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# Heat capacity change for ribonuclease A folding

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**Abstract**

The change in heat capacity $\Delta C_p$ for the folding of ribonuclease A was determined using differential scanning calorimetry and thermal denaturation curves. The methods gave equivalent results, $\Delta C_p = 1.15 \pm 0.08$ kcal mol$^{-1}$ K$^{-1}$. Estimates of the conformational stability of ribonuclease A based on these results from thermal unfolding are in good agreement with estimates from urea unfolding analyzed using the linear extrapolation method.

**Keywords:** differential scanning calorimetry; heat capacity change; protein folding; protein stability; ribonuclease A; urea denaturation

In a recent paper, we were puzzled by the wide range of values for $\Delta C_p$ for RNase A folding (Pace et al., 1998). For example, a recent compilation by Pfeil (1998) gives 37 values of $\Delta C_p$ ranging from 1.0 to 2.3 kcal mol$^{-1}$ K$^{-1}$ with an average of 1.39 ± 0.33 kcal mol$^{-1}$ K$^{-1}$. A selection of these values is given in Table 1. The $\Delta C_p$ values obtained from differential scanning calorimetry (DSC) based on $\Delta H_{cal}$ vs. $T_m$ measurements are generally smaller than $\Delta C_p$ values obtained by other methods based on $\Delta H_{vH}$. Some evidence suggested that a higher $\Delta C_p$ value (≈2 kcal mol$^{-1}$ K$^{-1}$) might be correct. For example, it gives better agreement with the observed temperature of maximum stability values $T_S$ (Equation 3) and with conformational stabilities measured by an analysis of solvent denaturation curves. Consequently, we decided to study the folding of RNase A under identical conditions by DSC and by thermal denaturation curves (TDC).

## Results

An analysis of the DSC scans gave values of $\Delta H_{cal}$, $\Delta H_{vH}$, and $\Delta H_{fit}$ that are in excellent agreement, although at each pH the $\Delta H_{cal}$ values are slightly larger: $\Delta H_{cal}/\Delta H_{vH} = 1.02 \pm 0.02$ and $\Delta H_{cal}/\Delta H_{fit} = 1.03 \pm 0.02$. An analysis of the TDC gave $\Delta H_{vH}$ values in reasonable agreement with the $\Delta H_{cal}$ values, but, again, the $\Delta H_{cal}$ were larger: $\Delta H_{cal}/\Delta H_{vH} = 1.07 \pm 0.04$. The presence of a small concentration of intermediate states at equilibrium would explain these differences (Jackson & Brandts, 1970; Freire & Biltonen, 1978). The $\Delta H_{cal}$ values from the DSC experiments and the $\Delta H_{vH}$ values from the TDC were plotted as a function of $T_m$ according to the Kirchhoff equation:

$$\Delta C_p = d(\Delta H)/d(T) \quad (1)$$

to estimate $\Delta C_p$, and the results are shown in Figure 1. The resulting $\Delta C_p$ values are given in Table 2. $\Delta C_p$ values based on plots of $\Delta H_{vH}$ and $\Delta H_{fit}$ from the DSC data, a global fit of all of the DSC data, and on the difference between the posttransition $C_p(D)$ and pretransition $C_p(N)$ baselines of the DSC experiments are also given. The $\Delta C_p$ value based on plots of $\Delta H_{cal}$ vs. $T_m$ in Table 2 is lower than the $\Delta C_p$ values determined in the same way that are given in Table 1. We are not sure why. It could be because we use only 10 mM buffer and most of the other studies used higher buffer concentrations. (See McCrary et al., 1998 for a possible explanation.) Table 2 also gives estimates of $\Delta C_p$ obtained by Privalov et al. (1973) using the same methods. The agreement is reassuring.

## Discussion

The difference in heat capacity between the folded $C_p(F)$ and unfolded $C_p(U)$ states of a protein, $\Delta C_p = C_p(U) - C_p(F)$, is important in determining the temperature dependence of the conformational stability $\Delta G = G(U) - G(F)$ (Becktel & Schellman, 1987; Privalov, 1990). This is illustrated in Figure 2, which shows the protein stability curve for ribonuclease A (RNase A) calculated using $\Delta C_p = 1.15$ kcal mol$^{-1}$ K$^{-1}$, a value consistent with the results in this paper, and $\Delta C_p = 2.2$ kcal mol$^{-1}$ K$^{-1}$, a value reported by Pace and Laurents (1989). These curves were calculated with the Gibbs–Helmholtz equation:

$$\Delta G(T) = \Delta H_m(1 - T/T_m) - \Delta C_p[T_m - T + T\ln(T/T_m)] \quad (2)$$

