Trypsin–Pancreatic Trypsin Inhibitor Association. Dynamics of the Interaction and Role of Disulfide Bridges†

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ABSTRACT: (1) The association between trypsin and the pancreatic trypsin inhibitor is unusually strong. The dissociation constant, K, of the 1:1 complex is 6×10^{-14} M at pH 8.0, 25°. The second-order rate constant for the association is 1.1×10^{6} M^{-1} sec^{-1}, and the first-order rate constant for the dissociation is 6.6×10^{-8} sec^{-1} corresponding to a half-life of about 17 weeks. (2) Temperature and pH dependencies of the second-order rate constant of association have been studied. (3) Disconnection of the specificity site of trypsin (Asp_{177}) from the catalytic site (His_{46} and Ser_{183}) in pseudotrypsin does not abolish association with the inhibitor but K increases to 9.0×10^{-9} M at pH 8.0, 25°. Ion-pair formation between the ϵ-ammonium of Lys_{15} in the active site of the inhibitor and the β-carboxylate of Asp_{177} in the specificity site of trypsin is not obligatory for association. (4) Particular attention has been given to the functional role of the Cys_{14}–Cys_{38} bridge adjacent to Lys_{15} in the inhibitor structure. This bridge, which is highly susceptible to reduction in native inhibitor is masked in the trypsin–inhibitor complex. Selective reduction of the bridge does not prevent association with trypsin but the dissociation constant of the resulting complex is $K = 1.8 \times 10^{-9}$ M, 3×10^{4} times higher than the dissociation constant of the trypsin–inhibitor complex. A number of derivatives were obtained by chemical modification of the thiol groups which appeared on reduction of the Cys_{14}–Cys_{38} bridge. In all cases, these derivatives contracted much looser associations with trypsin than native inhibitor. The integrity of the Cys_{14}–Cys_{38} bridge is essential for a very tight association with trypsin. A mechanism involving disulfide interchange between Cys_{14}–Cys_{38} (inhibitor) and Cys_{179}–Cys_{203} and/or Cys_{31}–Cys_{47} (trypsin) is postulated as a plausible interpretation of the data. The existence of such covalent interactions would be of general interest in effector–receptor associations.

Interactions between small ligands and proteins are well illustrated by associations between enzymes and their substrates. Dissociation constants of enzyme–substrate complexes range between 10^{-2}–10^{-3} M and 10^{-6}–10^{-7} M. As far as we are aware, the strongest interaction between a small ligand and a protein was observed for the association of estradiol to the estradiol-binding protein of calf uterine cytosol (Best-Belpomme *et al.*, 1970). The dissociation constant of the latter complex is of the order of 10^{-11} M.

Recognition of tRNAs by aminoacyl-tRNA synthetases is a well-studied example of protein–nucleic acid associations. Dissociation constants of these enzyme–substrates complexes are of the order of 10^{-8} M (see, for example, Rouget and Chapeville, 1971). The tightest interaction ever demonstrated for nucleic acids–protein associations was found in the *Lac*-operator–*Lac*-repressor complex. The dissociation constant of this complex is 10^{-13} M at 24°, pH 7.4 (Riggs *et al.*, 1970).

In spite of their importance in control systems, very few interactions between heterologous proteins or between peptides and proteins have been extensively studied. However, it is well known that many such associations are very tight. Protein and peptide hormones such as insulin (Cuatrecasas, 1971), the nervous growth hormone (Bewley *et al.*, 1969), or oxytocin (Jard *et al.*, 1960), for example, act at very low doses. This indicates very low dissociation constants (probably less than 10^{-10} M) for the complexes they form with their receptors.

The study of some effector–receptor systems has been undertaken in this laboratory. We report here the results obtained for the formation of the trypsin–pancreatic trypsin inhibitor (PTI)[1] complex. This association is one of the security devices which avoid accidental activation of trypsinogen in the pancreas. The dissociation constant of the trypsin–PTI complex was known to be lower than 10^{-10} M (Green and Work, 1953). Each of the partners in the trypsin–PTI complex is well characterized. Both their covalent and their three-dimensional structures in the crystalline state are available (Walsh and Neurath, 1964; Stroud *et al.*, 1971; Kassell and Laskowski, 1965; Chauvet *et al.*, 1964; Anderer and Hörnle, 1966; Huber *et al.*, 1970), and a considerable amount of work has been devoted to the analysis of the components of their active sites. The essential catalytic groups of trypsin are His_{46} and Ser_{183} (Neurath *et al.*, 1970). The essential element of the specificity site is Asp_{177} (Smith and Shaw, 1969; Steitz *et al.*, 1969; Eyl and Inagami, 1970). It can be disconnected from the catalytic site upon specific hydrolysis of the Lys_{176}–Asp_{177} bond. This transformation of trypsin into pseudotrypsin abolishes all specificity (Smith and Shaw, 1969).

The only known essential element in the active site of PTI is Lys_{15} (Chauvet and Acher, 1967; Kress and Laskowski, 1968; Fritz *et al.*, 1969).

Particular attention is given in this paper to the role of the disulfide bridge Cys_{14}–Cys_{38} which is adjacent to Lys_{15}, in the PTI sequence. This bridge connects two strands of a β sheet

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[1] The abbreviations used are: PTI, virgin basic pancreatic trypsin inhibitor; R*PTI, basic pancreatic trypsin inhibitor selectively reduced at S–S 14–38; RCAM*PTI, carboxamidomethylated R*PTI; RCOM*-PTI, carboxymethylated R*PTI; RAE*PTI, aminoethylated R*PTI; RCOM*trypsin, trypsin selectively reduced and carboxymethylated at S–S 179–203; Bz-L-ArgEt, α-*N*-benzoyl-L-arginine ethyl ester; *N*-Ac-L-TyrEt, α-*N*-acetyl-L-tyrosine ethyl ester; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); NPGB, *p*-nitrophenyl *p*′-guanidinobenzoate.

at the top of the pear-shaped inhibitor molecule (Huber *et al.*, 1971a). It can be very easily and selectively reduced (Kress and Laskowski, 1967). The conformational properties of this partially reduced inhibitor and of derivatives obtained by alkylation of SH groups formed on reduction have been previously described (Vincent *et al.*, 1971).

Materials and Methods

(*a*) *Materials.* The pancreatic trypsin inhibitor was a gift from Choay Laboratories. The protein is pure (Sach *et al.*, 1965) as judged by polyacrylamide gel electrophoresis, analytical ultracentrifugation, and stoichiometry of the inhibition with trypsin. Commercial bovine trypsinogen (Worthington) was purified before use as previously described (Vincent *et al.*, 1970). Trypsin with a specific activity of 50–51 Bz-L-ArgEt units per mg of protein was obtained by activation of pure trypsinogen (Abita *et al.*, 1969). Pseudotrypsin (Smith and Shaw, 1969) was kindly given to us by Dr. Shaw. Iodoacetic acid, Bz-L-ArgEt, and *p*-tosyl-L-arginine methyl ester were Sigma products. [^{14}C]Iodoacetic acid and [^{14}C]iodoacetamide were products of the Radiochemical Centre Amersham.

(*b*) *Preparation of RCOM*Trypsin.* Trypsinogen was selectively reduced on the Cys_{179}–Cys_{203} disulfide bond with sodium borohydride. The reduced derivative was then alkylated with iodoacetic acid and activated into RCOM*trypsin. All these operations were carried out by the techniques developed in Light's group (Light *et al.*, 1969). Titrations of the active site were carried out with NPGB (Chase and Shaw, 1967).

(*c*) *Preparation of Inhibitor Derivatives.* The disulfide bridge Cys_{14}–Cys_{38} was selectively reduced with sodium borohydride (Kress and Laskowski, 1967); RCAM*PTI, RCOM*PTI, and RAE*PTI were obtained by allowing the reaction of iodoacetamide, iodoacetic acid, and ethyleneimine, respectively, with R*PTI (Kress *et al.*, 1968; Vincent *et al.*, 1971). In some instances, reduction and alkylation were carried out at the same time. PTI was dissolved at a concentration of 0.3 mM in a medium at pH 8.3 containing 0.1 M sodium borohydride and 3.2 mM [^{14}C]iodoacetamide. After 2 hr at 1° and under nitrogen, the reaction was stopped by acidification to pH 2 which destroys sodium borohydride. RCAM*PTI was separated from excess reagent and reaction products by Sephadex G-25 chromatography at pH 2 (10 mM HCl). The method prevented reoxidation which might occur when R*PTI was isolated before being alkylated.

