Thermodynamics of the Binding of S-Peptide to S-Protein to Form Ribonuclease S'*

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ABSTRACT: The thermodynamics of the reaction between S-peptide and S-protein to form ribonuclease S' has been determined by equilibrium and calorimetric measurements over a wide temperature range. All the thermodynamic parameters for the reaction, $\Delta G°$, ΔH, $\Delta S°$, and ΔC_P, are found to be strong functions of the temperature. The variation of ΔH with temperature is largely the result of thermally induced conformational transitions undergone by S-protein and ribonuclease S'. At 5°, where these transitions make no contribution to the reaction enthalpy, the thermodynamic parameters have the values $\Delta G° = -12$ kcal mole^{-1}, $\Delta H = -24$ kcal mole^{-1}, $\Delta S° = -42$ cal deg^{-1} mole^{-1}, and $\Delta C_P = -700$ cal deg^{-1} mole^{-1}. The understanding of the thermodynamics of protein stucture is at present in too rudimentary a stage to permit a meaningful interpretation of these data in terms of the known three-dimensional structure of ribonuclease S (presumed to be the same as that of ribonuclease S').

Equilibrium and calorimetric study of the combination of Met(O_2)-13-S-peptide with S-protein has also been carried out. The substantial changes observed in the various thermodynamic quantities resulting from oxidation of the thioether group of methionine to the corresponding sulfone find no ready rationalization in terms of structure.

Richards and Vithayathil (1959) reported that when bovine pancreatic ribonuclease A (RNase A) is subjected to mild digestion with the enzyme subtilisin, the main product is a modified enzyme in which a single peptide bond, the one between Ala-20 and Ser-21, has been hydrolyzed (see also Doscher and Hirs, 1967). The N-terminal fragment, S-peptide, and the remainder of the molecule, S-protein, remain tightly bound to each other at neutral pH, and the product, RNase S, retains enzymic activity. The two components of RNase S can be separated at low pH, and neither one shows any enzymic activity. Activity is regained on mixing the two components at neutral pH; the product of this reaction, RNase S', appears to be very similar to, if not identical with, RNase S.

The recent determination of the three-dimensional structure of RNase S in crystalline form (Wyckoff *et al.*, 1967) increases the importance of quantitative study of the intramolecular interactions responsible for the binding of S-peptide to S-protein. If it may be assumed that RNase S and RNase S' have essentially the same structures, the thermodynamics of the reaction

$$\text{S-peptide} + \text{S-protein} \rightleftarrows \text{RNase S}' \qquad (1)$$

is of special interest in this connection. In this paper we report the results of equilibrium and calorimetric measurements on this reaction over a range of temperatures. Included also are similar measurements on the reaction

$$\text{Met}(O_2)\text{-13-S-peptide} + \text{S-protein} \rightleftarrows \text{Met}(O_2)\text{-13-RNase S}' \qquad (2)$$

in which the residue Met-13 of S-peptide has been oxidized to the corresponding sulfone.

Experimental Section

Materials. RNase S was prepared from bovine pancreatic ribonuclease (Light and Co., England) by the action of subtilisin according to the method of Richards and Vithayathil (1959). The progress of the digestion was followed by the differential assay described by Klee and Richards (1957), based on the fact that trypsin attacks RNase S much more rapidly than the native enzyme. RNase S was isolated by chromatography an Amberlite IRC-50 resin, dialyzed against H_2O, deionized by ion-exchange chromatography, and lyophilized for storage.

RNase S was separated into its S-protein and S-peptide components by gel filtration (Sephadex G-75) in 5% formic acid (pH $\approx$2). The S-protein peak was located by its absorption at 280 mμ and the S-peptide peak by means of the Folin protein test (Lowry *et al.*, 1951). Having located the two peaks, the decision as to which tubes to include in each fraction was based on enzymic activity after addition of an excess of S-peptide to the S-protein tubes, or of S-protein to the S-peptide tubes. Conservative cuts were made to ensure preparations of high purity. The preparations were lyophilized for storage.

The concentrations of solutions of S-peptide were calculated from the weight of material dissolved. For S-protein it was found that the concentration based on the absorbance at 280 nm (Potts *et al.*, 1964) was uniformly only 87 $\pm$ 4% of that based on the weight of lyophilized S-protein dissolved, indicating the presence of 13% of H_2O plus formic acid in the lyophilized material. Concentrations based on absorbance were used in all calculations.

S-Peptide was oxidized to Met(O_2)-13-S-peptide with 98% performic acid following the procedure of Hirs (1956) and Vithayathil and Richards (1960). After 2 hr at 0°, the reaction mixture was diluted with several volumes of H_2O and lyophilized. Amino acid analyses showed that the oxidation was complete and that no alteration of other residues had taken place.

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Enzyme Assays. Enzyme activites were determined by the spectrophotometric assay described by Crook *et al.* (1960), in which the rate of hydrolysis of cytidine 2′,3′-cyclic phosphate (*c*CMP) is determined by the rate of increase of optical density at 284 nm. All assays were performed in 0.1 M Tris-HCl, pH 7.0, containing 0.3 M NaCl, at a substrate concentration of 3×10^{-4} M.

Determination of Binding Constants. Since dissociation of RNase S into its components is accompanied by complete loss of enzymic activity, the equilibrium in the dissociation may be conveniently studied by means of activity determinations. Two general sources of error in such measurements must be considered. (1) The variation of enzymic activity with the concentrations of the reactants S-peptide, represented in the following discussion by p, and S-protein, represented by P, can be unambiguously interpreted in terms of the equilibrium

$$p + P \rightleftharpoons E \qquad (3)$$

where E stands for RNase S, only if it can be shown that the equilibrium is no more complex than indicated by eq 3. That this is the case is indicated by kinetic (Cathou and Hammes, 1965) studies, and by observations of the binding of competitive inhibitors (Hummel *et al.*, 1961; Nelson *et al.*, 1962; Hammes and Schimmel, 1965), all of which indicate that RNase A has only one binding site for nucleotides. It is safe to assume that the same holds for RNase S. The results presented below also support this view. (2) It is essential to employ assay conditions which do not appreciably perturb the equilibrium being measured. The Michaelis–Menten constant, K_m, for the substrate used, *c*CMP, and for experimental conditions similar to those employed here at 25°, has been variously reported to be 2.7×10^{-2} M (Vithayathil and Richards, 1961), 3.3×10^{-3} M (Witzel and Barnard, 1962), and 1.1×10^{-2} M (Herries *et al.*, 1962). Although we have no information concerning the variation of K_m with temperature, and although knowledge of the K_m does not necessarily permit an accurate estimate of the amount of enzyme bound in the enzyme–substrate complex, nevertheless it seems likely that the maximum substrate concentration used in our assays, 3×10^{-4} M, has little or no effect on the equilibrium of eq 3. This is further substantiated by the fact that a series of measurements at a substrate concentration of approximately 3×10^{-5} M led to the same value of the equilibrium constant as obtained with 3×10^{-4} M substate. Temperatures were controlled and measured to ±1°.

It is easily shown that the equilibrium constant for reaction 3 can be put into the form

$$K = \frac{a}{(P)_0(1 - a)(r - a)} \qquad (4)$$

where a is the ratio of observed activity, at a molar ratio $r = (p)_0/(P)_0$, to the activity at $r = \infty$, and $(p)_0$ and $(P)_0$ are the total concentrations of S-peptide and S-protein, respectively. The value of K for a set of experiments with varying r was calculated from the weighted average of $a/(1 - a)(r - a)$. In cases where the binding was relatively weak (S-peptide + S-protein at 45°; Met (O_2)-13-S-peptide + S-protein at 25° and 30°), the limiting activity was determined as the intercept of a plot of 1/activity *vs.* $1/r$ fitted by least squares. It can be seen from eq 4 that when $r \gg a$ and $a \ll 1$ such a plot should be linear.

