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EVALUATION OF THERMODYNAMIC PROPERTIES OF IRREVERSIBLE PROTEIN THERMAL UNFOLDING MEASURED BY DSC

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We assessed the applicability of the extrapolation procedure at infinite scanning rate to differential scanning calorimetry (DSC) data related to irreversible protein unfolding. To this aim, an array of DSC curves have been simulated on the basis of the Lumry–Eyring model N↔U→F. The results obtained confirmed that when the apparent equilibrium constant $K_{app\,(T=T1/2)}$ is lower than 3, the application of the extrapolation procedure provides accurate thermodynamic parameters. Although this procedure applies only to monomeric proteins for which the Lumry–Eyring model is a reasonable approximation, it will hopefully contribute to increase the potential of DSC in obtaining reliable thermodynamic information regarding the folding/unfolding equilibrium.

Keywords: *differential scanning calorimetry, extrapolation to infinite scan rate, reliability*

Introduction

During the last two decades differential scanning calorimetry (DSC) has significantly contributed to the development of our current understanding of the energetics and thermodynamic properties of protein folding-unfolding transitions [1–10]. However, the above types of analysis require that the experimental heat capacity data accurately reflect the protein equilibrium unfolding [11]. DSC analysis of a variety of proteins has shown that only few small (M_W<20000) globular proteins, in particular experimental conditions usually undergo reversible, two-state thermally induced folding/unfolding transitions [12]. Conversely, larger multidomain proteins in the majority of cases, exhibit a complex, irreversible unfolding pathway [13–19]. For these systems, therefore, thermodynamic parameters cannot be directly extracted from DSC analysis. Nevertheless, it has been widely held in the literature that irreversible alteration of the unfolded state does not significantly distort DSC transitions, but takes place at a somewhat higher temperature [20–25]. However, many experimental and theoretical works highly disfavour this claim. In fact, it has been shown that irreversible DSC transitions are distorted by time-dependent factors [26–29]. In an attempt to clarify this matter, two questions arise: *i*) to what extent is a given DSC curve affected by the occurrence of an irreversible process? *ii*) is it possible to obtain thermodynamic information from calorimetric experiments affected by an irreversible process? It has been already demonstrated that even in the case of an irreversible temperature-induced denaturation, it is still possible to obtain thermodynamic information about protein unfolding by using a method of analysis that takes into account the kinetics of the irreversible process. The theoretical basis for a detailed analysis of the kinetic models describing the irreversible denaturation of proteins and the mathematical procedure to extract thermodynamic information from the corresponding DSC curves have been reviewed elsewhere [30].

However, despite the promising initial results obtained by means of this procedure, very few papers dealing with the extrapolation of DSC data at infinite scan rate are available in the literature. One of the possible drawbacks in the widespread application of this methodology in DSC studies is that the range of applicability and the reliability of the extrapolated data have not yet been clearly defined.

In the present paper the equations used for the extraction of equilibrium data from irreversible DSC curves were studied to investigate: a) the reliability of the extrapolated thermodynamic data; b) what factors can negatively influence the extrapolation procedure; c) whether there is any interaction between the variables in affecting the procedure; d) if it is possible to define a general criterion to establish in what cases the extrapolation procedure may be correctly applied to experimental DSC curves.

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Theory and methods

The denaturation model

A description of the kinetic models relative to irreversible protein unfolding can be found in many papers [30–32]. In this section we report only the equations and the symbols that will be used throughout the paper. It has been proposed that irreversible denaturation occurs in two steps: *i*) the reversible unfolding of the native protein N↔U; *ii*) the irreversible alteration of the unfolded protein U to yield a final state (F), which is unable to fold back to the native one, according to the scheme:

$$N \overset{K}{\leftrightarrow} U \overset{k}{\rightarrow} F \tag{a}$$

which is known as the 'Lumry–Eyring' model [31].

The equilibrium constant K depends on temperature T according to the equation:

$$K = \frac{X_{\mathrm{U}}}{X_{\mathrm{N}}} = \exp\left[-\frac{\Delta H_{\mathrm{U}}}{R}\left(\frac{1}{T} - \frac{1}{T_{1/2}}\right)\right] \tag{1}$$

where X_{U} and X_{N} are the molar fractions of the unfolded and native state, ΔH_{U} is the enthalpy change associated with the reversible process, R is the gas constant, and $T_{1/2}$ is the temperature at which $K=1$. It is assumed that the irreversible step is under first-order kinetic control and that the kinetic constant k depends on T according to the Arrhenius equation:

