

Conformational stability of globular proteins

RIBONUCLEASE T₁ (RNase T₁) has proven to be an excellent model for investigating the conformational stability of globular proteins. (See also *TIBS* 14, 450–454, for a recent review on barnase, a close relative of RNase T₁.) The three-dimensional structure of RNase T₁ has been determined at high resolution¹ 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 α -helix, and an extended antiparallel β -sheet (Fig. 1). The folded conformation is stabilized in part by two disulfide bonds, by 87 intramolecular hydrogen bonds, and by hydrophobic interactions in which 85% of the nonpolar residues are removed from contact with water. Thus, RNase T₁ provides a good model for studies of the folded conformation. When the disulfide bonds are broken, RNase T₁ is unfolded under physiological conditions, also providing a good model for studies of the unfolded conformations². Recent studies of staphylococcal nuclease suggest that the unfolded conformations of a protein make more important contributions to the net conformational stability than previously thought³. Here we summarize our studies of the conformational stability of RNase T₁. They show that the conformational stability of this small globular protein can be varied experimentally over a range of almost 20 kcal mol⁻¹ (84 kJ mol⁻¹).

The conformational stability of globular proteins is remarkably low

We define the conformational stability of a globular protein as the difference in free energy between the folded and unfolded conformations under physiological conditions, and denote this as $\Delta G(H_2O)$ (Fig. 1). Estimates of the conformational stability of RNase T₁ and several other proteins that are under active experimental study at present are given in Table I. The conformational stability of almost all naturally occurring globular proteins is between 5 and 15 kcal mol⁻¹ (21–63 kJ mol⁻¹). It appears to be advantageous to living organisms to have proteins for which the

folded, biologically active conformation is only marginally more stable than unfolded, inactive conformations. The reasons for this are still not clear, but it may have to do with the protein's turnover¹⁵ or function¹⁶. Since a protein based on a four-helix bundle structure has been constructed with a conformational stability of 22.5 kcal mol⁻¹ (94.5 kJ mol⁻¹)¹⁷, it seems likely that evolution could have generated more stable proteins if they were needed.

Globular proteins can be unfolded by heating or cooling

At pH 7, RNase T₁ is most stable near -5°C and the stability decreases both at higher and at lower temperatures (Fig. 2). Becktel and Schellman¹⁶ refer to plots of ΔG vs temperature as protein stability curves and have discussed them in detail. This interesting temperature dependence is described by a form of the Gibbs-Helmholtz equation (Eqn 1):

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

where $\Delta G(T)$ is ΔG at temperature T , T_m is the midpoint of the thermal unfolding curve, ΔH_m is the enthalpy change for unfolding measured at T_m , and ΔC_p is the difference in heat capacity between the unfolded and folded conformations. The temperature of maximum stability (T_s) occurs at the temperature where $\Delta S = 0$, and is given by

$$T_s = T_m \exp(-\Delta H_m / [T_m \Delta C_p]).$$

T_s is between -10°C and 35°C for the proteins that have been studied to date. T4 lysozyme¹³ ($T_s = 12.5^\circ\text{C}$, and $T_m = -3$ and 28°C), and staph nuclease¹⁸ ($T_s = 18^\circ\text{C}$, and $T_m = -19$ and 57°C) provide interesting

examples where T_s occurs above 0°C so that both low and high temperature unfolding can be observed directly.

When a protein unfolds, the buried nonpolar side chains are brought into contact with water. In order to accommodate these side chains, they are surrounded by cages of water molecules so that the extent of hydrogen bonding is increased. The heat capacity of the unfolded protein is greater than that of the folded protein because now these cages must be melted in order to raise the temperature of the solution. Thus, the same effect that gives rise to hydrophobic interactions also gives rise to the larger ΔC_p that dominates the thermodynamics of protein folding. (See Baldwin¹⁹, and Privalov and Gill²⁰ for interesting discussions.)

Globular proteins are most stable near their isoelectric points

At 25°C, RNase T₁ is most stable at pH 4.5, and this is near the pH where the net charge is zero (Fig. 3). At zero net charge, RNase T₁ will have six positive charges (3 His, 1 Lys, 1 Arg and 1 α -amino) and a total of six negative charges from the 13 carboxyl groups. The charges on the surface of globular proteins are generally arranged so that there are more favorable than unfavorable electrostatic interactions among the charged groups; thus they should contribute favorably to the conformational stability²¹. When the protein unfolds, the charges will be further apart and the effective dielectric constant increased; consequently, electrostatic interactions will be substantially reduced, especially at moderate salt concentrations. Note that RNase T₁ is only about 6 kcal mol⁻¹ (25.2 kJ mol⁻¹) less stable at pH 10 where the net charge on the molecule is

