for the protein temperature denaturation process. Here, $C_p(T)$ is the heat capacity of all states that are present; $C_{p\text{I}}(T)$ and $C_{p\text{II}}(T)$ are the heat capacities for states I and II, respectively. At temperature T_{I} or T_{II}, it is assumed that only state I or state II exists. The experimentally "measured" enthalpy change associated with the transition from state I to state II is denoted as $\Delta H^{\text{expt}}(T)$. (**A**) The Jackson–Brandts method and (**B**) a smooth weighted baseline are shown.

One of the widely used methods for calculating the "measured" enthalpy change is that developed by Jackson and Brandts as shown in Figure 1A (Jackson & Brandts, 1970; Privalov, 1979; Tiktopulo et al., 1994; Gesierich & Pfeil, 1996). In the method of Jackson and Brandts, the "baseline" used to determine the heat capacity peak area consists of two lines that are linearly extrapolated from T_{I} and T_{II}, the temperatures at which only pure states I and II exist, to the temperature T. These lines represent hypothetical heat capacities for pure state I, $C_{p\text{I}}$, and for pure state II, $C_{p\text{II}}$, respectively. The quantity $\Delta H^{\text{expt}}(T)$ is determined from the equation (Jackson & Brandts, 1970)

$$\Delta H^{\text{expt}}(T) = \int_{T_{\text{I}}}^{T_{\text{II}}} C_p(T)\, dT - \int_{T_{\text{I}}}^{T} C_{pI}(T)\, dT - \int_{T}^{T_{\text{II}}} C_{p\text{II}}(T)\, dT, \tag{3}$$

which can be rewritten in term of enthalpies $H(T)$ as

$$\Delta H^{\text{expt}}(T) = H_{\text{II}}(T) - H_{\text{I}}(T) + H(T_{\text{II}}) - H_{\text{II}}(T_{\text{II}}) + H_{\text{I}}(T_{\text{I}}) - H(T_{\text{I}}) \tag{4}$$

since $(\partial H/\partial T)_p = C_p(T)$, by definition. Here, $H_{\text{I}}(T)$ and $H_{\text{II}}(T)$ are the enthalpies of the system in states I and II, respectively. Since we have assumed that only state I (state II) is present at temperature $T_{\text{I}}(T_{\text{II}})$, it follows that $H(T_{\text{II}}) \approx H_{\text{II}}(T_{\text{II}})$ and $H_{\text{I}}(T_{\text{I}}) \approx H(T_{\text{I}})$, which lead to $\Delta H^{\text{expt}}(T) = H_{\text{II}}(T) - H_{\text{I}}(T)$. Thus, $\Delta H^{\text{expt}}(T)$ is indeed an approximate way to obtain the two-state enthalpy change.

A weighted smooth baseline is also used in determining enthalpy changes (Fig. 1B). The experimentally measured $\Delta H^{\text{expt}}(T)$ is calculated from (Takahashi & Sturtevant, 1981; Sturtevant, 1987; Freire, 1995)

$$\Delta H_s^{\text{expt}} = \int_{T_{\text{I}}}^{T_{\text{II}}} [C_p(T) - f_{\text{I}} C_{p\text{I}}(T) - f_{\text{II}} C_{p\text{II}}(T)]\, dT, \tag{5}$$

rather than from Equation 3 where s denotes the weighted smooth-baseline method. Superficially, Equation 5 is physically more reasonable since it calculates the "true-excess" heat released during the transition from T_{I} to T_{II}. Mathematically, as we shall see, ΔH_s^{expt} for a two-state process is close or equal to $H_{\text{II}}(T_d) - H_{\text{I}}(T_d)$ where T_d is the middle transition temperature. However, there is no physical basis to assign ΔH_s^{expt} as $H_{\text{II}}(T) - H_{\text{I}}(T)$ at a well-defined temperature, such as T_d or T_m (the excess heat capacity maximum temperature), between T_{I} and T_{II} since the transition occurs over a range of temperatures (see below). The calorimetric criterion based on a weighted smooth baseline is often used in conjunction with the statistical deconvolution method based on a least-squares fit (Freire & Biltonen, 1978; Freire, 1995).

The van't Hoff enthalpy change and the "measured" enthalpy change are thus two different ways of obtaining the two-state enthalpy change regardless which baseline method is used. The equivalence of these two enthalpy changes, the basis of the calorimetric criterion, is not in general a sufficient condition for a thermodynamic two-state process. That the calorimetric criterion is satisfied could be a direct consequence of the implicit two-state assumption in the enthalpy evaluation. On the other hand, if the calorimetric criterion is not satisfied, it can indicate either that the transition is not a two-state process or that the baseline for the heat capacity curve is incorrect, as we point out below.

Simple statistical models

In this section, we demonstrate that (1) arbitrarily dividing a system into two states (one state plus the remainder) will automatically satisfy the calorimetric criterion and (2) the satisfaction of the calorimetric criterion does not mean that the population of intermediate states is negligible.

A: Formal two-state model

In the formal two-state definition, the conformational states are arbitrarily divided into state I and the remainder (state II). For simple protein models, such as the lattice models mentioned above (Dill et al., 1995; Karplus & Šali, 1995; Shakhnovich, 1996), one can define the native state as the lowest energy state and the remaining states as the denatured state. For actual proteins, the formal dichotomy is more complex in that the native "state" includes the cluster of conformations sampled at a certain temperature (say, 300 K), and the denatured state includes all other conformations. It should be noted that the formal two-state definition is not appropriate for cases where the existence of an intermediate is evident from the appearance of a shoulder in the main transition or even separate transition peaks. In other words, the formal definition is limited to the cases where only one well-defined heat-capacity peak exists.

