DSC Study of the Thermal Stability of S-Protein and S-Peptide/S-Protein Complexes†

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ABSTRACT: The thermal denaturation of S-protein is investigated at pH 7.0 by means of DSC measurements. The process is reversible and can be assimilated to a two-state transition. The low values of denaturation temperature and enthalpy, between 38.5 and 40.0 °C and 165 and 180 kJ mol^{-1}, respectively, demonstrate that the loss of S-peptide strongly decreases the structural stability. The interaction between S-peptide and S-protein is thermodynamically characterized, at pH 7.0, by studying the thermal stability of S-peptide/S-protein complexes at different molar ratios. A two-dimensional nonlinear regression analysis of the excess heat capacity surface as a function of temperature and S-peptide concentration enables us to determine the thermodynamic parameters of binding equilibrium. The values obtained are K_b(38.6 °C) = (1.10 ± 0.15) × 10^6 M^{-1}, $\Delta_b H$(38.6 °C) = (−185 ± 10) kJ mol^{-1}, and $\Delta_b C_p$ = (−3.5 ± 0.5) kJ K^{-1} mol^{-1}. These figures result in satisfactory agreement with literature values.

Molecular recognition is the preliminary step of almost all the biochemical and biophysical processes. Proteins possess the fundamental feature to interact with high affinity and specificity with various molecules both small and large. These interactions play a pivotal role in enzyme catalysis, hormone action, antibody specificity, and other protein−receptor mediated functions. But even the processes of transcription, replication, and restriction of DNA genetic code are ruled by strong and selective interactions between proteins and specific sequences of base pairs (Steitz, 1990). Any molecular description of such noncovalent interactions requires a deep understanding of both structural and thermodynamic details (Spolar & Record, 1994). The atomic level resolution of the formed complexes, by means of X-ray or NMR techniques, cannot clarify, by itself, the energetics of the recognition process. It is well established that most molecular recognition processes involve large changes of enthalpy, entropy, and heat capacity (Ha et al., 1989; Takeda et al., 1992; Jin et al., 1993; Lundback et al., 1993; Murphy et al., 1993; Foguel & Silva, 1994), which can be accurately determined by means of calorimetric methods. Isothermal titration calorimetry allows the performance of direct measurements of association constant and binding enthalpy and heat capacity (Eftink & Biltonen, 1980). However, to obtain reliable estimates, it is necessary that the product of the association constant times the protein concentration lies in the range 10−100 (Sturtevant, 1994). In other words, direct equilibrium methods are unable to characterize binding equilibria with association constants greater than 10^7 M^{-1}. But it is well-known that various recognition processes have binding constants greater than 10^9 M^{-1}. In such cases indirect methods are necessary to gain thermodynamic information on association.

Among indirect methods, differential scanning calorimetry is a very useful approach. By determining the thermal stability of the formed complex and comparing it to that of the macromolecule alone, DSC[1] allows one to obtain the thermodynamic parameters of binding equilibrium. To this purpose, it is necessary the development of suitable thermodynamic models to analyze DSC curves, as shown by Brandts and Lin (1990). Additionally, it is useful to increase the dimensionality of experimental data by determining a two-dimensional heat capacity surface as a function of temperature and ligand concentration, as brought out by Straume and Freire (1992). A global linkage analysis, by means of a nonlinear regression procedure on a complete set of DSC curves obtained at different ligand concentrations, solved simultaneously the energetics of intrinsic protein thermal stability and protein−ligand binding equilibrium.

We applied this approach to investigate the interaction between S-peptide and S-protein. The two parts originate from the proteolytic cleavage of the peptide bond between Ala20 and Ser21 in RNase A due to subtilisin (Richards & Vithayathil, 1959). Around neutral pH the two parts are tightly bound to form a noncovalent complex that is fully active and is called RNase S. The interaction between S-peptide and S-protein has been deeply studied with structural determinations (Wyckoff et al., 1970; Kim et al., 1992, Varadarajan & Richards, 1992), equilibrium measurements (Hearn et al., 1971; Connelly et al., 1990; Varadarajan et al., 1992; Thomson et al., 1994), and theoretical approaches (Simonson & Brunger, 1992). The results obtained with isothermal titration calorimetry represent a good reference to verify the validity of DSC as a tool to study protein−

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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; Nle, norleucine; DSC, differential scanning calorimetry; ITC, isothermal titration calorimetry; CD, circular dichroism; ASA, accessible surface area.

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ligand interactions. We have investigated, at pH 7.0, the thermal denaturation of S-protein and S-peptide/S-protein complexes at different saturating ratios. The binding thermodynamic parameters determined from the nonlinear regression procedure are in agreement with literature values.

MATERIALS AND METHODS

RNase S type XII S was purchased from Sigma and its purity confirmed by HPLC gel filtration. RNase S was separated in S-protein and S-peptide by gel filtration (Sephadex G-75) in 5% formic acid (pH ≈2). The S-protein peak was located by the absorption at 280 nm. Fractions corresponding to S-protein were collected, and the resulting solution was exhaustively dialyzed against water to eliminate any formic acid trace. Conservative cuts were made to ensure preparations of high purity. The preparations were lyophilized and stored at −18 °C (Hearn et al., 1971). S-protein purity was also checked by electrospray mass spectrometry (Fenn et al., 1989). To locate the S-peptide peak, the high eluation time fractions that did not absorb at 280 nm were frozen and dried *in vacuo* to eliminate formic acid. The solid residue was dissolved in bidistilled water, and its absorbance at 210 nm was determined. The collected and lyophilized fractions were stored at −18 °C.