Reaction Calorimetry. The enthalpy changes in reactions 1 and 2 were determined over the temperature ranges 5–41° and 1.5–35°, respectively, using a Beckman Model 190 microcalorimeter. This instrument, which has a capacity of about 15 ml of reactant solution, gives an accuracy of the order of 1–2% for a rapid reaction having a heat absorption or evolution of 20 mcal. The calorimeter was calibrated at 25° by means of the neutralization of HCl by excess NaOH, for which ΔH = 13.37 ± 0.01 kcal mole^{-1} (Hale *et al.*, 1963; Vanderzee and Swanson, 1963), and the neutralization of Tris by excess HCl, for which ΔH = 11.35 ± 0.05 kcal mole^{-1} (Öjelund and Wadsö, 1969). At other temperatures the value for the enthalpy of neutralization of HCl was calculated from the value at 25° given above and the temperature coefficient given by Harned and Owen (1958). The temperatures of the calorimetric experiments were known to ±0.1°.

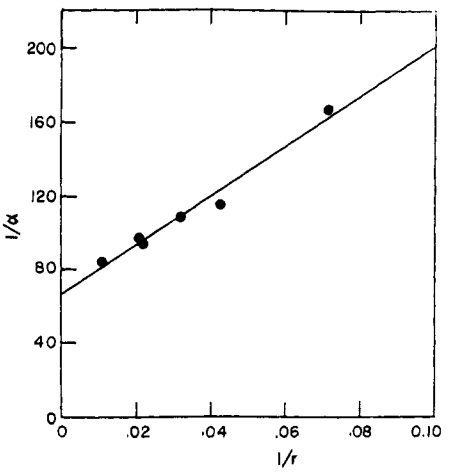

FIGURE 1: Double-reciprocal plot of $1/\alpha$ *vs.* $1/r$ for the S-peptide–S-protein interaction at 45°, where α is the enzyme activity in absorbance units min^{-1} and r is the ratio of total S-peptide to total S-protein concentration. The S-protein concentration was constant at 4.7×10^{-7} M. The intercept gives a limiting activity of 0.0149 ± 0.0009 absorbance unit min^{-1} at saturation of S-protein by S-peptide.

The reaction between S-peptide and S-protein was also studied at 25° in a flow modification of the Beckman Model 190 microcalorimeter (Sturtevant and Lyons, 1969). The same calibrating reactions were employed with this equipment.

Transition Calorimetry. The enthalpy changes in the thermally induced conformational transitions of RNase S and S-protein were determined in a highly sensitive differential scanning calorimeter (Danforth *et al.*, 1967; Tsong *et al.*, 1970). In this instrument, approximately 2-ml samples of the protein solution and of the solvent are heated in separate cells at a constant rate, and the extra heat which must be supplied to the solution cell to maintain zero temperature difference between the cells is measured as a function of temperature.

Results

The Equilibrium in the Binding of S-Peptide and Met(O_2)-S-Peptide to S-Protein. The enzymic activity data for the evaluation of the equilibrium constant of reaction 1 at 45° are summarized in the double-reciprocal plot of Figure 1. The least-squared line has an intercept giving for the limiting value of the activity at saturation of the protein by the peptide the value of 0.0149 ± 0.0009 optical density unit per min. This value, used in conjunction with eq 4, leads to the linearized plot in Figure 2A (filled circles). The value of K computed as outlined above from these data was used to calculate the theoretical

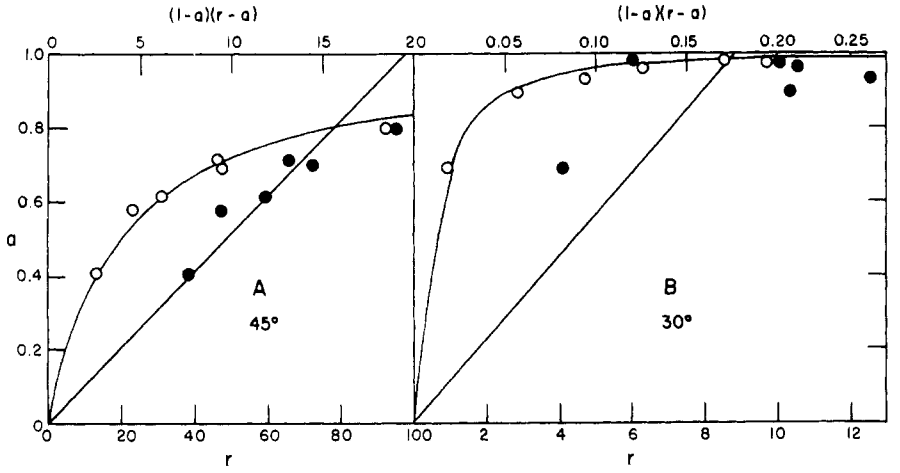

FIGURE 2: Equilibrium data for the interaction of S-peptide with S-protein at 45° (A) and 30° (B). Relative activities, a, are plotted against r, the ratio of total S-peptide to total S-protein concentration (open circles) and the product $(1 - a)(r - a)$ (filled circles). According to equation (4) the latter plot should be a straight line. The maximum rate at large excess of S-peptide at 45° was obtained as the intercept of the plot in Figure 1, and at 30° from the observed value at $r = 19.1$. The straight lines in A and B were obtained as outlined in the text, and the curves were calculated using the equilibrium constants evaluated from the slopes of the lines.

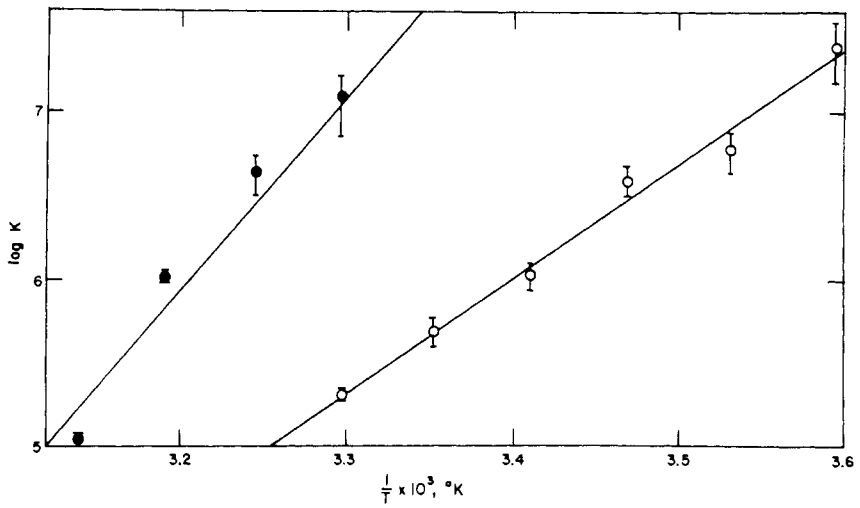

FIGURE 3: van't Hoff plots of the equilibrium constants listed in Table I for the interaction of S-peptide and S-protein (●) and of Met(O_2)-13-S-peptide and S-protein (○).

curve given in the figure (unfilled circles). It is seen that the observed activities are well accounted for by this treatment. A less favorable case is presented in Figure 2B, which illustrates the difficulty in evaluating the equilibrium constant in cases of rather tight binding. The association constants for the two systems, together with estimated standard deviations, are given in Table I. van't Hoff plots of these association constants are given in Figure 3.

Reaction Calorimetry–Batch Calorimeter. All measurements of the enthalpy change accompanying reactions 1 and 2 were made in unbuffered solutions adjusted to pH 7.0 and containing 0.3 M NaCl. After mixing of the reactant solutions the peptide [either unmodified or Met(O_2)-13] concentration was 1.0×10^{-4} M and the S-protein concentration was 7.5×10^{-5} M, the molar ratio being 1.33. According to the equilibrium data, the reaction with the S-peptide was essentially complete at all temperatures, whereas in the case of the Met(O_2)-13-S-peptide the reaction was only 83.7, 77.1, and 65.2% complete at 25, 30, and 35°, respectively. The observed enthalpies were appropriately corrected at these temperatures.

