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The paradox between *m* values and Δ*Cp*'s for denaturation of ribonuclease T1 with disulfide bonds intact and broken

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Abstract

Urea-induced denaturations of RNase T1 and reduced and carboxyamidated RNase T1 (RTCAM) as a function of temperature were analyzed using the linear extrapolation method, and denaturation *m* values, Δ*Cp*, Δ*H*, Δ*S*, and Δ*G* quantities were determined. Because both Δ*Cp* and *m* values are believed to reflect the protein surface area newly exposed on denaturation, the prediction is that the ratio of *m* values for RNase T1 and RTCAM should equal the Δ*Cp* ratio for the two proteins. This is not the case, for it is found that the *m* value of RTCAM is 1.5 times that of RNase T1, while the denaturation Δ*Cp*'s for the two proteins are identical. The paradox of why the two parameters, *m* and Δ*Cp*, are not equivalent in their behavior is of importance in the interpretations of their respective molecular-level meanings. It is found that the measured denaturation Δ*Cp*'s are consistent with Δ*Cp*'s calculated on the basis of empirical relationships between the change in surface area on denaturation (Δ*ASA*), and that the measured *m* value of RNase T1 agrees with *m* calculated from empirical data relating *m* to Δ*ASA*. However, the measured *m* of RTCAM is so much out of line with its calculated *m* as to call into question the validity of always equating *m* with surface area newly exposed on denaturation.

Keywords: denaturation heat capacity change; disulfide bonds; loop entropy; *m* values; protein stability; RNase T1; solvent-accessible surface area

It is generally thought that the sensitivity of proteins toward a particular denaturant (the *m* value) is proportional to the amount of newly accessible surface area exposed upon denaturation. The bases of this claim are largely derived from the theoretical work of Schellman, as well as from correlations observed between the *m* value, an experimentally determined parameter, and the change in accessible surface area (Δ*ASA*), calculated using a model of the denatured state (Schellman, 1978; Myers et al., 1995). Similarly, there has been success in the literature in establishing that heat capacity of denaturation, Δ*Cp*, can be parameterized in terms of the protein surface area newly exposed on denaturation (Murphy & Freire, 1992; Spolar et al., 1992). The correlation between Δ*Cp* and Δ*ASA* is high (correlation coefficient $R = 0.98$), whereas *m* values have been found to be more roughly proportional to Δ*ASA* (correlation coefficient $R = 0.90$) (Myers et al., 1995).

To investigate these correlations further, we evaluated Δ*Cp* and *m* values for two forms of the same protein: wild-type ribonuclease T1 (RNase T1) and ribonuclease T1 with its two disulfide bonds reduced and carboxyamidated (RTCAM). The primary difference in the solvent-induced denaturation of these two proteins is that the disulfide bonds are intact in RNase T1 but not in RTCAM. Identical Δ*Cp* values for RNase T1 (1.59 ± 0.10 kcal/mol) and RTCAM (1.56 ± 0.15 kcal/mol) obtained from Gibbs–Helmholtz analysis, as well as similar Δ*H* values of denaturation for the two proteins, indicate that both proteins expose equivalent amounts and character of surface area upon denaturation. By contrast, the urea-denaturation *m* value for RTCAM (1.95 kcal/mol·M) is found to be 50% larger than the *m* value for RNase T1 (1.24 kcal/mol·M), a result that is commonly attributed to a large change in surface area exposed on denaturation. Our purpose in this paper is to find a rational solution to the paradox in which thermodynamic parameters Δ*Cp* and Δ*H* suggest marginal differences in exposed surface areas of denatured RNase T1 and RTCAM, while the large *m* value differences suggest large Δ*ASA* differences.

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Abbreviations: Δ*ASA*, newly accessible surface area exposed to solvent on denaturation; Δ*Cp*, denaturation heat capacity change; $\Delta G^{\circ}_{N\text{-}D}$, Gibbs energy of denaturation in the limit of zero denaturant concentration; LEM, linear extrapolation method; *m*, defined as $d\Delta G/d$[denaturant]; RNase T1, ribonuclease T1; RTCAM, reduced and carboxyamidated RNase T1; TMAO, trimethylamine-N-oxide; UV-CD, ultraviolet circular dichroism.