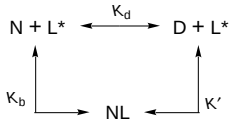
S-protein concentration was determined by absorption spectroscopy using a molar extinction coefficient at 280 nm of 9560 M^{-1} cm^{-1} (Connelly et al., 1990). The concentration of S-peptide solutions was calculated from the weight of dissolved material. Solutions for DSC measurements were exhaustively dialyzed by using Spectra Por MW 6000−8000 membranes against 10 mM Mops buffer, pH 7.0, and 200 mM NaCl and were filtered on Millipore membranes. Doubly deionized water was used throughout. The pH of all solutions was determined with a radiometer pHmeter model PHM 93 at 25 °C. The pH change due to the ionization of Mops on raising temperature is not so large as to affect the results.

Scanning Calorimetry. Calorimetric measurements were carried out on a second-generation Setaram Micro-DSC apparatus, interfaced with a data translation A/D board for automatic data accumulation. A scan rate of 0.5 K min^{-1} was chosen for the present study. All data analyses were accomplished with software developed in our laboratory (Barone et al., 1992a, 1994). The raw data were converted to an apparent molar heat capacity by correcting for the instrument calibration curve and the buffer−buffer scanning curve and dividing each data point by the scan rate and the number of moles of protein in the sample cell. Finally, the apparent molar heat capacity was converted to the excess molar heat capacity, $\langle\Delta C_p\rangle$, assuming the native state heat capacity as the reference baseline (Freire & Biltonen, 1978; Biltonen & Freire, 1978; Freire, 1994). For a reliable evaluation of baseline the DSC scans always started from 0 °C. The calorimetric enthalpy $\Delta_d H(T_d)$ was determined by direct integration of the area under the curve, and the van't Hoff enthalpy was calculated with the standard formula:

$$\Delta H^{vH}(T_d) = 4RT_d^2\langle\Delta C_p\rangle_{T_d}/\Delta_d H(T_d) \quad (1)$$

where T_d is the denaturation temperature corresponding to the maximum of DSC peak, $\langle\Delta C_p\rangle_{T_d}$ is the height of the excess molar heat capacity at T_d, and R is the gas constant. The unitary value of the ratio $\Delta_d H(T_d)/\Delta H^{vH}(T_d)$ is a necessary condition to state that a two-state transition takes place.

Thermodynamic Model. We consider a protein that denatures according to a two-state transition and can bind a specific ligand on one site of the native conformation. The overall process can be described by the following scheme:



where K_d is the equilibrium constant of the denaturation of S-protein, K_b is the equilibrium binding constant, and K' is the denaturation constant of the stoichiometric complex NL (i.e., RNase S). However, it is easy to show that K' is not an independent parameter, since $K' = K_d/K_b$. Both the equilibrium constants are temperature dependent according to

$$K_d = \exp -\{[\Delta_d H(T_d)/R][(1/T) - (1/T_d)] + (\Delta_d C_p/R)[1 - (T_d/T) + \ln(T/T_d)]\} \quad (2)$$

where $K_d = 1$ for $T = T_d$, and

$$K_b = K_b^\circ \exp -\{[\Delta_b H(T_d)/R][(1/T) - (1/T_d)] + (\Delta_b C_p/R)[1 - (T_d/T) + \ln(T/T_d)]\} \quad (3)$$

where $K_b = K_b^\circ$ for $T = T_d$. In these equations, T_d is the denaturation temperature, $\Delta_d H(T_d)$ and $\Delta_d C_p$ represent the denaturation enthalpy and heat capacity changes, and $\Delta_b H(T_d)$ and $\Delta_b C_p$ are the binding enthalpy and heat capacity changes. Clearly eqs 2 and 3 are exact in the assumption that $\Delta_d C_p$ and $\Delta_b C_p$ are temperature independent. Selecting the native state as reference (Robert et al., 1988; Brandts & Lin, 1990), the macroscopic canonical partition function for this system results:

$$Q = 1 + K_b[L] + K_d \quad (4)$$

where [L] represents the free ligand concentration in solution. The excess enthalpy function $\langle\Delta H\rangle$ can be readily obtained by a well-known statistical mechanical relationship:

$$\langle\Delta H\rangle = RT^2[\partial \ln Q/\partial T]_{p,[L]} \quad (5)$$

which gives

$$\langle\Delta H\rangle = [\Delta_d H(T_d) + \Delta_d C_p(T - T_d)](K_d/Q) + [\Delta_b H(T_d) + \Delta_b C_p(T - T_d)](K_b[L]/Q) \quad (6)$$

When the total ligand concentration is much greater than protein concentration, the free ligand concentration [L] can be considered constant at increasing temperature in a DSC measurement. But, if the total amount of ligand is lower than the saturation level of protein binding site, it is necessary to consider the mass balance equation for the ligand to determine the free ligand concentration [L]:

$$[L]_{tot} = [L] + [\bar{L}][P]_{tot} \quad (7)$$

where $[L]_{tot}$ and $[P]_{tot}$ are the total ligand and protein concentration, respectively, and $[\bar{L}]$ represents the binding

isotherm. In the case considered, the free ligand concentration can be calculated in the whole investigated temperature range, by simply solving a second degree equation (Brandts & Lin, 1990).

