The Association of Anhydrotrypsin with the Pancreatic Trypsin Inhibitors†

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ABSTRACT: The dynamic and thermodynamic properties of the complexes formed between anhydrotrypsin and the pancreatic trypsin inhibitors (Kunitz and Kazal) have been studied at different pH. They have shown that dehydration of Ser_{183} in the active site of trypsin hardly affects the binding properties of the enzyme to inhibitors. For example, at pH 8.0, 25°, the dissociation constant of the anhydrotrypsin–Kunitz inhibitor complex is 1.1×10^{-13} M as compared to 6.0×10^{-14} M for the trypsin–Kunitz inhibitor complex, and the dissociation constant of the anhydrotrypsin–Kazal inhibitor complex is 3.4×10^{-10} M as compared to 3.3×10^{-11} M for the trypsin–Kazal inhibitor complex. The same observations were made by comparing the binding properties of trypsin and anhydrotrypsin to derivatives of the Kunitz inhibitor modified on the Cys_{14}–Cys_{38} bridge. The conclusion of the work is that the tetrahedral adduct which forms between the enzyme and inhibitor active sites in the complex does not make an important contribution to the stabilization of the enzyme–inhibitor complex. The unusual stability of the trypsin–Kunitz inhibitor complex, which is mainly the result of a very low rate of dissociation ($t_{1/2} \simeq 17$ weeks) is only due to noncovalent interactions between the enzyme and inhibitor partners.

Because of their interest as models of heterologous protein–protein interactions, associations of trypsin with protein trypsin inhibitors have been the object of extensive investigations during the last years both from the part of biochemists and from that of crystallographers. Two systems have been particularly well studied: the association of trypsin with soybean trypsin inhibitor (for references see Finkenstadt *et al.*, 1974, Sweet *et al.*, 1974) and the association of trypsin with the pancreatic trypsin inhibitor[1] discovered by Kunitz (for references see Lazdunski *et al.*, 1974; Rühlmann *et al.*, 1974).

The three-dimensional structure of the basic pancreatic inhibitor (Kunitz) is now known with a resolution of 1.5 Å (Deisenhofer and Steigemann, 1974); that of the trypsin–PTI complex is known with a resolution of 2.8 Å (Rühlmann *et al.*, 1973). This complex is extremely stable; its dissociation constant is 6×10^{-14} M and the half-life measured for its dissociation is about 17 weeks at pH 8.0, 25° (Vincent and Lazdunski, 1972).

X-Ray analysis of the crystalline trypsin–PTI complex has shown that assembly of the partners was essentially stabilized by (i) 7 hydrogen bonds and about 200 van der Waals contacts, (ii) the formation of a salt bridge between the ϵ-ammonium of Lys_{15} in PTI and the β-carboxylate of Asp_{177}, the essential residue in the specificity site of the enzyme, (iii) the formation of a covalent bond which is part of a tetrahedral adduct between the -OH function of Ser_{183}, one of the essential residues in the catalytic site of trypsin, and the carbonyl carbon of Lys_{15} in PTI (Rühlmann *et al.*, 1973).

The work presented in this paper was carried out to evaluate how the formation of the tetrahedral adduct affects the stability and the kinetics of association and dissociation of the trypsin–PTI complex. This was done by measuring the association characteristics of anhydrotrypsin (Ako *et al.*, 1972) and PTI. A number of chemically modified derivatives of PTI drastically alter the binding properties to trypsin (Vincent and Lazdunski, 1972; Vincent *et al.*, 1974). For example, reduction of the Cys_{14}–Cys_{38} bridge in PTI (R★PTI) or reduction and carboxamidomethylation (RCAM★PTI) considerably decrease the strength of the interaction of the inhibitor with trypsin. We also present in this paper a comparison of the kinetic and thermodynamic properties of the trypsin–R★PTI and the anhydrotrypsin–R★PTI complexes on one hand and of the trypsin–RCAM★PTI and anhydrotrypsin–RCAM★PTI complexes on the other hand.

The acinar cell of the pancreas synthesizes two kinds of trypsin inhibitor. The first one, the Kunitz inhibitor (PTI), remains in the pancreatic cell whereas the Kazal inhibitor (PSTI) is secreted with all the zymogens into the pancreatic juice. The system trypsin–PSTI is well characterized; the active site is identified in the sequence of PSTI (Greene and Bartelt, 1969; Greene and Guy, 1971; Rigbi and Greene, 1968) and the dynamic and thermodynamic properties of the interaction have been elucidated (Schweitz *et al.*, 1973). A comparison of the trypsin–PSTI and anhydrotrypsin–PSTI complexes is also presented.

The idea of the use of anhydroproteases to demonstrate that a covalent bond of an acyl-enzyme type is unimportant for stabilization of serine proteases–protein inhibitor complexes was first proposed by Foster and Ryan (1965) then by Feinstein and Feeney (1966). The association of anhydrotrypsin with other types of inhibitors has been studied independently by Ako *et al.* (1972, 1974).

Experimental Section

(*a*) *Materials.* The bovine pancreatic trypsin inhibitors (Kunitz and Kazal) were gifts from Choay Laboratories. Both

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[1] Abbreviations used are: PSTI, pancreatic secretory trypsin inhibitor (Kazal inhibitor); PTI, basic pancreatic trypsin inhibitor (Kunitz inhibitor); R★PTI, basic pancreatic trypsin inhibitor selectively reduced at S-S 14–38; RCAM★PTI, carboxamidomethylated R★PTI; Bz-L-ArgOEt, α-N-benzoyl-L-arginine ethyl ester, $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride.