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**Table 1.** *$\Delta C_p$ values for RNase A folding from the literature*

| Group | Method[a] | $\Delta C_p$ (kcal mol$^{-1}$ K$^{-1}$) |
|---|---|---|
| Privalov Lab (1973–1995)[b] | $\Delta H_{cal}$ vs. $T_m$ | 1.27 |
| Schwarz and Kirchhoff (1988) | | 1.67 |
| Fujita and Noda (1991) | | 1.09 |
| Hinz et al. (1994) | | 1.08 |
| Makhatadze et al. (1995) | | 1.24 ± 0.07 |
| Liu and Sturtevant (1996) | | 1.74 ± 0.02 |
| Catanzano et al. (1997) | | 1.31 ± 0.14 |
| Average | | 1.34 ± 0.26 |
| Brandts and Hunt (1967) | $\Delta H_{vH}$ (global fit − pH) | 2.0 ± 0.04 |
| | $\Delta H_{vH}$ (global fit − urea) | 1.7 ± 0.3 |
| Salahuddin and Tanford (1970) | $\Delta H_{vH}$ (global fit − GdnHCl) | 2.2 ± 0.5 |
| Shiao et al. (1971) | $\Delta H_{vH}$ (global fit − pH) | 2.0 ± 0.2 |
| Hawley (1971) | $\Delta H_{vH}$ (pressure) | 1.7 ± 0.25 |
| Pace and Laurents (1989) | $\Delta G$'s from UDC (LEM) | 2.2 ± 0.3 |
| Yamaguchi et al. (1995) | $\Delta H_{vH}$ (pressure) | 1.8 |
| Arnold and Ulbrich-Hofmann (1997) | $\Delta G$'s from UDC (LEM) | 2.3 ± 0.06 |
| Average | | 2.0 ± 0.24 |

[a]The $\Delta C_p$ values in the top half were all determined from plots of $\Delta H_{cal}$ vs. $T_m$ using results from DSC. The $\Delta C_p$ values in the bottom half used $\Delta H_{vH}$ values determined by noncalorimetric methods.
[b]The value used is from Makhatadze and Privalov (1995).

where $\Delta G(T)$ is the conformational stability at temperature $T$, $T_m$ is the midpoint of the thermal denaturation curve, and $\Delta H_m$ is the enthalpy change at $T_m$. The temperature of maximum stability $T_S$ can be calculated using (Becktel & Schellman, 1987)

$$T_S = T_m \exp(\Delta H_m/(T_m \Delta C_p)). \quad (3)$$

Note that the curvature of the plots depends on $\Delta C_p$ and that the maximum stability ranges from −19 to +18 °C as $\Delta C_p$ increases from 1.15 to 2.2 kcal mol$^{-1}$ K$^{-1}$. Next, we will explain why the higher $\Delta C_p$ value reported by Pace and Laurents (1989) is wrong.

In the Pace and Laurents (1989) method, an analysis of urea denaturation curves (UDC) by the linear extrapolation method (LEM) (Greene & Pace, 1974)

$$\Delta G = \Delta G(H_2O) - m(\text{urea}) \quad (4)$$

is used to determine $\Delta G(H_2O)$, $\Delta G$ at 0 M urea, and $m$, a measure of the observed linear dependence of $\Delta G$ on urea concentration. The $\Delta G(H_2O)$ values are then used in Equation 2 along with values of $T_m$ and $\Delta H_m$ to calculate $\Delta C_p$. The $\Delta G(H_2O)$ values are determined at different temperature to reflect the curvature of the protein stability curve. In the original paper, $T_m$ = 42.6 °C and $\Delta H_m$ = 95 kcal/mol values at pH 2.8 from Freire and Biltonen (1978) were used, and this led to $\Delta C_p$ = 2.2 kcal mol$^{-1}$ K$^{-1}$. We have since found 10 values in the literature at the same pH and using the average $T_m$ = 44.0 ± 1.9 and $\Delta H_m$ = 83.7 ± 5.2 yields $\Delta C_p$ = 1.35 ± 0.45 kcal mol$^{-1}$ K$^{-1}$. Using the $T_m$ and $\Delta H_m$ values reported here (Table 3) gives $\Delta C_p$ = 1.28 ± 0.20 kcal mol$^{-1}$ K$^{-1}$ as shown in Table 2. Thus, the Pace and Laurents (1989) method is a valid, noncalorimetric method for estimating $\Delta C_p$, but it requires accurate values of $T_m$ and $\Delta H_m$ from TDC or DSC. The method has been shown to give good agreement with different methods in other studies (Scholtz, 1995).

