Thermodynamics of Protein–Peptide Interactions in the Ribonuclease S System Studied by Titration Calorimetry†

Patrick R. Connelly,‡ Raghavan Varadarajan,§ Julian M. Sturtevant,‡,§ and Frederic M. Richards*,§

Department of Chemistry and Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06511

Received December 27, 1989; Revised Manuscript Received March 28, 1990

ABSTRACT: Two fragments of pancreatic ribonuclease A, a truncated version of S-peptide (residues 1–15) and S-protein (residues 21–124), combine to give a catalytically active complex designated ribonuclease S. Residue 13 in the peptide is methionine. According to the X-ray structure of the complex of S-protein and S-peptide (1–20), this residue is almost fully buried. We have substituted Met-13 with seven other hydrophobic residues ranging in size from glycine to phenylalanine and have determined the thermodynamic parameters associated with the binding of these analogues to S-protein by titration calorimetry at 25 °C. These data should provide useful quantitative information for evaluating the contribution of hydrophobic interactions in the stabilization of protein structures.

Interactions involving buried hydrophobic residues are thought to be important determinants of protein structure and stability. However, quantitative information on the energetics of such interactions localized in specific portions of known protein structures is needed in order to clarify their role in stabilization. A number of recent studies have approached this problem by studying the effect that substitutions of buried hydrophobic amino acids have on the stability of proteins (Shortle & Meeker, 1986; Kellis et al., 1988; Matsumura et al., 1988; Yutani et al., 1987). In such studies, one proceeds by disrupting mutant and wild-type protein structures in solution by means of increasing temperature or concentration of denaturant, while following the reaction process optically or calorimetrically. Subsequently, one extracts the various thermodynamic parameters characterizing the disruption process and extrapolates the extracted parameters to a particular set of reference conditions to reveal the energetic differences in stability. However, it has been difficult to obtain quantitative values for specific interactions from such measurements, since one cannot ascribe the relative energetic contributions of the folded and unfolded states to the net stability differences. In addition, interpretation of such studies is often complicated by factors such as irreversible denaturation, the possible presence of folding intermediates, and the need for making large extrapolations (to zero denaturant concentration, neutral pH, or a reference temperature). We have avoided these complications in our approach by investigating the effects that specific substitutions of buried hydrophobic residues have on the simple binding reactions between S-protein and various S-peptide analogues.[1]

Bovine pancreatic ribonuclease A (RNase-A) may be cleaved with subtilisin at the peptide bond between residues 20 and 21 to give the S-protein and the N-terminal S-peptide (Richards & Vithyathil, 1959). These fragments may be separated, and can be reconstituted to give rise to the product ribonuclease S (RNase-S) that has a structure very similar to that of RNase-A (Wyckoff et al., 1970). Residues 3–13 of the peptide portion of RNase-S form an α-helix, just as these residues do in uncleaved RNase-A. Residues 16–20 are evidently not important for binding and are not clearly defined in the crystal structure of RNase-S (Wyckoff et al., 1970). A complex of a peptide containing only the first 15 residues of S-peptide (S15) with S-protein has been shown to be structurally identical with RNase-S (Taylor et al., 1981). The hydrophobic residues in S-peptide thought to be particularly important for binding are Met-13 and Phe-8 (Hearn et al., 1971; Scoffone et al., 1967). In order to study interactions among buried hydrophobic groups, peptides have been synthesized in which the methionine at position 13 has been replaced by seven other hydrophobic amino acids (glycine, alanine, α-amino-*n*-butyric acid, valine, leucine, isoleucine, phenylalanine). The thermodynamic properties of the reactions of these various peptide analogous with S-protein have been determined by titration microcalorimetry.

Probing the nature of stabilizing interactions in this manner can be done isothermally, without the use of denaturants, and in a simple 1:1, well-defined reaction. Changes in the structure of the unbound peptide (the equivalent of the denatured state) can be monitored by circular dichroism. There is no need to extrapolate the derived parameters to other conditions. Direct comparisons between the free energies, enthalpies, and entropies of the reactions for various peptide analogues are made under identical solution conditions. The stoichiometry of the reaction process automatically imposes the simplifying constraint of a two-state process. That is to say, the species present in solution are either complexed or dissociated. The two-state assumption has not been found to be universally applicable to the denaturation of proteins by means of heating or addition of a denaturant (Sturtevant, 1987; Kitamura & Sturtevant, 1989). Additionally, seven of the peptide protein complexes

† We acknowledge the support of the National Institute of General Medical Sciences, Grant GM-22778 (F.M.R.), and the National Science Foundation, Grant PCM-8417341 (J.M.S.). R.V. is supported by a postdoctoral fellowship from the Damon Runyon-Walter Winchell Cancer Fund.

‡ Department of Chemistry.

§ Department of Molecular Biophysics and Biochemistry.

[1] Abbreviations: RNase-A, bovine pancreatic ribonuclease; RNase-S, product of proteolytic cleavage of bond 20–21 in RNase-A; S-protein, protein component of RNase-S, residues 21–124; S-peptide, peptide component of RNase-S, residues 1–20; S15, a truncated version of S-peptide, residues 1–15 with a C-terminal amide; ANB, α-amino-*n*-butyric acid; M13X, usual nomenclature for single-site mutants at position 13.

0006-2960/90/0429-6108$02.50/0

have been crystallized and the structures are currently being determined by X-ray crystallographic analysis.