In the formal definition, the partition function Q can be expressed exactly as the sum of the contribution from state I, Q_{I}, and that from state II, Q_{II},

$$Q = Q_{\mathrm{I}} + Q_{\mathrm{II}}. \tag{6}$$

The system internal energy and heat capacity and the individual internal energies and heat capacities for states I and II can be obtained from Q, Q_{I}, and Q_{II} using (Friedman, 1985)

$$U(T) = -\frac{d\ln Q}{d\beta}, \qquad C_v(T)/k_B = \beta^2\frac{d^2\ln Q}{d\beta^2}, \tag{7}$$

$$U_{\mathrm{I}}(T) = -\frac{d\ln Q_{\mathrm{I}}}{d\beta}, \qquad C_{v\mathrm{I}}(T)/k_B = \beta^2\frac{d^2\ln Q_{\mathrm{I}}}{d\beta^2}, \tag{8}$$

$$U_{\mathrm{II}}(T) = -\frac{d\ln Q_{\mathrm{II}}}{d\beta}, \qquad C_{v\mathrm{II}}(T)/k_B = \beta^2\frac{d^2\ln Q_{\mathrm{II}}}{d\beta^2}. \tag{9}$$

where $\beta = 1/k_BT$. From Equations 6–9

$$U(T) = f_{\mathrm{I}}U_{\mathrm{I}}(T) + f_{\mathrm{II}}U_{\mathrm{II}}(T) \tag{10}$$

where $f_{\mathrm{I}}(\equiv Q_{\mathrm{I}}/Q)$ and $f_{\mathrm{II}}(\equiv Q_{\mathrm{II}}/Q)$ are the fraction of states in states I and II, respectively. Here $f_{\mathrm{II}} = 1 - f_{\mathrm{I}}$ since there are only two states.

In applying the calorimetric criterion, we use the standard internal energy change ΔU and the constant-volume heat capacity $C_v(T)$ rather than the standard enthalpy change ΔH and the constant-pressure heat capacity $C_p(T)$ because theoretical models are most conveniently described in the canonical ensemble where volume and temperature are the independent variables. This ensemble change makes no difference to the argument.

In the canonical ensemble, the two-state effective standard change of internal energy can be obtained by differentiating the effective two-state "chemical" equilibrium constant $K^{\mathrm{eff}}(T)$ with respect to temperature T at constant volume (compared with Equation 2 at constant pressure)

$$\Delta U^{\mathrm{eff}}(T) = k_BT^2\frac{d\ln K^{\mathrm{eff}}}{dT} = \frac{k_BT^2}{f_{\mathrm{I}}f_{\mathrm{II}}}\frac{df_{\mathrm{II}}}{dT} \tag{11}$$

where the second equality follows from Equation 1. Substituting $f_{\mathrm{II}} \equiv Q_{\mathrm{II}}/Q$ into Equation 11, we have (cf. Equations 7–9)

$$\Delta U^{\mathrm{eff}}(T) = U_{\mathrm{II}}(T) - U_{\mathrm{I}}(T). \tag{12}$$

The fraction of states in state II, f_{II}, can also be expressed exactly in terms of U using Equation 10 and $f_{\mathrm{I}} = 1 - f_{\mathrm{II}}$

$$f_{\mathrm{II}} = \frac{U(T) - U_{\mathrm{I}}(T)}{U_{\mathrm{II}}(T) - U_{\mathrm{I}}(T)}. \tag{13}$$

Substituting Equation 13 into Equation 11, we obtain another equation for $\Delta U^{\mathrm{eff}}(T)$

$$\Delta U^{\mathrm{eff}}(T) = k_BT^2\,\frac{C_v(T)[U_{\mathrm{II}}(T) - U_{\mathrm{I}}(T)] - C_{v\mathrm{I}}(T)[U_{\mathrm{II}}(T) - U(T)] - C_{v\mathrm{II}}(T)[U(T) - U_{\mathrm{I}}(T)]}{[U(T) - U_{\mathrm{I}}(T)][U_{\mathrm{II}}(T) - U(T)]}, \tag{14}$$

where we have used Equations 7, 8, and 9. At the middle point of the transition defined to be the temperature $T = T_d$ at which $f_{\mathrm{II}} = 1/2$, Equation 14 becomes (cf. Equation 13)

$$\Delta U^{\mathrm{eff}}(T_d) = 4k_BT_d^2\,\frac{\Delta C_v(T_d)}{[U_{\mathrm{II}}(T_d) - U_{\mathrm{I}}(T_d)]}, \tag{15}$$

where the excess heat capacity $\Delta C_v(T_d) \equiv C_v(T_d) - 1/2[C_{v\mathrm{I}}(T_d) + C_{v\mathrm{II}}(T_d)]$.

In experiments using the Jackson–Brandts method (Jackson & Brandts, 1970; Privalov, 1979; Tiktopulo et al., 1994; Gesierich & Pfeil, 1996), $\Delta U^{\text{eff}}(T_d)$ obtained from Equation 12 is called the "measured" standard change of internal energy while $\Delta U^{\text{eff}}(T_d)$ obtained from Equation 15 is called the "effective" two-state change of internal energy. The equivalence of Equation 12 to Equation 15 means that arbitrarily dividing a system into one plus the remainder will automatically satisfy the calorimetric criterion. Thus, the calorimetric criterion can be satisfied for any given heat capacity vs. temperature curve provided that baselines used to close the area of the heat capacity peak are consistent with the heat capacities of the individual states. The number of solutions for the heat capacities of the individual states that satisfy the calorimetric criterion is theoretically infinite since there are infinite number of ways to divide a system into two. Different solution have different baseline requirements, so that if the baseline is known accurately within the transition region, the number of such solutions will be limited to a few or even to one, in practice. However, in general, it is difficult to measure the thermodynamics of the transition region. (Also see the section on the three-helix bundle protein model.)

In experiments using the weighted smooth baseline method (Takahashi & Sturtevant, 1981; Sturtevant, 1987), the "measured" standard internal energy change is obtained from (cf. Equation 10)

$$\Delta U_s^{\text{expt}} = \int_{T_{\text{I}}}^{T_{\text{II}}} [C_v(T) - f_{\text{I}} C_{v\text{I}}(T) - f_{\text{II}} C_{v\text{II}}(T)]\, dT$$

$$= U_{\text{II}}(T_{\text{II}}) - U_{\text{I}}(T_{\text{II}}) - \int_{T_{\text{I}}}^{T_{\text{II}}} f_{\text{II}}[C_{v\text{II}}(T) - C_{v\text{I}}(T)]\, dT. \quad (16)$$

