Single Peptide Bond Hydrolysis/Resynthesis in Squash Inhibitors of Serine Proteinases. 2. Limited Proteolysis of *Curcurbita maxima* Trypsin Inhibitor I by Pepsin†

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ABSTRACT: Porcine pepsin hydrolyzes the Leu7–Met8 (P2′–P3′) peptide bond in *Cucurbita maxima* trypsin inhibitor I (CMTI I) in the pH range 2.0–4.8. The reaction proceeds to equilibrium between intact CMTI I and its cleaved form. The pH-independent value of the equilibrium constant ($K^0_{hyd} = 0.78$) indicates that both forms of the inhibitor have similar Gibbs energies. The pH dependence of this constant shows that the peptide bond hydrolysis does not perturb ionization constants of any preexistent groups. The same equilibrium values can also be reached from the cleaved inhibitor side through pepsin-catalyzed resynthesis of the Leu7–Met8 peptide bond. Catalytic rate constants for the forward (hydrolysis) and reverse (resynthesis) reactions are similar. Both catalytic rate constants are strongly pH dependent, approaching the highest values at pH 2.0. Michaelis constant values for hydrolysis and resynthesis reactions depend much less on pH and are within values typical for oligopeptide substrates of pepsin. The influence of the binding loop rigidity on slow proteolysis by pepsin and other proteinases is discussed.

Limited proteolysis plays a key role in the regulation of many physiological processes such as the blood coagulation cascade, fibrinolysis, complement system and zymogen activation, release of signal peptides, virus maturation, and hormone and growth factor processing (Neurath, 1985). In most of these events a hydrolyzed peptide bond has been successfully identified in an amino acid sequence of a precursor protein, in spite of the large size of a parent protein. In some spectacular instances, such as trypsinogen, chymotrypsinogen, pepsinogen, or procarboxypeptidase activation, processes could be described in structural terms due to determination of crystal structures for both a precursor and an active enzyme (Bode & Huber, 1986; James & Sielecki, 1986; Coll et al., 1991).

The availability of high-resolution structures served to correlate target proteolytic sites with their conformational features (Hubbard et al., 1991). Typically, such a peptide bond is located in a flexible part of the molecule, which forms a solvent-accessible loop. Unfortunately, stronger correlations are much more difficult to establish. Main-chain ϕ and φ angles in a nicksite region are markedly different. With a few exceptions, *e.g.*, trypsinogen activation (Garcia-Moreno et al., 1991), limited proteolysis reactions have not been analyzed in terms of steady-state parameters and substrate properties of hydrolyzed sites were not determined.

The only group of proteins for which limited proteolysis has been described in structural, thermodynamic, and kinetic terms is protein inhibitors of serine proteinases. Apart from serum inhibitors and hirudin, all other inhibitors share a probably common, so-called cannonical conformation of the main chain flanking the scissile P1–P1′ peptide bond. This range of proteins was intensively studied over the past two decades by X-ray diffraction techniques (Bode & Huber, 1992). It was observed that φ and ϕ dihedral angles for the P3–P3′ segment are similar, in spite of the fact that the inhibitors belong to otherwise sequentially and structurally unrelated proteins.

The complex formation with a proteinase does not change the binding loop conformation. Close conformational similarities of free and complexed inhibitor were observed for basic pancreatic trypsin inhibitor (Wlodawer et al., 1984), ovomucoid third domains (Bode et al., 1985), barley chymotrypsin inhibitor 2 (McPhalen & James, 1987), and *Cucurbita maxima* trypsin inhibitor I (CMTI I)[1] (Holak et al., 1989b). However, binding loops exhibit different levels of flexibility. A considerable flexibility observed in ovomucoid third domain in their uncomplexed state was significantly lowered upon complexation. Since the relative orientation of the binding loop is slightly different in complexes with different proteinases, it was postulated that the flexibility broadens an inhibitor specificity (Read & James, 1986). On the other hand, the binding loop of PTI displays a similar state of rigidity in unliganded and complexed state (Wlodawer et al., 1987).

