Single Peptide Bond Hydrolysis/Resynthesis in Squash Inhibitors of Serine Proteinases. 1. Kinetics and Thermodynamics of the Interaction between Squash Inhibitors and Bovine β-Trypsin†

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ABSTRACT: The substrate and inhibitory parameters are described for the interaction between *Cucurbita maxima* trypsin inhibitor I (CMTI I) and bovine β-trypsin. The data are fully consistent with the reactive site hypothesis and the standard mechanism proposed for the protein inhibitor–serine proteinase interaction. The second-order association rate constant (k_{on}) for the interaction of the intact inhibitor and trypsin is high, above 10^6 M^{-1} s^{-1}. The same value is only 22-fold lower for the reactive site hydrolyzed inhibitor. This result implicates a very low transition-state barrier for the hydrolysis of the Arg5–Ile6 reactive site peptide bond. The equilibrium constant K_a ($=1/K_{m,f}$) and K_{assoc} change by 6 orders of magnitude in the pH range 4.0–8.3. The steady-state parameters for the hydrolysis and resynthesis of the reactive site have been determined over the pH range 3.2–8.3. Catalytic rate constants, but not k_{cat}/K_m, exhibit strong pH dependence. The dependence of the hydrolysis constant (K_{hyd}) on pH fits the simplest form of the Dobry equation, indicating that after the hydrolysis of the reactive site, pK values of any preexistent groups are not perturbed. It is suggested that a major factor leading to high k_{cat}/K_m values is the presence of Arg or Lys residues at the P1 position. Low values of K_m result from a conservation of the ground-state conformation of the inhibitor binding loop upon the complex formation. The crucial stage of the reactive site hydrolysis seems to be associated with a change of basic side-chain interactions within the S1 binding pocket.

Protein inhibitors of serine proteases are of particular interest in the study of protein–protein interactions and enzyme catalysis. Fundamental aspects of inhibitor action are nicely linked with applied research, since inhibitors are potential and real drugs in many pathophysiological diseases. Over the past 30 years, a great amount of data has been accumulated concerning amino acid and nucleotide sequences and three-dimensional structures both for free inhibitors and for enzyme–inhibitor complexes.

In spite of these achievements, complete analysis of the serine proteinase–protein inhibitor interaction has been investigated only for the bovine β-trypsin–soybean trypsin inhibitor (STI)[1] system [reviewed by Laskowski and Kato (1980)]. The same mechanism of action for inhibitors representing other inhibitor families is usually anticipated. However, this conclusion is based on limited kinetic and thermodynamic data.

According to the STI–trypsin interaction model, each inhibitor molecule (or inhibitor domain) contains a single peptide bond formed between the P1 and P1′ residues [notation of Schechter and Berger (1967)] called the reactive site, which is selectively cleaved by a cognate enzyme. The serine proteinase–protein inhibitor interaction can be presented as

$$E + I \underset{k_{off}}{\overset{k_{on}}{\rightleftarrows}} C \underset{k^*_{off}}{\overset{k^*_{on}}{\rightleftarrows}} E + I^* \qquad (1)$$

(called the standard mechanism) where E is the proteinase, I is the intact inhibitor, I* is the reactive site cleaved inhibitor, C is a stable Michaelis-type inhibitor–proteinase complex, k_{on} and k^*_{on} are second-order rate constants for the formation of the complex between proteinase and reactive site intact or cleaved inhibitor, respectively, and k_{off} and k^*_{off} are first-order dissociation rate constants of the complex.

The mechanism of inhibition resembles hydrolysis of regular substrates with a few major exceptions (Laskowski & Kato, 1980). (i) The complex C is much more stable than the Michaelis ES complex. The complex exhibits all typical features of a protein–protein recognition (Janin & Chothia, 1990). (ii) The catalytic rate constant for hydrolysis of the reactive site is extremely low. (iii) This hydrolysis is reversible, *i.e.*, the cleaved inhibitor is active and forms the same complex with the enzyme as the intact form. (iv) The equilibrium value of [I*]/[I] (hydrolysis constant, K_{hyd}) is usually close to unity at neutral pH values. The last statement results from the inhibitor properties; all others are features of the particular enzyme–inhibitor interaction.

Since the enzyme–inhibitor association energy is very high, it is difficult to precisely measure. The same holds for most of the individual rate constants. Moreover, two steady-state equilibria occur during the complex formation (Finkenstadt & Laskowski, 1967). The first results from the intact inhibitor–proteinase interaction. It is described by association constant $K_a = [C]/[E][I]$. At this stage, kinetics and thermodynamics of association share features typical for other protein–protein recognition. The second equilibrium, described by $K_{assoc} = [C]/([E]([I] + [I^*]))$, is a global and final one, reflecting the presence of the cleaved inhibitor. Without careful kinetic analysis, it is not possible to decide which equilibrium constant is measured.

Crystallographic and NMR investigations of free inhibitors representing different families and proteinase–inhibitor complexes [reviewed by Bode and Huber (1992)] are comple-

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[1] Abbreviations: CMTI I, *Cucurbita maxima* trypsin inhibitor I; CPTI II, *Cucurbita pepo* trypsin inhibitor II; NPGB, 4-nitrophenyl 4-guanidinobenzoate; PTI, pancreatic trypsin inhibitor (Kunitz); STI, soybean trypsin inhibitor (Kunitz).

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mentary to the standard mechanism. The interactions between proteinase and inhibitor resemble a productive binding of peptide substrates. In particular, the carbonyl carbon of the P1 residue is in close proximity (2.6–2.8 Å) to the catalytic nucleophile O^{γ} of Ser195 (Read & James, 1986; Bode & Huber, 1992). The P1–P1′ peptide bond is intact in the stable complex. Structural investigations revealed that the conformation of the inhibitor main chain, which makes the strongest interactions with a proteinase (P3–P3′ segment), is very similar in different inhibitor families (so-called canonical conformation; Read & James, 1986; Bode et al., 1987; Bode & Huber, 1992). Thus, from the functional and structural point of view, it is possible to distinguish between scaffold, which is the major part of the inhibitor, and the solvent-exposed proteinase binding loop (Bode & Huber, 1991). A global three-dimensional architecture, secondary structure, and disulfide bonds pairing of scaffold are unique for each inhibitor family.