Modification of the trypsin–PTI complex was carried out using simultaneous reduction and alkylation. In this case reduction was not stopped by acidification as for the free PTI. Sodium borohydride was eliminated by chromatography on a Sephadex G-25 column (2 × 20 cm) equilibrated with 10 mM ammonium carbonate at pH 8.3.

(*d*) *Isolation of Trypsin–Inhibitor Complexes.* Trypsin, RCOM*trypsin, or pseudotrypsin (0.01 to 0.1 mM) was incubed at 25° and pH 8.0 with an excess (2 to 10 times) of inhibitor (native or chemically modified) in 3–4 ml of a Tris buffer (50 mM) containing 50 mM $CaCl_2$–0.1 M NaCl.

After a reaction time for a total association (15 to 120 min), the mixture was passed at 4° through a Sephadex G-75 column (3 × 48 cm) equilibrated at pH 8.0 with the Tris buffer just described. This chromatographic system separates well the complex (molecular weight 30,400) from excess free inhibitor (molecular weight 6500), but not free trypsin (molecular weight 23,900) from the trypsin–inhibitor complex.

(*e*) *Stoichiometry.* All stoichiometries of association involving trypsin and PTI or its derivatives were determined by measuring the decrease of *p*-tosyl-L-arginine methyl ester or Bz-L-ArgEt activity which followed addition of the inhibitor (Vincent *et al.*, 1970, 1971). Maximal trypsin activity is higher for *p*-tosyl-L-arginine methyl ester than for Bz-L-ArgEt (Lazdunski, 1965; Trowbridge *et al.*, 1963). Therefore, the *p*-tosyl-L-arginine methyl ester test was preferentially used each time a high sensitivity was necessary.

This technique could not be used with pseudotrypsin because of its very low catalytic activity toward these substrates. For this reason, rate assays of pseudotrypsin were replaced by active site titrations with NPGB (Smith and Shaw, 1969). Production of *p*-nitrophenol was followed in a Cary 14 spectrophotometer. Pure pseudotrypsin liberated 1 mole of *p*-nitrophenol per mole of enzyme in the presteady state of the reaction with NPGB (Smith and Shaw, 1969), whereas the complex pseudotrypsin–PTI did not react with NPGB.

(*f*) *Association Kinetics.* (i) COMPLEXES BETWEEN TRYPSIN OR RCOM*TRYPSIN AND PTI OR ITS DERIVATIVES. The kinetics were evaluated by following the decrease of trypsin activity (Green and Work, 1953). Enzymatic activities were determined in a pH-Stat Radiometer TTTIC equipped with an SBR2C recorder. *p*-Tosyl-L-arginine methyl ester (4 mM) was the substrate. The mixture contained 10 mM $CaCl_2$–0.2 M NaCl. All determinations (incubations and activity measurements) were carried out under nitrogen.

(ii) COMPLEX BETWEEN PSEUDOTRYPSIN AND PTI. The kinetics of the association of pseudotrypsin with PTI were followed by competition with trypsin. Trypsin (0.46 μM) and pseudotrypsin (0.4–8 μM) were incubated together at 25° and pH 8.0 in a Tris buffer (1 mM), containing 10 mM $CaCl_2$–0.2 M NaCl. The reaction was started by adding PTI (0.46 μM). Residual trypsin activity was evaluated after 2 min with a Radiometer pH-Stat using Bz-L-ArgEt (3 mM) as substrate.

(*g*) *Dissociation Kinetics.* (i) TRYPSIN SUBSTRATES CAN DISPLACE THE MODIFIED INHIBITOR FROM ITS ASSOCIATION WITH TRYPSIN IN THE COMPLEX. The complex formed between trypsin and the modified inhibitor (R*PTI or RAE*PTI) was isolated and incubated at a concentration of about 0.1 μM in a Tris buffer (1 mM) at pH 8.0 containing 10 mM $CaCl_2$–0.2 M NaCl. The complex was apparently devoid of catalytic activity with Bz-L-ArgEt (3.5 mM) during the first few seconds, but esterolytic activity appeared and increased with time. This behavior was due to a displacement of trypsin from the complex by Bz-L-ArgEt. The reaction was carried out in a pH-Stat which recorded automatically the appearance of free trypsin.

(ii) THE MODIFIED INHIBITOR IS DISPLACED FROM ITS ASSOCIATION WITH TRYPSIN BY THE VIRGIN INHIBITOR. The following technique was used to measure the rate of dissociation of the complex formed between trypsin and radioactive RCAM*-PTI. The complex was incubated at a concentration of 40 μM in a Tris buffer (50 mM) at pH 8.0 in the presence of 50 mM $CaCl_2$–0.1 M NaCl. The displacement was started by adding PTI (final concentration 40 μM). Aliquots were taken at different times and passed through a Sephadex G-75 column (3 × 48 cm) equilibrated at pH 8.0 with the Tris buffer already described. This technique separates the liberated inhibitor from the complex. Measurement of the radioactivity under the free inhibitor peak by scintillation counting in a Packard Tri-Carb spectrometer Model 3375 gave the extent of the displacement at any given time.

(iii) TRYPSIN DERIVATIVES ARE DISPLACED FROM THE COMPLEX BY NATIVE TRYPSIN. The following technique was used

for pseudotrypsin. The isolated pseudotrypsin–inhibitor complex (2 μM) was incubated at pH 8.0 in the Tris buffer described in ii. Displacement is started by adding pure trypsin to a concentration of 2.2 μM. Aliquots were taken at different times and used to determine the amount of free trypsin. Because of the negligible catalytic activity of pseudotrypsin (Smith and Shaw, 1969) this measurement could be done directly in a pH-Stat as usual, with Bz-L-ArgEt as a substrate. The RCOM*trypsin–inhibitor complex was studied similarly.

(*h*) *Evaluation of Dissociation Constants from Equilibrium Measurements.* (i) THE TRYPSIN–PTI COMPLEX. Trypsin (40 μM) was first incubated at pH 8.0 (Tris buffer described in g (ii)), 25°, 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 40 μM (*i.e.*, 100 times more dilute than RCAM*PTI). The displacement of RCAM*PTI by PTI was followed as previously described (g (ii)). The knowledge of the dissociation constant of the trypsin–RCAM*PTI complex and the measurement of the amount of the radioactive RCAM*PTI which remained bound to trypsin when equilibrium was attained allowed an evaluation of the dissociation constant for the trypsin–PTI complex.

(ii) THE TRYPSIN–RAE*PTI COMPLEX. The dissociation constant was high enough to permit a determination with the method previously used for measurements of stoichiometries of association (section e and Vincent *et al.*, 1970).

Results

(*a*) *Stoichiometries of Association.* It has been known for a long time (Kunitz and Northrop, 1936) that trypsin associates stoichiometrically with PTI. Figure 1 shows that 1:1 stoichiometries can also be observed between trypsin and R*PTI, between trypsin and RCAM*PTI, and between pseudotrypsin and PTI provided that the concentrations of both partners are much larger than the dissociation constants of the complexes.

(*b*) *Kinetics of Complex Formation.* (i) ASSOCIATION OF TRYPSIN, RCOM*TRYPSIN, AND PSEUDOTRYPSIN WITH NATIVE OR CHEMICALLY MODIFIED BASIC INHIBITOR. The association of trypsin (Tryp) with PTI was first studied kinetically by Green and Work (1953). It may be represented simply by eq 1.

$$\text{Tryp} + \text{PTI} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{Tryp–PTI} \qquad (1)$$

The rate of association is expressed by eq 2, where k_1 and

$$v = k_1\,[\text{Tryp}][\text{PTI}] - k_{-1}[\text{Tryp–PTI}] \qquad (2)$$

k_{-1} are rate constants for association and dissociation, respectively. At the very beginning of such an association reaction the concentration of complex is low as compared to concentrations of free trypsin and PTI. The second term of eq 2 vanishes. In fact, for PTI and most of its derivatives, the value of k_{-1} is so low, as will be seen later, that eq 2 always simplifies to

$$v = k_1[\text{Tryp}][\text{PTI}] \qquad (3)$$

Classical integration gives the following equation which describes the time course of most associations nearly to completion (about 90%).

$$\frac{1}{[\text{PTI}]_0 - [\text{Tryp}]_0} \ln \frac{[\text{PTI}]_0 - [\text{Tryp–PTI}]}{[\text{Tryp}]_0 - [\text{Tryp–PTI}]} = k_1 t + \ln \frac{[\text{PTI}]_0}{[\text{Tryp}]_0} \qquad (4)$$

For the association of RAE*PTI with native trypsin, eq 4 may be applied only to 50% combination. After that, the second term of eq 2 ceases to be negligible (less than 10%).