The protein inhibitors of serine proteinases share a common, so-called, standard mechanism of interaction with a cognate enzyme (Laskowski & Kato, 1980). Most probably this results mainly from similar binding loop conformations. The mechanism was carefully analyzed for the soybean trypsin inhibitor (STI)–bovine β-trypsin system (Finkenstadt et al., 1974) and less intensively for a few others (Quast et al., 1978; Tonomura et al., 1985). In the preceding paper (Otlewski & Zbyryt, 1994) we described the squash inhibitor–β-trypsin interaction. We analyzed kinetic and thermodynamic parameters which are affected both by the inhibitor and by the proteinase. Here we analyze a selective proteolytic cleavage of CMTI I inhibitor by porcine pepsin. This enzyme selectively hydrolizes the Leu7–Met8 (P2′–P3′) peptide bond which is located close to

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[1] Abbreviations: BAPNA, Bz-L-Arg 4-nitroanilide; CMTI I, *Cucurbita maxima* trypsin inhibitor I; CPTI II, *Cucurbita pepo* trypsin inhibitor II; NPGB, 4-nitrophenyl 4-guanidinobenzoate; OMCHI2, chicken ovomucoid second domain; OMTKY3, turkey ovomucoid third domain; PSTI, pancreatic secretory trypsin inhibitor (Kazal); PTI, pancreatic trypsin inhibitor (Kunitz); STI, soybean trypsin inhibitor (Kunitz); TLCK, N^{α}-4-tosyl-L-Lys chloromethyl ketone.

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the Arg5–Ile6 (P1–P1′) peptide bond hydrolyzed by trypsin. Contrary to the standard mechanism, pepsin cleavage does not proceed via a stable complex and the inhibitor binding loop can be considered more like a typical substrate. Thus, an analysis of the limited proteolysis of both sites provides information on how the conformation of the binding loop affects substrate and inhibitory behaviour of the protein.

MATERIALS AND METHODS

Materials. CMTI I was purified as described elsewhere (Otlewski et al., 1984). The inhibitor was homogeneous in a native polyacrylamide gel electrophoresis and in an analytical reversed-phase HPLC. The purity of the inhibitor was additionally confirmed by an amino acid analysis. The pepsin-cleaved form of CMTI I was obtained after a 48-h incubation of 5 mM CMTI I in 50 mM formic acid, pH 1.5, with 5 mol % porcine pepsin. At this pH, CMTI I is almost completely (>99%) converted into the cleaved form. The reaction mixture was subsequently applied on a Bio-Gel P-10 column (1.5 × 120 cm) equilibrated with 50 mM formic acid, pH 1.5. Fractions containing the cleaved inhibitor were combined and lyophilized.

Pepsin three times crystallized was purchased from Fluka AG. Anydrotrypsin was prepared according to the method of Ako et al. (1972) and additionally purified on STI–Sepharose. Traces of trypsin activity were blocked with TLCK. Trypsin was purchased from Worthington Chemical Corp. Bio-Gels P-10 and P-2 were obtained from Bio-Rad Laboratories. Tosyl-L-Lys chloromethyl ketone (TLCK), Bz-L-Arg 4-nitroanilide (BAPNA), 4-nitrophenyl 4′-guanidinobenzoate (NPGB), soybean trypsin inhibitor (STI), and pepstatin A were purchased from Sigma Chemical Co. All other chemicals were of reagent grade.

General Methods. Polyacrylamide gel electrophoresis at pH 8.6 was performed according to the procedure of Davis (1964). Amino acid compositions were determined with a Aminochrom II amino acid analyzer after hydrolysis of samples (50 μg) in 5.7 M HCl at 110 °C under vacuum for 24 and 48 h. The N-terminal amino acids were determined as PTH derivatives on a Waters C_{18} "resolved" column as described by Lottspeich (1980). Trypsin and anhydrotrypsin were immobilized on Sepharose 4B as described by Knight and Light (1974). Affinity chromatography on trypsin– and anhydrotrypsin–Sepharose was performed in 50 mM Tris buffer and 20 mM $CaCl_2$, pH 8.0. CMTI I and the Leu7–Met8 cleaved form of the inhibitor were resolved by isocratic HPLC chromatography (Waters) on a TSK DEAE 5PW column, 7.5 × 75 mm (Pharmacia LKB) in 0.12 M Tris buffer, pH 8.55.

