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ORIGINAL PAPER

Relationship between the wavelength maximum of a protein and the temperature dependence of its intrinsic tryptophan fluorescence intensity

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Abstract Proper determination of the temperature dependence of intrinsic tryptophan fluorescence intensity in native and denatured states is an essential prerequisite for extracting the free energy of protein unfolding from the thermal denaturation profile. The most common method employed in determining the temperature dependence of these conformations is through the determination of slopes of pre- and post-transition baselines. However, simulations of protein unfolding profiles suggest that this method does not work for marginally stable proteins. We show herein that the temperature dependence of the fluorescence intensity of *N*-acetyl tryptophanamide (NATA) in organic solvents and water may be used to represent the temperature dependence of the fluorescence intensity of tryptophan in native and denatured conformations of a protein, respectively. The wavelength of the emission maximum, λ_{max}, of *N*-acetyl tryptophanamide (NATA) in a particular solvent or tryptophan in proteins is related to the temperature dependence (m) of its fluorescence intensity by the equation: m $(K^{-1}) = (-0.000299 \pm 2.2 \times 10^{-5}$ K^{-1} $nm^{-1}) \times \lambda_{max}$ (nm) + (0.0919 ± 0.0025 K^{-1}).

Keywords Free energy of unfolding · Pre-transition baseline · Post-transition baseline · Simulation · Fluorescence

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Introduction

The stability of proteins is generally quantified in terms of ΔG_u^0, the free energy change for the native to unfolded transition. ΔG_u^0 is estimated from protein unfolding data monitored by calorimetric or spectroscopic methods (Becktel and Schellman 1987; Eftink and Wong 1994; Schellman 1987). The calorimetric method involves the use of a differential scanning calorimeter (DSC) to measure excess heat capacity with changing temperature, affording direct measurement of the change in enthalpy and heat capacity (Privalov 1979, 1989; Sturtevant 1974). These parameters are subsequently used to determine ΔG_u^0. In spectroscopic methods, the protein is first unfolded by changing the temperature, pH, or concentration of denaturants such as urea or guanidinium hydrochloride. Then we choose a spectroscopic signal which differs between the native and denatured state. This signal enables us to determine the relative population of native (*N*) and denatured (*U*) states and, in turn, the equilibrium constant and the free energy change for the unfolding transition.

Although differential scanning calorimeter (DSC) is unparalleled in its ability to provide a thermodynamic description of protein stability, thermodynamic parameters extracted using calorimetry experiments are particularly unreliable for marginally stable proteins (Haynie and Freire 1994; Straume and Freire 1992; Xie et al. 1991). In these cases, thermal unfolding profiles obtained using fluorescence intensity as a signal can be used as a viable alternative to extract thermodynamic parameters of protein stability. As a probe, fluorescence intensity has higher sensitivity (Eftink and Wong 1994). This allows low concentrations of proteins to be studied, thereby minimizing problems associated with protein aggregation.

Extrathermodynamic assumptions are generally employed during fluorescence data analysis, and the accuracy of parameters extracted using these assumptions is doubtful. Signals from native and denatured conformations of proteins (y_N and y_D) are temperature dependent, and assumptions are employed to determine the temperature dependence of the signals (m_N and m_D). There are two ways in which the temperature dependence of y_N and y_D can be extracted. In the first, the temperature dependence is obtained by measuring the slopes of the pre- and post-transition baselines (m_N^{app} and m_D^{app}) with the assumption that the pre-transition signal is entirely from native protein whereas the post-transition signal is entirely from denatured protein. This assumption is particularly invalid for marginally stable proteins, since a significant portion of the pre-transition signal is contributed by the denatured state (Haynie and Freire 1994; Straume and Freire 1992; Xie et al. 1991). Sometimes, even for stable proteins, inadequate spread of the pre- and post-transition data may lead to inaccurate determination of m_N and m_D. Another potential difficulty in determination of m_N and m_D is that the number of pre-transition data points influences the slope of the pre-transition baselines (Allen and Pielak 1998). The second way to extract the temperature dependence of the pre-transition and post-transition regions is by automated fitting of the entire protein unfolding profile with the slopes as a variable parameter. This method has the advantage that it does not assume that the pre-transition signal consists of a signal from the native state only and that the post-transition signal is due to the denatured state only. However, this method requires fitting of the unfolding profile with a greater number of variables, which increases the inaccuracy and confidence interval of the extracted values.