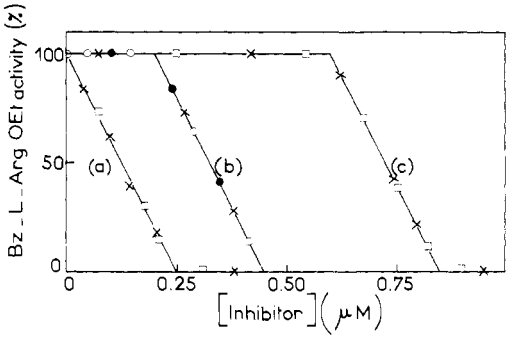

FIGURE 1: Stoichiometric association of trypsin or anhydrotrypsin to PSTI, PTI, R★PTI, or RCAM★PTI: (a) inhibition of trypsin (c = 0.25 μM) by PTI (×) and by PSTI (□); (b) interaction between anhydrotrypsin (c = 0.20 μM) and PTI (×), R★PTI (○), and RCAM★PTI (●) followed by back titration with trypsin (c = 0.25 μM); (c) interaction between anhydrotrypsin (c = 0.60 μM) and PTI (×) and PSTI (□) followed by back titration with trypsin (c = 0.25 μM). 25°, pH 8.0, 0.2 M NaCl.

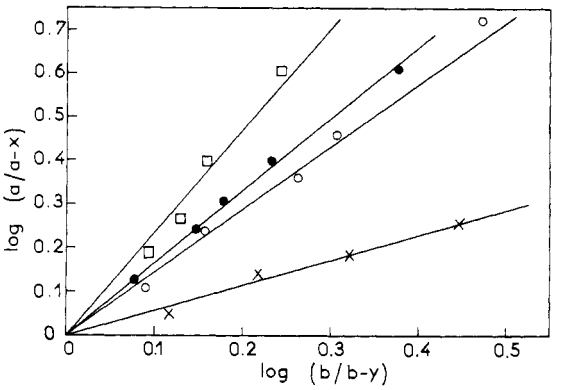

FIGURE 2: Analysis of the association of anhydrotrypsin with PTI, PSTI, R★PTI, or RCAM★PTI at 25°, pH 8.0. Competition between anhydrotrypsin and trypsin for PTI (○), PSTI (●), R★PTI (×), or RCAM★PTI (□). Incubation time is 2 min (○, ●) or 5 min (×, □). Data were treated according to eq 1.

inhibitors were pure as judged by polyacrylamide gel electrophoresis, analytical centrifugation, and stoichiometry of the inhibition with trypsin. Commercial bovine trypsin (Worthington) was purified before use by affinity chromatography in a PTI–Sepharose column as previously described (Vincent *et al.*, 1974). The specific activity of the purified enzyme was 50–51 Bz-L-ArgOEt units/mg of protein at 25°, pH 8.0. Bz-L-ArgOEt is a Sigma product, [^{14}C]iodoacetamide was obtained from the Radiochemical Centre (Amersham).

(*b*) *Preparation and Purification of Anhydrotrypsin.* Anhydrotrypsin was prepared and purified by $PhCH_2SO_2F$ treatment of trypsin, base elimination of $PhCH_2SO_2$ from the $PhCH_2SO_2$-trypsin derivative, and chromatography in a soybean trypsin inhibitor–Sepharose column as previously described (Ako *et al.*, 1972). Purified anhydrotrypsin was almost completely devoid of activity toward Bz-L-ArgOEt (less than 0.2% as compared with native trypsin). The purity of the anhydrotrypsin material is demonstrated by stoichiometric titrations with PTI and PSTI (see Results). The homogeneity of the anhydroenzyme is also indicated by the simple behavior observed in association and dissociation kinetics with both PTI (or its derivatives) and PSTI (see Results). A heterogeneous preparation of anhydrotrypsin would not be expected to give pure second-order processes of association or pure first-order processes of dissociation. The physicochemical properties of anhydrotrypsin prepared according to Ako *et al.* (1972) have been described very recently (Ako *et al.*, 1974).

(*c*) *Preparation of PTI Derivatives.* The disulfide bridge Cys_{14}–Cys_{38} in PTI was selectively reduced with sodium borohydride (Kress and Laskowski, 1967), RCAM★PTI was obtained by reacting R★PTI with iodoacetamide (Kress *et al.*, 1968; Vincent *et al.*, 1971; Vincent and Lazdunski, 1972).

(*d*) *Stoichiometries.* The stoichiometry of association between anhydrotrypsin and PSTI, PTI, or its derivatives was evaluated by back titration with trypsin of inhibitor molecules not associated with anhydrotrypsin. Anhydrotrypsin (0.2–0.6 μM) was incubated at 25°, pH 8.0, with various concentrations of inhibitor. The association was allowed to proceed during 2 min (this incubation time is largely sufficient for maximal association as will be seen later). Trypsin (0.25 μM) was then added in the incubation mixture and allowed to react during 2 more min with the remaining free inhibitor. The residual activity of trypsin was then measured at 25°, pH 8.0, in a Radiometer pH-Stat with Bz-L-ArgOEt (3 mM) as substrate. This procedure allowed an easy estimation of the fraction of inhibitor which has been associated with anhydrotrypsin during the first 2-min period. Dissociation of the anhydrotrypsin–inhibitor complexes by active trypsin was negligible during the time required to complete the whole experiment.