The data for the reaction involving the S-peptide are summarized in Table II. The ΔH values were fitted to a quadratic function of temperature by the least-squares procedure

$$\Delta H = A + BT + CT^2 = A' + B'\mathbf{T} + C'\mathbf{T}^2 \qquad (5)$$

where T is the Celsius temperature and $\mathbf{T}$ is the Kelvin temperature. The values for the constants are listed in Table II, and reproduce the observed values with a standard deviation of 1.3 kcal mole^{-1}. Integration of the Gibbs–Helmholtz equation gives

$$\Delta G = A' - B'\mathbf{T} \ln \mathbf{T} - C'\mathbf{T}^2 + D'\mathbf{T} \qquad (6)$$

where D' is an integration constant, selected to give the best fit with the values of $\Delta G°$ derived from the equilibrium measurements. Since the calorimetric experiments were performed at low molar concentrations of S-peptide and S-protein, and ΔH values are in general only weakly dependent on concentration,

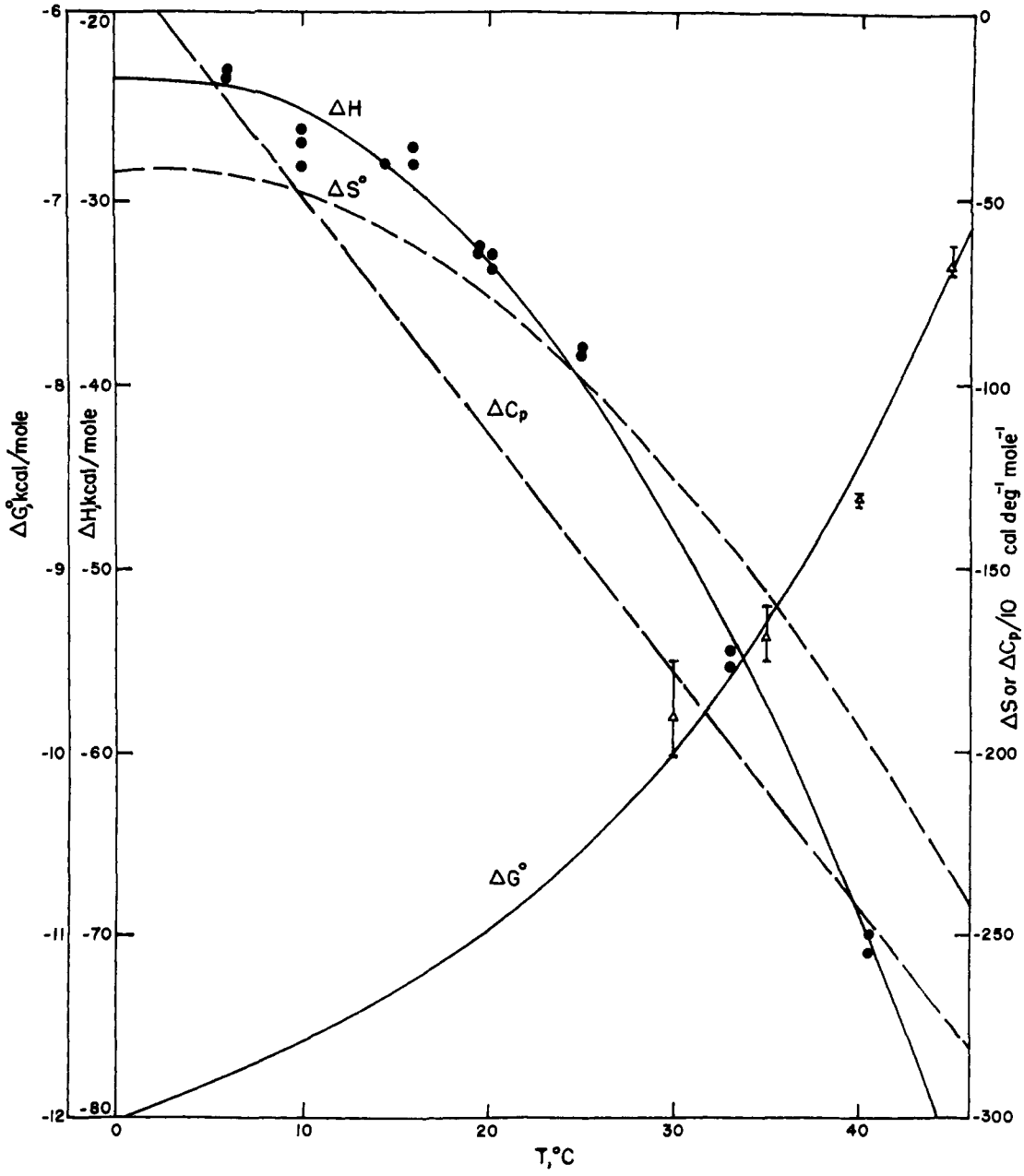

FIGURE 4: Summary of the thermodynamic parameters for the reaction of S-peptide with S-protein. The curve for ΔH is a least-squares fit of the experimental data (circles) to a quadratic in the temperature. The curve for ΔC_p is the temperature derivative of the ΔH curve. The curve for $\Delta G°$ was obtained by integration of the Gibbs–Helmholtz equation, the integration constant being determined by comparison to the experimental values (triangles).

it is permissable to compare directly the values of ΔG derived from the enthalpy data with the standard-state quantities derived from the equilibrium data. ΔH and $\Delta G°$ are given as functions of temperature, together with the values of ΔC_p and $\Delta S°$ derived from them, in Figure 4.

The corresponding data for the Met(O_2)-S-peptide reaction are summarized in Table III and Figure 5. The standard deviation of the fit of the observed data to the quadratic given in the table is 3.3 kcal mole^{-1}.

Since unbuffered solutions were employed, it was important to investigate whether any change in pH was produced by the reaction. S-peptide and S-protein solutions were adjusted to be close to pH 7.0 and were then mixed in the same concentrations as in the calorimetric experiments. The resulting pH was noted, and the solution titrated with alkali back to the

TABLE I: Association Constants for Reactions 1 and 2.

T (°C)	K_1 (M^{-1})	± Std Dev	K_2 (M^{-1})	± Std Dev
5			2.47×10^6	0.96×10^7
10			6.00×10^6	1.7×10^6
15			3.89×10^6	0.89×10^6
20			1.05×10^6	0.19×10^6
25			4.80×10^5	0.92×10^5
30	1.20×10^7	0.48×10^6	2.01×10^5	0.13×10^5
35	4.32×10^6	1.1×10^6		
40	1.01×10^6	0.06×10^6		
45	1.09×10^5	0.13×10^5		

TABLE II: Enthalpy Change in the Reaction of S-Peptide with S-Protein in 0.3 M NaCl at pH 7.

	T (°C)								
	5	10	14.5	16	19.5	20.2	25.0	33.0	40.6
$-\Delta H$, kcal mole^{-1}[a]	22.9	26.1	28.0	27.1	32.5	32.9	37.9	54.4	70.0
	23.3	26.9		28.0	32.8	33.7	38.2	55.3	71.0
		28.2							

[a] $\Delta H = -23.4 + 0.138T - 0.0318T^2 = -2433.7 + 17.509\mathbf{T} - 0.0318\mathbf{T}^2$ ($\mathbf{T}$ = Kelvin temperature).

TABLE III: Enthalpy Change in the Reaction of Met(O_2)-13-S-Peptide with S-Protein in 0.3 M NaCl at pH 7.

	T (°C)				
	1.5	15.1	25.8	29.6	35
$-\Delta H$, kcal mole^{-1}[a]	17.7	24.1	35.2	32.8	45.7
	20.0	26.1	37.4	44.9	50.5

[a] $\Delta H = -18.7 - 0.118T - 0.0204T^2 = -1508.5 + 11.027\mathbf{T} - 0.0204\mathbf{T}^2$ ($\mathbf{T}$ = Kelvin temperature).

TABLE IV: Liberation of Protons in the Reaction of S-Peptide with S-Protein in 0.3 M NaCl Solution.[a]

	Initial pH			
T (°C)	S-Peptide	S-Protein	Final pH	Mole of H^+ per Mole of Protein
10	6.994	6.986	6.845	0.40
21	7.008	7.010	6.942	0.45
21	7.00	7.03	6.948	0.27
40	7.039	7.007	6.938	0.18

[a] Final concentrations: 0.0977 mM S-peptide; 0.0733 mM S-protein.

TABLE V: Enthalpy Change in Reaction 1 as Determined by Flow Calorimetry at 25°, pH 7, in 0.3 M NaCl Solution.