The free ligand concentration changes with temperature, during the denaturation process, because ligand can bind only to native conformation. Therefore, in the case of subsaturating amounts of ligand, the free ligand concentration [L] is a physically significant variable to describe the system. In fact, $\langle \Delta H \rangle = f(T,[L])$ and $[L] = f(T)$, so it results:

$$\langle \Delta C_p \rangle = [\partial \langle \Delta H \rangle / \partial T]_{[L]} + [\partial \langle \Delta H \rangle / \partial [L]\,]_T (d[L]/dT) \quad (8)$$

The effect of subsaturating amounts of a strong ligand on DSC profiles is dramatic because two endothermic peaks appear. Shrake and Ross (1990, 1992), starting from their studies of the thermal denaturation of human serum albumin in the presence of fatty acids, carefully investigated the nature of the two peaks. Simulations have shown that the maximum distortion of DSC peak from the two-state transition profile happens when the molar ratio $[L]_{tot}/[P]_{tot}$ is equal to 0.5 (i.e., when the total ligand concentration is half the concentration of binding sites). Moreover, the complex shape of DSC profile does not depend on the value of binding constant alone but markedly depends on the product of total protein concentration times the association constant at T_d. This product is a dimensionless parameter, labeled $C \equiv K_b^{\circ}[P]_{tot}$ by Brandts and co-workers (Wiseman et al., 1989; Brandts & Lin, 1990). It is important to note that, even assuming $\Delta_b H = 0$, a single two-state transition gives rise to two distinct peaks when the C parameter is great enough.

Following the procedure devised by Freire and Straume (Straume & Freire, 1992; Straume, 1994), we constructed an experimental excess heat capacity surface as a function of temperature and ligand concentration. A nonlinear regression of the entire surface with respect to eq 8 was performed by means of the Levenberg−Marquardt algorithm (Morè, 1977), as implemented in the Optimization Toolbox of MATLAB. The regression was repeated until a convergence criterion was satisfied (Straume, 1994). Nonlinear, joint confidence intervals were determined for all estimated parameters according to the method developed by Johnson (Johnson & Frasier, 1985; Johnson, 1992). Such two-dimensional analysis of the excess heat capacity allows the calculation of a unique set of thermodynamic parameters characterizing both the thermal denaturation of the globular protein and the binding equilibrium of the ligand to the macromolecule (Straume & Freire, 1992).

RESULTS AND DISCUSSION

Analysis of S-Protein Denaturation. The thermal denaturation of S-protein is investigated at pH 7.0, 10 mM Mops buffer, and 200 mM NaCl. DSC measurements were performed at protein concentrations ranging between 0.115 and 0.320 mM. Lower concentrations could not be measured by scanning calorimetry due to instrument sensitivity. The values of thermodynamic parameters are collected in Table 1, whereas Figure 1 shows two experimental DSC profiles. In general the DSC peaks are very broad, and the overall process takes place over a temperature range of about 30 °C. But the broadening cannot be held as the proof of a poorly cooperative phenomenon. Indeed, it must be stressed that a decrease of $\Delta_d H(T_d)$ in a perfect two-state transition causes a broadening of DSC peak. In the selected conditions the thermal denaturation is highly reversible, as demonstrated by the recovery of the original signal in rescanning of the same sample. The denaturation temperature lies in the range 38.5−40.0 °C and is about 10−12 °C lower than that of RNase S. The smearing of T_d values can be ascribed to association of S-protein molecules, a phenomenon well documented in literature (Hearn et al., 1971). The denaturation enthalpy amounts to 165−180 kJ mol^{-1}, a very low value for a globular protein: the loss of S-peptide causes a strong decrease of thermal stability. The van't Hoff enthalpy values correspond to the calorimetric enthalpy ones, and the process can be assimilated to a two-state transition. However, at raising the S-protein concentration above 0.300 mM, the discrepancy between $\Delta_d H(T_d)$ and $\Delta H^{vH}(T_d)$ increases. The finding that the van't Hoff enthalpy is greater than the calorimetric one may be an indication of the association of S-protein molecules in solution, as concentration rises. It is worth noting that the values of denaturation temperature and enthalpy here reported agree with those determined by DSC and CD measurements by Hearn et al. (1971) and from a careful analysis of isothermal titration calorimetry data by Thomson et al. (1994).

Table 1: Thermodynamic Parameters of the Denaturation Process of S-Protein at pH 7.0, 10 mM Mops Buffer, and 200 mM NaCl[a]

$[P]_{tot}$ (mM)	T_d (°C)	$\Delta_d H(T_d)$ (kJ mol^{-1})	$\Delta_d C_p$ (kJ K^{-1} mol^{-1})	$\Delta H^{vH}(T_d)$ (kJ mol^{-1})
0.115	39.0	165	2.6	180
0.180	38.9	175	2.2	190
0.260	38.6	180	2.0	180
0.320	40.0	185	2.5	210

[a] Each figure represents the value averaged over three or four measurements. The error in T_d does not exceed 0.2 °C. The estimated (relative) uncertainties in $\Delta_d H(T_d)$ and $\Delta_d C_p$ amount to 5% and 10%, respectively, of reported values. The values of $\Delta H^{vH}(T_d)$ were calculated from experimental curves according to eq 1.

