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Thermodynamics and Kinetics of the Hydrolysis of the Reactive-Site Peptide Bond in Pancreatic Trypsin Inhibitor (Kunitz) by *Dermasterias imbricata* Trypsin 1†

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ABSTRACT: Incubation of pancreatic trypsin inhibitor (Kunitz) (PTI) with catalytic amounts of *Dermasterias imbricata* trypsin 1 leads to rapid yet highly specific hydrolysis of the Lys^{15}–Ala reactive-site peptide bond. Aside from providing a facile means for the preparation of modified inhibitor (PTI*), this finding also allows rapid measurement of the peptide bond hydrolysis equilibrium constant, K_{hyd}. The pH dependence of this constant over the pH 6.2–8.8 range shows that peptide bond hydrolysis does not perturb the pK values of any preexistent ionizable groups (in PTI) which ionize in this pH range. K_{hyd}^0 is 0.95, and the pK of the α-amino group of Ala^{16} in PTI* is 8.3. From the rate of equilibration, we calculate that k_{cat}/K_m is quite similar to k_{cat}/K_m for the hydrolysis of the same bond in PTI by bovine trypsin, while k_{cat} for the *D. imbricata* trypsin 1 catalyzed reaction is 10^6 times greater than the value of k_{cat} for the bovine trypsin-PTI reaction. The ratio of the dissociation rate constants for the enzyme–inhibitor complex (k_{-2}/k_3)—one to form virgin inhibitor and free enzyme (k_{-2}) and the other (k_3) to form modified inhibitor and free enzyme—is about 50 for the *D. imbricata* trypsin 1–PTI complex and about 90 for the bovine trypsin–PTI complex. These values are very similar even though the individual rate constants for the two enzymes differ by a factor of 10^6. The values of k_{cat}/K_m and k_{-2}/k_3 for the hydrolysis of the Arg^{63}–Ile reactive-site peptide bond in STI are also quite similar for *D. imbricata* trypsin 1 and bovine trypsin, although k_{cat} is 50 000 times greater for the starfish enzyme.

Niekamp et al. (1969) and Mattis & Laskowski (1973) have shown that hydrolysis of the reactive-site peptide bond in soybean trypsin inhibitor (Kunitz), STI[1], does not proceed to completion but that an equilibrium between virgin (reactive-site peptide bond intact) and modified (reactive-site peptide bond hydrolyzed) inhibitors, governed by the equilibrium constant K_{hyd}, becomes established. K_{hyd} is given by

$$K_{hyd} = [I^*]/[I] \quad (1)$$

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[1] Abbreviations used: STI, soybean trypsin inhibitor (Kunitz); STI*, modified STI in which the Arg^{63}–Ile reactive-site peptide bond has been hydrolyzed; PTI, bovine pancreatic trypsin inhibitor (Kunitz); PTI*, modified PTI in which the Lys^{15}–Ala reactive-site peptide bond has been hydrolyzed; GdnBzMum, 4-methylumbelliferyl *p*-guanidinobenzoate hydrochloride; MeSAcNHBzMum, 4-methylumbelliferyl *p*-[ω-(dimethylsulfonio)acetamido]benzoate bromide; Tris, tris(hydroxymethyl)aminomethane.

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where I is the virgin inhibitor and I* is the modified inhibitor. The existence of this equilibrium has been established for numerous inhibitors of serine proteinases (Laskowski & Sealock, 1971; Finkenstadt et al., 1974).

The overall mechanism for the interaction of a great number of protein proteinase inhibitors with serine proteinases was first put forth by Luthy et al. (1973) for the interaction of STI with bovine β-trypsin. It is given by

$$E + I \underset{K_L}{\rightleftharpoons} L \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} C \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} L^* \underset{K_{L^*}}{\rightleftharpoons} E + I^* \qquad (2)$$

where E is the proteinase, I and I* are the virgin and modified (reactive-site peptide bond hydrolyzed) inhibitor, respectively, and C is the stable complex. L and L* are loose, noncovalent complexes of the enzyme with either virgin or modified inhibitor. L and L* are in extremely rapid equilibrium with E + I and E + I*, respectively; thus, the equilibrium constants K_L and K_{L^*} are shown instead of the individual rate constants. It should be noted that this is the minimal mechanism; additional intermediates are likely to exist and in fact have been shown to exist in some systems (Quast et al., 1978a,b).

Although this mechanism was found to be obeyed by several other inhibitors of serine proteinases, proof of reactive-site hydrolysis in the case of PTI was difficult to obtain. Chauvet & Archer (1967) were able to show that Lys^{15} of PTI is specifically involved in the interaction of PTI with bovine trypsin, and X-ray studies by Huber et al. (1974) indicated that the Lys^{15}–Ala peptide bond interacted with the active site of bovine trypsin in the trypsin–PTI complex. Specific hydrolysis of the Lys^{15}–Ala peptide bond was first obtained by Kress & Laskowski (1968). They obtained hydrolysis by incubating bovine trypsin with PTI in which the Cys^{14}–Cys^{38} disulfide bond had been selectively reduced and carboxamidomethylated. Their product, however, was not active as an inhibitor. Tschesche et al. (1974) utilized bovine trypsin to hydrolyze the Lys^{15}–Ala peptide bond of selectively reduced (Cys^{14}–Cys^{38}) PTI. Upon air oxidation of the free sulfhydryls, they obtained modified PTI which was active as an inhibitor.