$$k = \exp\left[-\frac{E}{R}\left(\frac{1}{T} - \frac{1}{T^{*}}\right)\right] \tag{2}$$

where E is the activation energy and T^* is the temperature at which $k=1$ (the frequency factor is $A=\exp(E/RT^*)$). Being X_{F} the molar fraction of the final state F, the rate equation for the irreversible formation of F is:

$$\frac{\mathrm{d}X_{\mathrm{F}}}{\mathrm{d}t} = kX_{\mathrm{U}} \tag{3}$$

If the heating rate ν is assumed to be constant ($\nu=\mathrm{d}T/\mathrm{d}t$), integration of Eq. (3) from a low temperature, T_0, (where the reaction rate is negligible and X_{F} is zero), will give the temperature dependence of X_{F}:

$$X_{\mathrm{F}} = 1 - \exp\left(-\frac{1}{\nu}\int_{T_0}^{T}\frac{kK}{K+1}\mathrm{d}T\right) \tag{4}$$

Analogously, it is possible to obtain the following expressions for X_{U} and X_{N}:

$$X_{\mathrm{U}} = \frac{K}{K+1}\exp\left(-\frac{1}{\nu}\int_{T_0}^{T}\frac{kK}{K+1}\mathrm{d}T\right) \tag{5}$$

$$X_{\mathrm{N}} = \frac{1}{K+1}\exp\left(-\frac{1}{\nu}\int_{T_0}^{T}\frac{kK}{K+1}\mathrm{d}T\right) \tag{6}$$

The apparent excess enthalpy $<\Delta H>$ is defined as:

$$<\Delta H>=X_{\mathrm{U}}\Delta H_{\mathrm{U}}+X_{\mathrm{F}}\Delta H \tag{7}$$

where ΔH_{U} and ΔH are the enthalpies of states U and F, respectively (taking N as the reference state). The excess calorimetric enthalpy ΔH is the sum of two effects:

$$\Delta H=\Delta H_{\mathrm{F}}=\Delta H_{\mathrm{U}}+\Delta H_{\mathrm{ag}} \tag{8}$$

the first of which is ascribable to reversible unfolding (ΔH_{U}), while the second is associated with the irreversible step (ΔH_{ag}).

Equation (7) can be re-written as:

$$<\Delta H>=X_{\mathrm{U}}\Delta H_{\mathrm{U}}+X_{\mathrm{F}}(\Delta H_{\mathrm{U}}+\Delta H_{\mathrm{ag}}) \tag{9}$$

Taking into account the expressions of X_{U} and X_{F}, the first derivative with respect to temperature of Eq. (9) gives the excess heat capacity:

$$C_{\mathrm{p_{exc}}} = \left[\frac{K\Delta H_{\mathrm{U}}}{(\mathrm{K}+1)^2}\left(\frac{k}{\nu} + \frac{\Delta H_{\mathrm{U}}}{RT^2}\right) + \Delta H_{\mathrm{ag}}\frac{1}{\nu}\frac{kK}{K+1}\right] \exp\left(-\frac{1}{\nu}\int_{T_0}^{T}\frac{kK}{K+1}\mathrm{d}T\right) \tag{10}$$

When $\nu\rightarrow\infty$ Eq. (10) will transform into:

$$C_{\mathrm{p_{exc\infty}}} = \frac{K\Delta H_{\mathrm{U}}^{2}}{RT^{2}(K+1)^{2}} \tag{11}$$

which represents the $C_{\mathrm{p_{exc}}}$ function for a N↔U equilibrium.

In 1990 Freire *et al*. proposed a general mechanism for the description of proteins thermally-induced conformational transitions [32].

According to this model it was possible to define a reversible excess enthalpy ($<\Delta H>_{\mathrm{rev}}$) associated only to the intermediate states which are in thermodynamic equilibrium with each other. $<\Delta H>_{\mathrm{rev}}$ is related to the calorimetric enthalpy ΔH, according to the following equation:

$$\Delta H - <\Delta H> = (\Delta H - <\Delta H>_{\mathrm{rev}})\mathrm{e}^{-F(T)/\nu} \tag{12}$$

where $F(T) = \int_{T_0}^{T} k_{\mathrm{app}}\mathrm{d}T$ and $k_{\mathrm{app}} = \frac{kK}{K+1}$. It is evident from Eq. (11) that when $\nu\rightarrow\infty$, $<\Delta H>\rightarrow<\Delta H>_{\mathrm{rev}}$. In other words, the $C_{\mathrm{p_{exc}}}(T)$ curve extrapolated to infinite scanning rate will provide information relative only to the states in thermodynamic equilibrium [28]. In order to investigate the effect of the unfolding parameters, ΔH_{U}, $T_{1/2}$, E_{att}, T^* and ΔH_{ag} on the shape of DSC curves and, in turn, on the reliability of the extrapolated data, we devised a mathematical program whose