Results

Although Δ*Cp* has been firmly correlated with Δ*ASA*, the correlation of *m* to Δ*ASA* is not nearly as strong (Myers et al., 1995). To evaluate the relationship between Δ*Cp* and *m* values, we determine here Δ*Cp* and *m* for two proteins: RNase T1 (with its two disulfide bonds intact), and reduced and carboxyamidated RNase T1 (RTCAM). We evaluated the *m* values from urea-induced denaturation experiments by monitoring the intrinsic fluorescence upon excitation either at 295 nm or at 278 nm (Fig. 1). Excitation at 295 nm with emission at 319 nm monitors changes in the environment of the single Trp side-chain residue in the course of unfolding, whereas 278 nm excites the Trp along with nine Tyr residues and emission monitored at 319 nm arises both from excitation of Trp and from energy transfer from Tyr residues to the single Trp residue. Thus, the second excitation protocol links Tyr probes distributed throughout the protein as part of the denaturation detected by Trp fluorescence emission. The fluorescence-detected denaturation data were analyzed assuming two-state behavior, and the solid lines in Figure 1 represent the fits to the linear extrapolation method. This procedure gives *m* and $\Delta G^{\circ}_{N\text{-}D}$ as fitting parameters, where $m = d\Delta G/d[D]$ and $\Delta G^{\circ}_{N\text{-}D}$ is the denaturation Gibbs energy change at the limit of zero denaturant concentration. We find that the *m* values derived from monitoring excitation at the two wavelengths are in agreement with *m* values published previously with the Q25 forms of RNase T1 and RTCAM (Pace et al., 1988). As shown in Figure 2, the *m* values determined for denaturation of RTCAM are considerably larger than those for RNase T1. The average *m* value for RTCAM (1.95 ± 0.10 kcal/mol M^{-1}) is 1.5 times the average *m* evaluated for RNase T1 (1.24 ± 0.05 kcal/mol M^{-1}).

Through the parameterization methods of others, Δ*Cp* has been found to correlate well with Δ*ASA* exposed upon denaturation (Murphy & Freire, 1992; Spolar et al., 1992). It is important to note that the denatured state could vary, depending on whether solvent- or temperature-induced denaturation is used. Thus, to compare *m* and Δ*Cp*, both parameters must be obtained using the same solvent-denaturing conditions. Because *m* represents a change in Gibbs free energy with respect to solvent (urea) concentration, we determined Δ*Cp* from solvent-induced denaturation/folding experiments. To obtained Δ*Cp*, we determined Gibbs energy values ($\Delta G^{\circ}_{N\text{-}D}$) from the linear extrapolation method (LEM) at different temperatures and used a modified form of the Gibbs–Helmholtz equation given below to fit the data (Greene & Pace, 1974).

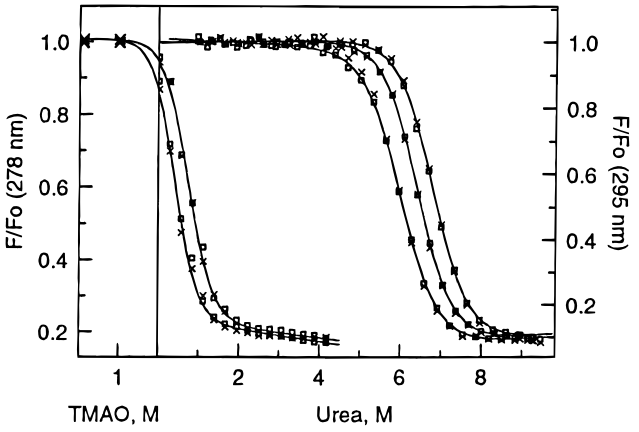

Fig. 1. Urea-induced equilibrium denaturations of RNase T1 and RTCAM as a function of temperature. Curves shown from left to right are RTCAM at 5 and 0 °C, and RNase T1 at 23, 19, and 15 °C. All transitions were monitored by intrinsic fluorescence at 319 emission with excitation at 278 nm (open squares) and 295 nm (×) in 30 mM MOPS, pH 7.0, 0.1 M NaCl, 2mM EDTA. The solid lines represent the results of nonlinear least-squares best fits of the data using the linear extrapolation method. F/F_o values for RTCAM denaturation were scaled relative to the upper limit of fluorescence measured in the presence of TMAO.

