Recombinant Chymotrypsin Inhibitor 2: Expression, Kinetic Analysis of Inhibition with α-Chymotrypsin and Wild-Type and Mutant Subtilisin BPN′, and Protein Engineering To Investigate Inhibitory Specificity and Mechanism†

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ABSTRACT: The serine protease inhibitor chymotrypsin inhibitor 2 (CI2 or BSPI2) has been expressed in *Escherichia coli* with the pINIIIompA3 expression vector to produce 20–40 mg/L of culture. Recombinant CI2 purified from this system has been characterized and found to be identical with CI2 from barley. Slow-binding kinetics were observed for the interaction between CI2 and subtilisin BPN′, with $K_i = 2.9 \times 10^{-12}$ M. Analysis of slow-binding data indicates that binding of the inhibitor follows the simplest model of E + I = EI with no kinetically detectable intermediate steps or proteolytic cleavage of the reactive site bond in CI2 (Met-59–Glu-60). This, in agreement with crystallographic data, indicates that the enzyme–inhibitor adduct is the Michaelis complex, which is not chemically processed by the enzyme. Three mutant CI2 molecules with new P_1 residues have also been examined with a range of serine proteases, including a mutant subtilisin. In agreement with earlier studies, we find the P_1 amino acid an important determinant of specificity. CI2 Met → Lys-59 was found to be a temporary inhibitor of subtilisin BPN′ but an effective inhibitor of subtilisin Carlsberg and subtilisin BPN′(Glu → Ser-156). The structural reasons for this are discussed in relation to mechanisms of inhibition of serine proteases.

The availability of high-resolution structural information on complexes of serine proteases with naturally occurring polypeptide inhibitors makes them attractive targets for studying protein–protein interactions. We are interested in performing such studies on complexes with subtilisin BPN′ because we have an expression system for the enzyme (Thomas et al., 1985). This opens up the possibility of an extended protein-engineering study utilizing mutants in both the enzyme and inhibitors. Here, we report the expression and characterization of a suitable inhibitor. CI2[1] is a small protein, M_r 9200 (Svendsen et al., 1980a), which is a potent inhibitor of chymotrypsin and the microbial serine protease subtilisin (Svendsen et al., 1980b). The protein, isolated from *Hiproly* barley, has been characterized in detail (Boisen et al., 1982), and crystal structures have been determined for the free inhibitor (McPhalen & James, 1987) and for inhibitor complexed with subtilisins BPN′ and Carlsberg (McPhalen & James, 1988). An NMR solution structure for the inhibitor has also been determined that is very similar to the crystal structure (Clore et al., 1987a,b). CI2 is a wedge-shaped disk where the narrow edge is formed by the reactive site loop which binds in the active site cleft of the serine protease. The reactive site bond, which may be slowly cleaved by the enzyme, is between Met-59 and Glu-60 [numbering as in McPhalen and James (1987)].

The structural information available on CI2 and CI2–subtilisin complexes makes it a suitable model system for the study of serine protease inhibition as well as protein–protein interactions in general. However, no detailed kinetic studies have been published on CI2 inhibitor–enzyme interactions, although binding to subtilisin and chymotrypsin is known to be tight (K_i is in the nanomolar range or less; Svendsen et al., 1980b). Despite much research in this area, there is still no clear idea of what structural features of these proteins are essential for their functioning as inhibitors and prevent them from being well-designed substrates. Common features among these families of inhibitors such as very tight binding (K_i values in the 10^{-8}–10^{-16} M range) and rigid, stable structures have led to proposals for general mechanisms of inhibitory action. Because of the very tight binding between enzyme and inhibitor, early proposals were for an adduct of a tetrahedral intermediate with a covalent bond between the P_1 carbonyl carbon of the inhibitor and the serine oxygen nucleophile [e.g., Finkenstadt and Laskowski (1967)]. This idea became untenable with the determination of highly refined crystal structures for many enzyme–inhibitor complexes [reviewed by Laskowski and Kato (1980) and Read and James (1986)]. The conclusion from most, if not all, of the crystal structures is that the inhibited complex is close to a Michaelis complex, where the inhibitor was bound with minimal change in its structure, with no evidence for any new covalent bond. This is supported, among other NMR studies, by ^{13}C NMR studies on the subtilisin–SSI complex, which was found to have a planar P_1–P_1' bond (Tonomura et al., 1985). Other proposals from kinetic studies have involved equilibrium between intact and active site cleaved inhibitor (also called modified inhibitor) to account for inhibition [e.g., see Laskowski and Sealock

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[1] Abbreviations: CI2, chymotrypsin inhibitor 2; P_1 and P_1', notation as in Schechter and Berger (1967); FPLC, fast protein liquid chromatography; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; IPTG, isopropyl thiogalactopyranoside; NMR, nuclear magnetic resonance; SD, standard deviation; CI2(wt), where CI2 wild type (methionine 59) is specifically discussed; CI1, chymotrypsin inhibitor 1; L-BAPNA, N_α-benzoyl-L-arginine-*p*-nitroanilide; NPGB, *p*-nitrophenyl guanidinobenzoate; PMSF, phenylmethanesulfonyl fluoride; SSI, *Streptomyces* subtilisin inhibitor. Mutant CI2 molecules are named MX59CI2, where X represents the amino acid replacing methionine at position 59. Similarly, BPN′ES156 denotes a mutant subtilisin BPN′ enzyme with a glutamate replacing serine at position 156.

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(1971) and Jencks (1980)]. This is possible since, following cleavage of the active site peptide bond, the newly formed termini are held in close proximity by many intramolecular bonds, so resynthesis can occur. Indeed, many cleaved inhibitors do remain active, so that when incubated with appropriate serine proteases, the active site (P_1–P_1') bond may be re-formed. However, this theory does not explain why chemically inactivated enzymes are still able to bind inhibitors (Haynes & Feeney, 1968). A central question for understanding protease inhibition is: Why does active site peptide bond hydrolysis take place so slowly or not at all?.

CI2 represents an excellent model system to address this question by protein engineering. We are also in a position to investigate factors controlling specificity of inhibitor–enzyme interactions. These findings may be generally applicable to other enzyme–inhibitor systems.

Materials and Methods

The gene for CI2 was provided as a full-length cDNA clone in a derivative of pBR322 (pcIC38), by M. Kreis (Williamson et al., 1987). The gene was transferred into M13mp8 (Pharmacia UK, Ltd.), and deletion mutagenesis of the gene was carried out to bring the *Eco*RI site in the polylinker region immediately upstream of the translational start codon (ATG) of the CI2 gene to reduce the distance between the Shine–Dalgarno sequence and ATG site in the subsequent expression constructs. Mutagenesis reactions were performed by the double-priming method, as described by Zoller and Smith (1983), using M13 universal primer and a 21-mer mutagenic primer, 5′-GAACTCATCCGAATTCGTAAT-3′ (synthesized on an ABI DNA synthesizer, Model 380B). Following synthesis of the second, mutated, strand of DNA, the duplex was transfected into the MutL strain of *Escherichia coli*, deficient in mismatch repair enzymes (Biggin, 1984). Positive clones were detected by hybridization screening using ^{32}P-labeled oligonucleotide as described by Maniatis et al. (1982). DNA sequencing of the M13 template was performed according to the dideoxy chain termination method of Sanger et al. (1977).