Initial Concentrations (mM)		No. of Expt.	Mean ΔH, (kcal mole^{-1})	Calculated "Fraction Converted"[a]
S-Protein	S-Peptide			
0.885	1.90	2	−32.8	0.94
0.126	0.733	1	−37.6	0.91
0.187	0.401	3	−36.3	0.78
0.179	0.401	4	−37.9	0.77
0.107	0.184	3	−34.1	0.63
0.268	0.301	1	−27.9	0.62
0.0630	0.147	2	−33.1	0.61
0.055	0.100	3	−28.6	0.51
0.0945	0.110	3	−27.6	0.48
0.0492	0.0835	2	−33.8	0.47
0.0311	0.0669	3	−29.9	0.43
0.0329	0.0560	3	−34.5	0.38
0.020	0.0429	2	−44.1	0.34
0.027	0.0286	1	−32.0	0.24
	Mean		33.6	
	Std error of mean		± 0.9	

[a] Calculated using the equilibrium constant reported by Woodfin and Massey (1968).

original pH. The data obtained at three different temperatures are summarized in Table IV. It is evident that the solutions were effectively buffered by the protein present.

Reaction Calorimetry-Flow Calorimeter. In order to ascertain whether the reaction between S-peptide and S-protein at 25° was complete as judged by calorimetric criteria under the conditions used in the batch calorimeter, a series of experiments over a wide range of concentrations was performed in the flow calorimeter. The data for these experiments are given in Table V. No trend is discernible in ΔH with varying concentration, which indicates that the reaction is complete at all concentrations used. The mean value of ΔH, -33.6 ± 0.9 kcal mole^{-1}, agrees fairly well with the value found by batch calorimetry.

Woodfin and Massey (1968) reported spectrophotometric measurements of the equilibrium in reaction 1 at 25° which led to an association constant, 1.4×10^4 M^{-1}, 3 orders of magnitude smaller than the value which can be estimated from the curve for $\Delta G°$ in Figure 5. According to the smaller association constant, the degree of completion of the reaction for the experiments in Table V should have ranged from 94% to as low as 24%, with a correspondingly large range of values for the calculated ΔH. Obviously the calorimetric results in the table are more in accord with our equilibrium measurements than with those of Woodfin and Massey. The source of this discrepancy is unknown.

Transition Calorimetry. It is known (see, for example, Tsong *et al.*, 1970) that both S-protein and RNase S′ undergo thermally induced conformational transitions similar to that shown by RNase A. It is therefore of interest to investigate the contributions which the enthalpy changes in these transitions make to the temperature dependence of the observed heats of reactions 1 and 2. Tsong *et al.* (1970) have reported a few measurements of the transitions of S-protein, RNase S, and RNase S′ at pH 7 in 0.2 M NaCl. They found the behavior of RNase S and RNase S′ in the transition calorimeter to be indistinguishable.

We have studied the transitions of S protein and RNase S at

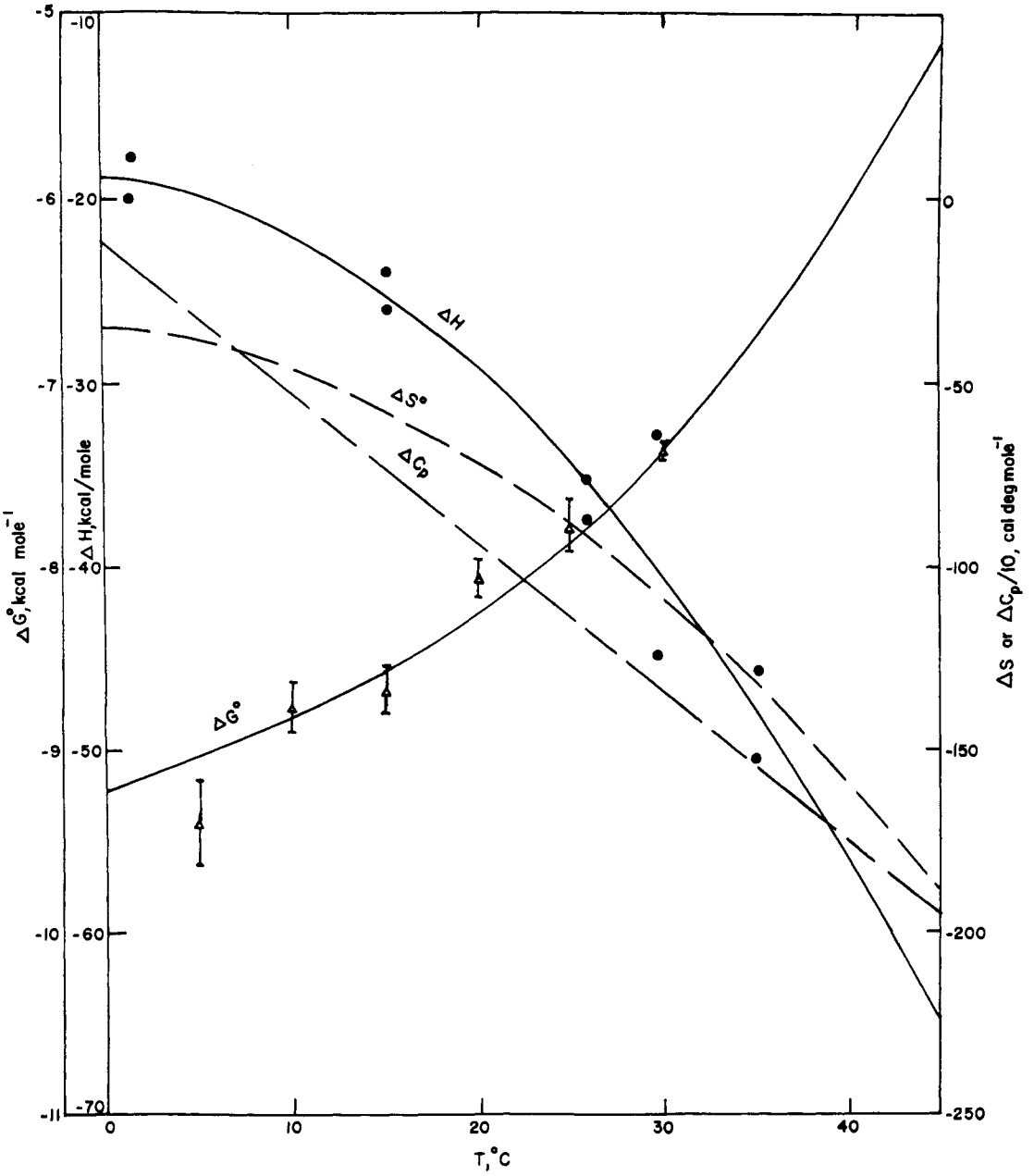

FIGURE 5: Summary of the thermodynamic parameters for the reaction of Met(O_2)-13-S-peptide with S-protein. See the legend of Figure 4 for the significance of the curves in the figure.

pH 7 in 0.3 M NaCl both calorimetrically and spectrophotometrically (Hearn, 1969). As is the case with RNase A, at pH 7, the transitions are incompletely reversible. For unknown reasons, the calorimetric data in the case of S-protein were particularly poorly reproducible. Allende and Richards (1962) reported sedimentation velocity data indicating that at neutral pH there is considerable aggregation of S-protein, and chromatographic evidence that the aggregation is reversed on the addition of an equimolar amount of S-peptide. It is possible that aggregation contributes to the irreproducibility mentioned above, since the aggregates, which may be present in variable amounts, are probably broken up by heating.

Typical calorimetric transition curves for S-protein, RNase S, and RNase S′ have been published by Tsong *et al.* (1970). Data for the transitions as reported by Tsong *et al.* and as determined in the present work are summarized in Tables VI and VII. Detailed discussion of the evaluation of the quantities given in Tables VI and VII is given by Tsong *et al.* (1970).[1] The enthalpy values, $\Delta H_{vH}(T_m)$, obtained from the slopes of the transition curves by application of the van't Hoff equation, must be considered as purely formal expressions for the

[1] Since the transitions of RNase S and RNase S′ are reactions of the type A $\rightleftarrows$ B + C, the equilibrium constant is $K = \alpha^2(A)_0/(1 - \alpha)$, where $(A)_0$ is the initial protein concentration. Differentiation of this expression gives

$$\Delta H_{vH}(T_m) = 6RT_m^2 \left(\frac{d\alpha}{dT}\right)_{T_m}$$

The factor 6 replaces the factor 4 appropriate to a process of the type A $\rightleftarrows$ B. The values of ΔH_{vH} for RNase S and RNase S′ given by Tsong *et al.* (1970) are incorrect since the factor 4 was used in their computation; they are given correctly in Table VII. The increase in T_m with increasing $(A)_0$ which is evident in Table VII is largely due to the fact that the transition is accompanied by dissociation. We are indebted to one of the referees of this paper for bringing this point to our attention.

