numbers, with no single step clearly having the smallest turnover number.

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Calorimetric Studies of the Binding of *Streptomyces* Subtilisin Inhibitor to Subtilisin of *Bacillus subtilis* Strain N′ †

Katsutada Takahashi* and Harumi Fukada

Laboratory of Biophysical Chemistry, College of Agriculture, University of Osaka Prefecture, Sakai, Osaka 591, Japan
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ABSTRACT: The binding of *Streptomyces* subtilisin inhibitor (SSI) to subtilisin of *Bacillus subtilis* strain N′ (subtilisin BPN′, EC 3.4.21.14) was studied by isothermal calorimetry at pH 7.0 and at various temperatures ranging from 5 to 30 °C. Thermodynamic quantities for the binding reaction were derived as a function of temperature by combining the data reported for the dissociation constant with the present calorimetric results. At 25 °C, the values are $\Delta G° = -57.9$ kJ mol^{-1}, $\Delta H = -19.8$ kJ mol^{-1}, $\Delta S° = 0.13$ kJ K^{-1} mol^{-1}, and $\Delta C_p = -1.02$ kJ K^{-1} mol^{-1}. The entropy and the heat capacity changes are discussed in terms of the contributions from the changes in vibrational modes and in hydrophobic interactions.

Many protein–ligand interactions are known to be accompanied by large changes in the entropy and heat capacity of the reacting species (Sturtevant, 1977; Hinz, 1983). For such systems, it seems probable that changes in hydrophobic interactions and in internal vibrational modes are major contributions to the large changes in thermodynamic quantities (Sturtevant, 1977). Hydrophobic interactions and vibrational modes are obviously very much dependent on protein conformation, and the process of specific interactions between protein molecules may thus be accompanied by large changes in them, leading to large entropy and heat capacity changes.

Streptomyces subtilisin inhibitor (Murao et al., 1972; Sato & Murao, 1973) is a unique protein with an M_r of 23 000 (dimer), which specifically and tightly binds to the alkaline proteases, especially subtilisin, to inhibit the catalytic activity, with a dissociation constant in the case of subtilisin of $K_d = 7.12 \times 10^{-11}$ mol dm^{-3} at pH 7.0 and 25 °C (Uehara et al., 1978). It has also been shown by a kinetic study (Uehara et al., 1980) that although the SSI[1] molecule exists as a dimer of identical subunits, the binding process can be interpreted by an independent site model. Thus, the dimerization state is not affected by the association with subtilisin.

In an earlier paper (Takahashi & Sturtevant, 1981), the thermal denaturation of the inhibitor and of the inhibitor–subtilisin complex was studied by differential scanning calorimetry, and it was found that the unfolding temperature of the enzyme is raised by about 20 K upon the binding of the SSI molecule at pH 7.00. The result was also obtained that the SSI molecule does not dissociate from the enzyme even after the complex is heated up to its denaturation temperature, 87 °C. These findings indicate that the binding of SSI to subtilisin BPN′ induces a large change in the molecular situation and that the process should thus be accompanied by large changes in the thermodynamic quantities. It would,

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[1] Abbreviations: SSI, *Streptomyces* subtilisin inhibitor; subtilisin BPN′, subtilisin of *Bacillus subtilis* strain N′.

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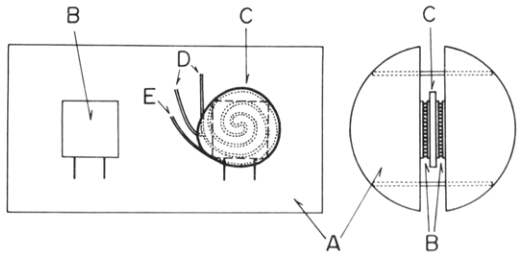

FIGURE 1: Schematic drawing of the flow calorimeter structure: (A) aluminum heat sink; (B) thermopile plate; (C) reaction chamber; (D) inlet tubing; (E) outlet tubing.

therefore, be of great interest to determine the thermodynamic parameters by means of direct calorimetric measurements in order to obtain useful information concerning the nature of the complex formation.

