Conformational Stability and Mechanism of Folding of Ribonuclease T1*

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Urea and thermal unfolding curves for ribonuclease T1 (RNase T1) were determined by measuring several different physical properties. In all cases, steep, single-step unfolding curves were observed. When these results were analyzed by assuming a two-state folding mechanism, the plots of fraction unfolded protein versus denaturant concentration were coincident. The independent dependence of the free energy of unfolding, $\Delta G$ (in kcal/mol), on urea concentration is given by $\Delta G = 5.6 - 1.21 (\text{urea})$. The parameters characterizing the thermodynamics of unfolding are: midpoint of the thermal unfolding curve, $T_m = 48.1 ^\circ \text{C}$, enthalpy change at $T_m$, $\Delta H_m = 97 \text{ kcal/mol}$, and heat capacity change, $\Delta C_p = 1650 \text{ cal/mol deg}$. A single kinetic phase was observed for both the folding and unfolding of RNase T1 in the transition and post-transition regions. However, two slow kinetic phases were observed during folding in the pre-transition region. These two slow phases account for about 90% of the observed amplitude, indicating that a faster kinetic phase is also present. The slow phases probably result from cis-trans isomerization at the 2 proline residues that have a cis configuration in folded RNase T1.

These results suggest that RNase T1 folds by a highly cooperative mechanism with no structural intermediates once the proline residues have assumed their correct isomeric configuration. At 25 °C, the folded conformation is more stable than the unfolded conformations by 5.6 kcal/mol at pH 7 and by 8.9 kcal/mol at pH 5, which is the pH of maximum stability. At pH 7, the thermodynamic data indicate that the maximum conformational stability of 8.3 kcal/mol will occur at $-6 ^\circ \text{C}$.

Ribonuclease T1 (RNase T1) is an excellent model for studying the energetics and mechanism of protein folding. The three-dimensional structure of this protein has been determined at high resolution and shows that the 104 amino acid residues fold to a compact globular conformation in which the hydrophobic core is sandwiched between a 4.5 turn $\alpha$-helix and an extended antiparallel $\beta$-sheet (1, 2). This conformation is stabilized in part by two disulfide bonds, 96 intramolecular hydrogen bonds, and by hydrophobic bonding involving the removal of about 85% of the nonpolar residues from contact with water on folding (1, 3). Thus, RNase T1 provides an excellent model for studies of the folded conformation. When the two disulfide bonds are broken, RNase T1 will fold, but $T_m$ is lowered by about 40 °C (4). This is important for two reasons. First, it allows folding to be studied in the presence or absence of the disulfide bonds, and, second, it allows an investigation of the unfolded conformations of RNase T1 under physiological conditions. Thus, RNase T1 is also an excellent model for studies of the unfolded conformations. Recent studies of staphylococcal nuclease (5) and results presented in the following paper (6) have made it clearer that the unfolded conformations of a protein may make important contributions to the net conformational stability.

The conformational stability of RNase T1 can be more than doubled by adding salts. This has been shown to result primarily from the preferential binding of cations and anions to the folded conformation (7). In contrast, the conformational stability can be decreased by over 9 kcal/mol by breaking the disulfide bonds (4). Thus, the conformational stability of this small globular protein can be varied over a range of about 15 kcal/mol.

In this and the following paper (6), we focus our interest on the conformational stability of RNase T1, i.e., how much more stable the folded, globular conformation is than unfolded conformations. In this paper, we present studies of the wild-type protein and show that the conformational stability is 5.6 kcal/mol at 25 °C in 30 mM MOPS1 buffer at pH 7. We also show how the conformational stability depends on temperature, and we present evidence that folding closely approaches a two-state mechanism. In the accompanying paper (6), we present studies of the conformational stability of three mutants of RNase T1.