(*e*) *Association Kinetics.* Kinetics of association of anhydrotrypsin with PSTI, PTI, or its derivatives were followed by competition with trypsin. This technique has already been described for association kinetics of pseudotrypsin and PTI (Vincent and Lazdunski, 1972). Trypsin (0.25 μM) and anhydrotrypsin (0.2–2.4 μM) were incubated together at 25°, pH 8.0, in a Tris buffer (1 mM) containing 0.2 M NaCl. The reaction was started by adding the inhibitor (0.2–2.5 μM). Residual trypsin activity was evaluated after 2 min (PSTI or PTI) or 5 min (R★PTI or RCAM★PTI) using the pH-Stat technique with Bz-L-ArgOEt (3 mM) as substrate.

(*f*) *Dissociation Kinetics.* Kinetics of dissociation were followed by displacement of anhydrotrypsin from the anhydrotrypsin–inhibitor complexes by trypsin. The method has already been described for the dissociation kinetics of the pseudotrypsin–PTI and chymotrypsin–PTI complexes (Vincent and Lazdunski, 1972, 1973). The anhydrotrypsin–inhibitor complexes were first prepared by mixing anhydrotrypsin and the inhibitor (PSTI, R★PTI, or RCAM★PTI) at equimolecular concentration (0.2–15 μM) at 25°, pH 8.0. The displacement was started by adding trypsin (final concentration identical with that of the complex). Aliquots were taken at different times and used to determine the amount of free trypsin. The replacement of anhydrotrypsin by trypsin within the anhydrotrypsin–inhibitor complex was followed by measuring the loss of Bz-L-ArgOEt activity in the pH-Stat (25°, pH 8.0, 0.2 M NaCl, 3 mM Bz-L-ArgOEt).

(*g*) *Evaluation of Dissociation Constants from Equilibrium Measurements.* (i) THE ANHYDROTRYPSIN–PTI COMPLEX. The technique has been used previously to determine the dissociation constant of the trypsin–PTI complex (Vincent and Lazdunski, 1972). Anhydrotrypsin (15 μM) was first incubated in a Tris buffer (50 mM) at 25°, pH 8.0, in the presence of 50 mM $CaCl_2$–0.1 M NaCl with a 100 molar excess of [^{14}C]RCAM★PTI. After 15 min of incubation, when association was complete, PTI was added to the mixture to a final concentration of 15 μM (*i.e.*, 100 times more dilute than RCAM★PTI). The displacement of RCAM★PTI by PTI was followed by chromatography of aliquots in a Sephadex G-75 column (3 × 85 cm) equilibrated at pH 8.0 with the Tris buffer just described. This technique separates the liberated inhibitor from the complex. The radioactivity was measured under both

TABLE I: Comparison between the Kinetic and Thermodynamic Characteristics of the Interaction of Trypsin and Anhydrotrypsin with PSTI, PTI, and Its Derivatives.[a]

Enzyme	Inhibitor	k_a (M^{-1} sec^{-1})	k_d (sec^{-1})	($t_{1/2}$)	K_d (M)	$\Delta G°_a$ (kcal mol^{-1})
Trypsin[b]	PTI	1.1×10^6	6.6×10^{-8}	(17 weeks)	6.0×10^{-14}	−18.1
Anhydrotrypsin		7.7×10^5	8.5×10^{-8}	(13 weeks)	1.1×10^{-13}	−17.8
Trypsin[c]	PSTI	6.8×10^6	2.2×10^{-4}	(50 min)	3.3×10^{-11}	−14.4
Anhydrotrypsin		4.0×10^6	1.4×10^{-3}	(8.5 min)	3.4×10^{-10}	−13.0
Trypsin[b]	R★PTI	3.2×10^5	5.7×10^{-4}	(20 min)	1.8×10^{-9}	−12.0
Anhydrotrypsin		5.5×10^5	2.2×10^{-3}	(5 min)	4.0×10^{-9}	−11.5
Trypsin[b]	RCAM★PTI	1.3×10^5	2.2×10^{-5}	(520 min)	1.7×10^{-10}	−13.4
Anhydrotrypsin		5.6×10^4	1.6×10^{-4}	(70 min)	2.9×10^{-9}	−11.7

[a] k_a and k_d are the rate constants for association and dissociation, respectively; $t_{1/2}$ is the half-life of the complex; K_d is the dissociation constant; $\Delta G°_a$ is the standard free energy of association. 25°, pH 8.0. [b] From Vincent and Lazdunski, 1972. [c] From Schweitz *et al.* (1973).

peaks by scintillation counting in a Packard TriCarb spectrometer, Model 3375. The knowledge of the dissociation constant of the anhydrotrypsin-RCAM★PTI complex and the measurement of the amount of the radioactive RCAM★PTI which remained bound to anhydrotrypsin when equilibrium was attained allowed an evaluation of the dissociation constant for the anhydrotrypsin-PTI complex.

(ii) THE ANHYDROTRYPSIN-R★PTI COMPLEX. The stability of the anhydrotrypsin-R★PTI complex in the presence of active trypsin was compared with the stability of the native enzyme-inhibitor complex in the presence of anhydrotrypsin. Anhydrotrypsin was displaced from the anhydrotrypsin-R★PTI complex (15 μM) by trypsin (15 μM) as described in (f). Conversely, trypsin was displaced from the trypsin-R★PTI complex by anhydrotrypsin under the same experimental conditions. The competition between trypsin and anhydrotrypsin for R★PTI was followed by measurement of enzymatic activity until equilibrium was attained. Knowledge of the dissociation constant for the trypsin-R★PTI complex (Vincent and Lazdunski, 1972) allowed an estimation of the dissociation constant for the anhydrotrypsin-R★PTI complex

Results

(*a*) *Stoichiometries of Association.* Figure 1 shows that both trypsin and anhydrotrypsin associate stoichiometrically, in a 1:1 ratio, with PSTI, PTI, R★PTI, or RCAM★PTI. This property is the best criterion for characterizing preparations of purified anhydroenzymes (Ako *et al.*, 1974).