TABLE VI: Calorimetric Data for the Thermal Transition of S-Protein at pH 7.0.

Concentration (mg ml^{-1})	T_m[c] (°C)	ΔH_{cal} (T_m)[d] (kcal mole^{-1})	ΔH_{vH}(T_m)[e] (kcal mole^{-1})	ΔC_p (kcal deg^{-1} mole^{-1})
16.1[a]	37.0	55	14	1.5
16.1[a]	35.3	57	16	1.2
15.9[a]	35.2	63	16	1.6
15.5[a]	36.1	43	18	1.1
13.9[a]	37.0	52	20	1.3
5.0[b]	37.6	55	30	1.4
Mean values	36.4	54	19	1.4
Std error of mean	±0.4	±3	±2	±0.1

[a] This work; 0.3 M NaCl. [b] Tsong *et al.* (1970); 0.2 M NaCl. [c] Temperature at which the transition is half-completed, the so-called melting temperature. [d] Calorimetric value for the enthalpy change at T_m. [e] Enthalpy value obtained from the slope of the calorimetric transition curve at T_m (Tsong *et al.*, 1970) by formal application of the van't Hoff equation d ln $K/dT = \Delta H°/RT^2$.

slopes, since it is manifestly incorrect to apply an equation valid only for a simple two-state equilibrium to the present cases. Of primary interest, for the application of Hess' law to be discussed later, is the actual excess heat absorption per mole in raising the temperature of S-protein and RNase S′ in solution. Average values derived from all the experiments in 0.3 M NaCl and in 0.2 M NaCl (Tsong *et al.*, 1970) are given in Table X. Q_P is the excess heat absorption by S-protein and Q_E that by RNase S′. Uncertainty limits were estimated for the experiments in 0.3 M NaCl from the scatter of the observed data; since only one experiment was run with each protein in 0.2 M NaCl, no uncertainty limits are given.

TABLE VII: Calorimetric Data for the Thermal Transition of Ribonuclease S at pH 7.0.

Concn (mg ml^{-1})	T_m[e] (°C)	ΔH_{cal}(T_m)[f] (kcal mole^{-1})	ΔH_{vH}-(T_m)[g] (kcal mole^{-1})	ΔC_p (kcal deg^{-1} mole^{-1})
11.4[a]	49.6	131	83	1.9
6.4[a]	48.8	122	90	1.7
6.3[a]	48.1	130	83	2.1
6.3[a]	48.3	132	84	2.0
5.0[a]	47.3	149	75	2.7
4.5[a]	47.2	134	80	2.7
5.0[b]	47.7	107	93	2.1
5.0[c]	47.1	111	95	2.1
Mean values	48.0	127	85	2.2
Std error of mean	±0.3	±5	±2	±0.1
5.0[d]	61.3	168	88	2.1

[a] This work; 0.3 M NaCl. [b] Tsong *et al.* (1970); 0.2 M NaCl. [c] Ribonuclease S′ (Tsong *et al.*, 1970); 0.2 M NaCl. [d] Ribonuclease A (Tsong *et al.*, 1970); 0.2 M NaCl. [e] Temperature at which the transition is half-completed, the so-called melting temperature. [f] Calorimetric value for the enthalpy change at T_m. [g] Enthalpy value obtained from the slope of the calorimetric transition curve at T_m (Tsong *et al.*, 1970) by formal application of the van't Hoff equation d ln $K/dT = \Delta H°/RT^2$.

TABLE VIII: Spectrophotometric Data for the Thermal Transition of S-Protein at pH 7.0.[a]

T_m,[b] °C	35.2	37.4	35.6
ΔH_{vH} (T_m),[c] kcal mole^{-1}	43	38	40

[a] Protein concentration 1.0 mg ml^{-1} in 0.3 M NaCl. [b] Temperature at which the spectral change is half-completed. [c] Enthalpy value obtained from the slope of the spectrophotometric transition curve at T_m by formal application of the van't Hoff equation d ln $K/dT = \Delta H°/RT^2$.

Spectrophotometric Melting Curves. Sherwood and Potts (1965a,b) studied the changes in the spectra of S-protein and RNase S, associated with the normalization of buried tyrosine residues, which occur on heating. We have redetermined the spectrophotometric melting curves under the experimental conditions used in our calorimetric experiments, for S-protein at 285 nm and for RNase S at 287 nm, at protein concentrations of approximately 1 mg ml^{-1}. In the case of S-protein, the transition appeared reversible as judged by recovery of the original absorbance on cooling, although it is known from the calorimetric experiments to be irreversible, whereas RNase S suffered a loss in absorbance of about 20% when heated above the transition and then cooled.

Three closely agreeing transition curves were run for S-protein and six for RNase S, and in each case the average curve was used to construct a plot of fraction converted as a function of temperature. As shown in Figures 6 and 7, spectrophotometry gives a significantly different estimate of the extent of reaction from that based on calorimetric observations. In each case the calorimetric transition is broader than the spectrophotometric one; thus the calorimetric transition curves give van't Hoff enthalpy values of 16 kcal mole^{-1} for S-protein and 55 kcal mole^{-1} for RNase S, whereas the spectrophotometric values are 39 and 67 kcal mole^{-1}, respectively. The data derived from the spectrophotometric curves are given in Tables VIII and IX.

Discussion

Thermal Transitions of S-Protein and RNase S. As noted in the preceding paragraph, the enthalpies of S-protein and RNase S at concentrations of 5 to 16 mg ml^{-1} at pH 7.0 are very different functions of the temperature from the absorbances at concentrations of 1 mg ml^{-1} at the same pH. This is clear evidence that these transitions are not simple two-state, fully cooperative processes (Lumry *et al.*, 1966) under either of these sets of conditions. It is possible that the greater steepness of the spectrophotometric transition curves is due at least in part to the lower concentration employed in these experiments, although the fact that the van't Hoff enthalpy values derived from the spectrophotometric data are much smaller than the actual calorimetric values at the respective melting temperatures (Tables VIII and IX) indicates that the