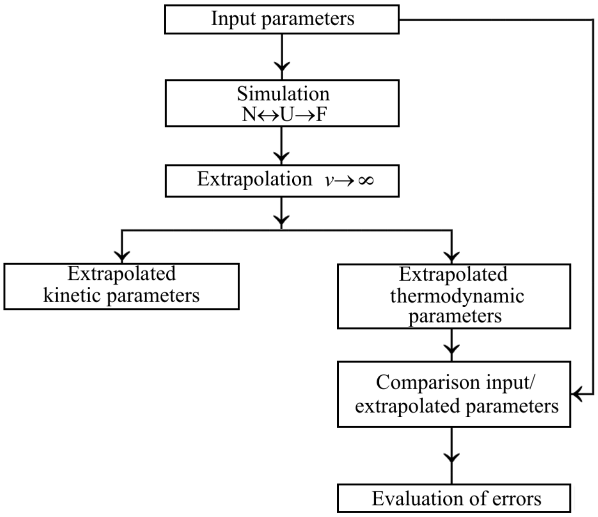

Fig. 1 Flowchart of the mathematical program developed for the analysis of the effectiveness of the extrapolation procedure of DSC curves at infinite scanning rate

flow chart is reported in Fig. 1. The entry values are ΔH_U, $T_{1/2}$, T^*, E_{att} and ΔH_{ag}. After having entered the values of these variables, the program simulates six different $C_{p_{exc}}(T)$ curves calculated at 0.1, 0.3, 0.5, 0.7, 0.9, 1.2 K min^{-1}. These curves are then extrapolated to infinite scanning rate as described elsewhere [28], and the results obtained are compared with the entry values. The differences between the entry values and the extrapolated values calculated for ΔH_U and $T_{1/2}$ are the numerical indicators of the efficiency of the extrapolation procedure.

Results and discussion

Excess heat capacity curves, each defined by the values of the unfolding parameters, ΔH_U, $T_{1/2}$, E_{att}, T^*, ΔH_{ag}, have been simulated by means of the Mathematica ver. 3.0 program. The ranges of entry values of the unfolding parameters have been chosen considering the values commonly observed for the majority of small globular proteins. In particular we have considered all the possible combinations of the following values: ΔH_U=300, 400, 500 kJ mol^{-1}; $T_{1/2}$=328.15, 338.15, 348.15, 358.15, 368.15 K; E_{att}=50, 150, 250 kJ mol^{-1}; T^*=323.15, 333.15, 343.15, 353.15, 363.15 K, ΔH_{ag}=0, –100 kJ mol^{-1}. Thus, 360 simulations were performed altogether. The results obtained for some simulations (30 of 360) are reported in Ta-

