Heat Capacity Changes for Protein–Peptide Interactions in the Ribonuclease S System†

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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. We have substituted the wild-type residue Met-13 with six other hydrophobic residues ranging in size from alanine to phenylalanine and have determined the thermodynamic parameters associated with binding of these analogues to S-protein by titration calorimetry in the temperature range 5–25 °C. The heat capacity change (ΔC_p) associated with binding was obtained from a global analysis of the temperature dependences of the free energies and enthalpies of binding. The ΔC_p's were not correlated in any simple fashion with the nonpolar surface area (ΔA_{np}) buried upon binding.

A significant feature in the processes of protein folding and the binding of certain ligands to proteins is the fact that a number of nonpolar amino acid side chains are removed from water and are packed together in the protein interior. These phenomena are a consequence of what have been called hydrophobic interactions. Such interactions are believed to be important in determining the compact structure and stability of proteins (Kauzmann, 1959). It is useful to recognize at the outset that hydrophobic interactions may be divided into two parts. One part, the hydration component, consists of the interactions of nonpolar groups with the solvent water. Another part, the packing component, consists of the interactions between the nonpolar groups in the interior of the protein. Knowledge of the nature and magnitude of the energetics of these interactions in proteins of known structure is needed in order to clarify their respective roles in protein stabilization.

Our approach to obtaining estimates of the energetics of such interactions has been to make substitutions of a buried hydrophobic amino acid residue in ribonuclease S (RNase-S)[1] (Connelly et al., 1990; Varadarajan et al., 1990; Richards & Vithyathil, 1959). This is composed of two fragments of pancreatic ribonuclease A, S-peptide (residues 1–20) and S-protein (residues 21–124), which combine in a reversible reaction to produce a catalytically active complex having a structure very similar to that of native ribonuclease A. Since residues 16–20 of S-peptide are not important for binding to S-protein, in our studies we have used a truncated version of S-peptide consisting of the first 15 residues (S15). We have substituted Met-13 in S15 with seven other hydrophobic residues ranging in size from glycine to phenylalanine, and we have recently reported estimates of the free energies, enthalpies, and entropies of the peptide–protein interactions at 25 °C (Connelly et al., 1990). It was found that over half of the stabilization energy of the complex could be lost by making the single amino acid change Met-13 → Gly.

Insight into the nature of interactions involving nonpolar groups in proteins has come from observing correlations between data on protein unfolding and the aqueous dissolution of hydrophobic model compounds (Sturtevant, 1977; Baldwin, 1986; Privalov & Gill, 1988). From these comparisons it is apparent that one thermodynamic quantity, the heat capacity change, plays a central role in characterizing hydrophobic interactions. The unfolding of small globular proteins and the dissolution of solid, liquid, and gaseous nonpolar substances in water are all characterized by large positive heat capacity changes. It has been argued that the heat capacity increment is associated primarily with the ordering of water molecules around exposed nonpolar groups (Baldwin, 1986; Spolar et al., 1989; Murphy et al., 1990). Expressions for the hydrophobic contribution to the free energy of protein stability have been developed largely on the basis of the observed heat capacity changes for protein unfolding and liquid hydrocarbon dissolution (Baldwin, 1986; Privalov & Gill, 1988). Since large changes in the free energy of binding were observed upon changing a buried hydrophobic amino acid in the RNase-S system, we set out to measure, and we herein report, the corresponding changes in heat capacities for peptide binding to S-protein.

Materials and Methods

Materials. RNase-S was obtained from Sigma. S-Protein and S-peptide analogues were obtained and purified as described earlier (Connelly et al., 1990). Peptide concentrations were determined by quantitative amino acid analysis at the Yale University Medical School Protein and Nucleic Acid Chemistry Facility. Peptide purity was checked by Dr. W. J. McMurray by fast atom bombardment mass spectrometry. In all the titration and differential scanning calorimetry (DSC) experiments described below, the buffer used was 50 mM

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[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.

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sodium acetate/100 mM NaCl, pH 6.0.

Titration Calorimetry. The calorimetric experiments were performed with the titration calorimeter from MicroCal, Inc. (Northhampton, MA) as described previously (Connelly et al., 1990). A series of injections of peptide into S-protein solution were performed, and the heat q_i evolved at each injection step i was measured. The shape of the resulting titration curve (Figure 1) is governed by the unitless parameter $c = K[M_T]_0$, where K is the binding constant of the peptide for S-protein and $[M_T]_0$ is the total macromolecule concentration in the titration cell (Wiseman et al., 1989). For optimum resolution of the binding constant (K) and enthalpy change (ΔH) for a reaction, Wiseman et al. suggest designing an experiment so that the value of c is between 1 and 1000. Shown in Figure 1 are three titration curves with c values of 1188, 67, and 3. In titrations with high c values (Figure 1, top), at each injection step all of the injected ligand reacts until a sharp endpoint is reached. From the injections made before the endpoint, ΔH may be obtained although it is difficult to obtain K. This type of titration was performed in order to calculate enthalpy changes at low temperatures (5 and 10 °C) for some of the peptides (S15, M13L, M13I, M13V), owing to their tight binding. For intermediate c values (Figure 1, middle), both K and ΔH can be determined using a nonlinear least-squares fit to the data as described previously (Connelly et al., 1990). For low c values (Figure 1, bottom), at every injection step only a fraction of the added ligand binds. It is difficult to obtain ΔH under these conditions.

