Increasing protein stability: Importance of ΔC_p and the denatured state

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Abstract: Increasing the conformational stability of proteins is an important goal for both basic research and industrial applications. *In vitro* selection has been used successfully to increase protein stability, but more often site-directed mutagenesis is used to optimize the various forces that contribute to protein stability. In previous studies, we showed that improving electrostatic interactions on the protein surface and improving the β -turn sequences were good general strategies for increasing protein stability, and used them to increase the stability of RNase Sa. By incorporating seven of these mutations in RNase Sa, we increased the stability by 5.3 kcal/mol. Adding one more mutation, D79F, gave a total increase in stability of 7.7 kcal/mol, and a melting temperature 28°C higher than the wild-type enzyme. Surprisingly, the D79F mutation lowers the change in heat capacity for folding, ΔC_p , by 0.6 kcal/mol/K. This suggests that this mutation stabilizes structure in the denatured state ensemble. We made other mutants that give some insight into the structure present in the denatured state. Finally, the thermodynamics of folding of these stabilized variants of RNase Sa are compared with those observed for proteins from thermophiles.

Keywords: protein stability; ΔC_p ; β -turns; denatured state ensemble; thermophiles; urea denaturation; thermal denaturation

Introduction

Organisms can be classified as mesophiles, thermophiles, or hyperthermophiles based on their optimal growth temperatures.^{1,2} Proteins from these organisms are of great interest because they provide insight into the general mechanisms used to stabilize proteins. Comparative studies show that thermophilic proteins and their mesophilic homologues often adopt similar three-dimensional structures,³ but have different thermodynamic properties. A number of factors have been identified that contribute to the increased stability of thermophilic proteins, but it

has been difficult to identify general principles because there are often many differences in the primary structure.^{4–6} It does appear that salt bridges or networks of salt bridges are present in many of the proteins from thermophiles,^{2,6–8} including the most stable protein observed.⁹ One objective of this article is to compare the thermodynamics of folding of a mesophilic protein stabilized by a series of mutations with those of proteins from thermophiles.

RNase Sa is one of the smallest enzymes with only 96 amino acid residues. The stability and thermodynamics of folding of RNase Sa¹⁰ and many variants have been studied.^{11–15} A number of mutations were found to increase the stability of RNase Sa. These can be categorized into three groups: (1) those that improve charge–charge interactions (D25K, E74K)¹¹; (2) those that improve entropic contributions to stability by modifying β -turns (S31P, S42G, S48P, T76P, and Q77G)¹⁴; and (3) a mutation that removes a buried charged side chain (D79F).¹³

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Figure 1. Ribbon diagram of the RNase Sa structure showing the mutated residues. The figure was generated using the YASARA program²⁰ and the 1.2 Å crystal structure of RNase Sa (PDB code: 1RGG).²¹ The colored labels indicate the type of mutation: red, surface charge; blue, β -turn; magenta, buried charge. An [interactive view](#) is available in the electronic version of the article.

Except for the last case, all the side chains mutated are >80% solvent exposed. The combination of such stabilizing mutations is often additive^{14,16,17}; therefore, we combined these mutations to design two RNase Sa variants one with only the seven surface mutations and one with all eight mutations. We will refer to the RNase Sa variant with seven mutations as 7S and the variant with eight mutations as 8S. The thermodynamic parameters of these proteins were measured to determine the most important factors contributing to the increased stability. The change in heat capacity, ΔC_p , was considerably smaller for 8S than 7S and this makes an important contribution to the difference in stability. This mechanism of stabilization was previously shown to be important in studies of the RNases H from a mesophile and a thermophile by the Marqusee laboratory.^{18,19} They showed convincingly that ΔC_p is lower for RNase H from the thermophile than from the mesophile because more structure is present in the denatured state ensemble (DSE). Similarly, in RNase Sa a single mutation, D79F, is capable of creating a “thermophilic” variant of RNase Sa with a lower ΔC_p and more structure in the DSE.