Materials and Methods

Materials. RNase-S was obtained from Sigma. S-protein was prepared from RNase-S by the method of Doscher and Hirs (1967) and stored either lyophilized or as a frozen solution at −20 °C. Peptides were synthesized as C-terminal amides by Multiple Peptide Systems Inc., San Diego, CA. Crude peptides were purified by reverse-phase HPLC using a Vydac C-18 preparative column. Peptide purity was checked by fast atom bombardment mass spectrometry. The concentrations of stock solutions of peptides were determined by quantitative amino acid analysis. Each stock solution was then divided into 500-μL aliquots, lyophilized, and stored at −20 °C. Prior to use each aliquot was redissolved in an appropriate amount of buffer. An extinction coefficient at 280 nm of 9.56 mM^{-1} cm^{-1} for S-protein in water was determined by quantitative amino acid analysis.

Titration Calorimetry. The calorimetric experiments were performed with the OMEGA titration calorimeter from MicroCal, Inc., Northampton, MA. S-Protein solution of known concentration (quantitated optically) was transferred to the reaction cell. The reference cell of the calorimeter serves only as a thermal reference to the sample cell and it was filled with water (Wiseman et al., 1989). A 100-μL injection syringe was filled with peptide solution, typically 30 times higher in concentration than the initial S-protein solution. After an initial equilibration period of approximately 10 min, stirring at 400 rpm was begun. After another equilibration period of 10 min was finished as judged by base-line stability, 5-μL injections were performed at intervals of approximately 4.5 min. Injections were continued until all of the protein had reacted. Control dilutions of the peptides into buffer and buffer into protein were performed and the total observed heat effects were corrected for these small contributions.

Data Analysis. The reaction heat observed at the ith step, q_i, of a titration experiment for a simple association reaction, $M + X \rightarrow MX$, is equal to the molar enthalpy of binding, ΔH, times the number of moles of product formed upon addition of the ligand (i.e., moles of product, MX, formed on going from step $i - 1$ to step i in the titration):

$$q_i = \Delta H[MX_i - MX_{(i-1)}] \quad (1)$$

The amount of product present at a step i, $[MX]_i$, is a function of the total concentration of peptide in the cell, $[X_T]_i$, the total concentration of protein, $[M_T]_i$, and the association constant, K. The values of protein and peptide concentration at each step are determined by the dilution factor and the initial concentrations of each reactant, $[X_T]_0$ and $[M_T]_0$. The dilution factor, D, is calculated from the volume of the reaction cell, V (1.3815 mL), and the injection volume, v, according to the expression $D = (V - v)/V$ (Parody-Morreale et al., 1987). In order to account for small uncertainties in the initial concentration of peptide, we have introduced a fitting parameter n. Thus, $[X_T]_i = n[X_T]_0(1 - D^i)$ and $[M_T]_i = [M_T]_0 D^i$. In order to use eq 1, the expression for $[MX]_i$ in terms of K, $[X_T]_i$, and $[M_T]_i$ must be substituted. This may be obtained from the conservation equation $[MX]_i = [X_T]_i - [X]_i$. The term $[X]_i$ is obtained as the positive real root of the quadratic expression $K[X]_i^2 + (1 + K[m_T]_i - K[X_T]_i)[X]_i - [X_T]_i = 0$.

The values of ΔH, K, and n are determined by a nonlinear least-squares fit to the data utilizing the Marquardt algorithm (Bevington, 1969). An average value of $n = 0.98 \pm 0.03$ was obtained from all experiments reported here for all peptides under the range of conditions employed, indicating that, in fact, the initial concentrations of peptide are quite accurately known.