Mutant CI2 Proteins, Synthesis, and Expression. Site-specific mutagenesis of the 5′-truncated CI2 gene in M13mp8 was performed with double priming (Zoller & Smith, 1983) to create proteins with new P_1 residues. The wild-type methionine P_1 residue was changed to alanine, lysine, and tyrosine with the following mutagenic oligonucleotides (synthesized on an ABI Model 380B DNA synthesizer):

MA59CI2: 5′-CGATATTCCGCGGTCACA-3′

MK59CI2: 5′-CGATATTCTTTGGTCACA-3′

MY59CI2: 5′-CGATATTCATAGGTCACA-3′

Wild-type and mutant CI2 genes were expressed from the secretion vector pINIIIompA3 (Grayheb et al., 1984). This is a high-level expression vector based on pBR322, which utilizes the lpp promoter and lac promoter–operator sequence to direct expression of the foreign protein as a fusion with the ompA signal peptide. CI2 genes were transferred from M13, as *Hin*dIII/*Eco*RI fragments, into pINIIIompA3; the wild-type construct was termed pAC4. The recombinant vector constructs were transformed into competent *E. coli* (Kramer et al., 1984) strains TG2 or JM101 as shown under Results. Clones were grown on rich agar plates with ampicillin selection. Colonies carrying the recombinant plasmid were detected by hybridization screening, using the ^{32}P-labeled oligonucleotide 5′-TGGCTTGTCCTGCAG-3′ (CI2Q1). This probe is complementary to a region in the middle of the CI2 gene. Positive clones were checked by restriction mapping to verify correct insertion of a single CI2 gene.

Protein Expression and Purification. E. coli containing plasmid was grown in 2 × TY media (Maniatis et al., 1982), containing ampicillin (50 μg/mL) and IPTG (0.5 mM) (Sigma Chemical Co. Ltd.), for induction of expression. Routinely, 2 L of culture was grown up overnight at 37 °C with vigorous shaking to an absorbance of approximately 6–8 at 650 nm. The time of addition of IPTG was found to be unimportant, so the inducer was added at the beginning of cell growth. Cells were concentrated by centrifugation (6500*g*, GSA rotor), and the cell paste was resuspended in approximately 50 mL of 10 mM Tris-HCl buffer, pH 8.0, containing 1 mM EDTA (TE8) for sonication in a Heat Systems ultrasonic sonicator, typically with a 0.5-in. probe and 3 × 1 min pulses on maximum power with cooling in ice–water. Cell debris was removed by centrifugation at 1100*g* (Beckman SS34 rotor) before the pH of the supernatant was lowered to 4.4 by the addition of 1 M acetic acid. The precipitate thus formed was removed by centrifugation at 25000*g*. To this supernatant was added ammonium sulfate to 40% (w/v) at 4 °C to precipitate unwanted proteins, which were removed by centrifugation at 25000*g*. CI2 was precipitated from this supernatant by the addition of ammonium sulfate to a concentration of 65% (w/v) and collected by centrifugation at 25000*g*. Protein pellets were redissolved in up to 15 mL of 10 mM ammonium carbonate buffer, pH 9.0, and the cleared solution was applied to a column (3 × 70 cm) of Sephadex G-75 (superfine grade) at a flow rate of approximately 10 mL/h. Fractions containing inhibitory activity were pooled and passed through a small (approximately 1 mL) column of DEAE-Trisacryl (LKB Ltd.) preequilibrated in 10 mM carbonate buffer, pH 9.0, to remove unwanted protein. The CI2 pool (approximately 100 mL) was then concentrated by lyophilization. The resulting solid was redissolved in up to 5 mL of 50 mM sodium acetate buffer, pH 4.4, for FPLC using a Mono S column (1 mL) (all FPLC equipment from Pharmacia UK, Ltd.) preequilibrated in the same buffer. Pure CI2 was eluted in a gradient of 0–1 M NaCl. Before further use or storage, pooled active protein was desalted into water on a column of Sephadex G-25.

General Kinetic Methods. Subtilisin BPN′ was prepared from a clone expressing the gene in *Bacillus subtilis* (Thomas et al., 1985). Enzyme assays were performed in 0.1 M Tris-HCl buffer, pH 8.6, at 25 °C. The chromogenic substrate was succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide (Sigma Chemical Co. Ltd.). Values of k_{cat} and K_m determined under these conditions are 55 s^{-1} and 0.15 mM (Russell, 1987). Enzyme concentrations were calculated from initial rates of substrate hydrolysis monitored at 412 nm. Similarly, bovine pancreatic α-chymotrypsin (Sigma Chemical Co. Ltd.) was assayed in 0.144 M Tris-HCl buffer, pH 7.78, at 25 °C containing succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide. k_{cat} and K_m values are 45 s^{-1} and 0.04 mM, respectively (Delmar et al., 1979). In some cases buffers also contained 0.05% Tween 80 (see Results). Constants for subtilisin Carlsberg are k_{cat} = 950 s^{-1} (K_m = 0.23 mM) and for subtilisin BPN′ES156 are k_{cat} = 55 s^{-1} (K_m = 0.05 mM) with the substrate succinyl-Ala-Ala-Pro-Phe nitroanilide (Russell, 1987). Bovine pancreatic trypsin (Sigma) was assayed in 0.144 M Tris-HCl buffer, pH 7.78, at 25 °C, with the L isomer of benzoylarginine *p*-nitroanilide (L-BAPNA) as substrate. Trypsin concentrations were determined by active site titrations using *p*-nitrophenyl *p*-guanidinobenzoate (NPGB) as described by Chase and Shaw (1967). Subsequently, values of k_{cat} and K_m for trypsin with L-BAPNA were used to calculate enzyme concentrations from initial rates. Porcine pancreatic elastase (Sigma) was assayed

in 0.2 M Tris-HCl buffer, pH 8.0, at 25 °C with succinyl-Ala-Ala-Ala *p*-nitroanilide (Calbiochem) as substrate. Enzyme concentrations were determined from initial rates with values of k_{cat} = 16.6 s^{-1} and K_m = 1.15 mM (Beith & Wermuth, 1973).

CI2 concentrations were determined from stoichiometric binding curves of subtilisin inhibition vs [I] (Laskowski & Sealock, 1971). Enzyme concentrations for complex formation were approximately 1 μM, and free enzyme was assayed up to 10 nM in 0.4 mM substrate. MY59CI2 was assayed by titration against chymotrypsin.