Equation 16 shows that even for a two state process, ΔU_s^{expt} in general does not equal to $U_{\text{II}}(T) - U_{\text{I}}(T)$ at a pre-assigned temperature, although there are special cases when it does. For example, $\Delta U_s^{\text{expt}} = U_{\text{II}}(T) - U_{\text{I}}(T)$ if $U_{\text{II}}(T) - U_{\text{I}}(T)$ is independent of temperature ($C_{v\text{II}}(T) - C_{v\text{I}}(T) = 0$). For proteins, $U_{\text{II}}(T) - U_{\text{I}}(T)$ is temperature dependent but $C_{v\text{II}}(T) - C_{v\text{I}}(T)$ can be approximated as independent of temperature in most cases (Privalov, 1979). [This approximation is often used in the deconvolution method (Freire & Biltonen, 1978; Freire, 1995) but is not true for all proteins (Makhatadze & Privalov, 1995).] In this case, $\Delta U^{\text{expt}} = U_{\text{II}}(T_d) - U_{\text{I}}(T_d)$ if $f_{\text{II}}(T)$ is a step function at T_d or $f_{\text{II}} - 0.5$ is symmetric around $T = T_d$. The "measured" standard internal energy change ΔU^{expt} in some cases (Sturtevant, 1987; Freire, 1995) has been assigned as $U_{\text{II}}(T_m) - U_{\text{I}}(T_m)$, where T_m is the temperature at which $C_v(T) - \mathrm{f}_{\text{I}} C_{v\text{I}}(T) - \mathrm{f}_{\text{II}} C_{v\text{II}}(T)$ exhibits a maximum. This assignment is not exact as has been shown by Privalov and Potekhin (1986). However, the error due to the assignment may be smaller than the experimental error, which is rarely better than 5%. Thus, although the Jackson–Brandts baseline is theoretically exact for a two-state process while the weighted smooth baseline method is only approximately so, the two methods are likely to be equivalent within experimental errors unless very precise measurements are available.

B: Simple three-state model

The thermodynamic definition of a two-state process corresponds to the requirement that there be a negligible population of intermediate states at the transition temperature. If there are no intermediate states, whether by definition or because of the form of the potential surface, the calorimetric criterion will be satisfied, as shown in the previous subsection. The important question in applying this type of approach to protein denaturation is whether the opposite is also true, i.e., does satisfying the calorimetric criterion demonstrate that intermediate states make a negligible contribution?

To answer this question in the simplest fashion, we introduce a three-state model. The three states for the model are: (1) the lowest energy state I, (2) an intermediate state *m*, and (3) the highest energy state II. The partition function for this three-state model is

$$Q = g_{\text{I}} e^{-\beta\epsilon_{\text{I}}} + g_m e^{-\beta\epsilon_m} + g_{\text{II}} e^{-\beta\epsilon_{\text{II}}} \quad (17)$$

where $\beta = 1/k_B T$, g_l and ϵ_l are the degeneracy factor and energy for the state *l*, respectively, and $\epsilon_{\text{I}} < \epsilon_m < \epsilon_{\text{II}}$. For proteins, state I can be thought of as the folded state, state II as the unfolded denatured state, and state *m* as the intermediate state. (To ensure that state *m* is a transition intermediate, only cases with a single heat capacity peak are considered.[4]) Since each state is a collection of many micro-states (Lumry et al., 1966; Chan et al., 1995), the energy level and degeneracy factor in the model represent average properties. For simplicity, we assume that the energy levels and degeneracy factors in Equation 17 are independent of temperature.

The three-state model can be analyzed by following the steps corresponding to a calorimetric experiment. In experiments, the only measurable quantity is the curve describing heat capacity as a function of temperature $C_v(T)$. Experiments normally assume that away from the transition region at $T = T_{\text{I}}$ and $T = T_{\text{II}}$ there exists only the state I or the state II and the heat capacity of the state I or the state II can be linearly extrapolated from $T = T_{\text{I}}$ and $T = T_{\text{II}}$, respectively. Here, we use $T_{\text{I}} = 0$, $T_{\text{II}} = \infty$, and the exact results $C_{v\text{I}}(T) = C_{v\text{II}}(T) = 0$. The use of exact results for $C_{v\text{I}}(T) = C_{v\text{II}}(T) = 0$ allows us to analyze the data without concern about the method used for fitting the baselines.

The experimentally measured internal energy change satisfies (cf. Equation 3)

$$\Delta U^{\text{expt}} = \int_0^\infty C_v(T)\, dT. \quad (18)$$

It should be noted that ΔU^{expt} is independent of temperature for this model. To calculate the effective van't Hoff two-state internal energy change, we also need to evaluate the fraction of the system in state II, f_{II}, which can be obtained from Equation 13. Equation 13 is applicable here because one has to assume a two-state approximation to obtain van't Hoff enthalpy changes. However, since $U_{\text{I}}(T)$ and $U_{\text{II}}(T)$ are not known in real experiments, we need to replace them with the experimentally measurable quantity $U(T)$; we include a temperature dependence for the energy and heat capacity for generality. The denominator in Equation 13, $U_{\text{II}}(T) - U_{\text{I}}(T)$, is taken to be $\int_0^\infty C_v(T)\, dT$ from Equation 18. The numerator in Equation 13, $U(T) - U_{\text{I}}(T)$, can be also exactly expressed as $U(T) - U(0)$ for our model here since $U(0) = U_{\text{I}}(0) = U_{\text{I}}(T)$. Thus, we have

$$f_{\text{II}} = \frac{\int_0^T C_v(T)\, dT}{\int_0^\infty C_v(T)\, dT}. \quad (19)$$

In other words, f_{II} can be approximated as the fraction of peak area that lies between the temperature T and temperature zero, a definition similar to that used in experiments (Privalov, 1979). This definition automatically satisfies the requirement that $f_{\mathrm{II}} = 0$ at $T = 0$ and $f_{\mathrm{II}} = 1$ at $T = \infty$. Using Equations 1 and 2, we obtain an expression for the effective van't Hoff two-state internal energy change as (Privalov, 1979)

$$\Delta U^{\mathrm{eff}}(T) = k_B T^2 \frac{C_v(T)}{f_{\mathrm{II}}(1-f_{\mathrm{II}})\int_0^\infty C_v(T)\,dT}. \tag{20}$$

At $f_{\mathrm{II}} = 1/2$, which defines the middle transition temperature T_d, we have the equation (cf. Equations 18 & 20)

$$\Delta U^{\mathrm{eff}}(T_d) = 4k_B T_d^2 \frac{C_v(T_d)}{\Delta U^{\mathrm{expt}}}, \tag{21}$$

which is identical to the equation used in experiments (Privalov, 1979). The ratio γ of the "measured" enthalpy change to the effective two-state enthalpy change is given by

$$\gamma \equiv \frac{\Delta U^{\mathrm{expt}}(T_d)}{\Delta U^{\mathrm{eff}}(T_d)} = \frac{[\Delta U^{\mathrm{expt}}]^2}{4k_B T_d^2 C_v(T_d)}. \tag{22}$$

The parameter $\gamma - 1$ measures the deviation from the calorimetric criterion.