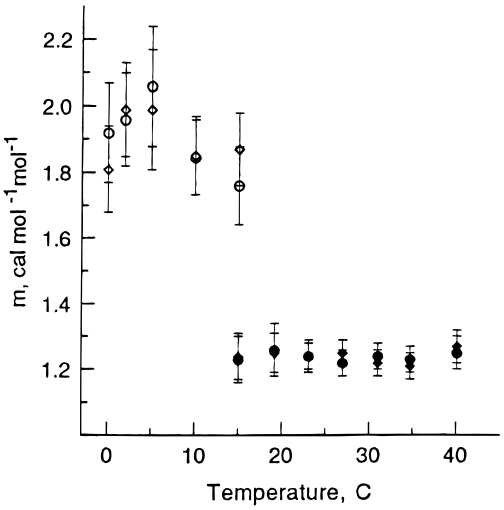

Fig. 2. The *m* values for RNase T1 and RTCAM as a function of temperature. The *m* values were derived from application of the LEM to the urea-induced denaturation data presented in Figure 1 and at other temperatures. RTCAM denaturation curves at 10 and 15 °C were artificially stabilized with 0.5 M $MgCl_2$. However, the RTCAM data at 10 and 15 °C are shown as points of reference and were not used in calculating the average *m* value for RTCAM. The *m* values for RNase T1 (filled symbols) and RTCAM (open symbols) are the result of monitoring the denaturation curves at an emission wavelength of 319 nm with excitation at 278 nm (circles) and 295 nm (diamonds).

$$\Delta G^{\circ}_{N\text{-}D}(T) = \Delta H_m(1 - T/T_m) - \Delta Cp[(T_m - T) + T \ln (T/T_m)], \quad (1)$$

In Equation 1, T_m is a reference temperature corresponding to the midpoint of denaturation, ΔH_m is the enthalpy change for unfolding measured at T_m, Δ*Cp* is the difference in heat capacity between the denatured and the native states, and $\Delta G^{\circ}_{N\text{-}D}$ is the Gibbs energy change in the limit of zero denaturant concentration (Greene & Pace, 1974).

In contrast with RNase T1, which is a stable protein, RTCAM is thermodynamically unstable at room temperature. Hence, RNase T1 unfolding was studied using urea denaturation, while RTCAM was studied using urea denaturation at low temperatures where the protein is more thermodynamically stable, and using osmolyte-induced folding over the full temperature range. Figure 3 shows examples of TMAO-induced folding curves for RTCAM measured at different temperatures using intrinsic fluorescence to monitor denaturation. Again, the fluorescence was monitored upon excitation at 278 and 295 nm, and these two excitation protocols were found to give identical *m* and $\Delta G^{\circ}_{N\text{-}D}$ values (Table 1). At 5 °C we measured both urea-induced denaturation and TMAO-induced folding for validation of the osmolyte-induced folding method to get

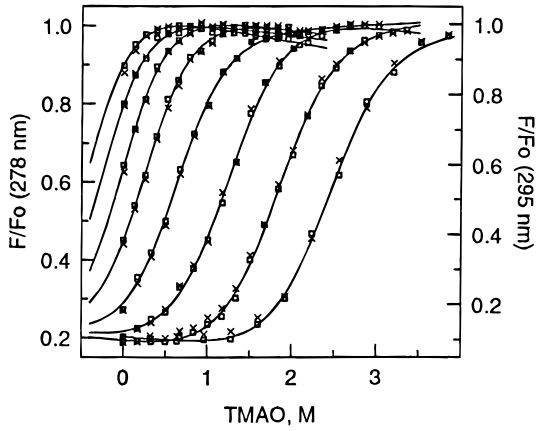

Fig. 3. Reversible TMAO-induced folding of RTCAM monitored at 319 nm emission with excitation at 278 nm (□) and 295 nm (×) at different temperatures. Transitions from left to right are at 5, 8, 12, 16, 20, 25, 30, and 35°. The solid curves represent the results of nonlinear least-squares best fits of the data using the LEM (Santoro & Bolen, 1988).

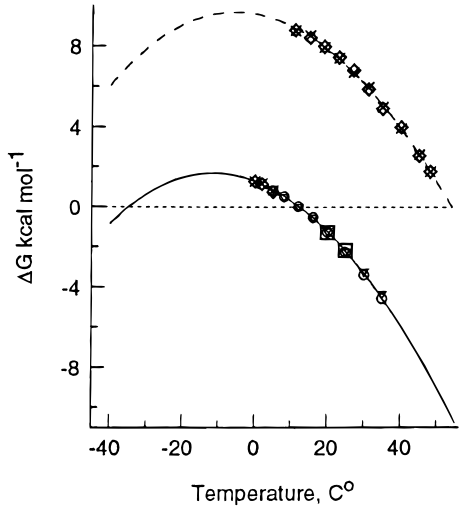

Fig. 4. Gibbs energy changes ($\Delta G^{\circ}_{N\text{-}D}$) of RNase T1 (dashed curve) and RTCAM (solid curve) are presented as a function of temperature at pH 7.0, 30 mM MOPS, 0.1 M NaCl, taking into account the ΔH ionization of the buffer to give pH 7.0. The data points are from TMAO-induced folding experiments: ○, excitation at 278 nm; △, excitation at 295 nm; □, sarcosine-induced folding; urea-induced unfolding (×), excitation at 278 nm; ◇, excitation at 295 nm. The solid and slashed curves represent the nonlinear least-squares best fit of the data to the Gibbs–Helmholtz equation.