Figure 2 presents some typical second-order kinetics of association between trypsin and PTI or its derivatives. These

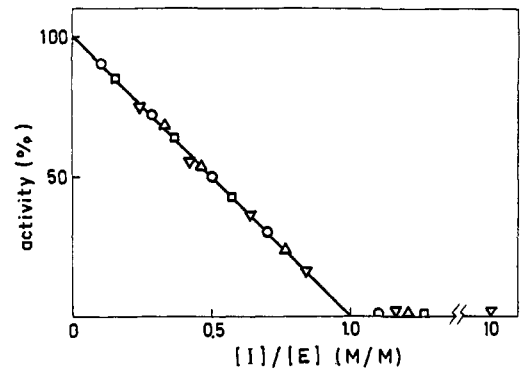

FIGURE 1: Stoichiometric inhibitions of trypsin or pseudotrypsin by PTI, R*PTI, or RCAM*PTI. Inhibition of trypsin (c = 0.4 μM) by PTI (○), by R*PTI (△), and by RCAM*RTI (▽). Inhibition of pseudotrypsin (c = 50 μM) by PTI (□), 25°, pH 8.0, 0.2 M NaCl.

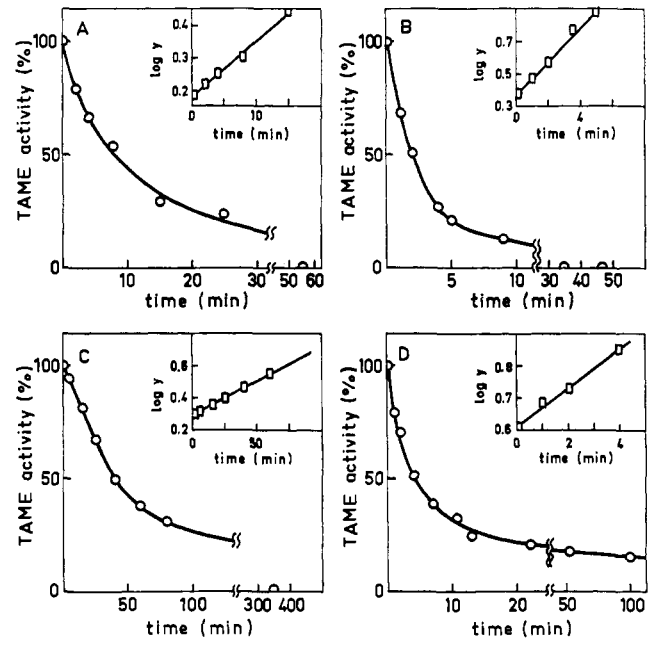

FIGURE 2: Kinetics of association of trypsin with PTI or its derivatives. Kinetics of association were evaluated by following the decrease of trypsin activity for *p*-tosyl-L-arginine methyl ester (TAME). Association between trypsin (1.2 nM) and PTI (1.8 nM) (A), between trypsin (9.3 nM) and R*PTI (22 nM) (B), between trypsin (1.2 nM) and RCAM*PTI (2.4 nM) (C), between trypsin (9.3 nM) and RAE*-PTI (38 nM) (D): 25°, pH 8.0, 0.2 M NaCl. Inserts present corresponding linear plots demonstrating second-order kinetics. Equation 4 with ([PTI]$_0$ − [Tryp − PTI])/ ([Tryp]$_0$ − ([Tryp − PTI]) = y was used to calculate k_1 values.

TABLE I: Kinetics and Thermodynamic Characteristics of the Interaction of Trypsin, Pseudotrypsin, and RCOM*Trypsin with PTI and Its Derivatives.[a]

Receptor	Inhibitor	k_1 (M^{-1} sec^{-1})	k_{-1} (sec^{-1})	K (M)
Trypsin	PTI	1.1×10^6	6.6×10^{-8}	6×10^{-14}
Trypsin	R*PTI	3.2×10^5	5.7×10^{-4}	1.8×10^{-9}
Trypsin	RCAM*PTI	1.3×10^5	2.2×10^{-5}	1.7×10^{-10}
Trypsin	RAE*PTI	8.2×10^4	7.5×10^{-4}	9.1×10^{-9}
Trypsin	RCOM*PTI	0		
Pseudotrypsin	PTI	7.0×10^4	6.3×10^{-4}	9.0×10^{-9}
RCOM*trypsin	PTI	2×10^4	1.2×10^{-4}	6.0×10^{-9}

[a] k_1 and k_{-1} are the rate constants for association and dissociation, respectively. K is the dissociation constant of the complex at 25°, pH 8.0.

derivatives were obtained by selective reduction of the Cys_{14}–Cys_{38} bridge of PTI (Figure 2B) and by carboxamidomethylation or aminoethylation of the newly formed SH groups (Figure 2C and 2D). The rate constants, k_1, obtained from these curves are complied in Table I. Under conditions described in Figure 2B, the association of trypsin with R*PTI was nearly completed after 5 min. It has been checked that this time is short enough to prevent significant reoxidation of the R*PTI partner (90% of the modified inhibitor remained in the reduced state after 5 min).

No association of RCOM*PTI with trypsin could be observed at pH 8.0, 25° even with a 20 molar excess of the modified inhibitor. This result is in agreement with previous data (Kress *et al.*, 1968). Table I also includes the rate constant of association of PTI with RCOM*trypsin.

The data in Figure 2 were based upon measurements of the loss of trypsin activity which followed the addition of the inhibitor to the enzyme. This technique cannot be applied to the association of the inhibitor with pseudotrypsin. The latter enzyme has extremely low activity toward specific substrates of normal trypsin. The transformation of trypsin into pseudotrypsin decreases k_{cat} by a factor of 100 and increases K_m by a factor of 10^4 when Bz-L-ArgEt is used as a substrate (Smith and Shaw, 1969).

As described in Materials and Methods, the association of pseudotrypsin with PTI was studied in competition experiments with trypsin. Our system can be schematized as follows.

$$\begin{array}{l} \qquad\qquad + \text{Tryp} \xrightarrow{k_1} \text{Tryp–PTI} \\ \qquad\qquad\quad (a-x) \qquad\quad (x) \\ \text{PTI} \\ (c-x-y) \\ \qquad\qquad + \psi\text{Tryp} \xrightarrow{k_1'} \psi\text{Tryp–PTI} \\ \qquad\qquad\quad (b-y) \qquad\quad (y) \end{array}$$

a, b, and c are the initial concentrations of trypsin, pseudotrypsin (ψTryp), and of PTI. x and y are the concentrations of complexes with trypsin or with pseudotrypsin (ψTryp–PTI) at a given time of the association. k_1' is the rate constant for the association of PTI with pseudotrypsin. Dissociation of the 2 types of complexes does not appear in this scheme. The rate constants for the dissociations will show later that under our experimental conditions the reverse reactions can be ignored.

Rate equations for the formation of the complexes are

$$v_1 = \frac{dx}{dt} = k_1(a-x)(c-x-y) \qquad (5)$$

$$v_2 = \frac{dy}{dt} = k_1'(b-y)(c-x-y) \qquad (6)$$

From eq 5 and 6 it follows that

$$\frac{k_1}{k_1'} = \frac{\log \dfrac{a}{a-x}}{\log \dfrac{b}{b-y}} \qquad (7)$$

Time and concentration of PTI do not appear in eq 7.

The time of incubation of the mixture tryspin–pseudotrypsin–PTI should be sufficiently long for complex formation of all the PTI with either trypsin or pseudotrypsin. It should also be sufficiently short to prevent any displacement of pseudotrypsin in the pseudotrypsin–PTI complex by free trypsin. The value of 2 min was chosen. It is long enough to ensure that the concentration of free PTI, $c - x - y$, is very low; that is, $c = x + y$. The time is short enough to ensure that even when the concentration of free trypsin, $a - x$, is high after 2 min, there is no significant displacement of pseudotrypsin from the complex pseudotrypsin–PTI. It will be seen later that the half-life for the dissociation of the complex is 18 min.

The value of $a - x$ and, in consequence, the value of x are easily determined from measurements of the residual trypsin activity after 2 min. Knowing a, b, c, $a - x$, and x, the value of y can be easily calculated from $y = c - x$.

If all the previously discussed conditions are filled, then eq 7 can be used to evaluate k_1/k_1' and then k_1', since k_1 is already known.

