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Contribution of Hydrogen Bonding to the Conformational Stability of Ribonuclease T1†

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ABSTRACT: For 30 years, the prevailing view has been that the hydrophobic effect contributes considerably more than hydrogen bonding to the conformational stability of globular proteins. The results and reasoning presented here suggest that hydrogen bonding and the hydrophobic effect make comparable contributions to the conformational stability of ribonuclease T1 (RNase T1). When RNase T1 folds, 86 intramolecular hydrogen bonds with an average length of 2.95 Å are formed. Twelve mutants of RNase T1 [Tyr → Phe (5), Ser → Ala (3), and Asn → Ala (4)] have been prepared that remove 17 of the hydrogen bonds with an average length of 2.93 Å. On the basis of urea and thermal unfolding studies of these mutants, the average decrease in conformational stability due to hydrogen bonding is 1.3 kcal/mol per hydrogen bond. This estimate is in good agreement with results from several related systems. Thus, we estimate that hydrogen bonding contributes about 110 kcal/mol to the conformational stability of RNase T1 and that this is comparable to the contribution of the hydrophobic effect. Accepting the idea that intramolecular hydrogen bonds contribute 1.3 ± 0.6 kcal/mol to the stability of systems in an aqueous environment makes it easier to understand the stability of the "molten globule" states of proteins, and the α-helical conformations of small peptides.

Most of the important tasks in living cells are carried out by proteins in which the polypeptide chain is tightly folded into a globular conformation that is essential for their biological activity. Consequently, there is great interest in the forces that stabilize globular proteins (Creighton, 1991; Dill, 1990). In early discussions of protein structure, hydrogen bonding was thought to be the most importance force contributing to the conformational stability. The main proponent of this view was Linus Pauling, who wrote with Mirsky (1936): "The importance of the hydrogen bond in protein structure can hardly be overemphasized." However, by the 1950's, the emphasis had shifted, and the importance of the hydrophobic effect was stressed first by Kauzmann (1959) and later by Tanford (1962), who used model compound data and calculations based on a simple model to conclude: "...the stability of the native conformation in water can be explained...entirely on the basis of the hydrophobic interactions of the non-polar parts of the molecule." The view that the hydrophobic effect makes a more important contribution than hydrogen bonding to globular protein stability is still widely held today. As recent examples, Kim and Baldwin (1990) state: "Stripping H_2O from nonpolar side chains to form a hydrophobic core provides the main source of free energy stabilizing a folded protein." Creighton (1990) states: "Nevertheless, the hydrophobic interaction is probably the major stabilizing factor." In a recent review that gives an excellent overview of the forces contributing to globular protein stability, Dill (1990) states: "More than 30 years after Kauzmann's insightful hypothesis, there is now strong accumulated evidence that hydrophobicity is the dominant force of protein folding, provided that "hydrophobic" is operationally defined in terms of the transfer of nonpolar amino acids from water into a medium that is nonpolar and

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Table I: Parameters Characterizing the Urea and Thermal Unfolding of Wild-Type RNase T1 (Lys25-RNase T1) and 12 Hydrogen-Bonding Mutants in 30 mM MOPS Buffer, pH 7[a]

	urea unfolding			thermal unfolding			
protein	m (cal/M)	$[\text{urea}]_{1/2}$ (M)	$\Delta(\Delta G)$ (kcal)	ΔH_m (kcal)	ΔS_m (cal/C)	T_m (°C)	$\Delta(\Delta G)$ (kcal)
RNase T1	1210	5.30		110	339	50.9	
Tyr11 → Phe	1270	3.56	−2.11	101	317	44.9	−2.03
Tyr42 → Phe	1170	6.24	1.14	106	325	54.3	1.15
Tyr56 → Phe	1260	4.65	−0.78	99	306	48.8	−0.71
Tyr57 → Phe	1285	4.88	−0.50	107	332	49.6	−0.44
Tyr68 → Phe	1320	4.17	−1.36	89	279	46.9	−1.35
Ser12 → Ala	1275	4.29	−1.23	99	309	47.7	−1.08
Ser17 → Ala	1215	5.85	0.67	109	334	52.6	0.57
Ser64 → Ala	1375	4.11	−1.44	104	324	46.3	−1.56
Asn9 → Ala	1275	4.56	−0.90	101	313	48.8	−0.71
Asn36 → Ala	1310	5.31	0.03	111	344	50.9	0.00
Asn44 → Ala	1300	3.59	−2.08	91	287	45.4	−1.86
Asn81 → Ala	1435	2.92	−2.87	91	287	42.3	−2.91

[a] m and ΔS_m are the slopes of plots of ΔG vs [urea] and T, respectively, and $[\text{urea}]_{1/2}$ and T_m are the midpoints ($\Delta G = 0$) of urea and thermal denaturation curves [see Pace et al. (1989)]. On the basis of many independent experiments, the uncertainties are estimated to be ±50 in m, ±0.05 in $[\text{urea}]_{1/2}$, ±15 in ΔS_m, and ±0.5 in T_m. These give rise to an uncertainty of about ±0.25 kcal/mol in the $\Delta(\Delta G)$ values. The enthalpy change at T_m, ΔH_m, was calculated from the entropy change at T_m, ΔS_m, using $\Delta H_m = T_m \Delta S_m$. For the urea data, $\Delta(\Delta G) = \Delta[\text{urea}]_{1/2} m$(wild type). This gives $\Delta(\Delta G)$ at a urea concentration between the respective $[\text{urea}]_{1/2}$ values. For the thermal data, $\Delta(\Delta G) = \Delta T_m \Delta S_m$(wild type). This gives $\Delta(\Delta G)$ at a temperature between the respective T_m values.

preferably capable of hydrogen bonding." This is the definition for "hydrophobic" used here.