Differential Scanning Calorimetry. Differential scanning calorimetry was carried out in the DASM-4 instrument (Biopribor, Pushchino, Moscow Region, USSR). Experimental conditions are described in the legend for Figure 4.

Accessible Surface Area Calculations. Let $A_{np}(Y)$ be the total nonpolar accessible area of species Y. All carbon and sulfur atoms in the protein were considered to be nonpolar. Let $\Delta A_{np}(M13X)$ denote the change in nonpolar accessible area that occurs on dissociation of the peptide–S-protein complex ($M13X_{comp}$) into free S-protein and free peptide ($M13X_{pep}$). The double difference $\Delta\Delta A_{np}$ stands for the change in $\Delta A_{np}(M13X)$ relative to the area change $[\Delta A_{np}(S15)]$ that occurs upon dissociation of the Met peptide.

$$\Delta A_{np}(M13X) = A_{np}(M13X_{pep}) + A_{np}(\text{S-protein}) - A_{np}(M13X_{comp}) \quad (1)$$

$$\Delta A_{np}(S15) = A_{np}(S15_{pep}) + A_{np}(\text{S-protein}) - A_{np}(S15_{comp}) \quad (2)$$

Subtracting eq 2 from eq 1 and rearranging gives

$$\Delta\Delta A_{np} = \Delta A_{np}(M13X) - \Delta A_{np}(S15) = [A_{np}(M13X_{pep}) - A_{np}(S15_{pep})] - [A_{np}(M13X_{comp}) - A_{np}(S15_{comp})]$$

$$\Delta\Delta A_{np} = \Delta A_{np}(M13X_{pep}) - \Delta A_{np}(M13X_{comp}) \quad (3)$$

where eq 3 provides the definitions of $\Delta A_{np}(M13X_{pep})$ and $\Delta A_{np}(M13X_{comp})$.

Since the structures of all of the free peptides in aqueous solution under the conditions under which the ΔC_p measurements were made are largely random coil (Connelly et al., 1990), we have approximated the accessibilities of an amino acid in the free peptide by the values for the corresponding amino acid in the model tripeptides A-X-A (Lee & Richards, 1971). Hence

$$\Delta A_{np}(M13X_{pep}) = A_{np}(\text{A-X-A}) - A_{np}(\text{A-M-A}) \quad (4)$$

This can be readily determined. Estimation of the second term in eq 3, $\Delta A_{np}(M13X_{comp})$, is complicated by the fact that there are several disordered surface residues in each complex. Since