Standardization of Enzyme and Inhibitor Stock Solutions. The concentration of CMTI I was determined by titration with NPGB-standardized trypsin as described elsewhere (Otlewski et al., 1990). To determine the concentration of the Leu7–Met8 hydrolyzed form of CMTI I, the pepsin-cleaved inhibitor was mixed with a known amount of CMTI I and applied to the analytical ion-exchange HPLC column (TSK 5PW DEAE, 7.5 × 75 mm, LKB) followed by absorbance monitoring at 280 nm. The ratio of two integrated peaks was used to calculate the concentration of the cleaved inhibitor. Molar absorbances at 280 nm of intact and cleaved forms of CMTI I were the same within experimental error.

A stock solution of pepstatin A was prepared by dissolving 1.0 mg of the peptide in 1.0 mL of DMSO. The concentration of pepsin was determined by titration with pepstatin. Increasing amounts of pepstatin (0–70 μL of 2.9 × 10^{-6} M solution) were added to the constant amount of pepsin (600 μL of *ca.* 4 × 10^{-7} M), and the samples of the total volume of 700 μL in 0.1 M formate buffer, pH 3.0, were incubated for 10 min at 37 °C. Residual pepsin activity were subsequently measured according to the method of Suzuki et al. (1989).

Determination of k_{cat} and K_m for Hydrolysis and Resynthesis of the Leu7–Met8 Peptide Bond. Hydrolysis/resynthesis of the Leu7–Met8 peptide bond was monitored by a loss/recovery of the antitrypsin activity of CMTI I. Appropriate volumes of pepsin stock solution were added to mixtures containing 9 × 10^{-4} M CMTI I (or the pepsin-cleaved form of CMTI I). The final concentration of enzyme never exceeded 5 mol %. The reactions were performed at 22 °C in the following buffer systems: 0.1 M sodium glycine (pH 2.0–2.8); 0.1 M sodium formate (pH 3.2–4.0); 0.1 M sodium acetate (pH 4.4–4.8). After adequate time increments, aliquots were withdrawn from the reaction mixture and added to trypsin solutions (varied from 2 × 10^{-7} to 2 × 10^{-6} M) in 0.1 M Tris buffer, 20 mM $CaCl_2$, and 0.005% Triton X-100, pH 8.3. The samples were incubated for 10 min, and then trypsin activity was measured with 1.3 × 10^{-4} M BAPNA as a substrate (Erlanger et al., 1961) on a HP 8452A diode array spectrophotometer (Hewlett-Packard) in the wavelength range from 380 to 410 nm. The initial velocities of BAPNA hydrolysis expressed concentrations of the uncleaved inhibitor. The concentration of cleaved inhibitor was calculated as a difference between total and intact inhibitor concentration. The experimental points were converted to α ($\alpha = [I]_{split}/[I]_{total}$), and the data were fitted using nonlinear least-squares analysis according to the integrated Haldane equation for a reversible one substrate–one product reaction (Estell et al., 1980; see also the preceding paper).

Michaelis constants for the hydrolysis reaction ($K_{m,f}$) were determined by measuring the initial rates of peptide bond hydrolysis vs CMTI I concentration. The data were fitted by nonlinear least-squares regression to the Haldane equation. The inhibitor concentration ranged from 5 × 10^{-5} to 4 × 10^{-3} M, and pepsin was used up to 5 mol %. The reactions were performed as described above.

Determination of the Hydrolysis Constant (K_{hyd}). After a prolonged time of incubation, the samples were withdrawn from the reaction mixtures and submitted to the analytical ion-exchange HPLC as described under General Methods. The ratio of two integrated peaks (cleaved to intact form) provided the K_{hyd} value. The hydrolysis constant was also estimated using the equilibrium data α_{eq} obtained from integrated Haldane equation (see above). The pH dependence of K_{hyd} was analyzed according to the equation of Dobry et al. (1952)

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

where K^0_{hyd} is the pH-independent hydrolysis constant, K_1 is the dissociation constant of the carboxyl group of Leu7, and K_2 is the ionization constant of the amino group of Met8.