In this paper, we suggest that hydrogen bonding and the hydrophobic effect make comparable contributions to the conformational stability of ribonuclease T1. This is based on studies reported here of mutants of RNase T1 designed to improve our understanding of hydrogen bonding, and on results from a number of other laboratories published recently (Fersht, 1987; Freier et al., 1986; Lesser et al., 1990; Bass & Cech, 1984; Tanner & Cech, 1987; Street et al., 1986; Steyaert et al., 1991; He & Quiocho, 1991).

Experimental Procedures

Wild-type RNase T1 and the mutants were prepared from genes expressed in *Escherichia coli* (Shirley & Laurents, 1990). The mutant genes were prepared by site-directed mutagenesis as described by Stanssens et al. (1989). Ultrapure urea was purchased from Schwarz/Mann Biotech, and stock solutions were prepared and their concentrations determined as described by Pace et al. (1989).

Urea denaturation curves were determined by measuring the intrinsic fluroescence intensity (278-nm excitation, 320-nm emission) of solutions containing ≈0.01 mg/mL RNase T1 in 1-cm quartz cuvettes thermostated at 25 ± 0.1 °C with a Perkin-Elmer MPF 44B spectrofluorometer. The solutions were incubated at least 6 h before the measurements. We have made two changes in our procedures that have improved the quality of the experimental results: first, we use battery-powered, motorized edp 2 pipets, obtained from Rainin Instruments for preparing our solutions, and, second, we now never remove the cuvette from the instrument during the course of the measurements. A vacuum system is used to remove old samples from the cuvettes. The resulting urea denaturation curves (see Figure 1, top panel) are the best we have ever obtained.

Thermal unfolding curves were determined by measuring the optical rotation at 295 nm of ≈1 mg/mL RNase T1 solutions in 1-cm cuvettes with a Cary 60 spectropolarimeter as described in more detail elsewhere (Pace et al., 1989). Typically, four measurements are made in the pretransition region, seven or eight in the transition region, and three in the posttransition region. With this approach, thermal unfolding was over 96% reversible.

Results

For our previous studies, wild-type RNase T1 had Gln at position 25, Gln25-RNase T1. This is the RNase T1 first isolated by Sato and Egami (1957) and used for most of the subsequent studies in countries other than Germany. However, all of the crystal structures determined by Saenger's group (Pace et al., 1991; Martinez-Oyanedel et al., 1991) have been determined with a RNase T1 variant with Lys at position 25, Lys25-RNase T1. For this reason and also because the solubility and conformational stability of Lys25-RNase T1 are greater than for Gln25-RNase T1 (Shirley et al., 1989), we plan to denote Lys25-RNase T1 as wild type in the future. Thus, RNase T1 and all of the mutants discussed here have Lys at position 25.

The unfolding of both Gln25-RNase T1 and Lys25-RNase T1 has been shown to closely approach a two-state folding mechanism (Thomson et al., 1989; Kiefhaber et al., 1990a), and the conformational stability has been carefully measured under a variety of conditions (Pace, 1990; Kiefhaber et al., 1990b). The differences in conformational stability between RNase T1 and the hydrogen-bonding mutants were determined using both urea and thermal unfolding experiments. Typical experimental results from urea unfolding curves are shown in the top panel of Figure 1. As explained elsewhere (Pace et al., 1989), an analysis of urea unfolding experiments yields ΔG as a function of urea concentration, and an analysis of thermal unfolding experiments yields ΔG as a function of temperature. From plots of these data (see bottom panel of Figure 1), the midpoints of the transitions (where $\Delta G = 0$), $[\text{urea}]_{1/2}$ or T_m, and measures of the steepness of the transitions, m or ΔS_m (the slopes of plots of ΔG vs [urea], m, or temperature, ΔS_m), can be determined. These parameters are listed in Table I along with estimates of the difference in stability, $\Delta(\Delta G)$, from both the urea and thermal unfolding experiments. It can be seen that the estimates from the two different approaches are in excellent agreement. This need not have been the case because the results apply to different sets of conditions: 25 °C in the presence of 2.9–6.3 M urea for the results from urea unfolding, and 42–55 °C in the absence of urea for the results from thermal unfolding. The close agreement between the two sets of data gives us considerable confidence that the measured differences in stability are quite reliable.