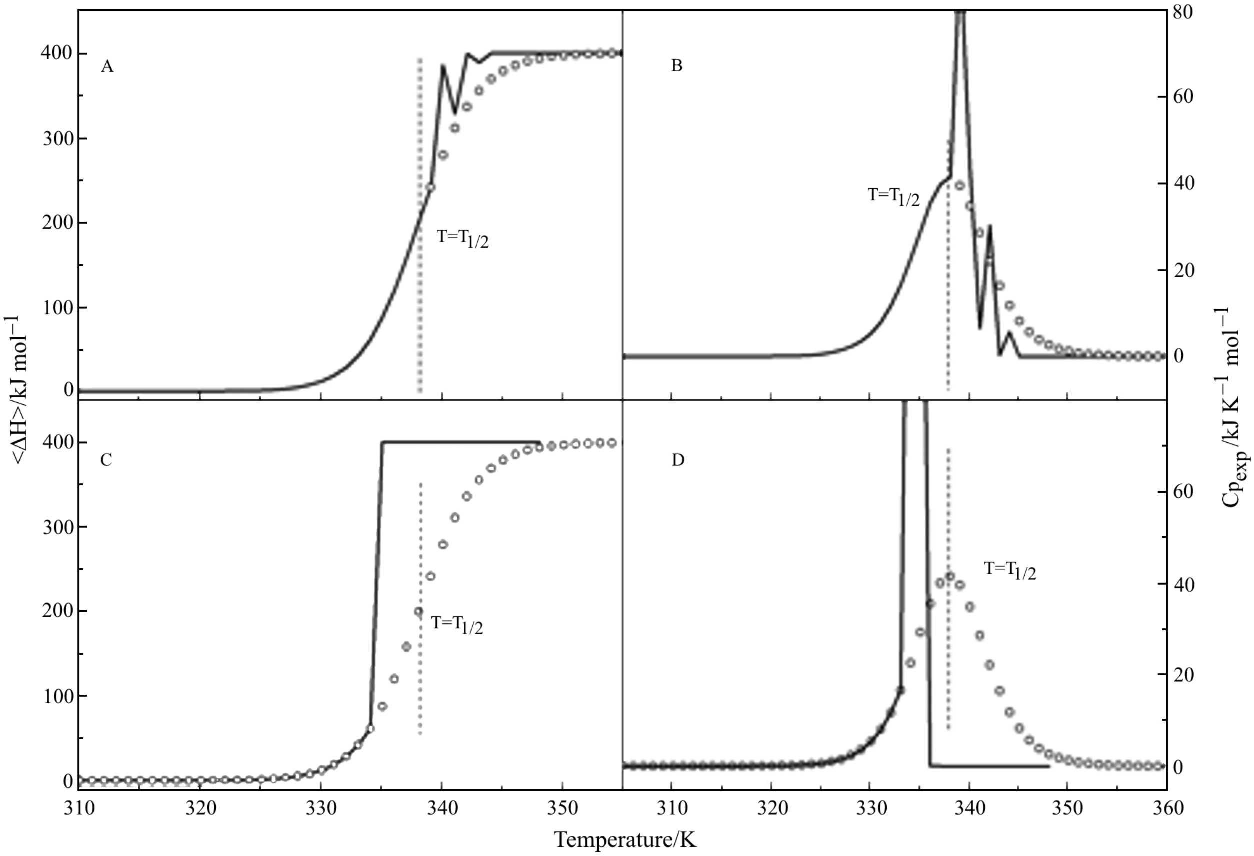

Fig. 2 Examples of reliable (panel A and B) and unreliable (panel C and D) extrapolations of DSC curves at infinite scanning rate

ble 1 as an example. For all the simulations performed, it has been observed that it is not possible to extract the trace $<\Delta H_{\mathrm{rev}}>(T)$ along the whole temperature range of the thermal transition. Nevertheless, considering that the enthalpy curve $<\Delta H_{\mathrm{rev}}>(T)$ is symmetrical with respect to $T_{1/2}$, if the procedure is correctly applicable as far as $T_{1/2}$, it is still possible to calculate by reflection the curves for temperatures above $T_{1/2}$ (Fig. 2, panels A and B). Left panels are the excess enthalpy values and right panels are their first derivative with respect to T, i.e. the excess heat capacity functions. Solid lines were obtained by the extrapolation of six simulated curves to infinite scanning rate, according to the procedure described in the text. Open circles were plotted by applying Eq. (11) to the adopted entry values. They are the expected $C_{\mathrm{p_{exc}}}(T)$ points for the simple N↔U equilibrium. In the case of reliable application of the extrapolation procedure, inaccurate results began to be provided beyond $T_{1/2}$. The entry values were: ΔH_{U}= 400 kJ mol^{-1}, $T_{1/2}$=338.15 K, E_{att}=150 kJ mol^{-1}, T^*=343.15 K, ΔH_{ag}=0. Similarly, when the extrapolation procedure could not be applied, inaccurate results started to be obtained before $T_{1/2}$. In this case entry values were: ΔH_{U}=400 kJ mol^{-1}, $T_{1/2}$=338.15 K, E_{att}=150 kJ mol^{-1}, T^*=323.15 K, ΔH_{ag}=0.

Table 1 Simulations of DSC curves carried out by modifying the entry values reported in columns 2–6. The corresponding extrapolated parameters (columns 7, 8), obtained as described in the text, allow to calculate the errors made by extrapolating the DSC curves (columns 9, 10)

