interaction between His-48 and ATP, whereas in the Asn and Gln mutants the increase is due only to an improved hydrogen bond. Alternatively, the increased affinity of ATP with TyrTS(Pro-51) could result simply from improved van der Waals' contacts. The total energy of interaction of His-48 with the transition state of the reaction in TyrTS(Pro-51), some 3 kcal/mol, is far larger than we have found in any of our experiments in which a simple hydrogen bond has been removed from the enzyme–substrate complex. These are generally 0.5–1.5 kcal/mol (Fersht et al., 1984, 1985). The high value implies that there are additional interactions superimposed on the energy of the hydrogen bond, either electrostatic or van der Waals'.

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We thank Professor D. M. Blow for making available the unpublished coordinates of the tyrosyl-tRNA synthetase.

Registry No. His, 71-00-1; Asp, 70-47-3; Gln, 56-85-9; TyrTS, 9023-45-4; Tyr, 60-18-4; ATP, 56-65-5.

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Thermodynamics of the Binding of *Streptomyces* Subtilisin Inhibitor to α-Chymotrypsin†

Harumi Fukada, Katsutada Takahashi, and Julian M. Sturtevant*

Laboratory of Biophysical Chemistry, College of Agriculture, University of Osaka Prefecture, Sakai, Osaka 591, Japan, and Department of Chemistry, Yale University, New Haven, Connecticut 06511

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ABSTRACT: The binding of *Streptomyces* subtilisin inhibitor (SSI) to α-chymotrypsin (CT) (EC 3.4.21.1) was studied by isothermal and differential scanning calorimetry at pH 7.0. Thermodynamic quantities for the binding of SSI to the enzyme were derived as functions of temperature from binding constants (S. Matsumori, B. Tonomura, and K. Hiromi, private communication) and isothermal calorimetric experiments at 5–30 °C. At 25 °C, the values are $\Delta G°_b = -29.9$ kJ mol^{-1}, $\Delta H_b = +18.7$ (±1.3) kJ mol^{-1}, $\Delta S°_b = +0.16$ kJ K^{-1} mol^{-1}, and $\Delta C_{p,b} = -1.08$ (±0.11) kJ mol^{-1}. The binding of SSI to CT is weak compared with its binding to subtilisin [Uehara, Y., Tonomura, B., & Hiromi, K. (1978) *J. Biochem. (Tokyo) 84*, 1195–1202; Takahashi, K., & Fukada, H. (1985) *Biochemistry 24*, 297–300]. This difference is due primarily to a less favorable enthalpy change in the formation of the complex with CT. The hydrophobic effect is presumably the major source of the entropy and heat capacity changes which accompany the binding process. The unfolding temperature of the complex is about 7 °C higher than that of the free enzyme. The enthalpy and the heat capacity changes for the unfolding of CT were found to be 814 kJ mol^{-1} and 17.3 kJ K^{-1} mol^{-1} at 49 °C. The same quantities for the unfolding of the SSI–CT complex are 1183 kJ mol^{-1} and 39.2 kJ K^{-1} mol^{-1} at 57 °C.

Streptomyces subtilisin inhibitor (SSI) (Murao et al., 1972; Sato & Murao, 1973) having a molecular weight of 23 000 (dimer) specifically binds to alkaline proteases to inhibit their proteolytic action. It inhibits subtilisin BPN′ (SBPN′) most strongly to form a complex, E_2I_2, with a dissociation constant as small as 10^{-11} mol dm^{-3} for each of two identical, independent sites (Uehara et al., 1978). The formation of the subtilisin complex with SSI has been studied by various methods such as X-ray crystallography (Mitsui et al., 1979; Hirono et al., 1984), reaction kinetics (Inoue et al., 1977; Uehara et al., 1980), and NMR spectroscopy (Kainosho et al., 1985).

In previous papers, we reported studies of the binding of SSI to SBPN′ at pH 7.0 by the methods of isothermal and differential scanning calorimetry (DSC) and reported the thermodynamic properties for the interaction between the two proteins (Takahashi & Sturtevant, 1981; Takahashi & Fukada, 1985). It was found that the inhibitor binds to SBPN′ so tightly that it does not dissociate from the enzyme even after the complex unfolds at about 87 °C and that the strong binding

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* Address correspondence to this author at the Department of Chemistry, Yale University.

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Table I: Isothermal Calorimetric Data for the Binding of *Streptomyces* Subtilisin to α-Chymotrypsin

t (°C)	no. of measurements	q_{obsd} (J dm^{-3})	e_0[a] (×10^{-6} mol dm^{-3})	i_0[b] (×10^{-6} mol dm^{-3})	K_b[c] (×10^5 dm^3 mol^{-1})	ΔH_b (kJ mol^{-1})
5.1	6	0.793 ± 0.039	89.1	24.4	0.716	40.8 ± 1.9
10.0	7	0.851 ± 0.044	157.5	26.8	0.954	35.8 ± 1.8
10.0	8	0.772 ± 0.060	82.2	28.7	0.954	33.3 ± 2.5
15.0	7	0.929 ± 0.041	97.4	31.5	1.21	34.3 ± 1.4
20.0	7	0.643 ± 0.025	92.6	34.2	1.47	22.0 ± 0.8
25.0	7	0.646 ± 0.017	81.7	42.4	1.70	18.0 ± 0.5
30.0	7	0.323 ± 0.019	85.8	27.9	1.89	13.2 ± 0.7

[a] Total active enzyme concentration after mixing determined by the method described in the text. [b] Total inhibitor concentration after mixing. [c] Binding constants calculated by means of eq 2 with the values for $K_b(298)$, $\Delta H_b(298)$ and $\Delta C_{p,b}$ given in Table II.