Since the contribution of the denatured state to the pre-transition signal is dependent on the thermodynamic parameters of unfolding of the protein, simulations were carried out to understand the effect of different thermodynamic parameters on the the pre- and post-transition baselines. A new protocol based on the temperature perturbation effect on spectroscopic signals of a model compound, *N*-acetyl tryptophanamide (NATA), is discussed in an attempt to model the temperature dependence of native and denatured states in the pre- and post-transition regions. NATA is generally used as a model compound to represent tryptophans in proteins. The protocol was tested with experimental data for the temperature-induced denaturation of chymotrypsin, trypsin, lysozyme, and cytochrome c. These proteins were chosen because their heat-induced denaturation is supposed to be a two-state process (Efimova et al. 2007; Kishore and Ranjana 2001; Privalov and Khechinashvili 1974; Santos et al. 2008), and simulation of their thermal denaturation profiles using DSC-observed thermodynamic parameters does not show any significant contribution of the denatured and native states to the pre- and post-transition baselines, respectively. Thus, we expect the slope in the pre- or post-transition region (m_N^{app}, m_D^{app}) to be equal to m_N and m_D.

Materials and methods

N-acetyl tryptophanamide (NATA) and cytochrome c (cyt-c) were purchased from Sigma; hen's egg-white lysozyme (HEWL), trypsin, chymotrypsin, hemin, and buffer reagents were purchased from Sisco Research Laboratory; potassium iodide, ethanol, and 2-octanol were purchased from Merck; methanol, 2-propanol, butanol, dioxane, and acetonitrile were purchased from Qualigens. Flourescence spectra of NATA and model proteins were obtained by using a Varian Cary eclipse fluorescence spectrophotometer with a Peltier-based temperature controller. The thermal dependence of the fluorescence intensity of tryptophan in protein and NATA in various solvents was measured with excitation wavelength of 295 nm with emission at its λ_{max} in the respective protein/solvent. Slit width of 10 and 2.5 nm was used for proteins and NATA, respectively. All data were normalized to 1.0 at 298 K before being used for fitting.

Thermodynamic models of protein denaturation

For thermally induced two-state transitions, nonlinear least-squares analysis using Eq. 1 was carried out to fit plots of signal versus temperature.

$$y(T) = \frac{y_N + y_D e^{\frac{-\Delta G}{RT}}}{1 + e^{\frac{-\Delta G}{RT}}}, \tag{1}$$

$$\Delta G = \Delta H_m \left(1 - \frac{T}{T_m}\right) - \Delta C_p \left((T_m - T) + T \ln\left(\frac{T}{T_m}\right)\right), \tag{1a}$$

where $y(T)$ is the experimentally observed spectroscopic property of the protein at temperature T, ΔG is the free energy of denaturation, ΔC_p is the specific heat capacity of denaturation, and ΔH_m is the enthalpy change at T_m, the mid-point of the thermal denaturation; y_N and y_D are the spectroscopic properties of the native and denatured state, respectively. y_N and y_D generally exhibit temperature dependence, which may be explained by two models:

$$\begin{aligned} \text{linear model}: y_N(T) &= y_N^0 + m_N \Delta T \;\&\\ y_D(T) &= y_D^0 + m_D \Delta T, \end{aligned} \tag{2a}$$

$$\begin{aligned} \text{parabolic model}: y_N(T) &= y_N^0 + a_N (\Delta T)^2 + b_N \Delta T \;\&\\ y_D(T) &= y_D^0 + a_D (\Delta T)^2 + b_D \Delta T, \end{aligned} \tag{2b}$$

where y_N^0 and y_D^0 are the spectroscopic values for native and denatured states, respectively, at 298 K, $\Delta T = T - 298$ K,