Figure 3 shows that a graph of $\log (a/(a-x))$ *vs.* $\log (b/(b-y))$ is perfectly linear as expected from eq 7. The rate constant k_1 for the association between trypsin and PTI is 1.1×10^6 M^{-1} sec^{-1}, but k_1', the rate constant for the association of pseudotrypsin is only 7×10^4 M^{-1} sec^{-1}.

(ii) pH AND TEMPERATURE DEPENDENCE OF THE KINETICS OF ASSOCIATION BETWEEN TRYPSIN AND PTI. The pH dependence of the association between trypsin and PTI is represented in Figure 4. There are 2 plateau regions. Maximal rate constants of about 10^6 M^{-1} sec^{-1} are obtained between pH values 8 and 10. The association proceeds at a slower rate at acidic pH.

Figure 5 shows an Arrhenius plot describing the temperature dependence of k_1. The activation energy for the association is 10.5 kcal/mole.

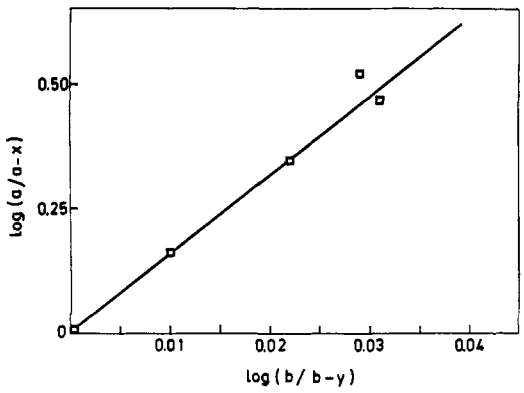

FIGURE 3: Associations of pseudotrypsin with PTI at 25°, pH 8.0. (□) Competition for PTI between pseudotrypsin and trypsin. Incubation time is 2 min. Data were treated according to eq 7.

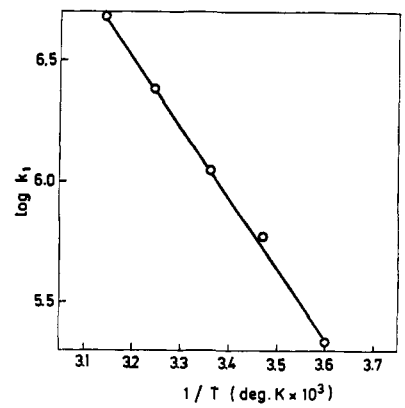

FIGURE 5: Arrhenius plot for the second-order rate constant, k_1, of the association of trypsin with PTI: pH 8.0, 0.2 M NaCl–10 mM $CaCl_2$. Temperatures were varied from 5 to 45°.

(c) *Kinetics of Complex Dissociation.* Kinetics of dissociation were always followed with complexes that had been isolated from excess inhibitor by Sephadex G-75 chromatography as described in Materials and Methods.

(i) COMPLEXES TRYPSIN–R*PTI AND TRYPSIN–RAE*PTI. In these cases the modified pancreatic trypsin inhibitors can be displaced from their association with trypsin by trypsin substrates themselves. This displacement can be recorded directly in a pH-Stat. A typical experiment showing the reappearance of trypsin activity with time is presented in Figure 6. The system evolves toward an equilibirum position where trypsin has been liberated from its interaction with the inhibitor. The association of substrate with free trypsin being extremely rapid, measurement of the rate of the displacement gives an easy evaluation of the first-order rate constant for the dissociation of the complex (Figure 6).

$$\begin{matrix}\text{Tryp–R*PTI} \\ \text{or} \\ \text{Tryp–RAE*PTI}\end{matrix} \xrightarrow{k_{-1}'} \text{Tryp} + \begin{matrix}\text{R*PTI} \\ \text{or} \\ \text{RAE*PTI}\end{matrix}$$

We have confirmed previous observations by Kress and Laskowksi (1967) that reoxidation of R*PTI is completely prevented inside of the trypsin–R*PTI complex even after long periods of time (more than 1 hr). However, reoxidation of a small fraction (15%) of R*PTI occurs during the substrate-induced dissociation of the trypsin–R*PTI complex. The presence of small quantities of native inhibitor formed by reoxidation of the dissociated R*PTI does not affect the determination of k_{-1}' for the trypsin–R*PTI complex. Association of PTI (native or modified) with trypsin is impossible in the presence of high substrate concentrations.

(ii) COMPLEX TRYPSIN–RCAM*PTI. In this case, the method described in i could not be used. There are 2 related reasons for that. First, the complex is so stable that even high concentrations of Bz-L-ArgEt cannot efficiently displace the modified inhibitor from its association with trypsin. Second, the dissociation rate of the complex is much too slow, as will be seen later.

RCAM*PTI was displaced from its association with trypsin by PTI itself the complex of which is more stable.

The complex between trypsin and the radioactive RCAM*-PTI was first isolated. This complex was then dissolved at pH 8.0 to a final concentration of 40 μM. Time zero of the experiment corresponded to the introduction of PTI to a final concentration of 40 μM. Aliquots were taken at intervals and chromatographed as described in Figure 7A to estimate the amount of radioactive RCAM*PTI displaced. After 10 days, the radioactive RCAM*PTI was nearly completely displaced (over 95%) by PTI. With long incubations a third peak devoid of inhibitory activity appeared in the chromato-

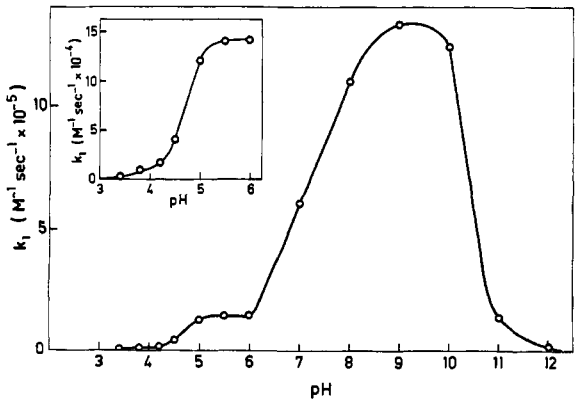

FIGURE 4: pH dependence of the second-order rate constant, k_1, for the association of trypsin with PTI: 25°, 0.2 M NaCl–10 mM $CaCl_2$. Insert: enlarged version of the acidic range.

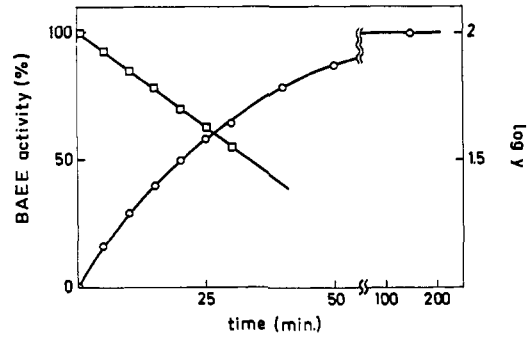

FIGURE 6: Dissociation kinetics of the trypsin–RAE*PTI complex. Displacement of RAE*PTI from the trypsin–RAE*PTI complex was achieved with Bz-L-ArgEt (BAEE) (final concentration 3.5 mM). (○) Time course of reappearance of trypsin activity. (□) Pseudo-first-order representation of the data. y = 100 − per cent of Bz-L-ArgEt activity: 25°, pH 8.0, 0.2 M NaCl. The same type of representation was obtained for the displacement of R*PTI from the trypsin–R*PTI complex.

