of histidine D to another aromatic residue in FIII similar to the interaction of His-48 and Tyr-52 in pancreatic phospholipase A2. Aguiar et al. (1979) observed an upfield shift of 1.57 and 0.4 ppm for the C-2 H and the C-4 H resonances of His-48, respectively, which could be explained on the basis of the X-ray structure as the ring current effect of Tyr-52.

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Thermal Denaturation of *Streptomyces* Subtilisin Inhibitor, Subtilisin BPN′, and the Inhibitor–Subtilisin Complex†

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ABSTRACT: The thermal unfolding of the microbial proteinase inhibitor *Streptomyces* subtilisin inhibitor (SSI) [Sato, S., & Murao, S. (1973) *Agric. Biol. Chem. 37*, 1067–1074), the bacterial proteinase subtilisin BPN′ (EC 3.4.21.14), and the complex formed by these two proteins has been studied by differential scanning calorimetry (DSC). The thermal denaturation of SSI at pH 7.00 is fully reversible while those of subtilisin BPN′ and its complex with SSI are not. The DSC data show that dimeric SSI remains dimeric as the temperature is raised until it unfolds and that it then dissociates during the unfolding process. The apparent specific heat of denatured SSI decreases rapidly with increasing temperature, a behavior not previously observed for proteins. The shape of the DSC curves observed with the enzyme–inhibitor complex suggests that the two components of the complex undergo their unfolding transitions more or less independently. The enthalpies of unfolding of mixtures of enzyme and inhibitor in various molar ratios indicate a substantially larger enthapy of interaction than that deduced from fluorescence titrations (Uehara, Y., Tonomura, B., & Hiromi, K. (1978) *J. Biochem. (Tokyo) 84*, 1195–1202).

Streptomyces subtilisin inhibitor (SSI), isolated from *Streptomyces albogriseolus*, strongly inhibits bacterial proteinases such as subtilisin BPN′, with inhibition constants as high as 10^{10} M. The inhibitor consists of two identical subunits, each containing a polypeptide chain of 113 amino acid residues with 2 intrachain disulfide bridges. Each subunit, of molecular weight 11 483, binds to one molecule of subtilisin to form a complex E_2I_2 (Inouye et al., 1978). The amino acid sequence of the inhibitor was determined by Ikenaka et al. (1974), and its three-dimensional structure has been recently determined at 2.3-Å resolution, along with that of the inhibitor–protein complex E_2I_2 at 4.3-Å resolution, by Mitsui et al. (1979).

The detailed characterization available for SSI and for its complex with subtilisin makes this system an attractive one for investigation by DSC. The results of such a study are presented in this paper.

Materials and Methods

Materials. Partially purified SSI was a gift from Professor Keitaro Hiromi of Kyoto University, Japan. The protein was further purified by the method of Sato & Murao (1973). Its concentration was determined from the absorbance at 280 nm by using a value for the absorptivity of 0.82 cm^2 mg^{-1}. Crystalline subtilisin BPN′ (molecular weight 27 500), purchased from Nagase Sangyo Co., Osaka, Japan, was also a gift from Professor Hiromi. Its concentration was determined

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spectrophotometrically by using an absorptivity of 1.041 cm^2 mg^{-1} at 280 nm. This enzyme was found by Uehara et al. (1980) to be 75.8% pure as judged by initial burst titration with *N-trans*-cinnamoylimidazole according to the method of Bender et al. (1966). It is not clear how our calorimetric data should be corrected for this deviation from purity, since the enzymically inactive protein present is likely to have spectroscopic and denaturation properties similar to those of the active enzyme. In view of this uncertainty, we have decided to apply no correction.

Solutions of SSI were prepared by first precipitating the protein from a solution of purified material by adjusting the pH of the solution to the isoelectric point, 4.5. The precipitate was then collected and a minimum amount of buffer solution added to obtain a clear solution, and this solution was exhaustively dialyzed against buffer. The dialyzed solution usually had a protein concentration of about 8×10^{-4} M and was used as a stock solution. The buffers employed were 0.04 M glycine and 0.04 M McIlvaine buffer at pH 2.84 and 0.06 M phosphate at pH 7.00. The McIlvaine buffer was 0.04 M citric acid containing sufficient Na_2HPO_4 to give pH 2.84 and gave results indistinguishable from those obtained with 0.04 M glycine.

Subtilisin solutions were prepared by dissolving the solid protein in buffer immediately before use, without any dialysis, in an effort to minimize autolysis.