**EXPERIMENTAL PROCEDURES**

RNase T1 was purified as previously described (8) from a crude Aspergillus oryzae extract obtained from Sigma (Product P 4755). RNase T1 concentrations were determined using an absorbance of 1.9 for a 1 mg/ml solution (9). "Ultrapure" urea was purchased from Schwarz/Mann Biotech. Urea stock solutions were prepared, and their concentration was determined as described elsewhere (10). Stock RNase T1 solutions were filtered using "Acrodisc" filters obtained from Gelman Sciences. All other solutions were filtered using medium porosity sintered glass filters.

Urea unfolding curves were determined by measuring the chosen spectral property as a function of urea concentration on RNase T1 solutions buffered at pH 7.0 with 30 mM MOPS and thermostatted at 25.0 ± 0.1 °C. Thermal unfolding curves were determined by similar measurements on solutions in which the temperature was monitored directly in the cuvette using a YSI telethermometer that had been calibrated against a National Bureau of Standards certified thermometer. The techniques used to determine and analyze urea and thermal unfolding curves have been discussed in detail elsewhere (11).

Intrinsic fluorescence intensity measurements on 0.01 mg/ml

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1 The abbreviation used is: MOPS, 4-morpholinepropanesulfonic acid.
RNase T1 solutions were made with a Perkin-Elmer MPF 48B spectrofluorometer using 1-cm path length cuvettes with excitation at either 278 nm or 295 nm and emission measured at 320 nm. The optical rotation of ~1 mg/ml RNase T1 solutions was measured at 295 nm with a Cary 80 spectropolarimeter using 1-cm path length cells. Circular dichroism measurements, 300 to 235 nm and 250 to 210 nm, were made with a JASCO J-600 A spectropolarimeter using 1- and 0.1-cm path length cuvettes, respectively. Difference spectral measurements were made on ~0.2 mg/ml RNase T1 solutions with a Cary 15 spectrophotometer using tandem 1-cm cells for urea unfolding studies and matched 1-cm cells for thermal unfolding studies with only the sample cell thermostatted.

The kinetics of folding and unfolding were followed by monitoring the change in fluorescence intensity (278 nm excitation and 320 nm emission) of 0.01 mg/ml RNase T1 solutions containing 30 mM acetate buffer, at pH 5.0, 25.0 ± 0.1 °C. Unfolding was initiated by mixing 0.2 ml of a RNase T1 stock solution with 2.8 ml of a urea solution. The dead time was approximately 30 s. Folding was initiated by diluting urea-denatured RNase T1 solutions with buffer. Unfolding was allowed to proceed for at least 30 min (seven relaxation times) prior to the initiation of folding. Both folding and unfolding were monitored until equilibrium was reached. All data were fit to the equation

\[ A_i = A_\infty + \sum A_i \exp(-t/\tau_i) \]  

where \( A_i \) is the total amplitude at time \( t \), \( A_\infty \) is the amplitude at infinite time, \( A_i \) is the amplitude of the individual phase, \( i \), and \( \tau_i \) is the associated relaxation time. The "best values" of \( A_\infty, A_i, \) and \( \tau_i \) were determined by an iterative nonlinear least squares program varying all parameters until minimization had occurred.

pH measurements were made at room temperature with a Radiometer PHM 26 pH meter.

RESULTS

The unfolding of RNase T1 was monitored using several spectroscopic techniques in order to gain information about the mechanism of folding and to obtain independent estimates of the conformational stability. The spectroscopic techniques and the wavelengths chosen were: optical rotation at 295 nm, circular dichroism at 238 and 284 nm, absorbance difference at 286 (thermal) or 287 (urea) and 292 nm, and intrinsic fluorescence at 320 nm after excitation at either 278 or 285 nm. The optical rotatory dispersion spectra of folded and unfolded RNase T1 and urea and thermal unfolding curves monitored using optical rotation measurements at 295 nm have been published previously (11, 12, 14). There is a 7-fold decrease in the optical rotation at 295 nm when the protein unfolds, making this a good wavelength for following unfolding. It is clear from the spectra that this change in the optical rotation depends mainly on the secondary structure of the protein rather than on changes in the contribution of the side chains of the aromatic residues. Circular dichroism spectra of folded and unfolded RNase T1 have been published previously (13, 14). The circular dichroism changes at 284 nm depend mainly on the environment of the side chains of the aromatic amino acids and, hence, on the tertiary structure, but the changes at 238 nm probably depend to a larger extent on the secondary structure of the protein (15).