Results

Design of mutations

Previous studies identified eight mutations that increased the stability of RNase Sa.^{11,13,14} In a variant containing all of these mutations, and assuming that the contributions to stability are completely additive, we would expect an increase in T_m of 33°C, and an increase in conformational stability of 9.5 kcal/mol. Consequently, we added these eight muta-

tions to RNase Sa in three steps. The variants prepared were designated 2S (D25K and E74K), 7S (2S + S31P, S42G, S48P, T76P, and Q77G), and 8S (7S + D79F). The seven mutations in 7S are on the surface, but in the D79F mutation in 8S, the carboxyl group is 85% buried (Fig. 1).

Structure characterization of variants

Circular dichroism (CD) spectroscopy was used to monitor changes in the structure induced by the mutations. The CD spectra of all the variants were very similar to that of wild-type RNase Sa (data not shown), suggesting that the overall structure is not changed by the mutations.

Thermal denaturation

The thermal unfolding of RNase Sa and its variants was followed by measuring the CD at 234 nm. The denaturation curves were analyzed to determine the midpoint of the denaturation curves, T_m , and the enthalpy change at T_m , ΔH_m , as previously described.²² The denaturation curves in Figure 2 show that all three variants unfold at higher temperatures than wild-type RNase Sa, indicating an increase in stability. The thermodynamic parameters for wild-type RNase Sa and the three variants are given in Table I. The values of T_m and ΔH_m for wild-type RNase Sa given here are in good agreement with previously reported values.^{10,13,14,23} The 8S variant has a T_m 28°C higher and a conformational stability 7.7 kcal/mol higher than the wild-type protein.

Because 7S and 8S were much more stable than wild-type RNase Sa and 2S, ΔC_p values were determined for 7S and 8S to see if this might contribute to the differences in stability. By measuring the stability of 7S and 8S as a function of pH, ΔH_m could be determined as a function of T_m , and used to

Figure 2. Thermal unfolding curves for RNase Sa WT (■), 2S (●), 7S (▲), and 8S (▼) in 30 mM MOPS, pH 7. The solid lines are curves calculated as previously described,²² using the thermodynamic parameters given in Table I.

Table I. Parameters Characterizing the Thermal Unfolding of RNase Sa and the Stabilized Variants in 30 mM MOPS, pH 7.0

Variant	ΔH_m^a (kcal/mol)	ΔS_m^b (cal/mol/K)	ΔC_p^c (kcal/mol/K)	T_m^d (°C)	ΔT_m^e (°C)	$\Delta\Delta G^\circ$ (60°C) ^f (kcal/mol)
WT	93	287	1.52	49.3	—	—
2S	92	284	n.d.	51.5	2.2	0.8
7S	105	309	1.68	66.6	17.3	5.3
8S	97	277	1.07	77.2	27.9	7.7

^a Enthalpy change at T_m . The error is ± 5 kcal/mol.

^b $\Delta S_m = \Delta H_m/T_m$.

^c Determined by Kirchoff analysis. The value of 1.52 for WT was taken from Ref. 10. The errors are ± 0.05 kcal/mol/K for 7S and 8S.

^d Midpoint of the thermal unfolding curve. The error is $\pm 0.3^\circ\text{C}$.

^e $\Delta T_m = T_m$ (variant) – T_m (WT).

^f $\Delta\Delta G^\circ = \Delta G^\circ$ (variant) – ΔG° (WT), calculated as described in Ref. 22 using a reference temperature of 60°C. The error is ± 0.3 kcal/mol. The positive values indicate increases in stability.

determine the heat capacity change for folding, ΔC_p , according to the Kirchoff equation^{24,25}:

$$d(\Delta H_m)/d(T_m) = \Delta C_p \quad (1)$$

The plots of ΔH_m as a function of T_m are shown in Figure 3a for 7S and in Figure 3b for 8S. The resulting ΔC_p values for the proteins are given in Table I. The ΔC_p values differ significantly: 1.52 for wild-type RNase Sa, 1.68 for 7S, and 1.07 kcal/mol/K for 8S. These large differences in ΔC_p suggest that the DSEs of the proteins might contain different amounts of structure. To investigate this possibility, two variants of 7S and four variants of 8S were prepared and the thermodynamic parameters, including ΔC_p , were measured (Table II). The ΔC_p results will be discussed below.

