Trypsin–Pancreatic Secretory Inhibitor (Kazal Inhibitor) Interaction. Kinetic and Thermodynamic Properties†

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ABSTRACT: The trypsin–Kazal inhibitor interaction has been studied under a variety of pH and temperature conditions, between pH values of 4 and 10 and between 1 and 37°. Typical values for the association constant (K_a), the second-order rate constant of association (k_a), and the first-order rate constant of dissociation (k_d) at 25° and pH 8.0 are 3.1×10^{10} M^{-1}, 6.8×10^{6} M^{-1} sec^{-1}, and 2.2×10^{-4} sec^{-1}, respectively. Thermodynamic values for complex formation are $\Delta G°_a = -14.4$ kcal/mol, $\Delta H°_a = -1.4$ kcal/mol, $\Delta S°_a = 43.6$ cal mol^{-1} deg^{-1}. The association of the secretory inhibitor with trypsin is an entropy-driven process. Comparisons are made between the trypsin–Kunitz inhibitor and the trypsin–Kazal inhibitor complexes. Some comparative structural data concerning the two inhibitors were therefore needed. Although the Kazal inhibitor has nearly the same number of amino acids as the Kunitz inhibitor (56 and 58 residues, respectively) and also the same number of disulfide bridges (three), its stability to thermal denaturation is quite different. It is completely unfolded at 85° and pH 8.0, whereas the Kunitz inhibitor remains completely folded under the same conditions. The trypsin–Kazal inhibitor complex is less stable than the trypsin–Kunitz inhibitor complex, which at pH 8.0 and 25° has a K_a of 1.6×10^{13} M^{-1}. This difference of stability of nearly three orders of magnitude is mainly due to differences in k_d. The trypsin–Kunitz inhibitor complex has a very low k_d 6.8×10^{-8} sec^{-1} ($t_{1/2}$ = 17 weeks as compared to 50 min for the trypsin–Kazal inhibitor association). It is of interest that both the kinetic and thermodynamic data found with the trypsin–Kazal inhibitor system are very similar to values obtained with other effector–receptor interactions involving a small protein effector, *e.g.*, the association of insulin with its receptor and the association of snake neurotoxins with the membrane-bound acetylcholine receptor.

Trypsin plays a central role in pancreatic zymogen activation because all activations of precursors (chymotrypsinogens, procarboxypeptidases, prophospholipase, proelastase) are initiated by trypsin-catalyzed proteolytic cleavages. The acinar cell of the pancreas synthesizes two kinds of trypsin inhibitor which function as security devices against accidental trypsinogen activation. The first one, the Kunitz inhibitor (PTI),[1] remains in the pancreatic cell whereas the second one, the Kazal inhibitor (PSTI), is secreted with all the zymogens into the pancreatic juice where it accounts for 1% of the total potential trypsin (Greene *et al.*, 1966). It is of interest that PSTI does not inactivate the other trypsinogen activating enzyme, enterokinase, which is attached to membranes of the duodenal brush border (Maroux *et al.*, 1971). This absence of inhibition is a necessary requirement for activations to be started in the intestine.

Each of the partners in the trypsin–PSTI association is well characterized. Their sequences are known (Walsh and Neurath, 1964; Greene and Bartelt, 1969; Greene and Guy, 1971) and the three-dimensional structure of trypsin is now available (Stroud *et al.*, 1971). The essential catalytic groups in trypsin are His_{46} and Ser_{183} (Neurath *et al.*, 1970); the essential residue of the specificity site is Asp_{177} (Smith and Shaw, 1969; Steitz *et al.*, 1969; Eyl and Inagami, 1970). One known essential element in the active site of PSTI from bovine origin is Arg_{18} (Rigbi and Greene, 1968). This residue is replaced by a lysine in porcine PSTI (Tschesche *et al.*, 1971).

Present information concerning the two partners in the complex allows the trypsin–PSTI interaction to serve as an excellent model for studies of heterologous protein–protein associations.

Extensive studies have been carried out concerning another very interesting model, the interaction of trypsin with the Kunitz inhibitor (PTI) (for references, see Laskowski and Sealock, 1971, Huber *et al.*, 1971, Liu *et al.*, 1971, and Vincent and Lazdunski, 1972). In comparison, much less is known about the trypsin–PSTI interaction.

This paper presents a systematic investigation of the dynamic and thermodynamic properties of the association of trypsin with PSTI. The data presented also permit a comparison of the structural properties of PTI and PSTI.