Table II: Thermodynamics of the Binding of SSI to α-Chymotrypsin and to Subtilisin BPN′ at pH 7.0 and 25.0 °C[a]

	K_b (dm^3 mol^{-1})	$\Delta G°_b$ (kJ mol^{-1})	ΔH_b (kJ mol^{-1})	$\Delta S°_b$ (kJ K^{-1} mol^{-1})	$\Delta C_{p,b}$ (kJ K^{-1} mol^{-1})
CT	1.70 × 10^5	−29.9	18.7 ± 1.9	0.160	−1.13 ± 0.11
SBPN′	1.40 × 10^{10}	−57.9	−19.8 ± 1.2	0.128	−1.02 ± 0.10

[a] The thermodynamic quantities are per mole of monomeric complex, EI.

of SSI to SBPN′ is characterized by a large entropy increase and a large enthalpy decrease.

SSI also binds to CT, though less tightly, to form an E_2I_2 complex, the dissociation constant of which is reported to be 4.50 × 10^{-6} mol dm^{-3} at pH 7.0 and 25 °C (S. Matsumori, B. Tonomura, and K. Hiromi, private communication), being larger than that of the SBPN′ complex by a factor of 10^5 (Inoue et al., 1979). It is therefore of interest to compare the thermodynamic properties of the SSI–CT complex with those of the SSI–subtilisin complex. In the present study, calorimetric measurements were made at various temperatures to determine the enthalpy and heat capacity changes associated with the binding of SSI to CT at pH 7.0. Excess heat capacity changes during thermal unfolding of the enzyme and the complex were determined by DSC.

Materials and Methods

Materials. Partially purified *Streptomyces* subtilisin inhibitor (SSI) was a gift from Prof. K. Hiromi of Kyoto University, Kyoto, Japan, and from Prof. S. Murao of the University of Osaka Prefecture, Osaka, Japan. It was further purified by the method of Sato & Murao (1973). SSI solutions used for the calorimetric measurements were prepared by exhaustive dialysis against 0.025 M potassium phosphate buffer at pH 7.00 and I = 0.1 M with KCl in the same manner as previously reported (Takahashi & Sturtevant, 1981; Takahashi & Fukada, 1985). The concentration was determined spectrophotometrically by using a value for the absorption coefficient of 0.829 cm^2 mg^{-1} at 276 nm.

Crystalline CT (lot CDI 30P881) was purchased from Worthington Biochemicals Co. and used without further purification. The enzyme concentration was determined by absorption measurements using an absorption coefficient of 2.05 cm^2 mg^{-1} at 282 nm. The activity of the enzyme was checked by titration with *N-trans*-cinnamoylimidazole according to the method of Schonbaum et al. (1961), and the active fraction was found to be 95.0%. A correction factor of 0.950 was therefore applied to all enzyme concentrations. Solutions were prepared by dissolving the crystalline protein in the SSI dialyzate immediately before use so that autolysis of the enzyme was minimized.

All other chemicals used were of reagent grade. Doubly distilled and deionized water was used for the preparation of solutions.

Methods. Isothermal calorimetry was performed in a flow calorimeter having gold tubing as a reaction chamber. The details of the apparatus and the mode of operation have been described in a previous paper (Takahashi & Fukada, 1985). The two reactant solutions were delivered from 5 cm^3 gas-tight syringes (Hamilton Co.) mounted on commercial dispenser pumps (Perfusor 137, B. Braun Melsungen, West Germany) and mixed in the calorimetric unit. The measurements were conducted with a stopped-flow procedure. The solutions were delivered from each syringe for 60–120 s at a flow rate of 4.581 × 10^{-3} cm^3 s^{-1}. The heat effect was estimated after correction, as previously described (Takahashi & Fukada, 1985), for dilution heats of both the enzyme and inhibitor solutions and for viscous heating. The SSI concentration was about 30 μM, and the concentration of the enzyme was about 90 μM, both after mixing. The total heat effects observed were in the range of 200–1300 μJ.

Measurements were made at 5.1, 10.0, 20.0, 25.0, and 30.0 °C. The calorimeter was calibrated at each temperature by using the neutralization of 0.004 N HCl with 0.01 N NaOH (Grenthe et al., 1970).

Differential scanning calorimetry was performed in the DASM-1M microcalorimeter described by Privalov et al. (1975) on solutions of CT and its complex with SSI at a heating rate of 1 K min^{-1}. The protein concentrations were in the range 0.2–7 mg mL^{-1}. The calorimetric enthalpies of unfolding were obtained from the DSC recordings of excess heat capacity changes according to the method described previously (Fukada et al., 1983).