Chemical denaturation of RNase Sa and its variants at pH 7, 25°C

The effect of mutations on the conformational stability of RNase Sa was also determined by urea and GuHCl denaturation. The 2S variant is more stable in urea but less stable in GuHCl than wild-type RNase Sa (Fig. 4). The two stabilizing mutations in 2S were designed to improve charge–charge interactions on RNase Sa, and their effect is diminished by the high-ionic strength in GuHCl solutions. The 7S and 8S variants were too stable to be unfolded by urea at pH 7. When GuHCl was used as a denaturant, both 7S and 8S unfolded at much higher GuHCl concentrations than the wild-type protein (Fig. 4b). Both urea and GuHCl curves were analyzed assuming a two-state folding mechanism to determine the unfolding free energy, ΔG , as a function of denaturant concentration. ΔG varied linearly with denaturant concentration so the data were analyzed using the linear extrapolation method:

$$\Delta G = \Delta G(\text{H}_2\text{O}) - m[\text{denaturant}] \quad (2)$$

where m is a measure of the dependence of ΔG on denaturant molarity, and $\Delta G(\text{H}_2\text{O})$ is an estimate of

the conformational stability of the protein.^{26–28} These parameters are given in Table III. Note that the 2S variant is 0.4 kcal/mol more stable than wild-type RNase Sa as determined by urea unfolding, but

Figure 3. ΔH_m plotted as a function of T_m for the stability variants, using data from thermal denaturation curves measured over a range of pH. (a) 7S (■) and variants 7S (D79A) (●), 7S (I92D) (▲). (b) 8S (●) and variants 8S (I92D) (●), 8S (I70D) (▲), 8S (I92A) (▼), 8S (Y80A) (◆). The lines are least-square fits to Eq. (1).

Table II. Parameters Characterizing the Thermal Unfolding of RNase Sa 7S and 8S Variants in 30 mM MOPS, pH 7.0

Variant	ΔH_m^a (kcal/mol)	ΔS_m^b (cal/mol/K)	ΔC_p^c (kcal/mol/K)	T_m^d (°C)	ΔT_m^e (°C)	$\Delta\Delta G^\circ$ (60°C) ^f (kcal/mol)
7S	105	309	1.68	66.6	—	—
7S (I92D)	73	237	1.41	34.0	−32.6	−9.6
7S (D79A)	102	293	1.15	75.3	8.7	2.2
8S	97	277	1.07	77.2	—	—
8S (I92D)	84	261	1.41	50.2	−26.9	−7.1
8S (I70D)	78	247	1.08	41.5	−35.6	−9.5
8S (I92A)	102	303	1.33	63.9	−13.3	−3.2
8S (Y80A)	87	263	1.29	58.0	−19.2	−4.8

^a Enthalpy change at T_m . The error is ± 5 kcal/mol.

^b $\Delta S_m = \Delta H_m/T_m$.

^c Determined by Kirchoff analysis. The errors are ± 0.05 kcal/mol/K.

^d Midpoint of the thermal unfolding curve. The error is $\pm 0.3^\circ\text{C}$.

^e $\Delta T_m = T_m$ (variant) $- T_m$ of 7S or 8S.

^f $\Delta\Delta G^\circ = \Delta G^\circ$ (variant) $- \Delta G^\circ$ of 7S or 8S, calculated as described in Ref. 22 using a reference temperature of 60°C. The error is ± 0.3 kcal/mol. Negative values indicate decreases in stability.

0.7 kcal/mol less stable as determined by GuHCl unfolding. By GuHCl unfolding, 7S is 3.7 and 8S 6.1 kcal/mol more stable than wild type. However, if we correct these values for the effect of ionic strength observed with 2S, then 7S would be 4.8 and 8S 7.2 kcal/mol more stable than wild-type RNase Sa. These corrected values are in better agreement with the estimates of 5.3 and 7.7 kcal/mol at 60°C from thermal denaturation.