a_N, b_N, a_D, and b_D are constants, m_N is the baseline slope for the plot of signal from native protein versus T, $\left(\frac{\partial y}{\partial T}\right)_N$, and m_D is the baseline slope of the plot of signal from denatured protein versus T, $\left(\frac{\partial y}{\partial T}\right)_D$. Generally, the temperature dependence of y_N and y_D is considered to be linear and, for these cases, $y(T)$ may be expressed as

$$y(T) = \frac{(y_N^0 + m_N \Delta T) + (y_D^0 + m_D \Delta T) e^{\frac{-\Delta G}{RT}}}{1 + e^{\frac{-\Delta G}{RT}}}. \quad (3)$$

Results and discussion

Eftink (1994) et al. have shown that the fluorescence intensity directly tracks the population of states in an unfolding transition, whereas λ_{max} skews the transition towards the dominant fluorescing species. Therefore, we utilized the fluorescence intensity as a signal for extracting the thermodynamic parameters of the unfolding of the proteins.

Dependence of y_N and y_D on temperature

The contribution of denatured state of protein to pre-transition data depends on the free energy of denaturation. Thermodynamics parameters such as ΔC_p, ΔH_m, and T_m are correlated with the free energy of denaturation and can affect the pre-transition data, and hence their temperature dependence. Simulation of the fluorescence intensity of proteins as a function of temperature using a range of values of ΔH_m, T_m, and ΔC_p was carried out with m_N and $m_D = -0.0006$ using Eq. 3. The value of m (m_N^{app}, m_D^{app}) obtained through linear fitting of the pre- and post-transition baselines differs from -0.0006 in a number of cases (see Supplementary Table 1). For proteins with high heat capacity of denaturation and/or lower enthalpy of denaturation and/or high denaturation temperature, the slopes of the pre- and post-transition baselines differ from the m_N and m_D values employed in the simulation. These are proteins with low free energy of denaturation, $\Delta G^0 < 2.5$ kcal/mol, at lower temperature (between 273 and 293 K). Hence, the fluorescence signal has a significant contribution from the denatured species. Apart from a group of wild-type proteins, most of the mutants fall into this category.

Comparison of the data analysis between 278–293 K and 283–293 K for marginally stable protein shows that, as points are eliminated from the low-temperature end, the slopes of the pre-transition baselines become more negative. Our simulation results are in agreement with those observed experimentally (Allen and Pielak 1998). While these investigators did not provide an explanation for their observations, our simulation attributes it to the increasing contribution of the signal from the denatured state to the pre-transition data at low temperature.

To examine the effect of using the slopes of the pre-and post transition baselines (i.e., m_N^{app} and m_D^{app}) as m_N and m_D, the different thermodynamic parameters were extracted by nonlinear fitting of the simulated protein unfolding profiles with m_N^{app} and m_D^{app} as m_N and m_D, respectively, during fitting. The extracted parameters are listed in Supplementary Table 1. It is evident that the values of the extracted thermodynamic parameters are significantly different from the values employed for simulation if m_N and m_D differ from m_N^{app} and m_D^{app}, respectively. In almost every case, there is underestimation of the thermodynamic parameters of unfolding.

Thermal studies of NATA in various solvents

The tryptophan environment is aqueous in the denatured state or in exposed regions of the native protein. Therefore, the fluorescence spectra of 2 μM solution of NATA in 50 mM acetate buffer (pH 5) and 25 mM phosphate buffer (pH 7) were recorded as a function of temperature. As expected, we observed a decrease in the fluorescence intensity of NATA at its emission wavelength λ_{max} (360 nm) with increasing temperature, indicating quenching (Fig. 1). Quenching is supposed to be due to an increase in the rates of various deexcitation processes (Kirby and Steiner 1970). We observed that the temperature dependence of the fluorescence properties of the model compound NATA at its emission λ_{max} is best fitted using a parabolic model (Eq. 2b). A similar observation has been reported for the temperature dependence of the absorption properties of L-trp (Sinha et al. 2000). However, the temperature dependence can be assumed to be linear in higher (333–368 K) and lower temperature regions (293–328 K; Fig. 1), the regions where denatured and native states are populated, respectively. The values of m (from the linear fitting using Eq. 2a), and a and b (from the parabolic fitting using Eq. 2b) obtained in this region are presented in Table 1. The temperature dependence does not change for different buffers.