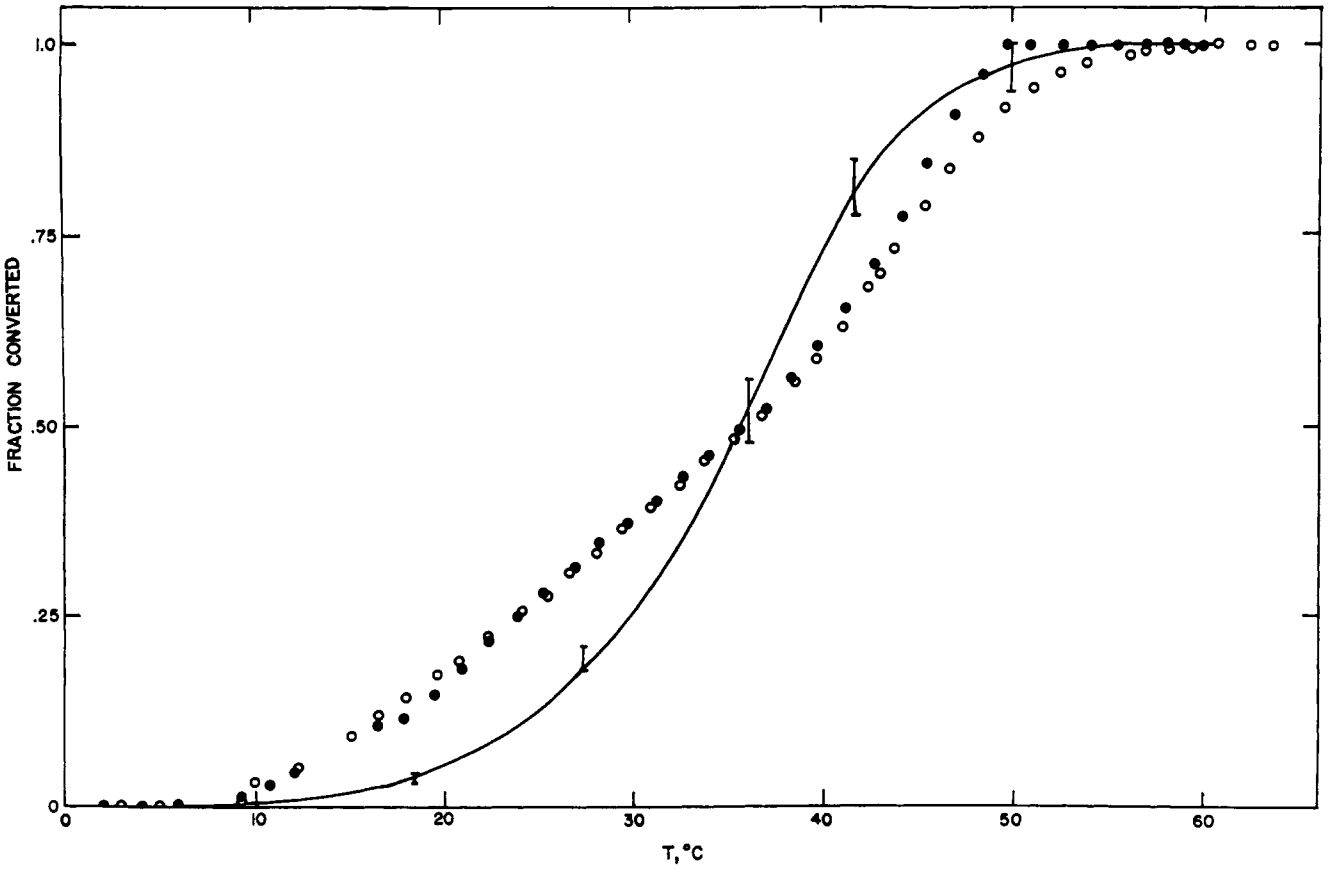

FIGURE 6: Comparison of the apparent fraction converted in the thermal transition of S-protein as observed spectrophotometrically and calorimetrically at pH 7.0 in 0.3 M NaCl. The curve is derived from the average of the three spectrophotometric runs (Table VIII), the open circles from the third calorimetric experiment listed in Table VI, and the filled circles from the fourth calorimetric experiment listed in the table.

transitions are not two-state even at the lower concentrations. In view of the finding (Tsong *et al.*, 1970) that the thermal transition of RNase A is not two-state at pH 7.0, it would be surprising if the same were not also true of S-protein and RNase S.

Temperature Variation of the Enthalpies of Reactions 1 and 2. As shown in Figures 4 and 5, the enthalpy changes observed for reactions 1 and 2 turned out to be surprisingly strong functions of the temperature. The enthalpy data may be expressed within experimental uncertainty as quadratics in the temperature, as listed in Tables II and III. Differentiation of the expressions for the enthalpy changes leads to very large values for the changes in apparent heat capacity, ΔC_p, which are shown graphically in Figures 4 and 5.

Very large values for ΔC_p have been found for a number of protein reactions (Tanford, 1969, 1970; Velick *et al.*, 1971); however, in no previous cases has ΔC_p been found to be more than slightly, if at all, dependent on temperature. The apparent temperature dependence of ΔC_p for reaction 1 is largely the result of the conformational transitions undergone by S-protein and RNase S′ as the temperature is raised. This is shown by the following argument.

Consider reaction 1 at two different temperatures, 5° and some higher temperature, T.

$$\begin{array}{ccc} p(5) + P(5) & \xrightarrow{\Delta H_5} & E(5) \\ {\scriptstyle Q_p}\downarrow & & \downarrow{\scriptstyle Q_P} \quad \downarrow{\scriptstyle Q_E} \\ p(T) + P(T) & \xrightarrow{\Delta H_T} & E(T) \end{array} \qquad (7)$$

In reaction 7, Q_p is the enthalpy difference between S-peptide at T and at 5°, and Q_P and Q_E are the corresponding quantities for S-protein and RNase S′. It follows from Hess' law that

$$\Delta H_5 = Q_p + Q_P - Q_E + \Delta H_T \qquad (8)$$

The quantities Q_P and Q_E were directly evaluated, unfortunately with rather poor reproducibility, in the transition calorimetry mentioned earlier. Each of the transitions was accompanied by a very large increase in the apparent heat capacity of the solute, amounting to approximately 1.3 kcal deg^{-1} mole^{-1} (this work) or 1.4 kcal deg^{-1} mole^{-1} (Tsong *et al.*, 1970) for S-protein, and 2.2 kcal deg^{-1} mole^{-1} (this work)

TABLE IX: Spectrophotometric Data for the Thermal Transition of Ribonuclease S at pH 7.0.[a]

T_m,[b] °C	46.1	45.9	46.1	46.1	46.2	46.7
$\Delta H_{vH}(T_m)$,[c] kcal mole^{-1}	70	74	71	74	71	75

[a] Protein concentration 1.1 mg ml^{-1} in 0.3 M NaCl. [b] Temperature at which the spectral change is half completed [c] Enthalpy value obtained from the slope of the spectrophotometric transition curve at T_m by formal application of the van't Hoff equation $d \ln K/dT = \Delta H°/RT^2$.

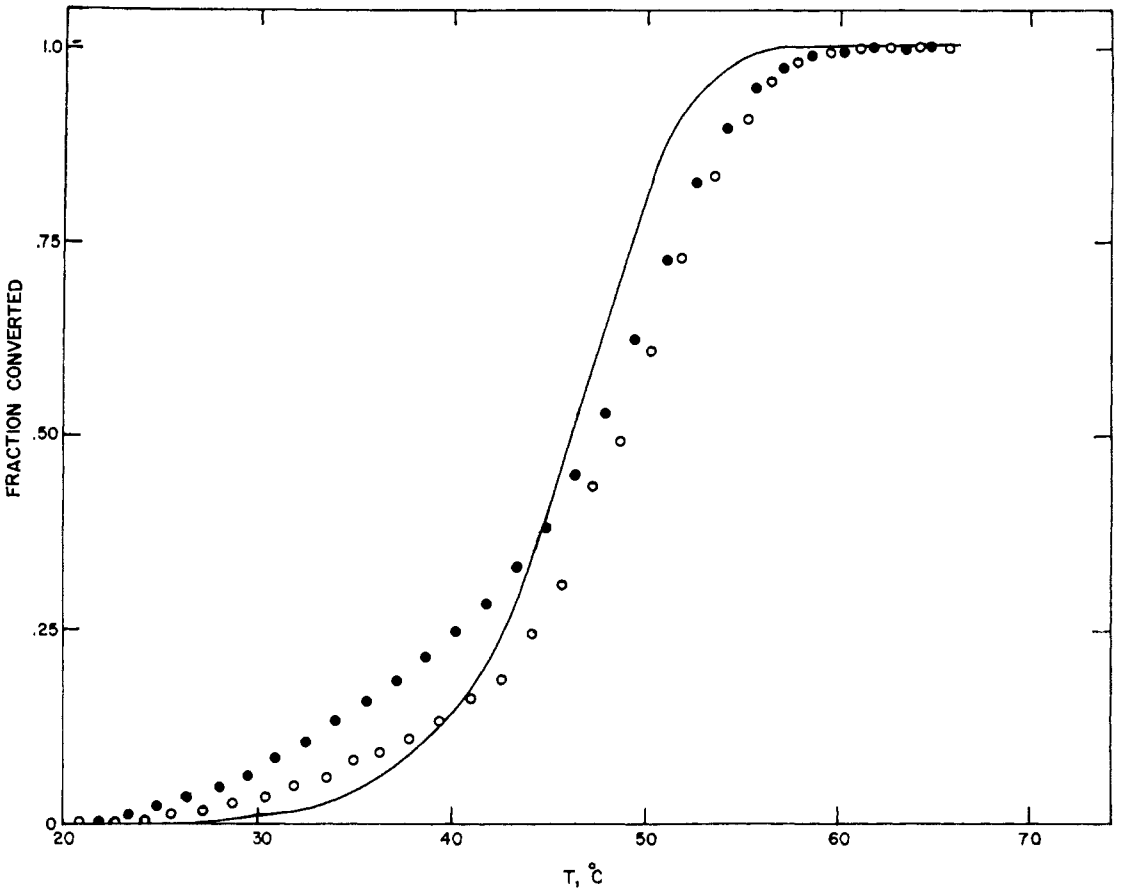

FIGURE 7: Comparison of the apparent fraction converted in the thermal transition of RNase S as observed spectrophotometrically and calorimetrically at pH 7.0 in 0.3 M NaCl. The curve is derived from the average of the six spectrophotometric runs (Table IX), the open circles from the second calorimetric experiment listed in Table VII, and the filled circles from the fifth calorimetric experiment listed in the table.