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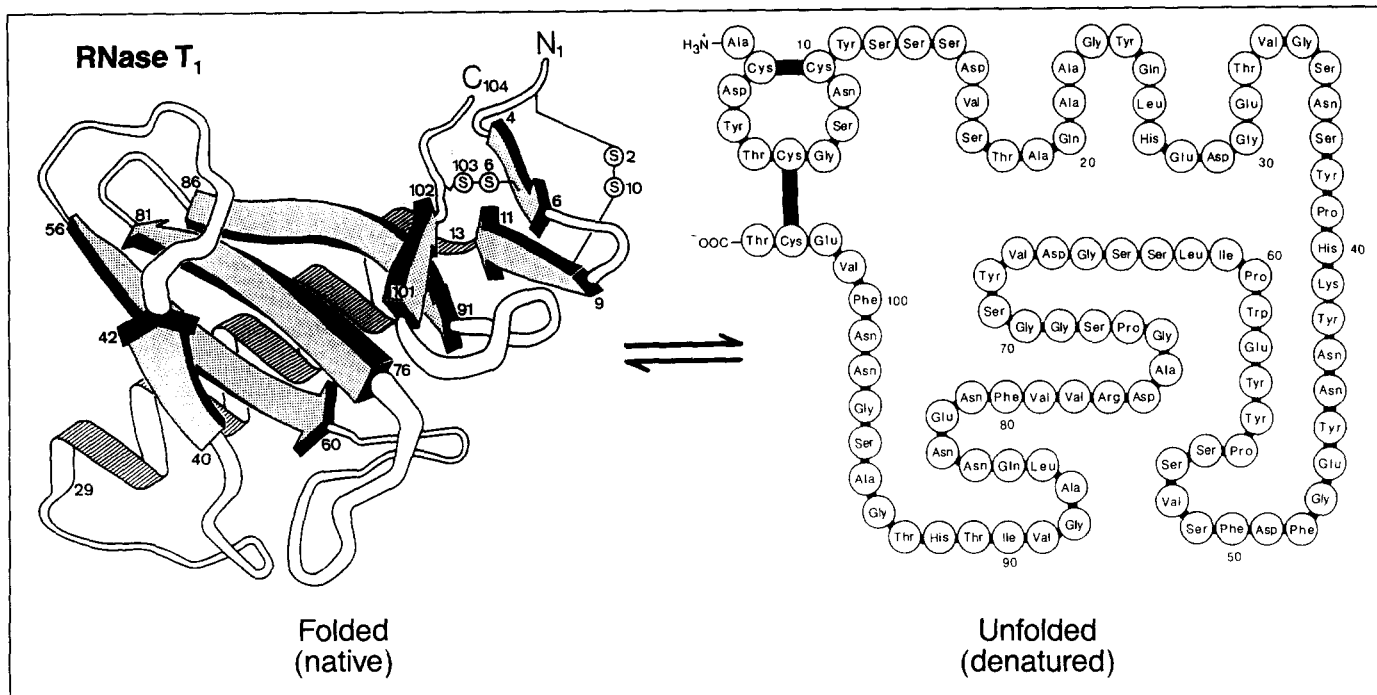


Figure 1

ΔG for this reaction under physiological conditions is the conformational stability, i.e. $\Delta G(\text{H}_2\text{O})$. The three-dimensional structure of RNase T_1 was determined in Saenger's laboratory¹ and is reproduced here with permission.

about -12 , and repulsive electrostatic interactions among the charges will predominate. This suggests that electrostatic interactions among the charges on the surface of a globular protein make only a small contribution to the conformational stability, probably much smaller than the contributions from hydrophobic interactions and hydrogen bonding.

Another important contribution to the pH dependence of $\Delta G(\text{H}_2\text{O})$ results from differences between the pK s of groups in the folded and unfolded conformations. The 6 kcal mol^{-1} (25.2 kJ mol^{-1}) increase in $\Delta G(\text{H}_2\text{O})$ between pH 10 and 5 results in part from the presence of groups that bind protons more tightly (higher pK s) in the folded than in the unfolded conformations, and thereby shift the conformational equilibrium toward the folded conformation. NMR titrations have shown that the pK s of the histidine residues in folded RNase T_1 are 7.9 (His92), 7.8 (His40), and 7.2 (His27)²², and a pK of about 6.4 would be expected for the histidine residues in the unfolded conformations. This alone would account for a change of over 3 kcal mol^{-1} (12.6 kJ mol^{-1}) in $\Delta G(\text{H}_2\text{O})$ with pH⁵. Higher pK s are expected when the net charge on the molecule is negative, and lower pK s are expected when the net charge is positive. Thus, the entire decrease in $\Delta G(\text{H}_2\text{O})$ at low pH can be explained by the presence of three aspartic acid residues with pK s of 2.7 in the folded

and 4.1 in the unfolded conformations. So, both pK differences and electrostatic interactions among the charged groups will tend to favor the maximum conformational stability occurring near the isoelectric pH, as is generally observed.