The environment of the tryptophan in each protein is different. In some proteins it is in a highly nonpolar environment, whereas in others it may be in a moderately nonaqueous environment. Therefore, fluorescence spectra of NATA in various organic solvents were recorded. The emission λ_{max} decreased with decreasing solvent polarity. Therefore, the emission λ_{max} can be an important indicator of the environment of tryptophan in the protein. Changes in the fluorescence intensity of NATA in various organic solvents as a function of temperature were also recorded at their respective λ_{max}. Again, the temperature dependence

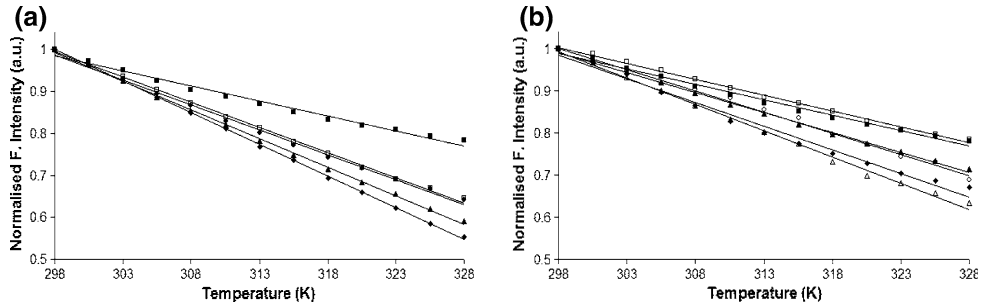

Fig. 1 Temperature dependence of the fluorescence intensity of NATA, as obtained from the slope of the linear fit of fluorescence intensity at the emission λ_{max} versus temperature: **a** in various solvents: 50 mM acetate buffer (pH 5) (*filled diamonds*), 100% MeOH (*filled squares*), 65% EtOH (*open squares*), 50% EtOH (*filled circles*), and 20% EtOH (*filled triangles*); **b** in other solvents: 80% EtOH (*filled diamonds*), 100% EtOH (*filled squares*),100% 2-PrOH (*filled triangles*), 100% BuOH (*open triangle*), dioxan (*open squares*), and acetonitrile (*open circles*)

Table 1 The wavelength of the emission maxima (λ_{max}) and the temperature dependence of the fluorescence intensity of NATA at their respective λ_{max} in different solvent systems

Solvent	λ_{max} (nm)	Linear slope, m (K^{-1})	Parabolic slope	
			a (10^{-5} K^{-2})	b (K^{-1})
25 mM Acetate buffer (pH 5)	359.1	$-0.0172^{a} \pm 0.0004$	7 ± 3	-0.0231 ± 0.002
		$-0.0082^{b} \pm 0.0003$		
25 mM Acetate buffer (pH 5) + heme	360	$-0.0114^{a} \pm 0.0004$	9 ± 2	-0.0218 ± 0.001
		$-0.0075^{b} \pm 0.0004$		
25 mM Acetate buffer (pH 5) + 0.5 M KI	360	-0.0103 ± 0.0007	5 ± 1	-0.0176 ± 0.001
Methanol	349.1	-0.0176 ± 0.0006	7 ± 2	-0.0168 ± 0.002
20% Ethanol	359.1	-0.0130 ± 0.0003	6 ± 1	-0.0174 ± 0.001
50% Ethanol	353.1	-0.0148 ± 0.0005	7 ± 2	-0.0171 ± 0.003
65% Ethanol	354.5	-0.0117 ± 0.0005	6 ± 1	-0.0161 ± 0.004
80% Ethanol	352	-0.0110 ± 0.0006	7 ± 2	-0.0157 ± 0.006
Ethanol	346.5	-0.0108 ± 0.0005	9 ± 3	-0.0140 ± 0.002
Propanol	347.1	-0.0100 ± 0.0006	6 ± 2	-0.0134 ± 0.005
Butanol	344.5	-0.0112 ± 0.0004	5 ± 1	-0.0157 ± 0.007
Dioxane	334.5	-0.0096 ± 0.0005	7 ± 1	-0.0127 ± 0.010
Acetonitrile	342.5	-0.0100 ± 0.001	4 ± 2	-0.0066 ± 0.002-
Acetonitrile + heme	342	-0.0028 ± 0.0003	10 ± 3	-0.0101 ± 0.004
100% EtOH + heme	347	-0.0129 ± 0.0005	10 ± 4	-0.0213 ± 0.002
80% EtOH + heme	352	-0.0158 ± 0.0005	10 ± 4	-0.0252 ± 0.005
50% EtOH + heme	356	-0.0153 ± 0.0007	9 ± 5	-0.0223 ± 0.008