The near UV absorption spectra of folded and unfolded (8 M urea) RNase T1 have been published previously (13, 14). The circular dichroism changes at 284 nm depend mainly on the environment of the side chains of the aromatic amino acids and, hence, on the tertiary structure, but the changes at 238 nm probably depend to a larger extent on the secondary structure of the protein (15).

The near UV absorption spectra of folded and unfolded (8 M urea) RNase T1 and the difference between them are shown in Fig. 1. RNase T1 contains a single Trp residue which is completely buried \(^1\) and 9 Tyr residues which are, on the average, 82% buried in the interior of the folded protein (1, 3). The exposure of these buried residues to solvent on unfolding is the major factor which contributes to the blue shift in the absorption spectrum that leads to the observed difference spectrum (16). The shoulder at 292 nm shows that the Trp side chain has changed its environment on unfolding, and the larger change at 287 nm shows that several of the Tyr residues have changed their environment on unfolding. Similar difference spectra result when RNase T1 is unfolded by heating, and Fig. 2 shows typical thermal unfolding curves monitored by following the absorbance differences measured at 286 and 292 nm.

The fluorescence emission spectra of folded and unfolded RNase T1 have been published previously (11, 13, 14). Fig. 3 shows typical urea unfolding curves followed using fluorescence measurements at 320 nm after excitation at either 278 or 295 nm. In folded RNase T1, several of the nine Tyr side chains are located near the single Trp residue (1); consequently, it is not surprising that Longworth (17) has suggested that about one-third of the energy absorbed by the Tyr residues is transferred to the Trp. The molar absorption coefficients of N-acetyltryptophan amide and N-acetyltirosine amide made in n-propyl alcohol are, respectively, 5005 and 1690 M\(^{-1}\) cm\(^{-1}\) at 278 nm and 2750 and 72 M\(^{-1}\) cm\(^{-1}\) at 295 nm.\(^2\) Assuming that the Trp and Tyr residues in folded and unfolded RNase T1 have these same absorption coefficients, 81% of the light at 295 nm, but only 27% of the light at 278 nm, is absorbed by the Trp residue. Thus, after 295 nm, the Trp fluorescence is monitored.

\(^{1}\) Although the accessibility program (3) classes the Trp residue as completely buried, the N of the indole side chain is hydrogen-bonded to a water molecule (1).

\(^{2}\) C. N. Pace, unpublished observations.
Conformational Stability of RNase T1

FIG. 2. Thermal unfolding curve for RNase T1 in 30 mM MOPS buffer, pH 7, monitored using the difference in absorbance at 286 nm (O) or 292 nm (C). The solid curves in the transition region were calculated using the parameters given in Table II. The lines shown in the pre- and post-transition region were used to determine the y0 and y∞ values used in Equations 2 and 3.

FIG. 3. Urea unfolding curves for RNase T1 at 25 °C, pH 7, in 30 mM MOPS buffer monitored by measuring the fluorescence intensity at 320 nm after excitation at 278 nm (C) or 295 nm (C). After 278 nm excitation, the decrease in the fluorescence intensity is due mainly to the change in environment of the Trp residue. After 278 nm excitation, the other hand, the transfer of energy from the Tyr residues to the Trp will make a considerably larger contribution to the fluorescence intensity of the folded and unfolded molecules. Consequently, measuring emission at 320 nm after excitation at 278 and 295 nm, which is very easy to do experimentally, provides two probes for monitoring unfolding which should respond differently to the presence of partially folded intermediate states. In retrospect, 298 nm might be a better wavelength to use than 295 nm since the absorption of Tyr is essentially zero at this wavelength in propanol. Both the changes in the absorption spectra (Figs. 1 and 2) and intrinsic fluorescence (Fig. 3) depend mainly on changes in the tertiary structure of RNase T1 on unfolding.