(*b*) *Kinetics of Complex Formation.* Association kinetics between anhydrotrypsin and the inhibitors were followed by competition experiments involving anhydrotrypsin, trypsin, and each one of the inhibitors, native or modified. The system has been previously used and described in detail for the determination of the association kinetics between pseudotrypsin and PTI (Vincent and Lazdunski, 1972). The competition can be schematized as

$$\begin{array}{rcl} & + \underset{(a-x)}{\text{Trp}} & \xrightarrow{k_a} \underset{(x)}{\text{Trp–I}} \\ \underset{(c-x-y)}{\text{I}} & & \\ & + \underset{(b-y)}{\text{AnhTrp}} & \xrightarrow{k_a'} \underset{(y)}{\text{AnhTrp–I}} \end{array}$$

where I is for inhibitor (PSTI, PTI, R★PTI, or RCAM★PTI), Trp for trypsin, AnhTrp for anhydrotrypsin; *a*, *b*, and *c* are the initial concentrations of trypsin, anhydrotrypsin, and inhibitor; x and y are the concentrations of complexes with

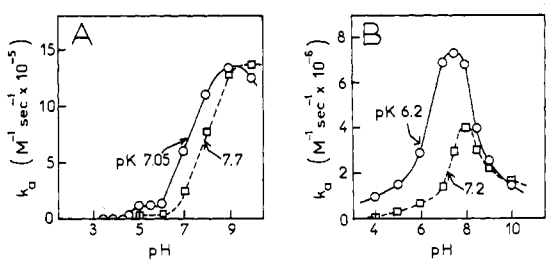

FIGURE 3: A comparison of the pH dependence of the second-order rate constants of association of trypsin and anhydrotrypsin with PTI (A) and PSTI (B): (——) trypsin-PTI and trypsin-PSTI complexes; (- - -) anhydrotrypsin-PTI and anhydrotrypsin-PSTI complexes. 25°, 0.2 M NaCl.

trypsin and anhydrotrypsin at a given time of the association; k_a and k_a' are the second-order rate constants of association of the inhibitor with trypsin and anhydrotrypsin, respectively. It has been previously stated that the system obeys the following relationship (eq 1) (Vincent and Lazdunski, 1972). The time of

$$k_a/k_a' = \log [a/(a-x)]/\log [b/(b-y)] \quad (1)$$

incubation of the mixture trypsin-anhydrotrypsin-inhibitor should be sufficiently long for complex formation of all inhibitor molecules with either trypsin or anhydrotrypsin. It should be sufficiently short to prevent any displacement by free trypsin of anhydrotrypsin in the anhydrotrypsin-PTI complex. The incubation time was chosen as follows: 2 min for competition kinetics involving PTI or PSTI as inhibitors, and 5 min when the inhibitor was R★PTI or RCAM★PTI. Values of x and y were evaluated from measurement of the residual trypsin activity after 2 (or 5) min. Figure 2 shows that each one of the graphs log $[a/(a-x)]$ *vs.* log $[b/(b-y)]$ obtained with the different inhibitors is perfectly linear as expected from eq 1. Equation 1 can be used to evaluate k_a/k_a' and then k_a' since k_a is already known (Vincent and Lazdunski, 1972; Schweitz *et al.*, 1973). Such values are compiled in Table I. The k_a/k_a' ratios range between 0.58 and 2.3 indicating that the kinetic characteristics for the association of trypsin and anhydrotrypsin with PSTI, PTI, or its derivatives are very similar.

Figure 3 presents a comparison of the pH dependence of the rates of association of trypsin and anhydrotrypsin with PTI (Figure 3A) and PSTI (Figure 3B). The first observation is that replacement of trypsin by anhydrotrypsin does not drastically modify the aspect of the k_a–pH profiles. Figure 3A shows that maximal values of k_a for the association between trypsin

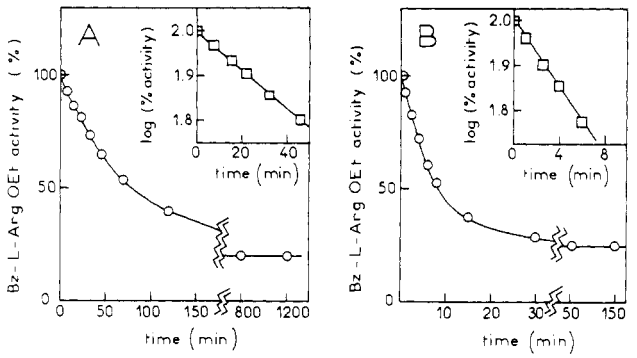

FIGURE 4: Dissociation kinetics of the complexes of anhydrotrypsin with RCAM★PTI and PSTI. Anhydrotrypsin was displaced from the anhydrotrypsin-RCAM★PTI complex (A) and from the anhydrotrypsin-PSTI complex (B) by an equimolecular concentration of trypsin, 25°, pH 8.0, 0.2 M NaCl: (○) time course of the dissociation; (□) pseudo-first-order representation of the data obtained during the initial part of the displacement (45 min in A and 6 min in B) gave the initial rate of the displacement.