In a previous study of RNase A, we determined the pH dependence of the stability by measuring $\Delta G(H_2O)$ as a function of pH (Pace et al., 1990). If we use seven of these $\Delta G(H_2O)$ values at pH values ranging from three to seven with the $T_m$ and $\Delta H_m$ values reported here, we can again calculate $\Delta C_p$ using Equation 2. The average $\Delta C_p$ value from this approach is in excellent agreement with the other values (Table 2).

As the pH is lowered from pH 7 to 2, the net charge on RNase A increases from 4 to 17. Does this increase in the net charge effect the conformation of the folded and unfolded states? The effect on the conformation of the folded state will probably be small as long as the protein does not begin to unfold. However, the average conformation of the ensemble of denatured states might change significantly with pH. We showed previously that the $m$ value (Equation 4) for urea denaturation doubles between pH 7 and 2, and concluded: "This suggests that the unfolded conformations of RNase A become more accessible to urea as the net charge on the molecule increases" (Pace et al., 1990). If this is correct, we would expect $\Delta C_p$ to increase at lower pH (Myers et al., 1995). There is some indication that this occurs based on $\Delta C_p$ values estimated from the baselines. The average $\Delta C_p$ based on the 12 DSC scans between pH 2 and 3 was 1.20 ± 0.32 kcal mol$^{-1}$ K$^{-1}$, and the average based on the 11 DSC scans between pH 3.5 and 5 is 0.86 ± 0.20 kcal mol$^{-1}$ K$^{-1}$. There is considerable uncertainty in estimating $\Delta C_p$ from the baselines, but these results from DSC are consistent with the conclusions based on studies of the pH dependence of urea unfolding. In contrast, for staphylococcal nuclease (Carra et al., 1994), $\alpha$-lactalbumin (Griko et al., 1994), and apomyoglobin (Griko & Privalov, 1994), the $\Delta C_p$ values from the baselines indicate that the unfolded state may become more compact at lower pH. In these cases, other evidence also indicates that