The conformational stability of the single mutants of 7S and 8S were also determined using GuHCl, since urea could not unfold them completely. These results are given in Table IV. All of the variants are less stable than their respective parent proteins, except 7S(D79A), which is 1.5 kcal/mol more stable than 7S.

NaCl dependence of the thermal stability of RNase Sa and the 2S variant

The conformational stabilities of RNase Sa and 2S determined by urea and GuHCl denaturation differed. This might result because GuHCl is a salt and urea is not. To test this, the stability of the two proteins was measured as a function of NaCl concentration. The results are shown in Figure 5. Both proteins are stabilized by NaCl, but the increase in stability is greater for wild type than it is for 2S. This explains why 2S is more stable than wild type in the absence of salt, but less stable in the presence of salt, as in GuHCl denaturation.

Discussion

Many proteins have been stabilized by improving charge–charge interactions on the surface of the protein.^{29,30} In a previous study, we showed that D25K and E74K both increased the stability of RNase Sa by ~ 1 kcal/mol.^{11,31} These increases were less than expected based on calculations with Coulomb's law using a dielectric constant of 80, and suggested that electrostatic interactions in the DSE can contribute

to protein stability.³² When these two mutations were made to create 2S, the stability only increased 0.8 kcal/mol, less than expected for either of the

Figure 4. Chemical denaturant unfolding curves for RNase Sa and the stability variants. (a) Urea and (b) GuHCl unfolding curves for WT (■), 2S (●), 7S (▲), and 8S (▼). The continuous lines are theoretical curves calculated using the Santoro and Bolen equation²⁸ and the parameters in Table III.

Table III. Parameters Characterizing the Urea and GuHCl Unfolding of RNase Sa and the Stabilized Variants in 30 mM MOPS, pH 7.0, 25°C

Variant	Denaturant	$D_{1/2}$ ^a (M)	$\Delta D_{1/2}$ ^b (M)	m^c (kcal/mol/M)	ΔG° (H ₂ O) ^d (kcal/mol)	$\Delta\Delta G^{\circ e}$ (kcal/mol)
WT	GuHCl	2.99	—	2.43	7.3	—
2S	GuHCl	2.70	-0.29	2.61	7.1	-0.7
7S	GuHCl	4.44	1.45	2.52	11.2	3.7
8S	GuHCl	5.40	2.41	2.51	13.6	6.1
WT	Urea	6.06	—	0.96	5.8	—
2S	Urea	6.51	0.45	1.02	6.6	0.4

^a Midpoint of the unfolding curve. The error is $\pm 1\%$.

^b $\Delta D_{1/2} = D_{1/2}(\text{variant}) - D_{1/2}(\text{WT})$.

^c The slope of plots of ΔG versus [denaturant]. The error is $\pm 10\%$.

^d $\Delta G^\circ(\text{H}_2\text{O})$ = the intercept of plots of ΔG versus [denaturant] at 0 M denaturant.

^e From $\Delta D_{1/2} \times$ the average m value of WT and the variants. Positive values indicate increases in stability.

single mutations (Table V). In retrospect, it was a bad idea on our part to add both mutations because the two carboxyl groups are only 11 Å apart in the folded structure.

Another method that is generally useful for increasing protein stability is to improve β -turns.^{14–16} We improved five of the β -turns in RNase Sa, and the increases in stability ranged from 0.7 to 1.3 kcal/mol per mutation. We added these five mutations to 2S to give us 7S, and the stability was increased by 4.5 kcal/mol (Table V). This agrees with the stability increase of 4.5 kcal/mol expected based on additivity (Table V).

The D79F mutation increases the stability of RNase Sa by 3.0 kcal/mol at pH 7, and 3.7 kcal/mol at pH 8.5.¹³ This is among the largest increases in stability observed for a single mutation. The carboxyl group of Asp79 is 85% buried and it has a $pK = 7.4$, which is why the stability change is greater at pH 8.5 than at pH 7.^{13,33} We added the D79F mutation to 7S to give us 8S, and the stability was increased 2.4 kcal/mol at pH 7 (Table V).