In the present paper thermodynamic quantities are reported that have been derived from flow calorimetric measurements at various temperatures on the binding of SSI to subtilisin BPN′.

Materials and Methods

Streptomyces Subtilisin Inhibitor. Partially purified *Streptomyces* subtilisin inhibitor was a gift from Prof. K. Hiromi of Kyoto University, Kyoto, Japan. It was further purified by the method of Sato & Murao (1973). SSI solutions used for 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. The dialyzed solution had a protein concentration of about 100 μmol dm^{-3}, which was then used after appropriate dilution. The concentration was determined from the absorption measurement of the solutions using a value of specific absorption of 0.829 cm^2 mg^{-1} at 276 nm.

Subtilisin BPN′. Crystalline subtilisin BPN′ (M_r 27 500) purchased from Nagase Sangyo Co., Osaka, Japan, was used without further purification. Its concentration was determined spectrophotometrically by using a value of specific absorption of 1.063 cm^2 mg^{-1} at 278 nm. The 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). The correction was made by using a factor of 0.758 to obtain the active enzyme concentration. Subtilisin solutions were prepared by dissolving the crystalline material in the SSI dialyzate immediately before use. No dialysis was made in order to minimize autolysis.

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

Isothermal Calorimetry. The flow calorimeter developed in this laboratory was used for the reaction heat measurements. The design is basically of the conduction principle with twin calorimetric units (Sturtevant & Lyons, 1969). The structure of the calorimeter is illustrated schematically in Figure 1. The reaction chamber is formed with gold tubing (0.8-mm inner diameter) and two thin copper plates (0.3-mm thickness) between which the gold tubing is wound flat and sandwiched. The tubing is 0.906 cm^3 in volume and has a Y-junction at one end that acts as a mixing part. Silver flux is embedded between the tubing and the plates so that good thermal conductivity is attained. The reaction chamber is fabricated in such a manner that it forms a disk with 3-mm thickness and about 4-cm diameter.

The two reactant solutions are introduced through two Teflon inlet tubings that are attached to the two openings of the Y-junction and are led to the outlet through a third Teflon tubing connected to the other end of the gold tubing. Two semiconducting thermopile plates (Melcor CP 1.4-71-06L, Melcor, Inc., Trenton, NJ) that act as sensors are placed in good thermal contact with the flat surfaces of the disk by using a small amount of thermal grease, thus forming a calorimetric unit. A copper disk of the same size to which two additional thermopile plates are applied forms a reference unit.

The two calorimetric units are sandwiched between two symmetrical half-cylinders made of aluminum that serve as an inner heat sink. This assembly is then placed in an aluminum cylindrical capsule that serves as an outer heat sink. The whole unit, having dimensions of 12-cm diameter and 24-cm length, is covered with a plastic-foam wall of 7-cm thickness and is contained in a brass submarine can that is submerged in a thermostated water bath. The temperature of the bath was maintained constant to ±0.01 K in the range of 0–45 °C.

The solutions were delivered from either 2.5- or 5-cm^3 gas-tight syringes (Hamilton 81401 or 81501, Hamilton Co., Reno, NV) mounted on commercial dispenser pumps (Perfusor 137, B. Braun, Melsungen, West Germany). The Teflon-tipped plungers were driven by gear-head synchronous motors that enabled delivery of the solutions in a wide range of flow rates from 1.144×10^{-5} to 2.290×10^{-2} cm^3 s^{-1} in 20 steps. The flow rate was frequently checked by weighing the effluent.

The measurements were conducted with the stopped-flow procedure. The solutions were delivered from each syringe for 60–120 s at a flow rate of 2.291×10^{-3} cm^3 s^{-1}. The heat effect was estimated from the total area under the curve drawn on the recorder by the calorimeter output signal. The SSI concentration was about 40 μmol dm^{-3} while the concentration of the enzyme was 60 μmol dm^{-3}, both after mixing. Because of the small value of the dissociation constant, all of the inhibitor present was in the bound form after mixing at these concentrations.