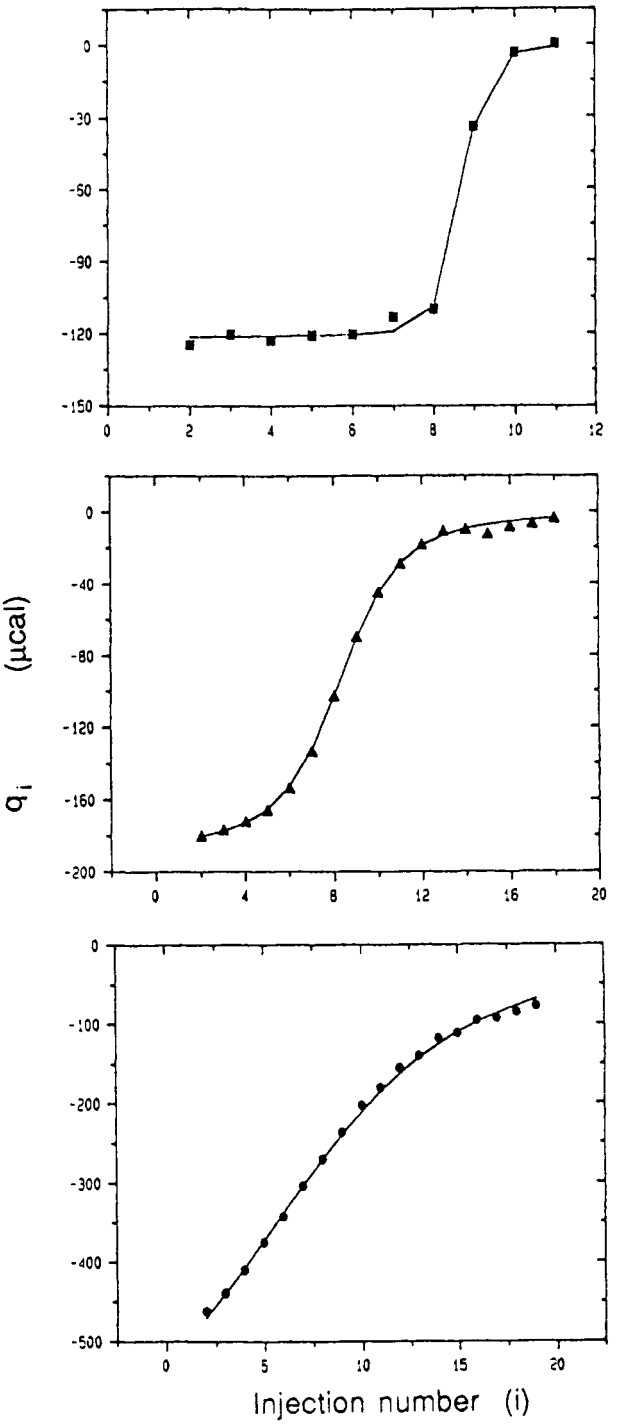

FIGURE 1: Calorimetric titrations for (top) tight ($K = 4 \times 10^7$ M^{-1}, $\Delta H = -22.8$ kcal/mol, $c = 1188$), (middle) intermediate ($K = 1.9 \times 10^6$ M^{-1}, $\Delta H = -24.5$ kcal/mol, $c = 67$), and (bottom) weak binding ($K = 1.9 \times 10^4$ M^{-1}, $\Delta H = -31.0$ kcal/mol, $c = 3$). The solid line represents the best fit of the heat for an injection (q_i) versus the injection step (i) using the procedure described in Connelly et al. (1990).

the electron density is not clearly defined for these residues, the refinement procedure used in the analysis of the crystallographic data places such residues in different conformations in the different mutant complexes. Accessibility differences at these positions are thus likely to be artifactual. Most (>-90%) of the atoms in the mutant complexes have accessibility differences of less than 5 Å^2, relative to the S15 complex. However, differences that arise as a result of conformational changes at disordered residue positions are typically greater than 5 Å^2. If no correction is made for this, $\Delta A_{np}(M13X_{comp})$

Table I: Thermodynamic Parameters[a,b] Obtained on Titrating S-Protein with Various S-Peptide Analogues at Temperatures from 5 to 25 °C

peptide										
M13A	T	5.0	10.0	14.8	19.8	25.0				
	$\Delta G°$	−6.6	−6.2	−6.2	−5.8	−5.4				
	ΔH	−16.6	−21.3	−23.8	−31.5	−35.3				
M13ANB	T	5.6	10.0	14.7	19.9	24.9				
	$\Delta G°$	−8.9	−8.9	−8.6	−8.4	−8.1				
	ΔH	−17.3	−20.3	−23.6	−25.6	−31.8				
M13V	T	5.2	10.3	15.2	19.6	24.8				
	$\Delta G°$				−10.0	−9.8				
	ΔH	−23.1	−25.8	−28.8	−31.6	−35.9				
M13I	T	4.7	9.5	14.7	20.3	24.6	25.2			
	$\Delta G°$			−9.9	−9.9	−9.7	−9.7			
	ΔH	−21.8	−24.0	−27.3	−30.2	−34.5	−35.5			
M13L	T	5.9	9.9	14.7	19.8	25.4				
	$\Delta G°$			−9.9	−9.5	−9.3				
	ΔH	−20.7	−21.7	−25.1	−29.7	−36.3				
S15	T	4.9	9.8	10.1	14.7	14.8	19.8	25.0	29.7	39.8
	$\Delta G°$				−10.3		−10.0	−9.4	−9.2	−7.5
	ΔH	−21.6	−27.5	−28.9	−30.0	−29.6	−34.2	−41.9	−49.6	−78.8
M13F	T	5.3	10.3	14.9	20.4	25.0				
	$\Delta G°$	−8.0	−7.9	−7.7	−7.4	−6.7				
	ΔH	−18.8	−23.6	−25.6	−30.2	−37.6				

[a] The measured values at 25 °C are somewhat different than those reported previously (Connelly et al., 1990). This is because the previous titration curves were fit using an incorrect cell volume of 1.3815 mL. The authors were made aware of the error shortly after publication, and the present data have been fit using the corrected cell volume of 1.3215 mL. The effect of this change was largely to increase the absolute values of ΔH by about 5%. $\Delta G°$ values are much less affected by the change in cell volume. The estimated errors are ±0.1 kcal/mol for $\Delta G°$ and ±0.6 kcal/mol for ΔH. These were calculated as standard deviations of repeated experiments performed at 25 °C (Connelly et al., 1990). Blank spaces indicate that binding was too tight for $\Delta G°$ to be reliably estimated at these temperatures. $\Delta G°$ and ΔH are expressed in kilocalories per mole. [b] $\Delta G° = -RT \ln K$.

values of up to 100 Å^2 are estimated. In order to overcome this problem, we used two different approaches.