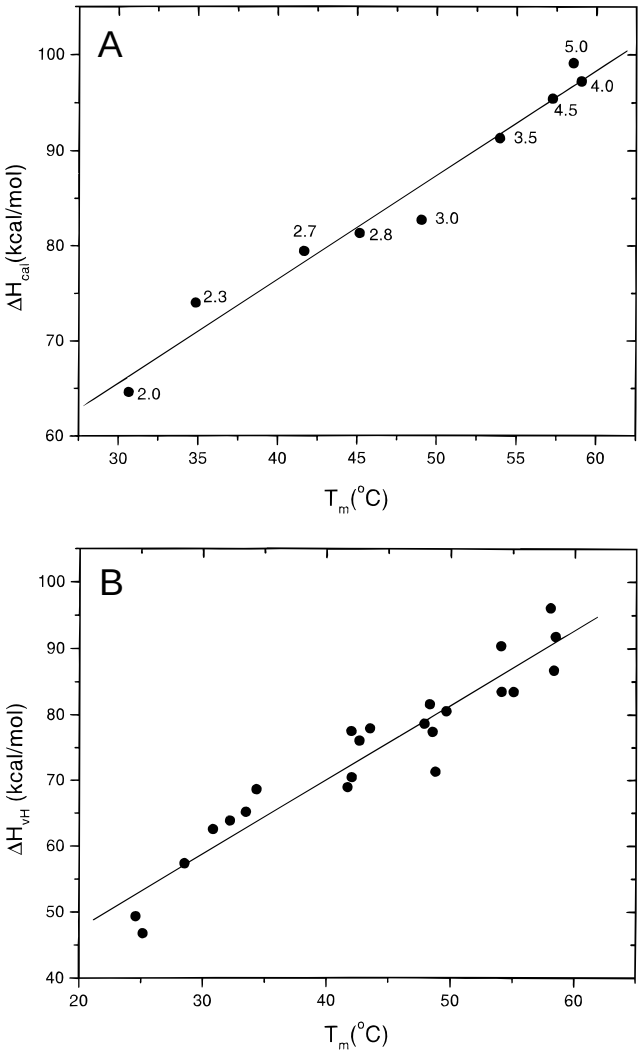


**Fig. 1.** $\Delta H_{cal}$ from DSC experiments (**A**) and $\Delta H_{vH}$ from TDC (**B**) as a function of $T_m$ for the folding of RNase A. The $\Delta H_{cal}$ values are averages based on duplicate or triplicate DSC runs at the pH values shown near the data points. The solid lines are based on least-squares fits of the data that yielded (**A**) $\Delta C_p = 1.09 \pm 0.07$ kcal mol$^{-1}$ K$^{-1}$ and (**B**) $\Delta C_p = 1.13 \pm 0.08$ kcal mol$^{-1}$ K$^{-1}$.

these proteins form compact denatured states in the low pH region, but this is probably not the case with RNase A (Yao & Bolen, 1995; Baskakov & Bolen, 1998).

In our previous studies, we analyzed UDC by the linear extrapolation method to determine $\Delta G(H_2O)$ as a function of pH and temperature (Pace & Laurents, 1989; Pace et al., 1990). A selection of these values is given Table 3 along with $\Delta G(T)$ values calculated with Equation 2 using values of $T_m$, $\Delta H_m$, and $\Delta C_p$ reported here. We use $\Delta C_p = 1.09$ kcal mol$^{-1}$ K$^{-1}$ so that the $\Delta G(T)$ values are based exclusively on DSC and TDC results. In all cases, the values are in remarkably good agreement (Table 3). This suggests the following: (1) The denatured state ensembles after thermal and urea denaturation are thermodynamically equivalent even though they do not appear to be structurally equivalent, as has been noted before (Tanford, 1968; Pfeil & Privalov, 1976); and (2) conformational stability estimates based on an analysis of urea denaturation curves by the linear extrapolation method are reliable.

**Table 2.** *$\Delta C_p$ values for RNase A folding*

| | $\Delta C_p$ (kcal mol$^{-1}$ K$^{-1}$) |
|---|---|
| DSC (this paper)[a] | |
| $\Delta H_{cal}$ vs. $T_m$ | 1.09 ± 0.07 |
| $\Delta H_{vH}$ vs. $T_m$ | 1.07 ± 0.08 |
| $\Delta H_{fit}$ vs. $T_m$ | 1.09 ± 0.10 |
| Global fit | 1.08 ± 0.42 |
| $C_p(D) - C_p(N)$ | 1.03 ± 0.32 |
| TDC (this paper)[b] | |
| $\Delta H_{vH}$ vs. $T_m$ | 1.13 ± 0.08 |
| DSC and TDC (Privalov et al., 1973) | |
| $\Delta H_{cal}$ vs. $T_m$ | 1.14 ± 0.11 |
| $\Delta H_{vH}$ vs. $T_m$ | 1.06 ± 0.11 |
| $C_p(D) - C_p(N)$ | 1.09 ± 0.09 |
| Pace and Laurents (1989) method | |
| $4\Delta G(H_2O)$ values from UDC, and $T_m$ and $\Delta H_m$ values from this paper[c] | 1.28 ± 0.20 |
| $\Delta C_p$ required to give $\Delta G(H_2O) = \Delta G$ (25 °C) | |
| $7\Delta G(H_2O)$ from UDC (Pace et al., 1990), and $T_m$ and $\Delta H_m$ values from this paper[d] | 1.12 ± 0.16 |
| Average[e] | 1.15 ± 0.09 |

[a] The $\Delta C_p$ values were determined as described in Materials and methods. The value for $\Delta H_{cal}$ vs. $T_m$ is based on the plot shown in Figure 1A.
[b] The $\Delta C_p$ value is based on the plot shown in Figure 1B.
[c] The four $\Delta G(H_2O)$ values used to estimate $\Delta C_p$ are based on urea denaturation curves (UDC) determined at four temperatures at pH 2.8 and reported in Pace and Laurents (1989). The $T_m = 44.85$ °C and $\Delta H_m = 79.4$ kcal/mol values used are the average of values determined by DSC and TDC in this paper.
[d] The seven $\Delta G(H_2O)$ values used to estimate $\Delta C_p$ were interpolated from Figure 6 in Pace et al. (1990). The pH 7 value and the pH 3.55 value are also given in Pace (1990). The $T_m$ and $\Delta H_m$ values used in Equation 2 to calculate $\Delta C_p$ are based on results reported here (Table 3).
[e] This is the average of the values of 1.09, 1.13, 1.28, and 1.12 kcal mol$^{-1}$ K$^{-1}$ that depend on the data in this paper; i.e., the three values from Privalov et al. (1973) were not included.