[a] In temperature range 298–368 K

[b] In temperature range 333–368 K

of NATA fluorescence at its λ_{max} can be assumed to be linear in the low temperature region (298–328 K; Fig. 1), the region where the native state is typically populated. The emission λ_{max} and m values at λ_{max} obtained using Eq. 2a are presented in Table 1. The value of m decreased with decreasing solvent polarity. The m values obtained from the temperature dependence of the fluorescence intensity at λ_{max} are plotted against λ_{max} in Fig. 2. There exists a linear relation between λ_{max} (in nm) and m (in K^{-1}) at λ_{max}. The equation describing this relationship is

$$m\left[\mathrm{K}^{-1}\right] = \left(-0.000299 \pm 2.2 \times 10^{-5}\,\mathrm{K}^{-1}\,\mathrm{nm}^{-1}\right) \times \lambda_{max}\,[\mathrm{nm}] + \left(0.0919 \pm 0.0025\,\mathrm{K}^{-1}\right). \quad (4)$$

The fluorescence intensity of NATA in various solvents as a function of temperature was also measured at a fixed wavelength (350 nm). The slopes were different than the

one obtained from the temperature dependence of NATA fluorescence at λ_{max}. Slopes at 350 nm showed poor correlation with λ_{max} (Supplementary Fig. 2).

Fluorescence spectra of proteins

The fluorescence spectra of 10 μM chymotrypsin and 10 μM cytochrome c in 50 mM acetate buffer (pH 4), 1 μM hen's egg white lysozyme (HEWL) in 50 mM acetate buffer (pH 5, 0.5 M KI), and 2 μM trypsin in 50 mM phosphate buffer (pH 3) were obtained at room temperature. Different values of λ_{max} indicate a different environment for tryptophan in a single tryptophan protein or the average environment in multitryptophan proteins. The emission λ_{max} suggests that the tryptophan environments in lysozyme, chymotrypsin, trypsin, and cytochrome c are similar to those of NATA in dioxane, 100% ethanol, 100% butanol, and 50% ethanol, respectively. Since heme may affect the temperature dependence of the intensity of tryptophan fluorescence, spectra of NATA were obtained in the presence of heme at different temperatures and in various solvents (50% ethanol, 80% ethanol, 100% ethanol, acetonitrile, and water), keeping in mind the environment of tryptophan in cyt-c. The temperature dependence of the fluorescence intensity was calculated from these spectra (Table 1).

Thermal unfolding profile of proteins

Fluorescence spectra of proteins were obtained as a function of temperature. Plots of fluorescence intensity at λ_{max} versus temperature are shown in Fig. 3. A sigmoidal decrease in fluorescence intensity is observed for all proteins except lysozyme. Absence of a sigmoidal profile for lysozyme makes it difficult to extract thermodynamic parameters with reasonable accuracy. The fluorescence quencher, KI, was added to lysozyme to aid in observing a sigmoidal unfolding profile. The values of m_N^{app} and m_D^{app} for the model proteins were obtained through linear fitting of the pre- and post-transition data and are listed in Table 2. In each case, m_N^{app} is close to m_N, which was