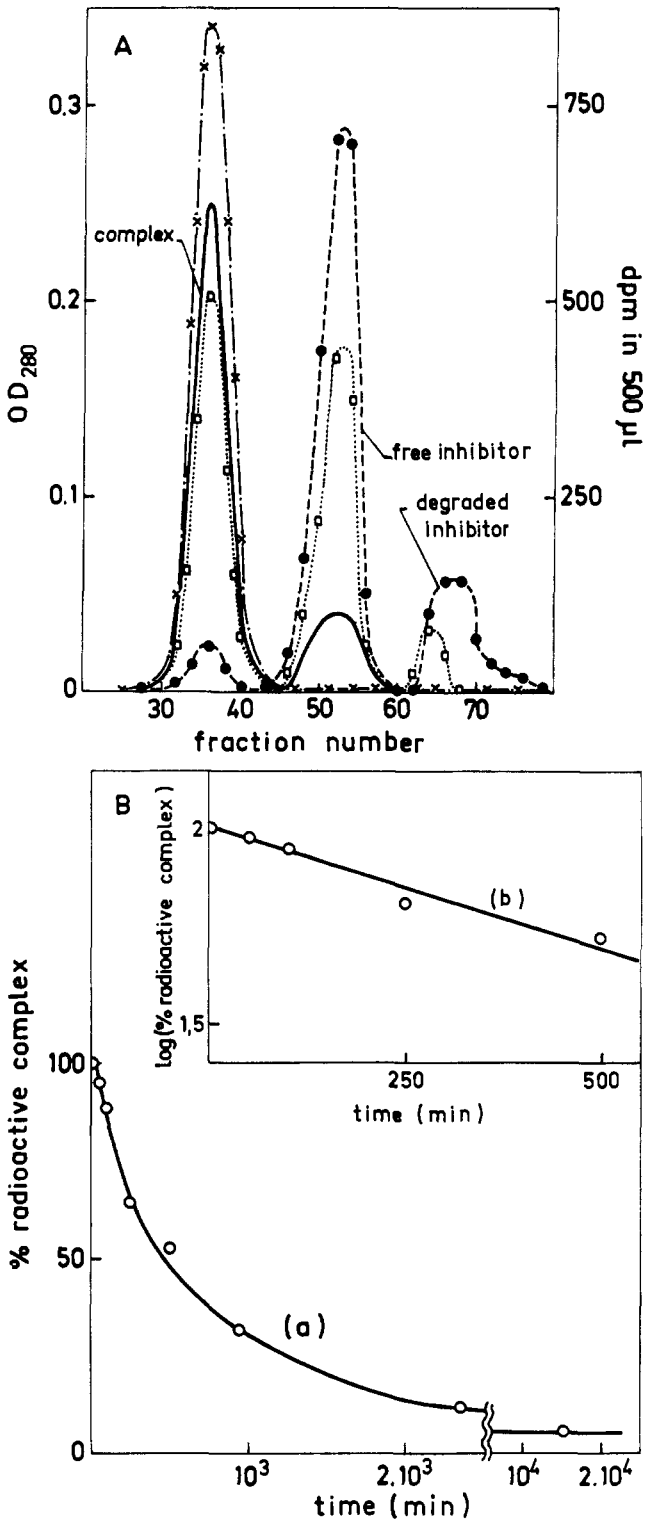

FIGURE 7: Dissociation of the trypsin-RCAM*PTI complex. Radioactive RCAM*PTI was displaced by PTI (40 µM) from the trypsin-RCAM*PTI complex (40 µM): 25°, pH 8.0, 0.1 M NaCl-50 mM $CaCl_2$. (A) G-75 Sephadex chromatography of aliquots taken at different times of the displacement. Broken lines represent radioactivity elution patterns at different times of the displacement: (×) time zero, (□) 500 min, (●) 10 days. The third peak appeared only after very long incubation (from 500 min); it corresponds to degraded RCAM*PTI devoid of inhibitory activity. Solid line: optical density profile measured at 280 nm. This profile is not time dependent up to 500 min. The third peak is hardly detectable by optical density even after longer incubation. (B) Kinetics of the dissociation. (a) Time course of the displacement. (b) Pseudo-first-order representation. Each point in these figures was calculated from the results presented in A and from other data not represented.

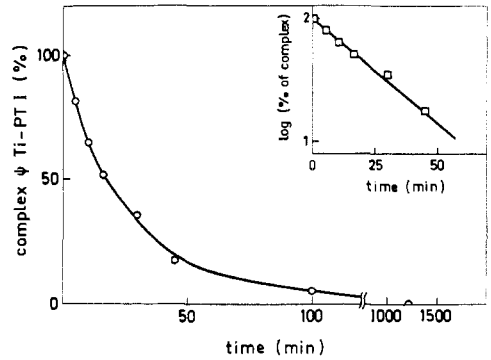

FIGURE 8: Dissociation kinetics of the pseudotrypsin-PTI complex. Trypsin-induced displacement of pseudotrypsin from the pseudotrypsin-PTI complex: 25°, pH 8.0, 0.1 M NaCl-50 mM $CaCl_2$. (○) Time course of the dissociation. (□) Pseudo-first-order representation. A similar approach gave similar results for the trypsin-induced displacement of RCOM*trypsin from the RCOM*trypsin-PTI complex.

gram (Figure 7A) and corresponded to degradation peptides of RCAM*PTI. Wilson and Laskowski (1971) had also noticed digestion of RCAM*PTI by trypsin. At the end of the reaction, the amount of degraded inhibitor does not exceed 20% of the initial concentration. Moreover, during the first two-thirds of the displacement, the material eluted in the last peak never represents more than 5% of the active inhibitory material eluted in the second peak. These observations indicate that the displacement of RCAM*PTI by PTI is not due to degradation of the modified inhibitor.

The kinetics of the displacement is presented in Figure 7B. Experiments shown in that figure can be schematized as follows.

$$\text{Tryp–RCAM*PTI} \underset{k_1'}{\overset{k_{-1}'}{\rightleftharpoons}} \underset{\text{RCAM*PTI}}{\text{Tryp} +} \xrightarrow[k_1]{\text{PTI}} \text{Tryp–PTI}$$

The half-life of the displacement reaction is about 500 min. This means that the formation of trypsin-PTI from trypsin-RCAM*PTI is limited by the rate of dissociation of trypsin-RCAM*PTI and not by the much faster association of trypsin with PTI. As expected, the displacement is a first-order reaction (Figure 7B). The value of $k_{-1}' = 2.2 \times 10^{-5}$ sec^{-1} is compared to other data in Table I.

(iii) COMPLEXES PSEUDOTRYPSIN-PTI AND RCOM*TRYPSIN-PTI. The stoichiometric complex pseudotrypsin-PTI was easily isolated on Sephadex G-75. This confirms the strong interaction which was already shown in Figure 1. In this case, neither of the 2 methods described in c (i) and c (ii) could be used. The simplest method is to displace pseudotrypsin from the pseudotrypsin-PTI complex by trypsin which forms more stable complexes. The reaction was carried out at pH 8.0, 25°. Trypsin (2.2 µM) was added to the complex pseudotrypsin-PTI (2 µM). Free pseudotrypsin has negligible activity. Therefore the loss of trypsin activity as a function of time gave an easy measure of the kinetics of the displacement.

The displacement is schematized as follows.

$$\psi\text{Tryp–PTI} \underset{k_1'}{\overset{k_{-1}'}{\rightleftharpoons}} \text{PTI} + \psi\text{Tryp} \xrightarrow[\text{Tryp}]{k_1} \text{Tryp–PTI}$$

Again, as shown in Figure 8, the displacement is a slow first-order reaction ($t_{1/2}$ = 18 min). The rate-limiting step is

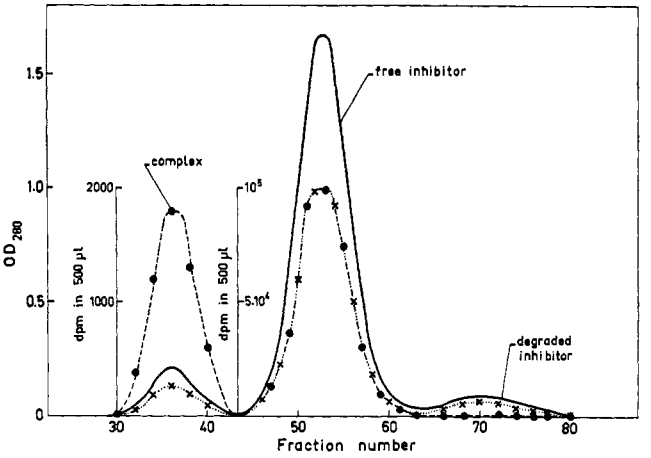

FIGURE 9: A competition experiment for the evaluation of the dissociation constant of the trypsin–PTI complex. Trypsin (40 μM) was first incubated at 25°, pH 8.0, with a 100 molar excess of [^{14}C]-RCAM*PTI. Association was completed after 15 min. At that time, dissociation of RCAM*PTI from the trypsin–RCAM*PTI complex was started by adding PTI at a final concentration of 40 μM. Aliquots were taken at different times and chromatographed as described in Figure 7. As shown in this figure, equilibrium was attained after 10 days. Solid line: optical density profile measured at 280 nm after 10 and 17 days. Broken lines: (●) radioactivity elution pattern at zero time, (×) radioactivity elution pattern after 10 and 17 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.