We are still puzzled by $T_S$. The results presented here give $T_S = -19$ °C. In contrast, the data of Brandts and Hunt (1967) who destabilized RNase A by pH and urea and of Hawley (1971) who destabilized RNase A by pressure indicate $T_S$ values near 0 °C. More recent studies of the pressure dependence of the thermodynamics of RNase A folding indicate a $T_S \approx 19$ °C (Yamaguchi et al., 1995). Salahuddin and Tanford (1970) observed a $T_S \approx 7$ °C in the presence of 3.1 M GdnHCl. We have confirmed this and find $T_S \approx 9$ °C near pH 3 in the presence of 2.5 M GdnHCl or 2.6 M urea. (The earlier studies of Foss and Schellman (1959) suggest an even higher $T_S$.) There is no clear trend in how $\Delta C_p$ varies with GdnHCl concentration (Pfeil & Privalov, 1976; Makhatadze & Privalov, 1992; Barone et al., 1994; Grantcharova & Baker, 1997; Kuhlman & Raleigh, 1998), or urea concentration (Pace & Tanford, 1968; Griko & Privalov, 1992; Makhatadze & Privalov, 1992; Barone et al., 1994; Scholtz, 1995; Nicholson & Scholtz, 1996; Chiti et al., 1998). Using Equation 2 and $T_m$, $\Delta H_m$, and $\Delta C_p$ values for RNase A folding determined in the presence of GdnHCl or urea leads to $T_S$ values of −18 °C (0 M), −11 °C (1 M urea), −6 °C (1 M GdnHCl), −7 °C (2 M urea), and +4 °C (2 M GdnHCl)

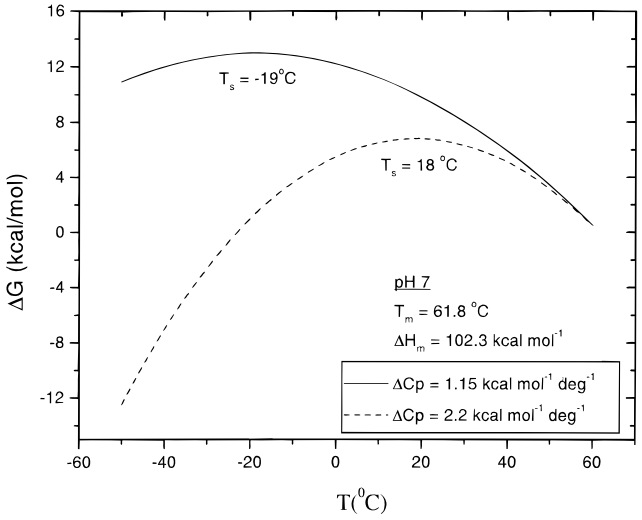


**Fig. 2.** $\Delta G$ for the unfolding of RNase A as a function of temperature. $\Delta G(T)$ was calculated using Equation 2 and the parameters shown.

(Makhatadze & Privalov, 1992). Thus, the trend with these data is clearly toward increased $\Delta C_p$ and $T_S$ values in the presence of GdnHCl and urea. We have experiments underway that may help solve this puzzle.

## Materials and methods

RNase A (Type XII-A, Catalog #5500) and the buffers were purchased from Sigma (St. Louis, Missouri). The buffers used were 10 mM glycine from pH 1 to 3.0, 10 mM acetate from pH 3.5 to 5.0, and 10 mM MOPS or HEPES at pH 7. Protein concentrations were determined from absorbance measurements at 276 nm (corrected for light scattering) using a molar absorption coefficient at 278 nm of 10,020 $M^{-1}$ $cm^{-1}$ for RNase A.