Many results collected over the past years emphasize a decisive role of the binding loop on inhibitory properties. The contribution of individual binding loop residues to the total association energy is predominantly additive (Laskowski et al., 1987; Wells, 1990). These additive effects were responsible for successful conversion of specificities of many different inhibitors into the new ones, which were directed against important physiological proteinases (Jallat et al., 1986; Beckmann et al., 1988; McWherter et al., 1989; Collins et al., 1990; Rolka et al., 1991).

The value of K_{hyd} can also be rationalized in structural terms (Ardelt & Laskowski, 1991). K_{hyd} is a local property of the binding loop and can serve as the binding loop rigidity index. K_{hyd} is hardly influenced by scaffold residues, unless they directly maintain its conformation. The highly conserved network of hydrogen bonds spanning from the hydrophobic core to the reactive site region was observed in several inhibitor families (McPhalen et al., 1985; Onesti et al., 1991). These structural elements clearly rigidify the binding loop, even after hydrolysis of the reactive site peptide bond. The crystallographic and NMR structures of cleaved inhibitors exhibit similar conformations to intact forms with obvious differences in the cleavage region (Rhyu & Markley, 1988; Musil et al., 1991; Krishnamoorthi et al., 1992).

In this and the following paper we compare thermodynamics and kinetics of the hydrolysis/resynthesis for two single peptide bonds within the proteinase binding loop of *Cucurbita maxima* trypsin inhibitor I (CMTI I), a representative of the squash inhibitor family [for a review, see Otlewski (1993)]. Due to their small size, their resistance to proteolysis, and the stability and rigidity of the molecule, squash inhibitors received great interest as potential therapeutic agents. Their natural variants inhibit serine proteases of ultimate physiological importance—human Hageman factor (Hojima et al., 1982; Wynn & Laskowski, 1990), human cathepsin G, and human plasmin (Otlewski et al., 1990). Moreover, since they are relatively easy to synthesize, many chemical variants active against pathogenic proteinases have been produced (McWherter et al., 1989; Favel et al., 1989; Rolka et al., 1989).

CMTI I consists of 29 amino acid residues cross-linked by 3 disulfide bonds. The high-resolution X-ray structure is available for the CMTI I–bovine β-trypsin complex (Bode et al., 1989). Moreover, the high-resolution NMR structure of unliganded CMTI I was recently elucidated (Holak et al., 1989a, 1991; Nilges et al., 1991). This paper strongly confirms that Arg5–Ile6 is the reactive site peptide bond and that the inhibitor obeys the standard mechanism. In the accompanying paper, we probed the properties of the reactive site loop by the highly selective cleavage of the Leu7–Met8 bond using an aspartic proteinase–porcine pepsin.

MATERIALS AND METHODS

Reagents. 4-Nitrophenyl 4-guanidinobenzoate (NPGB) was a product of Merck (Darmstadt, Germany). Turnover substrates of trypsin Bz-L-Arg 4-nitroanilide and Bz-L-Val-Gly-L-Arg 4-nitroanilide and Bz-L-Ile-L-Glu-Gly-L-Arg 4-nitroanilide from Kabi Vitrium. Bio-Gels were products of Bio-Rad, and SP-Sepharose was from Pharmacia. All other chemicals were of reagent or HPLC grade.

Proteins. Squash inhibitors were isolated and purified as described elsewhere (Otlewski et al., 1984). Their homogeneity was confirmed by native polyacrylamide gel electrophoresis and reversed-phase HPLC. Cleaved forms of the inhibitors (the reactive site peptide bond hydrolyzed) were prepared by a 48-h incubation of 70 mg of the inhibitor in 50 mM sodium formate and 20 mM $CaCl_2$, pH 2.8, with 0.2 mg of bovine trypsin (Worthington Chemical Corp.) in a total volume of 3 mL at 22 °C. Trypsin was subsequently removed by size exclusion chromatography on a Bio-Gel P-10 column (1.5 × 120 cm) equilibrated with 50 mM formic acid, pH 1.5. The fractions containing antitrypsin activity were combined and applied on a SP-Sepharose column (1 × 10 cm) equilibrated with 50 mM sodium formate, pH 3.2. Intact and cleaved inhibitors were resolved by a linear gradient of NaCl (0–0.3 M). Finally, the cleaved inhibitor was desalted on a Bio-Gel P-2 column (1 × 50 cm) in 50 mM formic acid, pH 1.5. Bovine β-trypsin was obtained according to a method of Liepnicks and Light (1974).

Standardization of Enzyme, Inhibitor, and Substrate Stock Solutions. Stock solutions of trypsin and β-trypsin (ca. 10^{-4} M) were prepared in 1 mM HCl containing 20 mM $CaCl_2$. The concentrations of trypsin and β-trypsin were determined by active-site titration with trypsin-specific burst titrant NPGB (Chase & Show, 1967). In turn, the enzyme solution was used to titrate the inhibitors. Stock solutions of the chromogenic substrates were made in dimethyl sulfoxide and standardized by complete hydrolysis of the substrate. The concentration of released 4-nitroaniline was calculated using a molar absorbance coefficient ϵ_{410} = 9620 M^{-1} cm^{-1}. The concentrations of the enzyme and substrate were always estimated just before measurements. The titrated inhibitors were kept at −20 °C for a few weeks.

Determination of the Second-Order Association Rate Constants k_{on} and k^{*}_{on}. The rate constants were measured according to the procedure of Beatty et al. (1985). An equimolar amount of β-trypsin (with respect to the inhibitor) was delivered to the reaction mixture containing 1.5–9.0 × 10^{-9} M squash inhibitor and 7.75 × 10^{-5} M (0.15K_m) D-Val-L-Leu-L-Lys pNA in 0.1 M Tris, 20 mM $CaCl_2$, and 0.005% Triton X-100, pH 8.3, at 22 °C. Typically, the cuvette volume consisted of 10 μL of the inhibitor solution, 10 μL of the enzyme solution, 30 μL of the substrate solution, and buffer up to 3.0 mL. Then, a release of 4-nitroaniline was continuously monitored at 410 nm on a HP 8452A diode array spectrophotometer (Hewlett-Packard). The experimental points were transferred to an IBM PC computer and fitted to the equation (Beatty et al., 1985)

$$[P] = [S_0](1 - \exp(-k_{cat}/K_m[E_0]t_{1/2}) \ln(1 + (t + d)/t_{1/2})) - [P_0] \quad (2)$$

$[P_0]$ is the concentration of product at $t = 0$, $[S_0]$ is the initial substrate concentration, $[E_0]$ is the total enzyme concentration, $t_{1/2}$ is the half-time of the second-order inhibitor–enzyme association, d is the dead-time of the method, and k_{cat} and K_m