Results and Discussion

Isothermal Calorimetry. The isothermal calorimetric results obtained at six different temperatures are summarized in Table I. The total concentrations of active enzyme and inhibitor in the reaction mixture after mixing are given in the fourth and fifth columns, respectively. Since under the experimental conditions employed a small amount of the reactants remained unreacted, the observed heat changes were corrected for the unbound fraction, usually amounting to about 10%, by using the known binding constants. The binding constant at each temperature was calculated by means of eq 2 as described below, using the values for $K_b(298)$, $\Delta H_b(298)$, and $\Delta C_{p,b}$ given in Table II, and is listed in the sixth column of Table I.

The number of protons released from the reactants during the binding process was determined in separate experiments by the pH-stat method. It was found that 0.2 ± 0.1 mol of H^+ is released during the formation of 1 mol of monomeric SSI–CT complex at 10 and 25 °C. To obtain the net enthalpy of reaction, the enthalpy contributions due to addition of protons to the buffer were deducted from the observed en-

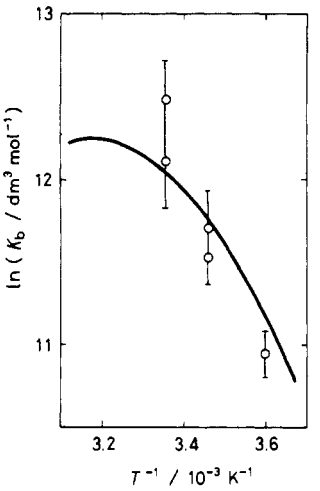

FIGURE 1: van't Hoff plot showing the temperature variation of the binding constant for the interaction of SSI and CT. The solid curve was calculated according to eq 2, and the open circles are the data of S. Matsumori et al. (private communication) obtained by fluorometric titration.

thalpies by using for the heat of deprotonation of phosphate values calculated from the relation given by Bates & Acree (1943):

$$\Delta H_i = 37.894 - 0.0003798T^2 \text{ kJ mol}^{-1} \quad (1)$$

where T is the absolute temperature. The number of protons taken up by the protein was assumed to be constant over the range of temperatures studied. The enthalpies corrected for buffer contributions and incomplete reaction are listed in column 7 of Table I.

Within experimental uncertainty, the enthalpy of reaction varies linearly with temperature. Least squaring the values for ΔH_b listed in Table I gives $\Delta H_b(298) = 18.7 \pm 1.1$ kJ mol^{-1} and $\Delta C_{p,b} = -(1.13 \pm 0.11)$ kJ K^{-1} mol^{-1}. We have here another example of the recurring phenomenon of extremely large changes in the apparent heat capacity accompanying reactions involving proteins (Sturtevant, 1977). The van't Hoff equation for a reversible reaction having a nonvanishing heat capacity may be put in the form

$$\ln \frac{K_b}{K_b(298)} = \frac{\Delta H_b(298) - 298.15\Delta C_{p,b}}{R}\left(\frac{1}{T} - \frac{1}{298.15}\right) + \frac{\Delta C_{p,b}}{R} \ln \frac{T}{298.15} \quad (2)$$

The only remaining adjustable parameter in this equation is ln $K_b(298)$, and selecting this to minimize the sum of the squares of the difference between ln K_b(obsd) and ln K_b(calcd) gives $K_b(298) = (1.70 \pm 0.21) \times 10^5$ dm^3 mol^{-1}. The mean value obtained by S. Matsumori et al. (private communication) was 2.22×10^5 dm^3 mol^{-1}. In Figure 1, the temperature variation of the calculated values of K_b is shown in the form of a van't Hoff plot. The open circles are the values obtained by Matsumori et al. by means of fluorescence titrations.

Calculation of the thermodynamic quantities for the binding of SSI to CT over the temperature range 0–50 °C, using the values at 25 °C summarized in Table II, gives the results shown in Figure 2. The data reported earlier (Takahashi & Fukada, 1985) for the binding of SSI to SBPN′ are included in Figure 2 (dashed lines) and in Table II for comparison.

It is interesting that the formation of the SSI–CT complex is accompanied by a large decrease in heat capacity which is equal within experimental uncertainty to that observed in the formation of the SSI–SBPN′ complex. Similarly, the relatively small increases in entropy for the two cases are equal within

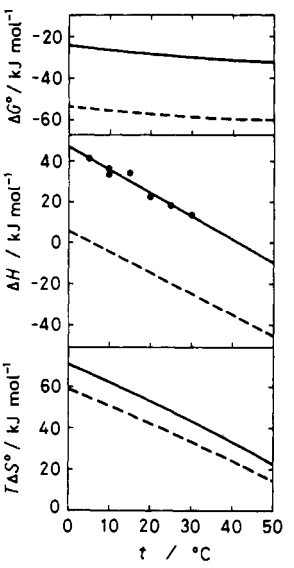

FIGURE 2: Thermodynamic parameters for the binding of SSI to CT as a function of temperature. The data previously reported for the SSI–subtilisin interaction (Takahashi & Fukada, 1985) are given (dashed lines) for comparison.