First, we considered only those residues involved in direct contacts in complex formation when evaluating A_{np}(M13X$_{comp}$). These residues were identified as those which showed any changes in accessibility when the areas in the complex were compared with those of the separate protein and peptide components in the same conformations that they have in the complex. This procedure was repeated for all the mutant complexes. The set of residues, each member of which was involved in complex formation in at least one of the eight complexes, consists of residues 4–5, 7–15, 25, 29, 33–35, 39, 41, 44–51, 54–55, 108, 111, and 116–120. Of these 35 residues, 32 are involved in the formation of all eight complexes. Accessibilites were summed over all nonpolar atoms in these residues to give A_{np}(M13X$_{comp}$). The net sum over all other residues is assumed to be identical for M13X$_{comp}$ and S15$_{comp}$.

Second, we examined if there were area changes distributed over the entire complex that contributed to $|\Delta\Delta A_{np}|$. Values of ΔA_{np}(M13X$_{comp}$) were determined using all protein atoms in the mutant and S15 complexes. All atoms in a given mutant which had an accessibility difference greater than 5 Å^2 relative to the S15 complex were identified. These were examined visually using the program FRODO (Jones, 1985) to determine whether the differences were real (due to a difference in conformation of a residue with clearly defined electron density in both mutant and the S15 complex) or artifactual (due to a difference in conformation of a disordered residue). The values of ΔA_{np}(M13X$_{comp}$) were then corrected by subtracting the contributions that resulted from altered conformations of these disordered residues.

Results

The measured values of ΔH and $\Delta G°$ for all peptide binding reactions are listed in Table I. The errors of these parameters listed in Table I are based on the standard deviation obtained from repeated experiments at 25 °C (Connelly et al., 1990). The values of ΔH for S15 agree reasonably well over the entire temperature range with those determined earlier by batch and flow calorimetry for the binding of the full 20-residue native peptide to S-protein (Hearn et al., 1971). Since the free energies of binding of the 20- and 15-residue peptides are also quite similar (Mitchinson & Baldwin, 1986), this implies that the entropies of binding are also similar and, consequently, that residues 16–20 are as disordered in the RNase-S complex as they are in the free peptide.

For the peptides M13F and M13A, it was possible to make measurements of both ΔH and $\Delta G°$ in the entire temperature range from 5 to 25 °C. In order to check the general consistency of results and to utilize all of the measurements in the determination of the thermodynamic parameters of the system, we have simultaneously fit the measured ΔH's and $\Delta G°$'s for M13A and M13F as follows. By assuming that ΔC_p is temperature independent within the temperature range 5–25 °C, one obtains (Privalov & Gill, 1988)

$$\Delta H(T) = \Delta H(T_0) + \Delta C_p(T - T_0) \quad (5)$$

$$\Delta G°(T) = \Delta H(T_0) + \Delta C_p(T - T_0) - T\{[\Delta H(T_0) - \Delta G°(T_0)]/T_0 + \Delta C_p \ln (T/T_0)\} \quad (6)$$

Equations 5 and 6 were used to fit the data with the use of $T_0 = 298.15$ K. The data sets were weighted according to their respective standard errors. The values of ΔC_p and ΔH(25 °C) so obtained are listed in Table II. The error estimates of these fitted parameters are obtained from the asymptotic covariance matrix (Bevington, 1969; Ratkowski, 1983) and are included in the table. The values of $\Delta G°$(25 °C) were identical within the experimental error (±0.1 kcal/mol) to the values obtained by carrying out titrations at 25 °C (Table I). Figure 2 shows a plot of the free energies and enthalpies for M13F binding to S-protein at 5-deg intervals in the range 5–25 °C. The solid lines represent the best fit of the data to eqs 5 and 6 using the parameters listed in Tables I and II.

For the remaining peptides, it was not possible to measure $\Delta G°$ at some of the lower temperatures because of the high binding constants involved. In these cases ΔH(25 °C) and ΔC_p were obtained by fitting the enthalpy data alone to eq 5. Errors in these parameters were determined as described above.