One of the most commonly used methods for estimating the conformational stability of proteins is the analysis of urea unfolding profiles such as those shown in Fig. 3 (10). By assuming a two-state mechanism for unfolding, the fraction of unfolded protein, Fu, can be calculated using the equation

\[ F_u = \frac{(y - y_{obs})}{(y_f - y_u)} \]  

where \( y_{obs} \) is the observed variable parameter, e.g. intrinsic fluorescence in Fig. 3 and \( y_f \) and \( y_u \) are the values of the variable parameter characteristic of the folded and unfolded conformations. The values of \( y_f \) and \( y_u \) in the transition region are obtained by extrapolation of the linear portions of the pre- and post-transition regions of the unfolding curve, respectively. By monitoring unfolding using different techniques, inferences can be made regarding the validity of the two-state assumption. Fig. 4 shows \( F_u \) values as a function of urea concentration using data obtained from four different spectroscopic techniques, two that reflect mainly changes in tertiary structure and two that reflect mainly changes in secondary structure. The coincidence of the plot is consistent with a two-state folding mechanism (18). Thermal unfolding was followed with all of the same techniques except fluorescence, and the plots of \( F_u \) as a function of temperature were also coincident within experimental error.

The difference in free energy between the folded and unfolded conformations, \( \Delta G \), can be calculated using the equation

\[ \Delta G = -RT \ln \left(\frac{F_u}{1 - F_u}\right) = -RT \ln \left(\frac{y - y_{obs}}{(y_f - y_u)}\right) \]  

where \( y_{obs} \) is the observed variable parameter, e.g. intrinsic fluorescence in Fig. 3 and \( y_f \) and \( y_u \) are the values of the variable parameter characteristic of the folded and unfolded conformations. The values of \( y_f \) and \( y_u \) in the transition region are obtained by extrapolation of the linear portions of the pre- and post-transition regions of the unfolding curve, respectively. By monitoring unfolding using different techniques, inferences can be made regarding the validity of the two-state assumption. Fig. 4 shows \( F_u \) values as a function of urea concentration using data obtained from four different spectroscopic techniques, two that reflect mainly changes in tertiary structure and two that reflect mainly changes in secondary structure. The coincidence of the plot is consistent with a two-state folding mechanism (18). Thermal unfolding was followed with all of the same techniques except fluorescence, and the plots of \( F_u \) as a function of temperature were also coincident within experimental error.
where \( R \) is the gas constant and \( T \) is the absolute temperature. For all of the results reported here, \( \Delta G \) was found to vary linearly with urea concentration (see Fig. 5, below). A least squares analysis was used to fit the data to the equation

\[
\Delta G = \Delta G(H_2O) - m(\text{urea})
\]

where \( \Delta G(H_2O) \) is the value of \( \Delta G \) in the absence of urea, and \( m \) is a measure of the dependence of \( \Delta G \) on urea concentration (10). Treatment of the data in this manner is supported by both experimental (19-22) and theoretical (23, 24) studies. For comparative purposes, it was necessary to have all the \( \Delta G(H_2O) \) values determined at the same pH. Since the pH of individual experiments differed slightly, \( [\text{urea}]_{1/2} \) values were corrected to a common pH. We have determined that for RNase T1 at 25°C near pH 7, \( \Delta G(\text{urea})_{1/2}/\Delta \text{pH} \) = 1.78 ± 0.02 M/pH unit.