Differential Scanning Calorimetry. The calorimeter developed by Privalov et al. (1975) was employed. Some modifications of the electronics associated with the calorimeter were made, but these changes did not alter the operation of the instrument. The volume of the sample cell was 1.086 mL. A scan rate of 1 K min^{-1} was in general employed. SSI concentrations ranged from 0.4 to 4.7 mg mL^{-1}, and those of subtilisin BPN′ ranged from 0.7 to 2 mg mL^{-1}. Calorimetric enthalpies were evaluated by planimeter integration of the curves of excess heat capacity recorded by the DSC, after establishment of a base line as discussed below.

Results and Discussion

DSC traces for the unfolding of SSI observed at various values of the pH are shown in Figure 1. Interpretable transition curves were obtained only at pH ≤3.0 and pH ≥5.5. At intermediate values of the pH, severe oscillations were observed which rendered the curves useless. The exact cause of those oscillations is unknown, although observations on numerous other systems have led us to believe that they are generally associated with precipitation of insoluble material in the calorimeter cell. In this connection it may be noted that the isoelectric pH of SSI, 4.5, is in the middle of the pH range within which oscillations were observed.

The reversibility of the unfolding process was checked at pH 2.84 and pH 7.00 by reheating the sample of protein after it had been cooled to room temperature in the calorimeter, for 8 and 48 h, respectively. The unfolding was found to be quantitatively reversible. The transition temperature at pH 7.00 is independent of the scan rate over the range 0.1–1.0 K min^{-1}. This behavior could not be accurately checked at pH 2.84 because of the breadth of the transition and its small amplitude. The peak temperature of the transition, T_p, but not the enthalpy, was found to be dependent on buffer concentration, T_p increasing from 80.5 to 86.8 °C when the buffer concentration was increased from 0.01 to 0.46 M. Thus, the stability of the protein relative to that of its unfolded form is increased by an increase in ionic strength.

Base Lines. A major problem in any DSC study is the establishment of appropriate chemical (as contrasted with instrumental) base lines. Typical low (pH 2.84) and high (pH 7.00) pH traces are shown in Figure 2. It is evident that at pH 7.00, the apparent heat capacities of both the native and denatured forms of the protein are strongly dependent on temperature. This behavior is less obvious in the curve for pH 2.84. We have no alternative but to assume that the apparently linear base lines observed before and after the transition can be extrapolated into the transition region, as indicated in Figure 2. On the assumption of two-state behavior, the true base line will then consist of a curve which changes from the one extrapolated base line to the other in proportion to the extent of the transition as determined by stepwise integration of the transition curve. Such calculated base lines are shown in the figure.

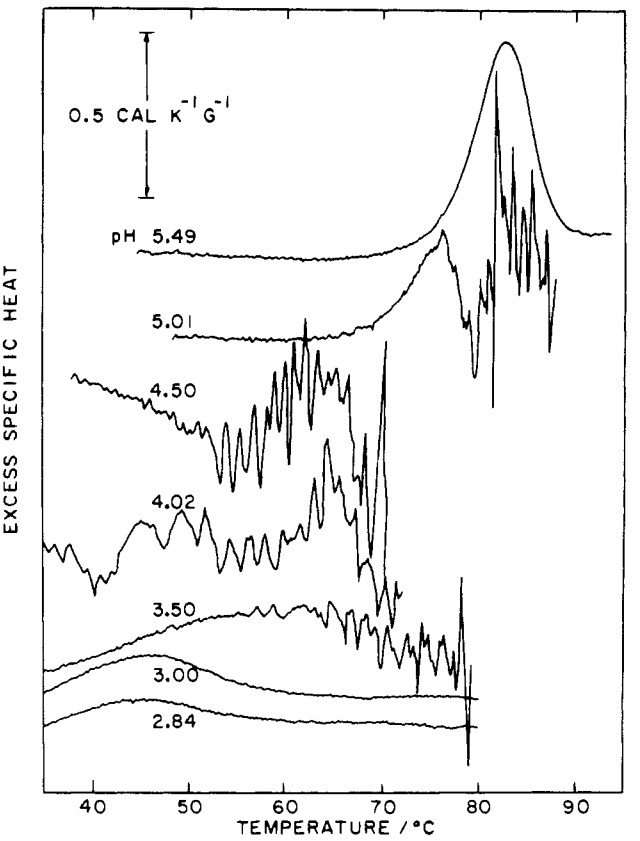

FIGURE 1: Tracings of DSC curves for the unfolding of SSI at the various values of pH indicated. Protein concentration was 2.35 mg mL^{-1}. Usable data were obtained only at pHs below 3.0 and above 5.5.