Table II: ΔH(25 °C) and ΔC_p for Peptide Binding to S-Protein in the Temperature Range 5–25 °C

peptide	ΔH(25 °C)[a] (kcal mol^{-1})	ΔH(25 °C)[b] (kcal mol^{-1})	ΔC_p[a] (kcal mol^{-1} K^{-1})	ΔC_p(25 °C)[b] (kcal mol^{-1} K^{-1})
M13A	−35.3 ± 1.0	−36.1 ± 0.9	−0.96 ± 0.08	−1.27 ± 0.11
M13ANB	−30.8 ± 0.9	−31.5 ● 0.9	−0.71 ± 0.07	−1.02 ± 0.11
M13V	−35.5 ± 1.1	−36.3 ± 0.9	−0.64 ± 0.03	−0.96 ± 0.11
M13I	−34.5 ± 0.5	−35.0 ± 0.7	−0.66 ± 0.05	−0.96 ± 0.10
M13L	−34.5 ± 1.4	−35.1 ± 0.9	−0.81 ± 0.12	−1.11 ± 0.10
S15	−40.3 ± 1.2	−41.3 ± 0.9	−0.91 ± 0.10	−1.20 ± 0.11
M13F	−35.9 ± 1.3	−36.7 ● 0.9	−0.89 ± 0.10	−1.20 ± 0.11

[a] Calculated under the assumption that ΔC_p is independent of temperature. Errors were estimated as described under Results. [b] Calculated under the assumption that ΔC_p is linearly dependent on temperature and that $d\Delta C_p/dT$ is the same for all the peptide binding reactions. From the fit, a value of $d\Delta C_p/dT$ of −0.031 ± 0.009 kcal mol^{-1} K^{-2} was obtained.

Table III: Difference Thermodynamic Parameters (Relative to S15–S-Protein Interaction) for Peptide Binding to S-Protein in the Temperature Range 5–25 °C

peptide	$\Delta\Delta G°$ (25 °C)[a] (kcal mol^{-1})	$\Delta\Delta H$ (25 °C)[b] (kcal mol^{-1})	$T\Delta\Delta S°$ (25 °C)[c] (kcal mol^{-1})	$\Delta\Delta C_p$[b] (kcal mol^{-1} K^{-1})
M13A	4.0	5.0	1.0	−0.05
M13ANB	1.3	9.5	8.2	0.20
M13V	−0.4	4.8	5.2	0.27
M13I	−0.3	5.8	6.1	0.25
M13L	0.1	5.8	5.7	0.10
S15				
M13F	2.7	4.4	1.7	0.02

[a] Derived from measurements at 25 °C listed in Table I. [b] Based on the values for ΔH and ΔC_p in Table II that were calculated under the assumption that ΔC_p is independent of temperature. However, these double difference values remain essentially unchanged if the values of ΔH and ΔC_p in Table II that were derived under the assumption of a temperature-dependent ΔC_p are used instead. [c] $T\Delta\Delta S° = \Delta\Delta H - \Delta\Delta G°$.

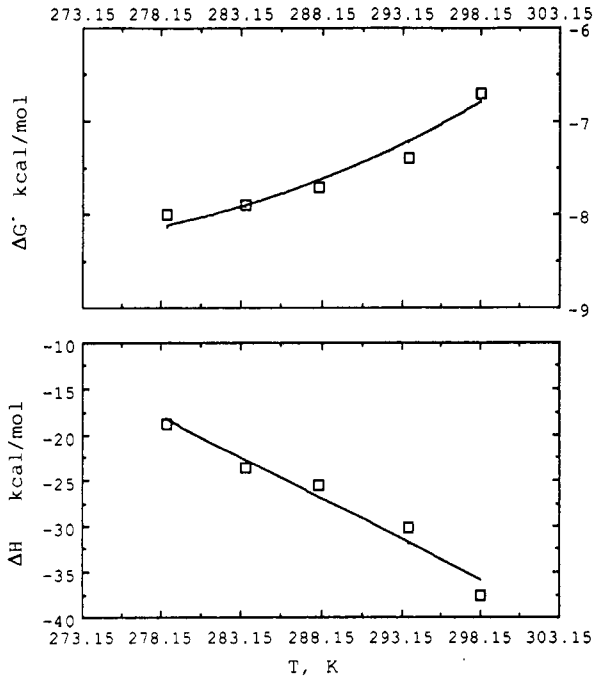

FIGURE 2: Free energies (top) and enthalpies (bottom) for M13F binding to S-protein in the temperature range 5–25 °C calculated under the assumption that ΔC_p is independent of temperature. Solid lines represent the best fits to the data upon constraining eqs 5 and 6 to assume the same value for the shared parameters. The parameters used are listed in Tables I and II.