Table 1: Kinetic and Thermodynamic Parameters for Hydrolyses of the Reactive Site Peptide Bonds in Protein Proteinase Inhibitors

inhibitor–enzyme	pH	K_{hyd}	k_{on} (M^{-1} s^{-1})	k^*_{on} (M^{-1} s^{-1})	k_{on}/k^*_{on}	$k_{cat,f} = k^*_{off}$ (s^{-1})	$K_{m,f}$ (M)	$k_{cat,r} = k_{off}$ (s^{-1})	$K_{m,r}$ (M)	$(k_{cat}/K_m)_f$ (M^{-1} s^{-1})	$k_{cat}/K_m)_f$ (M^{-1} s^{-1})
CMTI I–bovine β-trypsin	8.3	5.59	6.7×10^6	3.1×10^5	21.6	5.2×10^{-6}	2.5×10^{-12}	2.5×10^{-5}	1.0×10^{-10}	2.1×10^{-6}	2.5×10^5
CPTI II–bovine β-trypsin	8.3	4.92	5.8×10^6	2.5×10^5	23.2	2.1×10^{-6}	1.2×10^{-12}	7.4×10^{-6}	3.2×10^{-11}	1.8×10^{-6}	2.3×10^5
STI–*D. imbricata* trypsin 1[a]	8.0	3.4	1.1×10^6	1.1×10^5	10.2	1.2×10^{-1}	3.0×10^{-7}	3.4×10^{-1}	2.8×10^{-6}	4.0×10^5	1.2×10^5
STI–bovine β-trypsin[b]	8.0	3.4	1.0×10^7	5.5×10^5	18.0	2.5×10^{-6}	1.3×10^{-12}	1.3×10^{-5}	2.2×10^{-11}	2.0×10^6	5.9×10^5
PTI–*D. imbricata* trypsin 1[a]	8.2	1.8[c]	4.0×10^7	5.6×10^5	72.0	1.6×10^{-3}	1.6×10^{-9}	6.4×10^{-2}	1.1×10^{-7}	1.0×10^6	5.6×10^5
PTI–bovine β-trypsin[d]	7.5	1.1[c]	8.8×10^5	8.8×10^3	100.0	8.7×10^{-10}	9.6×10^{-14}	8.0×10^{-8}	8.8×10^{-12}	1.0×10^4	9.1×10^3

[a] Data obtained or calculated from the Estell et al. (1980). [b] Data obtained or calculated from Finkenstadt et al. (1974). [c] Data obtained or calculated from Siekmann et al. (1988). [d] Data obtained or calculated from Quast et al. (1978).

are the steady-state parameters for β-trypsin-catalyzed hydrolysis of D-Val-L-Leu-L-Lys pNA (see below). During minimization of the function $[P_0]$, $k_{cat}/K_m[E_0]$ and $t_{1/2}$ were treated as variable parameters. The rate constants were then calculated from the relation

$$k_{on} = 1/(t_{1/2}[E_0]_{cor}) \quad (3)$$

where $[E_0]_{cor} = [E_0]*(1 + [S_0]/K_m)^{-1}$ is the substrate-corrected total enzyme concentration. Under applied conditions, the method accounts for reversibility of the reaction and substrate competition. The specificity index (k_{cat}/K_m) for bovine β-trypsin with D-Val-L-Leu-L-Lys pNA was determined in the absence of the inhibitor. Starting conditions were exactly the same. The time course of the substrate depletion was fitted to

$$[P] = [S_0](1 - \exp(-k_{cat}/K_m[E_0]t)) \quad (4)$$

by applying a one-parameter (k_{cat}/K_m) minimization procedure. Evaluated constants are the mean values from three to five experiments.

Equilibrium Association Constants. The equilibrium association constants were determined at 22 °C, in the pH range 3.5–8.3, using the method of Green and Work (1954), as modified by Empie and Laskowski (1982). The following chromogenic substrates were used to monitor residual trypsin activity: pH 3.5, Bz-L-Ile-L-Glu-Gly-L-Arg 4-nitroanilide; pH 4.0–8.3, Bz-L-Val-Gly-L-Arg 4-nitroanilide. Michaelis constants for each pH were determined in two or three experiments using the integrated Michaelis–Menten equation. The time to achieve equilibrium for the enzyme–inhibitor association was optimized for each pH. Due to relatively high k_{on}/k^*_{on} values (~20, see Results), the time course of the complex formation displays two steady states, the so-called "overshoot" phenomenon (Finkenstadt & Laskowski, 1967). Therefore, the association was approached from the intact inhibitor side (the measured values are close to $K_a = [C]/[E][I]$), as well as from the cleaved inhibitor CMTI I* side [the measured equilibrium constants are close to the overall equilibrium constant, $K_{assoc} = [C]/[E]([I] + [I^*])$]. At pH 3.2 the association constant was established by a UV difference spectroscopy as described earlier for bovine trypsinogen–CMTI I association (Zbyryt & Otlewski, 1991). Total enzyme concentration was 9.0×10^{-5} M; initial inhibitor concentration ranged from 3.2×10^{-5} to 8.5×10^{-4} M.

Determination of the Steady-State Parameters and Hydrolysis Constants. Hydrolysis/resynthesis of the reactive site peptide bond in squash inhibitors (2×10^{-7} mol) with a catalytic amount of bovine β-trypsin (up to 50 mol %) was carried out in 0.1 M buffer at 22 °C in a total volume of 200 μL. The following buffers were used: sodium formate (3.2–4.0), sodium acetate (4.5–5.5), Mes (6.0–6.5), Hepes (7.0–7.4), and Tris (7.7–8.3). All buffers contained 20 mM $CaCl_2$. After various time increments appropriate volumes (usually 10 μL) were withdrawn from the reaction mixtures and

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               1       5 6*      10        15        20        25     29
CMTI I    R-V-C-P-R-I-L-E-M-E-C-K-K-D-S-D-C-L-A-E-C-V-C-L-E-H-G-Y-C-G
CMTI III  R-V-C-P-R-I-L-K-M-E-C-K-K-D-S-D-C-L-A-E-C-V-C-L-E-H-G-Y-C-G
CPTI II   R-V-C-P-K-I-L-E-M-E-C-K-K-D-S-D-C-L-A-E-C-I-C-L-E-H-G-Y-C-G
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FIGURE 1: Amino acid sequences of CMTI I, CMTI III, and CPTI II inhibitors from squash seeds (Wieczorek et al., 1985). The reactive site P1–P1 peptide bond is indicated with an asterisk.