The heats of dilution of both SSI and subtilisin BPN′ were measured by separate runs in which each of them was mixed with buffer (SSI dialyzate). Viscous heating was measured by mixing buffer with buffer. The dilution heats and the viscous heating were subtracted from the observed heat effect q_{obsd} due to the mixing of SSI with subtilisin to obtain the enthalpy of reaction q_b on the basis of the relation

$$q_b = q_{obsd} - q_{I+B} - q_{E+B} + q_{B+B} \quad (1)$$

where q_{I+B}, q_{E+B}, and q_{B+B} are the heat effects due to the mixing of SSI with buffer, subtilisin with buffer, and buffer with buffer, respectively. The total heat effect observed was in the range 40–400 μJ.

Measurements were made at 5.2, 15.1, 20.1, 25.1, and 30.0 °C. The calorimeter was calibrated at the each temperature by using the neutralization of 0.004 N HCl with 0.01 N NaOH. The standard heat of neutralization at each temperature was taken from the values reported by Grenthe et al. (1970).

Results and Discussion

In Table I, the observed enthalpies of reaction, ΔH_{obsd}, at the various temperatures are summarized. Since the binding process of the SSI dimer with the two subtilisin molecules is known to be interpreted by an independent model (Uehara et al., 1980), the thermodynamic quantities are given on a "mole of monomer" basis. The number of protons released during the binding process was measured by the pH-stat method and found to be 0.6 ± 0.1 at 25 and 10 °C. In order to obtain the net enthalpy change associated with the binding of SSI to

Table I: Observed Enthalpy Change for Reaction of Subtilisin BPN′ with SSI at pH 7.0 and at Various Temperatures[a]

temp (°C)	no. of measurements	ΔH_{obsd} (kJ mol^{-1})	ΔH (kJ mol^{-1})
5.2	9	−5.03 ± 1.15	0.05 ± 1.43
5.2	9	−3.93 ± 0.56	1.15 ± 1.02
15.1	7	−11.30 ± 0.75	−7.50 ± 0.98
20.1	8	−22.26 ± 0.75	−19.12 ± 0.91
25.1	10	−20.77 ± 0.43	−18.31 ± 0.59
25.1	7	−23.56 ± 0.64	−21.10 ± 0.76
30.0	7	−25.14 ± 0.63	−23.35 ± 0.70

[a] The net enthalpy change was calculated by eq 2 and the relation $\Delta H = \Delta H_{obsd} - n\Delta H_i$, where n is the number of protons taken up by the reaction system ($n = -0.6$). Precision in standard error. All quantities are for the formation of a monomeric complex.

subtilisin BPN′, a correction was made for the enthalpy change due to the proton uptake by the phosphate buffer that was obtained from the following relation reported for the enthalpy of phosphate ionization by Bates & Acree (1943)

$$\Delta H_i = 37.894 - 0.0003798T^2 \quad (2)$$

where T is the absolute temperature and ΔH_i is in kJ mol^{-1}. For the calculation the proton release was assumed to be constant over the temperature range studied. In the fourth column of Table I is given the net enthalpy change of the binding of the inhibitor to the enzyme. Using the basic thermodynamic relation

$$d\Delta H/dT = \Delta C_p \quad (3)$$

and assuming ΔC_p to be constant over the temperature range studied, we calculated the net heat capacity change associated with the inhibitor binding to the enzyme by a least-squares analysis on the data given in Table I to be $\Delta C_p = -1.02 \pm 0.10$ kJ K^{-1} mol^{-1} (−0.023 ± 0.003 J K^{-1} g^{-1}). This value is about the same magnitude with $\Delta C_p = -0.42 \sim -1.26$ kJ K^{-1} mol^{-1} (−0.009 ∼ −0.028 J K^{-1} g^{-1}) reported for the interaction between trypsin and soybean trypsin inhibitor (Barnhill & Trowbridge, 1975). Moreover, it should be noted that the present system is also accompanied by a large negative change in heat capacity like many other protein–protein interactions so far reported such as the Fc fragment of immunoglobulin G plus A-protein [$\Delta C_p = -4.31$ kJ K^{-1} mol^{-1} (Sjöquist & Wadsö, 1971)], immunoglobulin G, H chain plus L chain (κ) [$\Delta C_p = -10$ kJ K^{-1} mol^{-1} (Dorrington & Kortan, 1974)], H chain plus L chain (λ) [$\Delta C_p = -8.7$ kJ K^{-1} mol^{-1} (Dorrington & Kortan, 1974)], flagellin polymerization [$\Delta C_p = -12.5$ kJ K^{-1} mol^{-1} (Bode, 1974)], hemoglobin plus haptoglobin [$\Delta C_p = -7.86$ kJ K^{-1} mol^{-1} (Lavialle et al., 1974)], ribonuclease S-peptide plus S-protein [$\Delta C_p = -6.11$ kJ K^{-1} mol^{-1} (Hearn et al., 1971)], tubulin association [$\Delta C_p = -6.69$ kJ K^{-1} mol^{-1} (Hinz et al., 1979)], and reconstitution of tryptophan synthase from subunits $2\alpha + \beta_2 \rightarrow \alpha_2\beta_2$ [$\Delta C_p = -15.06$ kJ K^{-1} mol^{-1} (Wiesinger et al., 1979)].