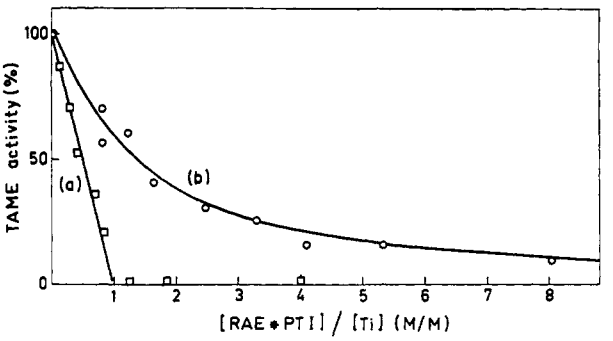

FIGURE 10: Inhibition of trypsin by RAE*PTI at 25°, pH 8.0, 0.2 M NaCl. Trypsin concentrations were 4 μM (a) and 9.3 nM (b), respectively. Direct evaluation of the stoichiometry with such a representation is only possible when the trypsin concentration is much higher than the dissociation constant of the complex. Solid lines are calculated curves using the value of K which gave the best fit, *i.e.*, $K = 8 \times 10^{-9}$ M.

the dissociation of the pseudotrypsin–PTI complex ($k_{-1}' = 6.3 \times 10^{-4}$ sec^{-1}).

The RCOM*trypsin–PTI complex was also first isolated on Sephadex. The RCOM*trypsin was displaced from its association with PTI by native trypsin. The operation procedure was the same as the one just described for the pseudotrypsin–PTI complex. The kinetics of the displacement indicates a first-order reaction with a value of $k_{-1}' = 1.2 \times 10^{-4}$ sec^{-1}.

(*d*) *Determination of the Dissociation Constants of Complexes Formed between Trypsin (or Pseudotrypsin or RCOM*-Trypsin) and Inhibitor (or Modified Inhibitor).* (i) THE USE OF KINETIC DATA. Dissociation constants could be evaluated easily when the rate constants for both association, k_1 or k_1', and dissociation, k_{-1} or k_{-1}', were known ($K = k_{-1}/k_1$ or k_{-1}'/k_1'). Such values are reported in Table I.

It is always possible without too much trouble to determine rate constants of association, but rate constants for dissociation are sometimes difficult to get directly. In such cases equilibrium constants, which characterize complex stability, were determined rather than rate constants.

(ii) THE DISSOCIATION CONSTANT OF THE COMPLEX BETWEEN NATIVE TRYPSIN AND PTI. To determine this dissociation constant, competition for trypsin between PTI and RCAM*PTI was used to obtain the ratio K/K' where K represents the dissociation constant of the trypsin–PTI complex and K' the dissociation constant for the trypsin–RCAM*PTI complex. From this ratio and the value of K' in Table I, K could be easily calculated.

Trypsin (40 μM) was first incubated at pH 8.0 and 25° with a hundredfold molar excess of radioactive RCAM*PTI (4 mM). Enough time was allowed for complete association of the partners and after 15 min an amount of PTI equal to that of trypsin (40 μM) was added to displace the modified inhibitor. From the chromatographic profiles of Figure 9 it was possible to determine the concentration of trypsin–PTI and trypsin–RCAM*PTI after 10 and 17 days. Equilibrium was attained after 10 days. The concentration of RCAM*PTI (which is the initial concentration) and that of free PTI are also easily determined.

$$\frac{K}{K'} = \frac{[\text{PTI}]}{[\text{RCAM*PTI}]} \times \frac{[\text{Tryp–RCAM*PTI}]}{[\text{Tryp–PTI}]}$$

Since $K/K' = 3.5 \times 10^{-4}$ and $K' = 1.7 \times 10^{-10}$ M, $K = 6 \times 10^{-14}$ M. When K and k_1 are known, k_{-1} could be easily calculated. Its value is 6.6×10^{-8} sec^{-1}.

(iii) DIRECT MEASUREMENT OF THE DISSOCIATION CONSTANT OF THE COMPLEX TRYPSIN–RAE*PTI. Titrations of native trypsin with RAE*PTI under two widely different conditions of concentration are represented in Figure 10. Stoichiometry was easily obtained when the trypsin concentration was much higher than the dissociation constant of the complex. The situation was markedly different when the trypsin concentration was of the same order of magnitude as the dissociation constant (curve b). In the latter case, the experimental points could be perfectly described by a calculated curve assuming a value of K, the dissociation constant, of 8×10^{-9} M. This value is in good agreement with the value of $K = 9.1 \times 10^{-9}$ M obtained from the evaluation of k_{-1}/k_1 (Table I).

(*e*) *Protection of the Disulfide Bridge Cys_{14}–Cys_{38} in the Association of PTI with Trypsin.* The results in Table I show that the integrity of the disulfide bridge Cys_{14}–Cys_{38} is important for a very tight association with trypsin. It was of interest to study the reactivity of the disulfide bridge in the complex formed between trypsin and PTI. Whereas two [^{14}C]carboxamidomethyl groups were incorporated into the free PTI after reduction and alkylation with [^{14}C]iodoacetamide, only 0.10 was incorporated, under the same conditions, into PTI associated with trypsin in the complex. This is direct evidence that the bridge Cys_{14}–Cys_{38} is masked in the complex. Independent evidence was obtained by Liu *et al.* (1971). These results are also in agreement with the latest crystallographic data (Huber *et al.*, 1971b).

Discussion

The second-order rate constant of association between trypsin and PTI is maximum near neutral pH, as was already

observed by Green and Work (1953) and by Putter (1967). This constant, 1.1×10^6 M^{-1} sec^{-1}, is similar to those found with other systems of macromolecular interactions such as the association of iodoinsulin with its membranous receptor (Cuatrecasas *et al.*, 1971), 3.5×10^6 M^{-1} sec^{-1}, or the association of leucyl-tRNA to leucine tRNA synthetase (0.9 –1) $\times 10^6$ M^{-1} sec^{-1} (Rouget and Chapeville, 1971). The rate constant is much lower than that found for the association of the *Lac*-repressor with the operator, 7×10^9 M^{-1} sec^{-1} (Riggs *et al.*, 1970) but the corresponding activation energies are very similar.

The variation of k_1 with pH is not simple. It is dependent upon the dissociation of essential ionizable groups borne both by the inhibitor and by the enzyme. The variation of the rate constant between pH 9 and pH 5.5 is controlled by the ionization of a single group with a pK of 7.05. This group has to be basic for the association to take place efficiently. The geometries of the active sites of trypsin (Lazdunski and Delaage, 1967) and of PTI (Vincent *et al.*, 1971) remain unchanged in this pH range. PTI has no imidazole side chain, and its α-amino group is masked (Vincent *et al.*, 1971). Therefore, the most reasonable interpretation is that the variation of k_1 between pH 9 and 5.5 is controlled by the ionization of the imidazole of His_{46} in the active site of trypsin. An interesting observation is that the protonation of the imidazole side chain does not nullify k_1 but only decreases it by a factor of 10. The decrease of k_1 which was observed below pH 5.5 is probably related to the structural change which affects trypsin in exactly the same pH range. This structural change is controlled by an ionizable group with an apparent pK of 4.6 at 25° (Lazdunski and Delaage, 1967). No conformational change of PTI has been found even at very acidic pH (Vincent *et al.*, 1971). The decrease of k_1 at pH values below 5.5 could also be due to the protonation of the carboxylic side chain of Asp_{177} in the specificity site of trypsin (Smith and Shaw, 1969). The pK of this group is still unknown. The rapid decrease of k_1 at alkaline pH is probably related to the deprotonation of the side chain of Lys_{15} in the active site of PTI and also to changes which alter the active conformation of trypsin and which are due to unmasking of the α-amino group of Ile_1 (Scrimger and Hofmann, 1967) and of some tyrosine side chains (Lazdunski and Delaage, 1967).

The rate of association is maximal between native trypsin and PTI (Table I). It decreases by a factor of 16 when pseudotrypsin is used in place of trypsin. In pseudotrypsin, the active site is disconnected from the specificity site. Reduction of the disulfide bridge Cys_{14}–Cys_{38} of PTI decreases the rate of association by a factor of only 3.5. Subsequent aminoethylation or carboxamidomethylation of the newly formed SH groups further decreases k_1 by a factor of 4 or 2.5, respectively.

The most striking conclusion from the data of Table I is the quasi-irreversibility of the trypsin–PTI complex. The dissociation constant, 6×10^{-14} M, is, as far as we are aware, the lowest constant ever determined for such an effector–receptor interaction. The half-life of the complex is more than 17 weeks. Such data favor considerably the existence of a covalent attachment of the inhibitor to the enzyme.