RESULTS

Identification of the Pepsin Cleavage Site. Incubation of CMTI I with a catalytic amount of pepsin generates one additional form of the inhibitor. The electrophoretic and chromatographic behavior of this form is identical with the reactive site cleaved form (see previous paper), thus suggesting that only one peptide bond is hydrolyzed by the enzyme. Since the amino acid compositions of both forms are the same (data not shown) and N-terminal amino acid analysis of CMTI I

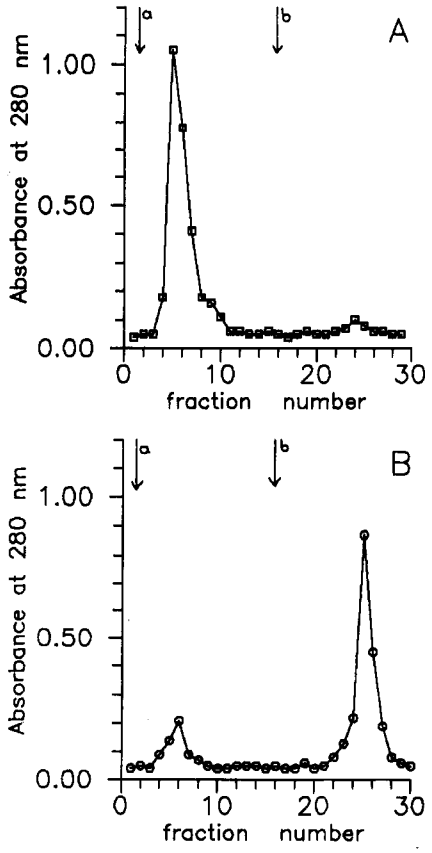

FIGURE 1: Affinity chromatography on anhydrotrypsin–Sepharose (A) and trypsin-Sepharose (B) of pepsin-cleaved CMTI I. In each case 12 mg of CMTI I cleaved at the Leu7–Met8 peptide bond was applied to a 1 × 7 cm column equilibrated with 50 mM Tris and 20 mM $CaCl_2$, pH 8.0. The anhydrotrypsin column was able to bind 15 mg of intact CMTI I. The trypsin column bound 22 mg of CMTI I. Arrows indicate elution buffers: (a) 50 mM Tris, 20 mM $CaCl_2$, pH 8.0; (b) 50 mM sodium formate, pH 3.0. Elution was conducted at a flow rate of 20 mL/h; 2-mL fractions were collected.

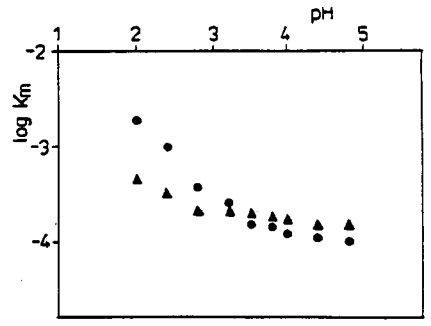

FIGURE 2: Plots of log $K_{m,f}$ (●) and log $K_{m,r}$ (▲) vs pH for the porcine pepsin catalyzed hydrolysis of the Leu7–Met8 (P2′–P3′) peptide bond in CMTI I.

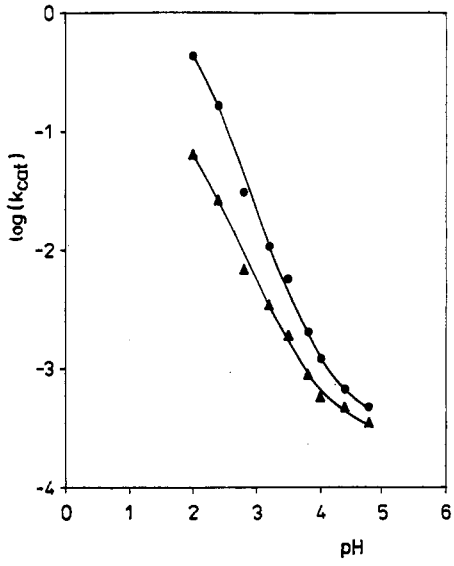

FIGURE 3: pH dependence of the catalytic rate constants $k_{cat,f}$ (●) and $k_{cat,r}$ (▲) for hydrolysis and resynthesis of the Leu7–Met8 peptide bond in CMTI I by pepsin.

digested by pepsin shows two peaks corresponding to Arg (the N terminus in the intact CMTI I) and Met residues (the only Met in the sequence), we have concluded that the cleavage occurs between Leu7 and Met8.