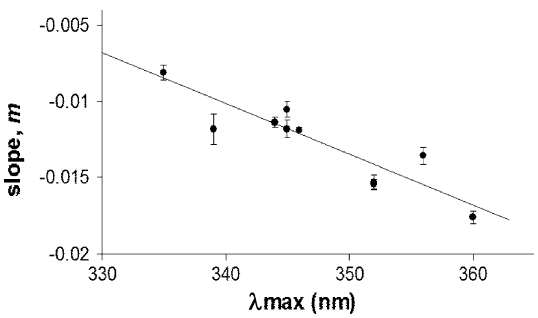

Fig. 2 Plot of m at λ_{max} versus λ_{max} of NATA in various solvents

obtained using Eq. 4. The value of m_D^{app} for the proteins was also equal to the m value obtained for NATA in water. Since KI was added to the lysozyme solution, the thermal dependence of NATA in buffer was investigated in the presence of KI to obtain m_D based on the emission λ_{max} of NATA data. A similar value for m_N^{app} and m_N indicates that the temperature dependence of the fluorescence intensity of NATA can be used to represent the temperature dependence of the fluorescence intensity of proteins.

A two-state fitting of the fluorescence data using Eq. 3 with m_N^{app} and m_D^{app} as m_N and m_D was carried out to extract thermodynamic parameters from the denaturation profiles. These parameters are listed in Table 2 along with the parameters obtained from differential scanning calorimetry (Efimova et al. 2007; Kishore and Ranjana 2001; Privalov and Khechinashvili 1974; Santos et al. 2008). The reported thermodynamic parameters of unfolding from analysis of the DSC data match, within experimental error, those obtained through analysis of the fluorescence intensity data. According to Becktel and Schellman (Becktel and Schellman 1987), the experimental error associated with calorimetric methods of determining ΔC_p depends upon the precision with which the difference between the specific heats of the native and denatured states can be obtained. They calculated that the minimum error in determining ΔC_p with a calorimeter was ±4%; however, the actual error is about ±10% in favorable cases, and considerably greater in typical cases (Pace and Laurents 1989; Privalov 1979). The two-state fitting of thermal denaturation profiles using Eq. 1 with the parabolic temperature dependence of fluorescence intensity of native and denatured states yields thermodynamic parameters (data not shown) that are similar to those obtained by fitting, using Eq. 1, the linear temperature dependence of the fluorescence intensity of native and denatured states.

The above experiments on proteins indicate that λ_{max} can be used to obtain accurate values of m_N. To understand the usefulness of an accurate value of m_N, we simulated the thermal profile of a typical marginally stable protein (taken from Supplementary Table 1). One thousand different simulated profiles were obtained from this ideal data by adding random errors generated using Monte Carlo simulation to each data point from a Gaussian distribution with mean of zero and standard deviation of 0.02 (two times the standard deviation observed in the case of nonlinear fitting of thermal profiles of lysozyme, trypsin, chymotrypsin, and cytochrome c). Nonlinear regression of simulated profiles was carried out in three different ways: (1) with m_N^{app} and m_D^{app} as m_N and m_D, (2) with m_N and m_D as variables, and (3) with the values of m_N and m_D as used in simulation (our method). The extracted parameters and their 95% confidence intervals are listed in Table 3. It is evident that the