Slow-Binding Kinetics. Inhibitors were investigated initially for slow-binding behavior [see Williams and Morrison (1979) and Cha (1975) for reviews]. Inhibitors were treated as slow binding when the attainment of the steady state could be observed over a time period of several minutes under the experimental conditions employed and data could be successfully fitted to the slow-binding equation (see below). For the simplest treatment of slow-binding data, experiments were carried out under pseudo-first-order conditions where lowest [I] ≥ 10[E]. The enzyme concentration was set at a suitably low level so as to give a measurable rate of substrate hydrolysis as well as an observable rate of inhibitor binding over the steady-state time scale. The range of inhibitor concentrations depends on the value of K_i for the system. The initial concentration of substrate was sufficiently high to allow the reaction to be followed for an adequate time for data collection, but with less than 10% of substrate being hydrolyzed by the end of the experiment. The length of these experiments was found to be critical for successful fitting of data, as discussed below. Optimized conditions for investigating slow-binding kinetics of CI2 with subtilisin BPN′ were as follows. Reactions were carried out in 0.1 M Tris-HCl buffer, pH 8.6, containing 0.05% Tween 80, at 25 °C. Enzyme concentration was approximately 0.25 nM. Inhibitor concentrations in reaction mixtures (1 mL) were 0, 5, 7, 9, 11, and 13 nM. Substrate concentration was 1 mM. Six cuvettes (1 mL) containing substrate and inhibitor were incubated at 25 °C in a Perkin-Elmer λ5 double-beam spectrophotometer fitted with automatic cell changer (2 × six cells). Six cuvettes containing 1 mL of 1 mM substrate were used as blanks. Reactions were initiated with the addition of enzyme solution (0.25 mL), preequilibrated at 25 °C. Cuvettes were mixed quickly and replaced in the cuvette holder such that mixing was completed in approximately 30 s. Data from the initial burst of substrate hydrolysis was collected as a data print out with a cycle time of 18 s for six cuvettes. After 30 min of reaction, cycle time was changed to 180 s, and data were recorded directly on to disk with purposely written software (S. Upstone, Perkin-Elmer). In this way the approach to steady state could be monitored for up to 20 h. On completion of the experiment, the two sets of data were combined (Lotus 123 spreadsheet, Lotus Corp.) and adjusted for mixing times. Up to 500 data points were read into Enzfitter, a nonlinear regression analysis program (Leatherbarrow, 1987) for fitting.

Data Analysis. Data from each curve at different inhibitor concentrations were fitted to eq 1, the integrated rate equation

$$A = v_s t + (v_o - v_s)(1 - e^{-k't})/k' + A_o \quad (1)$$

describing substrate hydrolysis in the presence of slow-binding inhibitor. A is the absorbance at 412 nm, and A_o is the initial absorbance. v_o and v_s are initial rate and final steady-state rate, respectively. k' is the apparent first-order rate constant for the transition to the steady-state rate. Curve fitting gives values for the four independent variables v_o, v_s, k', and A_o at each concentration of inhibitor. For the simplest model of complex formation, E + I = EI (see Discussion), a plot of k' vs [I] gives a straight line from which k_{on} and k_{off} can be determined according to eq 2. A plot of $(v_o - v_s)/v_s$ vs [I]

$$k' = k_{off} + k_{on}[I]/(1 + [S]/K_m) \quad (2)$$

can be used to determine K_i and should give a straight line passing through the origin according to eq 3. Where v_o could

$$(v_o - v_s)/v_s = [I]/[K_i(1 + [S]/K_m)] \quad (3)$$

not be determined accurately from fitting, eq 2 was not used. Equation 3 could still be used to calculate, K_i, but v_i, the uninhibited enzyme rate, was used instead of v_o.

Dissociation Kinetics. Direct determination of the dissociation rate constant (k_{off}) may be made by diluting preformed enzyme–inhibitor complex into concentrated substrate solution. For this to be successful, stoichiometric binding should be observed, but the complex should be diluted below its apparent K_i [$K_{i(app)} = K_i(1 + [S]/K_M)$]. The rate equation for complex dissociation is given by eq 4. Data were fitted to the integrated

$$[E] = [E_o](1 - e^{-k_{off}t}) \quad (4)$$

form, eq 5. $[E_o]$ was determined from the final steady-state

$$A = \{k_{cat}[E_o](k_{off}t + e^{-k_{off}t} - 1) + A_o\}/k_{off} \quad (5)$$

rate after complex dissociation. k_{off} for chymotrypsin–CI2 complex was determined in this way by diluting complex (1 μL) into 2 mM substrate (1 mL) to give a final $[E_o]$ of 1–2 nM. Release of free enzyme was monitored on a Perkin-Elmer λ5 spectrophotometer in conjunction with PECSS software (Perkin-Elmer) for up to 20 min. Data were fitted to eq 5 by nonlinear regression analysis (Enzfitter; Leatherbarrow, 1987).

Electrophoresis. Proteolytic cleavage of inhibitors by various enzymes was investigated by PAGE with the Tricine–SDS–PAGE system of Schagger and Von Jagow (1987). A 16.5% acrylamide separating gel was used overlayed with a 4% stacking gel. Samples (10 μL) of sample solution (complexes or inhibitor/enzyme individually) were incubated with 0.1 M PMSF in 2-propanol (2 μL) for 5–10 min, before sample buffer was added and the mixture heated to 100 °C for approximately 5 min. Proteins were stained with Coomassie Blue.

Additional Methods. Immunodiffusion assays using rabbit anti-CI2 serum (provided by F. Poulsen, Carlsberg Laboratories, Copenhagen, Denmark) were performed according to published methods (Ouchterlony, 1958). Isoelectric focusing was carried out in an LKB Multiphor flat-bed electrophoresis system in accordance with the manufacturer's instructions. NMR spectroscopy (500 MHz) was carried out on a Bruker AM500 spectrometer. N-terminal sequencing of purified CI2 was performed on approximately 1 nmol of protein with a modified form of gas-phase Edman degradation (Gros & Labouesse, 1969).

Results

Recombinant CI2 (wild type) was expressed in *E. coli* (TG2 strain) at approximately 20 mg/L, expressed in small-scale production (2 L in shake flasks). Larger scale preparations of CI2 from pAC4, using an MBR Bioreactor fermentation system, could achieve expression of 40 mg/L in 12 L of culture.

CI2 produced from pAC4 is directed to the periplasm, so theoretically, the protein could be liberated by osmotic shock. However, problems were encountered in concentrating the protein from the large volume of dilute solution produced in this way. Consequently, harvested cell paste was sonicated to release total cellular protein from which CI2 could be purified as described under Materials and Methods. By use of

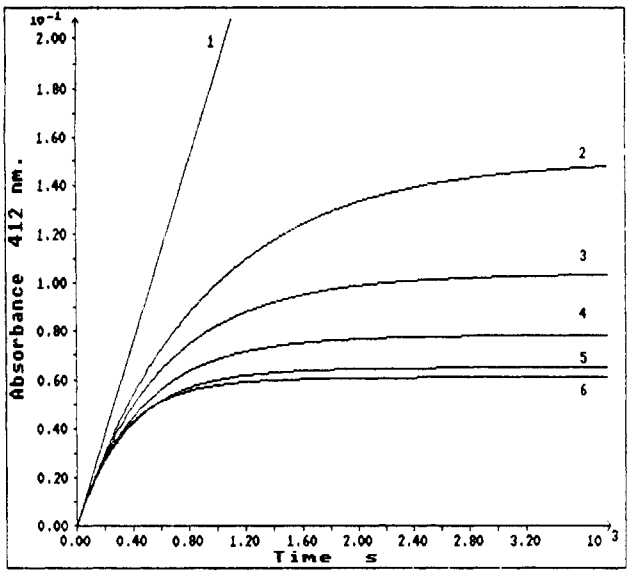

FIGURE 1: Family of slow-binding inhibition curves for subtilisin BPN′ and CI2. Each curve shows the course of enzyme inhibition at a different inhibitor concentration, and the line represents uninhibited enzyme. Data were collected for a total time of 16.6 h at 25 °C in 0.1 M Tris-HCl buffer, pH 8.6, containing 0.05% Tween 80. Substrate concentration was 1 mM (K_m = 0.15 mM), enzyme concentration was approximately 0.25 nM, and inhibitor concentrations were 0, 5, 7, 9, 11, and 13 nM (curves 1–6) giving increasing levels of inhibition with shorter burst phases. Reaction was initiated by the addition of enzyme soltuion (0.25 mL) to inhibitor and substrate (in 0.75 mL). Only the early stages of the reaction are shown for clarity.

this procedure, yields of pure protein of approximately 30% were usual.