Materials and Methods

Materials. Bovine pancreatic secretory trypsin inhibitor was kindly given to us by Dr. Greene. Bovine trypsin was obtained by activation of trypsinogen (Worthington) that had been purified as previously described (Vincent *et al.*, 1970). Bovine chymotrypsinogen A, Bz-L-ArgOEt, *N*-Ac-L-TyrOEt, and *p*-Tos-L-ArgOMet are Sigma products.

Methods. Activity measurements were carried out at 25°, pH 8.0 in a pH-Stat Radiometer TTT1C equipped with an SBR_2C recorder. Trypsin activities were usually measured with 3 mM Bz-L-ArgOEt or with 4 mM *p*-Tos-L-ArgOMet in the presence of 0.2 M NaCl.

Stoichiometry of the association between trypsin and PSTI were determined by following the decrease of *p*-Tos-L-

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[1] The abbreviations used are: PTI, basic pancreatic trypsin inhibitor (Kunitz inhibitor); PSTI, pancreatic secretory trypsin inhibitor (Kazal inhibitor); Bz-L-ArgOEt, α-*N*-benzoyl-L-arginine ethyl ester; *p*-Tos-L-ArgOMet, *p*-tosyl-L-arginine methyl ester; *N*-Ac-L-TyrOEt, α-*N*-acetyl-L-tyrosine ethyl ester.

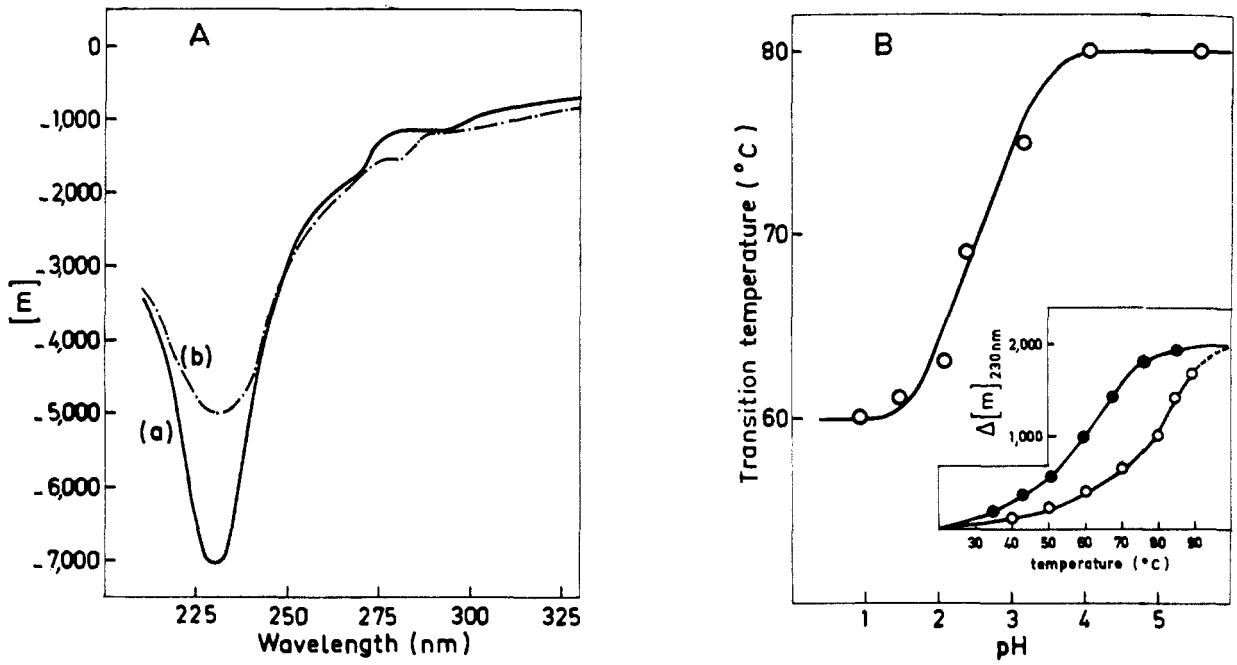

FIGURE 1: Thermal denaturation of the secretory trypsin inhibitor. (A) Optical rotatory dispersion spectrum of the inhibitor at 25° (a) and 85° (b). c = 0.10 mg/ml, pH 1.0. (B) Variation of the transition temperature of the inhibitor with pH. Insert presents thermal denaturation curves of the inhibitor obtained from optical rotatory dispersion measurements at 230 nm: (●) pH 1.0; (○) pH 5.1, c = 0.10 mg/ml.

ArgOMet activity after addition of the inhibitor (Vincent *et al.*, 1970, 1971).