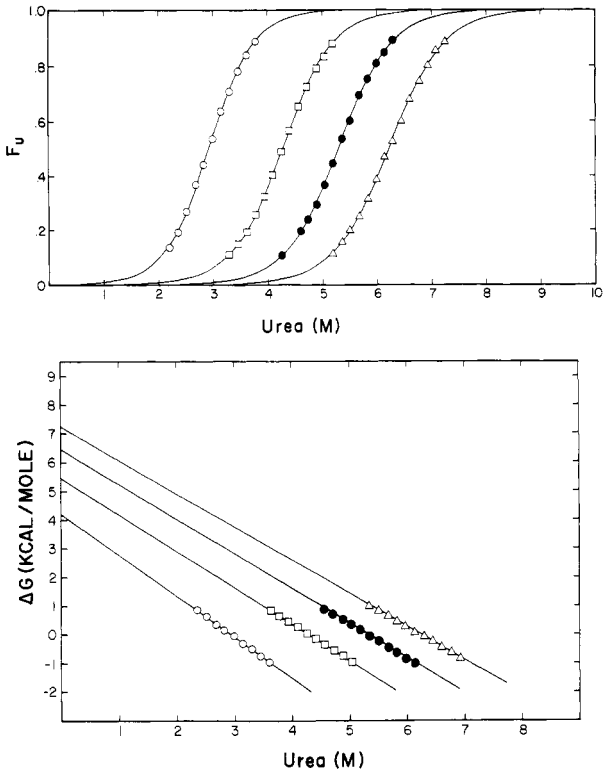

FIGURE 1: (Top) Fraction unfolded, F_U, and (bottom) ΔG as a function of urea molarity for RNase T1 (●) and the mutants Asn81 → Ala (○), Ser12 → Ala (□), and Tyr42 → Phe (△) in 30 mM MOPS buffer, pH 7.0 at 25 °C. The solid lines in both the top and bottom panels were calculated on the basis of the parameters characterizing the urea denaturation curves given in Table I. A two-state folding mechanism was assumed in order to calculate F_U and ΔG as a function of urea concentration from the urea denaturation curves (Pace et al., 1989). The data in the bottom panel were fit to the equation $\Delta G = \Delta G(H_2O) - m(\text{urea})$, where $\Delta G(H_2O)$ is the value of ΔG in the absence of urea and m is a measure of the dependence of ΔG on urea concentration. Values of m and $[\text{urea}]_{1/2}$, the midpoint of a urea unfolding curve, are given in Table I. Note that $[\text{urea}]_{1/2} = \Delta G(H_2O)/m$ so that $\Delta G(H_2O)$ can be calculated from the parameters given in Table I.

The m values range from 1170 to 1435 cal mol^{-1} M^{-1} with an average of 1285 ± 50 cal mol^{-1} M^{-1}. The fact that none of the mutants have an m value significantly less than wild-type RNase T1 is evidence that they also closely approach a two-state folding mechanism. The enthalpy values at T_m, ΔH_m, range from 89 to 111 kcal/mol with none of the mutants having a ΔH_m significantly higher than wild-type RNase T1 (Table I). This is reasonable since the number of intramolecular hydrogen bonds in the mutants will generally be less than or equal to the number in wild-type RNase T1, and hydrogen bonds are expected to contribute favorably to ΔH_m (Scholtz et al., 1991). However, the range of values observed is evidence that other factors make important contributions to ΔH_m.

Discussion

RNase T1 contains 293 nitrogen and oxygen atoms capable of donating or accepting hydrogen bonds, 205 in the backbone and 88 in the side chains. These atoms can form a total of 503 hydrogen bonds, 312 by the backbone and 191 by the side chains, based on the hydrogen-bonding capabilities given by Baker and Hubbard (1984). Using the geometric criteria suggested by these authors, the 1.5-Å resolution crystal structure determined by Martinez-Oyanedel et al. (1991), and a program written by Presta and Rose (1988), we find that folded RNase T1 contains 86 intramolecular hydrogen bonds

Table II: Hydrogen Bonding in RNase T1[a]

intramolecular hydrogen bonds		hydrogen bonds to H_2O	
type	no.	type	no.
peptide–peptide	52	peptide–H_2O	97
peptide–side chain	22		
side chain–side chain	12	side chain–H_2O	71
total	86	total	168
av length (Å)	2.95	av length (Å)	2.95

[a] This analysis was done using a program kindly provided by Dr. Leonard Presta [see Presta and Rose (1988)] and is based on the 1.5-Å crystal structure determined by Martinez-Oyanedel et al. (1991). A total of 121 H_2O molecules were included in the crystal structure. Only hydrogen bonds with lengths less than 3.5 Å and angles greater than 90° were included (Baker & Hubbard, 1984). Care was taken that "bifurcated" or "three center" hydrogen bonds such as those formed by some of the amide NH groups in the α-helix were counted only once (Martinez-Oyanedel et al., 1991).

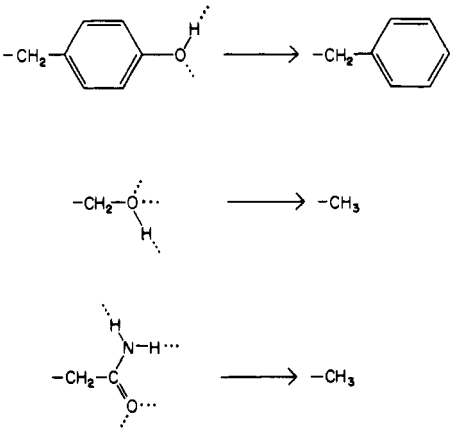

FIGURE 2: Hydrogen bonding of the amino acid side chains for Tyr → Phe (top), Ser → Ala (middle), and Asn → Ala (bottom) (Baker & Hubbard, 1984).

with an average length of 2.95 Å. A total of 168 hydrogen bonds to H_2O molecules can also be identified to the 121 H_2O molecules that were included in the crystal structure. A summary of the hydrogen bonding in RNase T1 is given in Table II. The objective of the research reported here was to estimate the contribution of a number of the individual hydrogen bonds to the stability and then to use this information to estimate the total contribution of the 86 intramolecular hydrogen bonds to the conformational stability of RNase T1.

The approach chosen was to select 12 of the side chains that participate in intramolecular hydrogen bonds and use site-directed mutagenesis to substitute an amino acid that removes the hydrogen-bonding capability. The substitutions made were: 5 (Tyr → Phe), 3 (Ser → Ala), and 4 (Asn → Ala). As shown in Figure 2, these groups are capable of participating in two, three, and four hydrogen bonds and can serve as either hydrogen bond donors or acceptors. Table III lists the side chains selected and relevant information about the hydrogen bonds of each in RNase T1. Note that nine of the groups form one hydrogen bond, one forms two hydrogen bonds, and two form three hydrogen bonds. The average donor–acceptor distance for these 17 hydrogen bonds is 2.93 Å.