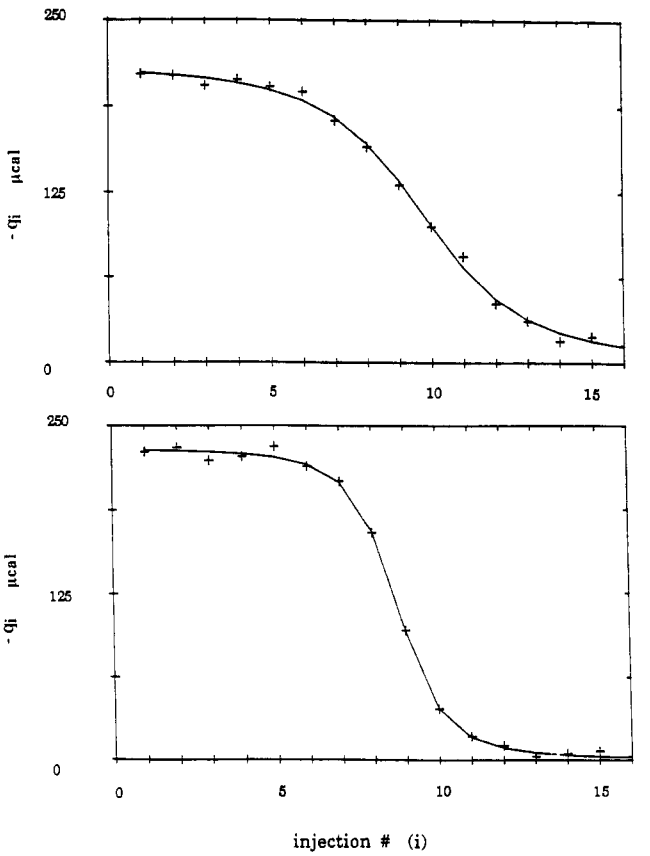

FIGURE 1: Calorimetric titrations of M13ANB (5-μL injections of 1.4 mM M13ANB into 0.050 mM S-protein, top) and M13L (5-μL injections of 1.4 mM M13L into 0.043 mM S-protein, bottom) at 25 °C, pH 6.0. The solid line represents the best fit of the heat of injection (q_i) versus the injection step (i) based on eq 1 with parameters $K = 7.1 \times 10^5$ M^{-1}, $\Delta H = -31.0$ kcal/mol, and $n = 1.00$ for M13ANB and $K = 3.2 \times 10^6$ M^{-1}, $\Delta H = -34.1$ kcal/mol, and $n = 1.00$ for M13L.

Wiseman et al. (1989) have pointed out that the shape of the titration curve is governed by the unitless parameter $c = K[M_T]_0$. They suggest designing an experiment so that the value of c is between 1 and 1000. The experiments reported here cover the range of c from 2 to 500, with the exception of the glycine-substituted peptide.

Results

Figure 1 shows two titration curves typical of the various S-peptide analogues. The solid lines were calculated by using the values of the best-fit parameters given in Table I. In all fits the standard error of a point (Bevington, 1969) is less than 0.03% of the total integral heat of binding. This compares favorably with the previously reported performance of the titration instrument employed here (Wiseman et al., 1989). Standard errors for each data set, calculated from the variance and number of degrees of freedom for the data set and fit, are listed in Tables I and II for pH 6 and 5, respectively. In addition, error estimates of the fitted parameters, ΔH and K, were obtained from the asymptotic covariance matrix (Bevington, 1969; Ratkowski, 1983) and are included in the tables. Errors on parameter averages were calculated as standard deviations from duplicate or triplicate experiments. In nearly all cases, repeat experiments gave parameter values within the range of asymptotic error estimates of the individual determinations. The binding parameters for the glycine-substituted peptide were considerably more difficult to determine than those for the other peptides due to its low binding affinity,

Table I: Thermodynamic Parameters Obtained upon Titrating S-Protein with Various S-Peptide Analogues at pH 6.0[a]

peptide	$[X_T]_0$ (mM)	$[M_T]_0$ (mM)	K (M^{-1})	$\Delta G°$ (kcal/mol)	ΔH (kcal/mol)	$T\Delta S°$ (kcal/mol)	sep (μcal)
M13G	7.12	0.317	2.0 (±0.8) × 10^3	−4.5 ± 0.2	−40.6 ± 3.2	−36.1 ± 4.0	2.7
M13A	8.73	0.198	8.9 (±0.7) × 10^3	−5.4	−33.9 ± 0.5	−28.5	3.3
	7.63	0.194	9.7 (±0.2) × 10^3	−5.4	−35.3 ± 1.2	−29.9	9.7
			9.3 (±0.4) × 10^3	**−5.4 ± 0.0**	**−34.6 ± 0.7**	**−29.2 ± 0.7**	
M13ANB	5.0	0.099	4.3 (±0.7) × 10^5	−7.7	−33.2 ± 0.6	−25.5	10.0
	1.40	0.050	7.1 (±1.5) × 10^5	−8.0	−31.0 ± 0.6	−23.0	4.4
			5.7 (±1.4) × 10^5	**−7.9 ± 0.2**	**−32.1 ± 1.1**	**−24.3 ± 1.3**	
M13V	2.36	0.051	4.0 (±1.7) × 10^6	−9.0	−36.7 ± 0.9	−27.7	10.0
	1.40	0.049	6.5 (±0.2) × 10^6	−9.3	−35.5 ± 0.6	−26.2	5.0
			5.3 (±1.3) × 10^6	**−9.2 ± 0.2**	**−36.1 ± 0.6**	**−27.0 ± 0.8**	
M13I	1.3	0.051	7.6 (±2.4) × 10^6	−9.4	−35.3 ± 0.7	−25.9	6.3
	1.4	0.051	5.9 (±2.2) × 10^6	−9.2	−33.7 ± 0.7	−24.5	6.5
			6.8 (±0.9) × 10^6	**−9.3 ± 0.1**	**−34.5 ± 0.8**	**−25.2 ± 0.7**	
M13L	1.40	0.043	3.5 (±0.6) × 10^6	−8.9	−33.2 ± 0.4	−24.3	3.5
	1.40	0.079	2.8 (±0.5) × 10^6	−8.9	−34.9 ± 0.4	−26.0	5.0
			3.2 (±0.4) × 10^6	**−8.9 ± 0.0**	**−34.1 ± 0.9**	**−25.2 ± 0.9**	
S15	0.89	0.025	1.0 (±0.4) × 10^7	−9.6	−40.1 ± 0.9	−30.5	5.4
	0.91	0.030	7.1 (±2) × 10^6	−9.3	−39.2 ± 0.8	−29.9	4.5
	1.00	0.029	9.5 (±4) × 10^6	−9.5	−38.5 ± 1.2	−29.0	5.0
			9.0 (±1.5) × 10^6	**−9.5 ± 0.1**	**−39.3 ± 0.6**	**−29.8 ± 0.5**	
M13F	4.80	0.196	8.9 (±0.9) × 10^4	−6.8	−35.4 ± 0.4	−28.6	8.8
	4.80	0.201	8.8 (±0.1) × 10^4	−6.7	−36.1 ± 0.5	−29.4	11.0
			8.9 (±0.1) × 10^4	**−6.8 ± 0.1**	**−35.8 ± 0.4**	**−29.0 ± 0.4**	

[a] All experiments were performed at 25 °C in 50 mM sodium acetate and 100 mM NaCl. Errors on fitted parameters, ΔH and K, are calculated for individual experiments as described under Materials and Methods. Average values of parameters are given in boldface with errors calculated as standard deviations of repeated experiments. $[X_T]_0$ is the peptide concentration in the syringe; $[M_T]_0$ is the initial concentration of protein in the cell; and sep is the standard error of a point in the fit for each data set (Bevington, 1969).