$\Delta G^{\circ}_{N\text{-}D}$. $\Delta G^{\circ}_{N\text{-}D}$ values derived from both urea-unfolding and TMAO-folding experiments are identical (Table 1) indicating that $\Delta G^{\circ}_{N\text{-}D}$ is independent of whether unfolding or folding experiments are used. To establish that $\Delta G^{\circ}_{N\text{-}D}$ values determined for RTCAM are a property of the protein and independent of the solvent we obtained $\Delta G^{\circ}_{N\text{-}D}$ using three different osmolytes—TMAO, sarcosine, and sucrose—at two temperatures, 20° and 25 °C. Table 1 shows that $\Delta G^{\circ}_{N\text{-}D}$ values determined using three osmolytes at fixed temperature are essentially indistinguishable from one another.

The data for RTCAM obtained from all sets of experiments on TMAO-, sarcosine-, and sucrose-induced folding and urea-induced unfolding in the range from 0–35 °C were analyzed according to the two-state mechanism of protein folding/denaturation and the $\Delta G^{\circ}_{N\text{-}D}$ values derived from the analyses are presented in Figure 4 together with the result of the fitting of $\Delta G^{\circ}_{N\text{-}D}$ vs. T. $\Delta G^{\circ}_{N\text{-}D}$ values of urea-induced denaturation for RNase T1 covers a temperature range from 11 up to 48 °C, and a least-squares fit of the $\Delta G^{\circ}_{N\text{-}D}$ vs. T data yields ΔCp for RNase T1 that is identical to ΔCp for RTCAM (Table 2).

$$\Delta H(T) = \Delta H(T_m) + (T - T_m)\Delta Cp. \quad (2)$$

The ΔH for denaturation can be calculated (Equation 2) assuming that denaturation heat capacity ΔCp does not depend upon temperature over the temperature range of interest. Using thermodynamic parameters listed in Table 2, we evaluated the functional dependencies of ΔH vs. temperature for both proteins. As shown in Figures 5A and 5B, the ΔH vs. temperature plots are similar for

Table 1. *Free energy change for RTCAM determined from urea-induced denaturation and TMAO-, sarcosine-, and sucrose-induced folding experiments*

T (°C)	Solute	ΔG° (278 nm)	ΔG° (295 nm)
0°	Urea	1.31 ± 0.08	1.32 ± 0.09
2°	Urea	1.20 ± 0.09	1.61 ± 0.08
5°	Urea	0.85 ± 0.07	0.79 ± 0.07
5°	TMAO	0.92 ± 0.04	0.86 ± 0.03
8°	TMAO	0.55 ± 0.03	0.54 ± 0.03
12°	TMAO	0.059 ± 0.012	0.039 ± 0.01
16°	TMAO	−0.49 ± 0.02	0.51 ± 0.02
20°	TMAO	−1.18 ± 0.06	−1.22 ± 0.05
25°	TMAO	−2.20 ± 0.15	2.24 ± 0.13
30°	TMAO	−3.19 ± 0.32	−3.38 ± 0.31
35°	TMAO	−4.32 ± 0.48	−4.56 ± 0.40
20°	Sarcosine	−1.29 ± 0.04	−1.23 ± 0.05
25°	Sarcosine	−2.14 ± 0.09	−2.14 ± 0.10
20°	Sucrose	−1.31 ± 0.05	−1.28 ± 0.04
25°	Sucrose	−2.26 ± 0.15	−2.30 ± 0.12

Table 2. *Thermodynamic parameters for denaturation of RNase T1 and RTCAM obtained by fitting of ΔG vs. T according to the Gibbs–Helmholtz equation* [a]

	ΔCp (kcal mol^{-1} K^{-1})	T_m (°C)	ΔH_m (kcal mol^{-1})	$\Delta H_{(60^{\circ})}$ (kcal mol^{-1})
RTCAM	1.59 ± 0.10	12.4 ± 0.2	39.9 ± 0.5	115.6 ± 1.0
RNase T1	1.56 ± 0.15	54.0 ± 0.5	102.7 ± 1.0	112.4 ± 1.0

[a] $\Delta H_{(60^{\circ})}$ represents the denaturation enthalpy change calculated from solvent-induced transitions of RNase T1 and RTCAM using the ΔCp provided. ΔCp's, the denaturation enthalpy changes ΔH_m, and corresponding T_m's (melting temperatures) are obtained as parameters from fitting the RNase T1 and RTCAM data in Figure 4 to the Gibbs–Helmholtz equation.