Table I summarizes the parameters characterizing the urea unfolding of RNase T1. The values given for \( I_f(278 \text{ nm}) \) are the average of seven independent experiments by five different graduate students. The \( m \) values ranged from 1180 to 1250 cal/mol/M with an average deviation of 17, and the \( [\text{urea}]_{1/2} \) values ranged from 4.38 to 4.66 M with an average deviation of 0.06 M. (For six of these experiments, the \( [\text{urea}]_{1/2} \) values fell between 4.51 and 4.66 M urea with an average deviation of 0.04 M.) \( \Delta G \) values from several of the experiments summarized in Table I are shown plotted as a function of urea molarity in Fig. 5. The solid line was calculated with Equation 4 using the average parameters given in Table I.

Another commonly used method of estimating the conformational stability of proteins is an analysis of thermal unfolding curves such as those shown in Fig. 2 (11). As for urea unfolding, a two-state mechanism was assumed in order to calculate \( \Delta G \) as a function of temperature. A least squares analysis of plots of \( \Delta G \) versus \( T \) yields the midpoint of the thermal unfolding curve, \( T_m = T \). The enthalpy change at \( T_m, \Delta H_m, \) and the difference in heat capacity between folded and unfolded RNase T1, the value of \( \Delta G \) at any temperature \( T, \Delta G(T) \), can be calculated using (22, 26):

\[
\Delta G(T) = \Delta H(1 - T/T_m) - \Delta C_p(T_m - T) - T\ln(T/T_m)
\]

A value of 1650 cal/mol deg was used for \( \Delta C_p \) (22). Parameters characterizing the thermal unfolding of RNase T1 are given in Table II. \( \Delta G \) values from individual experiments listed in Table II are plotted as a function of temperature in Fig. 6.

![Urea Molarity](image)

**Urea Molarity**

**Fig. 5.** \( \Delta G \) as a function of urea molarity. The \( \Delta G \) values were calculated with Equation 3 using data obtained with the techniques described in Table I to follow unfolding: \( I_f = \text{F}, \Delta A = \text{C}, \theta = \text{D} \), and \( \alpha = \text{A} \). The solid line represents Equation 3 with the average parameters given in Table I.

**TABLE II**

Parameters characterizing the thermal unfolding of RNase T1 at pH 7 in 30 mM MOPS buffer

| Technique* | \( \Delta H_m \) | \( T_m \) | \( \Delta G(25°C) \) |
|------------|----------------|----------|------------------|
| \( \Delta \text{A} \) (386 nm) | 98.5 | 4.8 | 5.5 |
| \( \Delta \text{A} \) (292 nm) | 96.9 | 4.8 | 5.5 |
| \( \theta \) (238 nm) | 97.1 | 4.7 | 5.5 |
| \( \theta \) (284 nm) | 96.3 | 4.3 | 5.5 |
| \( \alpha \) (295 nm) | 96.8 ± 0.2 | 4.8 ± 0.4 | 5.5 |
| Average | 97.1 ± 0.6 | 4.8 ± 0.3 | 5.6 ± 0.1 |

* \( \Delta \text{A} \) = difference spectroscopy, \( \theta \) = circular dichroism, and \( \alpha \) = optical rotation, as described in more detail in the text.

**TABLE I**

Parameters characterizing the urea unfolding of RNase T1 at 25°C, pH 7, in 30 mM MOPS buffer

| Technique* | \( m \) | \( [\text{urea}]_{1/2} \) | \( \Delta G(H_2O) \) |
|------------|------|----------------|------------------|
| \( I_f \) (278 nm) | 1220 ± 17 | 4.54 ± 0.06 | 5.5 |
| \( I_f \) (295 nm) | 1200 | 4.63 | 5.6 |
| \( \Delta \text{A} \) (287 nm) | 1250 | 4.63 | 5.8 |
| \( \Delta \text{A} \) (292 nm) | 1230 | 4.71 | 5.8 |
| \( \theta \) (238 nm) | 1165 | 4.60 | 5.4 |
| \( \theta \) (284 nm) | 1134 | 4.71 | 5.4 |
| \( \alpha \) (295 nm) | 1230 | 4.67 | 5.7 |
| Average | 1205 ± 30 | 4.64 ± 0.05 | 5.6 ± 0.15 |

* \( I_f \) = intrinsic fluorescence, \( \Delta \text{A} \) = difference spectroscopy, \( \theta \) = circular dichroism, and \( \alpha \) = optical rotation, as described in more detail in the text.