Very interesting models of interaction have recently been proposed by crystallographers. These models were built by assembling the structures of trypsin (Stroud *et al.*, 1971) or chymotrypsin (Blow *et al.*, 1972) with that of the pancreatic trypsin inhibitor (Huber *et al.*, 1970; Huber *et al.*, 1971a). In these models, the recognition of Lys_{15} (PTI) by Asp_{177} (trypsin) is followed by a split of the Lys_{15}–Ala_{16} bond concurrent with an acylation of Ser_{183} in the active site of trypsin. Deacylation is prevented by the absence of water molecules in the area of contact between trypsin and PTI. The covalent bond is then an ester bond between the carboxylate of Lys_{15} (PTI) and the alcohol function of Ser_{183} (trypsin). This type of interaction was suggested by the very extensive and elegant analysis done by Laskowski and his group on the association of trypsin with the soybean trypsin inhibitor (Laskowski *et al.*, 1971). Acylation of the enzyme with the inhibitor implies that the rate-limiting step for the dissociation of the complex is the deacylation. If that were the case, the dissociated pancreatic inhibitor would be a 2-chain molecule because of the splitting of the Lys_{15}–Ala_{16} bond. In fact a difficulty of the acylation mechanism is that no digestion of the pancreatic trypsin inhibitor occurs near neutral pH (Kress and Laskowski, 1968). This is in contrast with the situation for the soybean trypsin inhibitor. In that case, splitting occurs at the strategic Arg_{64}–Ile_{65} bond (Ozawa and Laskowski, 1966).

Cleavage by trypsin of the Lys_{15}–Ala_{16} bond of PTI cannot occur but it may occur with RCAM*PTI at pH 5.5 (Kress and Laskowski, 1968). This 2-chain pancreatic inhibitor is inactive (Kress and Laskowski, 1968), whereas the 2-chain soybean trypsin inhibitor is still active (Ozawa and Laskowski, 1966). Figure 7 showed that RCAM*PTI was fully active and therefore not cleaved by trypsin after displacement by native PTI. Degraded forms of RCAM*PTI appeared only after very long incubation indicating that the rate of displacement does not correspond to a deacylation step. Therefore, degradation of the modified inhibitor is not related to the mechanism of complex formation. Degradation of RCAM*PTI in the complex indicates that the associated trypsin has not completely lost its peptidase activity. This activity is extremely low when compared to trypsin activity for model peptides or for the hydrolysis of the Arg_{15}–Ile_{16} bond of chymotrypsinogen for example (Abita *et al.*, 1969). k_{cat} values in the latter cases range between 0.4 and 7 sec^{-1} whereas that evaluated from Figure 7 is of the order of 3×10^{-6} sec^{-1} for the degradation of RCAM*PTI in the complex.

Before discussing more extensively the mechanism of the covalent binding, it is necessary to evaluate the contribution of Lys_{15} to the inhibitor–enzyme association. Lys_{15} is thus far the only chemically identifiable element of the inhibitor active site (Chauvet and Acher, 1967; Kress and Laskowski, 1968; Fritz *et al.*, 1969). The α-amino side chain of this residue mimicks the structure of natural substrates of the enzyme and forms an ion-pair interaction with the side chain of Asp_{177}, the anionic part of the specificity site in trypsin. The Lys_{176}–Asp_{177} bond in trypsin can be split selectively to give rise to pseudotrypsin (Smith and Shaw, 1969). The data in Figure 1 concerning the association of pseudotrypsin with PTI show that the ion-pair interaction between Lys_{15} of PTI and Asp_{177} of the receptor is not necessary for a stoichiometric complex. However, the dissociation constant of the pseudotrypsin–PTI complex is 1.5×10^5 times higher than that of the trypsin–PTI complex. Disconnection of the anionic binding site increases the rate constant of dissociation of the complex by a factor of about 10^4. Complementary evidence that the salt bridge Lys_{15} (PTI)–Asp_{177} (trypsin) is not absolutely essential for the interaction stems from recent data (Rigbi, 1971) showing that the fully acetylated PTI in which Lys_{15} was modified still abolished trypsin activity. Chymotrypsin, which has a high degree of homology with trypsin (Hartley *et al.*, 1965; Neurath *et al.*, 1970) and which also cleaves some arginyl and lysyl ester bonds (Inagami and Sturtevant, 1960) although it has no Asp_{177} in its binding site,

also associates with PTI very efficiently. The kinetics of association and dissociation and the stability of the complex chymotrypsin–PTI are very smilar to those of pseudotrypsin–PTI. For example, $K = 9.10^{-9}$ M for the chymotrypsin–PTI complex (Vincent and Lazdunski, 1972). It is of interest to remark that all the essential components of the active sites of trypsin and PTI are in close proximity to disulfide bridges. The Cys_{14}–Cys_{38} bond of PTI is near the Lys_{15} residue of the active site. The Cys_{31}–Cys_{47} bond (trypsin) is near the essential His_{46} residue of the catalytic site of trypsin. The Cys_{179}–Cys_{203} bond (trypsin) is near both the active Ser_{183} and Asp_{177}, the essential element of the specificity site of the enzyme. Such proximity of critical amino acids from disulfide bridges appears to be much more than a simple coincidence.

Therefore, considerable attention has been given to the role of disulfide bridges in the association of trypsin with the inhibitor. Like Lys_{15} (Chauvet and Acher, 1967) the Cys_{14}–Cys_{38} disulfide bridge of PTI is masked in the trypsin–PTI complex. The data in Table I indicate the importance of this bridge in complex formation. Selective reduction of the disulfide bond increases the dissociation constant of the trypsin–PTI complex by a factor of 3×10^4. The rate constant of association is only slightly changed but the rate constant of the dissociation of the trypsin–R*PTI complex is 10^4 times greater than that of the complex formed from native partners. Chemical modification of the newly formed SH groups has different effects. Aminoethylation decreases the stability of the complex, whereas carboxamidomethylation increases it. The dissociation constants and rates of dissociation of the trypsin–RAE*PTI and the trypsin–RCAM*PTI complexes differ by one order of magnitude. Carboxymethylation is the only chemical modification that has a very drastic effect. As already noted by Kress *et al.* (1968), it abolishes the interaction.

It does not seem very probable that an appreciable decrease of complex stability would be due to a structural change in the inhibitor resulting from reduction of the Cys_{14}–Cys_{38} bond. It is well known that disulfide bridges do not determine protein conformations; they only stabilize them. The protein conformation specifies the correct positioning of cysteines for the oxidation that leads to disulfide bridge formation. The passive structural role of disulfide bridges is exemplified by assembly experiments of heavy and light chains of γ_G-immunoglobulins (Olins and Edelman, 1964). Accordingly, it was shown that the folded structures of PTI and RCAM*-PTI were indistinguishable by infrared or optical rotatory dispersion techniques. Moreover, the pK's of the masked α-amino group of Arg_1 were found to be identical in the two molecules and gave perfectly superimposable alkaline isomerization curves (Vincent *et al.*, 1971).[4] Finally, if selective reduction of the Cys_{14}–Cys_{38} bridge produced a structural change, the resulting alterations could be expected to be amplified by subsequent chemical modifications of the thiol functions. In fact, R*PTI, RCOM*PTI, RCAM*PTI, and RAE*PTI were found to have identical physicochemical properties (Vincent *et al.*, 1971), and RCAM*PTI even associates better with trypsin than R*PTI (Table I).

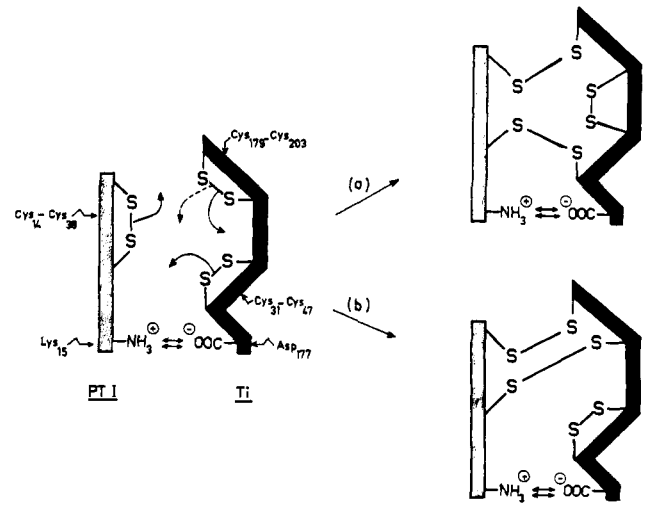

FIGURE 11: Disulfide interchange, a plausible explanation of the "quasi-irreversible" trypsin–PTI association. The "primary event" in this hypothetical mechanism is the recognition, through ion-pair formation, of the ϵ-amino group of Lys_{15} (PTI) by the β-carboxylate of Asp_{177} (trypsin). Disulfide bonds are then correctly positioned for the interchange. Two possible mechanisms were considered: (pathway a) a multiple interchange (solid arrows) involving Cys_{14}–Cys_{38} (PTI) and both Cys_{179}–Cys_{203} and Cys_{31}–Cys_{47} of trypsin; (pathway b) a simple interchange involving only Cys_{14}–Cys_{38} (PTI) and Cys_{179}–Cys_{203} (trypsin) (broken arrow). These 2 disulfide bridges have been found to be unusually reactive as compared to others (Kress and Laskowski, 1967; Light *et al.*, 1969). The final mechanism will have to wait for further X-ray crystallography data with the trypsin–PTI complex.