Recombinant CI2 produced in this way was verified as identical with native CI2 from barley (a gift from F. Poulsen, Carlsberg Laboratories, Copenhagen, Denmark). Both proteins were of the same M_r on SDS–PAGE, demonstrating that processing of the nascent CI2 protein had occurred to release mature CI2.

Recombinant and native (i.e., barley) CI2 were indistinguishable by reaction with antibodies to CI2 (a gift from F. Poulsen). Purified recombinant CI2 was also still active after prolonged treatment at high temperature (60 °C for 36 h) and after treatment at extremes of pH (6 h at pH 1 or 12). A general feature of these small protein inhibitors is their stability (Laskowski & Kato, 1980), and this has also been demonstrated for CI2 (Boisen et al., 1981). Isoelectric focusing of CI2 from barley has shown multiple forms of the protein, with p*I* values ranging between 6 and 8. Highly purified recombinant CI2 was found to give a single band on isoelectric focusing with a p*I* of 6.9. A sample of this material was also sequenced at the N-terminus to give Ser-Ser-Val-Glu-Lys-Lys-Pro-Glu, corresponding to the first eight residues of the published sequence of CI2 (Svendsen et al., 1980a).

Although called chymotrypsin inhibitor 2, it is known that CI2 is a more potent inhibitor of subtilisins (Svendsen et al., 1980b), but no values of K_i have been published. Detailed kinetic studies using recombinant CI2 demonstrated slow-binding behavior for the interaction between CI2 and subtilisin BPN′. A family of binding curves for a range of inhibitor concentrations under optimized conditions is shown in Figure 1. Data from this experiment were fitted to eq 1, as described under Materials and Methods, to give values for k', v_o, and v_s which were used to plot Figure 2. Figure 2A is a plot of k' vs [I] from which the slope can be used to calculate k_{on} according to eq 2 (k_{on} = 1.8 × 10^6 M^{-1} s^{-1}). Theoretically, k_{off} can be determined from the intercept on the y axis. In

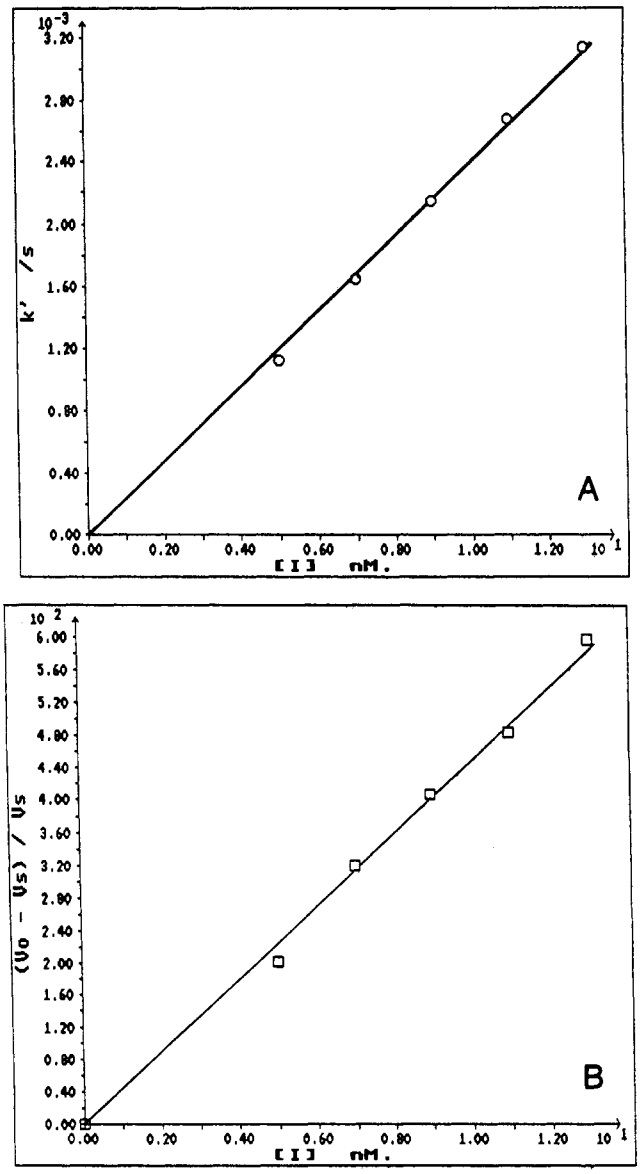

FIGURE 2: Analysis of data presented in Figure 1. (A) Plot of k' determined at each inhibitor concentration by fitting the data from Figure 1 to eq 1. From the slope and intercept on the ordinate, values for k_{on} and k_{off} can be determined, respectively. (B) The determination of K_i using values of v_o and v_s generated from eq 1.

this case, however, the intercept is so close to zero that we can only estimate k_{off} as being $<10^{-4}$ s^{-1}. Figure 2B is a plot according to eq 3 for the determination of K_i using initial rate (v_o) and final steady-state rates (v_s). As expected, this plot is a straight line passing through the origin. From the slope we can determine K_i = 2.9 × 10^{-12} M. Knowing k_{on} and K_i allows us to calculate k_{off} = 5.2 × 10^{-6} s^{-1}. Where equilibrium is reached slowly, it is important to run these experiments for a sufficient time so a good estimate for v_s is obtained from fitting. Otherwise, systematic errors are generated that can be misinterpreted as unusual binding. This is readily diagnosed from plots such as those shown in Figure 2B, which become nonlinear in these cases.

Initially, the same slow-binding kinetics approach was used to investigate the interaction between chymotrypsin and CI2. In this case, equilibrium between enzyme and inhibitor is achieved rapidly, with only a short phase before the final steady-state rate is achieved. When data from these experiments were fitted to eq 1, v_o and consequently k' are poorly estimated and cannot be used. However, v_s can be fitted accurately with low standard errors, which permits the de-

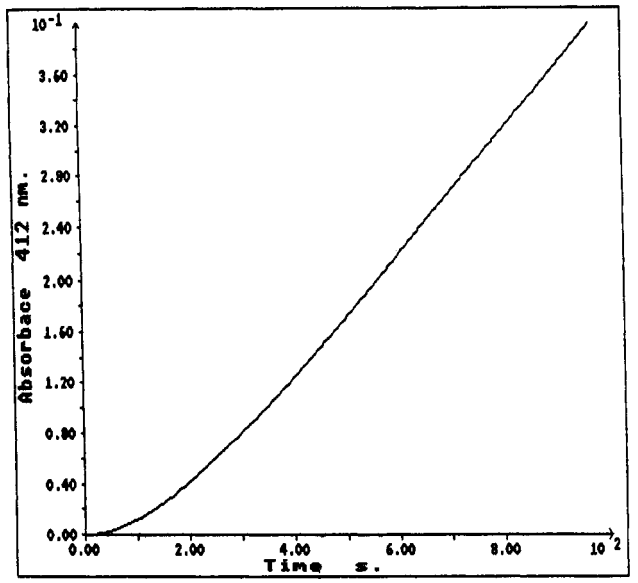

FIGURE 3: Dissociation of CI2–chymotrypsin complex. Preformed complex was diluted (final concentration 1–2 nM, determined from uninhibited rate) into substrate solution (1 mM) in a cuvette, and complex dissociation was followed by monitoring release of enzyme at 25 °C in 0.144 M Tris-HCl buffer, pH 7.78. Data were fitted to eq 5 as described under Materials and Methods for the direct determination of the dissociation rate constant, k_{off}.