Finally, in 1976, enzymatic hydrolysis of the reactive-site peptide bond in native PTI was achieved independently by two groups. Tschesche & Kupfer (1976) reported that plasmin, bovine β-trypsin, and bovine α-chymotrypsin hydrolyze the Lys^{15}–Ala reactive site of PTI but that the reactions proceed quite slowly, with the most rapid reaction, that of plasmin at pH 5.0, requiring 300 days to reach equilibrium. At the same time, studies in this laboratory (Estell et al., 1976) showed that the reactive site could be rapidly hydrolyzed (reaching equilibrium in 3–4 days) by a trypsinlike enzyme from the starfish *Dermasterias imbricata*.

In the preceding paper (Estell & Laskowski, 1980) we have described the isolation and characterization of this enzyme, designated *D. imbricata* trypsin 1. In this paper we present conclusive evidence that *D. imbricata* trypsin 1 provides a facile means of producing large amounts of modified (Lys^{15}–Ala peptide bond hydrolyzed) PTI. In addition, we determine the values of K_{hyd} over the pH range of 6.2–8.9, examine its pH dependence, and determine the kinetic parameters of this reaction.

The comparison of the kinetic parameters for bovine β-trypsin and for *D. imbricata* trypsin 1 in their interactions with PTI leads us to suggest that we need two molecular explanations for the interactions of enzymes with inhibitor reactive sites—one to account for the extreme specificity and high value of k_{cat}/K_m shown by both bovine trypsin and *D. imbricata* trypsin 1 and another to explain the exceptional stabilization of the enzyme–inhibitor complex shown by bovine trypsin but not by *D. imbricata* trypsin 1.

Experimental Procedures

Materials

Bovine pancreatic trypsin inhibitor (Kunitz), Trasylol, was a generous gift from Dr. E. Truscheit of Bayer A. G. *D. imbricata* trypsin 1 was prepared as described in the preceding paper (Estell & Laskowski, 1980). Bovine trypsin and α-chymotrypsin were obtained from Worthington Biochemical Corp. Bovine β-trypsin was purified by the method of Schroeder & Shaw (1968), as modified by Luthy et al. (1973).

The trypsin-specific burst titrant *p*-nitrophenyl *p*-guanidinobenzoate was a product of Nutritional Biochemical Corp. The fluorescent burst titrants 4-methylumbelliferyl *p*-guanidinobenzoate (Jameson et al., 1973) and 4-methylumbelliferyl *p*-[ω-(dimethylsulfonio)acetamido]benzoate bromide (Laskowski et al., 1974) were synthesized in this laboratory by Dr. P. Fankhauser, using methods similar to those given by Chase & Shaw (1967) and Wang & Shaw (1972) for the synthesis of the analogous *p*-nitrophenyl esters. For use these compounds were dissolved in dimethylformamide (1 mg/mL) and stored in ice.

Dansyl chloride [(*N*,*N*-dimethylamino)naphthalenesulfonyl chloride] was purchased from Pierce Chemical Co. Polyamide thin-layer sheets (Chen-Chin Trading Company, Ltd., Taipei, Taiwan) were obtained from Gallard-Schlesinger, New York. The carboxypeptidase B inhibitor, guanidinoethylmercaptosuccinic acid, was a generous gift from Dr. T. H. Plummer, Jr. (McKay & Plummer, 1978). All other chemicals were reagent grade or the best commercially available grade.

Methods

All incubations of inhibitor with *D. imbricata* trypsin 1 were carried out in solutions of 0.5 M KCl, 0.05 M $CaCl_2$, and 0.05 M Tris. This solvent system is consistently used in all quantitative studies of enzyme–inhibitor interactions in our laboratory. Inhibitor (6×10^{-7} mol of either PTI or PTI*) was incubated with either 1.3×10^{-9} mol of trypsin 1 (PTI) or 6.5×10^{10} mol of trypsin 1 (PTI*), in a total volume of 3 mL. PTI* is an unusually good substrate for carboxypeptidase B (Jehring & Tschesche, 1976), and some preparations of *D. imbricata* trypsin 1, in which no carboxypeptidase B activity could be detected by the standard hippuryl-L-arginine assay (Folk et al., 1960), were found to produce small amounts of de-Lys^{15}-PTI on long periods of incubation with PTI. For this reason, the carboxypeptidase inhibitor guanidinoethylmercaptosuccinic acid (5 mg) was added to each incubation mixture. Separate incubations of each inhibitor with trypsin 1 were carried out at pH 6.2, 6.6, 7.0, 7.4, 7.8, 8.2, 8.6, and 8.9, with temperature maintained at 21 ± 1 °C. At various times, 80-μL aliquots were withdrawn and treated with 40 μL of 0.1 M HCl to kill the reaction. These aliquots were then analyzed for PTI content and for total inhibitor content as described below.

Assay Procedures. Bovine β-trypsin concentrations were measured by active-site titration with GdnBzONp according to the method of Chase & Shaw (1967); the solutions thus assayed were used as reference standards for quantitation of the fluorescence burst titrations of bovine β-trypsin and α-chymotrypsin.