Table III: Hydrophobic and Vibrational Contributions to the Thermodynamics of Binding of SSI to α-Chymotrypsin at pH 7.0[a]

temp (°C)	$\Delta S°_u$	$\Delta S°_u$(h)	$\Delta S°_u$(v)	$\Delta C_{p,b}$	$\Delta C_{p,b}$(h)	$\Delta C_{p,b}$(v)
5	0.27	0.29	−0.02	−1.13	−1.12	−0.01
25	0.19	0.27	−0.08	−1.13	−1.06	−0.07
35	0.13	0.27	−0.11	−1.13	−1.03	−0.10

[a] All quantities in kilojoules per degrees kelvin per mole of monomeric complex (EI). $\Delta S°_u$ is the unitary entropy change obtained after correction for the cratic entropy, $\Delta S_c = 0.0334$ kJ K^{-1} mol^{-1}.

experimental uncertainty. As is necessarily the case, when the magnitude of ΔC_p is much larger than that of $\Delta S°$, the temperature variations of ΔH and $-T\Delta S°$ are nearly equal in magnitude and opposite in sign so that $\Delta G°$ is nearly independent of temperature. It is evident that the difference of 10^5 in the binding constants for CT and SBPN′ at 25 °C is due primarily to the difference of nearly 40 kJ mol^{-1} in the corresponding enthalpy changes. It is impossible at present, even in cases where detailed structural information is available (Fukada et al., 1983), to account in molecular terms for such large enthalpy differences.

The heat capacity and entropy changes for the SSI–CT interaction were split into hydrophobic (h) and vibrational (v) components by the empirical procedure proposed by Sturtevant (1977) with the results given in Table III. $\Delta S°_u$ is the unitary entropy change obtained from the observed entropy change by subtraction of the cratic entropy, $\Delta S°_c = +0.0334$ kJ K^{-1} mol^{-1} (Kauzmann, 1959). The values in the table are, as expected, quite similar to those obtained for the interaction between SSI and SBPN′ and indicate hydrophobic contributions to both $\Delta C_{p,b}$ and $\Delta S°_b$ which are much larger than the vibrational contributions.

Temperature Variation of Enzyme Activity. The loss of activity of CT on heating at 1 K min^{-1} was determined at concentrations of 79–97 μM (1.97–2.4 mg cm^{-3}) by removing 1 cm^3 aliquots at approximately 2 K intervals and adding these to 5 cm^3 of buffer solution at 0 °C. The activity was measured by observing an initial burst with *p*-nitrophenyl acetate as substrate according to the method of Bender et al. (1966).

The relative activity is shown in Figure 3. The temperature of half-inactivation is 47 °C, which is approximately equal to the peak temperature of the DSC curves discussed below. The van't Hoff plot in the inset in the figure shows that the loss

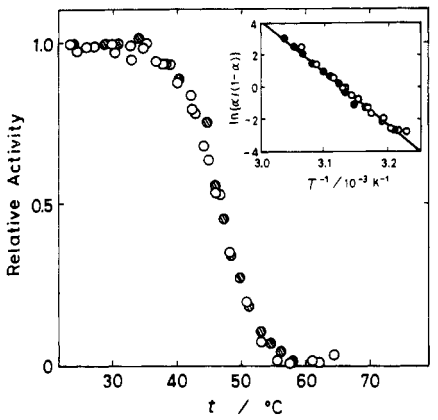

FIGURE 3: Change in the enzymic activity of CT during heating. The enzyme in 0.025 M potassium phosphate buffer, pH 7.0 and I = 0.1 M with KCl, was heated at a constant rate of 1 K min^{-1}. The activity of the enzyme was measured spectrophotometrically by observing the initial burst in the absorption at 405 nm when mixed with p-nitrophenyl acetate solution at 25 °C. The inset shows a van't Hoff plot of the inactivation data, from which the van't Hoff enthalpy of denaturation was evaluated to be $\Delta H_{vH}{}^{d}$ = 274 ± 6 kJ mol^{-1}. CT concentrations: (○) 79 × 10^{-6} mol dm^{-3}; (●) 97 × 10^{-6} mol dm^{-3}.

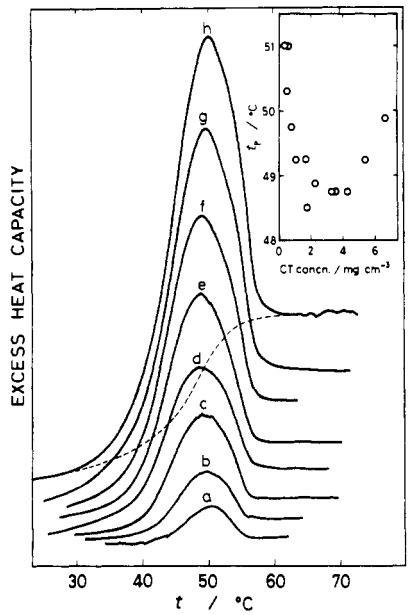

FIGURE 4: Excess heat capacity curves observed for CT in 0.025 M potassium phosphate buffer, pH 7.0 and I = 0.1 M with KCl. Heating rate 1 K min^{-1}. Protein concentrations: (a) 0.41, (b) 0.82, (c) 1.65, (d) 2.14, (e) 3.30, (f) 4.24, (g) 5.35, and (h) 6.59 mg cm^{-3}. The inset shows the concentration dependence of the apparent peak temperature. Ordinate: 15.2 kJ K^{-1} (mol of CT)$^{-1}$ cm^{-1} (curve h). The dashed curve is the base line for curve h calculated by assuming two-state behavior (Fukada et al., 1983). The procedure outlined by Fukada et al. was employed to establish the base lines for the other DSC curves in this paper.

of activity appears to follow two-state behavior despite the overall irreversibility of the denaturation (see below). The slope of the plot gives ΔH_{vH} = 274 ± 6 kJ mol^{-1}.