Temperature Dependence of the Apparent Heat Capacity. The apparent specific heat of SSI in the native (N) and denatured (D) forms can be expressed by

$$c_N = A + Bt \quad (1)$$

$$c_D = C + Dt \quad (2)$$

where t is the temperature in degrees Celsius. It is convenient to express the specific heats on a relative scale with the constant A set equal to zero. The other constants for the sample used for curve B in Figure 2 have the values $B = 0.00092$ cal K^{-2} g^{-1}, $C = 0.359$ cal K^{-1} g^{-1}, and $D = -0.00273$ cal K^{-2} g^{-1}. The value for B is similar to that observed for a number of globular proteins (Privalov, 1979), but the negative value for D is most unusual both in magnitude and especially in sign. A variety of causes for a negative temperature coefficient of specific heat can be suggested [see, for example, Sturtevant (1977)], but no basis exists at present for a choice between them. According to the van't Hoff equation, the negative

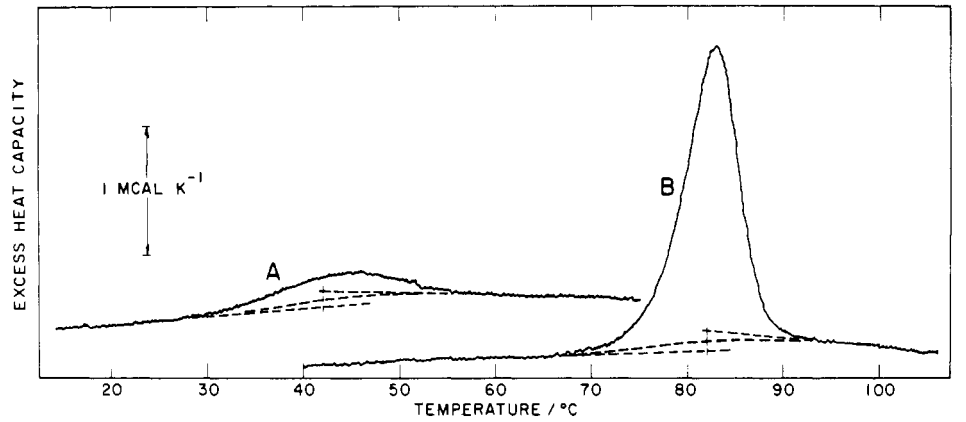

FIGURE 2: Tracings of typical DSC scans for SSI at pH 2.84 (curve A; protein concentration 2.35 mg mL^{-1}) and at pH 7.00 (curve B; protein concentration 2.81 mg mL^{-1}). The construction of base lines is illustrated; the dashed straight line segments are extrapolations of pre- and posttransitional base lines, and the dashed curves represent smooth transitions between these extremes, weighted at each temperature according to the integrated area of the DSC curve up to that temperature.

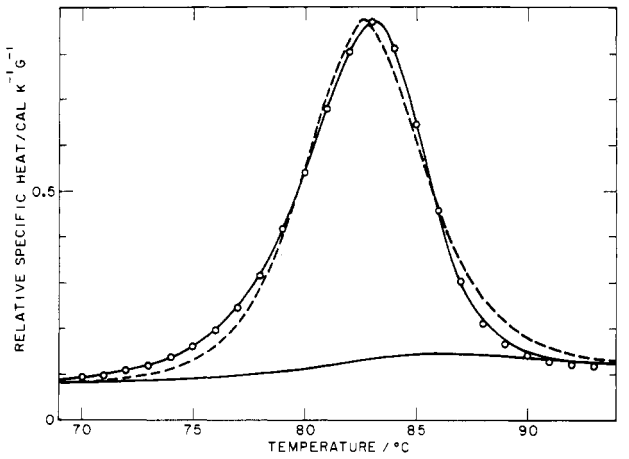

FIGURE 3: Illustration of the fitting of various types of two-state curves to experimental DSC data at pH 7.00; protein concentration 3.75 mg mL^{-1}. The circles are the experimental data. The dashed curve was calculated to give the best least-squares fit obtainable with a process of the form A ⇄ B and showed a standard deviation amounting to 3.7% of the maximal value of the total excess specific heat. The solid curve was the best one corresponding to the process A ⇄ 2B, with a standard deviation of 0.55% of the maximal excess specific heat.

temperature coefficient cannot result from an equilibrium exothermic process such as oligomerization.