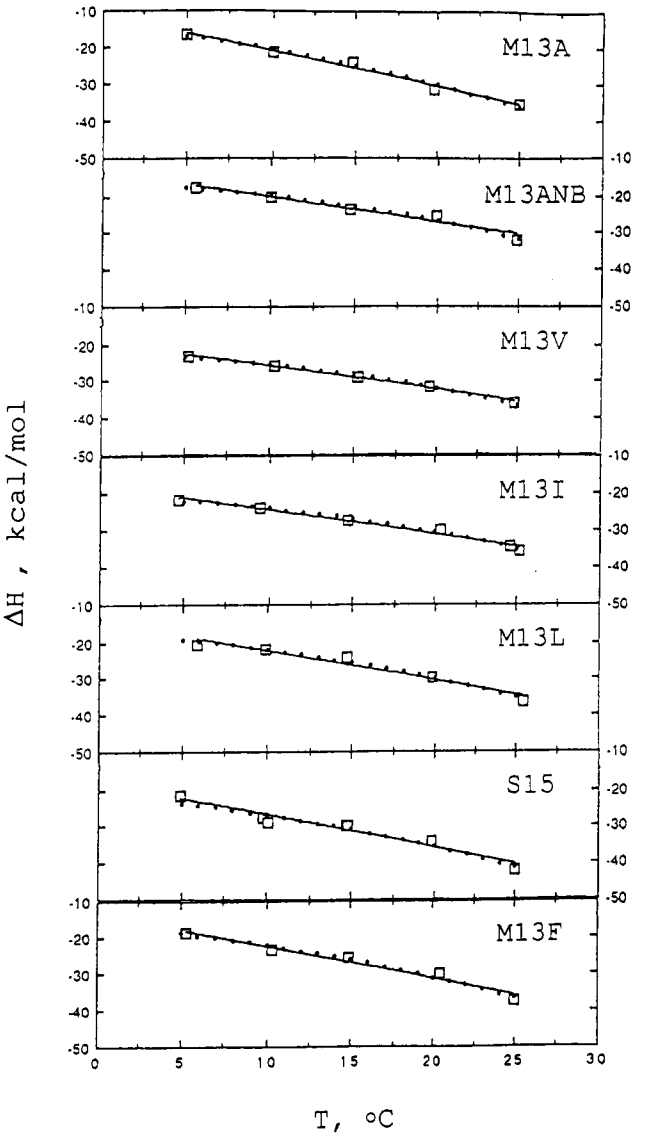

FIGURE 3: Enthalpies of binding of S-peptide analogues to S-protein in the temperature range 5–25 °C. The open squares represent the enthalpies that are obtained from titrations and are summarized in Table I. The solid lines represent the best fit to the individual data sets assuming a constant ΔC_p. The dotted lines represent the best fit, assuming that ΔC_p varies linearly with temperature with the constraint that the temperature dependence of ΔC_p is the same for all seven data sets shown.

Table III lists the values of the various thermodynamic binding parameters calculated relative to the S15 peptide so that, for $J = G°$, H, $S°$, or C_p, $\Delta\Delta J_X = \Delta J_{M13X} - \Delta J_{S15}$.

We have also calculated the ΔC_p's by assuming that ΔC_p has a component that is linearly dependent on temperature (Figure 3):

$$\Delta C_p(T) = \Delta C_p(T_0) + \alpha(T - T_0) \quad (7)$$

$$\Delta H(T) = \Delta H(T_0) + (\Delta C_p - \alpha T_0)(T - T_0) + \alpha/2(T^2 - T_0^2) \quad (8)$$

The number of data points was insufficient to fit both ΔC_p and α for each peptide. We therefore assumed that the parameter α was the same for all peptide binding reactions and performed a global fit of the data (ΔH as a function of temperature for all seven peptides) to obtain a value for α of −0.031 ± 0.009 kcal mol^{-1} K^{-2} as well as the ΔC_p(25 °C) for each peptide listed in Table II. Errors were estimated as described earlier in this section. We feel that the assumption that α is the same for all peptides is reasonable because any temperature dependence

Table IV: Accessible Surface Area Changes in the Peptide Dissociation Reaction[a]

peptide	A_{np}(A-X-A)	ΔA_{np}(M13X$_{pep}$)	ΔA_{np}-(M13X$_{comp}$)[b]		$\Delta\Delta A_{np}$(obsd)[c]	$\Delta\Delta C_p$(exptl)[d]	$\Delta\Delta A_{np}$(calcd)
M13G	38	−122	−9	+20	−113	–[e]	–
M13A	70	−90	−13	−43	−77	−0.05 ± 0.18	200
M13ANB	91	−69	−9	−17	−60	0.20 ± 0.17	−800
M13V	112	−48	−18	−7	−30	0.27 ± 0.13	−1080
M13I	141	−29	−25	−46	−4	0.25 ± 0.15	−1000
M13L	129	−31	−15	−19	−16	0.10 ± 0.23	−400
S15	160	–	–	–	–	–	–
M13F	163	3	6	+39	−3	0.02 ± 0.20	−80