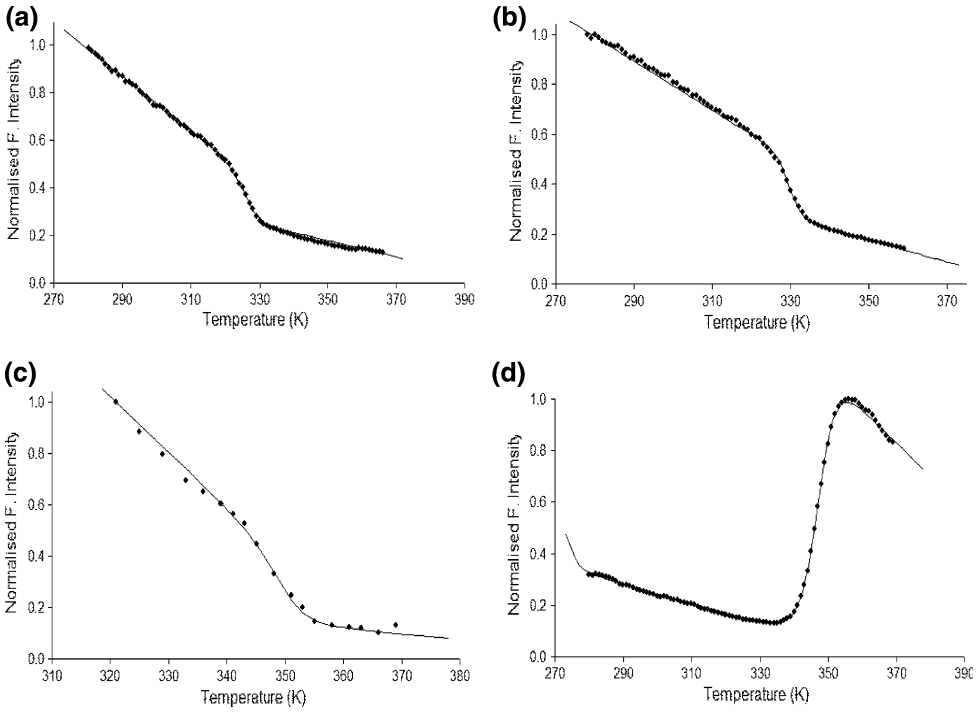

Fig. 3 Fluorescence intensity profiles of: **a** 2 μM trypsin in 50 mM phosphate buffer (pH 3), **b** 10 μM chymotrypsin in 50 mM acetate buffer (pH 4), **c** 1 μM lysozyme (HEWL) in 50 mM acetate buffer (pH 5, 0.5 M KI), and **d** 10 μM cytochrome c in 50 mM acetate buffer (pH 4) as a function of temperature at their respective λ_{max}. *Filled circles* indicate experimental data; *solid lines* indicate the linear fit of the thermal denaturation data of proteins

Table 2 Thermodynamic parameters extracted from the temperature dependence of the fluorescence intensity of tryptophans in different proteins at their respective λ_{max}

Protein	λ_{max} (±0.2 nm)	m_N^a (K^{-1})	m_N^b (K^{-1})	T_m(K)	ΔH_m (kcal/mol)	T_m^{cal} (K)	ΔH_m^{cal} (kcal/mol)
Trypsin (pH 3)	345	−0.0125	−0.013 ± 0.005	326 ± 0.5	99 ± 2	325.9	99.1
Chymotrypsin (pH 4)	346.5	−0.0127	−0.012 ± 0.01	330 ± 0.5	137 ± 9	331	140
Lysozyme (pH 5, 0.5 M KI)	334	−0.0092	−0.010 ± 0.008	350 ± 0.2	94 ± 10	349.6	83
Cytochrome c (pH 4)	350.5	−0.0145	−0.015 ± 0.01	347 ± 0.1	92 ± 5	344.2	87

m_D, obtained from linear fitting of post-transition data, is -0.0089 K^{-1} for all cases

^{cal} Transition temperature derived from calorimetric studies

^{a} From Eq. 4

^{b} From linear fitting of the pre-transition data

proposed method is better than the conventional methods in the determination of the extracted thermodynamic parameters in terms of both accuracy and confidence interval.

Conclusions

Our experiments with the model compound NATA and the proteins lysozyme, chymotrypsin, cytochrome c, and trypsin suggest that the thermal properties of NATA in alcohols and water can be used to represent the thermal properties of proteins in the pre- and post-transition regions, respectively. We propose the following method for the extraction of thermodynamic parameters: (1) record the emission spectrum of a given protein in its native condition to obtain the value of emission λ_{max}, (2) observe the change in fluorescence intensity at λ_{max} as a function of temperature to obtain a thermal unfolding profile of the protein, (3) calculate m_N from the relationship between λ_{max} and m_N using Eq. 4, and (4) carry out nonlinear fitting of the