Table I: Constants for the Binding of CI2 to Chymotrypsin and Subtilisin[a]

enzyme	K_i (M)	k_{off} (s^{-1})	k_{on} (M^{-1} s^{-1})
subtilisin BPN′	2.9×10^{-12}	5.2×10^{-6}	1.8×10^{6}
chymotrypsin	1.6×10^{-9}	6.1×10^{-3}	3.8×10^{6}

[a] k_{off} was calculated from K_i and k_{on} for subtilisin and k_{on} was calculated from K_i and k_{off} for chymotrypsin, by use of the relationship $K_i = k_{off}/k_{on}$.

termination of K_i from eq 3. Thus, the interaction between CI2 and chymotrypsin follows classical inhibition kinetics, but the approach used here is useful as a check to ensure a true steady-state rate has been reached at the time the rate is actually measured. Similar experiments were performed over a range of enzyme concentrations, 0.2, 0.5, 1.0, and 2.0 nM, all giving results in close agreement, $K_i = 1.67 \times 10^{-9}$, 1.57×10^{-9}, 1.40×10^{-9}, and 1.6×10^{-9} M, respectively (average $K_i = 1.56 \times 10^{-9}$ M).

Preformed complexes between chymotrypsin and CI2 could be dissociated by dilution into concentrated substrate solution. Results from one such experiment are shown in Figure 3, demonstrating a lag phase before a steady-state rate of enzyme activity is reached. These data were fitted to eq 5 to give a value of k_{off} directly. Results from a series of experiments gave $k_{off} = 6.05$ (±0.09) $\times 10^{-3}$ s^{-1} ($n = 5$) [average (±SD)]. The association rate constant for chymotrypsin and CI2 was calculated from K_i and k_{off} to be 3.8×10^{6} M^{-1} s^{-1}. Initial stopped-flow experiments using proflavin displacement from chymotrypsin gave a similar value of 4×10^{6} M^{-1} s^{-1} (S. Jackson, personal communication). Values for the constants determined for the binding of CI2 to subtilisin BPN′ and chymotrypsin are shown in Table I.

Mutant CI2 Proteins. Wild-type CI2 and the MK59CI2 mutant were both expressed well, to similar levels, >20 mg/L of culture. However, in the TG2 strain of *E. coli*, two other mutants, MY59CI2 and MA59CI2, were both very poorly expressed, approximately 0.1 mg/L of culture. A much-improved level of expression for MA59CI2 (15 mg/L) from strain JM101 was unexpected and cannot simply be the result of higher cell density for this faster growing strain.

Table II: Inhibition of Serine Proteases by CI2 and Its Mutants

CI2 residue at P_1 site (59)	K_i (nM) trypsin[a]	chymotrypsin[b]	elastase[c]
Ala	ND[d]	ND[d]	~600
Met (wt)	(hydrolysis)[e]	1.6[f]	~30
Lys	5.6	>>10	ND[d]
Tyr	ND[d]	0.47[g]	ND[d]

[a] 25 °C, 144 mM Tris (pH 7.78), and L-BAPNA as substrate. [b] As in footnote *a* but with succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide as substrate. [c] 200 mM Tris (pH 8.0) and succinyl-Ala-Ala-Ala-Ala *p*-nitroanilide as substrate. [d] Not determined. [e] Nonspecific hydrolysis. [f] $k_{on} = 3.8 \times 10^{6}$ M^{-1} s^{-1}; $k_{off} = 6.1 \times 10^{-3}$ s^{-1}. [g] $k_{on} = 2.0 \times 10^{6}$ M^{-1} s^{-1}; $k_{off} = 9.3 \times 10^{-4}$ s^{-1}.

Specificity Studies. Results from all the specificity studies are accumulated in Table II. Trypsin is not inhibited by CI2(wt). PAGE of mixtures of trypsin and CI2(wt) incubated at 25 °C under the usual conditions of 0.1 M Tris-HCl, pH 8.6, showed the protein to be progressively hydrolyzed in a nonspecific manner. The normally sharp protein band of CI2 was transformed to a diffuse smear of lower molecular weight (data not shown). Because CI2 has a large number of lysines and arginines, it is not unexpected that in the absence of inhibition the protein would be an excellent substrate. MK59CI2 was designed to be a potential inhibitor of trypsin on the basis of enzyme substrate specificity. This proved to be a new inhibitor of trypsin, with a low $K_i = 5.6 \times 10^{-9}$ M. Binding kinetics are not slow but follow classical inhibition kinetics. A dissociation rate constant could not be determined directly for the trypsin complex as done previously for chymotrypsin–CI2(wt) because of interference from unbound enzyme at the concentrations necessary for the assay of trypsin. PAGE analysis of trypsin–MK59CI2 complexes (not shown) initially showed no cleavage of the inhibitor; however, prolonged incubation of the complex (up to 16 h) showed some breakdown to a small number of discreet fragments. These may be the result of specific cleavage at the active site P_1–P_1' bond. Clearly, by this single mutation at the P_1 site we have made a new tight-binding inhibitor of trypsin and drastically reduced or eliminated nonspecific proteolysis of the inhibitor.

CI2(wt) is a potent inhibitor of chymotrypsin ($K_i = 1.6$ nM), but since chymotrypsin has a known preference for large hydrophobic residues at the P_1 site of substrates, we designed MY59CI2 to see what effect the substitution of a tyrosine for methionine would have on inhibition of this enzyme. As can be seen from Table II, MY59CI2 is an improved inhibitor of chymotrypsin ($K_i = 0.47 \times 10^{-9}$ M). The improvement in inhibition constant is accounted for by the dissociation rate constant (decreased 6.5-fold), which was measured directly by diluting the preformed complex in concentrated substrate. Interestingly, the onset of inhibition has also become more slow binding in nature, with a clearly discernible lag phase before the onset of the true steady-state rate (now shown). No evidence was found for any proteolysis by chymotrypsin with either CI2(wt) or MY59CI2 by PAGE analysis.