Within the (unknown) range of validity of eq 1 and 2, the change in specific heat accompanying denaturation is given by

$$\Delta c_p^d = (C - A) + (D - B)t \tag{3}$$

and the specific enthalpy of denaturation is then

$$\Delta h = \Delta h_0 + (C - A)t + \tfrac{1}{2}(D - B)t^2 \tag{4}$$

The enthalpy evaluated at pH 7.00 from curve B in Figure 2 is 5.41 cal g^{-1} and is to be assigned to the temperature, $t_{1/2}$, at which the denaturation is half completed, 82.2 °C in this case. The value of Δh_0 is thus −11.70 cal g^{-1}.

It is evident that eq 4 with the constants evaluated at pH 7.00 cannot be extrapolated to the low transition temperature observed at pH 2.84, since eq 3 predicts a value of Δc_p^d much larger than that observed. Thus, only part of the large difference in enthalpy observed at pH 2.84 and pH 7.00 can be accounted for as a purely heat capacity effect.

Stoichiometry of the Unfolding of SSI. Although it is well established that SSI is dimeric at neutral pH and room temperature, it is not known whether it is dimeric at high temperatures. Thus, the actual denaturation reaction might be of any one of the following types:

$$A_N \rightleftarrows A_D \tag{5}$$

$$A_{2,N} \rightleftarrows A_{2,D} \tag{6}$$

$$A_{2,N} \rightleftarrows 2A_D \tag{7}$$

Distinction between these possibilities can be made, when data with good signal-to-noise ratios are available, on the basis of a detailed comparison of the observed curve with theoretical curves based on the van't Hoff equation

$$\mathrm{d} \ln K/\mathrm{d}T = \Delta H_{vH}/(RT^2) \tag{8}$$

where K is the equilibrium constant for the denaturation process and ΔH_{vH} is the van't Hoff enthalpy in cal mol^{-1}. If we assume ΔH_{vH} to have the same temperature variation as Δh, setting

$$\Delta H_{vH} = \beta \Delta h \tag{9}$$

where β is a temperature-independent constant, then for the general process

$$A_{n,N} \rightleftarrows nA_D \qquad K = n[A_0]^{n-1}\alpha^n/(1-\alpha) \tag{10}$$

it can be shown that

$$R/\beta \ln (K/K_{1/2}) = A(1/T - 1/T_{1/2}) + B \ln (T/T_{1/2}) + C(T - T_{1/2}) \tag{11}$$

where

$$-A = \Delta h_0 - 273.15(C - A) + \tfrac{1}{2}(273.15)^2(D - B)$$
$$B = (C - A) - 273.15(D - B)$$
$$C = \tfrac{1}{2}(D - B) \tag{12}$$

In these equations, $[A_0]$ is the total molar concentration of SSI subunits, α is the extent of the reaction, $T_{1/2}$ is the absolute temperature at which $\alpha = 0.5$, $K_{1/2}$ is the value of the equilibrium constant at this temperature, and T is the absolute temperature at a point in the DSC scan. Equation 11 is used to calculate $K/K_{1/2}$ at a series of temperatures; α is obtained by solution of

$$\alpha^n = \frac{1 - \alpha}{2^{n-1}} \frac{K}{K_{1/2}} \tag{13}$$

and then the excess specific heat is calculated by

$$c_{ex} = \Delta h \frac{\mathrm{d}\alpha}{\mathrm{d}T} = \frac{\alpha(1 - \alpha)}{n - (n - 1)\alpha} \beta \frac{(\Delta h)^2}{RT^2} \tag{14}$$

Table I: Summary of DSC Data for SSI at pH 7.00 and pH 2.84 [a]

protein concn (mg mL^{-1})	t_p (°C)	ΔH_{cal} [kcal (mol of dimer)$^{-1}$]	ΔH_{vH} (kcal mol^{-1})	Δc_p^d (cal K^{-1} g^{-1})	c_{max} (cal K^{-1} g^{-1})
		SSI at pH 7.00			
0.469	80.79	143.0	178		0.761
0.938	81.50	121.8	204	0.0492	0.741
1.41	82.19	116.0	212	0.0328	0.730
1.88	82.35	129.1	192	0.0589	0.735
2.35	82.50	114.1	205	0.0409	0.695
2.81	82.90	124.5	206	0.0602	0.761
3.28	82.90	122.8	201	0.0475	0.730
3.75	83.09	122.0	205	0.0469	0.741
4.22	83.20	119.6	208	0.0436	0.735
4.69	83.40	124.3	205	0.0476	0.753
	mean:	122.2	202	0.0477	0.738
	SE:	±2.7	±3	±0.0028	±0.006
		SSI at pH 2.84			
1.88	44	28.3	69		0.073
2.35	44	28.0	72		0.075
2.35	44	30.3	65		0.074
2.35	44	25.6	62		0.059
2.81	44	24.1	54		0.048
	mean:	27.1	64		0.063
	SE:	±1.2	±7		±0.006