Table 3 Thermodynamic parameters extracted from nonlinear fitting of 1,000 simulated thermal unfolding profiles with: (a) m_N and m_D as variables, (b) actual m_N and m_D values (our method), and (c) m_N and m_D obtained from the slopes of the pre- and post-transition baselines

Methods	m_N	m_D	T_m	ΔH_m	ΔC_p
a	−0.0006	−0.00002	330 ± 0.4	65.2 ± 6.0	2.18 ± 0.3
b	−0.0006	−0.00006	330 ± 0.3	65.1 ± 4.4	2.19 ± 0.18
c	0.0078	0.000025	327 ± 0.3	38.1 ± 1.9	0.97 ± 0.06

These profiles were generated by adding random errors created through Monte Carlo simulation to data (using standard deviation of 0.02) of an ideal thermal profile obtained using Eq. 3 with $T_m = 330$ K, $\Delta H_m = 65$ kcal/mol, $\Delta C_p = 2.2$ kcal/mole/K, $m_N = -0.0006$, and $m_D = -0.0006$

protein unfolding profile using Eq. 3 with m_N obtained from the previous step and m_D as -0.0089 K^{-1}.

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References

Allen DL, Pielak GJ (1998) Baseline length and automated fitting of denaturation data. Protein Sci 7:1262–1263

Becktel WJ, Schellman JA (1987) Protein stability curves. Biopolymers 26:1859–1877

Efimova YM, Haemers S, Wierczinski B, Norde W, van Well AA (2007) Stability of globular proteins in H2O and D2O. Biopolymers 85:264–273

Eftink MR (1994) The use of fluorescence methods to monitor unfolding transitions in proteins. Biophys J 66:482–501

Eftink MR, Wong CY (1994) The use of fluorescence methods to monitor protein unfolding reactions. Biophys J 66:A164–A188

Haynie DT, Freire E (1994) Estimation of the folding/unfolding energetics of marginally stable proteins using differential scanning calorimetry. Anal Biochem 216:33–41

Kirby EP, Steiner EF (1970) The influence of solvent and temperature upon the fluorescence of indole derivative. J Phys Chem 74:4480–4490

Kishore N, Ranjana NK (2001) Thermodynamics of thermal denaturation of ribonuclease A and cytochrome c in the presence of 1,1,1,3,3,3-hexafluoro-2-propanol. J Chem Thermodyn 33:1325–1344

Pace CN, Laurents DV (1989) A new method for determining the heat capacity change for protein folding. Biochemistry 28:2520–2525

Privalov PL (1979) Stability of proteins: small globular proteins. Adv Protein Chem 33:167–241

Privalov PL (1989) Thermodynamic problems of protein structure. Annu Rev Biophys Biophys Chem 18:47–69

Privalov PL, Khechinashvili NN (1974) A thermodynamic approach to the problem of stabilization of globular protein structure: a calorimetric study. J Mol Biol 86:665–684

Santos AM, Santana MA, Gomide FT, Miranda AA, Oliveira JS, Boas FA, Vasconcelos AB, Bemquerer MP, Santoro MM (2008) Physical-chemical characterization and stability study of alpha-trypsin at pH 3.0 by differential scanning calorimetry. Int J Biol Macromol 42:278–284

Schellman JA (1987) The thermodynamic stability of proteins. Annu Rev Biophys Biophys Chem 16:115–137

Sinha A, Yadav S, Ahmad R, Ahmad F (2000) A possible origin of differences between calorimetric and equilibrium estimates of stability parameters of proteins. Biochem J 345(Pt 3):711–717

Straume M, Freire E (1992) Two-dimensional differential scanning calorimetry: simultaneous resolution of intrinsic protein structural energetics and ligand binding interactions by global linkage analysis. Anal Biochem 203:259–268

Sturtevant JM (1974) Some applications of calorimetry in biochemistry and biology. Annu Rev Biophys Bioeng 3:35–51

Xie D, Bhakuni V, Freire E (1991) Calorimetric determination of the energetics of the molten globule intermediate in protein folding: apo-alpha-lactalbumin. Biochemistry 30:10673–10678