**Table 3.** *Comparison of $\Delta G(T)$ values from DSC with $\Delta G(H_2O)$ values from UDC*

| pH | $T$ (°C) | $T_m$[a] (°C) | $\Delta H_m$[a] (kcal/mol) | $\Delta G(T)$[a] (kcal/mol) | $\Delta G(H_2O)$[b] (kcal/mol) |
|---|---|---|---|---|---|
| 2.8 | 17.1 | 44.9 | 79.4 | 5.6 | 5.4 |
| 2.8 | 21.1 | 44.9 | 79.4 | 4.9 | 4.9 |
| 2.8 | 24.9 | 44.9 | 79.4 | 4.3 | 4.3 |
| 2.8 | 27.8 | 44.9 | 79.4 | 3.7 | 3.5 |
| 2.8 | 25.0 | 44.9 | 79.4 | 4.3 | 4.3 |
| 3.0 | 25.0 | 49.1 | 82.7 | 5.2 | 5.2 |
| 3.6 | 25.0 | 54.5 | 91.5 | 6.7 | 6.4 |
| 4.0 | 25.0 | 56.1 | 94.2 | 7.2 | 7.3 |
| 5.0 | 25.0 | 58.6 | 99.1 | 8.1 | 7.9 |
| 6.0 | 25.0 | 60.3 | 100.7 | 8.5 | 8.6 |
| 7.0 | 25.0 | 61.8 | 102.3 | 9.0 | 9.1 |

[a]The $T_m$ and $\Delta H_m$ values are from this paper and were used with $\Delta C_p$ = 1.09 kcal $mol^{-1}$ $K^{-1}$ from Table 2 in Equation 2 to calculate the $\Delta G(T)$ values. Thus, the $\Delta G(T)$ values are based entirely on DSC and TDC data and the $\Delta G(H_2O)$ values are based on UDC data.

[b]The first four $\Delta G(H_2O)$ values are from Pace and Laurents (1989), and the last seven $\Delta G(H_2O)$ values are interpolated from Figure 6 in Pace et al. (1990). In a recent paper, Baskakov and Bolen (1998) found $\Delta G(H_2O)$ = 5.04 ± 0.15 kcal/mol at pH 3.0 using urea denaturation and the linear extrapolation method.

### *Differential scanning calorimetry (DSC)*

The DSC experiments were performed on VP-DSC calorimeter (Microcal Inc.) (Plotnikov et al., 1997). The RNase A solutions (0.5–3 mg/mL) were extensively dialyzed at 4 °C against the corresponding buffer using Spectrapor 3 dialysis membranes with a molecular weight cutoff of 3,500 Da. Insoluble material was removed by centrifugation for 15–20 min at 13,000 rpm. All experiments were performed at a heating rate of 1 °C/min as previously described (Makhatadze, 1998). Duplicate or triplicate scans were performed at the following pH values: 2.0, 2.3, 2.65, 2.8, 3.0, 3.5, 4.0, 4.5, 5.0, and 7.0. Calorimetric profiles were analyzed as described in Makhatadze (1998). Values of $\Delta H_{cal}$ were determined from the area under the excess heat capacity profiles. The van't Hoff enthalpy was calculated using the following relation:

$$\Delta H_{vH} = 4RT_m^2 C_{p,max}/\Delta H_{cal} \quad (5)$$

where $C_{p,max}$ is the maximum value of the excess heat capacity profile. $\Delta H_{fit}$ is the enthalpy of the DSC transition obtained by analyzing the transition by a two-state model in the same way that the TDC were analyzed as described below. In addition to the analyses of the individual profiles, a global fit of all of the data was performed using two sets of fitted parameters. The fitted parameters, in addition to the individual $T_{m,i}$ and $\Delta H_{cal,i}$ values, included the global temperature independent heat capacity change upon unfolding $\Delta C_p$ and the global linear functions for the heat capacities of the native $C_p^N(T) = C_p^N(T_m) + T \cdot \delta C_p^N$ and unfolded $C_p^U = C_p^U(T_m) + T \cdot \delta C_p^U$ states. In the first set, the value of $\Delta C_p$ (Table 2, fourth row) was independent of the $C_p^N(T)$ and $C_p^U(T)$ functions. In the second set, the global heat capacity change (Table 2, fifth row) was defined as $C_p^U(T_m) - C_p^N(T_m)$, where $T_m$ is an average transition temperature for all DSC profiles. The overall quality of the fit and the absolute values of the fitted parameters were comparable for both sets of the fitted parameters.

### *Thermal denaturation curves (TDC)*

Thermal denaturation curves were determined and analyzed as described in Pace et al. (1998). A total of 25 TDC were determined: 23 between pH 1.2 and 5, and 2 at pH 7. At low pH, $T_m$ is low and the pre-transition baselines are short and the post-transition baselines are long, but at high pH the situation is reversed. The curves were analyzed by both of the methods suggested by Allen and Pielak (1998), and the results did not differ significantly.

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