These values are the average of seven independent experiments by five different graduate students.

The kinetics of folding of RNase T1 have been studied in most detail at pH 5, the pH of maximum stability, and these results are shown in Fig. 7. Unfolding occurs as a single kinetic phase that accounts for the entire change in fluorescence intensity. The relaxation time, \( \tau \), for this phase depends on urea concentration with \( \tau \) values ranging from over 2500 s at 6.46 M urea to less than 200 s at high urea concentrations, as shown in Fig. 7. The folding of urea-denatured RNase T1 also shows a single kinetic phase at urea concentrations in the transition region (>3 M urea). However, two kinetic phases are observed at urea concentrations in the pre-transition region (<3 M urea). These two phases have almost identical amplitudes below 2 M urea and account for about 90% of the observed change in fluorescence intensity.

* Results qualitatively similar to those shown in Fig. 7 were observed at 25°C, pH 7 (J. Thomson, and N. Pace, unpublished observations), at 10°C, pH 8 (F. Schmid, personal communication, and (39)), and at 11°C, pH 6 (26).
Discussion

There is considerable interest in measuring the effect of small changes in amino acid sequence on the conformational stability of proteins (33, 34). This is best done through an analysis of urea (or GdnHCl) or thermal unfolding curves such as those shown in Figs. 2 and 3. This approach is generally more sensitive than attempting to measure differences in the conformational stability with a calorimeter. This approach works best when folding closely approaches a two-state mechanism, i.e., only the folded and unfolded states are present at significant concentrations at equilibrium. This simplifies the analysis of the data and leads to more clearcut interpretations of the results. In 1969, Brandts (18) stated: “The question of whether or not a protein undergoes an unfolding transition amenable to two-state theory can and should be answered from experimental data, rather than from theory, and should be answered specifically for each protein.” To date, all of the experimental data are consistent with a two-state folding mechanism for RNase T1.

First, both the urea and thermal unfolding curves show steep, single steps (Figs. 2 and 3). The unfolding curves of proteins with stable intermediates, e.g., tryptophan synthase (α-subunit) (35) and immunoglobulin light chain (36), sometimes show more than a single step in the unfolding profile. Second, the plots of F, versus denaturant are coincident no matter which physical technique is used to follow unfolding for both urea and thermal unfolding. This is sometimes referred to as the multiple variable test (18). The presence of stable intermediates may lead to noncoincidence in these plots, as observed with carbonic anhydrase (37) and α-lactalbumin (38). Third, there is no evidence from kinetic studies (30) to characterize the dependence of the rate constants on denaturant concentration. The free energy of activation, ΔG*, was calculated from k_f and k_u, using

$$\Delta G^* = -RT \ln(k_h/k_R T)$$  \hspace{2cm} (6)
of the presence of stable intermediates in the folding of RNase T1. There are two slow phases observed in the folding of RNase T1 (Fig. 7). However, proline residues 39 and 55 are known to be in the cis configuration in folded RNase T1 (1), and it has been shown that the rate of both of these slow phases is catalyzed, albeit at substantially different rates, by peptidylprolyl cis-trans isomerase (25, 39). Thus, the slow phases may result simply from the existence of unfolded states which differ from folded RNase T1 in the cis-trans isomerization state of 2 of the prolyl residues. This should have no significant effect on our analysis of the conformational stability of RNase T1. Another good method for showing that folding approaches a two-state mechanism is to show that ΔH (calorimetric) = ΔH (van't Hoff). Calorimetric studies of the unfolding of RNase T1 are in progress.