The pepsin-hydrolyzed CMTI I does not inhibit trypsin (Otlewski & Wilusz, 1985). The loss of antitrypsin activity results from the release of the Ile6–Leu7 dipeptide from the inhibitor after trypsin action on the Arg5–Ile6 reactive site peptide bond. To confirm this, we applied pepsin-digested CMTI I on trypsin- and anhydrotrypsin–Sepharose columns (Figure 1). The inhibitor was bound on anhydrotrypsin–Sepharose at pH 8.0, and it was eluted at pH 3.0. The eluted protein showed the same amino acid composition as the protein applied. However, when the pepsin-cleaved inhibitor was applied on the active-trypsin–Sepharose column at pH 8.0, the material was not bound. A subsequent size exclusion chromatography of the unbound material on Bio-Gel P-2 column yielded two peaks corresponding to des-[Ile6–Leu7]–CMTI I and Ile6–Leu7 dipeptide, as judged from the amino acid analyses (data not shown).

Kinetics and Thermodynamics of the Leu7–Met8 Peptide Bond Hydrolysis. Pepsin-catalyzed hydrolysis/resynthesis of the Leu7–Met8 pepetide bond is a reversible one substrate–one product reaction described by four steady-state parameters: $k_{cat,f}$, $K_{m,f}$, $k_{cat,r}$, and $K_{m,r}$. We determined these values using a procedure applied for the reactive site peptide bond hydrolysis/resynthesis by trypsin (see preceding paper). The values of Michaelis constants for the hydrolysis and resynthesis of the Leu7–Met8 peptide bond in the pH range 2.0–4.8 are similar to those of regular turnover substrates (10^{-3}–10^{-5} M). As shown in Figure 2, $K_{m,f}$ and $K_{m,r}$ are rather insensitive to pH. Slight varitions are observed only at low pH values; at higher pH the curves are flat. On the other hand, the forward and reverse catalytic rate constants vary by 3 orders of magnitude and reach maximal values of 0.43 and 0.21 s^{-1} at pH 2.0, respectively (Figure 3). The pH dependencies of $k_{cat,f}$ and $k_{cat,r}$ are very similar, although the catalytic rate constants for the reverse reaction are systematically 3–5 times lower than respective values of $k_{cat,f}$.

Figure 4 shows typical time courses of the fraction of cleaved inhibitor (α) for the reaction started with either the intact or cleaved inhibitor. At each single pH, the curves coincided at the same value of α, indicating that the equilibrium was attained. The equilibrium values of α, obtained from a three-parameter fit to the integrated Haldane equation, were used to calculate the hydrolysis constants from the relation $K_{hyd} = \alpha_{eq}/(1-\alpha_{eq})$. The values of K_{hyd} were also directly calculated from the chromatographic patterns of reaction mixtures as described under Materials and Methods. Both procedures yielded extremely similar (±2%) values of the equilibrium constants. Figure 5 presents the pH dependence of K_{hyd} for the Leu7–Met8 peptide bond. The experimental data were fitted to the equation of Dobry et al. (1952) to obtain values of pH-independent hydrolysis constant K^0_{hyd} and the ionization constant of the newly formed carboxyl group of Leu7 residue (K_1). We applied a two-parameter fit (only K^0_{hyd} and

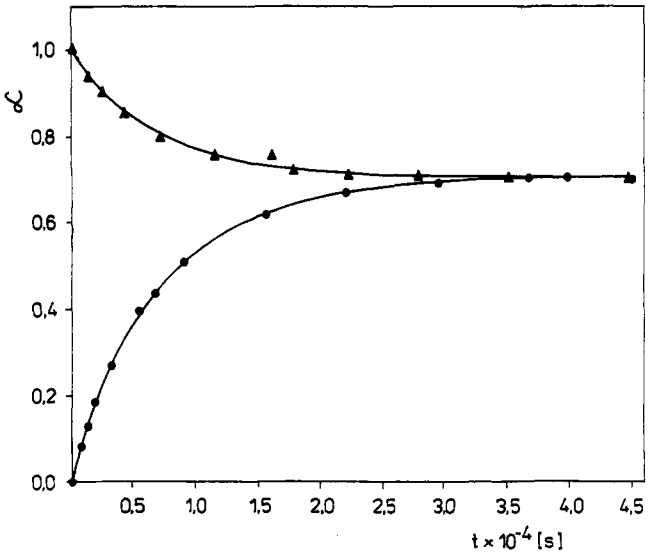

FIGURE 4: Time course of the hydrolysis (●) and resynthesis (▲) of the Leu7–Met8 peptide bond in CMTI I by porcine pepsin. A fraction of the cleaved inhibitor concentration to the total inhibitor concentration is expressed as α. The hydrolysis conditions are 0.1 M sodium formate, pH 3.5, 9.06×10^{-4} M, and 2.4 mol % pepsin at 22 °C. Conditions for resynthesis are the same except that the pepsin concentration is 3 mol %. Presented curves are recalculated to 5 mol % pepsin. The experimental points were fitted to the integrated Haldane equation.