A difficult problem in interpreting results from stability studies of mutant proteins is how to correct for the contribution of conformational entropy to the observed differences in stability. Conformational entropy can effect the $\Delta(\Delta G)$ values in several ways. First, the amino acid side chain can effect rotation around the N–C_α, ϕ, and C_α–$C_{carbonyl}$, ψ, bonds in the unfolded protein (Nemethy et al., 1966). This is especially important for substitutions involving Pro residues where ro-

Table III: RNase T1 Hydrogen Bonds Removed by Amino Acid Substitutions[a]

residue	H-bond and partner	length (Å)	angle (deg)	% buried[b]	
Tyr11	OH···OD2 Asp76	2.69	132	99	99
Tyr42	OH···OD1 Asn44	2.97	133	98	99
Tyr56	OH···O Val52	2.83	157	90	73
Tyr57	OH···OE2 Glu82	2.56	124	85	70
Tyr68	OH···O Gly71	2.68	139	99	98
Ser12	OH···OH Ser14	2.71	131	54	88
	OH···N Asp15	3.24	134		88
Ser17	OH···O Ser13	3.19	136	26	24
Ser64	OH···N Asp66	3.17	115	53	91
Asn9	ND2···OD1 Asp76	2.89	137	66	57
Asn36	OD1···OH Ser35	3.01	115	28	30
Asn44	ND2···O Phe48	2.90	137	81	76
	OD1···N Phe48	3.16	102		100
	OD1···OH Tyr42	2.97	133		100
Asn81	OD1···N Asn83	2.97	114	100	100
	OD1···N Gln85	2.91	143		100
	ND2···OE1 Gln85	2.94	126		99

[a] The analysis of the hydrogen bonding was done as explained in the footnote to Table II. [b] The accessibilities (percent buried) of the side chain (e.g., for Ser, $-CH_2-OH$) and of the individual group that actually forms the hydrogen bond (e.g., –OH) are respectively given in the last two columns. The accessibilities were estimated using the Lee and Richards program (Richards, 1977).

tation around ϕ is largely eliminated, and for substitutions involving Gly residues where rotation around both ϕ and ψ is much less restricted. This effect has been investigated experimentally with mutants of T4 lysozyme (Matthews et al., 1987). This effect should make an insignificant contribution for the three types of mutants studied here. Second, conformational entropy can contribute to $\Delta(\Delta G)$ for any amino acid substitution that changes the number of bonds in the side chain since rotation around these bonds will generally be more restricted in the folded protein than in the unfolded protein. Finally, conformational entropy can contribute to $\Delta(\Delta G)$ for any mutation that fills or creates a hole in the folded protein. Little is known about these latter two effects. Richards (1977) has shown that Tyr and Phe residues occupy almost the same volume in folded proteins, that Ser residues are ≈8 Å^3 larger than Ala residues, and that Asn residues are ≈44 Å^3 larger than Ala residues. For comparison, adding a $-CH_2-$ or CH_3 group increases the volume of the residue by ≈26 Å^3. On reflection, it seems likely that conformational entropy will make a smaller contribution to the measured $\Delta(\Delta G)$ values for Tyr → Phe and Ser → Ala mutants than for any other of the mutations that can be used to study hydrogen bonding or hydrophobic interactions, and may well be less than the experimental error for our $\Delta(\Delta G)$ values. For the Asn → Ala mutants, the contribution of conformational entropy should be larger, but the magnitude is unknown, and there is presently no reliable way to make corrections.

For each of the three types of amino acid substitutions reported here, the amino acid side chain in wild-type RNase T1 is less hydrophobic than the side chain in the mutant. This effect by itself is expected to increase the stability of the mutants relative to wild-type RNase T1, and will surely contribute to the measured $\Delta(\Delta G)$ listed in Table I. We will be able to get a more accurate assessment of the contribution of hydrogen bonding to the differences in stability if we can correct, at least approximately, for this effect. Measurements in several different solvent systems have been used to assess the magnitude of the hydrophobic effect for the amino acid side chains. In Table IVA, we give estimates of the free energy of transfer, ΔG_{tr}, of a $-CH_2-$ group from several different solvent systems to water. The question is which of these solvents is the best model for the interior of a protein. Hexane and cyclohexane are probably too nonpolar, and formamide and methanol are probably too polar. All of the other solvents give comparable ΔG_{tr} values, and, consequently, we have chosen to use the complete set of data available for 1-octanol to make the corrections described in Table V, and for the calculations presented below in Table VI. Note that the octanol data are consistent with the definition suggested by Dill (1990) (see the introduction). The octanol phase in the partitioning experiments used to determine the ΔG_{tr} values is capable of hydrogen bonding since it contains 2.5 M H_2O in addition to the octanol. thus, the ΔG_{tr} values should reflect only changes in hydrophobicity and not hydrogen bonding.