All fluorometric measurements were made by using a Perkin-Elmer MPF-2A fluorescence spectrometer with the following settings: excitation wavelength, 360 nm, band-pass, 6 nm; emission wavelength, 450 nm, band-pass, 10 nm. Enzyme solutions were assayed by adding either 20 nmol of GdnBzMum (β-trypsin) or 50 nmol of Me_2SAcNBzMum

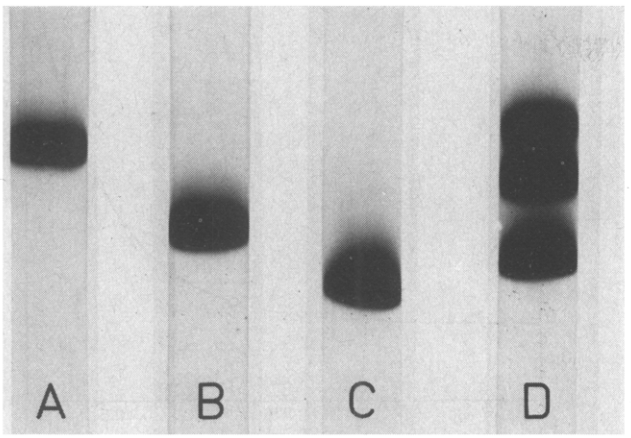

FIGURE 1: Polyacrylamide gel electrophoresis of PTI and its derivatives. Electrophoresis was at pH 9.3, with migration to the cathode at 0.003 A/gel for 120 min. (A) is 20 μg of de-Lys^{15}-PTI*; (B) is 20 μg of PTI*; (C) is 20 μg of PTI; (D) is 20 μg of de-Lys^{15}-PTI* plus 20 μg of PTI* plus 38 μg of PTI.

(α-chymotrypsin) to ~4 nmol of enzyme in 3 mL of 0.1 M veronal and 0.02 M $CaCl_2$, pH 8.3, in a 10-mm cuvette. After mixing, the increase in fluorescence as compared to an enzyme-free blank was measured, and the known concentration of β-trypsin was used to calibrate the signal in terms of nanomoles of enzyme per chart division. Because the same signal (release of 1 nmol of 4-methylumbelliferyl per 1 nmol of enzyme active site) was monitored for each enzyme, quantitation of the signal for β-trypsin also provided quantitation for the α-chymotrypsin signal. *D. imbricata* trypsin 1 concentrations were determined by GdnBzMum titration.

Concentrations of inhibitor solutions were determined by incubating up to 2 nmol of inhibitor with a known excess amount (~4 nmol) of enzyme for the required time and then analyzing by the above method. The ratio of virgin inhibitor concentration ([PTI]) to total inhibitor concentration ([PTI] + [PTI*]) was determined by utilizing the differences in association rates between PTI and PTI* in their interactions with bovine β-trypsin and α-chymotrypsin. For α-chymotrypsin, the second-order association rate constants for PTI and PTI* are respectively 6×10^5 and 8.5 M^{-1} s^{-1}; while, for β-trypsin, the corresponding values are 3×10^5 and 3×10^3 M^{-1} s^{-1} (Quast et al., 1978b). This disparity of rates indicates that, at the concentrations used for our assays, a 1-min incubation of a mixture of PTI and PTI* with α-chymotrypsin will permit only the formation of complex form PTI (at this time, >99.9% of the PTI and <0.1% of the PTI* have formed complex), thus giving a selective measure of the amount of PTI in the mixture. The total amount of inhibitor in the mixture can be determined by incubating an aliquot with β-trypsin for 2.5 h, which is sufficient time to permit complex formation from <99.99% PTI*.

Characterization of Proteins. Polyacrylamide disc gel electrophoresis of various components (Figure 1) and amino acid analyses (Tables I and II) were carried out as described in the preceding paper (Estell & Laskowski, 1980). Amino-terminal residues were determined with the use of dansyl chloride (Gray, 1967). The resulting dansyl amino acids were identified by thin-layer chromatography on polyamide sheets (Woods & Wang, 1967).

Results

*Preparation and Characterization of PTI**. PTI (2×10^{-5} mol in 40 mL of 0.03 M sodium borate, pH 10.0) was reacted with 2.4×10^{-9} mol of *D. imbricata* trypsin at 21 °C for 28

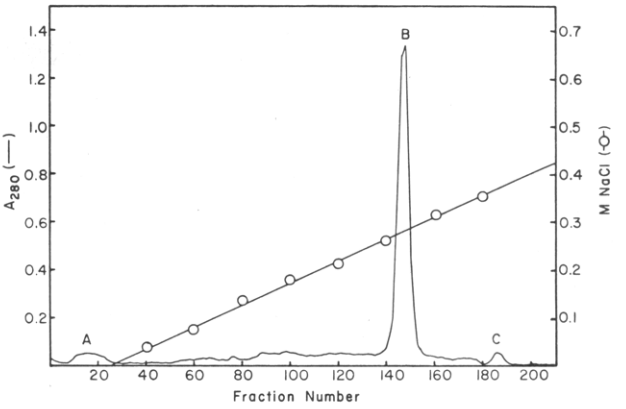

FIGURE 2: CM-Sephadex ion-exchange chromatography of a PTI → PTI* reaction mixture (reaction carried out at pH 10). Peak A is *D. imbricata* trypsin 1; peak B is PTI*; peak C is PTI.