[a] A_{np}(A-X-A) refers to the nonpolar accessible area of amino acid X in the tripeptide A-X-A (Lee & Richards, 1971). ΔA_{np}(M13X$_{pep}$) and ΔA_{np}(M13X$_{comp}$) refer to changes in nonpolar accessible area in the free peptide and in the complex relative to the corresponding values for the S15 peptide. $\Delta\Delta A_{np}$(calcd) is calculated using eq 9 and $\Delta\Delta C_p$(exptl). [b] The first set of values of ΔA_{np}(M13X$_{comp}$) was calculated using only residues involved in complex formation. The second set of values was calculated using all ordered residues. Details of all calculation procedures are outlined in the Materials and Methods section. [c] $\Delta\Delta A_{np}$(obsd) = ΔA_{np}(M13X$_{pep}$) − ΔA_{np}(M13X$_{comp}$). Values of ΔA_{np}(M13X$_{pep}$) and ΔA_{np}(M13X$_{comp}$) listed in columns 3 and 4 were used. [d] Errors in $\Delta\Delta C_p$ were obtained by adding the error estimates for the ΔC_p's of M13X and S15 listed in column 4 of Table II. [e] Values of ΔH and hence $\Delta\Delta C_p$ for M13G could not be determined because of the weak affinity of this peptide for S-protein.

of ΔC_p is likely to result primarily from the fact that the S-protein component (which is the same in all binding reactions) is undergoing a change in conformation in the 5–25 °C range. DSC experiments showed that S-protein has a broad transition centered around 42 °C (Tsong et al., 1970; J. M. Sturtevant, P. R. Connelly, and R. Varadarajan, unpublished results). In contrast, RNase-S and the M13I–S-protein complex have considerably sharper transitions centered at approximately 52 °C. We have measured by means of DSC the apparent C_p's of solutions of the peptide M13I, S-protein, and the M13I–S-protein complex in the temperature range 5–65 °C (Figure 4). In the range 5–25 °C, the mean values obtained for ΔC_p [C_p(M13I–S-protein) − C_p(S-protein) − C_p(M13I)] can be fitted to a straight line having a slope equal to −0.051 kcal K^{-2} mol^{-1}. The values of ΔC_p estimated from the DSC curves in Figure 4 are subject to considerably more uncertainty than those obtained in the titration experiments. However, it is encouraging that the slope of ΔC_p derived from DSC is of the same sign and approximately the same magnitude as that derived from titration calorimetry.

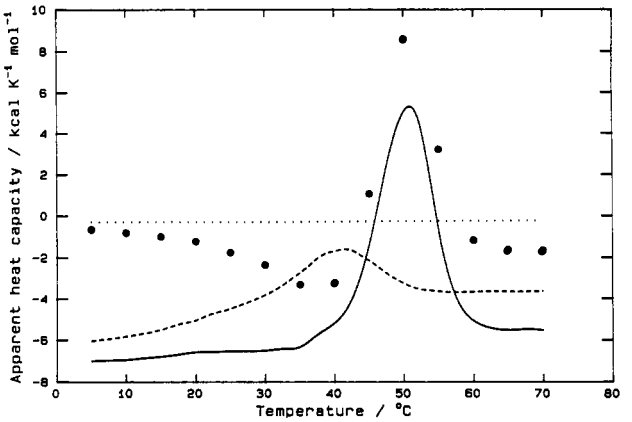

FIGURE 4: DSC curves showing the variation with temperature of the apparent heat capacities of the peptide M13I (···), S-protein (---), and the complex (—) formed from these two components. Solute concentrations of 5–6 mg/mL were employed at a scan rate of 1 K min^{-1}. The heat capacities are relative to that of the buffer as recorded in the baseline scan. Values of ΔC_p (●) for the reaction M13I + S-protein → M13I–S-protein are estimated from the three curves under the assumption that no significant volume change accompanies the reaction.

Discussion

In an attempt to understand the significance of the differences in the energetics of the reactions of the S-peptide analogues with S-protein, the binding reaction was conceptually broken up into two conformational events and an association event (Connelly et al., 1990): (1) a conformational change of the peptide from its state in solution (C) to the helical conformation that it assumes in the complex (H); (2) a conformational change of S-protein from its free state in solution (A) to the conformation that it assumes in the complex (B); and (3) the association of S-protein in state B and the peptide in state H. It was concluded that the third event served as the major contributor to the observed differences in the free energies, enthalpies, and entropies determined at 25 °C. Therefore, the relative stabilities of the complexes are governed by the differences in (a) removing residue 13 in the isolated S-peptide helix from its contact with water (dehydration) and (b) placing it into the hydrophobic core of the protein (packing).