Association kinetics were also evaluated by following the decrease of *p*-Tos-L-ArgOMet activity (Vincent and Lazdunski, 1972). Dissociation kinetics were followed using one of the techniques previously described for the complexes formed by trypsin with derivatives of the Kunitz inhibitor. The trypsin–PSTI complex was first prepared at 25° and pH 8.0 by mixing trypsin (4 μM) with a slight excess of PSTI (4.4 μM), then diluted tenfold in Tris buffer (1 mM) at pH 8.0 containing 0.2 M NaCl and 0.1 M Bz-L-ArgOEt. Under these conditions, Bz-L-ArgOEt reacted with free trypsin and displaced the equilibrium EI $\rightleftharpoons$ E + I on the direction of PSTI liberation. The displacement could be followed by the increase in Bz-L-ArgOEt activity and was directly recorded in the pH-Stat.

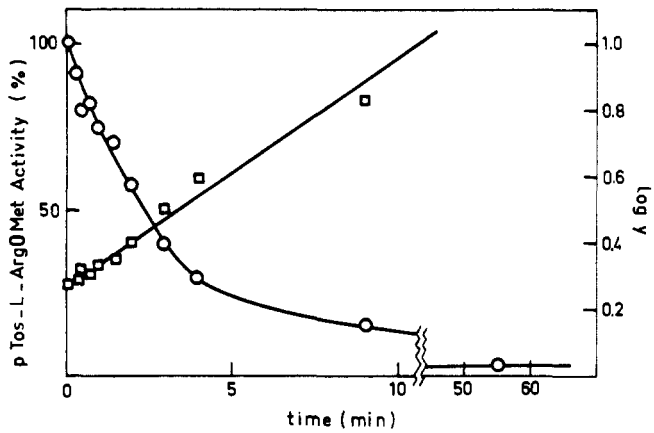

FIGURE 2: Kinetics of association of trypsin (Tryp) with PSTI. Kinetics of association were evaluated by following the decrease of trypsin activity for *p*-Tos-L-ArgOMet. (○) Time course of the association between trypsin (0.43 nM) and PSTI (0.81 nM): 25°, pH 8.0, 0.2 M NaCl. (□) Linear plot demonstrating second-order kinetics. Classical second-order equation

$$\frac{1}{[\text{PSTI}]_0 - [\text{Tryp}]_0} \ln \frac{[\text{PSTI}]_0 - [\text{Tryp–PSTI}]}{[\text{Tryp}]_0 - [\text{Tryp–PSTI}]} = k_a t + \frac{1}{[\text{PSTI}]_0 - [\text{Tryp}]_0} \ln \frac{[\text{PSTI}]_0}{[\text{Tryp}]_0}$$

with

$$y = ([\text{PSTI}]_0 - [\text{Tryp–PSTI}])/[\text{Tryp}]_0 - [\text{Tryp–PSTI}]$$

was used to calculate k_a values. No correction was made for the residual *p*-Tos-L-ArgOMet activity of the trypsin–PSTI complex, which is below 3% of the initial trypsin activity.

Optical rotatory dispersion measurements were carried out in a Fica Spectropol I spectropolarimeter. The cell could be thermostated between 5 and 90° ± 0.2°. Data are given as mean residue rotation $[m]_\lambda$ in deg × cm² × dmol⁻¹: $[m]_\lambda = \alpha_\lambda \overline{\omega r}/100cl$. The mean residue weight $\overline{\omega r}$ was 110 for PSTI. The concentration of the Kazal's inhibitor was determined at 280 nm with $E^{1\%}_{1\text{ cm}}$ = 5.89 (Greene *et al.*, 1966).

Results

Some Structural Properties of PSTI. As will be seen later, the dynamics of the interaction of trypsin with PSTI were followed over a wide pH range and at various temperatures. In order to interpret the kinetic data, it was necessary to determine the importance of structural changes which affect the inhibitor conformation under various conditions of pH and temperature.

Thermal denaturation of PSTI induces large perturbations of the optical rotatory dispersion spectrum. Considerable change was observed, particularly at 230 nm, the minimum of the trough (Figure 1A). This afforded an easy way to follow thermal denaturation (Figure 1B). The curves in Figure 1B indicate a very low cooperativity in the denaturation process. Denaturation starts at about 30° and is completed only at temperature higher than 90°. This behavior is similar to the

one observed with other small proteins containing disulfide bridges, such as the Kunitz inhibitor (Vincent *et al.*, 1971) or neurotoxins of snakes or scorpions (Chicheportiche and Lazdunski, 1970; Chicheportiche *et al.*, 1972). Under similar conditions, the denaturation of bigger disulfide-containing proteins, such as ribonuclease (Brandts, 1965), chymotrypsinogen (Brandts, 1964; Delaage *et al.*, 1968), and trypsinogen (Lazdunski and Delaage, 1967), occurs over a temperature range of only 20 or 30°.