Single mutations in other proteins have also been found to give large increases in stability: 2.8 kcal/mol for Q16L in Cro,³⁴ 3.3 kcal/mol for D57A in CheY,³⁵ 4.2 kcal/mol for N52I in iso-cytochrome c,^{36–38}

6.8 kcal/mol for H32Y in DsbA,³⁹ and 6.0 kcal/mol for R29W in gene-3-protein.⁴⁰ Most of these mutations were discovered by accident, but the last was identified by *in vitro* selection. Using *in vitro* selection to find stabilizing mutations,^{41–43} and structural⁴⁴ and theoretical studies^{45,46} to understand them will eventually give us a better foundation for increasing the stability of proteins.

Table V shows that the increase in T_m expected based on additivity was 32.8°C, and the observed increase was 27.9°C; and the expected increase in conformational stability was 9.5 kcal/mol, and the observed increase was 7.7 kcal/mol. Since the contribution of the five β -turn mutations to give 7S and the D79F mutation to give 8S made the major contributions to the stability increase, we will focus our attention on 7S and 8S for the rest of the article.

Figure 6a illustrates the three thermodynamic modifications to protein stability curves used by thermophilic proteins to increase their T_m .^{5,47–49} In method I, the stability curve is raised at all temperatures by improving enthalpic interactions. In method II, the stability curve is broadened by lowering ΔC_p . In method III, the stability curve is shifted to higher temperatures by improving entropic changes. Many thermophilic proteins use more than one method to

Table IV. Parameters Characterizing the GuHCl Unfolding of RNase Sa 7S and 8S Variants in 30 mM MOPS, pH 7.0, 25°C

Variant	$D_{1/2}$ ^a (M)	$\Delta D_{1/2}$ ^b (M)	m^c (kcal/mol/M)	ΔG° (H ₂ O) ^d (kcal/mol)	$\Delta\Delta G^{\circ e}$ (kcal/mol)
7S	4.44	—	2.52	11.2	—
7S (I92D)	0.83	-3.61	2.67	2.2	-9.1
7S (D79A)	5.05	0.61	2.40	12.1	1.5
8S	5.40	—	2.51	13.6	—
8S (I92D)	2.01	-3.39	2.73	5.5	-9.2
8S (I70D)	1.30	-4.10	2.87	3.7	-11.2
8S (I92A)	3.22	-2.18	2.78	9.2	-5.9
8S (Y80A)	2.74	-2.66	2.69	7.4	-7.2

^a Midpoint of the unfolding curve. The error is $\pm 1\%$.

^b $\Delta D_{1/2} = D_{1/2}(\text{variant}) - D_{1/2}(\text{7S or 8S})$.

^c The slope of plots of ΔG versus [GuHCl]. The error is $\pm 10\%$.

^d $\Delta G^\circ(\text{H}_2\text{O})$ = the intercept of plots of ΔG versus [GuHCl] at 0 M GuHCl.

^e From $\Delta D_{1/2} \times$ the average m value of either 7S and its variants, or 8S and its variants. A positive value indicates an increase in stability.

Figure 5. T_m as a function of NaCl concentration for RNase Sa WT (■) and the 2S variant (●). The lines are to guide the eye only.

increase stability.⁵ For example, method III is always used in combination with one of the other methods. The most common method observed is an increase in ΔG and a decrease in ΔC_p (methods I and II). The second most common is to just reduce ΔC_p (method II). Next, we discuss our results in terms of these methods.