It is clear that the energetics reflecting the two conformational events (events 1 and 2 above) involved in the binding process may change with temperature. For example, it is known that the isolated peptides have a small propensity to form helices as the temperature is lowered. Additionally, S-protein unfolds as the temperature is raised, having a transition centered at approximately 40 °C under the conditions of the experiments reported here. Since the heat capacity changes for the reactions here are given by the heat capacity of the complexes minus that of the free reactants, one might expect that the heat capacity changes would vary with temperature. We have fit the enthalpies derived from titration calorimetry by using a temperature-independent ΔC_p and also by using one that is linearly dependent on temperature as described above. Although the two fitting procedures give different final values of ΔC_p(25 °C), both give the same values of $\Delta\Delta C_p$(25 °C). The quality of the fit was comparable in both cases (Figure 3). The inclusion of the temperature-dependent parameter in the titration calorimetry fits was not significant at the 50% confidence level in an F-test although the temperature dependence was confirmed by DSC measurements (Figure 4). By performing titrations over an extended temperature range, it is clear that a sharp change in ΔC_p occurs at temperatures above 25 °C where the denaturation of S-protein becomes increasingly important. The DSC curves in Figure 4 show that a sharp change in ΔC_p toward positive values would be observed at temperatures above 40 °C, resulting from the onset of denaturation of the complex. The large enthalpy changes observed for S15 binding at higher temperatures (Table I) reflect the fact that the S-protein is induced to fold upon complexation with S15.

The heat capacity change (ΔC_p) for the transfer of gaseous, liquid, and solid nonpolar substances to water is proportional

to the amount of nonpolar surface area exposed to water (Gill & Wadso, 1976; Murphy et al., 1990). Recent observations (Spolar et al., 1989; Livingstone et al., 1991) have shown that the heat capacity change associated with protein denaturation is also proportional to the amount of nonpolar surface area (ΔA_{np}) exposed to water upon unfolding. It was observed that

$$\Delta C_p = (0.25 \pm 0.02)\Delta A_{np}/1000 \quad (9)$$

where ΔC_p is expressed in units of kilocalories per mole per Kelvin and ΔA_{np} is in units of square angstroms (Livingstone et al., 1991). The fact that the multiplying factor is very similar for all of the above processes has been taken as evidence that the observed ΔC_p is largely associated with solvation of nonpolar groups by water. Using eq 9, one can estimate the expected $\Delta\Delta A_{np}$ for a given peptide relative to that for S15 if $\Delta\Delta C_p$ is known. The structures of all of the peptide–S-protein complexes have been determined using X-ray crystallography (R. Varadarajan, unpublished results). Accessible area calculations on these complexes were performed as described in the Materials and Methods section and are summarized in Table IV. Regardless of which method is used to calculate $\Delta A_{np}(M13X_{comp})$, the $\Delta\Delta A_{np}$'s derived from the crystal structures are too small to account for the experimentally derived $\Delta\Delta C_p$'s. The $\Delta\Delta A_{np}$'s obtained from the crystal structures were compared with those calculated from eq 9 and with the use of the measured $\Delta\Delta C_p$'s. As can be seen from the values in Table IV, there are significant differences between these two values for some of the peptides. This suggests that factors other than ΔA_{np} can contribute measurably to the observed ΔC_p. At this time, we do not have any definitive evidence as to what these factors are.

However, an earlier analysis (Sturtevant, 1977) suggested that low-frequency vibrational modes of a protein may also contribute to ΔC_p. These modes are likely to be important in determining hydrogen-exchange rates between protein and aqueous solvent. It has been shown by Rosa and Richards (1981) that the rate of proton exchange in S-protein is decreased by a factor of 1000 on formation of the complex with S-peptide. This suggests a large change in the dynamic behavior of the S-protein component on formation of RNase-S. If the observed $\Delta\Delta C_p$'s are indeed due in significant amount to vibrational effects, then this should be reflected in differences in the rates of hydrogen exchange in the various complexes. These rates have yet to be determined.

The $\Delta\Delta S°$'s listed in Table III are all (with the exception of M13A)[2] large and positive. If only effects due to hydration are considered, then the burial of nonpolar surface area should result in a positive $\Delta S°$ that scales with ΔA_{np}. Such behavior is observed for the entropy of transfer of hydrocarbons from water to the liquid phase (Privalov & Gill, 1988). If such effects are important determinants of the $\Delta\Delta S°$'s in Table III, then these should also scale with $\Delta\Delta A_{np}$ (Table IV). Thus, $\Delta\Delta S°$ should be either close to zero (for M13I, M13L, and M13F) or negative (for the remaining peptides). Since this is clearly not the case, the observed $\Delta\Delta S°$'s are likely to be due largely to an increase in entropy of the mutant complexes relative to the S15 complex. Since the mean structures of all the complexes are quite similar (R. Varadarajan, unpublished results), this, like the $\Delta\Delta C_p$ data, suggests that there are differences in the vibrational dynamics of wild-type and mutant complexes.

In cases where there is a small or neglible $\Delta\Delta C_p$, all the observed $\Delta\Delta J$'s are relatively independent of temperature. Differences in the packing interactions within the peptide–protein complexes represent a possible origin for such temperature-independent effects. We are currently performing a detailed analysis of the crystallographic structures in order to clarify the role played by changes in packing in the various complexes.

[2] The crystal structure of the M13A complex shows that a water molecule occupies a part of the cavity created as a result of residue substitution. This may explain the small value of $\Delta\Delta S°$ for M13A. None of the other peptide complexes listed in Table III contain buried water molecules.

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