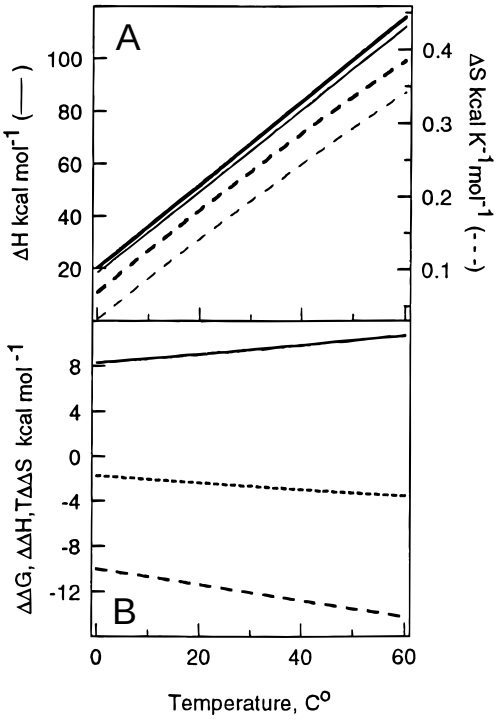

Fig. 5. A: Temperature dependence of the Δ*H* (solid lines) and Δ*S* (dashed lines) of denaturation of RNase T1 (thin lines) and RTCAM (bold lines). **B:** Temperature dependencies for $\Delta\Delta G$ (——) (where $\Delta\Delta G = \Delta G_{\text{RNase T1}} - \Delta G_{\text{RTCAM}}$), $\Delta\Delta H$ (---) (where $\Delta\Delta H = \Delta H_{\text{RNase T1}} - \Delta H_{\text{RTCAM}}$), and $T\Delta\Delta S$ (– – –) (where $T\Delta\Delta S = T\Delta S_{\text{RNase T1}} - T\Delta S_{\text{RTCAM}}$.

both proteins with Δ*H* for RNase T1 being 3.2 kcal/mol lower than the Δ*H* for RTCAM at 60 °C (see Table 2).

The data demonstrate that the denaturation Δ*H* and Δ*Cp* values are very close for RNase T1 and RTCAM, despite the differences between the proteins with respect to disulfide bonds. We evaluated the dimensions of the denatured ensembles for both proteins by size exclusion chromatography (Fig. 6), and as one might expect, RTCAM has a significantly more expanded denatured ensemble than does RNase T1, with a Stokes radius of 27.1 Å for RTCAM and 22.0 Å for RNase T1 (calculated from Fig. 1; Baskakov & Bolen, 1998b). The fact that these two proteins differ in their Stokes radii shows that disulfide bonds limit the conformations accessible to the denatured ensemble of RNase T1. To evaluate the effect of disulfide bonds on the entropy change of denaturation (Δ*S*), we calculated Δ*S* as a function of temperature using Equation 3 and parameters listed in Table 2. The results are given in Figure 5B.

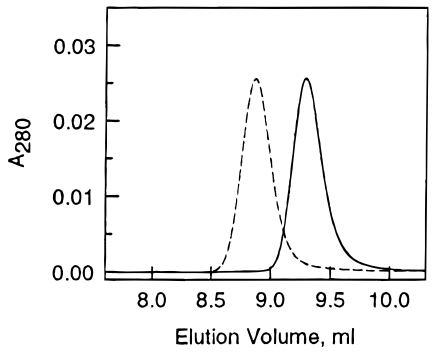

Fig. 6. Size-exclusion chromatography of RNase T1 (——) and RTCAM (– – –) in the presence of 8 M urea, 0.1 M NaCl at pH 7.0, 30 mM MOPS, 25 °C.

$$\Delta S(T) = \Delta H(T_m)/T_m + \Delta Cp \ln(T/T_m). \quad (3)$$

The entropy change on denaturation for RNase T1 is significantly less than the entropy change accompanying denaturation of RTCAM. The difference evaluated between the two $T\Delta S$ values ($T\Delta\Delta S = T\Delta S_{\text{RNase T1}} - T\Delta S_{\text{RTCAM}}$) is 11.7 kcal/mol at 25 °C and becomes more significant at higher temperatures.