It was not previously known whether CI2 was capable of inhibiting elastase. This may have some clinical relevance since elastases are an important group of enzymes in the development of inflammation in emphysema (neutrophil elastase) and pancreatitis (pancreatic elastase) (Travis & Salveson, 1983). New inhibitors of elastase are of interest, therefore, as potential therapeutic agents. We found that CI2(wt) was a reasonable inhibitor of porcine pancreatic elastase (K_i = approximately 30×10^{-9} M), and binding kinetics are not slow. A technical difficulty encountered with elastase experiments is enzyme

Table III: Inhibition of Subtilisins by CI2 and Mutant MK59CI2

	CI2(wt)		MK59CI2	
subtilisin	K_i (nM)	k_{on} (s^{-1} M^{-1} × 10^{-6})	K_i (nM)	k_{on} (s^{-1} M^{-1} × 10^{-6})
BPN′	2.9	1.9	hydrolysis	
Carlsberg	12.6	*a*	25	1.1
BPN′ES156	3.2	3.2	66	0.5

[a] True slow-binding kinetics not observed.

instability, presumably caused by autolysis, which has been reported previously, especially at pH values above neutrality (Beith & Wermuth, 1973). For this reason, estimates of K_i may be less reliable for this enzyme. However, a potentially significant finding from these inhibitor studies was that enzyme stability is improved in the presence of inhibitor. Thus, we found that over the course of our experiments the rate of reaction in the absence of inhibitor was eventually exceeded by rates in the presence of the inhibitor, which were maintained. Consequently, after prolonged incubation, more substrate had been hydrolyzed in solutions containing inhibitor. This may well have some clinical implications if proteolytic enzymes can be stabilized in vivo by exogenous inhibitors.

On the same principles of substrate specificity used in the trypsin and chymotrypsin work, MA59CI2 was designed to be an improved inhibitor of porcine pancreatic elastase, which is believed to have a specificity for small hydrophobic residues in the P_1 site (Beith & Wermuth, 1973). Binding to elastase was weaker than CI2(wt) binding, $K_i = 600 \times 10^{-9}$ M (20-fold weaker binding). This is unlikely to be the result of some gross structural perturbation since binding of the mutant inhibitor to subtilisin was still tight and stoichiometric binding curves were observed. The same phenomenon of enzyme stabilization in the presence of mutant inhibitor was also observed in this case with elastase, as with CI2(wt), above.

Subtilisin Studies. Three subtilisin enzymes were examined in this study with two CI2 proteins, to investigate inhibitory mechanism. Subtilisins are broad-specificity enzymes, which makes them useful in studying a range of substrates or inhibitors. Position 156 in subtilisin has been identified as an important residue for substrate binding (Wells et al., 1987a,b; Russell, 1987), so we examined inhibitor binding with subtilisin BPN′ (glutamate 156), subtilisin Carlsberg (serine 156), and subtilisin BPN′ES156 (a mutant BPN′, serine 156). These enzymes were studied with CI2(wt) (methionine 59) and MK59CI2 (lysine 59). The results from these kinetic studies are tabulated in Table III, and Figure 4 shows representative slow-binding curves for each interaction from families of curves produced by slow-binding inhibition experiments. Clearly, slow-binding-type kinetics are observed with each subtilisin and both inhibitors, but the replacement of methionine by lysine has made a less potent CI2 inhibitor of subtilisin in each case. The most striking behavior is seen in Figure 4B, curve 1, for the interaction between MK59CI2 and subtilisin BPN′. The data collected for this interaction could not be fitted to the slow-binding equation (eq 1), and the actual data points are shown. The curve shows that initially there is a lag phase before the onset of inhibition, but no steady-state is reached. This would be the expected pattern if inhibitor hydrolysis is taking place. Cleavage of MK59CI2 by subtilisin BPN′ was confirmed by PAGE, shown in Figure 5. A brief time course is shown for CI2(wt) and MK59CI2 incubated with subtilisin BPN′. Times for the curves shown in Figure 4 and for incubation of complex for PAGE are not directly comparable because slow-binding kinetics are pre steady state, largely due to the low concentrations of enzyme and inhibitor. Complex

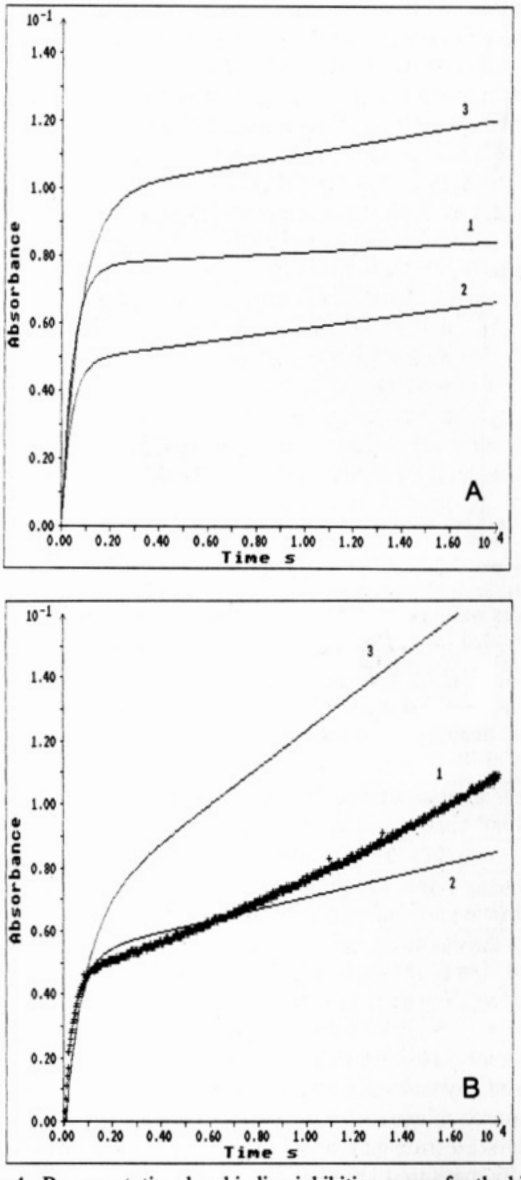

FIGURE 4: Representative slow-binding inhibition curves for the binding of CI2 to subtilisins. (A) Binding of CI2(wt) to subtilisin BPN′ ([I] = 10 nM) (curve 1); CI2(wt) binding to subtilisin Carlsberg ([I] = 9 nM) (curve 2); CI2(wt) binding to subtilisin BPN′ES156 [(I] = 10 nM) (curve 3). (B) Binding of CI2MK59 to the same three enzymes, respectively, where [I] = 12 nM (curve 1), [I] = 8 nM (curve 2), and [I] = 50 nM (curve 3). Curve 1 of panel B is the data points that would not fit to eq 1, due to inhibitor hydrolysis.

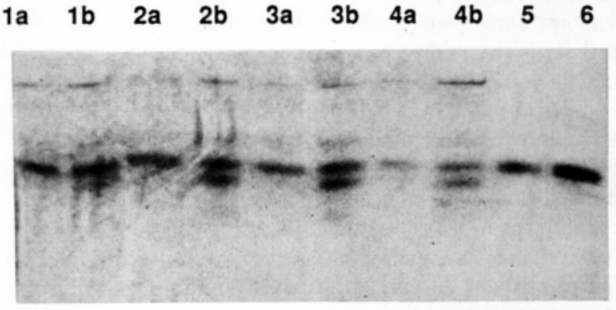

FIGURE 5: PAGE analysis of subtilisin BPN′ complexed with CI2(wt) (lanes a) or CI2MK59 (lanes b). Complexes were dissociated in SDS in the presence of PMSF after incubation at 25 °C for 0 min (lanes 1a and 1b), 10 min (lanes 2a and 2b), 30 min (lanes 3a and 3b), and 60 min (lanes 4a and 4b). Lanes 5 and 6 are standards of CI2(wt) and CI2MK59, respectively. The higher molecular mass band in lanes 1–4 is subtilisin (M_r 27 000). CI2MK59 is less stable as can be seen in lanes 1b to 4b, whereas no breakdown is observed with CI2(wt) in lanes 1a to 4a.