Proteins are stabilized by compounds that bind specifically to the folded conformation

The conformational stability of RNase T_1 can be almost doubled by adding $0.2 \text{ M Na}_2\text{HPO}_4$ (Table I). This remarkable increase in $\Delta G(\text{H}_2\text{O})$ also occurs in the presence of other salts, and has been shown to result primarily from the prefer-

ential binding to the folded protein of one divalent or two monovalent cations at a cation binding site and the binding of one HPO_4^{2-} ion at an anion binding site (the active site)⁷. The structure of both of these sites is now known through X-ray diffraction studies¹. The creation of specific cation and anion binding sites on the surface of a protein through genetic engineering might be a generally useful way of increasing the conformational stability. An obvious advantage of using ion binding for stabilization is that it need not interfere with the functioning of the active site of an enzyme. The use of specific binding to

Table I. Conformational stability [$\Delta G(\text{H}_2\text{O})$] of RNase T_1 and six other proteins^a

Protein	$\Delta G(\text{H}_2\text{O})$		Ref.
	(kcal mol^{-1})	(kJ mol^{-1})	
RNase T_1 ^b			
pH 7.0, 25°C	5.6	23.52	4
+0.2 M Na_2HPO_4	10.0	42.00	7
pH 7.0, -6°C	8.3	34.86	6
pH 4.5, 25°C	8.9	37.38	5
Gln25→Lys	6.5	27.30	8
Glu 58→Ala	4.8	20.16	8
BPTI ^c	14.3	60.06	9
Barnase	9.4	39.48	10
RNase A	9.0	37.80	5
Nuclease	6.1	25.62	11
DHFR	5.9	24.78	12
T4 Lysozyme	12.8	53.76	13

^a Methods used to measure conformational stability are described in Ref. 14.

^b For wild-type RNase T_1 , and for the two mutants, 30 mM MOPS buffer (pH 7.0, 25°C) was used.

^c Abbreviations: BPTI, bovine pancreas trypsin inhibitor; DHFR, dihydrofolate reductase; MOPS, 3-(*N*-morpholino) propanesulfonic acid.

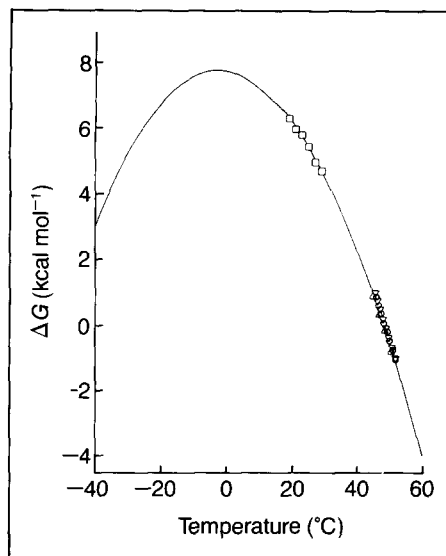


Figure 2

ΔG as a function of temperature for the unfolding of RNase T₁ at pH 7. The data points below 30°C are from urea unfolding curves, and those above 40°C are from thermal unfolding curves. The solid line was calculated with Eqn 1 using the thermodynamic parameters given here; $T_m = 48.25^\circ\text{C}$, $\Delta H_m = 95.2 \text{ kcal mol}^{-1}$ (399.84 kJ mol⁻¹), $\Delta C_p = 1.72 \text{ kcal mol}^{-1} \text{ deg}^{-1}$ (7.22 kJ mol⁻¹ deg⁻¹). Adapted from Ref. 6.

stabilize a protein is not a new idea. One hundred years ago in a remarkable 98 page paper, O'Sullivan and Tompson reported²³: '... we have shown that invertase when in the presence of cane sugar will stand without injury a temperature fully 25°C higher than in its absence. This is a striking fact, and, as far as we can see, there is only one explanation of it, namely, the invertase enters into combination with the sugar'.