Differential Scanning Calorimetry. DSC traces for the unfolding of CT at various concentrations are shown in Figure 4. The excess heat capacity curves have an asymmetry which is primarily due to the large increases in heat capacity ($\Delta C_{p,d}$). The plot of the peak temperature (t_m) vs. protein concentration shown in the inset in Figure 4 further illustrates the complexity of the denaturational behavior of CT under the present experimental conditions. The dashed curve included with curve h in the figure is the base line calculated on the assumption of two-state behavior (Takahashi & Sturtevant, 1981).

Table IV: Summary of DSC Data for α-Chymotrypsin at pH 7.0

e_0 (mg cm^{-3})	$t_m{}^a$ (°C)	$\Delta H_d{}^b$ (kJ mol^{-1})	$\Delta H_{vH}{}^c$ (kJ mol^{-1})	$\Delta C_{p,d}$ (kJ K^{-1} mol^{-1})	$\Delta H_d(322)^d$ (kJ mol^{-1})
6.59	49.9	819	284	24.1	803
5.35	49.3	792	291	22.8	787
4.24	48.8	819	287	23.4	823
3.55	48.8	770	304	17.2	774
3.30	48.7	830	290	23.4	835
2.14	48.9	794	304	20.0	796
1.86	48.5	696	334	9.9	704
1.65	49.2	881	336	25.8	877
1.07	49.3	818	335	9.9	813
0.82	49.7	894	327	31.9	882
0.59	51.0	741	386	3.5	707
0.49	50.3	865	382	9.6	843
0.41	51.0	974	384	4.3	940
			mean 326	17.3	814
			SE ±10	±2.5	±18

^{a} t_m is the temperature of maximal excess heat capacity. ^{b} ΔH_d is the molar enthalpy of denaturation. ^{c} $\Delta H_{vH} = 4.00RT_m{}^2(c_{max}/\Delta h_d)$. ^{d} $\Delta H_d(322) = \Delta H_d + \Delta C_{p,d}(T_m - 322)$.

Rescanning of heated sample showed the denaturation to be overall irreversible under these experimental conditions.

The calorimetric enthalpy and heat capacity changes were estimated from the DSC curves as previously described (Fukada et al., 1983), with the results given in columns 3 and 5 of Table IV. Both the specific enthalpy of denaturation, 32.6 ± 0.7 J g^{-1}, and the apparent specific heat change, 0.69 ± 0.10 J K^{-1} g^{-1}, are rather large as compared with other globular proteins (Privalov & Khechinashvili, 1974; Tischenko et al., 1974; Privalov, 1979, 1982).

For a strictly two-state reversible process in which no dissociation takes place, the van't Hoff enthalpy can be estimated from the DSC curve for the process by means of the relation

$$\Delta H_{vH} = 4RT_m{}^2(c_{max}/\Delta h_d) \quad (3)$$

where c_{max} is the maximal value of the excess specific heat, Δh_d is the specific enthalpy change, and T_m is the absolute temperature at which the excess specific heat reaches its maximal value (Velicelebi & Sturtevant, 1979). Application of this equation to the DSC curves in Figure 4 gives the enthalpy values listed in the fourth column of Table IV. The fact that the mean of these values is less than half the calorimetric value suggests that intermediate states in the denaturational process are significantly populated. The discrepancy may also be due in part to other complications such as the fact that the van't Hoff equation has been applied here to an apparently irreversible process. It is, however, interesting that the value of ΔH_{vH} deduced from the loss of enzyme activity on heating, 274 kJ mol^{-1}, is approximately equal to the DSC value, 326 kJ mol^{-1}.

The enthalpy of denaturation after correction to a constant temperature (column 6, Table IV) is constant within experimental uncertainty over a 16-fold range of protein concentration. Since it would be expected that autolysis would be more extensive at high than at low enzyme concentrations, we may conclude either that extensive autolysis does not take place or that the enthalpy change associated with autolysis is small compared to the denaturational enthalpy and the autolysis products have the same denaturational enthalpy as the intact enzyme.