The internal energy $U(T)$ of the three-state model system is given by (cf. Equation 17)

$$U(T) = -\frac{\partial \ln Q}{\partial \beta} = \frac{\sum_l g_l \epsilon_l e^{-\beta\epsilon_l}}{Q} \tag{23}$$

and the expression for the constant-volume heat capacity $C_v(T)$ is

$$C_v(T) = \left(\frac{\partial U}{\partial T}\right)_v = \frac{1}{k_B T^2}\left\{\frac{\sum_l g_l \epsilon_l^2 e^{-\beta\epsilon_l}}{Q} - [U(T)]^2\right\}. \tag{24}$$

Taking the limit $T \to 0$ and $T \to \infty$, we have (cf. Equation 23)

$$U(0) = \epsilon_{\mathrm{I}}, \qquad U(\infty) = \frac{\sum_l \epsilon_l g_l}{\sum_l g_l}, \tag{25}$$

and (cf. Equation 18)

$$\Delta U^{\mathrm{expt}} = \int_0^\infty C_v(T)\,dT = U(\infty) - U(0). \tag{26}$$

Since $f_{\mathrm{II}} = 1/2$ at $T = T_d$, we also have (cf. Equation 19)

$$U(T_d) = \tfrac{1}{2}[U(0) + U(\infty)]. \tag{27}$$

Equation 27 can be expressed as a nonlinear equation for the middle transition temperature T_d. Substituting Equations 24, 25, 26, and 27 into Equation 22, we obtain a simple expression for the ratio γ

$$\frac{1}{\gamma} = 1 + \frac{2[\epsilon_{\mathrm{II}} - U(\infty)]}{\Delta U^{\mathrm{expt}}} - \frac{4(\epsilon_m - \epsilon_{\mathrm{II}})(\epsilon_{\mathrm{I}} - \epsilon_m)P_m(T_d)}{[\Delta U^{\mathrm{expt}}]^2} \tag{28}$$

where $P_m(T)$ ($\equiv g_m e^{-\beta\epsilon_m}/Q$) is the population of intermediate state m. In Equation 28, the second term, which is greater than zero, is proportional to the difference between the energy of state II and the energy of the whole system at $T = \infty$. The third term, which is less than zero, is proportional to, but not equal to, the population of intermediate state m. Equation 28 shows that the deviation from the calorimetric criterion, as measured by $\gamma - 1$, is not simply equal to the population of intermediate state m as required by the sufficient condition.

We find that the calorimetric criterion works reasonably well if the interval between the energies of state II and state m is the same as the interval between the energies of state m and state I (i.e., $\epsilon_{\mathrm{II}} - \epsilon_m = \epsilon_m - \epsilon_{\mathrm{I}} = \epsilon$), and if the degeneracy factor for state II is much greater than the sum of the degeneracy factors for state I and for intermediate state m, i.e., $g_{\mathrm{II}} \gg g_{\mathrm{I}} + g_m$. In this case, Equation 28 becomes (cf. Equations 25 & 26)

$$\frac{1}{\gamma} = 1 + \frac{2(2g_{\mathrm{I}} + g_m)}{(2g_{\mathrm{II}} + g_m)} - \frac{4P_m(T_d)(g_{\mathrm{I}} + g_m + g_{\mathrm{II}})^2}{(2g_{\mathrm{II}} + g_m)^2}. \tag{29}$$

Because $g_{\mathrm{II}} \gg g_{\mathrm{I}} + g_m$, we further obtain

$$\gamma \approx \frac{1}{1 - P_m(T_d)} \approx 1 + P_m(T_d). \tag{30}$$

Equation 30 agrees with the common presumption that the deviation from the calorimetric criterion is equal to the population of intermediate states (Tanford, 1968; Privalov, 1979; Sturtevant, 1987). If Equation 30 were exact, the calorimetric criterion would be the necessary and sufficient condition for a two-state process. This is clearly not the case, in general, even for the simple three-state model. For example, we obtain $\gamma = 1.003$ for a hypothetical model with parameters $g_{\mathrm{I}} = 1$, $g_m = 2$, $g_{\mathrm{II}} = 20$, $\epsilon_{\mathrm{I}} = -2\epsilon$, $\epsilon_m = -\epsilon$, and $\epsilon_{\mathrm{II}} = 0$ despite the fact that the heat capacity vs. temperature curve has only one well-defined peak and the population of the intermediate state at the middle transition temperature $T_d^* = 0.617$ is 18%. Although the model described here is not based on protein data, its simplicity allows us to establish the principle that for a given heat capacity curve, $\gamma \sim 1$ does not necessarily signal a two-state process.

C: Thermodynamic three-state model

A more realistic model for proteins is the thermodynamic model that is used to fit the protein calorimetric data in the deconvolution method (Freire & Biltonen, 1978; Freire, 1995). Here, we use notations appropriate for a canonical ensemble as above. A partition function Q for a three-state system can be written as

$$Q = Q_{\mathrm{I}} + Q_m + Q_{\mathrm{II}} \tag{31}$$

where the three states correspond to the native, intermediate, and denatured states, respectively. With state I as the reference state, Equation 31 becomes

$$Q/Q_{\mathrm{I}} = 1 + Q_m/Q_{\mathrm{I}} + Q_{\mathrm{II}}/Q_{\mathrm{I}} = 1 + e^{-\beta\Delta A_m(T)} + e^{-\beta\Delta A_{\mathrm{II}}(T)} \tag{32}$$

where $\Delta A_m(T)$ and $\Delta A_{\mathrm{II}}(T)$ are excess Helmholtz free energy over the reference state I for states m and II, respectively. Assuming that the excess heat capacities relative to the reference states (ΔC_{vm} and $\Delta C_{v\mathrm{II}}$) are independent of temperature, $\Delta A_m(T)$ and $\Delta A_{\mathrm{II}}(T)$ can be determined by

$$\begin{aligned}\Delta A_m(T) &= \Delta U_m(T_{ref}) + \Delta C_{vm}(T - T_{ref}) \\ &\quad - T[\Delta S_m(T_{ref}) + \Delta C_{vm}\ln(T/T_{ref})]\end{aligned} \tag{33}$$

and

$$\begin{aligned}\Delta A_{\mathrm{II}}(T) &= \Delta U_{\mathrm{II}}(T_{ref}) + \Delta C_{v\mathrm{II}}(T - T_{ref}) \\ &\quad - T[\Delta S_{\mathrm{II}}(T_{ref}) + \Delta C_{v\mathrm{II}}\ln(T/T_{ref})].\end{aligned} \tag{34}$$