Table II: Thermodynamic Parameters Obtained upon Titrating S-Protein with Various S-Peptide Analogues at pH 5.0[a]

peptide	$[X_T]_0$ (mM)	$[M_T]_0$ (mM)	K (M^{-1})	$\Delta G°$ (kcal/mol)	ΔH (kcal/mol)	$T\Delta S°$ (kcal/mol)	sep (μcal)
M13ANB	3.00	0.099	2.4 (±0.3) × 10^5	−7.3	−35.8 ± 0.5	−28.5	5.7
M13V	1.37	0.049	3.7 (±0.8) × 10^6	−9.0	−40.1 ± 0.5	−31.1	4.5
M13L	1.84	0.049	2.2 (±0.4) × 10^6	−8.7	−36.9 ± 0.5	−28.2	4.0
S15	0.65	0.035	2.6 (±0.6) × 10^6	−8.8	−44.2 ± 0.9	−35.4	4.8
	1.11	0.025	4.6 (±0.8) × 10^6	−9.1	−43.9 ± 0.8	−34.8	4.8
	1.14	0.034	2.6 (±0.6) × 10^6	−8.8	−40.7 ± 0.8	−31.9	5.8
			3.4 (±0.8) × 10^6	**−8.9 ± 0.1**	**−42.9 ± 1.7**	**−34.0 ± 1.4**	
M13F	3.45	0.197	4.1 (±0.8) × 10^4	−6.3	−37.0 ± 1.2	−30.7	8.9
	1.38	0.054	5.4 (±1.1) × 10^4	−6.5	−39.8 ± 1.4	−33.3	2.3
			4.8 (±0.7) × 10^4	**−6.4 ± 0.1**	**−38.4 ± 1.4**	**−32.0 ± 1.3**	

[a] All experiments were performed at 25 °C in 50 mM sodium acetate and 100 mM NaCl. Errors have been calculated as described in the legend to Table I.

hence the larger errors of the parameters characterizing M13G binding.

Table III gives the values of the different thermodynamic parameters calculated relative to the S15 peptide so that for $J = G$, H, or S, $\Delta\Delta J_X = \Delta J_{M13X} - \Delta J_{S15}$ where X is the single letter code for the substituted amino acid. The differences in enthalpies, free energies, and entropies do not change appreciably with pH, although the absolute quantities do, as has been reported previously (Schreier & Baldwin, 1976). The thermodynamic properties of the reactions are determined by the energetic differences between the products and reactants—the RNase-S complex and the S-protein and S-peptide. Therefore, the differences listed in Table III reflect the net energetic differences between the complexes and the respective free peptides.

A direct comparison of the thermodynamic parameters described above requires knowledge of the conformation of the reactants. The S-protein component is the same for each experiment, but the S-peptide variants may differ in their helical content. The extensive literature on this system (Fillipi et al., 1981; Shoemaker et al., 1987; Mitchinson & Baldwin, 1986) does not happen to include the sequences used in this study. A measure of the differences in helical stability can be obtained by monitoring the mean residue ellipticities of the peptides at 222 nm by circular dichroism as a function of

Table III: Difference Thermodynamic Parameters for the Interaction of S-Peptide Analogues with S-Protein Relative to S-Peptide/S-Protein Interactions[a]

	$\Delta\Delta G^0$ (kcal/mol)		$\Delta\Delta H$ (kcal/mol)		$T\Delta\Delta S°$ (kcal/mol)	
peptide	pH 6	pH 5	pH 6	pH 5	pH 6	pH 5
M13G	5.0		(−1.3)		(−6.3)	
M13A	4.1		4.7		0.6	
M13ANB	1.6	1.6	7.2	7.1	5.5	5.5
M13V	0.3	−0.1	3.2	2.8	2.8	2.9
M13I	0.2		4.8		4.6	
M13L	0.6	0.2	5.2	6.0	4.6	5.8
M13F	2.7	2.5	3.5	4.5	0.8	2.0

[a] Tabulated values were calculated from average values of parameters listed in Table I (pH 6.0) and Table II (pH 5.0). Average errors for the various difference thermodynamic parameters can be approximated by adding errors of ΔJ_{S15} and ΔJ_{M13X} for each $\Delta\Delta J$. This leads to average errors of ±0.2 for $\Delta\Delta G°$, ±0.8 for $\Delta\Delta H$, and ±0.9 for $T\Delta\Delta S°$. Enthalpy and entropy differences for M13G are given in parentheses due to the larger uncertainties in these parameters.