Privalov & Khechinashvili (1974) and Tischenko et al. (1974) studied the thermal denaturation of CT in the pH range 2–4. They obtained the values 22.6 J g^{-1} for the enthalpy of denaturation at 50 °C and 0.50 J K^{-1} g^{-1} for the change in apparent specific heat, both considerably smaller than the

Table V: Enthalpy Change for the Unfolding of the SSI–α-Chymotrypsin Complex at pH 7.0

e_0 (μmol dm^{-3})	i_0 (μmol dm^{-3})	[complex](57 °C)[a] (μmol dm^{-3})	q^{I}[b] (mJ)	q^{II}[c] (mJ)	$\Delta H_d^{complex}$[d] (kJ mol^{-1})	ΔH_d^{SSI}[e] (kJ mol^{-1})
230.1	109.1	104.4	204.8	20.6	981	189
184.1	109.1	102.1	171.3	23.8	1024	218
181.8	109.6	102.3	179.1	21.3	1117	194
138.1	109.1	96.2	146.0	29.5	1163	271
96.2	109.6	80.6	99.9	22.7	1082	207
92.0	109.1	77.9	107.1	34.7	1227	318
72.3	108.4	64.1	81.5	31.8	1167	293
57.9	108.4	52.6	71.2	33.0	1273	304
48.1	109.6	44.3	49.6	26.7	1050	244
43.4	108.4	40.1	52.4	34.5	1240	318
42.8	109.6	39.6	51.8	28.2	1241	257
28.9	108.4	27.0	36.4	34.7	1291	320
23.8	109.6	22.4	31.9	29.7	1373	271
19.2	109.6	18.1	22.2	30.6	1175	279
14.5	108.4	13.7	20.5	36.7	1449	338
9.1	109.6	8.6	11.5	33.9	1291	309
76.3	220.6	73.5	101.3		1348	
76.3	109.1	67.3	89.8	25.2	1226	232
74.5	81.6	59.4	78.2	21.1	1109	259
76.3	54.2	45.8	76.1	12.6	1120	233
74.5	49.1	41.9	68.9	10.6	1011	217
76.3	43.9	38.2	80.6	10.3	1295	235
169.4	330.4	163.9	188.1		1120	
169.4	150.2	131.0	177.4	36.0	1115	240
169.4	75.1	71.0	156.6	13.8	1077	184
					mean 1183	258
					SE ±24	±10

[a] Concentration of EI complex at 57 °C calculated from eq 2 on the basis of data given in Table II. [b] Heat absorption corresponding to peak I. [c] Heat absorption corresponding to peak II. [d] Unfolding enthalpy of the EI complex calculated from peak I by correcting for the unbound fraction as described in the text. [e] Unfolding enthalpy of SSI calculated from peak II on the basis that the native SSI molecule is dissociated from the denatured enzyme, the mean value of which is close to that found for free SSI in a previous study (Takahashi & Sturtevant, 1981).

values observed in the present work at pH 7. They also found that the van't Hoff and calorimetric enthalpies agreed within 5%, indicating that the denaturation at low pH is very close to a two-state process. It is thus evident that the denaturational process as observed at neutral pH is a very different process and may well include a significant amount of autolysis.

DSC traces for a series of mixtures containing a constant amount of SSI and increasing amounts of CT and for a series containing a constant amount of CT and increasing amounts of SSI are shown in Figures 5 and 6, respectively.

The excess heat capacity curves for the mixture of the two proteins are characterized by two peaks, the first of which (peak I) is presumably mainly due to the unfolding of the SSI–chymotrypsin complex. The peak temperature of peak I is around 57 °C, being slightly dependent on the concentrations of enzyme and inhibitor present. The asymmetric shape of peak I is due to the presence of unbound enzyme, the unfolding of which overlaps that of the complex.

The peak temperature of peak II is approximately 82 °C, which is close to that of SSI (Takahashi & Sturtevant, 1981), and the unfolding enthalpy calculated from the area under peak II on the basis of total inhibitor present in the system was found to be $\Delta H_d = 258 \pm 10$ kJ mol^{-1}. This value, the average of 23 measurements, is very close to that found previously for SSI unfolding, $\Delta H_d^{SSI} = 255.5 \pm 5.6$ kJ mol^{-1} (Takahashi & Sturtevant, 1981). This result, together with the results of reheating experiments where only the second peak was observed, indicates that the native SSI molecule is dissociated from the enzyme during denaturation of the complex, in contrast to the situation with the SSI–subtilisin complex (Takahashi & Sturtevant, 1981). This being the case, we should expect to see in Figure 5 an increase in the t_m of peak I as increasing concentrations of enzyme are added to a constant concentration of SSI (Fukada et al., 1983), whereas a small decrease is actually observed. This may be due to the increasingly large overlapping endotherm due to the free CT present. The expected increase is observed when increasing amounts of SSI are added to a constant amount of CT (Figure 6).

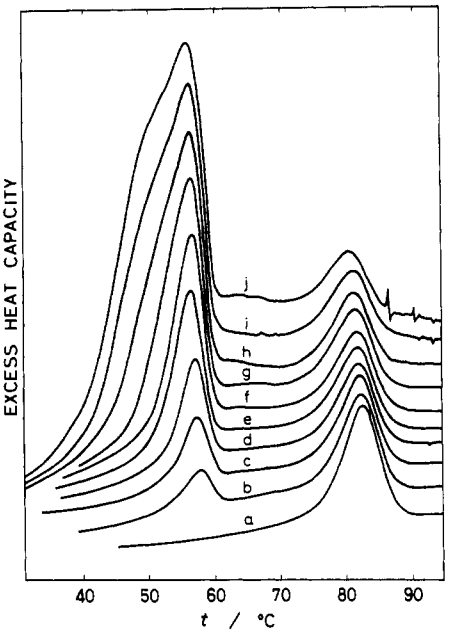

FIGURE 5: Excess heat capacity curves observed for mixtures containing a constant amount of SSI and increasing amounts of CT in 0.025 M potassium phosphate buffer, pH 7.0 and $I = 0.1$ M with KCl. Heating rate 1 K min^{-1}. SSI concentration 1.26 mg cm^{-3}. Concentrations of CT: (a) 0, (b) 0.18, (c) 0.36, (d) 0.59, (e) 1.09, (f) 1.45, (g) 2.30, (h) 3.45, (i) 4.60, and (j) 5.75 mg cm^{-3}. Ordinate: 47.1 kJ K^{-1} (mol of SSI dimer)$^{-1}$ cm^{-1}.