Three constants are required for defining each state. They are the excess heat capacity ΔC_v, the excess internal energy $\Delta U(T_{ref})$, and the excess entropy $\Delta S(T_{ref})$, at a certain reference temperature T_{ref}. The deconvolution method of Freire (1995) and Privalov et al. (1995) makes use of a least-squares fit of the measured heat capacity curve by increasing the number of states from a minimum of two (i.e., I and II) to the number required to obtain an accurate fit. Note, that if $\Delta C_{v\mathrm{II}} = 0$ and $\Delta C_{vm} = 0$, the thermodynamic three-state model reduces to the simple three-state model described in the previous subsection.

Due to increased complexity of the thermodynamic model, a simple expression for γ, similar to Equation 28, does not exist. An example to illustrate that the intermediate population is not identical to the deviation from the γ value has been given by Freire (1995). Here, we use a similar model with $\Delta U_m(55\,^\circ\mathrm{C}) =$ 40 kcal/mol, $\Delta C_{vm} = 500$ cal/K/mol, $\Delta S_m(55\,^\circ\mathrm{C}) = 121.9$ cal/K/mol, $\Delta U_{\mathrm{II}}(50\,^\circ\mathrm{C}) = 200$ kcal/mol, $\Delta C_{v\mathrm{II}} = 2{,}000$ cal/K/mol, and $\Delta S_{\mathrm{II}}(50\,^\circ\mathrm{C}) = 618.9$ cal/K/mol. The temperature dependence of the heat capacity and populations of states for the three-state model is shown in Figure 2. The intermediate concentration is ~20% around the transition temperature, thus, is non-negligible. The model yields 1.1 for the ratio of the two ΔUs, suggesting only a small deviation from a two-state transition. This confirms that the magnitude of the deviation from the simple calorimetric criterion is not sensitive to the population of intermediates (Freire, 1995).

With the deconvolution method, the C_v curve can also be fitted very accurately by a two-state model using the same value for $\Delta C_{v\mathrm{II}}$ (= 2,000 cal/K/mol) but different values for $\Delta U_{\mathrm{II}}(50\,^\circ\mathrm{C})$ (= 190.7 kcal/mol) and $\Delta S_{\mathrm{II}}(50\,^\circ\mathrm{C})$ (= 589.5 cal/K/mol). Only a small discrepancy between the original heat capacity curve and the fitted curve is found at the edge of the transition ($T \sim 40\,^\circ\mathrm{C}$). This small error may well be masked by uncertainties in experimental data. As the data improve, deviations obtained with over-simplified (two-state) descriptions become more significant. The model described here indicates that because of the large number of parameters in the deconvolution method (three per state), its application is meaningful only if very precise data are available. It should be emphasized that the analysis in here and the next subsections is independent of the choice of baseline methods since the exact baselines are used.

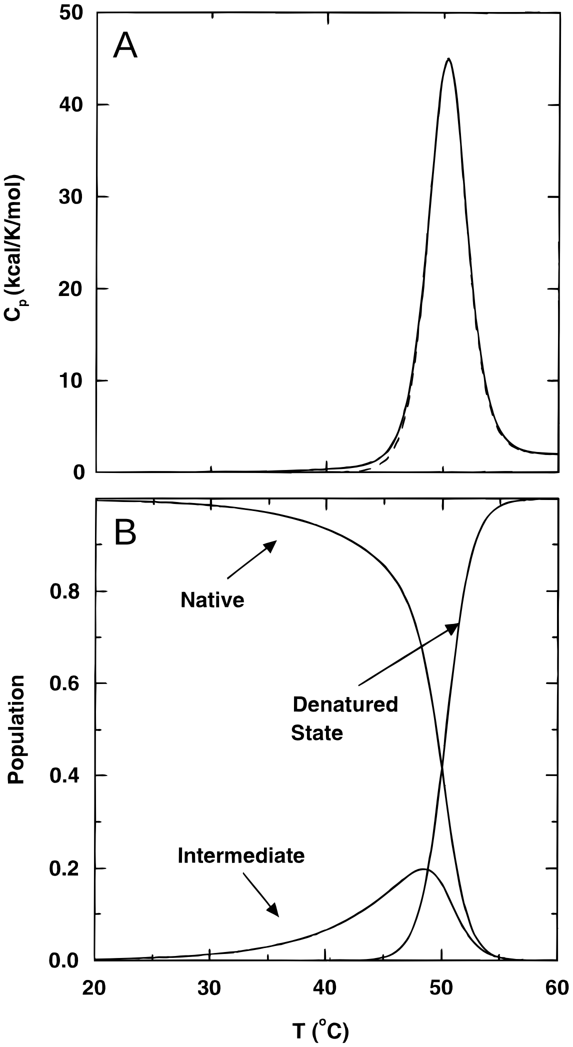

Fig. 2. (**A**) The temperature dependence of the heat capacity and (**B**) the populations for a three-state model described in text. The dashed line shown with the heat capacity curve (**A**) is a fit using the deconvolution method with a two-state model for the three-state system.

Homonuclear square-well tetramer

To obtain a clearer understanding of the results presented so far, it is useful to apply them to simple examples for which an exact analysis is possible. Since the calorimetric criterion has been used to analyze experiments on homopolymers (Tiktopulo et al., 1994, 1995), we first illustrate the criterion by using a model for an isolated homonuclear square-well tetramer. The square-well chain model is chosen for this purpose because it is perhaps the simplest yet reasonably-realistic off-lattice model of a homopolymer and because exact results for the thermodynamics of isolated trimers and tetramers are available (Taylor, 1995). Recently, it has been shown that homonuclear square-well chains are accurate models for alkanes ranging from methane to eicosane (Bokis et al., 1994).