The pH dependence of the transition temperature is presented in Figure 1B (transition temperature, midvariation of $\Delta[m]_{230nm}$ with temperature). Thermal denaturation of PSTI is much easier at very acidic pH than it is at neutral or moderately acidic pH. Similar observations have been made with many other proteins and in particular, with the Kunitz inhibitor (Vincent *et al.*, 1971). The simplest interpretation of such data is that there exists an equilibrium between two-folded isomeric forms of PSTI between pH values of 1 and 7 at temperatures below 30°. Form I is stable at neutral pH and down to pH 4; form II exists in large quantities only at very acidic pH, below pH 2. Form I is more resistant to thermal denaturation with a transition temperature at 80°; form II, being less resistant, has its transition temperature at 60°. All efforts to characterize the isothermal transition I $\rightleftharpoons$ II directly using spectropolarimetry have failed. The optical rotatory dispersion properties of the two forms are identical suggesting that in spite of a large difference in thermal stability, they have only a very limited difference in their folding. Recent work with the Kunitz inhibitor has revealed variation of $[m]_{230nm}$ between pH 8 and 11 at 25°. This variation was related to an alkaline isomerization triggered by the deprotonation of the masked α-amino group of the protein molecule (Vincent *et al.*, 1971). No change of the ORD spectrum of PSTI has been observed between pH 1 and 11 under the same conditions.

Dynamics of the Interaction of Trypsin with PSTI. Systematic work at different pH values confirmed previous observations that trypsin (Tryp) associates stoichiometrically with PSTI.

$$\text{Tryp} + \text{PSTI} \underset{k_d}{\overset{k_a}{\rightleftharpoons}} \text{Tryp–PSTI}$$

KINETIC ASPECTS OF THE ASSOCIATION PROCESS. Figure 2 shows a typical representation of the kinetics of association. Complex formation follows second-order kinetics very closely. The second-order rate constant for the association is $k_a = 6.8\ 10^6\ M^{-1}\ sec^{-1}$ at 25° and pH 8.0; it is six times greater than the rate of association of trypsin with the Kunitz inhibitor, $k_a = 1.1\ 10^6\ M^{-1}\ sec^{-1}$ (Vincent and Lazdunski, 1972).

The variation of k_a with pH is presented in Figure 3. The second-order rate constant of association is maximum at pH 7–8 and decreases at both acidic and alkaline pH values. The variation of the rate is dependent upon the ionization of two groups, one with an apparent pK of 6.2 ± 0.3 which controls the acidic variation, the other with an apparent pK of 8.5 ± 0.3 which controls the alkaline decrease of the rate. The pH dependence of k_a between pH 4 and 7 seems to be linked to the ionization of the imidazole side chain of a histidine residue. No such residue exists in the inhibitor sequence. Therefore, the imidazole side chain could belong to the essential His_{46} of the active site of trypsin. A rapid association would necessitate that the imidazole side chain be in the basic form. A similar decrease of the rate of association of PTI with trypsin (Vincent and Lazdunski, 1972) and chymotrypsin[2] was observed in the same pH range and tentatively interpreted in the same terms.

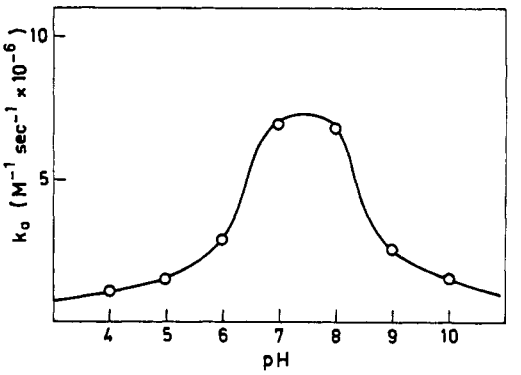

FIGURE 3: pH dependence of the second-order rate constant, k_a, for the association of trypsin with PSTI: 25°, 0.2 M NaCl–10 mM $CaCl_2$.