or 2.1 kcal deg^{-1} mole^{-1} (Tsong *et al.*, 1970) for RNase S or RNase S′. Since RNase S′ is undoubtedly dissociated after completion of its transition, it is reasonable to assume that the difference between these figures is the increase in apparent heat capacity undergone by the S-peptide, and probably to a lesser extent by the S-protein, when the S-peptide is dissociated from the S-protein. For convenience this excess heat capacity is arbitrarily assigned entirely to the S-peptide, and it is taken to equal $+0.7 \pm 0.1$ kcal deg^{-1} mole^{-1}. This large increase in heat capacity is presumably due mainly to two contributions: (a) exposure to the solvent of nonpolar groups involved in hydrophobic bonding between S-peptide and S-protein, with concomitant orientation of water molecules around them; and (b) increase in the number of excitable internal degrees of freedom. A negative contribution to ΔC_p, amounting to -30 to -50 cal deg^{-1} mole^{-1}, may result from exposure to the solvent of the charged groups involved in a salt linkage in RNase S′ between Asp-14 and Arg-33. The magnitude of ΔC_p is not exceptionally large for a protein system; for example, there is a change in apparent heat capacity of -500 cal deg^{-1} mole^{-1}, independent of temperature over the range 5–40°, when 1 mole of NAD^+ is bound by yeast glyceraldehyde phosphate dehydrogenase (Velick *et al.*, 1971).

If we make the reasonable assumption that the excess heat capacity of free S-peptide is independent of temperature, and that there is no thermally-induced change in the conformation of S-peptide which absorbs a significant amount of heat in the temperature range of interest, we may set Q_p in eq. 8 equal to (0.7 ± 0.1) (T-5) cal mole^{-1}. The latter assumption is supported by the observation of Klee (1968) and Scatturin *et al.* (1967), and further verified in the present work (Hearn, 1969), that the circular dichroism of S-peptide is very nearly independent of temperature, at least over the range of interest here. Table X lists values of Q_p calculated in this way, together with average values of Q_P and Q_E obtained from several runs (columns four and five), and values reported by Tsong *et al.* (1970) (columns seven and eight). The fact that ΔH_5(calcd) (columns six and nine) is constant within experimental uncertainty indicates that the entire temperature dependence of the enthalpy change in reaction 1 is adequately accounted for in terms of the quantities Q_p, Q_P, and Q_E. It is probable that a similar treatment for reaction 2 would hold if the requisite data were available.

We are thus led to the conclusion that the value of ΔC_p for reaction 1 involving completely folded S-protein and RNase S′ is close to -0.7 kcal deg^{-1} mole^{-1}, so that $\Delta H < 0$ at all accessible temperatures, and the reaction is always energy driven.

Temperature Variation of the Equilibrium Constants for Reactions 1 and 2. The equilibrium constants for reactions 1 and 2 were found to be strongly dependent on temperature, as shown in the van't Hoff plots of Figure 3. It is important to note that the van't Hoff plots are linear within experimental uncertainty, although the enthalpy changes as determined calorimetrically for both reactions change rapidly with temperature. The slopes of the van't Hoff plots correspond to $\Delta H = -59.5$ and -31.6 kcal mole^{-1} for reactions 1 and 2, respec-

TABLE X: Application of Hess' Law to the Enthalpy Data for Reaction 1.[a]

T (°C)	Mean ΔH_T	Q_P	Q_P[b]	Q_E[b]	ΔH_5(Calcd)[d]	Q_P[c]	Q_E[c]	ΔH_5(Calcd)[d]
5.0	−23.1	0	0	0	−23	0	0	−23
10.0	−27.0	3.5 ± 0.5	0.5 ± 0.2	0	−23 ± 1	0	0	−23
14.5	−28.0	6.6 ± 1.0	2.7 ± 1.0	0	−19 ± 1	0.8	0	−21
16.0	−27.6	7.7 ± 1.1	3.5 ± 2.0	0	−16 ± 2	1.0	0	−19
19.5	−32.7	10.1 ± 1.5	6.4 ± 3	0	−16 ± 3	3.0	0.8	−20
20.2	−33.3	10.6 ± 1.5	7.0 ± 3	0.4 ± 0.2	−16 ± 4	3.5	1.3	−20
25.0	−38.1	14.0 ± 2	12.0 ± 4	1.7 ± 0.8	−14 ± 5	7.8	2.3	−19
33.0	−54.9	22.5 ± 5	24.4 ± 5	7.5 ± 3	−16 ± 7	21.3	7.5	−22
40.6	−70.5	24.9 ± 4	39.8 ± 7	23.0 ± 6	−29 ± 10	43.4	18.1	−20
				Mean	−19		Mean	−21
				Std deviation	±5		Std deviation	±2

[a] All quantities are in kcal mole^{-1}. [b] This work. [c] Tsong *et al.* (1970). [d] Calculated according to eq 8.

TABLE XI: Summary of Thermodynamic Data for the Reaction of S-Peptide with S-Protein, 1, and of Met(O_2)-S-Peptide with S-Protein, 2, in 0.3 M NaCl, pH 7.

	ΔH (kcal mole^{-1})		$\Delta G°$ (kcal mole^{-1})		$\Delta S°$ (cal deg^{-1} mole^{-1})		ΔC_P (cal deg^{-1} mole^{-1})	
T (°C)	1	2	1	2	1	2	1	2
0		−18.9		−9.2		−35		(−120)
5	−23.6	−19.8	−11.8	−9.0	−42	−39	(−170)[a]	(−320)
10	−25.2	−21.9	−11.6	−8.8	−48	−46	(−490)[a]	(−530)
15	−28.4	−25.0	−11.3	−8.5	−60	−57	−810	−730
20	−33.3	−29.2	−11.0	−8.2	−76	−72	−1130	−930
25	−39.8	−34.4	−10.5	−7.8	−98	−89	−1460	−1140
30	−47.9	−40.6	−10.0	−7.3	−125	−110	−1780	−1340
35	−57.5	−47.8	−9.3	−6.7	−157	−133	(−2100)	(−1550)
40	−68.8	−56.0	−8.4	−6.0	−193	−160	(−2420)	(−1750)

[a] As explained in the text, it is felt that −700 cal deg^{-1} mole^{-1} is a more reliable figure for ΔC_P of reaction 1 at low temperatures than the values given here.

tively; the range of calorimetric values of ΔH in the temperature range of equilibrium measurements is −48 to −82 kcal mole^{-1} for reaction 1, and −20 to −41 kcal mole^{-1} for reaction 2. These data illustrate the great difficulty of obtaining reliable enthalpy data by the van't Hoff method. As shown by the comparison in Figures 4 and 5 between standard free energies calculated from the equilibrium constants and by integration of the Gibbs–Helmholtz equation, the equilibrium data are consistent with the calorimetric data although they give apparently linear van't Hoff plots.

Thermodynamic Parameters for Reactions 1 and 2. The empirical equations expressing the enthalpy changes in reactions 1 and 2 given in Tables II and III and the equilibrium data for the reactions can be combined to give the thermodynamic parameters listed in Table XI. Some bias may be introduced by the arbitrary decision to fit the enthalpy data to quadratic expressions in the temperature. It is obvious that the values of the apparent heat capacity changes obtained by differentiation of these expressions are subject to particularly large uncertainties at each end of the temperature range covered, as indicated by parentheses in the table. We believe that the value $\Delta C_p = -700$ cal deg^{-1} mole^{-1} for reaction 1, based as outlined in the first section of the discussion on the transition calorimetric data, is a more probable value for ΔC_p at low temperatures than those given in Table XI, although it is not possible to be certain of this at present.