The square-well tetramer consists of four freely-jointed beads; i.e., there are four spheres, which could represent extended atom alkane (CH_2 or CH_3) groups that are joined by rigid bonds. The bead-bead interaction potential u_{ij} for beads that are not bonded to each other is defined as

$$u_{ij}(r) = \begin{cases} \infty, & r/\sigma < 1, \\ -\epsilon, & 1 < r/\sigma < \lambda, \\ 0, & r/\sigma > \lambda, \end{cases} \tag{35}$$

where σ is the hard-sphere bead diameter, $\lambda\sigma$ is the square-well diameter, ϵ is the square-well depth, and i and j refer to any two nonbonded beads.

Exact results of thermodynamics for isolated square-well tetramers are known (Taylor, 1995). A square-well tetramer has four micro-states with reduced energy levels ϵ_l/ϵ ranging from -3 to 0. Its partition function is

$$Q = g_0 + g_1 e^{\beta^*} + g_2 e^{2\beta^*} + g_3 e^{3\beta^*} \tag{36}$$

where $\beta^* = 1/T^*$ with the reduced temperature $T^* = k_B T/\epsilon$ and g_l is the degeneracy factor for energy level $\epsilon_l = -l\epsilon$ where $l = 0$, 1, 2, and 3. The values of the normalized degeneracy factors g_l vary with the square-well diameter $\lambda\sigma$ and are tabulated in Table 1.

Figure 3 shows an example of the tetramer heat capacity vs. temperature curve. It has a single peak that represents the heat released during transition. We can make the same calorimetric analysis of the tetramer heat capacity vs. temperature curve as for the three-state model. Using the fact that the heat capacities of the initial state I and the final state II are zero, the ratio γ of the "measured" internal energy change to the effective van't Hoff internal energy change can be obtained from Equation 22 after the nonlinear Equation 27 for the middle transition temperature T_d is solved. The results are also listed in Table 1. As the square-well diameter increases from 1.1σ to 1.8σ, the ratio γ decreases monotonically from 2.4 to 0.69, reflecting the fact that the peak area (ΔU^{expt}) decreases faster than ΔU^{eff} estimated from the C_v peak height. However, the intermediate population is 56.5% even when γ ($= 1.08$) is close to 1 at $\lambda = 1.6$. Thus, the calorimetric criterion can be satisfied even in the presence of significantly populated intermediate states. In addition, Table 1 shows that γ can even be less than 1. This would be not be possible if $\gamma - 1$ were interpreted as the population of intermediate states. In real calorimetric experiments, $\gamma < 1$ would be interpreted as an indication of intermolecular cooperative transition (Sturtevant, 1987). This is not applicable here since there is only one molecule. However, the C_v curve with $\gamma = 1.08$ cannot be fitted using a simple two-state model that has a temperature independent g_{I}, g_{II}, ϵ_{I}, and ϵ_{II}. Thus, the two-state fitting used in a deconvolution method can eliminate some false positive results.

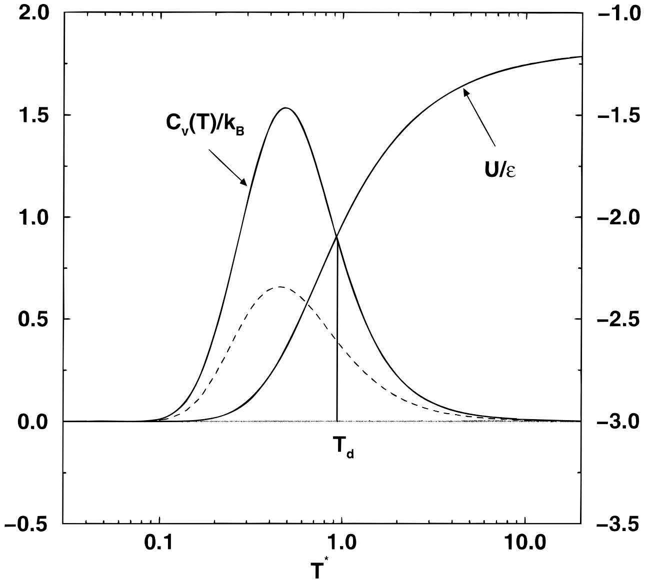

Fig. 3. The reduced heat capacity $C_v(T)/k_B$ and reduced internal energy as a function of reduced temperature $T^* = k_B T/\epsilon$ for a square-well tetramer at the square-well diameter $\lambda = 1.6$. T_d^* denotes the middle transition temperature defined in the text. The dashed line denotes the C_v curve with $\lambda = 1.8$.

Three-helix bundle protein model

To further illustrate the calorimetric criterion, we apply it to a two-state-like transition of a model three-helical fragment of *Staphylococcus aureus* protein A; details of the calculations are given in

Table 1. *The degeneracy factors g_l for the four reduced energy levels ($\epsilon_l/\epsilon = -3, -2, -1$, and 0) for isolated square-well tetramers at various square-well diameters $\lambda\sigma$ (Taylor, 1995) with corresponding middle transition temperature T_d^*, internal energy ratio γ, and the population of intermediates P_m ($= P_1 + P_2$)*

λ	g_3	g_2	g_1	g_0	T_d^*	γ	P_m
1.1	0.000122	0.004554	0.065336	0.463868	0.356	2.40	0.696
1.2	0.001064	0.018018	0.121262	0.393536	0.469	2.15	0.691
1.3	0.00397	0.039654	0.166486	0.323768	0.574	1.90	0.678
1.4	0.01064	0.06756	0.20011	0.25557	0.681	1.63	0.655
1.5	0.02467	0.09650	0.222457	0.190251	0.806	1.34	0.614
1.6	0.04871	0.12362	0.231958	0.129594	0.938	1.08	0.565
1.7	0.08238	0.15361	0.220877	0.077011	1.05	0.87	0.515
1.8	0.12489	0.19174	0.18115	0.0361	1.12	0.69	0.461
ϵ_l/ϵ	-3	-2	-1	0			