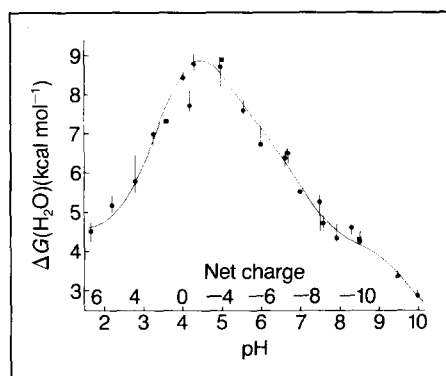


Figure 3

$\Delta G(\text{H}_2\text{O})$ as a function of pH for the unfolding RNase T₁ at 25°C. The approximate net charge given is based on the titration curve. The solid curve was calculated assuming that certain groups have different pKs in the folded and unfolded conformations as described in Ref. 5. Reproduced, with permission, from Ref. 5.

Disulfide bonds stabilize proteins by raising the free energy of the unfolded state

In Table I, the three proteins that contain disulfide bonds unfold when the disulfide bonds are broken. When the temperature is lowered, however, RNase T₁ will refold and retains its enzyme activity even when the SH groups are masked with bulky blocking groups (Fig. 4 and Table II). This is surprising because one disulfide bond is completely buried and only about 12 Å from the active site. It will be interesting to see how the carboxymethyl groups are accommodated in the folded protein without disrupting the active site. Note that breaking the 2 to 10 disulfide bond lowers the conformational stability by over 3 kcal mol⁻¹ (12.6 kJ mol⁻¹), and breaking both disulfide bonds lowers the stability by from 7 to 9 kcal mol⁻¹ (29.4–37.8 kJ mol⁻¹) depending on the blocking group. Since the melting temperature is below 25°C for both RCM- and RCAM-RNase T₁, the unfolded states existing under physiological conditions can be compared with the unfolded

equation predicts that the conformational stability of a protein will be raised by 3, 4 and 5 kcal mol⁻¹ (12.6, 16.8 and 21 kJ mol⁻¹) by adding loops of 15, 45 and 135 residues, respectively. Thornton²⁴ has shown that 49% of the disulfide bonds in globular proteins are separated by less than 24 residues and that 15 residues is the average separation. However, it has proven difficult to stabilize subtilisin BPN' by introducing disulfide bonds²⁵. Seven different disulfide bonds have been tried and none significantly enhances the stability to irreversible inactivation. In part, this indicates that it is difficult to add a disulfide bond that does not increase the free energy of the folded state due to strain or other factors.

Proteins can be stabilized or destabilized by changes in amino acid sequence

The new found ability to construct proteins to order has generated great interest in many areas of biochemistry²⁶. In the protein folding field, these new approaches

Table II. Activity, melting temperature and conformational stability of RNase T₁ and derivatives (see Fig. 4) with one or both disulfide bonds broken^a

Protein	Activity ^b (%)	T_m^c (°C)	$\Delta G(\text{H}_2\text{O})^d$		$\Delta(\Delta G)$	
			(kcal mol ⁻¹)	(kJ mol ⁻¹)	(kcal mol ⁻¹)	(kJ mol ⁻¹)
RNase T ₁	100	59.3	10.2	42.84	–	–
(2–10)-RCM-T ₁	86	53.3	6.8	28.56	3.4	14.28
R-T ₁	53	26.7	3.0	12.60	7.2	30.24
RCAM-T ₁	43	21.2	1.5	6.30	8.7	36.54
RCM-T ₁	35	16.6	0.9	3.78	9.3	39.06

^a 0.1 M sodium formate, pH 5.0, 0.25 M NaCl.

^b RNA hydrolysis at 5°C.

^c Midpoint of thermal denaturation curve.

^d Conformational stability at 12.5°C.