The significance of the decrease of k_a at alkaline values is not clear. The only groups in the active site of the two partners which might ionize at pH higher than 8 are the masked α-amino group of trypsin (Scrimger and Hofmann, 1967) or the guanidine function belonging to Arg_{18}, the essential element of the active site in the Kazal inhibitor (Rigbi and Greene, 1968). A pK value of about 8.5 would imply a very unusual reactivity for the guanidine function since pK for such side chain is usually higher than 12. No decrease in the rates of association was observed between pH 8 and 10 in the trypsin–PTI interaction, although in that case the active site of PTI contains a lysine residue (Lys_{15}) which could be expected to ionize in a lower pH range than an arginine residue. More information concerning the three-dimensional structure of the trypsin–PSTI complex will be needed for a conclusive identification of the ionizable group with an apparent pK of about 8.5.

Activation energies E_a for the association process were easily obtained from Arrhenius plots at different pH values (Figure 4). The activation energy, 8.6 kcal mol^{-1}, appears to be invariant between pH 5 and 8.

KINETICS ASPECTS OF THE DISSOCIATION PROCESS. The secretory inhibitor can be displaced from its association with trypsin by trypsin substrates. This displacement can be recorded in a pH-Stat. A typical experiment showing the reappearance of trypsin activity with time is presented in Figure 5. In this experiment, with a concentration of 84 mM Bz-L-ArgOEt, the system evolves toward an equilibrium position in which 60% of the initial trypsin was liberated from its interaction with the inhibitor. The association of substrate with free trypsin being extremely rapid, the initial rate of the displacement (insert of Figure 5) is directly measured to give an easy evaluation of the first-order rate constant for the dissociation of the complex, k_d. At pH 8.0 and 25°, $k_d = 2.2 \times 10^{-4}\ sec^{-1}$.

The pH dependence of $1/k_d$ is presented in Figure 6. The rate of dissociation of the complex is lowest and invariant between pH 7.5 and 10. It increases considerably when the pH becomes more acidic.

Variation of k_d with pH depends upon the ionization of a group with an apparent pK of 6.0 ± 0.3. This could be the same imidazole group that seems to be involved in the association process. The protonation of the side-chain His_{46} in the

[2] J. P. Vincent and M. Lazdunski, to be published.

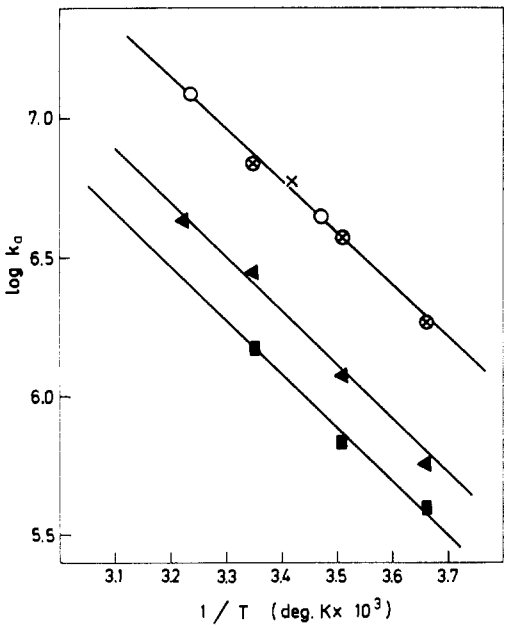

FIGURE 4: Arrhenius plots of the second-order rate constant, k_a, of the association of trypsin with PSTI: (■) pH 5.0; (▲) pH 6.0; (×) pH 7.0; (○) pH 8.0, 0.2 M NaCl–10 mM $CaCl_2$. Temperatures were varied from 1 to 37°.

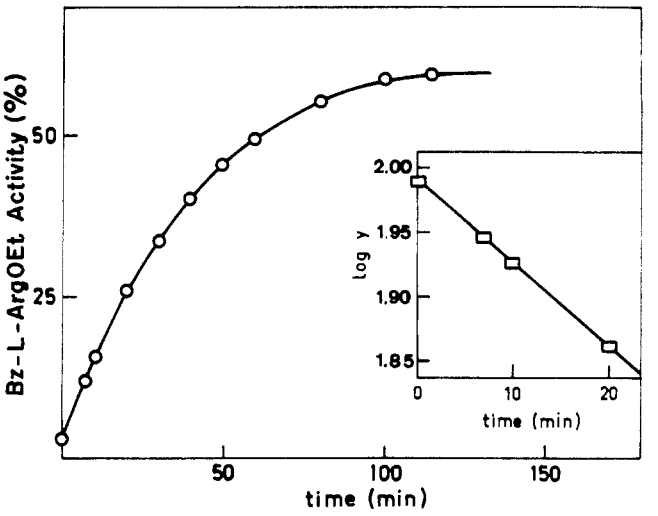

FIGURE 5: Dissociation kinetics of the trypsin–PSTI complex. Displacement of PSTI from the trypsin–PSTI complex was achieved with Bz-L-ArgOEt (final concentration 84 mM). Less than 10% of the initial Bz-L-ArgOEt concentration was transformed into products after 120 min. (○) Time course of reappearance of trypsin activity; (□) pseudo-first-order representation of the data obtained during the first 20 min gave the initial rate of the displacement. y = 100 − %Bz-L-ArgOEt activity; 25°, pH 8.0, 0.2 M NaCl–10 mM $CaCl_2$. No correction was made for the residual *p*-Tos-L-ArgOMet activity of the trypsin–PSTI complex.