Zhou and Karplus (1997). The model consists of 46 freely-jointed beads each of which represents an amino acid residue that can interact with other residues via a square-well potential. Such a square-well potential mimics standard interactions (e.g., van der Waals and hydrogen bonds), but is much faster for simulations by the use of discrete molecular dynamics (Liu et al., 1994). The global minimum structure of the model is shown in Figure 4. The square-well depth is $-\epsilon$ if the interaction pair involves a native contact and is zero otherwise (Taketomi et al., 1975). The detailed thermodynamics (phase diagram) of this model has been calculated and shown to correspond to experiments on proteins. It is thus a useful system for illustrating the calorimetric criterion. We focus on the transition from a random coil to a molten globule. The molten globule state is found to have well-defined three-helix structure on average with large liquid-like fluctuations. This transition is found to be a two-state transition as characterized by a bimodal distribution in the potential energies near the transition temperature (i.e., a free energy barrier separates the two states, each of which consists of many configurations (Fig. 5)). Although there are population overlaps between the two states, the actual population of intermediates is low; it is below 10% even when all configurations that have an energy in the range from -100ϵ to -120ϵ (the region of small population) are assumed to be intermediates.

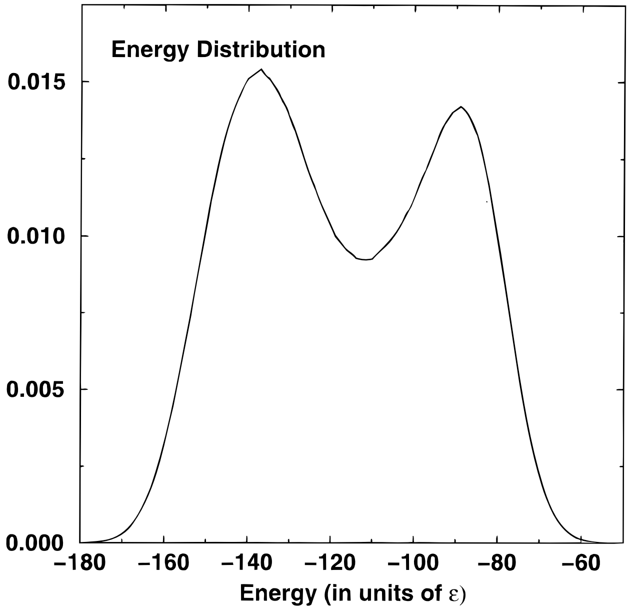

Fig. 5. The distribution of potential energies at $T^* = 0.898$ for the model three-helix bundle protein. The overlaps between the two states, the actual population of intermediates, are low; it is below 10% even when all configurations that have an energy in the range from -100ϵ to -120ϵ (the region of small population) are assumed to be intermediates.

The heat capacity vs. temperature curve for the collapse transition shown in Fig. 6 is obtained from equilibrium simulations at 21 reduced temperatures ranging from 0.1 to 5.0 via the weighted histogram method (Ferrenberg & Swendsen, 1989; Zhou et al., 1997). The curve shows that the heat capacity of random coils that are stable at $T^* > 1.1$ is smaller than that of the compact globule state that exists at $T^* = 0.5 - 0.7$. This reflects the fact that random coils have many similar stable energy states and, thus, the energy changes slowly with temperature. Such behavior is different from proteins (see Fig. 6) and homopolymers (Tiktopulo et al., 1994, 1995)[5] where the random-coil state has a higher heat capacity than the compact state. This disagreement with the experimental curve is probably due to the absence of temperature-dependent protein-solvent interactions in the model. Experimentally, the increased heat capacity upon unfolding is attributed to the exposure of hydrophobic residues to water (Kauzmann, 1959; Privalov & Khechinashvili, 1974; Murphy & Gill, 1991). However, this difference in behavior does not affect the arguments given below.

Based on the standard experimental approach (Privalov, 1979), the heat capacities for two individual states are extrapolated linearly from high or low temperatures. The middle transition temperature (T_d^*) is obtained by solving the nonlinear Equation 13 for $f_{\mathrm{II}} = 0.5$. The experimentally "measured" standard change of internal energy ΔU^{expt} calculated from Equation 12 is found to be (remember that ΔU^{expt} is simply another way to obtain ΔU^{eff}, see also Equation 4).

$$\Delta U^{\mathrm{expt}}(T_d) = U_{\mathrm{II}}(T_d) - U_{\mathrm{I}}(T_d) = 1.26N\epsilon. \qquad (37)$$

The effective standard change of internal energy can be calculated from Equation 15:

$$\Delta U^{\mathrm{eff}}(T_d) = 0.787N\epsilon. \qquad (38)$$

The value of γ, obtained from the ratio of the two ΔUs (Equation 22), is 1.60. The use of a weighted smooth baseline yields an essentially the same γ value. Thus, we have shown that even for a

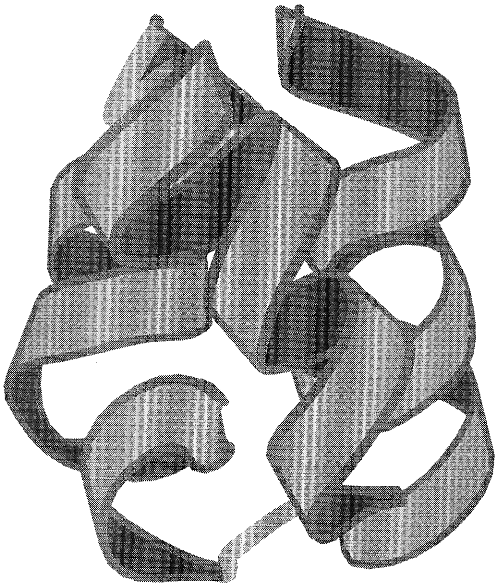

Fig. 4. The global minimum structure of the model three-helix bundle protein.

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[5] We note that poly(N-isoproylacrylamide) and poly(N-isopropylmethacrylamide) are more compact at high temperatures.