[a] t_p = temperature of maximal excess specific heat; ΔH_{cal} = enthalpy obtained by integrating the DSC trace: $\Delta H_{vH} = 5.83RT_p^2 \cdot (c_{max}/\Delta h_{cal})$, where 5.83 is the factor appropriate for a process of the the form of eq 7 and $\Delta h_{cal} = \Delta H_{cal}$/molecular weight; Δc_p^d = change in apparent specific heat due to denaturation at t_p; c_{max} = maximal excess specific heat. For calculation of the listed mean values, the individual values were weighted by the protein concentration, and the small variations of ΔH_{cal} and Δc_p^d with temperature were ignored.

The second equality in this expression is obtained by differentiation of K as given by eq 10 and introducing eq 8.

Finally, the total specific heat

$$c_{tot} = c_{ex} + c_{av} = c_{ex} + (1 - \alpha)(A + Bt) + \alpha(C + Dt) \quad (15)$$

is compared with the observed data, and the standard deviation of the observed points from the calculated points is computed. We have employed a program on the HP-41C calculator which adjusts the three parameters, $t_{1/2}$, Δh_0, and ΔH_{vH}, to minimize the standard deviation, employing 20 or more data points for each DSC scan.

The results of such a calculation are shown in Figure 3 for an experiment at pH 7.00 with a protein concentration of 3.75 mg mL^{-1}. In this case the constants in eq 1 and 2 had the values $A = 0$, $B = 0.00114$, $C = 0.44$, and $D = -0.00339$, in reasonable agreement with those given above for curve B in Figure 2. The filled circles in Figure 3 are the experimental data. The dashed curve was calculated with $n = 1$, $t_{1/2} = 82.45$ °C, $\Delta h_{1/2} = 5.17$ cal g^{-1}, and $\Delta H_{vH} = 139$ kcal mol^{-1} and gave a standard deviation of ±0.032 cal K^{-1} g^{-1}, or 3.7% of the maximal value of c_{tot}. A much better fit, the solid curve in Figure 3, was obtained with $n = 2$, $t_{1/2} = 82.25$, $\Delta h_{1/2} = 5.25$ cal g^{-1}, and $\Delta H_{vH} = 202$ kcal mol^{-1}. The standard deviation in this case was only ±0.0049 cal K^{-1} g^{-1}, or 0.55% of the maximal value of c_{tot}. A calculation with $n = 3$ gave a standard deviation of 0.016 cal K^{-1} g^{-1}. These results constitute strong support for the validity of eq 7 in this case.

A similar establishment of the stoichiometry cannot be based on the data at pH 2.84 since the transitions are very broad and of low signal-to-noise ratio.

Summary of DSC Data for SSI. The DSC data for SSI obtained at pH 7.00 and 2.84 are listed in Table I. The molar enthalpies are independent of concentration, with an average value at pH 7.00 of 122.2 ± 2.7 kcal (mol of dimer)$^{-1}$, or 5.31 ± 0.12 cal g^{-1}. This latter figure is similar to that observed with other globular proteins (Privalov, 1979). This is also true for the mean value of Δc_p^d, 0.0477 ± 0.0028 cal K^{-1} g^{-1}. When these mean values were calculated, no attention was paid to their small temperature variations over the narrow range of temperatures involved. The fact that the van't Hoff enthalpy is 1.65 times as large as the calorimetric enthalpy can only be accounted for on the basis of some degree of intermolecular (dimer–dimer) cooperation, since in the absence of such cooperation the van't Hoff enthalpy cannot exceed the calorimetric value.

The unfolding enthalpy of SSI at pH 2.84 is unusually small, 27.1 ± 1.2 kcal (mol of dimer)$^{-1}$, or less than 1 cal g^{-1}. As mentioned earlier, this sharp decrease from pH 7.00 can be accounted for only in part as a heat capacity effect. The fact that ΔH_{vH} at pH 2.84 is 2.4 times the calorimetric value indicates an even greater degree of intermolecular cooperation in the unfolding of SSI at pH 2.84 than at pH 7.00.