active site of trypsin would apparently destabilize considerably the association with the inhibitor. Similar results have been observed in an independent study carried out with the chymotrypsin–PTI complex. In contrast with what was observed with K_a, no variation of k_d was observed between pH 8 and 10. That means either that the deprotonation of the group with an apparent pK of about 8.5 in the free inhibitor, or in the free enzyme, is without influence upon complex dissociation, or that complex formation between trypsin and PSTI considerably shifts the apparent pK of this group toward much higher values.

Activation energies, E_d for the dissociation process were evaluated at various pH values from the Arrhenius plots presented in Figure 7. The activation energy 10 kcal mol^{-1} is again practically invariant between pH 5 and 8.

Equilibrium Characteristics of the Association Trypsin–PSTI. If k_d and k_a are known, the association constant $K_a = k_a/k_d$ of the complex can be calculated. $K_a = 3.1 \times 10^{10}$ M^{-1} at pH 8 and 25°; the corresponding value of $\Delta G°_a$ is −14.4 kcal mol^{-1}. The $\Delta H°_a$ can be calculated from the difference in activation energies for the association and dissociation processes, $E_a - E_d$; $\Delta H°_a = -1.4$ kcal mol^{-1}. $\Delta S°_a = (\Delta H°_a - \Delta G°_a)/T = 43.6$ cal mol^{-1} deg^{-1}.

Values of K_a, $\Delta G°_a$, and $\Delta S°_a$ at several pH values are given in Table I. $\Delta H°_a$ is practically invariant with pH.

TABLE I: Thermodynamic Parameters of the Trypsin–PSTI Association at Several pH Values and 25°.[a]

pH	K_a (M^{-1})	$\Delta G°_a$ (kcal mol^{-1})	$\Delta H°_a$ (kcal mol^{-1})	$\Delta S°_a$ (cal mol^{-1} deg^{-1})
4.0	1.1×10^9	−11.0	−1.4	32.2
5.0	1.1×10^9	−12.4	−1.4	37.0
6.0	8.0×10^9	−13.6	−1.4	41.0
7.0	2.8×10^{10}	−14.3	−1.4	43.3
8.0	3.1×10^{10}	−14.4	−1.4	43.6
10.0	6.5×10^9	−13.4	−1.4	40.3

[a] The standard deviations are ±0.5 kcal mol^{-1} for $\Delta G°_a$ and $\Delta H°_a$ and ±2 cal mol^{-1} deg^{-1} for $\Delta S°_a$.

Discussion

The structural properties of the Kunitz inhibitor have been found to be unusual. The folded conformation of this small protein remains unaltered in 6 M guanidine-HCl at pH 7.5 or at 75° at pH 2 (Vincent *et al.*, 1971). Although both Kazal and Kunitz inhibitors are one-chain proteins with about the same number of amino acids (56 for Kazal's inhibitor and 58 for Kunitz's inhibitor) (Greene and Bartelt, 1969; Kassell and Laskowski, 1965; Chauvet *et al.*, 1964; Anderer and Hörnle, 1966) and three disulfide bridges, and although they inhibit the same enzymatic reaction, they do not show any analogy in sequence.

Differences in sequences produce considerable differences in molecular properties. At 90° and pH 5, thermal denaturation has not started with the Kunitz inhibitor; the molecule remains perfectly folded (Vincent *et al.*, 1971); under the same conditions, the Kazal inhibitor is totally unfolded (Figure 1). The stability of the Kazal inhibitor is similar to that of derivatives of the Kunitz inhibitor obtained after the selective cleavage of the Cys_{14}–Cys_{38} disulfide bridge (Vincent *et al.*, 1971).