Because the denaturation Δ*H* values for the two proteins are very similar, the large Gibbs energy stability differences between RNase T1 and RTCAM are entropic and largely originate from the gain in conformational entropy that occurs on the release of the disulfide restraints in denatured RTCAM. The magnitude of $T\Delta S$ expected from releasing disulfide restraints for several proteins has been estimated by several research groups using Equation 4 (see references in Pace et al., 1988).

$$\Delta S = -a - (3/2)R \ln n. \quad (4)$$

Here, *n* represents the number of residues in the loop formed by the disulfide bond, and *a* is a constant determined by whatever spherical volume element is assumed to be sufficient to get the two ends of a chain together in forming a disulfide bonded loop. The values of the constant *a* that these groups have used to calculate the loop entropy effect range from 2.1 to 7.9, giving a corresponding range of conformational entropy ($T\Delta\Delta S$) contributions to $\Delta\Delta G$ at 25 °C of −7.3 to −10.7 kcal/mol (Pace et al., 1988). It can be seen in Figure 3 that the experimentally determined (25 °C) $T\Delta\Delta S$ is −11.7 kcal/mol, a quantity that includes both the conformational entropy as well as any other entropic contributions that occur as part of the experimental measurement. These results show that the loop entropy effect is the major contributor to the entropy and Gibbs energy differences in the denaturations of RNase T1 and RTCAM.

Discussion

There is ample evidence that the two experimental parameters, Δ*Cp* and *m*, are proportional to surface area newly exposed on protein denaturation. For a group of 45 proteins for which data exist, Myers et al. found that Δ*Cp* is much more strongly correlated with Δ*ASA* (correlation coefficient = 0.98) than *m* vs. Δ*ASA* (correlation coefficient = 0.90), although it is clear from the data that both parameters track with Δ*ASA*, a quantity calculated from the structure of the native protein and an extended model of the denatured state (Myers et al., 1995). On comparing the urea denaturation of disulfide intact and disulfide free forms of the protein RNase T1, it is surprising to find that the Δ*Cp*'s for denaturation of both proteins are identical, while the *m* values for urea denaturation of the proteins differ from one another by 50%! If we assume that releasing the disulfide restraints should result in increasing the surface area exposed on denaturation of RTCAM over that of the disulfide intact denatured state, the increase in *m* value can be readily understood, but the lack of change in Δ*Cp* makes little sense. On the other hand, if the disulfide intact denatured state is

highly solvent exposed, and the disulfide-free denatured state contributes little to increasing the surface area, it is easy to understand how both ΔCp's would be identical but difficult to rationalize why the m value is increased so significantly if m is only a measure of surface area change on denaturation. These results provide an important paradox to the current view of the interrelationships of ΔCp, m, and ΔASA, a paradox that presents the opportunity to more clearly define the relationships of these experimental parameters to molecular properties.

To understand the root causes of the paradox, it is important to consider that the m value can reflect molecular properties additional to the change in surface area on denaturation. DeKoster and Robertson have pointed out that, depending on solution conditions, m values for the same protein can vary by a factor of 2 (DeKoster & Robertson, 1997). In addition, Soulages (1998) and Carra and Privalov (1996) have discussed how the presence of intermediate states will lower the m value of solvent-induced denaturation. The implication is that the smaller m value observed for RNase T1 denaturation relative to that of RTCAM denaturation may reflect the occurrence of equilibrium intermediates in RNase T1 solvent-induced denaturation. A number of reports, however, present a strong case that solvent-induced denaturation of RNase T-1 exhibits two-state denaturation (Thomson et al., 1989; Kiefhaber et al., 1990; Plaza del Pino, 1992; Yu et al., 1994). In addition, Mücke and Schmid have devised a novel kinetic test demonstrating that RTCAM solvent-induced denaturation is two state in character (Mücke & Schmid, 1994). Because the experimental data accumulated so far demonstrate that the two-state model describes the denaturation transitions of both RNase T1 and RTCAM, it seems highly unlikely that the large (1.5-fold) difference between the m values for RTCAM and RNase T1 originates from the presence of an intermediate in RNase T1 denaturation and yet not be detectable. Neither can the differences in m value be attributed to different surface areas exposed in the native states of RNase T1 and RTCAM.