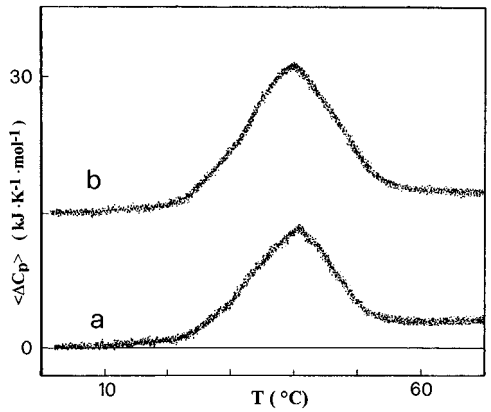


FIGURE 1: DSC profiles of S-protein at pH 7.0, 10 mM Mops buffer, and 200 mM NaCl. S-protein concentration is equal to 0.115 mM (curve a) and to 0.320 mM (curve b). Excess heat capacity values have been shifted along the *y*-axis for ease of presentation.

The denaturation heat capacity change is about 2.0−2.6 kJ K^{-1} mol^{-1}, a very low value. It has been demonstrated by various authors that $\Delta_d C_p$ can be calculated, with accuracy, from the knowledge of native protein tertiary structure and the specific contributions to the heat capacity of polar and nonpolar groups (Murphy & Freire, 1992; Privalov & Makhatadze, 1992; Spolar & Record, 1994; Barone et al., 1995a). The values of $\Delta_d C_p$ for S-protein calculated with these models lie in the range 5.0−5.5 kJ K^{-1}

mol^{-1}. The strong discrepancy between the experimental and calculated values can be explained by considering that S-protein in solution does not possess a very compact tertiary structure. A large fraction of its accessible surface area (ASA) is not buried but exposed to water. This picture may be also useful to explain the very low value of $\Delta_d H(T_d)$. Indeed, the loosening of the cooperative network of noncovalent interactions that give rise to the native three-dimensional folding pattern well accounts for the experimental findings. To validate this conclusion the thermodynamic parameters of S-protein can be compared to those of other globular proteins. For instance, RNase T1, a protein of ribonuclease family comprised of 104 residues (e.g., the same of S-protein) and possessing two disulfide bridges (e.g., S-protein has four disulfide bonds) has $T_d = 55.0$ °C, $\Delta_d H(T_d) = 460$ kJ mol^{-1}, $\Delta_d C_p = 6.0$ kJ K^{-1} mol^{-1} at pH 7.0, in 100 mM Mops buffer (Barone et al., 1992; Yu et al., 1994).

CD spectra allowed the determination of the content of secondary structure of S-protein in solution: at pH 6.8 there is 12% α-helix and 33% β-sheet (Labhardt, 1982). These values are only slightly lower than those found in the crystals of RNase A and RNase S: 19% α-helix and 38% β-sheet. Even though the secondary structure is preserved, S-protein does not have a very compact tertiary structure, judging from the denaturation temperature and denaturation enthalpy and heat capacity changes. However S-protein cannot be regarded as a "molten globule" (Ptitsyn, 1992), because its thermal denaturation is a cooperative process. Indeed, various authors (Pfeil et al., 1986; Yutani et al., 1992; Griko & Privalov, 1994) have shown that the thermal transition from molten globule to random coil happens with no heat absorption and can be assimilated to a second-order phase transition, in agreement with theoretical prediction (Finkelstein & Shakhnovic, 1989).

Analysis of the Interaction between S-Peptide and S-Protein. The interaction of S-peptide with S-protein was studied at pH 7.0, in 10 mM Mops buffer, 200 mM NaCl, by means of detailed DSC measurements, keeping the concentration of S-protein fixed at 0.260 mM, in order to reduce the number of independent variables. The concentration value was selected to obtain a good signal to noise ratio, because the heat effects are low. This noncovalent interaction is strongly specific: a value of the molar ratio r = [S-peptide]/[S-protein], equal or slightly greater than 1, is sufficient for complete recombination of the two parts, giving rise to the reconstitution of RNase S. On the other hand, by performing measurements at concentrations of S-peptide subsaturating with respect to S-protein, DSC profiles result very complex due to the concomitant presence in solution of S-protein, S-peptide, and reconstituted RNase S. But in all cases the process is reversible according to the reheating criterion. The experimental curves, for $r = 0.25$ and 0.45, respectively, that emphasize the complexity of calorimetric profile are shown in Figure 2. The height of the high temperature peak, representing the denaturation of reconstituted RNase S, clearly rises at increasing the value of r, while the low temperature peak decreases. A complete set of DSC profiles, ranging from $r = 0$ to $r = 1.10$, is reported in Figure 3. The corresponding thermodynamic parameters are collected in Table 2. In this table are reported the values of T_{max}, the temperature corresponding to the maximum of DSC profiles (i.e., for these measurements the notation T_{max} is to be preferred to T_d). Clearly T_{max} increases from 38.6 °C at $r = 0$ to 51.0 °C at $r = 1.10$. Similarly, the denaturation enthalpy and heat capacity changes go from the values proper of S-protein to those of RNase S.

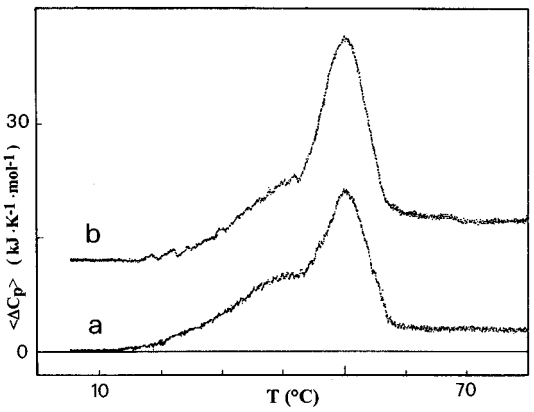


FIGURE 2: DSC profiles of S-peptide/S-protein complexes at pH 7.0, 10 mM Mops buffer, 200 mM NaCl, and different saturating ratios: $r = 0.25$ (curve a) and $r = 0.45$ (curve b). Excess heat capacity values have been shifted along the y-axis for ease of presentation.