Exp. N°	Entry values					Extrapolated parameters			
	ΔH_{U}/ kJ mol^{-1}	E_{att}/ kJ mol^{-1}	$T_{1/2}$/K	T^*/K	ΔH_{ag}/ kJ mol^{-1}	$T_{1/2}$/K	ΔH_{U} / kJ mol^{-1}	$\Delta(\Delta H_{\mathrm{U}})$/ kJ mol^{-1}	$\Delta T_{1/2}$/K
1	500	50	328.15	323.15	0	328.15	500.00	0	0
2	500	50	328.15	323.15	–100	328.15	500.00	0	0
3	500	50	338.15	323.15	0	338.15	500.00	0	0
4	500	50	338.15	323.15	–100	338.15	500.00	0	0
5	500	50	348.15	323.15	0	348.15	500.00	0	0
6	500	50	348.15	323.15	–100	348.15	500.00	0	0
7	500	50	358.15	323.15	0	357.15	384.58	–115.42	–1
8	500	50	358.15	323.15	–100	357.15	384.58	–115.42	–1
9	400	150	328.15	323.15	0	328.15	400.00	0	0
10	400	150	328.15	323.15	–100	328.15	400.00	0	0
11	400	150	328.15	333.15	0	328.15	400.00	0	0
12	400	150	328.15	333.15	–100	328.15	400.00	0	0
13	400	150	338.15	343.15	0	338.15	400.00	0	0
14	400	150	338.15	343.15	–100	338.15	400.00	0	0
15	400	150	358.15	323.15	0	350.15	35.52	–364.48	–8
16	400	150	358.15	323.15	–100	350.15	35.52	–364.48	–8
17	400	150	358.15	333.15	0	352.15	73.64	–326.36	–6
18	400	150	358.15	333.15	–100	352.15	73.64	–326.36	–6
19	400	150	358.15	343.15	0	356.15	255.90	–144.10	–2
20	400	150	358.15	343.15	–100	356.15	255.90	–144.10	–2
21	300	250	328.15	323.15	0	328.15	300.00	0	0
22	300	250	328.15	323.15	–100	328.15	300.00	0	0
23	300	250	338.15	323.15	0	333.15	26.74	–273.26	–5
24	300	250	338.15	323.15	–100	333.15	26.74	–273.26	–5
25	300	250	348.15	343.15	0	348.15	300.00	0	0
26	300	250	348.15	333.15	–100	348.15	300.00	0	0
27	300	250	348.15	353.15	0	348.15	300.00	0	0
28	300	250	348.15	353.15	–100	348.15	300.00	0	0
29	300	250	348.15	363.15	0	358.15	300.00	0	0
30	300	250	348.15	363.15	–100	358.15	300.00	0	0

By applying this methodology, we found that the values of the extrapolated thermodynamic data coincide with the entry values for the majority of the simulations performed (280 of 360). For 80 of 360 simulations the extrapolation procedure failed to provide reliable results before $T_{1/2}$ (Fig. 2, panels C and D), and the extrapolated values of ΔH_U and $T_{1/2}$ were much smaller than the entry values. Consequently, we have evaluated which of the input variables could affect the extrapolation procedure most negatively. According to the data reported in Table 1, ΔH_{ag} did not affect the applicability of the extrapolation procedure. In fact, the errors both in ΔH_U and in $T_{1/2}$ were independent from the value assumed by this variable in the range 0/–100 kJ mol^{-1}.

Conversely, $T_{1/2}$ and T^* affected significantly the reliability of the extrapolated data. In fact, the procedure provided correct extrapolated values only when, independently from the values of ΔH_U and E_{att}, T^* was set higher than $T_{1/2}$, or, in other words, for all cases in which the irreversible step U→F becomes significantly rapid before the equilibrium between the states N and U is established. For all simulations in which $T_{1/2}$ was set higher than T^*, the reliability of the extrapolated data was also dependent on the values of ΔH_U and E_{att}. This means that an interaction does exist between the unfolding parameters and that the poor applicability of the procedure is related to some particular combinations of the 4 variables. It is thus mandatory to search for a criterion that can provide information about these critical combinations. Taking into account that the apparent constant, k_{app} is a function of these 4 parameters, we have firstly considered this quantity as a possible indicator of the applicability of the extrapolation procedure. In Fig. 3, the upper panel reports the error in ΔH_U ($\Delta\Delta H_U$) and the lower panel reports the error in $T_{1/2}$ ($\Delta T_{1/2}$), *vs.* the value of k_{app} calculated at $T=T_{1/2}$. It was observed that when the value of k_{app} at $T=T_{1/2}$ is higher than 3, the extrapolation procedure does not give satisfactory results, i.e. $\Delta\Delta H_U$ and $\Delta T_{1/2}$ are not 0. On the contrary, when k_{app} at $T=T_{1/2}$ is lower than 3, extrapolate values are always equivalent to entry values. Thus, the values of k_{app} at $T=T_{1/2}$ are related to the distribution of the populations of states N, U and F in the whole temperature range investigated.