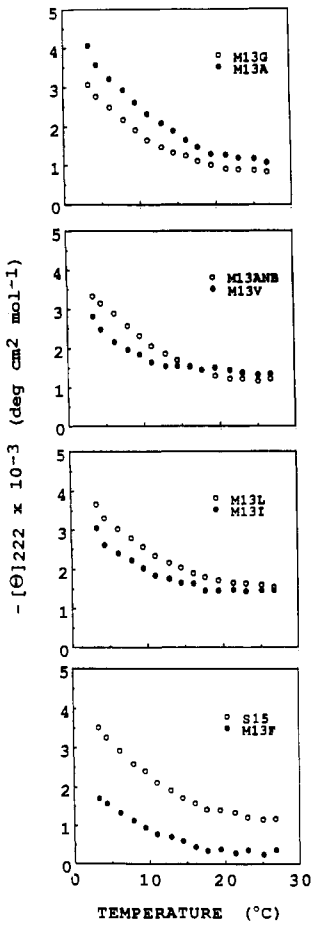

FIGURE 2: Mean residue ellipticities of S15 and its analogues as a function of temperature.

Table IV: Estimates of Peptide Helicity at 3.3 °C

peptide	$-[\theta]_{222} \times 10^{-3}$ (deg cm^2 dmol^{-1}) at 25 °C (A)	$-[\theta]_{222} \times 10^{-3}$ (deg cm^2 dmol^{-1}) at 3.3 °C (B)	% helicity at 3.3 °C [100(B − A)/(29.2 − A)][a]
M13G	0.82	3.06	7.9
M13A	1.06	4.06	10.7
M13ANB	1.21	3.33	7.6
M13V	1.34	2.81	5.3
M13L	1.52	3.66	7.7
M13I	1.46	3.04	5.7
S15	1.17	3.52	8.4
M13F	0.35	1.68	4.8

[a] Values are calculated by assuming that 100% helix has an ellipticity of 29 200 deg cm^2 dmol^{-1} (Mitchinson & Baldwin, 1986) and that all peptides have 0% helicity at 25 °C.

temperature. At pH 5.3, Mitchinson and Baldwin report that S15 peptide is 15% folded at 3 °C and appears to be fully unfolded at 25 °C. We have carried out such measurements here for all of the peptides and the results are summarized in Figure 2 and Table IV.

Discussion

Comparison with Previous Results. In order to check the general consistency of the data available on RNase-S, we have made three comparisons of our data with results from previous work. First, we measured the heat of association of S-peptide with S-protein at pH 7.0, 25 °C, in 50 mM phosphate buffer and 100 mM NaCl and obtained $\Delta H = -40.2$ kcal/mol. At pH 7.02, 25 °C, Hearn et al. (1971) reported a value of $\Delta H = -39.8$ kcal/mol in an unbuffered solution. Second, we have estimated the number of protons released in the association reaction of S15 with S-protein from our measurements at the

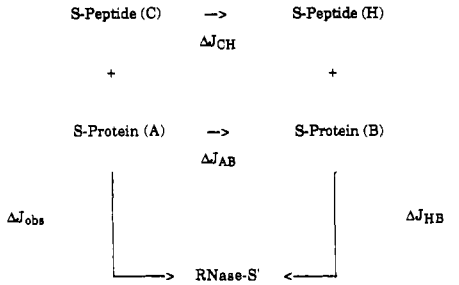

FIGURE 3: Reaction scheme for S-peptide binding to S-protein showing three contributions to the overall observed thermodynamic state properties, ΔJ_{obs}: (1) the coil to helix transition of the peptide, ΔJ_{CH}; (2) the conformational change in S-protein associated with peptide binding, ΔJ_{AB}; (3) the association of helix and protein in the B conformation, ΔJ_{HB}.

two values of pH, obtaining 0.42 mol of protons released/mol of reaction. Hearn et al. (1971) determined a value of 0.27 by measuring the pH change directly in an unbuffered solution after mixing S-peptide and S-protein and then back-titrating. Schreier and Baldwin (1976) reported a value of 0.4 for S-peptide at 0 °C from measurements of association constants of S-peptide and S-protein obtained over a range of pH. Third, on the basis of the enthalpy reported here and the heat capacity reported by Hearn et al. (1971), we estimated the free energy of binding of S15 at 35 °C and pH 5.0. Interpolating to pH 5.3 on the basis of the number of protons released upon binding enables a comparison with the results of Mitchinson and Baldwin (1986) that were obtained at this pH using a circular dichroism technique in which protein concentrations of a few micromolar were used. They obtained $\Delta G = -8.4$ kcal/mol, a value only slightly higher than ours (−7.8), presumably due to the fact that phosphate was used in their buffer system. Phosphate is known to stabilize the complex (Mitchinson & Baldwin, 1986). Additionally, the S15 peptide used by them contained a C-terminal carboxylate, in contrast to our peptides in which the C terminus was amidated.

Our titration experiments were performed at different concentrations of protein (25–300 μM). All data sets for a given peptide fit well to a simple single-site reaction model having the same values of resolved parameters within experimental error. The lack of concentration dependence argues that there is no significant change in the aggregation state of the protein upon binding peptide and hence that S-protein is monomeric in solution under the conditions we have used.