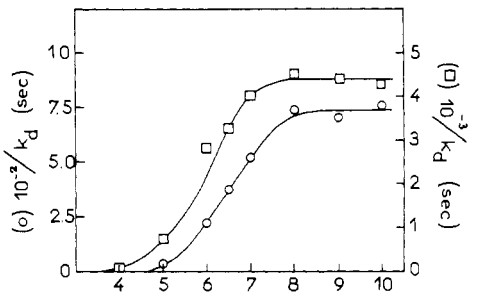

FIGURE 5: A comparison of the pH dependence of the first-order rate constant of dissociation of the trypsin-PSTI complex (□) and of the anhydrotrypsin-PSTI complex (○); 25°, 0.2 M NaCl.

or anhydrotrypsin and PTI are identical: $k_a = 1.4 \times 10^6$ M^{-1} sec^{-1}. Both association processes are dependent upon the ionization of a group, which shall be in the basic form for complex formation. The apparent pK of this essential function is 7.05 for the association between trypsin and PTI; it has been tentatively identified as the imidazole side chain of His_{46} in the catalytic center of trypsin (Vincent and Lazdunski, 1972). In the case of the anhydrotrypsin-PTI complex the apparent pK is 7.7 instead of 7.05. Such a variation is to be expected if these pK values correspond to the ionization of the imidazole side chain of His_{46} in the active site of the enzyme. The histidine side chain forms a hydrogen bond with Ser_{183} in native trypsin; this bond is part of the Asp_{90}–His_{46}–Ser_{183} charge relay system (Shotton, 1971; Blow *et al.*, 1969); the His_{46}–Ser_{183} hydrogen bond is no longer possible after transformation of Ser_{183} into dehydroalanine. There is a plateau region between pH 5 and 6 in the k_a-pH profile obtained for the trypsin-PTI association. This plateau persists when trypsin is replaced by anhydrotrypsin; its value is 4.0×10^4 M^{-1} sec^{-1} for the anhydrotrypsin-PTI complex instead of 1.2×10^5 M^{-1} sec^{-1} for the trypsin-PTI complex.

The k_a-pH profiles obtained for the association of PSTI with trypsin and anhydrotrypsin are very similar (Figure 3B). The pK value of 6.2 observed for the trypsin-PSTI association has also been attributed in a previous paper (Schweitz *et al.*, 1973) to the imidazole side chain of His_{46}. This pK is again shifted to the higher value of 7.2 for the anhydrotrypsin-PSTI association. The variation of k_a with pH between pH 8 and 10 both for the trypsin-PSTI and the anhydrotrypsin-PSTI association is controlled by a group with an apparent pK of 8.5–9. This ionizable group has not yet been identified. The maximal value of k_a is only decreased by a factor of 1.8 at the optimum of the k_a-pH curve when anhydrotrypsin replaces trypsin in the complex.

(*c*) *Kinetics of Complex Dissociation.* Anhydrotrypsin (AnhTrp) can be displaced from its association with native or modified inhibitors (I) by native trypsin (Trp). The dissociation process can be schematized as

$$\text{AnhTrp–I} \underset{k_a'}{\overset{k_d'}{\rightleftharpoons}} \text{AnhTrp} + \text{I} \xrightarrow[\text{Trp}]{k_a} \text{Trp–I}$$

Since anhydrotrypsin is devoid of catalytic activity, the displacement can be followed by measuring the loss of Bz-L-ArgOEt activity. Figure 4 presents typical dissociation kinetics of anhydrotrypsin–inhibitor complexes. When active trypsin is added in an equimolecular amount to the anhydrotrypsin-RCAM★PTI complex (Figure 4A), the system evolves toward an equilibrium position in which 80% of the initially complexed anhydrotrypsin is liberated from its interaction with RCAM★PTI and replaced by native trypsin in the complex. The half-life of the displacement in that case is about 50 min. The association of trypsin with RCAM★PTI being much more rapid than the displacement observed in Figure 4A (Vincent and Lazdunski, 1972), the initial rate of this displacement gives an easy evaluation of the first-order rate constant, k_d', for the dissociation of the anhydrotrypsin-RCAM★PTI complex (insert of Figure 4A). $k_d' = 1.6 \times 10^{-4}$ sec^{-1} at pH 8.0 and 25°.

The same technique has been applied to the anhydrotrypsin-PSTI complex (Figure 4B). In that case the equilibrium position is attained when 75% of anhydrotrypsin in the initial complex has been replaced by trypsin. $k_d' = 1.4 \times 10^{-3}$ sec^{-1} at pH 8.0 and 25°. These values of first-order dissociation rate constants k_d' for anhydrotrypsin–inhibitor complexes are compared in Table I to k_d values for the corresponding trypsin–inhibitor complexes.

The pH-dependence of $1/k_d'$ for the anhydrotrypsin-PSTI complex is compared in Figure 5 to the pH dependence of $1/k_d$ for the trypsin-PSTI complex. The profiles are very similar. For both types of complexes, the first-order rates of dissociation are invariant between pH 8 and 10 and increase considerably when the pH becomes more acidic. The plateaus correspond to k_d' and k_d values of 1.4×10^{-3} and 2.2×10^{-4} sec^{-1}, respectively. Replacement of trypsin by anhydrotrypsin shifts the pH profiles toward alkaline pH.

(*d*) *Determination of Dissociation Constants of Complexes Formed between Anhydrotrypsin and PSTI, and Anhydrotrypsin and PTI or Its Chemically Modified Derivatives.* (i) THE USE OF KINETIC DATA: Dissociation constants can be evaluated easily when the rate constants for both associations, k_a', and dissociation, k_d', are known, $K_d' = k_d'/k_a'$. Such values are reported in Table I.