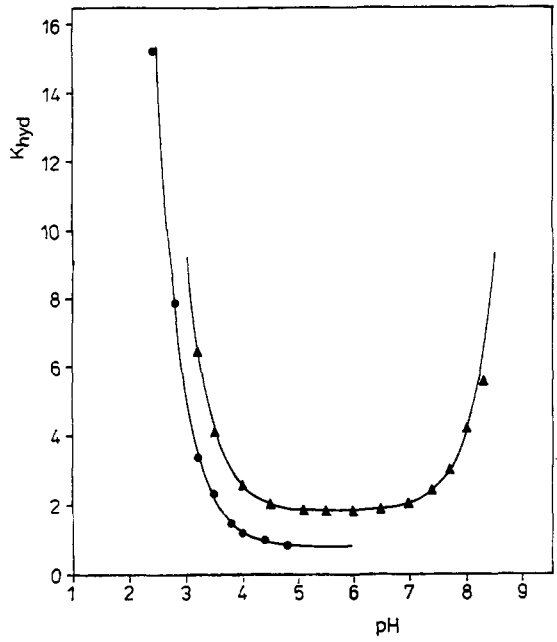

FIGURE 5: pH dependence of the equilibrium constant (K_{hyd}) for the hydrolysis/resynthesis of the Leu7–Met8 (●) and Arg5–Ile6 (▲) (preceding paper) peptide bonds in CMTI I. The experimental values of K_{hyd} were analyzed by nonlinear least squares to the Dobry equation.

K_1 were treated as parameters) and assumed that the value of K_2 (ionization constant of the newly formed NH_3^+ group of Met8) is 7.8. Table 1 shows ionization constants and hydrolysis constants for the Leu7–Met8 and Arg5–Ile6 peptide bond cleavages in CMTI I inhibitor and also for the reactive sites in other protein inhibitors.

DISCUSSION

This paper describes the kinetics and thermodynamics of pepsin action on CMTI I inhibitor. At low pH values (below 5.0), the enzyme selectively hydrolyzes the Leu7–Met8 peptide bond. This bond is located close to the reactive site (Arg5–Ile6) within the same covalent loop cross-linked by the Cys3–Cys20 disulfide bond. The Arg5–Ile6 peptide bond is selectively and slowly hydrolyzed by bovine trypsin—an enzyme strongly inhibited by CMTI I. According to X-ray (Bode et al., 1989) and NMR (Holak et al., 1989a; Habazettl et al., 1992) structures of the inhibitor, both peptide bonds are on the surface of the molecule and are accessible to a proteolytic cleavage. The region of inhibitor from Arg1 to Met8 is highly complementary to trypsin and forms the extended and hydrophobic trypsin binding loop. The conformation of this loop in complex with trypsin is extremely well-defined, and its main-chain atoms exhibit the lowest temperature *B* factors in the whole molecule (Bode et al., 1989). The NMR solution structure of the inhibitor shows that the uncomplexed loop has almost identical conformation (Holak et al., 1989b). However, the configurations of its side chains were difficult to determine due to high accessibility to a solvent (Holak et al., 1991).

Table 1: K^0_{hyd} and pK Values for the Leu7–Met8 Peptide Bond Hydrolysis/Resynthesis in CMTI I and Reactive Site Peptide Bonds in Protein Proteinase Inhibitors

inhibitor	hydrolyzed peptide bond	K^0_{hyd}	pK_1	pK_2
CMTI I[a]	Leu7–Met8	0.78	3.2	
CMTI I[b]	Arg5–Ile6	1.83	3.81	7.80
CPTI II[b]	Lys5–Ile6	1.61[c]		
PTI[d]	Lys15–Ala16	0.90	3.1	8.22
STI[e]	Arg63–Ile64 (His71$^+$)	5.66	3.56	7.89
STI[e]	Arg63–Ile64 (His71^0)	1.56	3.56	7.89
PSTI[e]	Arg18–Ile19	0.26	3.17	
OMCHI2[e]	Arg18–Ala19	1.85	3.82	7.77
OMTKY3[f]	Leu18–Glu19	0.97	3.27	7.83

[a] This work. [b] Preceding paper. [c] Estimated from K_{hyd} value at pH 6.0 = 1.61. [d] Siekmann et al. (1988). [e] Finkenstadt et al. (1974). [f] Ardelt and Laskowski (1991).