Effect of Protein Concentration on T_p. The value of T_p, the absolute temperature of maximal excess heat capacity, for SSI at pH 7.00 increases slightly with increasing protein concentration (Table I), as is expected for a process of the form of eq 7. Differentiation of c_{ex} (eq 14) and setting the derivative equal to zero gives

$$\frac{n(1 - 2\alpha_p) + (n - 1)\alpha_p^2}{[n - (n - 1)\alpha_p]^2} = 2\frac{RT_p}{\beta\Delta h_p}\left(1 - \frac{T_p}{\Delta h_p}\Delta c_p\right) = 2\frac{RT_p\,\Delta h_0}{\beta\Delta h_p^2} \quad (16)$$

where the subscript p refers to the temperature at which c_{ex} has its maximal value. This result would be only slightly altered if we were to differentiate $c_{ex} + c_{av}$ instead of c_{ex} alone.

The quantity on the right side of eq 16 changes very little over the range of values of T_p encountered in experiments where the concentration is varied by 10-fold or more. Numerical solution of eq 16 shows that α_p is insensitive to the value of the right-side term in the equation, and we may thus take α_p to be very nearly constant and conclude that to a sufficient approximation $K_p/[A_0]^{n-1}$ is also constant. This being the case, we can write

$$\Delta H_{vH}/(RT_p) + (n - 1)\ln [A_0] = \text{constant} \quad (17)$$

so that a plot of ln $[A_0]$ vs. $1/T_p$ should be linear with slope $-\Delta H_{vH}/[(n - 1)R]$. Such a plot with $n = 2$ for our data at pH 7.00 is shown in Figure 4 and leads to the result ΔH_{vH} = 220 ± 10 kcal mol^{-1}, in reasonable agreement with the value obtained by curve fitting as given above. This result gives further support to the validity of eq 7 for the unfolding of SSI.

Unfolding of Subtilisin BPN′ and of Its Complex with SSI. DSC experiments with subtilisin gave rather irreproducible results, presumably because of antolysis (Zahnley, 1980). DSC traces at pH 7.00 for subtilisin BPN′ (curve 1) and for a series of mixtures containing a constant amount of the enzyme and increasing amounts of the inhibitor (curves 2–8) are shown in Figure 5. Reheats of the samples giving curves 1 and 8 showed that both of these denaturation reactions are irreversible. This observation is surprising in the case of the complex (curve 1) in view of the fact that the unfolding of SSI alone was found to be reversible and suggests that the SSI is not dissociated from the subtilisin in the course of the denaturation, for if it were it would be expected to refold on cooling as it does in the absence of the enzyme. A similar situation

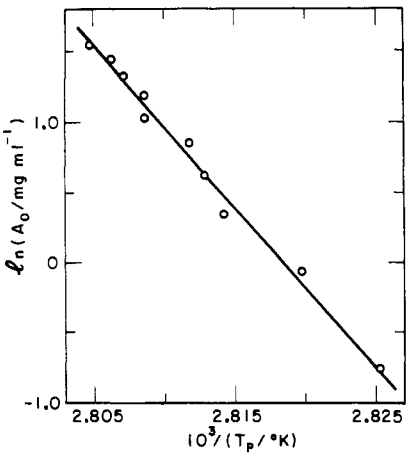

FIGURE 4: The variation of T_p, the temperature of maximal excess specific heat, with protein concentration. SSI at pH 7.00. The slope of the plot, with $n = 2$ (eq 17), gives the value $\Delta H_{vH} = 220 \pm 10$ kcal mol^{-1}, in satisfactory agreement with the values deduced from the DSC curves and listed in Table I.