Differences are observed not only between the structural stabilities of Kunitz and Kazal inhibitors but also between their kinetic and equilibrium parameters of association with trypsin. Although both Kazal and Kunitz inhibitors form stoichiometric complexes with trypsin, the stabilities of these associations are quite different. The association constant K_a for the formation of the trypsin–PTI complex is 1.6×10^{13} M^{-1} (Vincent and Lazdunski, 1972), that is a little more than 500 times greater than K_a for the formation of the trypsin–

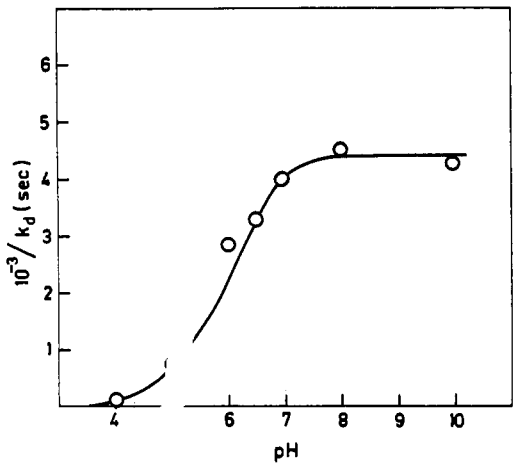

FIGURE 6: pH dependence of the first-order rate constant, k_d, of the dissociation of the trypsin–PSTI complex: 25°, 0.2 M NaCl–10 mM $CaCl_2$.

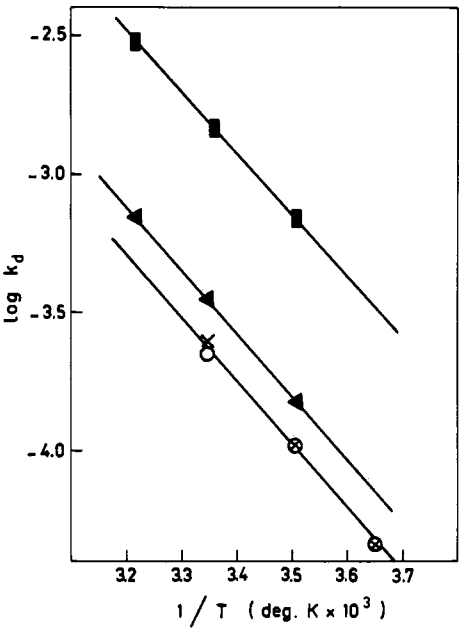

FIGURE 7: Arrhenius plot for the first-order rate constant, k_d, of the dissociation of the trypsin–PSTI complex: (■) pH 5.0; (▲) pH 6.0; (×) pH 7.0; (○) pH 8.0; 0.2 M NaCl–10 mM $CaCl_2$. Temperatures were varied from 1 to 37°.

PSTI complex. The 500-fold smaller K_a is observed in spite of a second-order rate of association of PSTI with trypsin, k_a, which is six times higher than the corresponding value for PTI under the same conditions at neutral pH. The difference in stability is due essentially to differences in k_d values. The first-order dissociation constant of the trypsin–PSTI complex, k_d, is 3000 times greater than the constant for the dissociation of the trypsin–PTI complex. In terms of half-lives, the half-life of the trypsin–PTI complex is more than 16 weeks (Vincent and Lazdunski, 1972) whereas the half-life of the other complex is about 50 min.

The primary specific interaction between trypsin and the secretory inhibitor is most probably the formation of an ion pair between the side-chain carboxylate of Asp_{177} in the specificity site of trypsin and the guanidinium function of Arg_{18} in the active site of PSTI. Many other noncovalent interactions are certainly involved. Model building by assembling the X-ray crystallographic structures of chymotrypsin and the Kunitz inhibitor permitted the identification of about 200 Van der Waals contacts in the complex (Blow *et al.*, 1972). Extensive studies on the trypsin–soybean trypsin inhibitor interaction lead Laskowski and his group (Laskowski *et al.*, 1971) to propose that a bond is formed between the carboxylic part of Arg_{64}, the active site of the soybean inhibitor, and Ser_{183} in the active site of trypsin. Generalization of this mechanism would imply bond formation between Arg_{18} (PSTI) and Ser_{183} (trypsin) in the trypsin–PSTI complex. The enzyme–inhibitor complex would then be either an acyl-enzyme or a tetrahedral intermediate. If formation of an acyl-enzyme could take place there would be a splitting of the Arg_{18}–Ile_{19} bond, and the dissociated inhibitor would be a two-chain molecule.