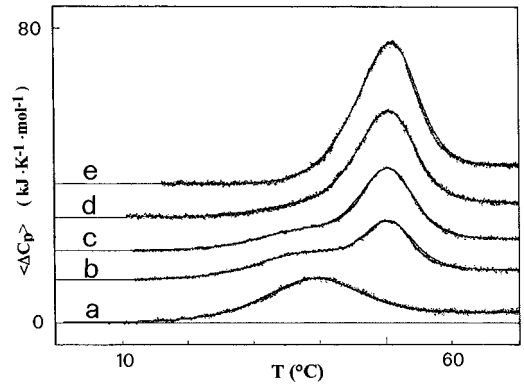


FIGURE 3: Complete set of DSC profiles of S-peptide/S-protein complexes corresponding to $r = 0$ (curve a), $r = 0.25$ (curve b), $r = 0.45$ (curve c), $r = 0.67$ (curve d), and $r = 1.10$ (curve e). S-protein concentration was fixed at 0.260 mM. The solid lines represent the best fit of the experimental curves calculated from the nonlinear two-dimensional regression with respect to eq 8. See text for more details. Excess heat capacity values have been shifted along the y-axis for ease of presentation.

Table 2: Thermodynamic Parameters of S-Peptide/S-Protein Complexes at Different Molar Ratios, r, pH 7.0, 10 mM Mops Buffer and 200 mM NaCl[a]

r	T_{max} (°C)	$\Delta_d H$ (kJ mol^{-1})	$\Delta_d C_p$ (kJ K^{-1} mol^{-1})
0.00	38.6	180	2.0
0.25	49.9	245	2.5
0.45	50.2	295	3.1
0.67	50.8	340	3.9
1.10	51.0	430	5.1

[a] Each figure represents the value averaged over three or four measurements. The error in T_{max} does not exceed 0.2 °C. The estimated (relative) uncertainties in $\Delta_d H$ and $\Delta_d C_p$ amount to 5% and 10%, respectively, of reported values. S-protein concentration was fixed at 0.260 mM.

To apply the developed thermodynamic model to this system, the following conditions must hold: (i) S-protein thermal denaturation is a two-state transition; (ii) S-peptide is a strong ligand of S-protein, but it dissociates during denaturation. S-protein does not possess a compact conformation in solution, but its thermal denaturation can be regarded as a two-state transition. The second condition is

supported by DSC measurements of RNase S, which unequivocally demonstrate that its two constituents dissociate during denaturation (see the accompanying paper). The nonlinear regression procedure with respect to eq 8 is simultaneously applied to all DSC curves in order to obtain a more reliable evaluation of thermodynamic parameters from a statistical point of view. The calculated curves are shown in Figure 3, as solid lines, superimposed on the experimental ones: the agreement is satisfactory, taking in mind the peculiarity of the system. The best set of thermodynamic parameters determined from the nonlinear regression is $T_d = 38.6 \pm 0.3$ °C, $\Delta_d H = (182 \pm 10)$ kJ mol^{-1}, $\Delta_d C_p = (1.6 \pm 0.5)$ kJ K^{-1} mol^{-1}, $\Delta_b H = (-185 \pm 10)$ kJ mol^{-1}, $\Delta_b C_p = (-3.5 \pm 0.5)$ kJ K^{-1} mol^{-1}, and $C = 295 \pm 25$. The standard deviation of the fit for all five DSC profiles is given by $\sigma = 4548$ J K^{-1} mol^{-1}. These results are in substantial agreement with the values calculated by us for a smaller set of DSC curves in a previous work (Barone et al., 1995b).

The thermodynamic values of denaturation process of S-protein are only slightly different from those determined by direct DSC measurements (see Table 1). This is a first evidence of the reliability of the two-dimensional nonlinear regression. The binding of S-peptide is very exothermic, and this suggests that, apart the coil to helix transition of S-peptide, a substantial conformational change of S-protein structure also happens, induced by the presence of S-peptide. It is worth noting that the binding enthalpy and heat capacity changes determined from the nonlinear regression procedure are in agreement with literature values. Indeed Sturtevant and co-workers (Hearn et al., 1971; Connelly et al., 1990) determined by means of direct isothermal calorimetric measurements that $\Delta_b H = -168$ kJ mol^{-1} and $\Delta_b C_p = -3.8$ kJ K^{-1} mol^{-1} at 25 °C, pH 7.0, and 300 mM NaCl, either in buffered or unbuffered solutions. On the other hand, the value of $\Delta_b H = -290$ kJ mol^{-1} at 40.0 °C, pH 7.0, and 300 mM NaCl determined by isothermal titration calorimetry (ITC) (Hearn et al., 1971) is very large and greater than our value $\Delta_b H = -185$ kJ mol^{-1} at 38.6 °C. Furthermore, at pH 6.0, 50 mM sodium acetate, and 100 mM NaCl, the binding enthalpy was -330 kJ mol^{-1} at 39.8 °C for S15-peptide (Varadarajan et al., 1992) and -315 kJ mol^{-1} at 40.0 °C for M13Nle S-15 peptide (Thomson et al., 1994). The strong discrepancy can be rationalized. DSC measurements, being an indirect method, allow calculation of the binding enthalpy by studying the denaturation process of S-peptide/S-protein complexes at different saturating ratios, and these complexes at 38.6 °C are not unfolded. Instead, by titrating S-protein with S-peptide around 40 °C, ITC measures also the heat release associated with the refolding of S-protein that at this temperature is largely unfolded.