formation for PAGE is carried out with more concentrated protein solutions, so the reaction is essentially instantaneous, on a steady-state time scale. Another, inherent practical difficulty with this approach generally arises from the way in which the complex is dissociated for electrophoresis. We have observed that adding denaturing PAGE sample buffer to subtilisin solutions invariably leads to severe autolysis of the enzyme, and no intact protein is subsequently detectable on gels. Preincubation with PMSF, to inactivate the enzyme chemically, prevents this. However, it is still uncertain what happens when denaturing buffer is added to complex, even in the presence of PMSF, as in this experiment. What we can conclude from the gels we have run is that MK59CI2 is much less stable than CI2(wt) in the presence of subtilisin BPN′ and that specific cleavage of inhibitor proceeds with time, producing a readily detectable fragment which would be expected to have a M_r of approximately 6000. Further incubation of CI2MK59 with subtilisin BPN′, or shorter incubation at 37 °C rather than at 25 °C, leads to complete loss of the native, intact protein band (not shown). It should also be pointed out that the results shown in Figure 5 cannot be explained by CI2MK59 being a structurally altered inhibitor, making it more susceptible to hydrolysis, since it is only in combination with one enzyme (subtilisin BPN′) that any significant hydrolysis was observed.

Discussion

The secretion vector pINIIIompA3 containing the truncated gene was found to produce high levels of CI2 that could be easily purified. Large-scale preparations could provide on the order of several hundred milligrams, sufficient for all our planned uses. This is an unusually high level of expression for a small protein.

Kinetics of inhibition of CI2 with subtilisin or chymotrypsin appear at first sight to be markedly different. Inhibition of subtilisin BPN′ follows slow-binding kinetics whereas inhibition of chymotrypsin is by classical kinetics. The difference in kinetics does not necessarily, however, imply a difference in mechanism of inhibition. The difference in the K_i values between the two systems is accounted for by the dissociation rate constant, k_{off}, (see Table I). The half-lives for dissociation of the complexes of subtilisin and chymotrypsin are 37.0 h and 1.8 min, respectively, whereas the association rate constants for the two systems are of the same order. This illustrates the point that the term "slow-binding inhibition" does not imply slow association of two partners during complex formation but may simply be a consequence of k_{off} (see eq 2). Apparent slow binding here is caused by the combination of low dissociation rate and equilibrium constants. The observed rate constant for the binding of an inhibitor under pseudo-first-order conditions is $k_{obs} = k_{off} + k_{on}[I]$, and so if k_{off} is low and [I] is low when the low values of K_i are measured, k_{obs} can be low even when k_{on} is at the diffusion-controlled limit.

There are many examples of slow-binding inhibition that result from a very low value for k_{on}, unlike here where k_{on} is within 2 orders of magnitude of the diffusion-controlled limit for the association of large molecules. It has been suggested by Morrison and Walsh (1988) that most, if not all, of the slow-binding inhibitors with low values of k_{on} bind to proteins and induce a conformational change. Information about the mechanism of binding may be derived from analysis of slow-binding data, e.g., for the three common mechanisms A–C.

mechanism A

$$E + I \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} EI$$

mechanism B

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EI'$$

mechanism C

$$E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E' \underset{k_{-2}}{\overset{k_2[I]}{\rightleftharpoons}} E'I$$

The plot of k' vs [I] shown in Figure 2A is a straight line, which is consistent with mechanism A. Mechanism B should give a hyperbolic plot, leveling off at high [I]. Mechanism C gives decreasing values of k' with increasing [I] [see Cha (1975) and Williamson and Morrison (1979)]. Shapiro and Riordan (1984) have shown that a plot of $1/(k' - k_x)$ vs $1/[I]$, where k_x is k_{off} or k_{-2}, is linear for mechanisms A and B, respectively. Such a plot (not shown) is a straight line passing through the origin as expected for mechanism A. Further, we find v_o to be independent of [I], which is expected for mechanism A. Thus, all our data are indicative of a simple bimolecular association of E and I (mechanism A) over the range of inhibitor concentrations we studied. However, mechanism B will give the same kinetics as mechanism A when the range of inhibitor concentrations is insufficient to give saturation of binding at the first step in B. At increasing concentrations of inhibitor, "docking" of the two proteins might show up as mechanism B. Further work is required to establish the mechanism of binding of CI2 to subtilisin BPN′ at higher concentrations of inhibitor, although other techniques will be required since it is not technically possibly to increase [I] much more in the slow-binding experiments described here.

These findings also have important implications for the mode of action of these small protein inhibitors generally. Following on from findings that many active site cleaved inhibitors can be resynthesized in native form by proteolytic enzymes, mechanisms of inhibition have been proposed involving equilibria between virgin and cleaved forms of inhibitors [e.g., see Laskowski and Sealock (1971) for a review]. Processing of the bound inhibitor to a cleaved form would be expected to show up in the slow-binding inhibitor studies discussed above as mechanism B or an extension thereof, but clearly does not. Significantly, all highly refined crystal structures of enzyme–inhibitor complexes published to date show the inhibitor peptide bond to be intact with little or no distortion from planarity [see Laskowski and Kato (1980) and Read and James (1986) for review]. The simplest interpretation of our kinetic results and the crystallographic data is that inhibitor binding occurs with minimal change in inhibitor structure such that the EI adduct represents a frozen Michaelis complex or something very closely related.

It is also apparent from the data presented in Figure 1 that, following binding of the inhibitor to subtilisin, a steady-state rate is established that is stable over the length of the experiment. Thus, there is no loss of inhibitor activity due to breakdown of the inhibitor. Using this slow-binding kinetics approach enables this type of behavior to be detected when it occurs.

The P_1 residue has long been appreciated as the major determinant of specificity in protease–ligand interactions (Bode et al., 1989; Carrell & Travis, 1985). Initially, we made three mutants with single amino acid substitutions at the P_1 residue to investigate inhibitory specificity in CI2. Two of these, MK59CI2 and MY59CI2, gave clear results that specificity can be tailored to an enzyme on the basis of rational considerations of substrate specificity. However, this was not so clear with MA59CI2, which is a weaker inhibitor of porcine pancreatic elastase than is CI2(wt). Results from a number of

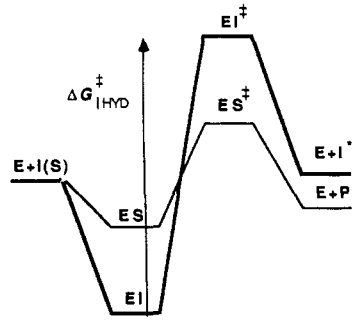

FIGURE 6: Reaction profile for the interaction of a serine protease with substrate (S) or with protein inhibitor (I). Inhibitor binds tightly to the enzyme leading to a very stable complex, EI, equivalent to the Michaelis complex. The energy of the transition state EI* is raised due to inherent inhibitor stability. Thus, $\Delta G^{\ddagger}_{I(hyd)}$ is large, and production of cleaved inhibitor I* is unfavorable. Inhibition comes about by two mechanisms: (1) lowering of the energy of EI (Michaelis complex) by enzyme–inhibitor complementarity; (2) raising of the energy of EI* (transition state) by having an inflexible peptide in the substrate binding cleft. In this way, the EI complex is in a "thermodynamic pit" with a large barrier preventing hydrolysis.

studies have shown that small hydrophobic P_1 residues are generally preferred by elastases, with neutrophil elastase having a slightly more hydrophobic S_1 site than porcine pancreatic elastase. However, this preference is dependent on the type of substrate or inhibitor [e.g., Bode et al. (1989)]. In agreement with these studies we must also conclude that designing new inhibitors may be more involved than simply matching the P_1 site to the known substrate specificity of the enzyme, and other sites of interaction may have to be taken into account.