Table I: Amino Acid Compositions of Modified Inhibitor Species[a]

AA	composition		
	PTI	PTI*	de-Lys^{15}-PTI*
Lys	4.12 (4)	4.16 (4)	3.05 (3)
Arg	6.08 (6)	6.16 (6)	5.91 (6)
Asp	5.05 (5)	5.06 (5)	4.93 (5)
Thr	2.90 (3)	3.03 (3)	2.83 (3)
Ser	1.10 (1)	1.10 (1)	0.96 (1)
Glu	3.00 (3)	3.00 (3)	3.00 (3)
Pro	4.06 (4)	4.02 (4)	4.09 (4)
Gly	5.96 (6)	6.01 (6)	5.84 (6)
Ala	5.92 (6)	5.96 (6)	5.70 (6)
Cys/2	6.08 (6)	5.55 (6)	5.50 (6)
Val	0.77 (1)	0.84 (1)	0.70 (1)
Met	0.82 (1)	0.79 (1)	0.80 (1)
Ile	1.66 (2)	1.82 (2)	1.74 (2)
Leu	1.90 (2)	1.92 (2)	1.86 (2)
Tyr	3.94 (4)	3.90 (4)	3.83 (4)
Phe	3.98 (4)	4.04 (4)	3.88 (4)
total:	58	58	57

[a] Average of 20- and 48-h hydrolyses except as follows: serine and threonine, zero-time corrected for destruction; isoleucine and valine, 48-h value. Based on glutamic acid = 3.00.

days. Analysis at this time by gel electrophoresis revealed the near-complete conversion of PTI to an electrophoretically different species (gel B, Figure 1). The reaction mixture was adjusted to pH 8.6 and charged onto a 1.5 × 68 cm column of CM-Sephadex C-25 equilibrated with the same buffer. The column was developed with a linear gradient of NaCl in 0.03 M sodium borate, pH 8.6 (Figure 2). The majority of the protein eluted as a single symmetric peak at 0.28 M NaCl. Only a small amount of protein was found at 0.37 M NaCl, the previously determined elution position of PTI. Fractions containing PTI* were pooled, adjusted to pH 2.2 with HCl, and desalted on a 2.5 × 84 column of Sephadex G-25 fine eluted with 0.01 M HCl. The desalted protein was then lyophilized.

Purified PTI* revealed a single band upon gel electrophoresis and was found to have an amino acid composition identical with that of PTI (Table I). It was found to be as fully active an inhibitor of bovine trypsin as virgin PTI, although its association rate was much slower. Treatment of PTI* with carboxypeptidase B resulted in its rapid inactivation, producing a third, more anionic species, de-Lys^{15}-PTI*. N-Terminal analysis of PTI* revealed two end groups, arginine (present in virgin PTI) and alanine, which is present since the Lys^{15}–Ala reactive-site peptide bond had been hydrolyzed. Additional proof that this particular peptide bond had been hydrolyzed was obtained by oxidizing PTI* with performic

Table II: Amino Acid Analyses[a] of the Peptides Obtained from Oxidized Modified PTI*

AA	composition small[b]	expected residues 1–15	large[c]	expected residues 16–58
Lys	1.01 (1)	1	3.07 (3)	3
Arg	1.01 (1)	1	5.03 (5)	5
Asp	1.00 (1)	1	4.00 (4)	4
Thr	0.95 (1)	1	1.92 (2)	2
Ser	0 (0)	0	0.90 (1)	1
Glu	0.99 (1)	1	2.09 (2)	2
Pro	3.50 (4)	4	0.42 (0)	0
Gly	1.00 (1)	1	4.90 (5)	5
Ala	0.06 (0)	0	5.71 (6)	6
Cys/2	1.90 (2)	2	3.56 (4)	4
Val	0 (0)	0	0.93 (1)	1
Met	0 (0)	0	0.93 (1)	1
Ile	0 (0)	0	1.56 (2)	2
Leu	0.94 (1)	1	1.09 (1)	1
Tyr	0.96 (1)	1	2.80 (3)	3
Phe	0.99 (1)	1	3.00 (3)	3
total:	15	15	43	43

[a] Analyses of 20-h hydrolysates of the oxidized peptides. [b] Determined by using Asp = 1.00. [c] Determined by using Asp = 4.00.

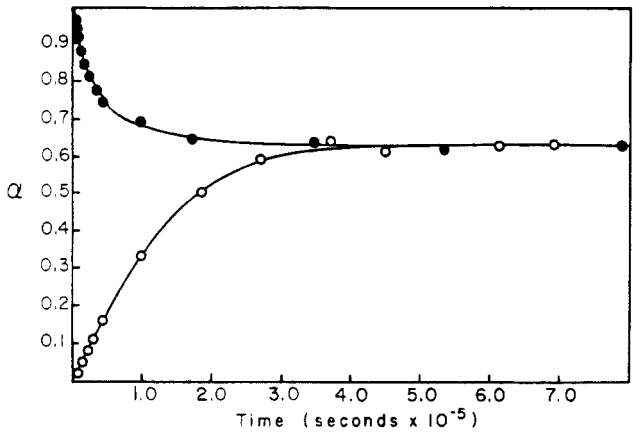

FIGURE 3: Dependence of the fraction (α) of modified inhibitor upon the time of incubation for the reaction of inhibitor with *D. imbricata* trypsin 1 in 0.5 M KCl and 0.05 M $CaCl_2$, pH 8.2, at 21 °C. (○) Initial mixture contained virgin inhibitor and 0.2 mol % trypsin 1; (●) initial mixture contained modified inhibitor and 0.1 mol % trypsin 1.

acid (Hirs, 1967) and separating the resulting fragments on Sephadex G-25 in 0.1 M HCl. The isolated fragments corresponded to residues 1–15 and 16–58 of PTI (Table II). When the above procedures were applied to de-Lys[15]-PTI*, the fragments were found to correspond to residues 1–14 and 16–58 of PTI.