It is also important to stress the large negative heat capacity change associated with the binding of S-peptide to S-protein. On the basis of firmly established theoretical and experimental results, the burial of nonpolar accessible surface area, ASA_{np}, from water contact makes a strong negative contribution to the heat capacity, instead the burial of polar accessible surface area, ASA_p, makes a positive contribution (Murphy & Freire, 1992; Privalov & Makhatadze, 1992; Graziano & Barone, 1996). But in this case it is difficult to establish a quantitatively right correlation between $\Delta_b C_p$ and ΔASAs because the tertiary structure of S-protein undergoes a large conformational rearrangement on binding of S-peptide. Indeed, Spolar and Record (1994), by assuming that S-protein in solution has the same structure determined from the X-ray diffraction of RNase S crystals, and that S-peptide undergoes a coil to helix transition, calculated $\Delta_b C_p = (-0.84 \pm 0.24)$ kJ K^{-1} mol^{-1}, a value in strong contrast with the experimental determinations. Moreover, Varadarajan et al. (1992) found that the values of $\Delta_b C_p$ were not correlated in any simple fashion with ASA_{np} buried upon binding of S15-peptide analogs to S-protein.

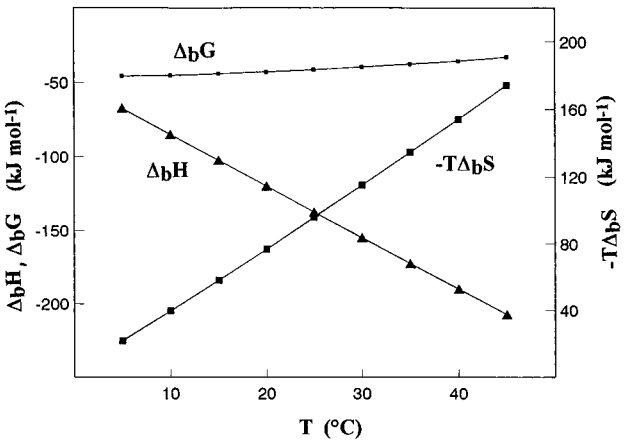


FIGURE 4: Thermodynamics of the S-peptide/S-protein interaction at pH 7.0, 10 mM Mops buffer, and 200 mM NaCl. The values are calculated by means of the parameters determined from the two-dimensional nonlinear regression.

From the value of $C \equiv K_b^\circ[P]_{tot} = 295 \pm 25$ and the knowledge of S-protein concentration in solution, $[P]_{tot} = 0.260$ mM, it is possible to calculate the binding constant of S-peptide at 38.6 °C. It results $K_b^\circ(38.6\ ^\circ\text{C}) = (1.10 \pm 0.15) \times 10^6$ M^{-1}. From the van't Hoff equation with the determined values of $\Delta_b H$(38.6 °C) and $\Delta_b C_p$, it results in $K_b(25\ ^\circ\text{C}) = 1.9 \times 10^7$ M^{-1} and $K_b(15\ ^\circ\text{C}) = 1.0 \times 10^8$ M^{-1}. These values are in agreement with those of Sturtevant and co-workers (Hearn et al., 1971), who determined $K_b(35\ ^\circ\text{C}) = (4.3 \pm 1.1) \times 10^6$ M^{-1} and $K_b(40\ ^\circ\text{C}) = (1.0 \pm 0.1) \times 10^6$ M^{-1} at pH 7.0, 100 mM Tris HCl buffer, and 300 mM NaCl, by means of substrate turnover measurements, exploiting the fact that the complex is catalytically active, while S-protein itself has no activity. The same authors determined, from isothermal calorimetric measurements, that $K_b(25\ ^\circ\text{C}) = 5.0 \times 10^7$ M^{-1} and $K_b(15\ ^\circ\text{C}) = 3.7 \times 10^8$ M^{-1} at pH 7.0, 300 mM NaCl. Recently, Varadarajan et al. (1992) determined by ITC that $K_b(25\ ^\circ\text{C}) = (7.8 \pm 1.5) \times 10^6$ M^{-1} and $K_b(15\ ^\circ\text{C}) = (6.5 \pm 1.5) \times 10^7$ M^{-1} at pH 6.0, 50 mM acetate buffer, and 100 mM NaCl, for the binding of S15-peptide (e.g., a truncated version of S-peptide constituted by the first 15 residues). In this respect, it has been demonstrated that only the first 15 residues are necessary to give a catalytically active complex with S-protein (Finn & Hofmann, 1973), and that the complex of S15-peptide with S-protein is structurally identical with RNase S (Taylor et al., 1981; Kim et al., 1992). In conclusion, the thermodynamic parameters associated with the binding of S-peptide calculated from the two-dimensional nonlinear analysis of DSC data are in agreement with literature values, obtained with more direct approaches.