Uehara et al. (1978) determined the dissociation constant for the enzyme–inhibitor complex by the method of fluorescence titration. Using the reported value of $K_d = 7.12 \times 10^{-11}$ mol dm^{-3} at 25 °C, we derived the thermodynamic association quantities by using the equations

$$\Delta G° = -RT \ln (1/K_d) \quad (4)$$

$$\Delta S° = (\Delta H - \Delta G°)/T \quad (5)$$

The values are summarized in Table II. Uehara et al. (1978) obtained a value of $\Delta H = +17.8$ kJ mol^{-1} for the enthalpy of SSI binding to subtilisin BPN′ from a van't Hoff plot of dissociation constants that were determined by fluorometric titration at three different temperatures, i.e., 5, 15, and 25 °C. The large discrepancy between their indirectly obtained value and the present calorimetric result is obviously due to the fact that the heat capacity change accompanying the complex formation was ignored in their calculation as is generally the case when the van't Hoff relation is applied to derive indirectly the enthalpy change. In fact, the uncertainty limit of the dissociation constants is so large that the data are found to lie in a reasonable range when they were fit to the form of the van't Hoff curve required by the values of ΔH and ΔC_p that were obtained in the present calorimetric study. This fact clearly indicates that if the van't Hoff relation is applied to equilibrium data with a large uncertainty, an erroneous conclusion may be reached.

Table II: Thermodynamic Quantities for Binding of SSI to Subtilisin BPN′ at pH 7.0 and 25 °C[a]

K_d (mol dm^{-3})	$\Delta G°$ (kJ mol^{-1})	ΔH (kJ mol^{-1})	$\Delta S°$ (kJ K^{-1} mol^{-1})	$-T\Delta S°$ (kJ mol^{-1})	ΔC_p (kJ K^{-1} mol^{-1})
7.1×10^{-11}	−57.9	−19.8 ± 1.2	0.13	−38.1	−1.02 ± 0.10

[a] All quantities are for the formation of a monomeric complex.

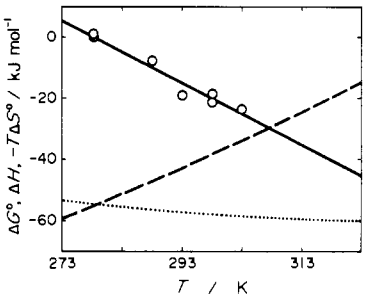

FIGURE 2: Thermodynamic parameters of the binding of SSI to subtilisin BPN′ at pH 7.0 as a function of temperature: ΔH (—); $-T\Delta S°$ (– –); $\Delta G°$ (···). The open circles are the calorimetrically obtained data.