Recently, we found that proteinase K predominantly cleaves the Ile6–Leu7 peptide bond (unpublished data), whereas thermolysin hydrolyzes the reactive site peptide bond Arg5–Ile6 at 49 °C (Stachowiak et al., 1990). Searching for proteolytic sites outside the binding loop segment in native CMTI I using different proteinases never succeeded (Stachowiak et al., 1990). Thus, the loop seems to be a choice target for specific hydrolysis of neighboring peptide bonds by different proteinases. This is reminiscent of the so-called bait region of the 1450 amino acid residue α_2-macroglobulin chain. Only a narrow segment of nine peptide bonds in the central part of this protein is specifically cleaved by several proteinases of different specificity (Sottrup-Jensen, 1989). Similarly, the reactive site loop of α_1-proteinase inhibitor appeared to be very sensitive to limited proteolysis (Mast et al., 1992). However, in the case of serum inhibitors the proteolysis is irreversible and leads to a major structural rearrangement of the inhibitor molecule manifested in a huge increase of conformational stability (Powell & Pain, 1992). As discussed below, these features are not relevant to trypsin and pepsin cleavages within the proteinase binding loop of CMTI I.

The hydrolysis of the Leu7–Met8 peptide bond is reversible, as is the trypsin-catalyzed proteolysis of the reactive site (see the preceding paper). Thus, the native form of the inhibitor can be converted into its cleaved form and *vice versa*. The hydrolysis/resynthesis equilibria are proteinase-independent and result from the thermodynamic equilibrium between two inhibitor forms. We showed that the equilibrium constant (K_{hyd}) can be measured either by separating both inhibitor forms at an equilibrium on an analytical anion-exchange

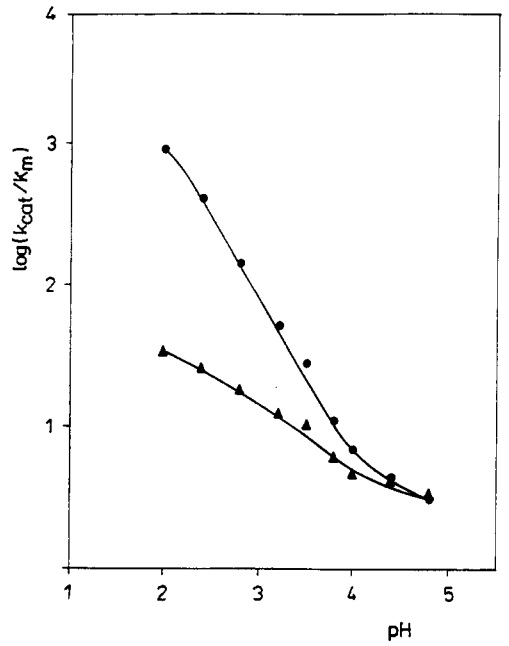

FIGURE 6: Plot of the specificity indices vs pH for hydrolysis ($k_{cat}/K_m)_f$ (●) and resynthesis ($k_{cat}/K_m)_r$ (▲) of the Leu7–Met8 peptide bond in CMTI I by porcine pepsin.

column or by measuring changes in the inhibitory activity resulting from hydrolysis (resynthesis) of the Leu7–Met8 peptide bond. Both methods provide similar values of K_{hyd}.

K_{hyd} values are strongly pH-dependent. Due to the denaturation of pepsin at pH values above 5, we could only measure this constant in an acidic region. A two-parameter fit of the K_{hyd} data (Figure 5) yielded the values of K^0_{hyd} and pK of the carboxyl group of Leu7. The value of K^0_{hyd} (0.78) is similar to the value for the reactive site (Arg5–Ile6) in CMTI I inhibitor (1.84). This indicates that both inhibitor forms have similar Gibbs energies, and therefore, probably no major conformational changes occurs after hydrolysis at either of these two sites.