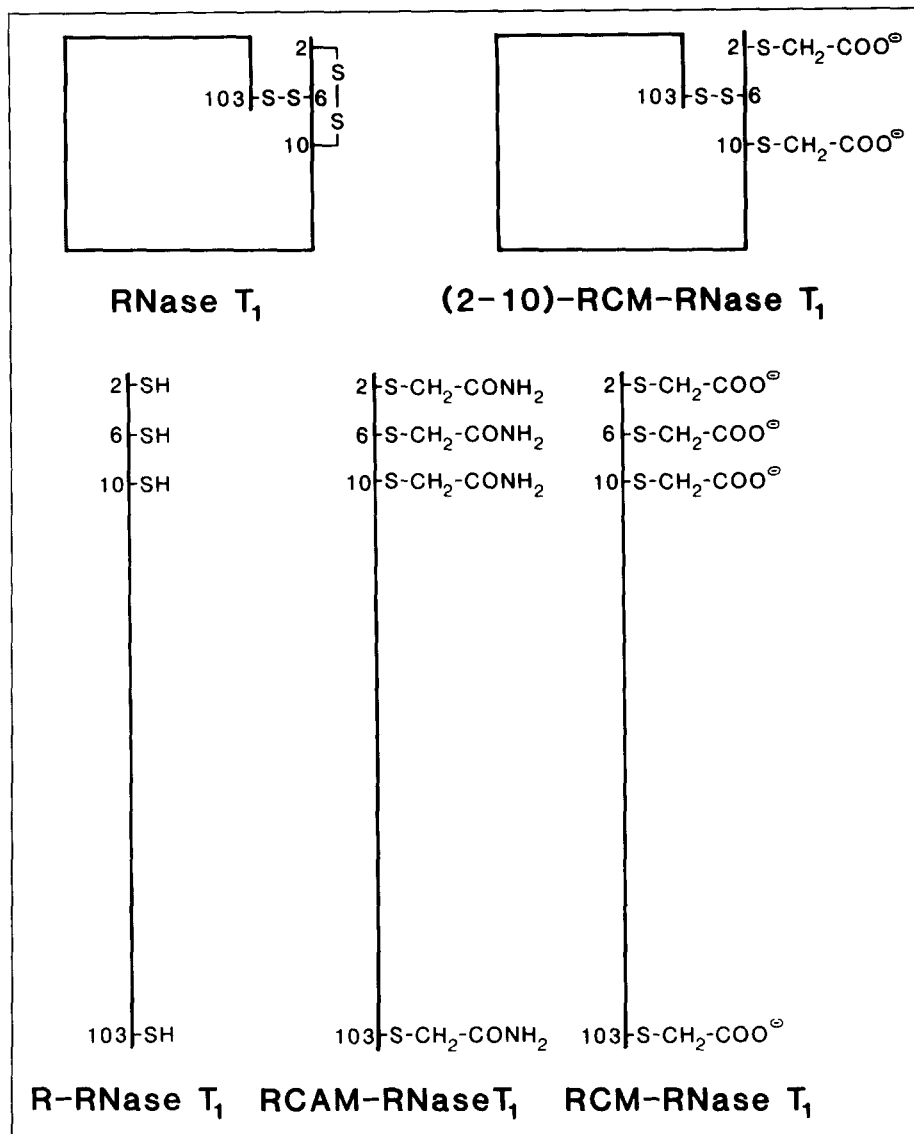
states at higher temperatures and in the presence of urea and GdnHCl. These studies are under way at present, and should improve our understanding of the unfolded states of proteins in the absence of denaturing conditions.

Disulfide bonds increase the conformational stability mainly by constraining the unfolded conformations of the protein and thereby decreasing their conformational entropy. Based on our results and other experimental studies in the literature, we have proposed a new equation for predicting the effect of a crosslink on the conformational entropy (ΔS_{conf}) of a protein:

$$\Delta S_{\text{conf}} = -2.1 - (3/2)R \ln n \quad (2)$$

where n is the number of residues in the loop forming the disulfide bond². This

should allow us to gain a better understanding of the forces that stabilize globular proteins²⁷. On the basis of studies of barnase mutants created by site-directed mutagenesis, for example, Kellis *et al.*¹⁰ have suggested that the contribution of hydrophobic interactions to protein stability '... exceeds by severalfold the values obtained from model experiments...' and studies with λ repressor by Lim and Sauer²⁸ have given us a much better understanding of the 'packing rules' for a hydrophobic core. Results with a mutant of RNase T₁ that increases the conformational stability by about 0.9 kcal mol⁻¹ (3.78 kJ mol⁻¹), and one that decreases the stability by about 0.8 kcal mol⁻¹ (3.36 kJ mol⁻¹) are shown in Table I. Much larger changes in the conformational stability are possible. In the Gln16 → Leu mutant of λ repressor, T_m is increased by 14°C, and a



Schematic representation of RNase T₁ and the derivatives with one or both disulfide bonds broken that are characterized in Table II.

$\Delta G(\text{H}_2\text{O})$ by 2.8 kcal mol⁻¹ (11.76 kJ mol⁻¹)²⁹. To date, the largest increase in conformational stability resulting from a single change in amino acid sequence is the Asn57 → Ile mutant of yeast iso-1-cytochrome *c* where T_m is increased by 17°C, and $\Delta G(\text{H}_2\text{O})$ by ≈4.2 kcal mol⁻¹ (17.64 kJ mol⁻¹)³⁰. So far, nature and random mutagenesis have proven much better at finding stable mutants than biochemists working from the three-dimensional structures of folded proteins,

and, despite considerable progress, this is likely to remain the case for some time to come. This is surely an exciting time to be studying proteins, and especially the reaction that is the 'secret of life', protein folding.

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