Since at pH 8.6 *D. imbricata* trypsin 1 is strongly anionic, it does not bind to the CM-Sephadex column and may be totally recovered from the breakthrough fractions (see Figure 2). The enzyme may then be reused by simply dissolving PTI in the pooled breakthrough fractions and proceeding as described above. In this manner, a small amount of enzyme will suffice to produce very large amounts of PTI*. Although our initial preparations of PTI* were made at pH 10, due to the large value of K_{hyd} at that pH, we now routinely carry out preparations at pH 8.8. At this pH K_{hyd} is still large and, since we reuse the enzyme, we prefer the milder conditions of this pH.

pH Dependence of K_{hyd}. When the above reaction is carried out at pH values less than 10, a measurable equilibrium between PTI and PTI* is attained. Figure 3 is an example of

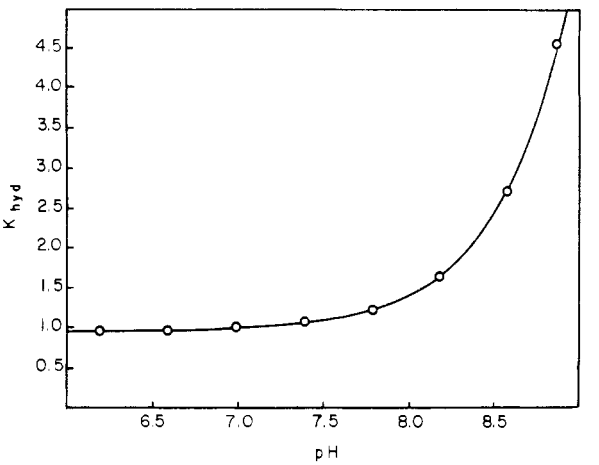

FIGURE 4: Equilibrium constant K_{hyd} for the PTI ⇌ PTI* reaction as a function of pH. The solid line is calculated from eq 4 with the parameters described in the text.

Table III: K_{hyd}^0 and pK_a Values for Reactive-Site Peptide Bonds[a]

peptide bond	K_{hyd}^0	pK_1	pK_2
Lys^{15}-Ala PTI	0.95	ND[e]	8.28
Arg^{63}-Ile STI (His^{71} unprotonated)[b]	1.56	3.56	7.89
Arg^{63}-Ile STI (His^{71} protonated)[b]	5.66	3.56	7.89
Arg^{89}-Ala chicken ovomucoid[c]	1.85	3.82	7.77
Arg^{18}-Ile bovine pancreatic secretory inhibitor[d]	0.26	3.17	ND

[a] All values listed were determined in 0.5 M KCl and 0.05 M $CaCl_2$. [b] Mattis & Laskowski (1973). [c] Schrode (1974). [d] Sealock & Laskowski (1973). [e] ND, not determined.

the results for the incubation of PTI and of PTI* with *D. imbricata* at pH 8.2. The quantity α is the ratio of [PTI] to the sum of [PTI] + [PTI*]. It is clear that the forward and reverse reactions coincide at equilibrium and define

$$K_{hyd} = [PTI^*]/[PTI] = \alpha/(1 - \alpha) \quad (3)$$

Analogous curves were obtained at each pH value, and the resultant K_{hyd} values are plotted in Figure 4. If the peptide bond hydrolysis does not perturb the pK values of any preexistent ionizable groups on the inhibitor (Mattis & Laskowski, 1973), the pH dependence of K_{hyd} should follow the expression first developed by Dobry et al. (1952):

$$K_{hyd} = K_{hyd}^0[1 + ([H^+]/K_1) + (K_2/[H^+])] \quad (4)$$

Here K_{hyd}^0 is the pH-independent equilibrium constant for formation from virgin inhibitor of a modified inhibitor with its Lys^{15} carboxyl group in the COO^- form and with its Ala^{16} amino group in the NH_3^+ form. K_1 and K_2 are the respective ionization constants of these groups. As is shown in Figure 4, the fit of the data to eq 4 is quite satisfactory. This fit was performed as described in Mattis & Laskowski (1973) by using the statistical weighting factor $1/(1 + K_{hyd})^4$ in order to correct for the larger experimental error inherent in large values of K_{hyd}. The best fit parameters are $pK_2 = 8.3$ and $K_{hyd}^0 = 0.95$. Due to instability of the starfish enzyme at low pH, K_1 could not be determined. Table III shows a comparison of these data with the known K_{hyd} and pK values for other inhibitors.