Significance of the Differences in Thermodynamic Properties of the Reactions. The thermodynamic properties of the protein/peptide reaction may be conceptually broken up into three parts (Figure 3). The reaction scheme illustrates that a given observed thermodynamic state property for the overall reaction, ΔJ_{obs}, is given by the sum of two conformational events and an association event: (1) the coil to helix conformational change of the peptide ΔJ_{CH}; (2) the change in conformation of the S-protein from the state it assumes when no peptide is bound (state A) to the conformation that it assumes in RNase-S (state B), ΔJ_{AB}; (3) the association of helix with protein in conformation B, ΔJ_{HB}.

Shown in Figure 2 are mean residue ellipticities of the peptides as a function of temperature. Increasing the temperature up to 35 °C resulted in no further change in ellipticity from the value observed at 25 °C, indicating that each of the peptides is fully unfolded at 25 °C. Table IV contains estimates of peptide helicities at 3.3 °C. The data summarized in Figure 2 and Table IV indicate that the first contribution, ΔJ_{CH}, is likely to be small and approximately the same for the various peptides.

All seven of the complexes so far crystallized yield crystals with identical morphology. The six complexes (native, M13ANB, M13V, M13L, M13I, M13F) for which X-ray data have been collected belong to the same space group and have very similar unit cell parameters. These facts as well as preliminary electron density maps suggest that the conformation of complexed S-protein and hence the ΔJ_{AB} term are likely to be very similar in the different complexes.

The *relative* stabilities are therefore governed by the difference in the energetics of taking the residue in the isolated peptide helix from the aqueous solution and placing it in contact with the hydrophobic core of the protein. The observed thermodynamic differences may be broken into three components: (a) changes in the hydration of residue 13 in the free peptides relative to S15, (b) changes in the hydration of residue 13 in the complexed peptides relative to the S15 complex, and (c) changes in the packing interactions of residue 13 with the hydrophobic core of the protein in the various complexes.

The contribution of term a is temperature dependent. According to Privalov and Gill (1988) the free energy of hydration of nonpolar substances ($\Delta G_{hydration}$) may be estimated as follows:

$$\Delta G_{hydration} = \Delta C_p[(T - T_s) - T \ln (T/T_s)] \quad (2)$$

where T_s has been asserted to be the temperature at which the influence of exposed nonpolar groups on water vanishes (Privalov & Gill, 1988). Estimates of this temperature range from about 380 to 410 K (Baldwin, 1986; Privalov & Gill, 1988). The experimentally observable quantity ΔC_p is the change in heat capacity at constant pressure associated with transfer of the nonpolar substance to water. Equation 2 calculates the contribution of the hydration component to the overall experimentally measured free energy of transfer and was derived by making the following assumptions. First, there exists a temperature T_s defined above. Second, the ΔC_p associated with the transfer process is independent of temperature and solely reflects contributions due to hydration.

The heat capacity change ΔC_p, for the dissolution of various nonpolar substances (solids, gases, and liquids) in water is proportional to their surface area (Gill & Wadsö, 1976; Privalov & Gill, 1988; Murphy et al., 1989). Recently, Spolar et al. (1989) have shown that the heat capacity change associated with protein denaturation correlates with the amount of nonpolar surface area, ΔA_{np}, exposed to water upon unfolding. For transfers of nonpolar substances from gases, liquids, and protein interiors into water it has been observed that

$$\Delta C_p = 0.28\Delta A_{np} \quad (3)$$

In eq 3, ΔC_p is expressed in units of cal K^{-1} mol^{-1} and ΔA_{np} in units of angstroms squared. The fact that the multiplying factor is the same for gases, liquids, and proteins has been taken as evidence that the observed heat capacity change in all three cases is largely associated with solvation by water (Privalov & Gill, 1988).

Substituting the above expression for ΔC_p, $T = 298$ K, and $T_s = 385$ K in eq 2, one obtains

$$\Delta G_{hydration}(M13G) - \Delta G_{hydration}(S15) = -2.99[\Delta A_{np}(M13G) - \Delta A_{np}(S15)] \quad (cal\ mol^{-1}) \quad (4)$$

Assuming that residue 13 is fully buried in complexes of both M13G and S15 with S-protein and using the static accessibilities of G and M in the model tripeptides Ala-Gly-Ala and Ala-Met-Ala (Lee & Richards, 1971) to approximate the solvent-exposed surface areas of these amino acids in the free peptides M13G and S15, we estimate $\Delta G_{hydration}(M13G) - \Delta G_{hydration}(S15)$ to be 0.26 kcal/mol. Thus the hydration term appears to stabilize binding of M13G to S-protein relative to S15 by 0.26 kcal/mol. For other peptides the absolute magnitude of this term would be even smaller, suggesting that the observed thermodynamic differences are due primarily to differences in the packing and/or hydration of residue 13 in the various complexes.

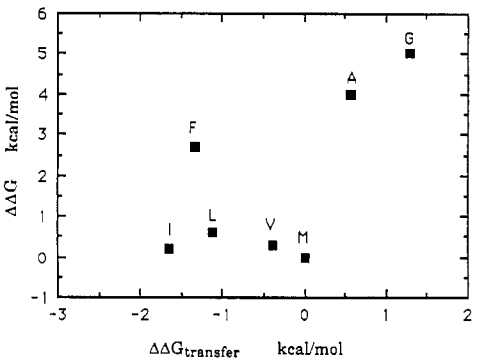

FIGURE 4: Free energy of binding ($\Delta\Delta G°$) of peptide analogues at pH 6.0, 25 °C, plotted against the free energy of transfer ($\Delta\Delta G°$ transfer) of amino acids from water to ethanol (Tanford, 1962). In both cases free energies are plotted relative to the values for Met.