(ii) THE DISSOCIATION CONSTANT OF THE ANHYDROTRYPSIN-PTI COMPLEX. To determine this dissociation constant, competition for anhydrotrypsin between PTI and RCAM★PTI was used to obtain the ratio K_d'/K_{d^*}' where K_d' represents the dissociation constant of the anhydrotrypsin-PTI complex and K_{d^*}' the dissociation constant for the anhydrotrypsin-RCAM★PTI complex. From this ratio and the value of K_{d^*}' in Table I, K_d' could be easily calculated (Vincent and Lazdunski, 1972).

Anhydrotrypsin (15 μM) was first incubated at pH 8.0 and 25° with a 100-fold molar excess of radioactive RCAM★PTI (1.5 mM). Enough time was allowed for complete association of the partners and after 15 min an amount of PTI equal to

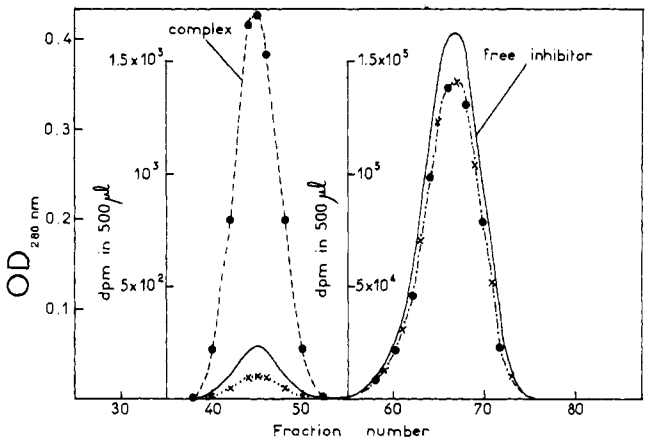

FIGURE 6: A competition experiment for the evaluation of the dissociation constant of the anhydrotrypsin-PTI complex. Anhydrotrypsin (15 µM) was first incubated at 25°, pH 8.0, with a 100 molar excess of [^{14}C]RCAM★PTI. Association was completed after 15 min. At that time, dissociation of RCAM★PTI from the anhydrotrypsin-RCAM★PTI complex was started by adding PTI at a final concentration of 15 µM. Aliquots were taken at different times and chromatographed as described in the Experimental Section. As shown in this figure, equilibrium was attained after 5 days: (——) optical density profile measured at 280 nm; this profile is not time dependent; (- - -) (●) radioactivity elution pattern at zero time, (×) radioactivity elution pattern after 5 and 8 days. Because of the great excess of [^{14}C]RCAM★PTI the amount of radioactivity under the peak of free inhibitor hardly varied (less than 1%) during the displacement.

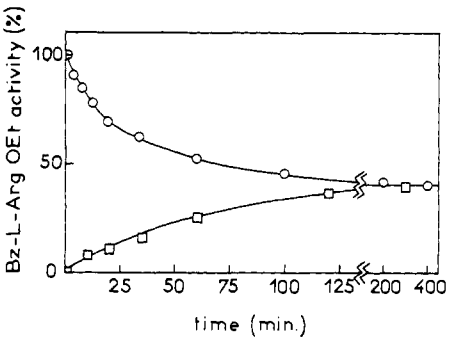

FIGURE 7: A competition experiment for the evaluation of the dissociation constant of the anhydrotrypsin-R★PTI complex: (○) time course of the displacement of anhydrotrypsin from the anhydrotrypsin-R★PTI complex by an equimolecular concentration of trypsin; (□) time course of the displacement of trypsin from the trypsin-R★PTI complex by an equimolecular concentration of anhydrotrypsin. 25°, pH 8.0, 0.2 M NaCl.

that of anhydrotrypsin (15 µM) was added to displace the modified inhibitor. From the chromatographic profiles of Figure 6 it was possible to determine the concentration of anhydrotrypsin-PTI and anhydrotrypsin-RCAM★PTI after 5 and 8 days. Equilibrium was attained after 5 days. The concentration of RCAM★PTI (which is the initial concentration) and that of free PTI are also easily determined. Since $K_d'/K_{d*}' = 3.8 \times$

$$\frac{K_d'}{K_{d*}'} = \frac{[\text{PTI}]}{[\text{RCAM}\bigstar\text{PTI}]} \frac{[\text{AnhTrp}-\text{RCAM}\bigstar\text{PTI}]}{[\text{AnhTrp}-\text{PTI}]}$$

10^{-5} and $K_{d*}' = 2.9 \times 10^{-9}$ M, $K_d' = 1.1 \times 10^{-13}$ M. When K_d' and k_a' are known, k_d' could be easily calculated. Its value is 8.5×10^{-8} sec^{-1}.

(iii) THE DISSOCIATION CONSTANTS OF THE COMPLEXES FORMED BETWEEN ANHYDROTRYPSIN AND R★PTI, RCAM★PTI, OR PSTI. The evaluation of the equilibrium constant corresponding to the dissociation of the anhydrotrypsin-R★PTI complex stems from the data presented in Figure 7. As expected, the displacement of anhydrotrypsin from the anhydrotrypsin-R★PTI complex by an equimolecular concentration of trypsin gives the same equilibrium position as that obtained when trypsin is displaced from the trypsin-R★PTI complex by equimolecular amounts of anhydrotrypsin. The equilibrium position corresponds to 60% of free anhydrotrypsin and 40% of free trypsin. These data allow an estimation of the ratio K_d/K_d' where K_d and K_d' represent dissociation constants of the trypsin-R★PTI and anhydrotrypsin-R★PTI complexes, respectively. Since $K_d/K_d' = 0.45$ and $K_d = 1.8 \times$

$$\frac{K_d}{K_d'} = \frac{[\text{Trp}]}{[\text{AnhTrp}]} \frac{[\text{AnhTrp}-\text{R}\bigstar\text{PTI}]}{[\text{Trp}-\text{R}\bigstar\text{PTI}]}$$

10^{-9} M (Vincent and Lazdunski, 1972), $K_d' = 4.0 \times 10^{-9}$ M. K_d' and k_a' being known, k_d' can now be evaluated, $k_d' = 2.2 \times 10^{-3}$ sec^{-1} (Table I).