Because of the rather high value indicated for the pK of the amino group of the new N-terminal Ala, independent confirmation of this pK value was sought. To this end, difference titration studies of PTI* vs. PTI were performed. Titrations were carried out by using 9.60×10^{-7} mol of inhibitor in 3 mL of deionized water or in 3 mL of 0.5 M KCl and 0.05 M $CaCl_2$. Delivery of carbonate-free NaOH (0.0640 M) was

Table IV: Kinetic Parameters for Hydrolyses of Reactive-Site Peptide Bonds[a]

enzyme	pH	k_{-2} (s^{-1})	k_3 (s^{-1})	k_{-2}/k_3	k_3/K_I (s^{-1} M^{-1})
for STI					
trypsin 1 (*D. imbricata*)	8.0	0.34	0.12	3	4×10^5
β-trypsin[b] (bovine)	8.0	1.3×10^{-5}	2.5×10^{-6}	5	2×10^6
for PTI					
trypsin 1 (*D. imbricata*)	7.4	0.17	0.0029	60	
	7.8	0.065	0.0020	30	
	8.2	0.064	0.0016	40	1×10^6
β-trypsin[c] (bovine)	7.5	8×10^{-8}	8.7×10^{-10} [d]	90	10^4
α-chymotrypsin[d] (bovine)	7.5	9×10^{-4}	1.4×10^{-8} [d]	6.4×10^4	7

[a] $k_{-2} = k_{cat,r}$; $k_3 = k_{cat,f}$; $k_3/K_I = k_{cat,f}/K_{m,f}$. [b] Mattis (1974). [c] Quast et al. (1978a). [d] The original values for k_3 were calculated assuming a value for K_{hyd} at pH 7.5 of 0.38. The values reported here were recalculated from the data of Quast et al. (1978a) by using the value $K_{hyd} = 1.1$ at pH 7.5 reported in this paper.

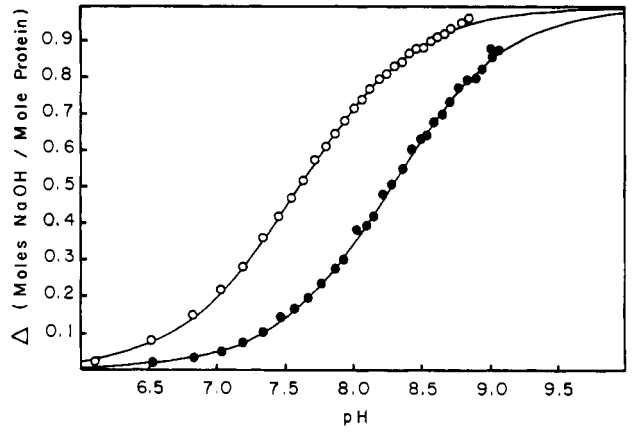

FIGURE 5: Difference titration curves for PTI* vs. PTI in water (○) and in 0.5 M KCl and 0.05 M $CaCl_2$ (●). The curves plotted were obtained by subtracting the titration curves for PTI from the titration curves for PTI*.

by a Radiometer autoburet, Model ABU 13, and the pH was monitored by using an Orion Research Model 601 digital ionalyzer. The number of nanomoles of base added per nanomole of inhibitor was recorded as a function of pH, and the values for PTI were subtracted from those for PTI* to give the difference titration curves (Figure 5). Computer analysis of the data indicates that (in the pH range investigated) PTI* contains one more ionizable group than does PTI and that this group exhibits a pK of 7.61 in deionized water[2] and a pK of 8.28 in 0.5 M KCl and 0.05 M $CaCl_2$. The value of 8.28 in the high ionic strength solvent system agrees quite well with the value of 8.3 calculated from the K_{hyd} data. The excellent fit of the difference titration data to titration curves for a single ionizable group provides further evidence that pK values for other groups in PTI are not perturbed.

Kinetics. Figure 3 provides not only equilibrium data but also data for the kinetics of the attainment of equilibrium. We are dealing here with a one substrate/one product enzyme-catalyzed reaction. The Michaelis–Menten expression for such a reaction is

$$\frac{d[PTI^*]}{dt} = \frac{[(k_{cat,f}/K_{m,f})[PTI] - (k_{cat,r}/K_{m,r})[PTI^*]][E]_0}{1 + [PTI]/K_{m,f} + [PTI^*]/K_{m,r}} \quad (5)$$

Integration of this equation, followed by simplification, yields

$$-(B + 1) \ln (1 - \alpha/\alpha_{eq}) - B\alpha/\alpha_{eq} = At \quad (6)$$

[2] Deionized water here means that the only salt contributed is by the titrant. This difference titration curve was obtained at low, but not constant, ionic strength.

For the forward reaction, the parameters A and B are given by

$$A_f = k_{cat,f}[E]_0/[\alpha_{eq}(K_{m,f} + [PTI]_0)] \quad (7)$$

and

$$B_f = [(K_{m,f}/K_{m,r} - 1)][PTI]_0\alpha_{eq}/(K_{m,f} + [PTI]_0) \quad (8)$$

where $[E]_0$ and $[PTI]_0$ are the initial enzyme and inhibitor concentrations. Equivalent parameters A_r and B_r were used for fits to the reverse data. This type of analysis was first applied by Mattis (1974) to the STI–bovine β-trypsin system, with the results reported in Finkenstadt et al. (1974). It is an adaptation of the earlier formulation by Peller & Alberty (1959), which describes the time course of reversible one substrate/one product enzyme-catalyzed reactions.