immediately submitted to the analytical ion-exchange (TSK 5PW DEAE, 7.5 × 75 mm column, LKB) HPLC chromatography (Waters M625) resolved isocratically with 0.12 M Tris buffer, pH 8.55. The ratio of two integrated peaks at 280 nm corresponding to intact and cleaved forms of inhibitor was used to calculate the fraction of cleaved inhibitor $\alpha = 1/(1 + [I]/[I^*])$. Absorbances at 280 nm of both inhibitor forms were identical as checked by UV difference spectrum. The time dependence of α was fitted to the equation (Estell et al., 1980)

$$-(B + 1)\ln(1 - \alpha/\alpha_{eq}) - B(\alpha/\alpha_{eq}) = At \quad (5)$$

The parameters for the forward reaction were defined as follows:

$$A_f = k_{cat,f}[E_0]/(\alpha_{eq}(K_{m,f} + [I_0])) \quad (6)$$

$$B_f = (K_{m,f}/K_{m,r} - 1)[I_0]\alpha_{eq}/(K_{m,f} + [I_0]) \quad (7)$$

Analogous parameters were used to analyze the reverse (resynthesis) reaction. $[E_0]$ and $[I_0]$ are the total enzyme and inhibitor concentrations; k_{cat} and K_m are the catalytic rate constant and the Michaelis constant, respectively, for hydrolysis (resynthesis) of the reactive site peptide bond; α_{eq} is the equilibrium value of α. The hydrolysis constant of the reactive site peptide bond was obtained from the relation $K_{hyd} = \alpha_{eq}/(1 - \alpha_{eq})$. The pH dependence of K_{hyd} was analyzed by a three-parameter minimization algorithm according to the relation of Dobry et al. (1952):

$$K_{hyd} = K^0_{hyd}(1 + [H^+]/K_1 + K_2/[H^+]) \quad (8)$$

K^0_{hyd} is a pH-independent hydrolysis constant and K_1 and K_2 are the dissociation constants of the reactive site carboxyl and amino groups, respectively.

RESULTS

Enzyme–Inhibitor Association. Table 1 summarizes the values of the association rate constants of squash inhibitors with bovine β-trypsin. We have examined three members of the squash inhibitor family—CMTI I, CMTI III, and CPTI II as well as their reactive site cleaved forms. Amino acid sequences of these inhibitors are shown in Figure 1. CMTI I differs from CPTI II by two substitutions: Arg → Lys at P1 and Val → Ile at P16′. The conformations of CMTI I and CPTI II are virtually identical as judged from 2D NOESY spectra of both inhibitors (J. Otlewski and T. A. Holak, unpublished results). Therefore, the CMTI I to CPTI II comparison reveals that the Arg → Lys substitution at the P1

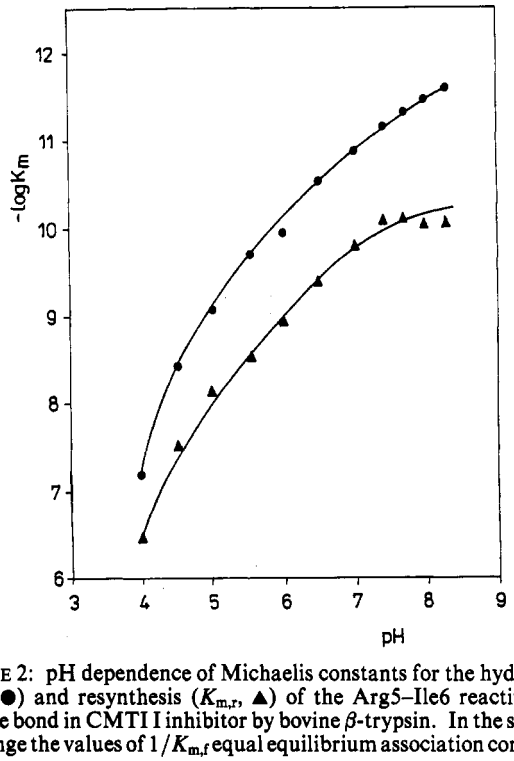

FIGURE 2: pH dependence of Michaelis constants for the hydrolysis ($K_{m,f}$, ●) and resynthesis ($K_{m,r}$, ▲) of the Arg5–Ile6 reactive site peptide bond in CMTI I inhibitor by bovine β-trypsin. In the studied pH range the values of $1/K_{m,f}$ equal equilibrium association constants K_a.

position does not affect the association rate constant. Also, the Lys → Glu substitution at position P4′ (CMTI I → CMTI III) does not change the rate of association (k_{on} and k^*_{on} values for CMTI III at pH 8.3 are, respectively, 4.8×10^6 and 2.3×10^5 M^{-1} s^{-1}), although this substitution causes a large change in the charge of the side chain. For all three inhibitors k^*_{on} values are about 20-fold lower than the rate constants measured for intact forms. We never observed systematic deviations from the second-order kinetics at enzyme and inhibitor concentrations used to determine k_{on} and k^*_{on}. Therefore, in this paper we do not consider loose complexes L and L*, which accumulate at high reactant concentrations (Finkenstadt et al., 1974).

The association equilibrium constant ($K_a = 1/K_{m,f} = [C]/[E][I]$) measured for CMTI I–bovine β-trypsin drops 6–7 orders of magnitude in the pH range 3.2–8.3 (Figure 2). Also, K_{assoc} exhibits a similar pH dependence, since both constants are linked by the simple relation $K_a = K_{assoc}/(1 + K_{hyd})$ (Finkenstadt et al., 1974). The pH dependence of K_a exhibits a multiproton transition below pH 5.0, similar to STI- and BPTI–β-trypsin associations (Finkenstadt et al., 1974). At higher pH, a single proton transition attributed to ionization of His-57 in the enzyme (Antonini et al., 1983) was observed.