The Cys_{179}–Cys_{203} bridge of trypsin can also be selectively reduced and alkylated (RCOM*trypsin) (Light *et al.*, 1969; Hatfield *et al.*, 1971) without destruction of the active site. Again stoichiometric association with PTI persists but the strength of the association decreases considerably. The dissociation constant increases by a factor of 10^5; while the rate of dissociation increases by a factor of about 2×10^8.

For all these reasons, a functional role of the Cys_{14}–Cys_{38} bond has been considered. A tentative model of interaction involving multiple disulfide interchange is presented in Figure 11. This figure implies a classical recognition of the Lys_{15}–Ala_{16} bond of PTI by the specificity site of the enzyme. Noncovalent interactions permit the positioning of the Cys_{14}–Cys_{38} disulfide bond so that interchange can occur with the critical disulfide bonds of the enzyme molecule to give a covalently stabilized complex. It has been tentatively assumed that the bonds involved in the disulfide rearrangement are Cys_{14}–Cys_{38} (PTI), Cys_{179}–Cys_{203}, and Cys_{31}–Cys_{47} (trypsin). The trypsin–PTI complex would also have the intrinsic property of catalyzing the interchange. In such a picture the mode of action of PTI resembles considerably that of covalent affinity labeling reagents. Disulfide interchange has already been mentioned by Kassell (1968) as a possible mechamism of interaction between trypsin and PTI. This type of interaction of PTI with the enzyme prevents the acylation of Ser_{183} (trypsin) by the carboxylic function of Lys_{15} (PTI) which would be the first step in the cleavage of the Lys_{15}–Ala_{16} bond of the inhibitor molecule. A disulfide interchange involving only 2 disulfide bonds would certainly give sufficient cross-linking. Although no information is available at the moment concerning the Cys_{31}–Cys_{47} bond, it has been incorporated in the interchange mechanism because of its proximity to His_{46} and also because proteolytic degradation of the complex

[4] Reduction of the Cys_{14}–Cys_{38} bridge decreases both thermal stability and resistance to denaturing agents (Vincent *et al.*, 1971). This effect obviously means that cleavage of the disulfide bridge provokes an increase of the conformational entropy of the denatured form, *i.e.*, a structural difference of the unfolded forms of PTI and R*PTI or RCAM*PTI. It does not indicate a conformational difference of the folded forms.

gave a high molecular weight fragment comprising the undigested inhibitor apparently covalently bound to a fragment of trypsin which contained the sequence Ala_{146}–Ser_{198}, that is Ser_{183} and Asp_{177} of the active site, as well as the 2 histidines His_{29} and His_{46} (Dlouha *et al.*, 1968). The fragment of the trypsin molecule remained bound to the intact inhibitor so firmly that only performic oxidation could lead to complete dissociation (Dlouha *et al.*, 1968). The trypsin–PTI complex seems to behave in this situation as a single molecule made of 2-chains cross-linked by disulfide bridges.

Although the three disulfide bridges involved in the interchange mechanism are all in the area of contact between the inhibitor and the enzyme (Huber *et al.*, 1971b), they are not necessarily close enough for a direct interchange and a conformational rearrangement may be needed.

The disulfide bridges play an important role in the interaction. However, the argument that these bonds participate in an interchange rests on indirect evidence and a final mechanism of association will probably be obtained only through the X-ray analysis of the crystal structure of the trypsin–PTI complex which is presently being carried out (Huber *et al.*, 1971b). However because it does not make use of the special catalytic properties of the enzymatic receptor, a disulfide interchange mechanism could occur in a number of effector–receptor systems and, in the first place, in other associations involving protein inhibitors and degradative (nonproteolytic) enzymes such as desoxyribonucleases or phospholipases, for example (Lesca and Paoletti, 1969; Vidal and Stoppani, 1971).

Snake neurotoxins have structural properties similar to those of PTI. They are small proteins of 60 to 71 amino acids cross-linked internally by 4 or 5 disulfide bridges. Studies of *Naja haje* neurotoxin I indicated 2 lysines in the active-site Lys_{26} and Lys_{46} (Chicheportiche *et al.*, 1972). Lys_{26} is very near the disulfide bridge Cys_3–Cys_{23} (Botes and Strydom, 1969). Snake neurotoxins form quasiirreversible complexes with the acetylcholine receptor of excitable membranes (Changeux *et al.*, 1970; Miledi and Potter, 1971). This suggests that a covalent bond is formed between the neurotoxin and the receptor. Here again it is tempting to postulate that the formation of an ion pair between the side chains of neurotoxin active-site lysines and the anionic site of the receptor, which normally recognizes the positive charge of acetylcholine, is followed by disulfide interchange involving critical disulfide bonds of the neurotoxin and of the receptor. In this respect, it is extremely interesting that the receptor protein of acetylcholine has a superreactive disulfide bond in the vicinity of the acetylcholine binding site. This disulfide bond can be selectively reduced by dithiothreitol. Such a reduction of the receptor inhibits the response to carbamylcholine (Karlin and Winnik, 1968). The reactive disulfide bond may well undergo a disulfide interchange with the neurotoxin molecule.

Most protein hormones such as insulin (Sanger *et al.*, 1955), growth hormone (Li *et al.*, 1966), nerve growth factor (Angeletti and Bradshaw, 1971), etc., contain one or more disulfide bridges. This is also true for a number of polypeptide hormones such as oxytocin or vasopressin, for example. The importance of disulfide bridges for the biological activity of human pituitary growth hormone has already been discovered (Bewley *et al.*, 1969). Both disulfide bridges of the hormone can be reduced and alkylated without causing significant changes in viscosity, tyrosine titration, and circular dichroism. The effects of different alkylating agents after reduction were found to be similar to those found with PTI. When the four thiol functions were alkylated by iodoacetamide, full biological activity was retained. Alkylation with iodoacetic acid suppressed growth-promoting activity (Bewley *et al.*, 1969). Here again, the role of the hormone disulfide bridges might be a functional one.

Acknowledgments

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Binding and Adenosine Triphosphatase of Flagellar Proteins from Sea Urchin Sperm†

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ABSTRACT: The extracted ATPase from sea urchin sperm flagella, dynein, is found to rebind spontaneously to the outer fiber from 60 to 90% of the solubilized ATPase. The aggregate of 4S, 10S, and 13S components of crude dynein formed in 1 mM $CaCl_2$–30 mM KCl–20 mM Tris-HCl (pH 8.0) has the binding activity. The bound and unbound dyneins have different properties. K^+ ion activates the Ca-activated ATPase of dynein, but inhibits that of axoneme, and the high concentration of ATP inhibits the ATPase of axoneme, but does not inhibit that of dynein.

The glycerinated model of sperm showed the movement of flagella by the addition of ATP, suggesting that the movement of sperm tail was coupled to phosphohydrolysis of ATP (Hoffmann-Berling, 1955; Bishop and Hoffmann-Berling, 1959; Kinoshita, 1958, 1959; Brokaw, 1967). Flagella and cilia have usually the same arrangement of microtubles called the "9 + 2" system. Gibbons (1963) isolated the protein "dynein" having the ATPase activity from *Tetrahymena* cilia. This protein was identified as the arm projecting on the outer fiber of cilia and found to rebind specifically the outer fiber (Gibbons, 1963, 1965a). Some of its biochemical natures were examined (Gibbons, 1966). Cilia which rebound dynein showed the superprecipitation-like phenomenon by the addition of ATP (Gibbons, 1965b). It is certain that

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