Similar experiments have already been presented in Figure 4 for the anhydrotrypsin-RCAM★PTI and anhydrotrypsin-PSTI complexes. The equilibrium positions and the already reported values of K_d (the dissociation constant for the corresponding complex with trypsin) (Vincent and Lazdunski, 1972; Schweitz *et al.*, 1973) permits an evaluation of K_d' values which are in very good agreement with values already calculated from ratios of the rate constants for the association and dissociation processes (Table I and paragraph d (i)). The values found from equilibrium positions are $K_d' = 2.7 \times 10^{-9}$ M for the anhydrotrypsin-RCAM★PTI complex and 3.0×10^{-10} M for the anhydrotrypsin-PSTI complex.

Discussion

The presently favored structure for the association of trypsin with PTI is one in which enzymatic catalysis is involved in inhibitor action. The work of Huber and his colleagues (Rühlmann *et al.*, 1973, 1974) has shown that a tetrahedral adduct is formed between the Oγ oxygen atom of Ser_{183} in the enzyme and the carbonyl carbon of Lys_{15} in the inhibitor. A tetrahedral adduct or an acyl-enzyme bond (Blow *et al.*, 1972) obviously cannot be formed between anhydrotrypsin and trypsin inhibitors. However, it is clear from the results presented in Table I that transformation of Ser_{183} into dehydroalanine in trypsin hardly affects complex formation with PTI or with PTI derivatives. Difference in standard free energy of association between trypsin-PTI and anhydrotrypsin-PTI complexes is only 0.3 kcal mol^{-1} (Table I). One also observes a negligible difference in free energy of interaction by comparing the stabilities of the trypsin-R★PTI and anhydrotrypsin-R★PTI complexes. A significant although small effect of the dehydration of Ser_{183} is only observed with RCAM★PTI complexes.

The important conclusion of this work is that the elimination of the alcoholic side chain of Ser_{183}, the side chain most often invoked as essential, or at least important, in the interaction of the trypsin inhibitors with trypsin has hardly any effect on the stability of the trypsin-PTI complex. In contrast with that situation, chemical modifications of other residues which are in the contact area between the trypsin and the PTI partners, such as Lys_{15} (PTI), Asp_{177} (trypsin), Cys_{14}-Cys_{38} (PTI), or Cys_{179}-Cys_{203} (trypsin) (Chauvet and Acher, 1967; Kress and Laskowski, 1968; Fritz *et al.*, 1969; Huber *et al.*, 1972; Vincent and Lazdunski, 1972), have very drastic effects on the

complex stability. A good example is that of the pseudotrypsin–PTI interaction. Autolytic cleavage of the Lys_{176}–Asp_{177} bond in trypsin leads to the formation of pseudotrypsin, a trypsin derivative in which the carboxylate side chain has been disconnected from the specificity site (Smith and Shaw, 1969). Pseudotrypsin still forms a 1:1 complex with PTI but the dissociation constant in that case has been considerably altered; it is 1.5×10^5 times higher that that of the native trypsin–PTI association (Vincent and Lazdunski, 1972). This variation of the dissociation constant represents a decrease of 7.1 kcal mol^{-1} of the standard free energy of interaction after chemical modification of the specificity site of trypsin. Selective guanidination of Lys_{15} into homoarginine, although it preserves the positive charge of this essential side chain and the possibility of forming a salt-bridge with Asp_{177} (trypsin), also decreases the stability of the complex formed with trypsin (Vincent *et al.*, 1974). The ratio of the dissociation constants for the trypsin–guanidinated PTI and trypsin–PTI complexes is 800; it indicates a decrease of 4 kcal mol^{-1} of the standard free energy of interaction after guanidination of Lys_{15}. Finally, and again in contrast with dehydration of Ser_{183} in trypsin, chemical modifications of disulfide bridges Cys_{14}–Cys_{38} in PTI (a near neighbor of Lys_{15}), and of Cys_{179}–Cys_{203} in trypsin (a near neighbor of Asp_{177} and of Ser_{183}) also have very important effects on the stability of the inhibitor–enzyme complex. The data in Table I, for example, indicate that selective reduction of Cys_{14}–Cys_{38} in R★PTI decrease the stability of the complex with trypsin by a factor of 30,000, that is by 6.1 kcal mol^{-1}.

Substitution of trypsin by anhydrotrypsin decreases the stability of the complex formed with PSTI by a factor of 10, that is by 1.4 kcal mol^{-1}. Although the effect is larger than that observed with PTI, it shows that in that case also tetrahedral adduct formation hardly contributes to the stability of the complex with trypsin.