In Fig. 4, the relative populations of states N, U and F calculated at a scan rate of 1.0 K min^{-1} in the 300–360 K temperature range are reported for two simulations in which the application of the extrapolation procedure either failed (A) or was applicable (B). The upper panel reports an example of thermal unfolding for which the extrapolation procedure is not applicable; entry values were: ΔH_U=400 kJ mol^{-1}, $T_{1/2}$=328.15 K, E_{att}=150 kJ mol^{-1}, T^*=343.15 K,

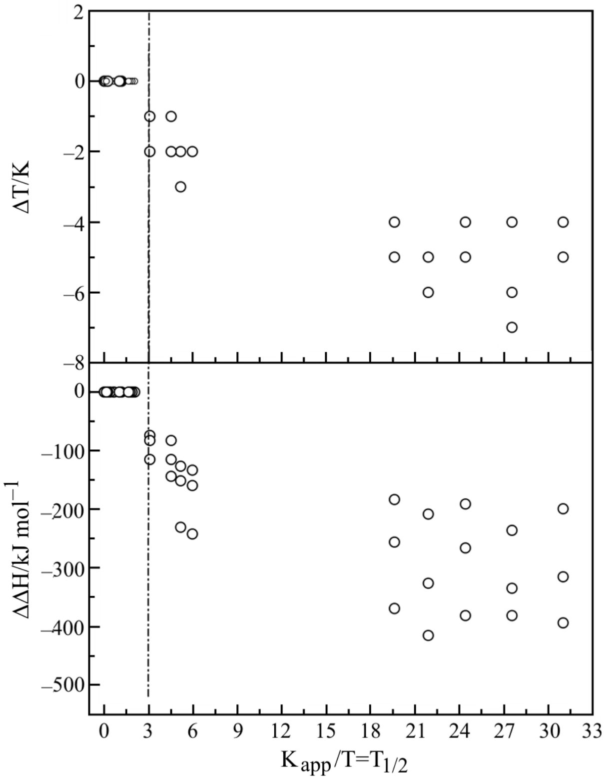

Fig. 3 Plots of errors in calculating $T_{1/2}$ (upper panel) and ΔH_U (lower panel) as a function of K_{app} calculated at $T=T_{1/2}$ ($K_{app}T=T_{1/2}$). Only when $K_{app}T=T_{1/2}<3$ the errors are negligible and the extrapolation procedure works correctly

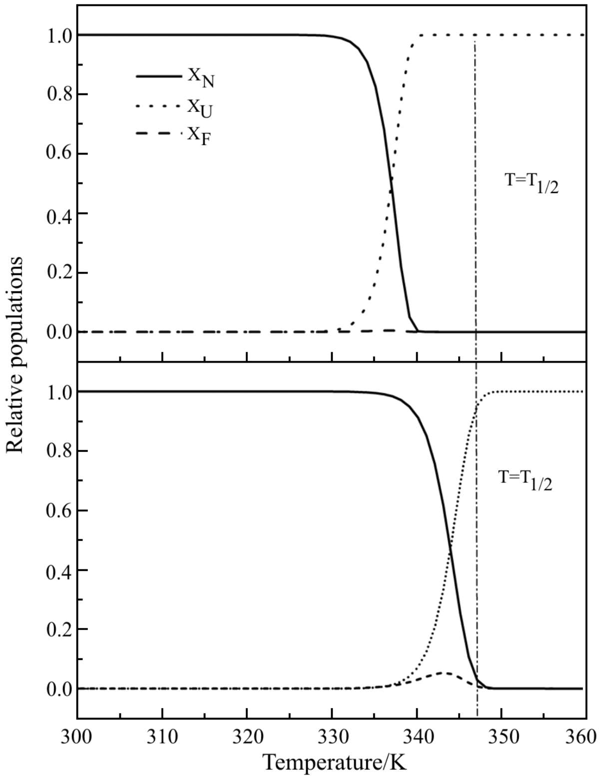

Fig. 4 Relative populations of the native (N), unfolded (U), and final (F) state as a function of temperature

ΔH_{ag}=0. In contrast, the lower panel reports an example of thermal unfolding for which the extrapolation procedure works effectively. Entry values were: ΔH_U=400 kJ mol^{-1}, $T_{1/2}$=328.15 K, E_{att}=150 kJ mol^{-1}, T^*=323.15 K, ΔH_{ag}=0. It can be noted that the reliability of the extrapolated data is related to the relative population of state U. In fact, when this is too low and only states N and F are significatively populated, the procedure did not provide correct extrapolated data. In these cases irreversible thermal unfolding can be described according only to the irreversible model N→F. In order to show the effect of the relative population of the unfolded state U on the reliability of extrapolated data, in Fig. 5 we report a plot of the errors made in calculating the unfolding enthalpy ($\Delta\Delta H$) *vs.* X_U, calculated at T=$T_{1/2}$ and at a heating rate of 1 K min^{-1}. We found that it was not possible to apply the procedure and therefore obtain the correct extrapolated values when the value of X_U was lower than $3\cdot10^{-4}$.