Recently, De Young and Dill (1990) and Sharp et al. (1991a,b) suggested that if measured values of ΔG_{tr} such as those shown in Table IV are corrected to take into account the relative size of the solute and solvent molecules, then they yield a more reasonable estimate of the contribution of the hydrophobic effect. This approach almost doubles the ΔG_{tr} values for a $-CH_2-$ group, as shown by the values in par-

Table IV: (A) ΔG_{tr} (Solvent → Water) Values for a $-CH_2-$ Group Based on Studies with Model Compounds and (B) $\Delta(\Delta G)$ Values for a $-CH_2-$ Group Based on Ile → Val Mutants

(A) ΔG_{tr} values (kcal/mol)		(B) $\Delta(\Delta G)$ values (kcal/mol)		
solvent	ΔG_{tr}	protein	residue	$\Delta(\Delta G)$
formamide[a]	0.32	S. nuclease[h]	15	0.8
N-methylacetamide[b,f]	0.74		18	1.1
acetone[a]	0.67		72	1.8
methanol[a]	0.60		92	0.5
ethanol[a]	0.67		139	1.5
butanol[a]	0.73	barnase[f]	88	1.3
heptanol[a]	0.73		96	1.2
octanol[c,f]	0.73 (1.4)[g]	gene 5 protein[j]	35	0.3
cyclohexane[d,f]	0.88 (1.5)[g]		47	1.2
hexane[e,f]	1.00	T4 lysozyme[k]	3	0.5

[a] Cohn & Edsall (1965). [b] Damodaran & Song (1986). [c] Fauchere & Pliska (1983). [d] Radzicka & Wolfenden (1988). [e] Fendler et al. (1975). [f] The average of the difference between the ΔG_{tr} values for Leu and Val and for Ile and Val was used for these solvents. [g] Corrected as described by Sharp et al. (1991b). [h] Shortle et al. (1990). [i] Kellis et al. (1989). [j] Sandberg & Terwilliger (1991). [k] Matsumura et al. (1988a).

Table V: Estimate of the Contribution of Hydrogen Bonding to the Measured Differences in Stability

protein	no. of H-bonds[a]	$\Delta(\Delta G)^b$ (kcal/mol)	$\Delta(\Delta G)^c$ (kcal/mol)	$\Delta(\Delta G)$/H-bond (kcal/mol)
Tyr11 → Phe	1	−2.07	−3.2	−3.2
Tyr42 → Phe	1	1.15	0	0
Tyr56 → Phe	1	−0.75	−1.8	−1.8
Tyr57 → Phe	1	−0.47	−1.4	−1.4
Tyr68 → Phe	1	−1.36	−2.5	−2.5
Ser12 → Ala	2	−1.16	−1.4	−0.7
Ser17 → Ala	1	0.62	0	0
Ser64 → Ala	1	−1.50	−1.7	−1.7
Asn9 → Ala	1	−0.81	−1.2	−1.6
Asn36 → Ala	1	0.02	−0.2	−0.6
Asn44 → Ala	3	−1.97	−2.5	−1.0
Asn81 → Ala	3	−2.89	−3.5	−1.4

[a] The number of intramolecular hydrogen bonds in wild-type RNase T1 (see Table III). [b] This is the average of the $\Delta(\Delta G)$ values in Table I from urea and thermal unfolding experiments. [c] The $\Delta(\Delta G)$ values in the preceding column have been corrected for the effect of differences in hydrophobicity between the amino acid in wild-type RNase T1 and the mutants by multiplying the $\Delta(\Delta G_{tr})$ values −1.1 (Tyr → Phe), −0.4 (Ser → Ala), and −1.2 (Asn → Ala) (in kilocalories per mole) by the accessibility of the side chain in the wild-type protein given in Table III, as described in the text. In addition, for the Ser17 → Ala mutant, a −0.5 kcal/mol correction was applied to correct for the differential effect that the two residues have on ther stability of the α-helix, as described in the text.

entheses for octanol and cyclohexane in Table IV. Both the corrected and uncorrected ΔG_{tr} values for the octanol data are listed below in Table VI.

Studies of the hydrophobic effect based on experimental results from mutant proteins generally show reasonable correlations with either the octanol data (Matsumura et al., 1988a; Kellis et al., 1989, Pakula & Sauer, 1990) or the ethanol data (Yutani et al., 1987; Matsumura et al., 1988b), but the magnitude of the effect may be larger than predicted (Kellis et al., 1989; Shortle et al., 1990; Sandburg & Terwilliger, 1991). The best results to consider are for Ile → Val mutants since they differ by only a $-CH_2-$ group, and this will minimize the size of the potential hole in the mutant, and possible conformational entropy effects. (Leu → Val mutants are more likely to have unfavorable steric effects.) Results for 10 Ile → Val mutants from 4 different proteins are given in Table IVB. The $\Delta(\Delta G)$ values range from 0.3 to 1.8 kcal/mol with an average of 1.0 kcal/mol. This is intermediate between the corrected and the uncorrected ΔG_{tr} values for octanol given in Table IVA. Nicholls et al. (1991) considered results for 45 mutants (Leu, Ile, and Val → Ala, and Leu and Ile → Val) and showed that the corrected ΔG_{tr} values for octanol give considerably better agreement with the experimental results than the ΔG_{tr} values that have not been corrected.

The corrections for the differences in hydrophobicity are shown in Table V. The corrections take into account the difference between the ΔG_{tr} values for the pairs: Tyr-Phe (1.1 kcal/mol), Asn-Ala (1.2 kcal/mol), and Ser-Ala (0.4 kcal/mol), and the accessibility of the side chain in the wild-type protein estimated using the Lee and Richards procedure (Richards, 1977). (The accessibility of each of the side chains in RNase T1 is given in Table III.) One further correction was made for the Ser17 → Ala mutant because it occurs at a largely exposed site in the α-helix of RNase T1. Recent studies of peptides have provided quantitative estimates of the helix-forming tendencies of the amino acids (Padmanabhan et al., 1990; Lyu et al., 1990; Merutka & Stellwagen, 1990; O'Neil & Degrado, 1990). The Ser17 → Ala substitution is expected to stabilize the mutant because Ala has a greater helix-forming tendency than Ser. The predicted stabilization is 0.51 kcal/mol based on the data of Lyu et al. (1990), 0.46 kcal/mol based on the data of Merutka and Stellwagen (1990), and 0.42 kcal/mol based on the data of O'Neil and DeGrado (1990). A value of 0.5 kcal/mol was used for the correction of the Ser17 → Ala data in Table V.