We have previously shown that the "native" folded form of RTCAM has the same fluorescence emission, far UV-CD, and near UV-CD spectral signatures as RNase T1, and it is observed to have 20% of the activity of RNase T1, despite accommodating four carboxyamido groups (Baskakov & Bolen, 1998a). These results strongly indicate that the native states of RNase T1 and RTCAM are very similar in terms of their structures.

The similar structural characteristics of native RNase T1 and RTCAM provide a view of their urea-induced denaturations that is quite consistent with their observed thermodynamic quantities, ΔH, ΔCp, ΔG, and ΔS. The fact that there is relatively little difference between the denaturation enthalpy changes for RNase T1 and RTCAM (Table 2; Fig. 5) over a broad range of temperatures indicates that the intramolecular interactions that are disrupted on denaturation of either RNase T1 and RTCAM are very similar in the two proteins. That is, the nearly identical denaturation ΔH changes for the two proteins strongly suggest that RNase T1 and RTCAM break the same number and kinds of intramolecular interactions and then make the same number and kinds of interactions between the denatured protein fabric and solvent. A consequence of the denaturation ΔH values being essentially the same for both proteins is that the degree of solvent exposure is virtually identical for the denatured states of RNase T1 and RTCAM. Because ΔCp is known to be very strongly correlated with the change in surface area exposed on denaturation, the fact that the denaturation ΔCps for RNase T1 and RTCAM are identical gives additional strength to the interpretation that the degrees of solvent exposure are quite similar in the denatured states of RNase T1 and RTCAM. [Using the experimental ΔCp and ΔH values determined here, the parameterized equations of Murphy and Freire and Spolar et al. can be used to calculate the polar and apolar surface areas exposed on denaturation of RNase T1 and RTCAM (Murphy & Freire, 1992; Spolar et al., 1992; Hilser et al., 1997). Based on these calculations, it is found that within ±2%, the same amount and character of surface area are exposed in denaturation of RNase T1 as are exposed in denaturation of RTCAM.]

In contrast to the denaturation model for RNase T1 and RTCAM suggested by the thermodynamics, the large differences in m values for denaturation of RNase T1 and RTCAM suggest that the degree of solvation of denatured RTCAM is significantly greater than that of denatured RNase T1. Thus, the root of the paradox appears to hinge on the degree to which the disulfide bonds decrease surface area exposure. Myers et al. have postulated that the disulfide bonds in RNase T1 decrease the comparative surface area exposed in the extended protein by 1,248 Å^2 (Myers et al., 1995). Using this surface area decrease along with the correlations of ΔCp and m with ΔASA, we can attempt to determine which measured parameter, ΔCp or m, is the one responsible for the paradox.

$$\Delta Cp = -119\ (\pm 110) + 0.2\ (\pm 0.007)\ (\Delta ASA). \qquad (5)$$

The equation obtained by Myers et al. for the fit of ΔCp to ΔASA from a database of 45 proteins is given in Equation 5 with a correlation coefficient of 0.98. Using ΔASA values of 7,255 (Å^2) and 8,503 (Å^2) for RNase T1 and RTCAM from Tables 1 and 2 of Myers et al. gives calculated ΔCps of 1.33(±0.12) and 1.58(±0.12) kcal/mol deg for RNase T1 and RTCAM, respectively (Myers et al., 1995). Comparison of our experimentally determined ΔCp's with the calculated ΔCp's give 1.56 (±0.15) and 1.33(±0.12) for RNase T1 and 1.59(±0.10) and 1.58(±0.12) kcal/mol deg for RTCAM. It is clear that our experimental values agree, within error, with the strong correlation of ΔCp with ΔASA. Thus, while there is nothing unusual about the measured values of ΔCp for RNase T1 and RTCAM in comparison with the established relationship of ΔCp with ΔASA, there is a problem with agreement between the observed m value for RTCAM and the relationship between m and ΔASA.