Since for these reactions $[PTI]_0$ is much smaller than K_m ($K_m = 1 \times 10^{-9}$ M; Estell & Laskowski, 1980), the forward and reverse rate constants, $k_{cat,f}$ and $k_{cat,r}$, could readily be calculated from the values of A_f and A_r. Assuming that k_3 (C → L* in eq 2) represents the rate-determining step in the conversion of PTI to PTI* and that k_{-2} (C → L in eq 2) represents the rate-determining step in the conversion of PTI* to PTI, $k_{cat,f}$ may be equated with k_3 and $k_{cat,r}$ may be equated with k_{-2}. These assumptions have been shown to hold true at neutral pH for the bovine trypsin–STI and bovine trypsin–PTI systems (Finkenstadt et al., 1974; Quast et al., 1978a). The data obtained in the preceding paper (Estell & Laskowski, 1980) for the *D. imbricata* trypsin 1 catalyzed hydrolysis of the Arg^{63}–Ile reactive site in STI were subjected to similar analyses. The rate constants obtained in this way are listed in Table IV, along with the equivalent values for bovine β-trypsin and α-chymotrypsin (Quast et al., 1978b). It is evident that, for the Lys^{15}–Ala reactive site of PTI, *D. imbricata* trypsin 1 catalyzes both the foward and the reverse reactions ~10^6 times more rapidly than does bovine β-trypsin.

From the mechanism given in eq 1, k_s, the dissociation rate constant for the enzyme–inhibitor complex, should be equal to the sum of k_{-2} and k_3. It can be seen (Table IV) that, for the *D. imbricata* trypsin 1-PTI complex, the sum of k_{-2} and k_3 at pH 8.2 agrees reasonably well with the value of k_d = 0.064 s^{-1} as determined at pH 8.3 in the preceding paper (Estell & Laskowski, 1980). For the *D. imbricata* trypsin 1–STI complex, the sum of the two dissociation rate constants yields a k_d which is too great to measure by the methods employed in that paper.

In the preceding paper (Estell & Laskowski, 1980), we reported the values of K_I for the interactions of STI and PTI with *D. imbricata* trypsin 1. According to the mechanism given in eq 2, these should correspond to $K_{m,f}$ for reactive-site peptide bond hydrolysis in the inhibitors. The values of $k_{cat,f}/K_{m,f}$ derived in this way are shown in Table IV in com-

parison with the values of $k_{cat,f}/K_{m,f}$ for the interactions of bovine β-trypsin with the same inhibitors. It is remarkable how similar the values are, in view of the dramatic differences in the $k_{cat,f}$ values for the two enzymes.

Discussion

The use of *D. imbricata* trypsin 1 clearly provides a facile means for the preparation of large quantities of PTI*. The method described here is much simpler than the indirect chemical method previously developed by Tschesche et al. (1974), which involves selective reduction of disulfide bonds, limited proteolysis by bovine trypsin, and reoxidation of free sulfhydryl groups. Since only a small quantity of *D. imbricata* trypsin 1 is required to catalyze the reaction and the enzyme is easily recoverable and may be reused essentially indefinitely, our method is well suited to the continued preparation of large quantities of PTI*.

The value of K_{hyd}^0 reported in this paper is 0.95 (Table III). Tschesche & Kupfer (1976) reported the value of $K_{hyd} = 0.33$ at pH 5.0. The two values are qualitatively quite similar in that both indicate that the free energies of virgin and of modified inhibitor are essentially the same. The two values are clearly different, however, within the limits of what we expect to be our experimental error in K_{hyd}^0 (roughly ± 0.04). While we were unable to measure K_{hyd} at pH 5, we regard such a sudden deviation from the equation of Dobry et al. (1952), eq 4, as highly unlikely. The cause of the discrepancy arises either as a result of the different ionic strengths used in the experiments—quite low in the experiment of Tschesche & Kupfer (1976) and high in ours—or to some systematic errors arising from the extremely long incubation times (on the order of 1 year) used by Tschesche & Kupfer (1976).

The values of K_{hyd}^0 can be compared to two different sets of values. One is the set of presumed values of K_{hyd}^0 for normal cyclic[3] peptide bonds in globular proteins. These are very poorly known. The general opinion is that most currently known limited proteolyses are "operationally irreversible". We estimate that such a statement means that hydrolysis is at least 95% complete; i.e., that K_{hyd}^0 is greater than 20. A somewhat better, but still questionable, estimate of $K_{hyd}^0 = 20$ was recently made by Homandberg & Laskowski (1979) for the Ala^{20}–Ser bond involved in the conversion of ribonuclease A to ribonuclease S. Niekamp (1971) [see also Laskowski (1970)] evaluated K_{hyd} for the reactive site of heat-denatured STI as 40; clearly, K_{hyd}^0 for PTI is much smaller than these values. We have interpreted, and continue to interpret, this difference as a strong indication that the reactive-site peptide bonds of inhibitors are very rigid and that conformational transitions after hydrolysis of this bond are very limited [Finkenstadt et al. (1974)—note, however, that the estimate of K_{hyd} for PTI given in that paper is grossly incorrect].

The second comparison of K_{hyd}^0 for PTI is to the values of K_{hyd}^0 for reactive sites of other protein proteinase inhibitors, some of which are listed in Table III. Here, the value of PTI is clearly intermediate. This is somewhat surprising, since two lines of reasoning suggest that it should be relatively large. First, Deisenhofer & Steigemann (1975) report that the reactive-site peptide bond and surrounding peptide bonds are strained in virgin PTI. This strain would presumably be relieved upon reactive-site peptide bond hydrolysis, leading to an increase in K_{hyd}^0 as compared to a situation in which no strain was involved. Second, comparisons of solvent perturbation, circular dichroism, fluorescence, and proton NMR spectra reveal that several amino acid residues gain rotational freedom upon PTI → PTI* conversion (Quast et al., 1975; Markley et al., 1976). This again should provide a driving force in the hydrolysis direction. It should be pointed out, however, that there may be no real basis for comparison, since the other inhibitors listed in Table III have not been subjected to similar analyses.