Although the thermal conformational transitions of S-protein and RNase S′ are not two-state reversible processes, it is of interest to note that application of equilibrium thermodynamics to the transition calorimetric data leads to a not unreasonable estimate for the standard entropy change in reaction 1 at 5°. In the transition of RNase S′, S-peptide, and S-protein bound together in constrained form are dissociated to essentially random, unfolded species; in the transition of S-protein, the protein is converted from its unconstrained low temperature form to the random unfolded form. Thus the difference between these two processes amounts to reaction 1, if we assume as we did earlier that the conformation of S-peptide is not significantly affected by temperature.

Possible Effect of Aggregation on the Thermodynamic Parameters for Reactions 1 and 2. Allende and Richards (1962) found from measurements of sedimentation velocity that S-

protein aggregates at neutral pH. We also have determined sedimentation coefficients which indicate aggregation, to a lesser extent at 35° than at 5°. On the basis of the sharpness of chromatographic peaks on carboxymethylcellulose, Allende and Richards (1962) concluded that the S-protein polymers are dissociated by combination with S-peptide. It is thus possible that the thermodynamic parameters for reactions 1 and 2, particularly at low temperatures, include contributions from the dissociation of S-protein aggregates. In this connection, it is significant that no appreciable variation of ΔH with concentration was observed in the flow calorimetric experiments at 25°, although concentrations of S-protein as low as 0.2 mg ml^{-1} were used. This result indicates either that the aggregation of S-protein involves insignificant changes in enthalpy or that the S-protein association constants are so high that aggregation is essentially unaffected by dilution to 0.2 mg ml^{-1}.

Comparison of Observed and Predicted Values of the Thermodynamic Parameters. From the known three-dimensional structure of RNase S, which we assume to be the same as that of RNase S′, it is possible to identify at least qualitatively some of the probable interactions between the S-peptide and the S-protein. It appears that the binding would be accompanied by the formation of 16 hydrogen bonds, as follows: (1) 7 in the S-peptide α-helical region; (2) 1 between the α-carbonyl group of Met-13 and the guanidino group of Arg-33. (3) 1 between Glu-11 and Asn-44; and (4) 7 or 8 between S-peptide and S-protein. However, it is quite possible that on dissociation of the S-peptide, the hydrophobic regions in the remaining cleft of the S-protein would move together causing rupture or weakening of hydrogen bonds elsewhere in the S-protein, so that the net formation of hydrogen bonds on association of the S-peptide would be larger than enumerated above. Hydrophobic interactions would be expected to result from the close contacts of Ala-4, Phe-8, His-12, and Met-13 in the S-peptide with the nonpolar core of the S-protein. Finally, Asp-14 and Arg-33 are so located as to suggest the existence of a strong electrostatic interaction, perhaps also involving His-48.

In addition to the interactions listed above, the S-peptide undoubtedly loses much of its conformational freedom on being bound to the S-protein. From the RNase S structure it can be estimated that at least residues 3 to 14 on the peptide are immobilized by the interaction. Brant *et al.* (1967) have estimated the conformational entropy of a randomly coiled polypeptide backbone to be 9–11 cal deg^{-1} (mole of residue)$^{-1}$, so that this contribution to the entropy change in reaction 1 might amount to as much as -100 cal deg^{-1} $mole^{-1}$.

The difference of 12° between the melting temperatures of RNase S′ and S-protein shows that the S-peptide stabilizes the S-protein, so that there may well be some loss of conformational freedom in the S-protein as well. There is, however, no basis presently available for estimating how important this contribution may be, since nothing is known concerning the structure of S-protein.

Any attempt to achieve a quantitative correlation between the previously mentioned sources of enthalpic and entropic contributions to the free-energy change in reaction 1 and the observed data is beset by very serious difficulties. (a) There is no information concerning the three-dimensional structures of the reactants S-peptide and S-protein. (b) There is no consensus as to the magnitude of the individual contributions associated with the various types of interaction currently thought to be important in determining protein structure. (c) Such values for the individual contributions as have been proposed apply to 25°, at which temperature the conformational transition of S-protein already makes a major contribution to the observed enthalpy.

It happens that if we assign to the hydrogen bonds and hydrophobic interactions listed above the mean values proposed by Kauzmann (1959), -100 deg^{-1} cal $mole^{-1}$ for the conformational entropy change, and $\Delta H = 10$ kcal $mole^{-1}$ and $\Delta S_{\text{unitary}} = -10$ cal deg^{-1} $mole^{-1}$ to the postulated electrostatic interaction, we "predict" $\Delta H = -38$ kcal $mole^{-1}$ (including $Q_P = 10$ kcal $mole^{-1}$, Table X) and $\Delta S = -81$ cal deg^{-1} $mole^{-1}$ (including a cratic entropy contribution of -8 cal deg^{-1} $mole^{-1}$). Although the predicted ΔH and ΔS are, respectively, in good and in fair agreement with the observed values, we believe that it is premature to take such numerology seriously.

The simplest possible view of the difference between reactions 1 and 2 is that the postulated hydrophobic interaction involving Met-13 would be weakened when the more polar and bulky Met(O_2) residue is present. Unfortunately for this view, the differences in ΔH and ΔS are both in the wrong direction, and the difference in ΔC_p, although of the expected sign, is unexpectedly large. Of course, this comparison suffers greatly from the fact that we have no information concerning the energetics of the conformational changes in the reactants and product in reaction 2, which probably are even more important in reaction 2 at 25° than in reaction 1.

We do not as yet even approach the position of being able to predict thermodynamic quantities for proteins on the basis of structural information. Far more quantitative data for a wide range of systems are needed, including experimental values of ΔH, ΔS, and ΔC_p for the transfer of a great variety of important types of molecules and groups from nonpolar to aqueous media.

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Binding of Cations to Caseins. Site Binding, Donnan Binding, and System Characteristics*

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ABSTRACT: Ion–protein interactions in the formation of core polymers and cores of casein micelles are determined by examining α_s-casein, β-casein, and their unit weight ratio mixture at final pH 6.6 and 37°. Increasing protein association is correlated with monomer net charge, protein solvation, and site binding of calcium and hydrogen ions. To obtain these data, isoionic proteins are used and proton releases are obtained (a) as monovalent cation concentration, I, is brought to 0.04, 0.08, and 0.16 M, and (b) at each I, as calcium concentration is brought progressively to 0.08 M. Protein solvations are measured for precipitates, and calculated from apparent monomer net charge for protein in solution. Apparent molar calcium binding, $\bar{\nu}_{Ca,A}$, is measured after equilibrium dialysis. Since net fixed charge occurs on short, solvent-accessible acidic peptides, Donnan binding of calcium ion is examined. A model consistent with the theory of Overbeek is used to obtain monomer net charge, and Donnan binding corrections, $\bar{\nu}_{Ca,D}$. Site-bound calcium is obtained by difference. Results and conclusions are: $\bar{\nu}_{Ca,D}$ are small at low $\bar{\nu}_{Ca,A}$ (~15%) and decrease as ionic strength and/or $\bar{\nu}_{Ca,S}$ increase. For α_s-casein, β-casein, and the mixture; and independent of I, (a) precipitation is initiated at the same $\bar{\nu}_{Ca,S}$ and $\bar{Z}$ (respectively, 9.3, 5.4, and 10.4, and −9, −6, and −1.5), and (b) at 0.08 M calcium, proton release and $\bar{\nu}_{Ca,S}$ are essentially constant (6.6, 4.8, and 6.3, and 20, 11.3, and 16.3). At these $\bar{\nu}_{Ca,S}$ there is apparently a reversal of $\bar{Z}$ in precipitate (to approximately +10.4, +4.4, and +8.9). Since solubility and solvation do not reverse significantly, core polymer cross-linking by calcium is proposed as a source of interaction energy. The mixture is not intermediate in precipitate characteristics of $\bar{\nu}_{Ca,S}$, $\bar{Z}$, and solvation. In the mixture, comingling of monomers in core polymers produces interactions which convert potential interpolymer cross-linking sites into intrapolymer cross-linking sites.

Casein micelles, according to the model of Waugh and Noble (1965), consist of cores of insoluble α_s- and β-caseinates stabilized by a coat layer containing κ-casein. Cores are variable in size, and this leads to a micelle size distribution. The coat-core model suggests that the properties of the core can be determined through investigation of the precipitation characteristics of α_s- and β-caseins. Waugh *et al.* (1970), using individual proteins or a unit weight ratio mixture, have shown that an increasing degree of monomer association is produced both by increasing ionic strength due to mono-

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