Hydrolysis and Resynthesis of the Reactive Site Peptide Bond. As mentioned above, CMTI I* rapidly inhibited catalytic activity of trypsin. Therefore, the Arg5–Ile6 peptide bond hydrolysis/resynthesis could not be followed by a loss/recovery of the inhibitory activity. However, intact and cleaved forms could be baseline-separated by anion-exchange chromatography at pH 8.55 due to an extra negative charge at the newly released carboxyl group of the modified inhibitor. Since many (over 500) injections were required to investigate the kinetics of hydrolysis/resynthesis, isocratic conditions (0.12 M Tris, pH 8.55) have been chosen. Under applied conditions, intact and cleaved forms were fully and rapidly separated without significant broadening of the peaks (retention times about 6 and 13.5 min, respectively) and small amounts (up to 5 mol %) of trypsin–inhibitor complex were eluted in the dead volume (Figure 3). A typical time course of the cleavage and resynthesis of the reactive site peptide bond in CMTI I by bovine β-trypsin is shown in Figure 4. Both curves coincide at a very similar value of α_{eq}, suggesting that the reaction approached equilibrium from both sides. The hydrolysis/resynthesis reactions were carried out in the 3.2–8.3 pH range. Figure 5 presents the pH dependence of the hydrolysis constant (K_{hyd}) for the Arg5–Ile6 peptide bond (reactive site) in CMTI I. The values of K_{hyd} were obtained from the equilibrium ratio of [I*]/[I], as described above. The experimental data fit well to the three-parameter equation of Dobry et al. (1954), suggesting that after the reactive site hydrolysis, preexistent ionizable groups in the inhibitor are not perturbed. The values of the pH-independent hydrolysis constant K^0_{hyd} and ionization constants of the newly formed C- and N-terminal groups in the inhibitor were calculated to be 1.84, 3.81, and 7.80, respectively. The ionization constant of the amino group of Ile6 is very close to the pK value obtained from kinetic studies

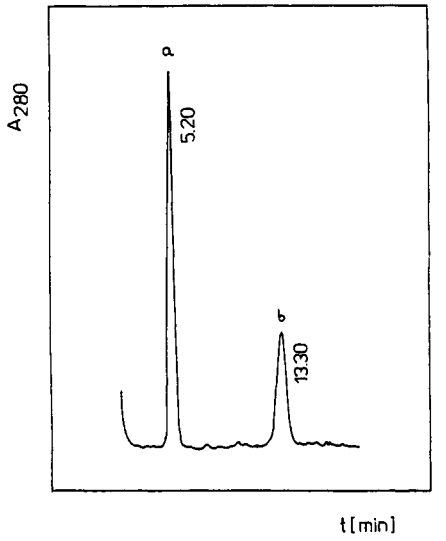

FIGURE 3: Separation of intact (peak A) and reactive site hydrolyed (peak B) forms of CMTI I inhibitor by the analytical anion-exchange HPLC. For experimental procedures, see Materials and Methods.

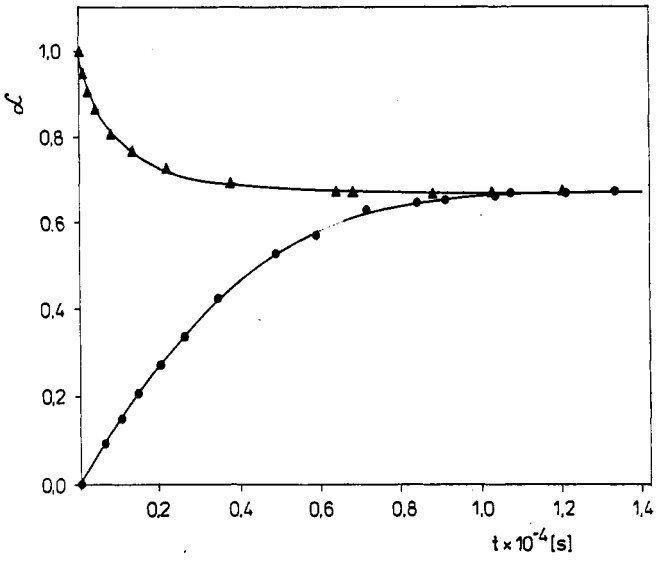

FIGURE 4: Time course of the hydrolysis (●) and resynthesis (▲) of the Arg5–Ile6 reactive site peptide bond in CMTI I by bovine β-trypsin. The conditions for the reactions are: 0.1 M sodium acetate, pH 4.5, 1.0×10^{-3} M CMTI I, and 1.8 mol % trypsin (forward reaction) or 0.5×10^{-3} M CMTI I and 0.3 mol % trypsin (reverse reaction). The points were analyzed according to the integrated Haldane equation. Both forward and reverse curves are recalculated to 5 mol % enzyme.

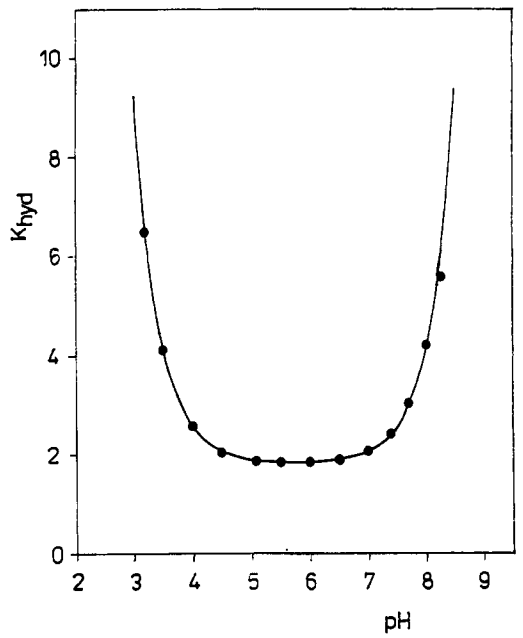

FIGURE 5: pH dependence of the equilibrium constant (K_{hyd}) for the hydrolysis/resynthesis of the Arg5–Ile6 reactive site peptide bond in CMTI I. The experimental values of K_{hyd} were fitted by a nonlinear least squares to the Dobry equation.

of the trypsinogen–Ile-Val–CMTI I* interaction (pK = 7.9; Zbyryt & Otlewski, 1991), indicating that the three-parameter model is satisfactory.

The K^0_{hyd} for hydrolysis of CPTI II reactive site (Lys5–Ile6) was not calculated from the pH dependence. Instead, it was approximated to be 1.62 by the value of K_{hyd} measured at pH 6.0 (Table 1). This approximation seems to be reasonable, since at pH 6.0 $[H^+]/K_1 \ll 1$ and $K_2/[H^+] \ll 1$ (see eq 8). Moreover, in the case of CMTI I, we did not detect any group ionizing around pH 6.0 which perturbs the K_{hyd} dependence on pH.