Over the observed pH range, the pH dependence of K_{hyd} obeys eq 4, indicating that in this pH range none of the preexistent ionizable groups in PTI have their pK values perturbed by the reactive-site bond hydrolysis. This conclusion is not only supported by the pH dependence of K_{hyd} but also supported by the shapes of the difference titration curves between PTI* and PTI. Clearly, the only difference is the addition of the newly formed amino-terminal group of Ala^{16} in PTI*. The conclusion of no additional pK perturbation is of interest, since Brown et al. (1978) report that for PTI in solution (not in the crystal) there is a salt bridge between the $-NH_3^+$ of Arg^1 and the $-COO^-$ of Ala^{58} and that the existence of this salt bridge perturbs the pK values of the participating groups. Our results therefore indicate that the hydrolysis of the Lys^{15}–Ala peptide bond has no effect on this salt bridge. The conformational transitions described by Quast et al. (1975) and by Markley et al. (1976) must be quite limited.

The pK value for the newly formed $-NH_3^+$ of Ala^{16} is measured as 8.3 by both the pH dependence of K_{hyd} and the difference titration curve (Figure 5) at high ionic strength. This value is higher than other values of reactive-site amino-terminal pK values. We are somewhat surprised by this result since the reactive-site region of PTI is very positive (Deisenhofer & Steigemann, 1975) and one might therefore expect the pK for this group to be lower than normal. The observed shift in pK, however, from 7.6 at very low ionic strength to 8.3 at high ionic strength is of the expected magnitude and direction for a positively charged reactive-site region in PTI*.

Some time ago we (Finkenstadt et al., 1974) pointed out that for the bovine trypsin catalyzed hydrolysis of reactive-site peptide bonds in protein trypsin inhibitors (such as STI and PTI), at neutral pH, $k_{cat,f}$ values are very small compared to those for normal substrates, while $k_{cat,f}/K_{m,f}$ ratios are quite normal or even high. The transition from a substrate to an inhibitor is made by a simultaneous decrease, by many orders of magnitude, of both $k_{cat,f}$ and $K_{m,f}$. Since for *D. imbricata* trypsin 1 the $k_{cat,f}$ values for STI and PTI are 5 and 6 orders of magnitude greater than those for bovine β-trypsin, it is of interest to examine the ratios of $k_{cat,f}/K_{m,f}$ for the hydrolysis of these reactive-site bonds by *D. imbricata* trypsin 1. Surprisingly, they are quite similar to the ratios for bovine β-trypsin. Even the relatively small changes may have a rather trivial explanation. STI and *D. imbricata* trypsin 1 are anionic, while bovine β-trypsin and PTI are strongly cationic. It is clear from Table IV that the reactions between like-charged proteins are relatively slower than the reactions between unlike-charged proteins. A slight cloud is cast on this explanation when we recall that these experiments were carried out at high ionic strength, where charge effects are likely to be masked, but we still believe the explanation to be credible. Thus (eliminating small effects or charge effects), $k_{cat,f}/K_{m,f}$ remains approximately the same in going from bovine β-trypsin to *D. imbricata* trypsin 1, while both $k_{cat,f}$ and $K_{m,f}$ rise dramatically. The same approximate conclusion is also reached when we compare bovine and human trypsins (Laskowski et al., 1974; Wilson, Sealock, and Laskowski, unpublished experiments).

In eq 2, we see that the enzyme–inhibitor complex can dissociate by two possible first-order reactions: k_{-2}, which

[3] The term "cyclic peptide bonds" is applied to those bonds whose hydrolysis produces only a single product, not two separate fragments.

produces the enzyme and virgin inhibitor, and k_3, which produces enzyme and modified inhibitor. As we move from bovine β-trypsin to *D. imbricata* trypsin 1, both k_{-2} and k_3 increase dramatically, but their ratio (k_{-2}/k_3) is almost unaffected, as can be seen in Table IV. Again, a similar conclusion is reached when bovine and human trypsins are compared, but, in this case, the rates themselves change less dramatically. It should be noted that the approximate constancy of k_{-2}/k_3 and of $k_{cat,f}/K_{m,f}$, in spite of tremendous changes in the parameters themselves, is not a general characteristic of all serine proteinases in their interactions with inhibitors. The above ratios are strikingly different for the interaction of bovine α-chymotrypsin with PTI (Table IV). The phenomenon we are dealing with appears to hold only for the interactions of trypsin and trypsinlike enzymes with trypsin-specific reactive sites. The exact definition of a trypsin in this context is as yet not very clear to us.

Let us now consider what we have learned. When *D. imbricata* trypsin 1 and bovine trypsin interact with STI or with PTI, they seek out *only one* of many possible Lys–X and Arg–X bonds—the reactive site. They hydrolyze this bond with a very high value of $k_{cat,f}/K_{m,f}$, another indication of a high degree of specificity. When they form the stable enzyme–substrate complex, this complex dissociates to reactants and to products at roughly the same relative rates. In the case of bovine trypsin, however, the complex is exceptionally stabilized against dissociation, while, in the case of the homologous *D. imbricata* trypsin 1, the complex is not. We believe that it will be a matter of great importance to find out the molecular basis for this difference. Unfortunately, neither the complete amino acid sequence nor the three-dimensional structure for either *D. imbricata* trypsin 1 or human trypsin (a less dramatic example, but a far more available protein) is as yet known.

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