Another problem which plagues folding studies is aggregation of the folded (40), unfolded (41), or partially folded states (42). We have shown that the elution volume of folded RNase T1 from a Sephadex G-50 column does not vary over a concentration range from 0.06 to 4.6 mg/ml in the buffer used for the experiments reported here. This indicates that the folded protein does not self-associate to a significant degree. Both urea and GdnHCl unfolding are completely reversible: RNase T1 left unfolded in 6 M GdnHCl for over 3 months will refold completely. Thermal unfolding is almost completely reversible if RNase T1 is exposed to unfolding temperature only long enough to allow complete unfolding. However, thermal unfolding becomes increasingly irreversible the longer RNase T1 is exposed to unfolding temperatures. After a thermal unfolding experiment with 10 measurements in the transition and post-transition regions and a maximum temperature of 71 °C, the reversibility was 95%. In an experiment with 22 measurements in the transition and post-transition regions and a maximum temperature of 89 °C, the reversibility was 78%. The observed irreversibility may result from the aggregation of unfolded RNase T1 molecules or other processes (41). The behavior is similar to that observed with other small globular proteins (43).

Three methods have been used for extrapolating data such as those shown in Fig. 5 in order to obtain ΔG(H2O), an estimate of the conformational stability (10). In Table 1, we have given only the ΔG(H2O) value obtained with the linear extrapolation model. The denaturant binding model (44) and Tanford's model (45) lead to estimates of ΔG(H2O) which are about 1 kcal/mol higher than the 5.6 kcal/mol obtained by linear extrapolation. Schellman (23, 46) has suggested that the linear dependence of ΔG on denaturant may be expected on theoretical grounds. Santoro and Bolen (21) have given only the ΔG(H2O) value obtained with the linear extrapolation model.

The agreement between our estimates of the conformational stability from urea and thermal unfolding experiments is consistent with this idea.

The solid line in Fig. 6 shows that dependence of ΔG on temperature calculated using Equation 5 and the parameters given in Table II. Note that the experimental results from the thermal and urea unfolding experiments are in good agreement with the calculated values. Becktel and Schellman (25) have referred to these plots as protein stability curves and have shown that the temperature of maximum stability, Tm, can be calculated with

\[ T_m = T_c \exp(-\Delta H_m/(R \Delta C_p)) \]

using the data from Table II leads to \( T_m = -6 \) °C for the unfolding of RNase T1. For the proteins investigated to date, \( T_m \) falls between -6 and 35 °C (18, 25, 47-56). So, RNase T1 joins RNase A (47, 54) and hen lysozyme (47), as proteins whose maximum stability occurs below freezing.

As noted above, the conformational stability of RNase T1 is maximal near pH 5 where ΔG(H2O) = 8.9 kcal/mol (Table III). ΔG(H2O) can be lowered by about 4.3 kcal/mol at either higher or lower pH by 3 pH units. Oobatake et al. (57) have shown previously that \( T_m \) is also maximal at pH 5. Note in Fig. 6 that the conformational stability of RNase T1 varies only over a small range at pH 5, but becomes much less stable in the other pH surroundings. The conformational stability of RNase T1 can be varied over a wide range using just pH and temperature. It must be kept in mind that the reaction under study could change with these variables, i.e. the folded conformation may undergo a conformational change between two different folded conformations as a function of pH (see, for example, Ref. 58) or the composition of the unfolded state may change as a function of pH or temperature (59). One project underway in our laboratory at present is a detailed study of the unfolded conformations of RNase T1.

We now have a reasonably good understanding of the conformational stability of RNase T1 and of how the stability depends on pH, temperature, salt concentration (7), and on the number of intact disulfide bonds (4). In the following paper (6), we report studies of the conformational stability of three mutants of RNase T1.

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