Kinetics. Besides K_{hyd} values, a time course of hydrolysis and resynthesis reactions provided data for the calculation of the steady-state parameters. Experimental points were fitted to the integrated form of the Haldane equation for a reversible one substrate–one product reaction described by four steady-state parameters—$k_{cat,f}$, $k_{cat,r}$, $K_{m,f}$, and $K_{m,r}$. For each pair of curves, a set of five parameters were generated: A_f, B_f, A_r, B_r and α_{eq}, which were subsequently used to calculate the steady-state parameters (Estell et al., 1980). Since only three of them are independent (the Haldane equation), Michaelis constants for the forward reaction were established by approximation: $K_{m,f} = 1/[K_{assoc}(1 + K_{hyd})]$. This is true above pH 4.0, where kinetics of hydrolysis and resynthesis are dominated by the stable complex C and other intermediates do not accumulate (Finkenstadt et al., 1974).

Unlike regular turnover substrates, Michaelis constants for the interaction of CMTI I with trypsin are extremely low at neutral pHs. Over the pH range 4.0–8.3, $K_{m,f}$ and $K_{m,r}$ change several orders of magnitude, reaching values of 10^{-11}–10^{-12} M at pH 8.3 (Figure 2). The ratio of $K_{m,f}$ to $K_{m,r}$ also decreases with pH. The catalytic rate constants for I → I* and I* → I conversion decrease dramatically with pH (Figure 6). At higher pH (above 4.0), the rate-limiting step is the dissociation of the stable complex. Over this pH range, the catalytic rate constant for the forward reaction, $k_{cat,f} = k^*_{off}$, whereas for the reverse reaction, $k_{cat,r} = k_{off}$. These conclusions are not relevant at lower pH. The $k_{cat,r}$ vs pH dependence shows a maximum, which results from the opposite runs of two curves describing the pH dependence of rate constants for two

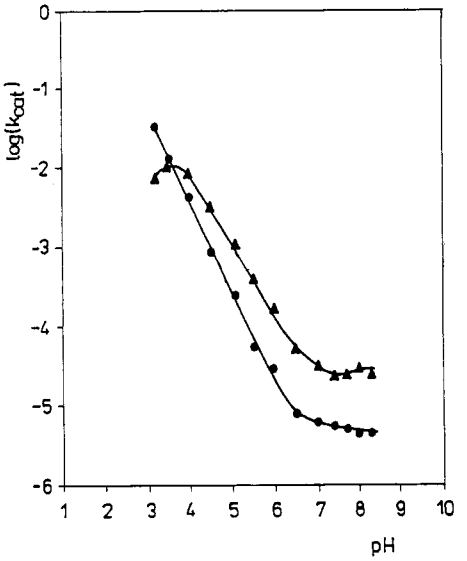

FIGURE 6: pH dependence of the catalytic rate constants for trypsin-catalyzed hydrolysis ($k_{cat,f}$, ●) and resynthesis ($k_{cat,r}$, ▲) of the Arg5–Ile6 peptide bond in CMTI I.

different steps. It means that at low pH two single steps of the overall reaction (eq 1) proceed with comparable rate and, therefore, the catalytic rate constant, $k_{cat,r}$, cannot be approximated by the dissociation rate constant of the stable complex. The specificity index k_{cat}/K_m for CMTI I–bovine β-trypsin interaction is very high, but contrary to regular substrates, this is mainly due to extraordinarily low values of K_m, rather than high values of k_{cat}. The value of k_{cat}/K_m for CPTI II is very similar (Table 1). The specificity indices for the reverse reactions are about 8-fold lower; again, both inhibitors exhibit very similar effects. Thus, small but opposite effects of the Lys → Arg substitution on K_m and k_{cat} cancel, giving (almost) identical values of the specificity constant for both directions of reaction. Although separated constants K_m and k_{cat} are strongly pH-dependent, the opposite directions of their changes cause the k_{cat}/K_m vs pH dependence to be similar to those obtained for oligopeptide substrates (Figure 7).

DISCUSSION

Squash Inhibitors Obey the Standard Mechanism. Although many "small" protein inhibitors of serine proteinases have been isolated and sequenced, detailed kinetic and thermodynamic data have been presented for only a few of them (Finkenstadt et al., 1974; Quast et al., 1978; Tonomura et al., 1985). This paper provides strong evidence that squash inhibitors obey the standard mechanism of interaction. The Arg5–Ile6 peptide bond in CMTI I inhibitor is selectively hydrolyzed by bovine trypsin. This peptide bond can be, in turn, resynthesized using the same proteinase. The data are in agreement with a 2.0-Å crystal structure of the bovine β-trypsin–CMTI I complex, which shows specific accommodation of the Arg5 side chain in the S1 binding pocket of the enzyme (Bode et al., 1989).

Besides this qualitative evidence, we provide more rigorous proofs. (i) The extent of conversion of the reactive site peptide bond (α) from the hydrolysis and resynthesis directions was within ±2% (Figure 4). (ii) Experimental and calculated values of the association constant and the hydrolysis constant agree within ±30% (Table 2). Taking into account the large differences in powers of individual rate constants and conceptually different methods of their measurements, we think that the agreement is very good.

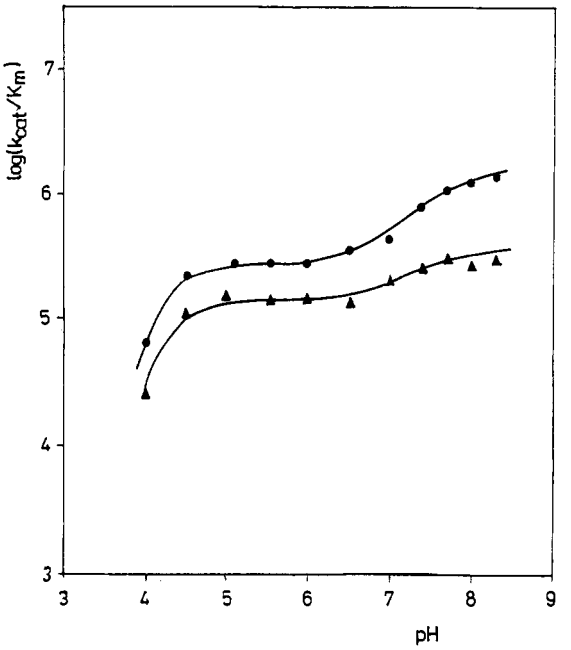

FIGURE 7: pH dependence of the specificity indexes k_{cat}/K_m for the hydrolysis (●) and resynthesis (▲) of the Arg5–Ile6 reactive site in CMTI I by β-trypsin.