Before the corrections, three of the $\Delta(\Delta G)$ values were positive; i.e., the mutants were more stable than wild-type RNase T1. After the corrections, two of the $\Delta(\Delta G)$ values are zero, and the rest are negative, ranging as high as −4.1 kcal/mol for the Asn81 → Ala mutant that potentially removes three hydrogen bonds. On a per hydrogen bond basis, the values range from 0 to −3.2 kcal/mol with an average value of −1.3 kcal/mol. Note that the average for the three Ser → Ala mutations, −0.8 kcal/mol, and the average for the four Asn → Ala mutations, −1.2 kcal/mol, are considerably smaller than the average for the five Tyr → Phe mutations, −1.8 kcal/mol. So, we think the $\Delta(\Delta G)$ values given in the last two columns in Table V are due mainly to changes in the hydrogen bonding in the mutants.

The results in Table V cannot be interpreted with any certainty until high-resolution, three-dimensional structures are available for the mutant proteins. (All of the mutants have enzyme activity, suggesting that their conformations do not differ too much from wild-type RNase T1.) A key question is what happens to the hydrogen-bonding partner in the mutants: Does it form a new intramolecular hydrogen bond, does it form a hydrogen bond to a water molecule, or does it fail to form any hydrogen bonds? In the first case, the $\Delta(\Delta G)$ value should be close to zero if the new hydrogen bond is approximately equivalent to the hydrogen bond in wild-type RNase T1. This may be the case for the two mutants for which $\Delta(\Delta G) \approx 0$. In the second case, the $\Delta(\Delta G)$ value would give the estimate that we are most interested in, namely, what the formation of a single intramolecular hydrogen bond contributes to the conformational stability of the protein. We argue below that this is the case for most of the mutants studied here. [This is observed, for example, when Thr154 is replaced by Gly in T4 lysozyme (Alber et al., 1987).] In the final case, the $\Delta(\Delta G)$ values should be considerably more negative because now both the loss of the intramolecular hydrogen bond and the formation of hydrogen bonds to water in the unfolded protein by the unpaired partner will contribute to the decrease in conformational stability. This may be the reason that the $\Delta(\Delta G)$ value for Tyr11 → Phe is considerably more negative than any of the other values.

For the Tyr11 hydrogen bond, neither the hydrogen bond donor nor the hydrogen bond acceptor forms a hydrogen bond with water in wild-type RNase T1. Furthermore, the side chains of Tyr11 and Asp76 are almost completely buried (99%). Thus, it is certainly possible that the carboxyl group of Asp76 may not be able to form a hydrogen bond in the Tyr11 → Phe mutant and that this contributes to the very negative $\Delta(\Delta G)$ value that is observed. In addition, the hydrogen bond lost is a good hydrogen bond, and it is probably to a charged acceptor since Asp76 is likely to be ionized at pH 7. There is no evidence from the titration curve for RNase T1 (Iida & Ooi, 1969), or from the pH dependence of the stability (Pace et al., 1990) for the presence of a carboxyl group with a pK of 7 or higher. Note that the hydrogen bonds of Tyr57 and Asn9 are also probably to charged acceptors.

For all of the other hydrogen bonds considered here, either the hydrogen bond donor or the hydrogen bond acceptor forms a hydrogen bond with a water molecule in addition to the intramolecular hydrogen bonds described in Table III. For this reason and because more room is available for hydrogen bonding to water molecules in the mutants, we think that the hydrogen-bonding partner will most often form a hydrogen bond to a water molecule in the mutant. Since the same group should also hydrogen bond to water molecules in the unfolded protein, they should no longer contribute significantly to the conformational stability through hydrogen bonding. On this basis, we assume that the nine $\Delta(\Delta G)$ values between −0.6 and −2.5 kcal/mol in the last column of Table V give the contribution of single hydrogen bonds to the conformational stability of RNase T1. The average of these 9 values is −1.4 kcal/mol which does not differ significantly from the average of −1.3 kcal/mol for all 12 of the $\Delta(\Delta G)$ values. We will see below that this estimate is in good agreement with estimates from completely different systems.

The longstanding question has been whether the formation of intramolecular hydrogen bonds in folded proteins makes a net favorable contribution to protein stability; i.e., is ΔG favorable for the reaction:

$$(>C{=}O\cdots(H_2O)_2 + H_2O\cdots H{-}N<)_{water} \leftrightarrow (>C{=}O\cdots H{-}N<)_{protein}$$

Pauling and Corey (1954) thought so: "With proteins in an aqueous environment the effective energy of hydrogen bonds is not so great, inasmuch as the difference between the energy of the system with the N–H···O hydrogen bonds surrounded