Mechanism of Inhibition. The magnitudes of the binding constants of the protease inhibitors were considered in the past to be surprisingly high, and so special mechanisms of binding were sought. However, it is now realized that there is ample binding energy available for tight binding of large ligands to proteins and that observed values of K_m often underestimate the utilization of binding energy, which is generally manifested more fully in the binding of transition states (Fersht, 1985). It has long been believed that inhibitors bind to enzymes in the manner of a good substrate [e.g., see Read et al. (1983), McPhalen and James (1988), and Bode et al. (1989)], and this can be clearly seen in the crystal structure of subtilisin BPN′ complexed with CI2(wt), where many interactions stabilize the complex. The current problem concerning protease inhibitor–protease inhibition is thus not to explain the tight binding but why turnover of the inhibitors is so slow.

One reason for slow hydrolysis, illustrated in Figure 6, is that the enzyme may bind the inhibitor in such a way that favorable noncovalent bonds in the enzyme–inhibitor complex have to be disturbed on formation of the enzyme–transition state complex—the opposite of the use of binding energy in normal enzyme catalysis [see Fersht (1985)]. This is why crystal structures of inhibitor–enzyme complexes are the Michaelis complex (or close to it) and inhibitors bind well to catalytically inactive enzymes (Haynes & Feeney, 1968). Subsequently, the rigid structure of the inhibitor can raise the energy level of the transition state. CI2, in common with other protease inhibitors, has a binding loop stabilized by many intramolecular bonds [e.g., Laskowski and Kato (1980) and McPhalen and James (1987)]. Circumstantial evidence for the importance of rigidity of the binding loop comes from inhibition studies using CI1, a protein closely related to CI2, but without one of the two stabilizing arginines (arginine 67) believed to be responsible for holding the active site loop in place. CI1 is more readily cleaved than CI2 and provides an example of temporary inhibition (Jonassen & Svendsen, 1982; Boisen et al., 1981). CI1 is a poorer inhibitor than CI2 although CI1 may have broader specificity as a consequence.

A second reason stems from the amino group that is released on cleavage of the peptide bond remaining attached to the enzyme–inhibitor complex. In the hydrolysis of normal substrates, formation of the acyl enzyme and release of amine is usually written as occurring simultaneously. But, there must be a transient acyl enzyme–amine complex, as in eq 6.

$$\mathrm{E + RCONHR' \overset{K_S}{\rightleftharpoons} E{\cdot}RCONHR' \overset{k_2}{\underset{k_{-2}}{\rightleftharpoons}}}$$
$$\mathrm{E{-}COR{\cdot}NH_2R' \overset{k_{diss}}{\rightleftharpoons} E{-}COR + NH_2R' \rightleftharpoons}$$
$$\mathrm{E + RCO_2^- + H^+} \quad (6)$$

Normally, the rate and equilibrium constants for the dissociation of the complex are high, and so it can be ignored. If, however, the amino group is held rigidly in the position it occupies immediately on the formation of the E–COR·NH_2R' complex, as could occur on cleavage of a protease inhibitor, then the equilibrium between E·RCONHR′ and E–COR·NH_2R' (k_{-2}/k_2) must be considered. This equilibrium should greatly favor the uncleaved peptide as shown from the following analysis based on the data of Fastrez and Fersht (1973), who determined the free energy profile for the hydrolysis of Ac-Phe-AlaNH_2 to Ac-Phe + AlaNH_2 catalyzed by chymotrypsin. k_2 is 5 s^{-1}, the dissociation constant of AlaNH_2 from the acyl enzyme (K_{diss}) is $\gg 1$ M, and k_{-2}/K_{diss} is 6340 M^{-1} s^{-1}. Thus, $k_{-2} \gg 6340$ s^{-1} and so $k_{-2}/k_2 \gg 1000$. For a normal substrate, $k_{diss} \gg k_{-2}$ and k_{cat} for peptide hydrolysis = k_2. If, however, k_{diss} is less than k_{-2}, then, $k_{cat} = k_{diss}(k_2/k_{-2})$. That is, k_{cat} will be $\ll k_{diss}/1000$ for the values determined by Fastrez and Fersht (1973). In other words, if the amino group released on cleavage of the inhibitor is held in a position that is productively bound for attack on the acyl enzyme, then an equilibrium is set up between acyl enzyme and virgin inhibitor that greatly favors the virgin inhibitor and so the overall hydrolysis rate is greatly decreased. Hydrolysis of the inhibitor requires that the released amino group can move from its position next to the acyl group to another site in the complex. This must occur in those situations where there is a measurable equilibrium between enzyme-bound virgin and cleaved inhibitors [see Laskowski and Sealock (1971)]. The relative importance of these two mechanisms, before and after cleavage, is not known. However, in the case of CI2 our kinetic studies show a simple bimolecular association with no evidence of further processing.

The observed hydrolysis of MK59CI2 catalyzed by subtilisin BPN′ (Figures 4 and 5) may be explained with these ideas. It should be stressed that although hydrolysis is observed, MK59CI2 may still be classified as an inhibitor and is a very poor substrate. This type of inhibition is usually termed temporary inhibition [cf. Laskowski and Kato (1980)]. That hydrolysis is not due to a structural alteration in the inhibitor per se is indicated by the lack of hydrolysis in the presence of subtilisin Carlsberg and BPN′156. The hydrolysis of MK59CI2 catalyzed by subtilisin BPN′ is caused by small structural alterations in the structure of the enzyme-bound complex(es) from the interaction between Lys-59 (inhibitor) and Glu-156 (enzyme). This could perturb the difference in energy levels between the EI complex and the EI* transition state, reducing the energy barrier for hydrolysis, and/or allow the released $-NH_2$ group more freedom to move from the acyl–serine bond.

The action of other, unrelated inhibitors may also be explained with these mechanisms. For example, it is known that

some plasma inhibitors (serpins) are cleaved and that the virgin (I) and modified (I*) inhibitor structures can be quite different (e.g., α_1-proteinase inhibitor, antithrombin III, α_1-antichymotrypsin, and C1 inhibitor) with termini widely separated (Bruch et al., 1988). However, cleavage is a slow event, and it is the initial binding and inhibition prior to cleavage that are biologically important. The initial binding of those serpins can be accounted for by the processes outlined in Figure 6. If so, serpin–protease complexes would have structures similar to those of other serine protease inhibitor–protease complexes. Proof of this must await the solution of a crystal structure of a complex.

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Registry No. Chymotrypsin, 9004-07-3; subtilisin, 9014-01-1; trypsin, 9002-07-7; elastase, 9004-06-2.

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