Second-Order Association Rate Constants. The values of k_{on} and k^*_{on} were measured at the beginning of this study, since they determine incubation times required for equilibrium measurements. Second-order association rate constants for the interaction of intact forms of squash inhibitors with β-trypsin are above 10^6 M^{-1} s^{-1} (Table 1). These are also typical values for many other protein–protein/ligand associations, which occur without significant conformational changes (Fersht, 1985). The k_{on} parameter is insensitive to the two charge substitution Glu → Lys at the (contact) P4′ position, as mentioned under Results. For squash inhibitor–β-trypsin interactions, association rate constants from the cleaved inhibitor side are about 20-fold lower than those from the intact side (Table 1). This is very similar to the ratio of association rate constants observed for the STI–β-trypsin and only 5-fold lower than for the PTI–β-trypsin system (Table 1). Thus, the k_{on}/k^*_{on} value is similar for all investigated trypsin–inhibitor interactions, in spite of severe sequential differences in these three inhibitors (the P3–P2 sequence is Ser-Tyr, Pro-Cys, and Cys-Pro in STI, PTI, and CMTI I, respectively). Some serine proteinases (notably chymotrypsin and elastase) bind 10^5–10^6-fold faster with intact inhibitors than with their cleaved forms (Ardelt & Laskowski, 1985). Therefore, since k_{on} in all cases is rather invariable, k^*_{on} seems to be a much more intriguing parameter.

The meaning of k^*_{on} can be shown after simple manipulation of the following equation (Ardelt & Laskowski, 1991):

$$(k_{cat}/K_m)_f/(k_{cat}/K_m)_r = K_{hyd} = (k_{on}/k^*_{on})/(k_{off}/k^*_{off}) \quad (9)$$

Since $K_{m,f} = k_{off}/k_{on}$, $k_{cat,f} = k^*_{off}$, and $k_{cat,r} = k_{off}$ [see Results and Finkenstadt et al. (1974)], $k^*_{on} = k_{cat,r}/K_{m,r}$. Thus, k^*_{on} is a specificity constant for the resynthesis reaction of the reactive site peptide bond; *i.e.*, it describes the energy difference between a cleaved inhibitor and a free proteinase and a transition state for this reaction. Since the K_{hyd} value is close to 1 for most small proteinase inhibitors, the k^*_{on} value also describes the transition-state barrier on the intact inhibitor side (see left side of eq 9). It follows that the transition-state barrier for trypsin-catalyzed hydrolysis/resynthesis reactions are very low for all investigated trypsin inhibitors. Most probably these result from low subsite specificities of trypsin. The specificity index hardly varies when the enzyme is probed with different P1-Arg (McRae et al., 1981) or P1-Lys (Morihara & Oka, 1973) oligopeptide substrates. In agreement with the recent protein engineering studies (Graff et al., 1988; McGrath et al., 1992) the P1-Lys/Arg–Asp189 ion pair interaction in the S1 pocket is critical for the transition-state stabilization. In other serine proteinases, stabilization of the transition state is more complex. For chymotrypsin (Imperiali & Abeles, 1987; Schellenberger et al., 1991), subtilisin (Wells, 1990), and elastase (Thompson & Blout, 1973) other positions flanking the hydrolyzed peptide bond contribute (predominantly additively) to the transition-state energy. Large differences in the k^*_{on} parameter observed for association of inhibitors with these proteinases (Ardelt & Laskowski, 1985) probably reflect sequence-dependent stabilization of the transition state. We expect that for highly specific "trypsin-like" serine proteinases (*e.g.*, coagulation factors) k^*_{on} will be generally much lower than for trypsin. These proteinases are much more sequence-specific than trypsin (in terms of k_{cat}/K_m), since they utilize binding energy from several subsites to increase catalysis.

Thermodynamics of the Interaction. Relatively high values of k_{on}/k^*_{on} suggest that association constants measured for the β-trypsin and uncleaved form of squash inhibitors describe a simple equilibrium: E + I ⇌ C (K_a = [C]/[E][I]) (Finkenstadt & Laskowski, 1967; Finkenstadt et al., 1974). To verify this, we measured the association constant between the reactive site cleaved inhibitor (CMTI I*) and trypsin in the pH range 3.5–8.3. In this case, the measured constant describes the overall equilibrium among I, I*, and E, and C (K_{assoc} = [C]/[E]([I] + [I*]) = $K_a/1 + K_{hyd}$). K_{assoc} values measured directly and calculated from K_a and K_{hyd} were very similar (Table 2). This indicates that measured K_a values describe a real and simple equilibrium of two interacting proteins.

Although the numerical difference between K_a and K_{assoc} is rather small (a factor of $1 + K_{hyd}$), we think that it contains the basic aspects of inhibitor action. It shows that the inhibition is a permanent process notwithstanding the enzymatic hydrolysis of the reactive site peptide bond. We feel that this is a key feature of the standard mechanism. Neither formation of a tight complex between inhibitor and enzyme nor selective hydrolysis of a peptide bond is a crucial condition for the standard mechanism. The nature of the proteinase–inhibitor recognition is similar to many other protein–protein interactions. A specific hydrolysis of the single peptide bond accompanies also the inhibition mechanism of serum proteinase inhibitors (Travis & Salvesen, 1981) and α_2-macroglobulins (Sottrup-Jensen, 1989). In these cases, however, the proteolysis is an irreversible process. A low value of the hydrolysis constant guarantees that inhibition is a reversible and, despite the cleavage reaction, a permanent process.

The values of K_a ($=1/K_{m,f}$) for the CMTI I–β-trypsin interaction increase by 6 orders of magnitude in the pH range 4.0–8.3 (Figure 2). At pH 8.3 K_a reaches a value 4×10^{11} M^{-1}. The plot shows a multiproton cooperative transition with a midpoint around pH 4 and a single proton transition (attributed to imidazole ring of His57) at neutral pH. Generally, the shape of the K_a vs pH plot resembles analogous curves for STI–β-trypsin and PTI–β-trypsin interactions (Finkenstadt et al., 1974). Since amino acid sequences of contact positions in CMTI I, STI, and PTI are completely different, the major factor contributing to unusual stability of the trypsin–inhibitor complex is canonical conformation of the main chain of the binding loop. Linear peptides encompassing the binding loop of PTI (Deshpande et al., 1991) and