The magnitudes of these components will depend on the structural details of the arrangement of protein and aqueous solvent around residue 13. A quantitative analysis of the relative importance of these terms will only be feasible once refinement of the structures of the complexes is complete.

In attempts to understand the differences in thermodynamic properties of mutant proteins, several authors have argued for and/or reported on a linear correspondence between the free energy differences between proteins engineered with specific hydrophobic alterations and the free energy of transfer of amino acids from organic solvents to water (Kellis et al., 1989; Matsumura et al., 1988; Yutani et al., 1987). The problems presented by such an analysis have been discussed previously (Bello, 1978; Privalov & Gill, 1988). As can be seen from Figure 4, we do not observe such a linear correlation. Additionally, neither the enthalpic nor entropic contributions to the observed free energy change (Table III) show such a correlation. The energetics of packing interactions of residue 13 with its surroundings are highly distance dependent. Such interactions would not generally be expected to scale simply with the surface area or hydrophobicity of the buried residue. They will depend on the detailed structural changes in both protein and aqueous solvent caused by the amino acid substitution.

Comparison of Peptide–Protein Interactions with Protein Denaturation. The reaction between S-peptide and S-protein is an intermolecular process. This contrasts to the denaturation or folding of a protein, which takes place intramolecularly. As a means of establishing a formal comparison of these two types of processes, we make use of a generalized equilibrium constant for a reaction at constant pressure (Gill et al., 1985; Wyman, 1984). More specifically, we wish to compare reactions of the form

$$A_0 \rightarrow A_1 \quad (5a)$$

with the generalized equilibrium constant

$$\Lambda_A = [A_1]/[A_0] \quad (5b)$$

to reactions of the form

$$B_0X_0 \rightarrow B_1 + X_1 \quad (6a)$$

with the generalized equilibrium constant

$$\Lambda_B = [B_1]/[B_0X_0] \quad (6b)$$

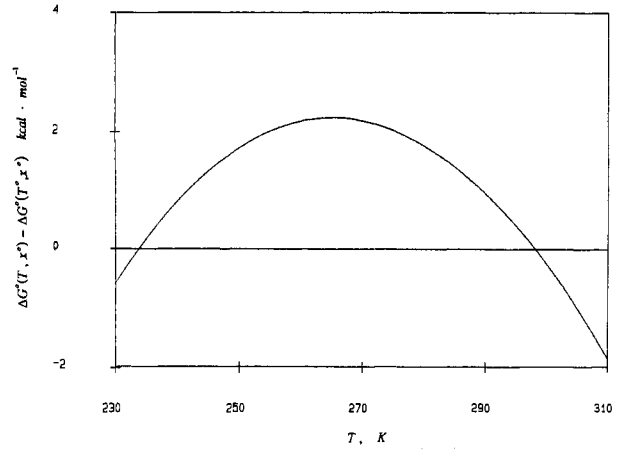

FIGURE 5: Free energy of dissociation of the S15–S-protein complex as a function of temperature relative to its value at 298 K. The curve was calculated by using eq 7 with ΔH = 39.3 kcal/mol (Table I), ΔC = 1.1 kcal mol^{-1} K^{-1} (Hearn et al., 1971), and $x = x°$. The reference state parameters $T°$ and $x°$ are chosen such that $T°$ = 298 K and $x°$, the ligand concentration at half-saturation and $T = T°$, is 0.11 μM. At low temperatures one predicts a phenomenon that might be described as the intermolecular analogue of cold denaturation—cold dissociation.

The first reaction, which we call reaction A (eq 5a), is intramolecular and depends only on temperature (for a given buffer, pH, and fixed activity of any other ligands). The second reaction, which we call reaction B (eq 6a), is intermolecular and depends explicitly on ligand activity (taken as concentration here) as well as temperature. The generalized equilibrium constant for both processes may be expressed as follows:

$$\Lambda_Z = \Lambda_Z^\circ \frac{x°}{x} \exp\left\{\left[\frac{-\Delta H_Z^\circ}{R}\left(\frac{1}{T} - \frac{1}{T°}\right)\right] + \left[\frac{T°\Delta C_Z}{R}\left(\frac{1}{T} - \frac{1}{T°}\right)\right] - \left[\frac{\Delta C_Z}{R} \ln \frac{T}{T°}\right]\right\} \quad (7)$$

where Λ_Z° is the equilibrium constant at $T = T°$ and $x = x°$ and ΔC_Z is the heat capacity change at constant pressure for the process and Z = A or B. Typically, for protein denaturation, $T°$ is chosen as the temperature at which half of the protein species are denatured and half are in the native conformation so that $\Lambda_A(T°) = \Lambda_A^\circ = 1$. Also $x = x°$ (constant ligand activity) so that the ligand-binding term does not contribute to Λ_A. For reaction B, we must choose a reference free ligand concentration as well as a reference temperature. We choose $x°$ to be the concentration of ligand at half-saturation and $T = T°$ so that $\Lambda_B(T°, x°) = \Lambda°_B = 1$.