Table VI gives the thermodynamic parameters characterizing the denaturation of RNase Sa, 7S, and 8S. These were used to calculate the protein stability curves shown in Figure 6b. The temperature of maximum stability, T_S , is increased in 7S and this raises T_m . In contrast, the T_S values for RNase Sa and 8S are quite similar. The change in properties that shifts the stability curve to higher temperatures for 7S is counterbalanced by changes in 8S. The proline residues inserted in 7S would be expected to lower ΔS , but in 8S a buried charge is replaced by a hydrophobic residue and the thermodynamic consequences of this are difficult to predict.^{24,25}

For both 7S and 8S, the stability at T_S , ΔG_S , is greater than wild-type RNase Sa. For 7S, the

increase in stability is mostly due to changes in the entropy of unfolding.^{14–17} For 8S, the unfavorable contribution of Asp79 to the stability appears to result from the Born self-energy of burying a charge and, more importantly, from unfavorable charge–charge interactions.¹³ ΔC_p is higher than wild type for 7S and lower for 8S. This will decrease the T_m for 7S and increase T_m for 8S. These results will be discussed further below.

The value of ΔC_p can also be determined by a global fit of stability measurements from thermal and urea denaturation curves.⁵⁰ We used this method to confirm the large difference in ΔC_p between 7S and 8S. It was necessary to do these experiments at pH 3 where the proteins are less stable so that urea denaturation curves could be determined. The values of ΔC_p from this approach are also shown in Table VI. The ΔC_p values are lower than those determined by the Kirchoff method at pH 7, but the value for 7S is still 46% greater than the value for 8S. The ΔC_p for 7S may be lower because Asp79 will not be ionized at pH 3, but it will be partially ionized at pH 7. The factors that contribute to ΔC_p have been studied experimentally^{24,25} and theoretically.⁵¹

The ΔC_p values for the mutants of 7S and 8S shown in Table II were determined to probe for structure in 8S that is not present in 7S. Our approach is similar to that used by the Marqusee laboratory.^{18,19} ΔC_p is lowered from 1.68 in 7S to 1.15 in 7S (D79A). This suggests the removing the charge on Asp79 is important in allowing the structure to form and that more structure is formed in 8S (7SD79F) than in 7S (D79A). The side chains of Ile70 and Ile92 are both 100% buried, but Ile92 is close to Asp79 and Ile70 is not. ΔC_p is identical for 8S and 8S (I70D) suggesting that Ile70 is not part of the structure formed in 8S. However, ΔC_p increases from 1.07 in 8S to 1.33 in 8S (I92A) and to 1.41 in 8S (I92D). This suggests that Ile92 is part of the structure in 8S and it is disrupted more when it is replaced by Asp than by Ala, as expected if the

Table V. Mutations Introduced to Make RNase Sa 8S and Their Expected Contribution to Stability

Type of mutation	Mutation	ΔT_m (°C) ^a	ΔT_m (°C) ^b (observed)	$\Delta \Delta G^a$	$\Delta \Delta G^{cb}$ (observed)
Surface charge	D25K	3.0	—	0.9	—
	E74K	3.9	—	1.1	—
Additive	2S	6.9	2.2	2.0	0.8
β-Turn	S31P	2.5	—	0.7	—
	S42G	2.6	—	0.7	—
	S48P	4.5	—	1.3	—
	T76P	3.5	—	1.0	—
	Q77G	2.9	—	0.8	—
Additive	—	16.0	15.1	4.5	4.5
Subtotal	7S	22.9	17.3	6.5	5.3
Buried charge	D79F	9.9	10.6	3.0	2.4
Total	8S	32.8	27.9	9.5	7.7

^a From Refs. 13–15.

^b From Table 1.

Figure 6. Stability curves for RNase Sa and the stability variants. (a) Thermodynamic modifications to a protein stability curves that thermophilic proteins use to increase their T_m : reference curve (solid line), method I (dashed line), method II (dotted line), and method III (dash-dotted line). See text for details. (b) Stability curves for WT (solid line), 7S (dash-dotted line), and 8S (dashed line) RNase Sa at pH 7 constructed using the modified Gibbs–Helmholtz equation²² and the parameters from Table VI. The two vertical lines are at -6.2°C , the T_s of WT RNase Sa (solid line) and 25°C (dotted line).

structure were a hydrophobic pocket. The ΔC_p is increased from 1.07 kcal/mol/K in 8S to 1.29 in 8S (Y80A), suggesting that Tyr80 may also contribute to the stability of the structure formed in 8S. Taken together, these results suggest that residual structure exists in the DSE of 8S that is not present in the DSE of 7S. They also suggest that three residues (Y79, Y80, and I92) that are in close proximity in the folded protein are part of this structure. Consequently, the structure present in the DSE of 8S may be a nativelike hydrophobic pocket that forms when D79 is removed.