Table VI: Estimates of the Contribution of the Hydrophobic Effect to the Conformational Stability of RNase T1

side chain	no. present	no. buried[a]	ΔG_{tr}[b] a	ΔG_{tr}[b] b	(no. buried)ΔG_{tr}[c] a	(no. buried)ΔG_{tr}[c] b
Trp	1	1.0	3.1	6.1	3.1	6.1
Phe	4	3.8	2.4	5.0	9.2	19.0
Ile + Leu	5	4.3	2.4	4.9	10.3	21.1
Val	8	6.7	1.7	3.5	11.4	23.5
Tyr	9	7.7	1.3	3.9	10.0	30.0
Cys	4	3.2	1.3	3.2	4.2	10.2
Pro	4	3.2	1.0	2.5	3.2	8.0
Thr	6	3.3	0.4	1.5	1.3	5.0
Ala	7	4.7	0.4	1.0	1.9	4.7
His	3	2.2	0.2	1.9	0.4	4.2
Ser	15	3.9	0.0	0.6	0.0	2.3
Gln	2	1.3	−0.3	1.5	−0.4	2.0
Asn	9	5.0	−0.8	0.4	−4.0	2.0
$-CH_2-$[d]	29	19.4	0.7	1.4	13.6	27.2
					total 64.3	165.2

[a] Calculated with the Lee and Richards accessibility program (Richards, 1977). [b] Column a gives the ΔG_{tr} values for 1-octanol from Fauchere and Pliska (1983), and column b gives the same values corrected as described by Sharp et al. (1991b), both in kilocalories per mole. [c] Column a gives the values calculated with the ΔG_{tr} values from column a, and column b gives the values calculated with the values from column b, both in kilocalories per mole. [d] $-CH_2-$ groups from the Lys, Arg, Glu, and Asp side chains.

by water and a system with the N–H group and the O atom forming hydrogen bonds with water molecules may be no more than around 2 kcal/mole." However, a variety of model compound studies failed to give an unequivocal answer, and some even suggested that the hydrogen bonds with water would be favored [see Dill (1990) for a review of this literature]. These studies strengthened the idea that hydrophobic interactions are the dominant force in protein folding.

With the advent of site-directed mutagenesis, it has been possible to estimate ΔG for the hydrogen-bonding reaction shown above more directly. Fersht (1987) summarized these studies and concluded: "...an individual uncharged hydrogen bond contributes some 0.5 to 1.8 kcal/mole to binding energy...". Similar estimates had been obtained much earlier in studies with nucleic acids. Crothers and Zimm (1964) suggested that each hydrogen bond between the bases in double-helical nucleic acids contributes 1 kcal/mol to the stabilizing free energy. This estimate has since been substantiated by extensive studies by Turner's group (Freir et al., 1986). More recent studies of enzyme–substrate (Steyaert et al., 1991), protein–sulfate (He & Quiocho, 1991), and protein–nucleic acid (Lesser et al., 1990) hydrogen bonds give comparable estimates. Why the intramolecular hydrogen bonds are more favorable than the hydrogen bonds to water is not clear. It could be due to stronger electrostatic interactions in the more nonpolar, solidlike environment of the intramolecular hydrogen bonds or differences in entropy effects (Creighton, 1991; Dill, 1990). In any event, the estimates of the contribution of individual hydrogen bonds to the conformational stability of RNase T1 given in Table V are in good agreement with other studies of hydrogen bonding in an aqueous environment.

The major force favoring the unfolding of proteins is conformational entropy. In the folded protein, rotation around the bonds in the backbone and side chains will generally be more limited than in the unfolded protein. Kauzmann (1954) made a rough guess that this would favor unfolding by about 1.2 kcal/mol per residue, and Privalov (1979) has pointed out that this is consistent with one interpretation of experimental results on the thermodynamics of unfolding of small, monomeric globular proteins. Using this estimate, conformational entropy would favor unfolding by 125 kcal/mol for RNase T1. Experimental studies (Pace et al., 1988) have shown that this would be lowered by about 7 kcal/mol due to the restraints imposed by the two disulfide bonds in RNase T1. Thus, this admittedly crude estimate suggests that conformational entropy favors unfolding by ≈118 kcal/mol. How is this overcome?

The 17 intramolecular hydrogen bonds described in Table III are typical of the 86 intramolecular hydrogen bonds observed in folded RNase T1. On the basis of the results in Table V, we suggest that the average hydrogen bond in RNase T1 contributes 1.3 kcal/mol to the conformational stability. This leads to the conclusion that hydrogen bonding contributes 112 kcal/mol to the conformational stability of RNase T1.

One approach used to estimate the contribution of the hydrophobic effect to the conformational stability of RNase T1 is shown in Table VI. We assume that all of the side chains are fully exposed in the unfolded protein and estimate the number of side chains buried in the folded protein using the Lee and Richards method (Richards, 1977). The number of groups of each type buried is then multiplied by the appropriate ΔG_{tr} value using the 1-octanol data. When the Fauchere and Pliska (1983) ΔG_{tr} values are used directly, an estimate of 64 kcal/mol for the contribution of the hydrophobic effect is obtained. However, when the corrected values from Sharp et al. (1991b) are used, the estimate of the contribution of the hydrophobic effect is 165 kcal/mol. Spolar et al. (1989) have used a related approach based on a different set of model compound data (ΔC_p measurements of a series of hydrocarbons) to conclude that the contribution of the hydrophobic effect can be estimated using: ΔG(hydrophobic effect) = (80 ± 10)ΔC_p [see also Livingstone et al. (1991)]. Values of ΔC_p = 1220 (Kiefhaber et al., 1990b), 1240 and 1590 (C. Q. Hu and J. M. Sturtevant, unpublished observations), and 1650 (Pace & Laurents, 1989) cal mol^{-1} K^{-1} have been determined for RNase T1 unfolding using different approaches. These ΔC_p values lead to estimates of the contribution of the hydrophobic effect to the stability of RNase T1 ranging from 98 to 132 kcal/mol. All of these estimates should be regarded as upper limits of the contribution of the hydrophobic effect to the stability because they assume that the nonpolar side chains are completely accessible to solvent in the unfolded protein. There is mounting evidence that RNase T1 (Pace et al., 1990; unpublished observations) and other proteins (Dill & Shortle, 1991) do not unfold completely after urea or thermal denaturation.