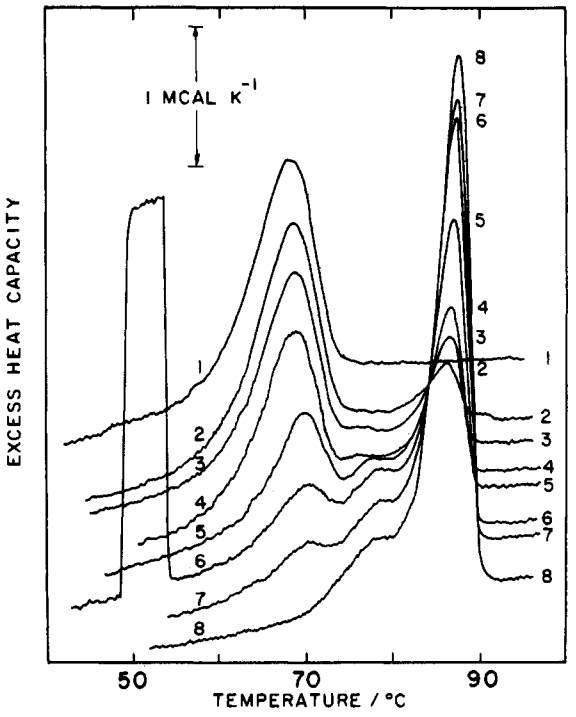

FIGURE 5: Tracings of DSC curves for mixtures of subtilisin BPN' with various amounts of SSI obtained at pH 7.00. In each experiment 2.32 mg mL^{-1} of subtilisin was mixed with SSI to give mole ratios as follows: curve 1, mole ratio = 0; curve 2, 0.243; curve 3, 0.365; curve 4, 0.486; curve 5, 0.608; curve 6, 0.729; curve 7, 0.802; curve 8, 0.973. The step in curve 6 is a calibration signal produced by introducing 250 μW of excess power into the reference cell. Such calibration signals were included in most of the experiments reported in this paper.

was reported by Donovan & Beardslee (1975) for the β-trypsin–ovomucoid complex.

The DSC data for subtilisin BPN' are given in Table II. Because of the irreversibility of the unfolding and the relatively low accuracy of the data, we have not calculated Δc_p^d for the process and have not attempted curve fitting to ascertain the stoichiometry. We also have not analyzed the apparent trend of t_p with protein concentration in the manner outlined in the preceeding section. In calculating the van't Hoff enthalpy, an operation of doubtful validity in the case of an irreversible process, we have assumed the enzyme to be monomeric. The small value for ΔH_{vH} suggests that intermediate states in the unfolding are significantly populated.

Table II: Summary of DSC Data for Subtilisin BPN' at pH 7.00[a]

protein concn (mg mL^{-1})	t_p (°C)	ΔH_{cal} (kcal mol^{-1})	ΔH_{vH} (kcal mol^{-1})	c_{max} (cal^{-1} K^{-1} g^{-1})
0.731	66.5	229	111	1.01
1.25	67.7	190	96	0.72
2.34	68.3	204	82	0.66
	mean:	208	92	0.80
	SE:	±11	±9	±0.11

[a] Column headings are as for Table I, except that $\Delta H_{vH} = 4.00RT_p^2(c_{max}/\Delta h_{cal})$, where 4.00 is the factor appropriate for a process of the form of eq 5.

Table III: Enthalpy of Denaturation of the Subtilisin BPN-SSI Complex at pH 7.00[a]

mole ratio SSI/enzyme	heat absorbed (mcal)	heat absorbed (calcd) (mcal)	difference
0	19.1	19.7	0.6
0.243	19.5	21.8	2.3
0.365	20.4	22.8	2.4
0.486	21.2	23.9	2.7
0.608	22.7	24.9	2.2
0.729	23.6	26.0	2.4
0.802	25.2	26.6	1.4
0.851	26.3	27.0	0.7
0.973	27.8	28.1	0.3
		mean:	1.7

[a] In each experiment subtilisin at a concentration of 2.32 mg mL^{-1} was mixed with an amount of SSI to give the listed mole ratio. The heat absorbed is given in column 2. The values in column 3 were calculated on the assumption of no heat of interaction between the two proteins, taking the heat for free enzyme to be 7.56 cal g^{-1} and that for bound enzyme ($t_p = 87.4$ °C) to be $7.56 + 0.065(87.4 - 67.5) = 8.85$ cal g^{-1} (the value of $\Delta c_p^d = 0.065$ cal K^{-1} g^{-1} used here is a rough estimate, probably with an uncertainty of ±25%) and the heat for bound SSI to be $5.31 - 0.0477(80.7 - 78.5) = 5.20$ cal g^{-1}.