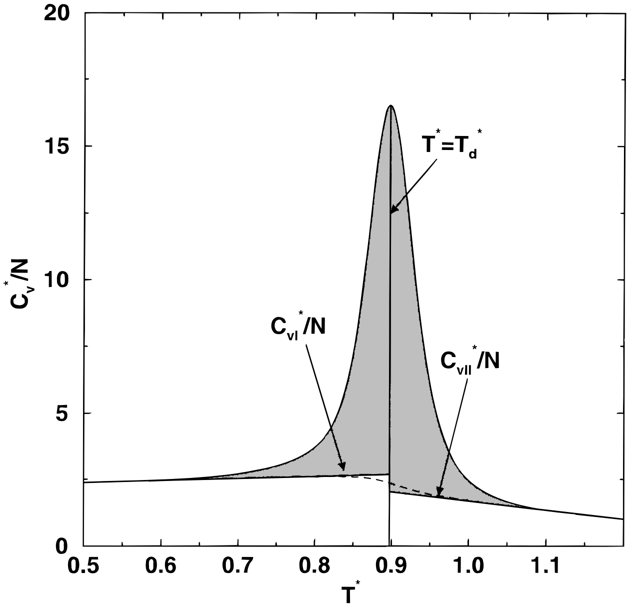

Fig. 6. The reduced heat capacity $C_v(T)/k_B$ per bead as a function of reduced temperature T^* for a model three-helix bundle protein. $T_d^* = 0.898$. The dashed lines are weighted smooth baselines.

thermodynamic two-state transition, the calorimetric criterion may not be satisfied if the standard linear interpolation of baselines is employed.

As pointed out earlier, one way to make $\gamma = 1$ is to arbitrarily divide the system into two parts (“formal” definition). For example, we can assign the states with energy less than or equal to -112ϵ as state I and the rest as state II. This yields nonlinear baselines (solid lines) of C_{vI} and C_{vII} vs. temperature curves as shown in Figure 7. The large change in baselines within the transition region is observed when $E \leq -120\epsilon$ is used to separate the state I from the rest (state II) (Fig. 5). This demonstrates that the detailed interpretation of the heat capacities for individual states, which is represented in the choice of the baselines, makes an essential difference in the result obtained from the calorimetric criterion. Obviously, the number of such solutions that lead $\gamma = 1$ depends on the knowledge of the baselines within the transition region.

Conclusions

We have shown that the calorimetric criterion for a two-state process ($\gamma = \Delta U^{\mathrm{expt}}(T_d)/\Delta U^{\mathrm{eff}}(T_d) = 1$) can be satisfied even when there is a significant population of intermediate states. Conversely, γ can deviate significantly from unity even when the transition is a thermodynamic two-state process. The above statement, however, does not preclude that under certain conditions (e.g., $\epsilon_{\mathrm{II}} - \epsilon_m = \epsilon_m - \epsilon_{\mathrm{I}} = \epsilon$, $g_{\mathrm{II}} \gg g_{\mathrm{I}} + g_m$ for the simple three-state model) the deviation from the calorimetric criterion is correlated with the population of intermediates. It is not clear whether proteins belong to this category, though the degeneracy criterion is expected to be satisfied. Even if they do, there is still an uncertainty regarding the meaning of the calorimetric criterion; i.e., for any given heat capacity vs. temperature curve, regardless of whether it originates from a two-state process or not, one can always find baselines (both linear or nonlinear) that make $\gamma = 1$ by simply dividing the system into two parts. Conversely, seemingly reasonable baselines can make γ deviate significantly from unity for a two-state process as we shown in Figure 6. This illustrates the importance of using the correct baseline from the theoretical point of view. Experiments have also shown that errors in baselines of weakly stable proteins yield incorrect results concerning the two-state character of transition (Haynie & Freire, 1994). Multiple experiments with different solvent conditions have also been used to obtain baselines for a wider temperature range (Makhatadze & Privalov, 1995). The deconvolution method (Freire & Biltonen, 1978; Freire, 1995) improves over the simple criterion since it compares the entire curve rather than one temperature point. However, unlike the simple criterion, additional assumptions regarding the behavior of individual states have to be made. In at least one case, a good fit does not necessary mean a low population for the intermediate even with correct baselines (Fig. 2).

Although the simple calorimetric criterion is not a sufficient condition for the existence of a two-state transition, the criterion has been applied to proteins in cases where its results are consistent with the results from other methods, including gel electrophoresis (Creighton, 1986), size exclusion chromatography (Uversky, 1993), and hydrogen-deuterium exchange (Yi & Baker, 1996). This suggests that the criterion is a useful tool for studying folding transition though it is important to be confirmed it by other measurements. This is particularly true when the baseline within the transition region is not accurately known.

Recently, Sturtevant and his coworkers (Liu & Sturtevant, 1995, 1997; Naghibi et al., 1995) have found significant deviations between van’t Hoff and calorimetric enthalpies for a wide variety of processes that are believed to be two-state like (e.g., the reaction of n-heptanoic acid with n-heptanoate in dodecane solution and of sodium heptanoate with α-cyclodextrin in aqueous solution). The results in this paper cannot be used to interpret the discrepancy

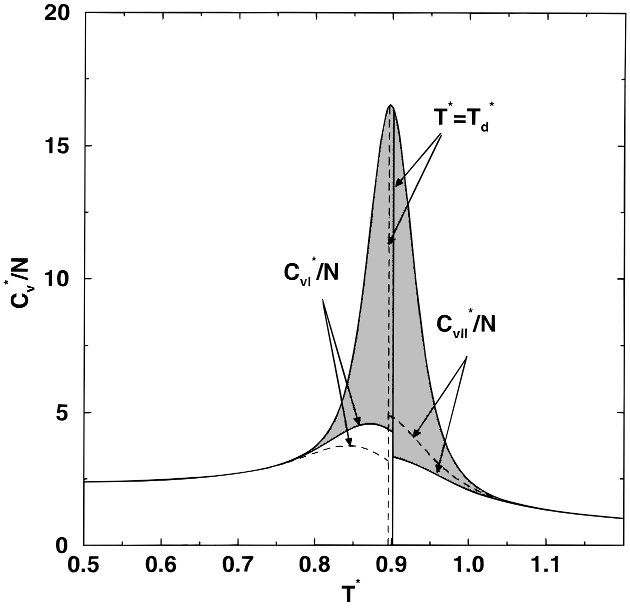

Fig. 7. As in Figure 6 but C_{vI} and C_{vII} calculated by dividing all states into two states shown in Figure 5. Solid lines: $T_d^* = 0.902$ and two states are divided at $E = -112\epsilon$. Dashed lines: $T_d^* = 0.896$ and two states are divided at $E = -120\epsilon$.

found by them since their experiments involve calorimetric enthalpies determined at a single temperature.

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