Myers et al. (1995) cite Equation 6 as the relationship obtained from fitting m to ΔASA values derived from their 45 protein data base, a fitting giving a correlation coefficient of 0.90. Applying the ΔASA values given above to Equation 6 gives the respective calculated and observed m values of 1.17 ± 0.14 and 1.24 ± 0.05 kcal mol^{-1} M^{-1} urea for RNase T1 along with 1.30 ± 0.14 and 1.95 ± 0.10 kcal mol^{-1} M^{-1} urea for RTCAM. The data show that calculated and observed m values for urea denaturation of RNase T1 agree well within error, but the observed m value for RTCAM falls far short of agreement with m calculated from the m vs. ΔASA relationship of Myers et al. The observed m for RTCAM is in excess of the calculated value by ≥2.5 standard deviation units, a deviation so extreme that some consider it justifiable to cast out such a data point for statistical reasons (Taylor, 1982). It is important to note that it is the m value of RTCAM, not RNase T1, that is the outlier, and that RTCAM's m value is much higher than the fitted line relating m to ΔASA.

$$m = 368\ (\pm 132) + 0.11\ (\pm 0.01)\ (\Delta ASA). \qquad (6)$$

The paradox whereby the Δ*Cp*'s for denaturation of RNase T1 and RTCAM are identical, while their *m* values differ from one another by 50%, follows from the common assumption that both *m* and Δ*Cp* reflect the newly exposed surface area on denaturation. The paradox brings into focus the question of whether Δ*Cp* and/or *m* are directly proportional to surface area and do not deviate from that proportionality. The results show that Δ*H* of denaturation for both proteins are essentially identical, and that disulfide bond corrected Δ*Cp*'s calculated from surface areas exposed on denaturation and the experimentally measured Δ*Cp*'s are, within error, identical for the two proteins. These data provide strong evidence that Δ*Cp* is directly proportional to newly exposed denatured protein surface area and that disulfide bond restraints can be appropriately accounted for. The same conclusion cannot be drawn for the *m* value effects exhibited by RNase T1 and RTCAM. Although the *m* value for RNase T1 calculated on the basis of newly exposed denaturation surface area agrees with our experimental *m* values, the experimentally determined *m* value for RTCAM is 50% greater than *m* calculated on the basis of surface area is simply too large to account for. These results indicate that *m* values can deviate significantly from direct proportionality with surface area and that *m*-value–based assessments of denatured surface areas of closely related proteins can lead to incorrect conclusions. A more direct demonstration of *m*-values not being proportional to surface area was recently shown using staphylococcal nuclease (SN) and the *m*+ SN mutant protein A69T (Baskakov & Bolen, 1998b). The *m* value for A69T is larger than that for wt SN, but it is observed that the Stokes radius of urea-denatured A69T at the midpoint of the denaturation transition is measurably smaller than the denatured ensemble of wt SN at the midpoint of its transition (Baskakov & Bolen, 1998b); that is, the measured sizes of the denatured states of A69T and wt SN not only do not correlate with the *m* values, their spherical surface areas are exactly opposite what one would predict based on the commonly assumed proportionality of *m* with Δ*ASA*.

Materials and methods

Trimethylamine-N-oxide, iodoacetamide were purchased from Sigma (St. Louis, Missouri); ultrapure urea was from Nacalai Tesque Inc. (Kyoto, Japan); sarcosine was from Fluka (Buchs, Switzerland); sucrose was from Mallinckrodt (Paris, Kentucky).

Solutions of urea and TMAO were prepared as described by Baskakov and Bolen (1998c). RNase T1 (K25 form) was generously provided by Dr. C. Nick Pace. Except for substituting iodoacetamide for iodoacetate, reduction and carboxyamidation of RNase T1 was performed as described by Mücke and Schmid (1992). RTCAM with all four cysteines carboxyamidated migrated as a single band in native polyacrylamide gel electrophoresis and as a single peak when chromatographed on Phenomenex Biosep (Torrance, California) SEC-S3000 gel filtration column. The assay for free thiols with Ellman's reagent was negative.

The *m* and Δ*G* values were obtained from urea-induced denaturation and osmolyte-induced folding experiments performed in 30 mM MOPS (pH 7.0), 0.1 M NaCl, 2 mM EDTA. Equilibrium denaturation/folding of RNase T1 and RTCAM were monitored and analyzed as described by Baskakov and Bolen (1998a). RNase T1 and RTCAM samples (10 μg/mL) were incubated for 4–48 h (depending on temperature) before measurements of equilibrium folding/unfolding at the corresponding temperature.

Gel-filtration chromatography was carried out using a Phenomenex Biosep SEC-S3000 HPLC column 300 × 7.80 mm, equilibrated in buffer (30 mM MOPS, 8 M urea, 0.1 M NaCl, 2 mM EDTA, pH 7.0) at 25 °C. Prior to their injection, RNase T1 and RTCAM samples were preincubated in the same buffer for 12 h at 25 °C.

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