Concluding Remarks

For RNase Sa, we were able to increase the T_m by 28°C by making eight mutations. Two general methods were used that can be applied to other proteins. One is to improve electrostatic interactions on the protein, and this is best done using the approaches developed by the Makhatadze laboratory.⁵² The second is to reduce the conformational entropy of the denatured state by adding proline residues in β -turns and at other locations in proteins. This approach was pioneered by the Suzuki laboratory,⁵³ and the general strategy has been described.¹⁵ This method can even be used to stabilize proteins from hyperthermophiles.⁵⁴ We were fortunate to have a single mutation (D79F) that increased the T_m by 10°C and the stability by 3 kcal/mol, in part by lowering ΔC_p through the formation of structure in the denatured state.

Materials and Methods

All buffers and chemicals were of reagent grade. Urea was from Amresco or Nacalai Tesque (Kyoto, Japan) and GuHCl was from MP Biomedicals or ICN Biomedicals. Both were used without further purification. The plasmids for RNase Sa and the variants were derived from the pEH100 plasmid previously described.⁵⁵ The expression host was *E. coli* strain C41 (DE3). Oligonucleotide primers for mutagenesis were from Integrated DNA Technologies (Coralville, IA). Site-directed mutagenesis was

Table VI. Parameters Characterizing the Stability of RNase Sa, 7S, and 8S

Variant	T_m^a ($^\circ\text{C}$)	ΔH_m^b (kcal/mol)	$\Delta G(60^\circ\text{C})^c$ (kcal/mol)	T_s^d ($^\circ\text{C}$)	ΔG_s^e (kcal/mol)	ΔC_p^f (kcal/mol/K)	ΔC_p^g (kcal/mol/K)
WT	49.3	93	-3.4	-6.2	8.2	1.52	—
7S	66.6	105	1.9	9.5	9.1	1.68	1.47
8S	77.2	97	4.3	-4.4	11.7	1.07	1.01

All values were determined at pH 7.0 except the ΔC_p values for 7S and 8S in last column, which were determined at pH 3.

^a From Table I.

^b Enthalpy change at T_m , from Table I.

^c Calculated using the modified Gibbs–Helmholtz equation²⁰ and the T_m , ΔH_m , and ΔC_p (column 7) values from this table.

^d T_s , Temperature of maximum stability, calculated as described in Ref. 48 using the ΔC_p values in column 7.

^e ΔG_s , stability at T_s , calculated as described in footnote c.

^f From Table 1.

^g Determined by a global fit of urea and thermal denaturation data at pH 3. The errors are ± 0.05 kcal/mol/K for both 7S and 8S. See Ref. 22.

performed with a QuikChangeTM Site-Directed Mutagenesis Kit from Stratagene (La Jolla, CA). Mutant plasmids were sequenced by the Gene Technologies Laboratory, Texas A&M University, and the integrity of each gene was confirmed through the sequence.

RNase Sa, and the stabilized variants were expressed and purified as described previously,⁵⁵ with slight modifications. Because of the increased stability of the 7S and 8S variants, a mutation (H85Q) was introduced into their active sites to decrease RNase activity and thus increase expression. It was shown previously that this mutation does not change the stability of RNase Sa.⁵⁶ The variants were expressed using the Studier autoinduction procedure.^{57,58}

Urea and thermal denaturation curves were determined using either an AVIV 62DS or 202SF spectropolarimeter (Aviv Instruments, Lakewood, NJ) to follow unfolding. These methods for RNase Sa have been described previously.¹⁰ The analysis of urea and thermal denaturation curves assumed a two-state unfolding model and was performed as described elsewhere.²² The data were analyzed using Microcal Origin 7.0 (Microcal Software, Northampton, MA).

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