After having evaluated the reliability of the extrapolated data and the range of applicability of the extrapolation procedure, we have investigated about the possibility of finding a method that would allow us to establish the reliability of the extrapolated data for experimental cases for which the theoretical thermodynamic parameters are not known *a priori*. In a DSC experiment when the van't Hoff ratio (r^{VH}=$\Delta H_{cal}/\Delta H_{VH}$) is close to unity, only two states (N and U) are in equilibrium with each other [12]. In the case of small globular proteins, for which oligomerization processes and the existence of a stable intermediate state during thermal unfolding can be ruled out, only kinetic factors are supposed to decrease the van't Hoff ratio below unity. It is thus expectable, in the case of irreversible unfolding, that the value of r^{VH} can provide useful information about the role played by kinetic factors in the distortion of DSC curves and, consequently, on the reliability of unfolding data extrapolated from the experimental curve. The effectiveness of the extrapolation procedure may be calculated by comparing the r^{VH} calculated for some DSC curves simulated at ν=0.5 K min^{-1} with the corresponding r^{VH} calculated after extrapolation to infinite heating rate. In Table 2 we report, as an example, the values of the van't Hoff ratio and the errors in the extrapolated data calculated for some (30 of 360) of the $\Delta H_U(T)$ profiles obtained using the extrapolation procedure. The same entry values given in Table 1 were adopted for the 30 simulations reported. Columns 4 and 5 report the errors made in calculating ΔH_U and $T_{1/2}$ from the DSC curves simulated at v=0.5 K min^{-1}. These errors were obtained by subtracting the calculated value at 0.5 K min^{-1} from the chosen input parameter. The corresponding van't Hoff ratios, reported in column 6, are in any case lower than one, thus evidencing that the straightforward calculation of ΔH_U and $T_{1/2}$ from non-extrapolated DSC curves can be misleading. The decrease in errors made in calculating ΔH_U and $T_{1/2}$ by extrapolation, as reported in columns 9 and 10, and the corresponding increase in the corresponding van't Hoff ratios can help to distinguish the situations in which the extrapolation of DSC curves to infinite scan rates is applicable. The corresponding values calculated at a scan rate of 0.5 K min^{-1} are also reported. It can be noted that all the van't Hoff ratios calculated at a scan rate of 0.5 K min^{-1} are lower than one, confirming the irreversibility of the DSC traces simulated and the need to apply the extrapolation procedure to obtain the thermodynamic parameters. As expected, the r^{VH} values calculated from the extrapolated curves were close to 1 only for those simulations for which extrapolated parameters were equal to the entry values. Moreover, it has been observed that after extrapolation, the van't Hoff ratios become closer to one if compared with the corresponding ratios calculated at a scan rate of 0.5 K min^{-1}. It should also be noted that when the extrapolation procedure is applicable, the errors made in ΔH_U and $T_{1/2}$, are much smaller than those obtained using the values calculated at a scan rate of 0.5 K min^{-1}.

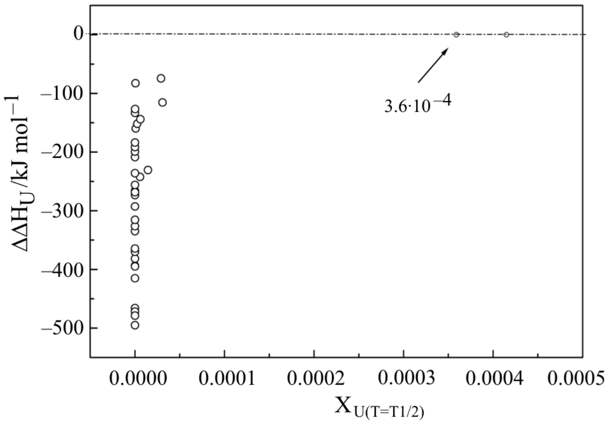

Fig. 5 Plots of calculation errors by the extrapolation of ΔH_U as a function of the maximum relative population of the unfolded state ($X_{U(T=T1/2)}$). Only when $X_{U(T=T1/2)}$>3.6 10^{-4} the errors are negligible and the extrapolation procedure works correctly