The complex, curve 8 in Figure 5, has a transition temperature about 20 °C above that of subtilisin, indicating a considerable stabilization of the complex relative to its denatured form as compared to the enzyme relative to its unfolded form. Similar effects have been observed with other enzyme–inhibitor complexes (Zahnley, 1979, 1980; Donovan & Mihalyi, 1974; Donovan & Beardslee, 1975). The total transition enthalpy is 304 kcal per mol of monomeric complex, EI. The shoulder on the low-temperature side of the transition curve can be considered as being due to a peak with an area about 20% of the total area, so that the areas of the major and minor peaks are approximately in the same ratio as the denaturational enthalpies of subtilisin and SSI. This suggests that the minor peak reflects the unfolding of SSI in the complex and the major peak that of subtilisin. On the other hand, as suggested by one of the referees of this paper, the minor peak might be due to a relatively loose complex of the inhibitor with inactive enzyme. A comparison of the widths of curve 1 and of the main peak of curve 8 suggests that the binding of the inhibitor to the enzyme increases the cooperativity of the unfolding of the latter.

The curves in Figure 5 are consistent with the results of the fluorescence titrations of Uehara et al. (1978) in that in the presence of a small excess of inhibitor, the enzyme peak completely disappears. It is not possible to obtain accurate enthalpy data from these curves because of the impossibility of laying down reliable base lines for such complicated curves. We consider the enthalpies listed in column 2 of Table III to be uncertain by as much as ±10%. The heats in the third

column of the table were calculated as outlined in Table III on the basis of no heat of interaction between the enzyme and the inhibitor. Although these heats differ from the observed values on average only by the estimated uncertainty in the measured values, the fact that all the differences are in the same direction suggests that there is a significant heat of interaction between the two proteins which is more positive in the native state than in the denatured state. Uehara et al. (1978) reported an enthalpy of interaction of 4.18 kcal mol^{-1} at 25 °C, based on fluorescence titrations over a range of temperatures. This corresponds to only 0.4 mcal at a mole ratio of 1.0 in the experiments under consideration here. The DSC results indicate a considerably larger interaction enthalpy. Whether the apparent difference between the calorimetric and fluorometric results can be accounted for on the basis of a heat capacity change in the denaturation of the complex is difficult to decide on the basis of the complex DSC curves. The fluorometric data gave no indication of a nonvanishing change in heat capacity accompanying the enzyme–inhibitor interaction.

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Erythrocruorin from the Crustacean *Caenestheria inopinata*. Quaternary Structure and Arrangement of Subunits†

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ABSTRACT: The subunit structure of erythrocruorin from the crustacean *Caenestheria inopinata* was studied. The native protein was found to have a sedimentation coefficient of 12.0 S and a molecular weight, as determined by sedimentation equilibrium, of 302 000. Iron and heme determinations gave 0.346 and 3.98% corresponding to minimal molecular weights of 16 100 and 15 500, respectively. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis gave one band with mobility corresponding to a molecular weight of 30 000. The molecular weight of the polypeptide chain was determined to be 30 500 by sedimentation equilibrium in 6 M guanidine hydrochloride and 0.1 M 2-mercaptoethanol. Dissociation of the 12S molecule was observed at acidic and alkaline pH. A dissociation species of 2.7 S was isolated and its molecular weight determined to be 28 000 by sedimentation equilibrium. On a molecular weight basis, the native molecule is composed of ten 2.7S subunits, each of which consists of a single polypeptide chain carrying two hemes. We propose a model for the molecule composed of ten spheres, each representing a 2.7S subunit, arranged in two layers stacked in an eclipsed orientation, the five spheres of each layer occupying the vertices of a regular pentagon. Support for this arrangement is provided by a comparison of projections of the model with molecular profiles seen in the electron microscope.

In Crustacea, erythrocruorin occurs mainly among the four orders of the subclass branchiopoda: anostraca, notostraca, cladocera, and conchostraca (Fox, 1957; Redmond, 1971). In a recent study (Ilan & Daniel, 1979a), it was found that erythrocruorins from species belonging to these four orders differ from each other with respect to the size of the native molecule, the size of the constituent polypeptide chains, or both. This means that different structures of erythrocruorin exist in the branchiopod crustaceans. This paper describes a detailed study of one of these structures, the structure of erythrocruorin from a conchostracan, the clam shrimp *Caenestheria inopinata*.

Materials and Methods

Preparation of Erythrocruorin. Caenestheria inopinata specimens were gathered during spring and early summer from a seasonal pond at Migdal Sedek, Israel. For the preparation of erythrocruorin, about 100 live animals were washed with cold distilled water, dried in air, and then placed in 10-mL plastic syringes (about 15 animals in each). Crude hemolymph was squeezed out by gentle application of pressure, diluted with an equal volume of 0.1 M sodium phosphate buffer, pH 6.8, and centrifuged for 20 min at low speed to remove particulate

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