So, this reasoning suggests that hydrogen bonding and hydrophobic interactions make comparable contributions to the conformational stability of RNase T1. Note that the sum of

these stabilizing interactions is considerably greater than our estimate of the destabilizing contribution from conformational entropy. There are, of course, a number of other forces that will contribute to the conformational stability that we have not considered. We will mention two that favor unfolding.

As noted above, unfilled hydrogen bonds in the folded protein should decrease the conformational stability because hydrogen bonding to water molecules will occur when the protein unfolds. In terms of groups, 41 of the 293 groups capable of hydrogen bonding do not form intramolecular hydrogen bonds and do not appear to form hydrogen bonds to water molecules in the crystal structure. (In terms of hydrogen bonds, 165 of the 503 possible hydrogen bonds are not formed.) However, the extent of hydrogen bonding with water molecules in solution is sure to be greater because only those water molecules that are largely immobilized will be observed in the crystal structure. Out of the 41 groups that are not hydrogen-bonded, only 1 (the N–H group of Val52) is completely buried, and 7 are actually hyperexposed compared to the model tripeptide used to estimate the accessibility (Richards, 1977). Thus, it seems likely that most of these 41 groups will be at least partially hydrogen-bonded to water molecules in the folded protein. Nevertheless, unfilled hydrogen bonds in the folded protein will surely contribute unfavorably to the conformational stability, but it is not possible to estimate the magnitude of this contribution at present.

Another factor that will favor unfolding is the transfer of peptide groups from a more nonpolar environment to water on unfolding. For RNase T1, about 70% of the peptide groups are buried in the folded protein. On the basis of model compound data, the ΔG_{tr} of a hydrogen-bonded peptide group from octanol to water is about −1.1 kcal/mol, even without application of the correction suggested by Sharp et al. (1991b). Thus, this is potentially a large contribution favoring unfolding that is generally not considered.

In summary, the evidence is strong that most intramolecular hydrogen bonds contribute 1.3 ± 0.6 kcal/mol to the stability of structures such as globular proteins and double-helical nucleic acids in an aqueous environment. Accepting this makes it easier to understand the stability of "molten globules" (Kuwajima, 1989) and of the α-helical conformations observed with some small peptides (Kim & Baldwin, 1990; Scholtz et al., 1991). In addition, it leads to the conclusion that hydrogen bonding and hydrophobic interactions make large but comparable contributions to the stability of globular proteins.

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Mechanism of the Conformational Transition of Melittin†

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ABSTRACT: It is known that, while melittin at micromolar concentrations is unfolded under conditions of low ionic strength at neutral pH, it adopts a tetrameric α-helical structure under conditions of high ionic strength, at alkaline pH, or at high peptide concentrations. To understand the mechanism of the conformational transition of melittin, we examined in detail the conformation of melittin under various conditions by far-UV circular dichroism at 20 °C. We found that the helical conformation is also stabilized by strong acids such as perchloric acid. The effects of various acids varied largely and were similar to those of the corresponding salts, indicating that the anions are responsible for the salt- or acid-induced transitions. The order of effectiveness of various monovalent anions was consistent with the electroselectivity series of anions toward anion-exchange resins, indicating that the anion binding is responsible for the salt- or acid-induced transitions. From the NaCl-, HCl-, and alkaline pH-induced conformational transitions, we constructed a phase diagram of the anion- and pH-dependent conformational transition. The phase diagram was similar in shape to that of acid-denatured apomyoglobin [Goto, Y., & Fink, A. L. (1990) *J. Mol. Biol. 214*, 803–805] or that of the amphiphilic Lys, Leu model polypeptide [Goto, Y., & Aimoto, S. (1991) *J. Mol. Biol. 218*, 387–396], suggesting a common mechanism of the conformational transition. The anion-, pH-, and peptide concentration-dependent conformational transition of melittin was explained on the basis of an equation in which the conformational transition is linked to proton and anion binding to the titratable groups.

The conformational stability of monomeric α-helical peptides has been useful for elucidating the factors stabilizing or destabilizing the α-helices of globular proteins (Marqusee & Baldwin, 1987; Shoemaker et al., 1987; Bradley et al., 1990; Lyu et al., 1990; Padmanabhan et al., 1990). To extend our understanding of protein folding, it is important to study peptides which form an oligomeric structure stabilized by long-range interactions, because such interactions are also important in determining the conformational stability of peptides and proteins (DeGrado & Lear, 1985; Kim & Baldwin, 1990).

Melittin, a bee venom toxin, is a suitable model for this purpose. Melittin consists of 26 amino acid residues, 5 of which are basic but none of which are acidic (Habermann, 1972). The conformational properties of melittin have been studied extensively in relation to the interaction of peptides with biological membranes (Talbot et al., 1979; Knoppel et al., 1979; Lauterwein et al., 1980; Brown et al., 1980; Bello et al., 1982; Quay & Condie, 1983; Inagaki et al., 1989). It has been shown that, whereas melittin assumes a monomeric helical structure in methanol or in membranes, its conformation in aqueous solution depends on ionic and pH conditions. Whereas melittin at micromolar concentrations is unfolded under conditions of low ionic strength at neutral pH, it adopts a tetrameric helical structure under conditions of high ionic strength or at alkaline pH. The X-ray structure of tetrameric melittin crystallized from aqueous solution has been reported (Terwilliger & Eisenberg, 1982a,b). Because the conformational transition accompanies a monomer-to-tetramer reaction, high peptide concentrations also favor the helical structure

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