From the values of K_b and $\Delta_b H$ at 38.6 °C and assuming $\Delta_b C_p$ temperature independent, it is possible to calculate the thermodynamics of S-peptide/S-protein interaction in the range 5−45 °C. In Figure 4 are plotted the functions $\Delta_b H$, $\Delta_b G$, and $-T\Delta_b S$ versus temperature. The binding free

energy is little affected by temperature changes because an enthalpy−entropy compensation happens. A similar behavior of thermodynamic functions has been found in various systems, involving not only biological macromolecules (Blokzijl & Engberts, 1993). Furthermore, the binding process results enthalpically driven at all temperatures. In fact, residues 3−13 of S-peptide undergo a coil to helix transition, a great number of noncovalent interactions are turned on between S-peptide and S-protein residues, and a large conformational rearrangement of S-protein itself occurs. The binding entropy is always negative. Probably the entropy cost due to the association of the two parts and the loss of conformational freedom of S-peptide residues overwhelms the positive contribution due to the release of structured water molecules in the hydration shell of nonpolar moieties. Usually hydrophobic interactions are expected to play a significant role in molecular recognition processes (Harrison & Eftink, 1982; Blokzijl & Engberts, 1993). Apart a large positive entropy change upon complexation, the existence of a large negative heat capacity change is generally considered a decisive criterion for the occurrence of hydrophobic interactions. But, recently, examples of enthalpically controlled complexation processes have been found (Ferguson et al., 1988; Harata et al., 1988; Smithrud & Diederich, 1990). In general, the thermodynamic parameters of molecular recognition processes must be interpreted with care, because the association can cause conformational changes of the host and guest molecules (Spolar & Record, 1994). This seems to be the case of S-peptide/S-protein interaction, which drives large conformational changes of both components.

With the developed thermodynamic model it is also useful to simulate DSC curves at $r = 1.0$ for different total concentrations of S-protein. Keeping fixed the value of K_b(38.6 °C) $= 1.1 \times 10^6$ M^{-1}, the C parameter passes from 5.5 for $[P]_{tot} = 0.005$ mM, to 55 for $[P]_{tot} = 0.050$ mM, to 295 for $[P]_{tot} = 0.260$ mM, and 968 for $[P]_{tot} = 0.880$ mM. The shape of DSC curves correctly resembles that of RNase S profiles, and the location of the maximum is right. Indeed, the values of denaturation temperature result in 43.0, 47.5, 50.9, and 53.2 °C, respectively, in agreement with DSC measurements on RNase S (see Table 2 of the accompanying paper). Therefore the thermodynamic parameters calculated from the two-dimensional nonlinear regression well reproduce the thermal stability of RNase S, demonstrating the self-consistent character of our investigation.

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APPENDIX

It must be noted that, when $\Delta_b C_p$ is large negative, eq 8 produces $\langle \Delta C_p \rangle$ values lower than zero before the transition peak. This surprising fact requires an explanation. Clearly, when $\Delta_b C_p$ is negative, the binding enthalpy and, as a consequence, the excess enthalpy are temperature dependent, even though the binding site is already filled. Therefore the excess heat capacity function $\langle \Delta C_p \rangle$, given by eq 8, is not zero but negative in the temperature region before the transition peak.

To further clarify this point, we have assumed the ligated native state NL as reference. The corresponding partition function is given by

$$Q_{NL} = 1 + (1/K_b[L]) + (K_d/K_b[L]) \qquad \text{(A1)}$$

The excess enthalpy function results

$$\langle \Delta H \rangle_{NL} = [\Delta_d H(T_d) + \Delta_d C_p(T - T_d) - \Delta_b H(T_d) + \Delta_b C_p(T - T_d)][(K_d/K_b[L])/Q_{NL}] - [\Delta_b H(T_d) + \Delta_b C_p(T - T_d)](1/K_b[L])/Q_{NL}] \qquad \text{(A2)}$$

Inserting this expression into eq 8, one readily calculates $\langle \Delta C_p \rangle$. It is worth noting that eqs 6 and A2 generate the same $\langle \Delta C_p \rangle$ profiles when $\Delta_b C_p$ is zero. However, when $\Delta_b C_p$ is negative, the $\langle \Delta C_p \rangle$ values calculated from eq A2 are greater than zero before the transition peak, whereas those calculated from eq 6 are lower than zero. Actually the two profiles are simply shifted along the vertical axis, and this fact is only due to the choice of the reference state. Therefore we used eq 6 to simulate DSC curves, and we shifted the various profiles from the respective negative values up to zero. The simulations have shown that this procedure is correct. In fact, the thermodynamic parameters obtained from the analysis of the calculated DSC curves perfectly correspond to the theoretical ones fixed in the simulations.