The total area under peak I in Figures 5 and 6 presumably corresponds to the sum of the unfolding enthalpies of the free enzyme and of the complex. An approximate evaluation of

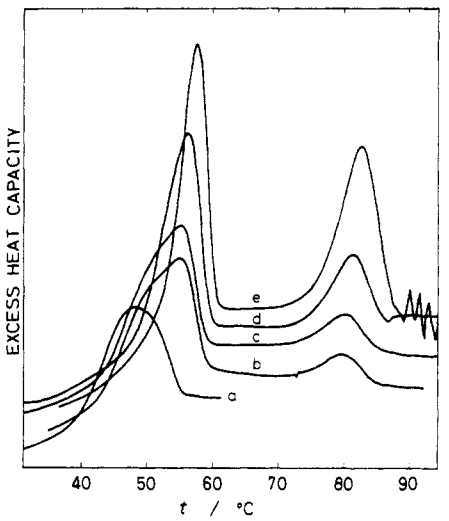

FIGURE 6: Excess heat capacity curves observed for mixtures containing a constant amount of CT and increasing amounts of SSI in 0.025 M potassium phosphate buffer, pH 7.0 and I = 0.1 M with KCl. Heating rate 1 K min^{-1}. CT concentration 1.91 mg cm^{-3}. Concentrations of SSI: (a) 0, (b) 0.51, (c) 0.62, (d) 1.26, (e) 2.54 mg cm^{-3}. Ordinate: 53.1 kJ K^{-1} (mol of CT)$^{-1}$ cm^{-1}.

this latter quantity at 57 °C was made by correcting the observed heats listed in column 4 of Table V as follows: the concentration of complex present at 57 °C (column 3, Table V) was estimated by means of eq 2 using the thermodynamic parameters listed in Table II. The enthalpy due to the free enzyme present was then calculated by using ΔH_d^{CT} = 814 kJ mol^{-1} at 49° C, the temperature at which the enzyme is denatured in the absence of SSI, and was subtrated from the corresponding observed heat, and the resulting quantities were divided by the concentration of complex to give the molar enthalpies listed in column 6 of Table V. The mean value of these enthalpies is 1183 kJ mol^{-1}, with a standard error of 2.0%. The corresponding heat capacity change is estimated to be 39.2 ± 3.3 kJ K^{-1} mol^{-1}.

If the complex actually undergoes dissociation associated with unfolding, the unfolding enthalpy observed for the complex should be the sum of the enthalpies for the dissociation of SSI from native CT and the unfolding of the free enzyme. The heat of dissociation of SSI at 57 °C calculated from the values for $\Delta H_b(298)$ and $\Delta C_{p,b}$ given above is 17.5 kJ mol^{-1}, and the unfolding enthalpy of the enzyme also calculated to 57 °C is 953 kJ mol^{-1}. The sum of these figures, 970 kJ mol^{-1}, is 213 kJ mol^{-1} smaller than expected. Part of this discrepancy may be due to the long extrapolation of $-\Delta H_b$ to 57 °C based on the assumption of the constancy of $\Delta C_{p,b}$, but it is unlikely that this can account for the entire difference. The cause of this discrepancy is unknown.

A comparison of the widths of the DSC curves for the free enzyme in Figure 3 and the complex in Figures 5 and 6 clearly suggests that the binding of SSI to chymotrypsin increases the cooperativity of unfolding of the enzyme protein. A similar situation has already been observed with the SSI–subtilisin interaction (Takahashi & Sturtevant, 1981). However, it is interesting to see that the unfolding temperature of CT is raised by only about 7 K when it is bound with SSI, while the SSI–subtilisin complex has a transition temperature about 20 K above that of the free enzyme. Thus, the stabilization of the complex relative to its "denatured form" as compared to the enzyme relative to its unfolded form is much less with the SSI–chymotrypsin complex than with the SSI–subtilisin complex. The same trend can be seen in the differences in the changes of Gibbs energy and enthalpy for the binding of SSI to the two enzymes as measured by fluorescence titration and flow calorimetry.

In conclusion, we point out here those data developed in this paper which we consider to be most reliable in the sense of their being essentially free from dependence on assumptions or models. These include the thermodynamic data for the binding of SSI to CT at 25 °C as listed in Table II, the values for t_m, ΔH_d, and $\Delta C_{p,d}$ for the denaturation of CT given in Table IV, and the denaturational enthalpies for the SSI–CT complex shown in columns 4 and 5 of Table V. The quantities in Table III depend on an empirical scheme for separating hydrophobic and vibrational entropies and heat capacities (Sturtevant, 1977); the quantities in columns 6 and 7 of Table V depend in part on an extrapolation of the thermodynamic values given in Table II from 25 to 57 °C and in part on assumptions concerning the nature of peaks I and II in the denaturation of the SSI–CT complex.