Digestion of the Kazal inhibitor has in fact been observed with a selective splitting of the Arg_{18}–Ile_{19} bond (Rigbi and Greene, 1968). However, the rates for the formation of this two-chain inhibitor are unrelated to the rates of complex dissociation determined in this study. Moreover, pH conditions for formation of high amounts of two-chain inhibitor are also not related to pH conditions of optimal stability of the complex. Both the rates and the extent of the formation of the two-chain PSTI are very low at pH 8. The inhibitor remains intact even after a 24-hr incubation with trypsin (Laskowski *et al.*, 1971) whereas, under the same conditions, the half-life for the dissociation of the trypsin–PSTI complex is only 50 min. Appreciable splitting of the Arg_{18}–Ile_{19} bond occurs only at acidic pH; both rate and extent increase dramatically between pH 4.5 and 2.7 (Rigbi and Greene, 1968). Since the percentage of catalytically active trypsin decreases considerably in this pH range because of protonation of the imidazole side chain of His_{46}, an essential component of the active site, there appears to be only one explanation for this observation. The folded isomeric form I of the inhibitor, which predominates at alkaline pH, cannot be a trypsin substrate for cleavage at the level of Arg_{18}–Ile_{19}; only from II which predominates at acidic pH (Figure 1) can be split by trypsin at this bond. For these reasons we tend not to favor the idea of acyl-enzyme formation between trypsin and its pancreatic secretory inhibitor around pH 7. Formation of a tetrahedral adduct necessitates no cleavage of the Arg_{18}–Ile_{19} bond; it represents therefore a more probable type of interaction. Recent crystallographic data obtained by Rühlmann *et al.*[3] strengthen this interpretation. The crystal structure analysis of the trypsin–PTI complex at 2.8-Å resolution reveals that the complex is a tetrahedral adduct with a 2.0-Å bond between the carbonyl carbon of Lys_{15} of the inhibitor and the γ-oxygen of Ser_{183} of the enzyme. The peptide bond between Lys_{15} and Ala_{16} (PTI) is not cleaved in the complex.

Although there is no direct proof of it, conformational changes seem to occur in the association of trypsin with PSTI. This is suggested by the very low value of the second-order rate constant of association measured at alkaline pH. This value is at least two orders of magnitude lower than would be expected for a diffusion-controlled process (Benson, 1960; Haynes and Feeney, 1968). The activation energy for the association is also too high, 8.6 kcal mol^{-1}, as compared to 3 kcal mol^{-1} for a classical diffusion step (Benson, 1960). The interaction of trypsin with PSTI to form the inactive complex is likely to occur in at least two steps: $E + I \rightleftarrows$

[3] A. Rühlmann, D. Kukla, P. Schwager, K. Bartels, and R. Huber, submitted for publication.

EI* $\rightleftharpoons$ EI with a rapid second-order absorption of the inhibitor to the enzyme complementary area (E + I $\rightleftharpoons$ EI*), followed by a slow first-order rearrangement of the complex, EI* $\rightleftharpoons$ EI. Similar proposals have been already presented for the interaction of trypsin with ovomucoid and lima bean inhibitors (Haynes and Feeney, 1968). The slow first-order rate of dissociation of the trypsin–PSTI complex might well be related to the conformational change EI $\rightleftharpoons$ EI*. Thermodynamic data for the association of PSTI with trypsin indicate that complex formation is essentially entropy driven. The large value of $\Delta S°_a$ (43.6 cal mol^{-1} deg^{-1}) is probably related, in part, to expulsion of bound water molecules during complex formation, but it may well be indicative also of structural changes affecting the effector and/or receptor conformations within the complex.

Low values both for the second-order rate of association and the first-order rate of dissociation have been found with other systems involving interaction between a mini-protein effector and a protein receptor of higher molecular weight. The association constant of insulin (mol wt 6,000) with its membrane-bound receptor is about 10^{10} M^{-1}. The rate of association, k_a = 2–3 × 10^6 M^{-1} sec^{-1} and the rate of dissociation, k_d = 4 × 10^{-4} sec^{-1} (Cuatrecasas, 1972), are very similar to the rates presented in this paper. The association constant of snake (*Naja naja*) neurotoxin (mol wt 7000) with the membrane-bound acetylcholine receptor of the electric organ of *Electrophorus electricus* is 2.3 × 10^9 M^{-1}; the rate of association is 1.7 × 10^5 M^{-1} sec^{-1} and the rate of dissociation is 7.2 × 10^{-5} sec^{-1} (Fulpius *et al.*, 1972). Again, these values are of the same order of magnitude as those found with our system. Formation of the neurotoxin–acetylcholine receptor complex is also entropy driven; the entropy change at 25° is 74 eu (Fulpius *et al.*, 1972). At least in the case of the interaction of insulin with its receptor, it is reasonable to assume that some kind of conformational change occurs and is transmitted to membrane structure to allow glucose permeation.

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