A plot of the free energy of dissociation of the S15–S-protein complex, $\Delta G_B = -RT \ln \Lambda_B$, as a function of temperature, relative to its value at 25 °C and with $x = x°$, is shown in Figure 5. This representation is analogous to the stability curves for protein denaturation discussed by Becktel and Schellman (1987). The curve was calculated by using the value of ΔH_B measured at 25 °C, pH 6.0 (Table I), and the known value of ΔC_B (Hearn et al., 1971). The large ΔC_B of the reaction ensures the observed temperature dependence of the stability seen in Figure 5. Presumably, the exposure of the nonpolar groups upon dissociation of RNase-S is the origin of the large heat capacity change of the reaction, as has been proposed for protein denaturation (Kauzmann, 1959; Privalov & Gill, 1988; Spolar et al., 1989). Just as in the case of protein denaturation, there is a narrow temperature range within which the complex is stable. Upon increasing or decreasing temperature, proteins undergo denaturation. Similarly, as one increases the temperature from 25 °C, the binding of S15 to the S-protein becomes weaker. At lower temperatures, we predict that binding would become progressively stronger until just below 0 °C. Upon further lowering of the temperature, we predict what might be described as the intermolecular analogue of cold denaturation—cold dissociation. Cold dissociation is predicted to occur at a substantially higher temperature than the analogous process of cold denaturation in RNase-A. This is because of the higher ΔC_p to ΔH ratio for the former process. Calorimetric experiments at various temperatures and refined structural data for the peptide–protein complexes may provide a sharper picture of these phenomena and further clarify the general energetic features of the RNase-S complex.

ACKNOWLEDGMENTS

We thank the Yale University Medical School Protein and Nucleic Acid Chemistry Facility for performing the amino acid analysis and Dr. W. J. McMurray for performing the mass spectrometry.

REFERENCES

Baldwin, R. L. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 8069–8072.
Becktel, W. J., & Schellman, J. A. (1987) *Biopolymers 26*, 1859–1877.
Bello, J. (1978) *Int. J. Pept. Protein Res. 12*, 38–41.
Bevington, P. R. (1969) *Data Reduction and Error Analysis in the Physical Sciences*, p 236, McGraw-Hill, New York.
Doscher, M. S., & Hirs, C. H. W. (1967) *Biochemistry, 6*, 304–312.
Fillipi, B., Chessa, G., & Borin, G. (1981) *J. Mol. Biol. 147*, 597–600.
Gill, S. J., & Wadsö, I. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2955–2958.
Gill, S. J., Richey, B., Bishop, G., & Wyman, J. (1985) *Biophys. Chem. 21*, 1–14.
Hearn, R. P., Richards, F., Sturtevant, J. M., Watt, G. D. (1971) *Biochemistry 10*, 806–816.
Kauzmann, W. (1959) *Adv. Protein Chem. 14*, 763–764.
Kellis, J. T., Nyberg, K., Sali, D., & Fresht, A. (1988) *Nature 333*, 784–786.
Kellis, J. T., Nyberg, K., & Fersht, A. (1989) *Biochemistry 28*, 4914–4922.
Kitamura, S., & Sturtevant, J. (1989) *Biochemistry 28*, 3788–3792.
Lee, B., & Richards, F. M. (1971) *J. Mol. Biol. 55*, 379–400.
Matsumura, M., Becktel, W. J., & Matthews, B. W. (1988) *Nature 334*, 406–410.
Mitchinson, C., Baldwin, R. L. (1986) *Proteins 1*, 23–33.
Murphy, K. P., Privalov, P. L., Gill, S. J. (1989) *Science 247*, 559–561.
Parody-Morreale, A., Robert, C. H., Bishop, G. A., Gill, S. J. (1987) *J. Biol. Chem. 262*, 10994–10999.
Privalov, P. L., Gill, S. J. (1988) *Adv. Protein Chem. 39*, 191–234.
Ratkowski, D. (1983) *Nonlinear Regression Analysis*, p 41, Marcel Dekker, Inc., New York.
Richards, F. M., & Vithyathil, P. J. (1959) *J. Biol. Chem. 234*, 1459–1465.
Schreier, A. A., & Baldwin, R. L. (1976) *J. Mol. Biol. 105*, 409–426.

Scoffone, E., Rocchi, R., Marchiori, F., Moroder, L., Marzotto, A., & Tamburro, A. (1967) *J. Am. Chem. Soc. 89*, 5450.

Shoemaker, K. R., Kim, P. S., York, E. J., Stewart, J. M., & Baldwin, R. L. (1987) *Nature 326*, 563–567.

Shortle, D., & Meeker, A. K. (1986) *Proteins 1*, 81–89.

Spolar, R. S., Ha, J., & Record, T. M. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 8382–8385.

Sturtevant, J. (1987) *Annu. Rev. Phys. Chem. 38*, 463–488.

Taylor, H. C., Richardson, D. C., Richardson, J. S., Wlodawer, A., Komoriya, A., & Chaiken, I. M. (1981) *J. Mol. Biol. 149*, 313–317.

Wiseman, T., Williston, S., Brandts, J., & Lin, L. (1989) *Anal. Biochem. 179*, 131–137.

Wyckoff, H. W., Tsernoglu, D., Hanson, A. W., Knox, J. R., Lee, B., & Richards, F. M. (1970) *J. Biol. Chem. 245*, 305–328.

Wyman, J. (1984) *Q. Rev. Biophys. 17*, 453–488.

Yutani, K., Ogasahara, K., Tsujita, T., & Sugino, Y. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 4441–4444.