Rather small differences in K^0_{hyd} values may result from the different chemical nature of hydrolyzed bonds, as observed in model dipeptides (Giletto et al., 1993) and ovomucoid third domains (Ardelt et al., 1991). The rigidity of the binding loop manifested in similar K^0_{hyd} values for the P1–P1′ and P2′–P3′ peptide bonds is a global feature of this polypeptide segment. According to our data, the hydrolysis of the Leu7–Met8 peptide bond does not perturb the dissociation constant of any other ionizable group in the inhibitor. This can be judged by good agreement of our data to the Dobry equation (Figure 5). Similarly, we did not observe any influence of the Arg5–Ile6 peptide bond hydrolysis on pK of other ionizing groups in CMTI I. Recently, Krishnamoorthi et al. (1992) reported that the pK of His25 is changed by 0.25 unit upon the reactive site peptide bond hydrolysis. We could not confirm this perturbation.

The time course of hydrolysis and resynthesis reactions catalyzed by pepsin provided kinetic values of k_{cat} and K_m for hydrolysis (forward) and resynthesis (reverse) reactions. The $k_{cat,f}$ value is strongly pH-dependent, changing by 3 orders of magnitude in the pH range from 2.0 to 4.8 (Figure 6). The constant reaches a maximum value 0.27 s^{-1} at pH 2.0. This is *ca.* 3 orders of magnitude lower than those for good oligopeptide substrates of pepsin (Fruton, 1976).

The specificity index (k_{cat}/K_m) for pepsin-catalyzed hydrolysis and resynthesis of the Leu7–Met8 peptide bond in CMTI I reaches a maximal value *ca.* 10^3 M^{-1} s^{-1} at pH 2.0 (Figure 6). This value is 1–2 orders of magnitude lower than those for good oligopeptide substrates of pepsin (Fruton, 1976). This may be partially explained by the presence of an Arg residue at P3 (by definition Leu7–Met8 is the P1–P1′ peptide bond), which is poor for porcine pepsin (Dunn et al., 1985). Also, aliphatic residues at the P1 and P1′ positions are significantly less favorable, as compared to Phe and Trp at P1 and P1′, respectively (Fruton, 1970). We suppose that rigidity of the cleaved region might also contribute to rather low values of kinetic parameters.

It has been suggested that a rigid body movement of both pepsin domains plays a part in the catalysis (Sali et al., 1992). In the former paper we have postulated that slow hydrolysis of the reactive site by trypsin might result from rigidification of the binding loop upon the complex formation, which blocks domain–domain movement in the enzyme (Dufton, 1990). The same can be referred to pepsin hydrolysis. Moreover, thermolysin-catalyzed hydrolysis of the reactive site proceeds very slowly (Stachowiak et al., 1990). Very recent X-ray analysis of this enzyme suggests that the conformational change plays an essential part in catalysis (Holland et al., 1992). In summary, we assume that enzyme flexibility is critical for the effective hydrolysis of amide bonds in different classes of proteinases. The rigid conformation of the substrate mediates against catalysis.

The catalytic rate constant measured for the reverse (resynthesis) reaction is slightly lower than that for the hydrolysis reaction in the whole pH range. It should be emphasized that, contrary to trypsin, maximal values of steady-state parameters k_{cat} and K_m are reached at pHs where pepsin is the most active. In the case of trypsin-catalyzed hydrolysis of the reactive site, these relations were inverse as a result of accumulation of the strong Michaelis complex. We failed to demonstrate any inhibition of pepsin by CMTI I, even at millimolar concentrations of the inhibitor.

In conclusion, we showed for the first time the selective proteolytic cleavage within the proteinase binding loop of serine proteinase inhibitors, which does not occur at the P1–P1′ site. We have determined kinetic and thermodynamic constants for the Leu7–Met8 peptide bond hydrolysis by the aspartic proteinase–porcine pepsin. Similar to the trypsin-catalyzed reaction at P1–P1′ (Arg5–Ile6), pepsin also resynthesizes the peptide bond, and the Gibbs energies of pepsin- and trypsin-cleaved forms are similar. Thus, the phenomenon of a reversible proteolysis is probably a common feature of the binding loop. As we have discussed in the previous paper, this is a prerequisite for the standard mechanism. CMTI I–pepsin interaction does not proceed through a stable complex. As a result, the pH dependencies of the steady-state parameters are similar to those of regular substrates.

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