Table 2: Thermodynamic Parameters for the CMTI I/CPTI II–Bovine β-Trypsin Interaction

inhibitor	pH	K_a^{obs} [a] (M^{-1})	K_a^{calc} [b] (M^{-1})	K_{assoc}^{obs} [c] (M^{-1})	K_{assoc}^{calc} [d] (M^{-1})	K_{hyd}^{obs}	K_{hyd}^{calc} [e]
CMTI I	8.3	3.3×10^{11}	2.7×10^{11}	5.2×10^{10}	4.0×10^{10}	5.59	4.49
CPTI II	8.3	8.9×10^{11}	7.8×10^{11}	nd[f]	1.5×10^{10}	4.92	6.58
CMTI I	6.0	8.7×10^{9}	nd	5.9×10^{9}	3.1×10^{9}	1.84	nd
CPTI II	6.0	9.0×10^{9}	nd	nd	3.4×10^{9}	1.62	nd

[a] Measured for the association of intact inhibitor with trypsin after equilibrium time = $10t_{1/2}$. [b] Calculated from $K_a = k_{on}/k_{off}$ (assuming $k_{off} = k_{cat,r}$). [c] Measured for the association of the reactive site cleaved form with trypsin after equilibrium time = $10t^*_{1/2}$. [d] Calculated from $K_{assoc} = K_a/(1 + K_{hyd})$. [e] Calculated from $K_{hyd} = (k_{on}/k^*_{on})/(k_{off})/(k^*_{off})$ (assuming $k_{off} = k_{cat,r}$ and $k^*_{off} = k_{cat,f}$). [f] Not determined.

of eglin C (Okada et al., 1989) bind two proteinases 10^5–10^6-fold more weakly than the whole protein inhibitors. According to one- and two-dimensional NMR spectroscopy, they are highly flexible and do not exhibit stable conformation (Deshpande et al., 1989). Instead, the conformation of the binding loop of free CMTI I is defined in NMR solution structure (Holak et al., 1989a, 1991). Thus, as it has been already postulated (Read & James, 1986; Bode & Huber, 1991), high association energy results from a rigid conformation of the binding loop. Upon complexation with trypsin, the binding loop of CMTI I does not change its conformation (Holak et al., 1989b), yet in contrast to the free state, it exhibits the smallest temperature *B* factors. Fixation of a binding loop in a conformation that already exists in a free state is probably a major factor leading to extremely tight binding.

Inhibitors as Substrates. Although the k_{cat}/K_m index for trypsin-catalyzed hydrolysis of the reactive site peptide bond in CMTI I is high (Figure 7), it does not lead to the fast hydrolysis of the reactive site peptide bond. The reason lies in an extremely low value of the Michaelis constant, which effectively reduces the catalytic rate constant to extraordinarily low values (Figure 6). In fact, unlike regular substrates of serine proteinases, the dependence of $k_{cat,f}$ on pH is dominated by the presence of the Michaelis complex and not by the p*K* of the catalytic His57. Synthetic oligopeptide substrates and (most probably) natural protein substrates exhibit completely different partitioning of k_{cat} and K_m parameters than squash inhibitors. Both parameters are increased 10^7–10^9-fold, leading to an equivalent increase in the reaction rate. In this case the rate enhancement results from weak binding of the ground state and strong binding of the transition state of the substrate. We think that k_{cat} better describes substrate properties of inhibitors than specificity constant. The catalytic rate constant is a more real parameter since it shows how hydrolysis is slowed by the accumulation of the tight Michaelis complex.

Extremely low values of Michaelis constants make protein inhibitors unusual, not only among proteinase substrates but probably among all enzyme substrates. High values of Michaelis constants are favorable for regular substrates, since they lead to faster reaction rates and prevent enzymes from being overloaded in the ES complex (Fersht, 1985). This should be particularly true for digestive enzymes, which are exposed to high protein substrate concentration in the duodenum. The conformational analysis of tryptic proteolysis nicksites in several proteins revealed the lack of canonical conformation. Although these nicksites are also located in exposed loops, a gross conformational change is required to correctly orientate the scissile bond to the catalytic apparatus (Hubbard et al., 1991). We suspect that similar situations occur with physiological substrates. The energetic cost of such a radical conformational change upon formation of the Michaelis complex should effectively increase the K_m value. In this respect, protein inhibitors cannot be recognized as typical substrates.

The ground-state canonical conformation of the binding loop is particularly well stabilized by trypsin. Almost half of the trypsin–CMTI I contacts originate from the P1 Arg5 side-chain interaction within the S1 binding pocket (Bode et al., 1989), which are considered to be a major factor stabilizing the tetrahedral transition state (Graff et al., 1988). We suppose that these interactions are not fully developed in the inhibitor–proteinase complex. This is based on a comparison of ground-state and transition-state energies for P1 Lys (CPTI II) and P1 Arg (CMTI I) inhibitors. The ratio of the $K_{m,f}$ index for these inhibitors equals 2.7 and of $k_{cat,f}/K_{m,f}$ index equals 1.17 (Table 1). We conclude that the interactions of basic side chains within the S1 binding pocket are changed as the reaction approaches the transition-state tetrahedral intermediate.

Enzymatic hydrolysis of the reactive site peptide bond is probably a highly dynamic process involving a cooperative conversion of the binding energy into catalytic energy (Hedstrom et al., 1992). The dynamics of this process might consist in subtle domain–domain movement, as has already been suggested (Dufton, 1990). This conversion is much simpler for ester substrates due to rotation of the chemical bond to be hydrolyzed. In the case of oligopeptide substrates and regular protein substrates, the conversion is facilitated by the highly flexible character of the polypeptide segment, which is manifested in high Michaelis constants. The binding energy can be easily utilized to distort the peptide bond. The canonical conformation of protein proteinase inhibitors exhibits opposite features. It is highly complementary to the ground state of the reaction, thus locking the proteinase in a highly populated noncatalytic state, despite the fact that for trypsin inhibitors plenty of transition-state stabilization energy (high index of $k_{cat,f}/K_{m,f}$) is available. In other words, the slow hydrolysis of the reactive site peptide bond fully arises from the peculiar conformation of the inhibitor binding loop.

The rigid character of the binding loop in the complex mediates against hydrolysis also during further stages of catalysis. Longstaff et al. (1990) considered the highly unfavorable situation for the hydrolysis, due to difficulties with the release of the amino group during formation of the acyl enzyme. This should greatly facilitate the resynthesis reaction. Indeed, we observe that the specificity constant or second-order rate constant for the reaction from the cleaved inhibitor side is only about 1 order of magnitude lower than that for the hydrolysis reaction (Table 1, Figures 6 and 7).

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