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Binding of Benzo[*a*]pyrene by Purified Cytochrome P-450$_c$[†]

Craig B. Marcus, Christopher R. Turner,[‡] and Colin R. Jefcoate*
Department of Pharmacology, University of Wisconsin Medical School, Madison, Wisconsin 53706
Received June 18, 1984; Revised Manuscript Received April 10, 1985

ABSTRACT: Benzo[*a*]pyrene (BP) fluorescence-emission intensities in phospholipid micelles are quantitatively described over a broad range of lipid and BP concentrations by excitation that is linearly dependent upon BP concentration and an offsetting excimer quenching that is dependent upon the square of the BP concentration. The fluorescence of BP is quenched by the presence of cytochrome P-450$_c$ in proportion to the concentration of the cytochrome in the micelles and in accord with stoichiometric complex formation. Parallel optical titrations indicate a change in spin state of P-450$_c$ to a predominantly high-spin state that correlates directly with the percentage fluorescence quenching of complexed BP. Neither change occurs with five other purified forms of rat liver P-450 that have low activity in BP metabolism. *N*-Octylamine, a ligand that binds to the heme of P-450, competitively inhibits both the spin-state changes and the fluorescence quenching in equal proportion. The K_d for the interaction of BP with P-450$_c$ is exceptionally low (10 nM) relative to the K_m for monooxygenation (ca. 1 μM). Decreasing the concentration of either dilauroylphosphatidylcholine or dioleoylphosphatidylcholine concomitantly increases the high-spin state (from 30% to 80%) of fully complexed P-450$_c$ and the fluorescence quenching (50–100%) of the complexed BP (half-maximal at 80 μg of lipid/mL). It is concluded that spin state and fluorescence quenching both reflect the same changes in the interaction of the BP with the P-450 heme. These changes also occur in parallel with a 3-fold decrease in BP metabolism as LPC increases from 7.5 to 200 μg/mL and a 20-fold increase in the affinity of P-450$_c$ for membrane-bound BP. The interaction of BP with P-450$_c$ is sensitive to the P-450$_c$:lipid ratio in a manner that directly affects enzyme turnover and may involve different aggregation states of the cytochrome.

Microsomal cytochrome P-450 exists in many forms of broad but nonetheless differing substrate specificity (Ryan et al., 1982; Guengerich et al., 1982). Substrate binding to cytochrome P-450, in most cases, can be measured by changes in its optical spectrum (Remmer et al., 1966; Peterson, 1971; Schenkman et al., 1967). These changes in the optical spectrum correlate with changes in the electron spin resonance spectrum of the cytochrome (Tsai et al., 1970; Stern et al., 1973; Grasdalen et al., 1975) and reflect changes in the spin state of the P-450 heme. Substrate-free cytochrome P-450 generally adopts a low-spin state (absorption maximum at 417 nm), although for a few forms a high-spin state is the preferred configuration in the absence of substrate (Friedrich et al., 1979; Ryan et al., 1980). Liver forms of microsomal P-450 typically adopt a mixed-spin state upon binding substrate. The mixed-spin state of these complexes is substrate dependent, and this has been attributed to a perturbation of an equilibrium between high- and low-spin configurations (Sligar & Gunsalus, 1979; Ristau et al., 1979). Transition of the heme to the high-spin configuration is also frequently associated with an increased rate of reduction of the cytochrome and with increased monooxygenation rates (Backes et al., 1982).

Membrane phospholipids may play a key role in determining both substrate binding and activity of P-450. Since most substrates are lipid soluble, the partitioning of such substrates between the aqueous and lipid phases becomes a major determinant of binding (Al-Gailany et al., 1978; Parry et al., 1976; Canady et al., 1974). In addition, cell membranes may contain regions of differing fluidity that may in turn be perturbed by lipophilic compounds (Sanioto & Schreir, 1975; Shimchick & McConnel, 1973; Ebel et al., 1978; Stier, 1976). Membrane lipids themselves may have direct effects on P-450 itself, as well as on the interaction between P-450 and its reductase (Schenkman et al., 1980; Miwa & Lu, 1981).

The binding of the fluorescent polycyclic hydrocarbon pyrene to cytochrome P-450 results in a quenching of pyrene fluorescence. This approach has been employed to establish the 1:1 stoichiometric binding of pyrene to rabbit hepatic cytochrome P-450 LM$_4$ (Imai, 1982). However, binding of benzo[*a*]pyrene (BP)[1] to LM$_4$ does not induce a spin-state

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[‡] Present address: U.S. Naval Medical College, Bethesda, MD 20814.

[1] Abbreviations: PAH, polycyclic aromatic hydrocarbon(s); BP, benzo[*a*]pyrene; MC, 3-methylcholanthrene; LPC, dilauroylphosphatidylcholine; OPC, dioleoylphosphatidylcholine; cmc, critical micelle concentration; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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