REFERENCES

Barone, G., Del Vecchio, P., Fessas, D., Giancola, C., & Graziano, G. (1992a) *J. Thermal Anal. 38*, 2779.
Barone, G., Del Vecchio, P., Fessas, D., Giancola, C., Graziano, G., Pucci, P., Riccio, A., & Ruoppolo, M. (1992b) *J. Thermal Anal. 38*, 2791.
Barone, G., Del Vecchio, P., Fessas, D., Giancola, C., Graziano, G., & Riccio, A. (1994) *J. Thermal Anal. 41*, 1263.
Barone, G., Del Vecchio, P., Giancola, C., & Graziano, G. (1995a) *Int. J. Biol. Macromol. 17*, 251.
Barone, G., Catanzano, F., Del Vecchio, P., Giancola, C., & Graziano, G. (1995b) *Pure Appl. Chem. 67*, 1867.
Biltonen, R. L., & Freire, E. (1978) *Crit. Rev. Biochem. 5*, 85.
Blokzijl, W., & Engberts, J. B. F. N. (1993) *Angew. Chem., Int. Ed. Engl. 32*, 1545.
Brandts, J. F., & Lin, L. N. (1990) *Biochemistry 29*, 6927.
Connelly, P. R., Varadarajian, R., Sturtevant, J. M., & Richards, F. M. (1990) *Biochemistry 29*, 6108.
Eftink, M., & Biltonen, R. L. (1980) in *Biological Microcalorimetry* (Beezer, A. F., Ed.) p 343, Academic Press, London.
Fenn, J. B., Mann, M., Meng, C. K., Wong, S. F., & Whitehouse, C. M. (1989) *Science 246*, 64.
Ferguson, S. B., Seward, E. M., Diederich, F., Sanford, E. M., Chou, A., Innocencio-Szweda, P., & Knobler, C. B. (1988) *J. Org. Chem. 53*, 5593.
Finkelstein, A. V., & Shakhnovich, E. I. (1989) *Biopolymers 28*, 1681.
Finn, F. M., & Hofmann (1973) *Acc. Chem. Res. 6*, 169.
Foguel, D., & Silva, J. L. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 8244.
Freire, E. (1994) *Methods Enzymol. 240*, 502.
Freire, E., & Biltonen, R. L. (1978) *Biopolymers 17*, 481.
Graziano, G., & Barone, G. (1996) *J. Am. Chem. Soc. 118*, 1831.
Griko, Y. V., & Privalov, P. L. (1994) *J. Mol. Biol. 235*, 1318.
Harata, K., Tsuda, K., Uekama, K., Otagiri, M., & Hirayama, F. (1988) *J. Inclusion Phenom. 6*, 135.
Ha, J. H., Spolar, R. S., & Record, M. T., Jr. (1989) *J. Mol. Biol. 209*, 801.
Harrison, J. C., & Eftink, M. R. (1982) *Biopolymers 21*, 1153.
Hearn, R. P., Richards, F. M., Sturtevant, J. M., & Watt, G. D. (1971) *Biochemistry 10*, 806.

Jin, L., Yang, J., & Carey, J. (1993) *Biochemistry 32*, 7302.
Johnson, M. L. (1992) *Anal. Biochem. 206*, 215.
Johnson, M. L., & Frasier, S. G. (1985) *Methods Enzymol. 117*, 301.
Kim, E. E., Varadarajan, R., Wyckoff, H. W., & Richards, F. M. (1992) *Biochemistry 31*, 12304.
Labhardt, A. M. (1982) *J. Mol. Biol. 157*, 331.
Lundback, T., Cairns, C., Gustafsson, J,A., Carlstedt-Duke, J., & Hard, T. (1993) *Biochemistry 32*, 5074.
Morè, J. J. (1977) in *Numerical Analysis* (Watson, G. A., Ed.) p 105, Springer-Verlag, New York.
Murphy, K. P., & Freire, E. (1992) *Adv. Protein Chem. 43*, 313.
Murphy, K. P., Xie, D., Garcia, K. C., Amzel, L. M., & Freire, E. (1993) *Proteins: Struct., Funct., Genet. 15*, 113.
Pace, C. N., & McGrath, T. (1980) *J. Biol. Chem. 255*, 3862.
Pfeil, W., Bychkova, V. E., & Ptitsyn, O. B. (1986) *FEBS Lett. 198*, 287.
Privalov, P. L., & Makhatadze, G. I. (1992) *J. Mol. Biol. 224*, 715.
Ptitsyn, O. B. (1992) in *Protein Folding* (Creighton, T. E., Ed.) p 243, W. H. Freeman and Company, New York.
Richards, F. M., & Vithayathil, P. J. (1959) *J. Biol. Chem. 234*, 1459.
Robert, C. H., Gill, S. J., & Wyman, J. (1988) *Biochemistry 27*, 6829.
Schwarz, F. P. (1988) *Biochemistry 27*, 8429.
Shrake, A., & Ross, P. D. (1990) *J. Biol. Chem. 265*, 5055.
Shrake, A., & Ross, P. D. (1992) *Biopolymers 32*, 925.
Simonson, T., & Brunger, A. T. (1992) *Biochemistry 31*, 8661.
Smithrud, D. B., & Diederich, F. (1990) *J. Am. Chem. Soc. 112*, 339.
Spolar, R. S., & Record, M. T., Jr. (1994) *Science 263*, 777.
Steitz, T. A. (1990) *Q. Rev. Biophys. 23*, 205.
Straume, M. (1994) *Methods Enzymol. 240*, 530.
Straume, M., & Freire, E. (1992) *Anal. Biochem. 203*, 259.
Sturtevant, J. M. (1994) *Curr. Opin. Struct. Biol. 4*, 69.
Takeda, Y., Ross, P. D., & Mudd, C. P. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 8180.
Taylor, H. C., Richardson, D. C., Richardson, J. S., Wlodaver, A., Komoriya, A., & Chaiken, I. M. (1981) *J. Mol. Biol. 149*, 313.
Thomson, J., Ratnaparkhi, G. S., Varadarajan, R., Sturtevant, J. M., & Richards, F. M. (1994) *Biochemistry 33*, 8587.
Varadarajan, R., & Richards, F. M. (1992) *Biochemistry 31*, 12315.
Varadarajan, R., Connelly, P. R., Sturtevant, J. M., & Richards, F. M. (1992) *Biochemistry 31*, 1421.
Wiseman, T., Williston, S., Brandts, J. F., & Lin, L. N. (1989) *Anal. Biochem. 179*, 131.
Yu, Y., Makhatadze, G. I., Pace, C. N., & Privalov, P. L. (1994) *Biochemistry 33*, 3312.
Yutani, K., Ogasahara, K., & Kuwajima, K. (1992) *J. Mol. Biol. 228*, 347.

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