Conclusions

The applicability of the previously developed mathematical procedure used for the evaluation of protein equilibrium unfolding data from irreversible DSC curves was analyzed in order to evaluate the reliability of the data obtained. The comparison of the thermodynamic parameters (ΔH_U and $T_{1/2}$) obtained using this extrapolation procedure at infinite scan rate with

Table 2 The comparison of the unfolding parameters calculated for a DSC curve simulated at a scan rate of 0.5 K min^{-1} with the extrapolated parameters

Exp N°	Parameters calculated at ν=0.5 K min^{-1}					Extrapolated parameters				
	$T_{1/2}$/K	ΔH_U / kJ mol^{-1}	$\Delta(\Delta H_U)$/ kJ mol^{-1}	$\Delta T_{1/2}$/K	r^{VH}	$T_{1/2}$/K	ΔH_U / kJ mol^{-1}	$\Delta(\Delta H_U)$/ kJ mol^{-1}	$\Delta T_{1/2}$/K	r^{VH}
1	325.15	500	0	–3	0.73	328.15	500.00	0	0	1.03
2	325.15	400	–100	–3	0.58	328.15	500.00	0	0	1.03
3	334.15	500	0	–4	0.70	338.15	500.00	0	0	1.02
4	334.15	400	–100	–4	0.56	338.15	500.00	0	0	1.02
5	343.15	500	0	–5	0.67	348.15	500.00	0	0	1.02
6	343.15	400	–100	–5	0.54	348.15	500.00	0	0	1.02
7	352.15	500	0	–6	0.66	357.15	384.58	–115.42	–1	0.63
8	352.15	400	–100	–6	0.53	357.15	384.58	–115.42	–1	0.63
9	324.15	400	0	–4	0.61	328.15	400.00	0	0	1.02
10	324.15	300	–100	–4	0.45	328.15	400.00	0	0	1.02
11	327.15	400	0	–1	0.72	328.15	400.00	0	0	1.02
12	326.15	300	–100	–2	0.52	328.15	400.00	0	0	1.02
13	336.15	400	0	–2	0.70	338.15	400.00	0	0	1.01
14	336.15	300	–100	–2	0.51	338.15	400.00	0	0	1.01
15	345.15	400	0	–13	0.52	350.15	35.52	–364.48	–8	0.05
16	345.15	300	–100	–13	0.39	350.15	35.52	–364.48	–8	0.05
17	348.15	400	0	–10	0.53	352.15	73.64	–326.36	–6	0.10
18	348.15	300	–100	–10	0.40	352.15	73.64	–326.36	–6	0.10
19	351.15	400	0	–7	0.54	356.15	255.90	–144.10	–2	0.47
20	351.15	300	–100	–7	0.41	356.15	255.90	–144.10	–2	0.47
21	324.15	300	0	–4	0.47	328.15	300.00	0	0	1.01
22	323.15	200	–100	–5	0.33	328.15	300.00	0	0	1.01
23	329.15	300	0	–9	0.41	333.15	26.74	–273.26	–5	0.01
24	329.15	200	–100	–9	0.27	333.15	26.74	–273.26	–5	0.01
25	343.15	300	0	–5	0.46	348.15	300.00	0	0	1.01
26	339.15	200	–100	–9	0.28	348.15	300.00	0	0	1.01
27	347.15	300	0	–1	0.63	348.15	300.00	0	0	1.01
28	346.15	200	–100	–2	0.41	348.15	300.00	0	0	1.01
29	348.15	300	0	0	0.90	348.15	300.00	0	0	1.01
30	347.15	200	–100	–1	0.45	358.15	300.00	0	0	1.01

the theoretical parameters (entry values) leads to the followings: *i*) the values of the thermodynamic data obtained by applying the extrapolation procedure coincide with those of the input (i.e. $\Delta\Delta H_U$ and $\Delta T_{1/2}$=0) for most of the simulations performed; *ii*) however, some limits of applicability exist for this procedure and it cannot be used simply as a matter of course; *iii*) the limits of applicability of the extrapolation procedure are strictly related to the relative population of state U (X_U): in fact, when X_U is lower than 0.03% the extrapolation procedure cannot be applied correctly. In these cases irreversible thermal unfolding is best described on the basis of the simple two-state model N→F and no thermodynamic information can be extracted from DSC data.

In conclusion, the results here have, on one hand confirmed the validity of the extrapolation procedure at infinite scan rate to evaluate thermodynamic information on protein unfolding from irreversible DSC data, and, on the other hand, defined the limits of its applicability.

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