Besides crystallographic results, other data taken in recent years to suggest the importance of the formation of a covalent bond of the acyl-enzyme type between trypsin and its inhibitors included the consideration of the pH dependence of the association rates. k_a–pH profiles such as those shown in Figure 3 obviously present analogies with rate–pH profiles obtained both with synthetic substrates (Bender *et al.*, 1964) and with natural protein substrates (Abita *et al.*, 1969). This similarity was therefore very reasonably taken as an indication that His_{46} in the active site of trypsin was involved in catalysis of a covalent bond between the alcoholic side chain of Ser_{183} (trypsin) and the carbonyl carbon of Lys_{15} (PTI). The present data indicate that such an interpretation should be considered with some caution since the k_a–pH profiles observed for the trypsin–PTI or the trypsin–PSTI association are qualitatively similar to those observed with anhydrotrypsin.

The demonstration of the formation of a tetrahedral adduct between trypsin and PTI (Rühlmann *et al.*, 1973) provides a better knowledge of the type of complexes formed in the course of trypsin catalysis. However, it is clear from the data presented in this paper that such a covalent bond does not participate to the stability of the trypsin–PTI complex. A tetrahedral adduct is also formed in the trypsin–soybean trypsin inhibitor interaction (Finkenstadt *et al.*, 1974; Sweet *et al.*, 1974). It is of interest that Ako *et al.* (1974) have demonstrated independently that the binding of soybean trypsin inhibitor by anhydrotrypsin was nearly as strong as the binding of the inhibitor by trypsin. Competitions between trypsin and anhydrotrypsin for ovomucoid and lima bean inhibitor were also studied by Ako *et al.* (1974). Although trypsin association with both inhibitors is favored as compared to anhydrotrypsin association, the dissociation constants for the anhydrotrypsin–inhibitor complexes are only 40–130 times larger than the dissociation constants of the trypsin–inhibitor complexes at pH 8.0. These results were also interpreted to demonstrate that covalent bond formation involving Ser_{183} (trypsin) is not an essential requirement for tight binding in trypsin–inhibitors complexes. In fact, Ako *et al.* (1974) have even found that lima bean inhibitor was bound more strongly by anhydrochymotrypsin than by chymotrypsin.

Complex formation between protein inhibitors of trypsin and the enzyme is generally believed to be a multistep process (Haynes and Feeney, 1968; Laskowski *et al.*, 1971; Schweitz *et al.*, 1973). The analogies of k_a and k_d values observed at pH 8.0, 25°, for the trypsin–PTI and anhydrotrypsin–PTI associations (Table I) strongly suggest that the rate-limiting step in association and dissociation processes is the same before and after dehydration of Ser_{183} in the enzyme active site. The similarities between the pH dependence of k_a and k_d for trypsin and anhydrotrypsin–inhibitor complexes (Figures 3 and 5) strengthen this view.

Because of the close proximity between the Cys_{14}–Cys_{38} (PTI) and Cys_{179}–Cys_{203} (trypsin) bridges on one hand and of the essential recognition elements in trypsin and PTI on the other hand, because these bridges are the only ones easily reducible in both partners, and because of the drastic effects in stability observed after selective modification of these bridges, a disulfide interchange was also proposed as a possible mode of interaction between trypsin and PTI (Vincent and Lazdunski, 1972). Such a mechanism is now improbable since it does not appear to be consistent with the present X-ray crystallographic data of the trypsin–PTI complex (Rühlmann *et al.*, 1973, 1974).

The very high free energy of interaction between trypsin and the pancreatic trypsin inhibitors is mainly due to noncovalent bonds. Crystallographers have described 7 hydrogen bonds and about 200 van der Waal's contacts between trypsin and PTI (Blow *et al.*, 1972; Rühlmann *et al.*, 1973). It is clear now that the unusual properties of the trypsin–PTI complex are not due to covalent interactions. These unusual properties include the low dissociation constant, 6.0×10^{-14} M, the very slow first-order rate constant for its dissociation ($k_d = 6.6 \times 10^{-8}$ sec^{-1}, $t_{1/2}$ = 17 weeks) (Vincent and Lazdunski, 1972), and the resistance of the complex to dissociation in 8 M urea (Levilliers *et al.*, 1970) or in 6 M guanidine-HCl solutions (Lazdunski *et al.*, 1974) after 2 hr. It remains, however, to understand why this protein assembly is more stable than those formed for example in oligomeric enzymes in which subunits are generally associated in a symmetrical way and by a number of noncovalent bonds higher than that observed for the trypsin–PTI complex (Rühlmann *et al.*, 1973). In fact when monomers associate in a multisubunit enzyme, an important part of the energy of interaction due to stabilizing noncovalent interprotomers bonds is probably used up to compensate for the thermodynamically unfavorable entropic change due to the "freezing" of protomers, within the oligomer, in a conformation more folded and rigid than that they had in the nonassociated state. Such a "loss" of free energy in the form of conformational entropy would not occur in the case of a perfect fit between two rigid partners. This perfect or nearly perfect fit between rigid partners is probably what happens for the association of trypsin with PTI (and PSTI). Both partners have rigid structures in the nonassociated state, particularly in their active site region; this is due to extensive cross-linking by disulfide bridges (three disulfide bridges in PTI and PSTI and six disulfide bridges in bovine or porcine trypsin). Moreover, X-ray crystallography of the trypsin–

PTI complex (Rühlmann *et al.*, 1973) has shown that only small conformational changes occur during association.[2]

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[2] The very drastic effects on complex stability due to reduction and subsequent chemical modification of the Cys_{14}–Cys_{38} bond in PTI or of Cys_{179}–Cys_{203} in trypsin might then be simply due to a decrease of rigidity (*i.e.*, a change in conformational entropy which does not necessarily imply a change of conformation (Vincent *et al.*, 1971)) in the active site region of the inhibitor or enzyme partners.

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