From Table II it appears that at 25 °C the entropy term contributes to the ease of inhibitor binding much more than the energetic stabilization does. The temperature variations of the thermodynamic quantities were derived from eq 5 and the equations

$$\Delta H = \Delta H_{298} + \Delta C_p(T - T_0) \quad (6)$$

$$\ln K_d = \frac{\Delta H_{298}}{R}\left(\frac{1}{T_0} - \frac{1}{T}\right) + \frac{\Delta C_p}{R}\left(\ln \frac{T}{T_0} + \frac{T_0}{T} - 1\right) + \ln K_{d,298} \quad (7)$$

where ΔH_{298} and $K_{d,298}$ are respectively the enthalpy change for the binding of SSI to subtilisin and the dissociation constant of the SSI–subtilisin complex at $T_0 \equiv 298.15$ K. The results are shown in Figure 2. The open circles in Figure 2 are the net enthalpy change obtained experimentally at each temperature (Table I). It is obvious that like many other protein–ligand interaction systems the present process is characterized by a negative heat capacity change that is significantly larger than $\Delta S°$ in magnitude, giving an almost constant value for the Gibbs energy change over the ordinary temperature range. From eq 6, ΔH is known to be 0 at 5.59 °C. Below this temperature, although the enthalpy term contributes unfavorably, formation of the SSI–subtilisin complex is driven by the entropy term alone and the binding is still very tight.

At above 5.59 °C, although the enthalpy change becomes much more negative as the temperature is higher, the contribution of the entropy term still overcomes that of the en-

Table III: Hydrophobic and Vibrational Contributions to the Thermodynamics of Binding of SSI to Subtilisin BPN′ at pH 7.0[a]

temp (°C)	$\Delta S°_u$	$\Delta S°_u$(h)	$\Delta S°_u$(v)	ΔC_p	ΔC_p(h)	ΔC_p(v)
5	0.24	0.26	−0.02	−1.02	−1.00	−0.02
25	0.16	0.24	−0.08	−1.02	−0.94	−0.08
35	0.13	0.24	−0.11	−1.02	−0.92	−0.10

[a] All quantities are in kJ K^{-1} (mol of monomer)$^{-1}$.

thalpy term up to 34.0 °C. Thus, at ordinary temperatures around 25 °C, the process is controlled by a positive entropy change more favorably than the negative enthalpy change.

According to the X-ray crystallographic results of Mitsui et al. (1979) and Hirono et al. (1984), the area on the contact regions of the two proteins amounts to about 650 Å^2; thus the surface area of both proteins exposed to the solvent is being reduced a great deal during their binding. It may be assumed that this decrease in surface area results in a decrease in the number of exposed nonpolar residues of both proteins. Vibrational freedom may also decrease upon the interaction of the two proteins, thus giving a decrease in the number of easily excitable internal modes of vibration.

In consideration of the above situation, an attempt was made to interpret the observed heat capacity and entropy data in terms of the contributions of the changes in hydrophobic interaction and vibrational modes. In Table III, hydrophobic and vibrational contributions to the entropy and heat capacity changes calculated according to the method proposed by Sturtevant (1977) are given. The suffixes h and v refer to the "hydrophobic" and "vibrational" contributions, respectively. For the calculation, the contribution resulting from changes in available conformations of the proteins was neglected, since this is presumably of major importance only in processes involving protein unfolding (Sturtevant, 1977). $\Delta S°_u$ is the unitary entropy obtained from the observed entropy change of reaction corrected for the cratic entropy, $\Delta S°_c$ = +0.0334 kJ K^{-1} mol^{-1} (Kauzmann, 1959).

The positive values for $\Delta S°_u$(h) and the negative values for $\Delta S°_u$(v), ΔC_p(h), and ΔC_p(v) give a strong indication that associated with the present reaction are significant decreases in the number of soft vibrational modes and in the number of exposed hydrophobic groups. So far as the magnitudes of the entropy and heat capacity changes are concerned, it appears that the hydrophobic contribution is predominant over the vibrational effect. It has been shown by X-ray crystallography (Mitsui et al., 1979; Hirono et al., 1984) that several hydrophobic amino acid residues are involved in the contact region of the SSI molecule: Val-69, Met-70, Pro-72, Met-73, Val-74, and Tyr-75. On this basis it may be reasonably concluded that the strong binding of SSI to subtilisin BPN′ is due mainly to hydrophobic effects involving the nonpolar groups in the contact regions of the two proteins.

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Registry No. SSI, 37205-61-1; subtilisin BPN′, 9014-01-1.

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