Design of a Small Peptide-Based Proteinase Inhibitor by Modeling the Active-Site Region of Barley Chymotrypsin Inhibitor 2

Robin J. Leatherbarrow* and Henryk J. Salacinski‡

Department of Chemistry, Imperial College of Science, Technology and Medicine, South Kensington, London SW7 2AY, U.K.

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ABSTRACT: A synthetic peptide-based proteinase inhibitor was constructed by modeling the regions responsible for inhibition in barley chymotrypsin inhibitor 2 (CI-2). The 18-residue peptide was designed by molecular modeling, based on the crystal structure of CI-2. The amino acid sequences that interact with the proteinase were preserved, as well as residues that maintain the structure of the inhibitory loop. A disulfide bridge was introduced to force the peptide to adopt a cyclic structure. Kinetic studies on binding of the cyclic peptide to subtilisin BPN′, subtilisin Carlsberg, chymotrypsin, and pancreatic elastase show that the cyclic peptide retains both the inhibition properties, the kinetic mechanism, and the specificity of the original protein inhibitor. Formation of a cyclic structure was found to be essential, and activity was abolished by reduction of the disulfide. As with CI-2, tightest binding is found to subtilisin BPN′, where the K_i value for the cyclic peptide was 28×10^{-12} M, compared with 29×10^{-12} M for CI-2 under identical conditions. This remarkable result shows that it is possible to use a short synthetic peptide to model the molecular recognition properties of the intact protein, in this case obtaining full functionality with just 18 residues instead of 83 for CI-2.

Proteinases are involved in numerous biological processes, including digestion of food, the cascade systems of blood clotting and complement, activation of hormones, and degradation of endogenous proteins within cells. It is very important to regulate the activity of these enzymes and prevent uncontrolled proteolysis, which can result in tissue damage (Hörl, 1989). The most common mechanism employed in vivo is via specific proteinase inhibitors. Inhibition by proteinase inhibitors is essentially irreversible and involves the formation of a tight noncovalent 1:1 enzyme–inhibitor complex, which resembles a Michaelis complex. Although the inhibitor binds in the same manner as a good substrate, the energy barrier for hydrolysis is large and unfavorable, resulting in extremely low rates of hydrolysis (Laskowski & Kato, 1980; Read & James, 1986; Longstaff et al., 1990). The portion of the inhibitor that interacts with the proteinase is usually an external loop. In all proteinase inhibitors studied, there are many intramolecular interactions that stabilize the binding loop, which is thought to reduce the rate of cleavage. Most proteinase inhibitor families have disulfide bridges flanking the reactive site bond (Laskowski & Kato, 1980), although many members of the potato inhibitor 1 family lack disulfides but have an extensive network of hydrogen bonds and electrostatic interactions that are thought to perform a similar role (McPhalen & James, 1987, 1988).

In this paper, we aim to produce a synthetic inhibitor that incorporates the structural portions from a proteinase inhibitor that are responsible for inhibition. From the previous description, we felt that retention of the structure around the reactive site loop, together with the interactions that stabilize this loop structure, may be enough to retain the inhibitory activity. We have used the inhibitor chymotrypsin inhibitor 2 (CI-2) as a model on which to test this hypothesis because it has well-characterized structure and kinetics. CI-2 is a small 83-residue protein isolated from *Hiproly* barley (Jonassen, 1980; Svendsen et al., 1980a) and is a potent inhibitor of chymotrypsin and subtilisin (Svendsen et al., 1980b). The kinetics of inhibition have been extensively characterized (Longstaff et al., 1990). Although initially described as an inhibitor of chymotrypsin, CI-2 binds subtilisin far more tightly, and so is sometimes referred to as barley serine proteinase inhibitor-2, or BSPI-2. CI-2 is a member of the potato inhibitor 1 family of proteinase inhibitors (Laskowski & Kato, 1980; Svendsen et al., 1982), which usually lack stabilizing disulfide bonds. Crystal structures have been determined for the free inhibitor (McPhalen & James, 1987) and for the complex with subtilisin BPN′[1] (McPhalen & James, 1988). In addition, a solution structure has been calculated from NMR data and is very similar to the crystal structure (Clore et al., 1987a,b). The reactive site bond is between Met59 and Glu60 of the inhibitor [numbering as in McPhalen and James (1987)], with Met59 occupying the P_1 position [nomenclature as in Schechter and Berger (1967)].

Materials and Methods

Materials. Subtilisin Carlsberg, bovine pancreatic α-chymotrypsin, and porcine pancreatic elastase were purchased from Sigma Chemical Co. Purified recombinant subtilisin BPN′ was a gift from Dr. A. J. Russell and Prof. A. R. Fersht. CI-2 was purified from a clone expressing the gene in *Escherichia coli* (Longstaff et al., 1990) and isolated as described previously (Jandu et al., 1990). Activity assays for subtilisin and chymotrypsin used the chromogenic substrate *N*-succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide (Sigma). Corresponding assays for elastase used *N*-succinyl-Ala-Ala-Ala *p*-nitroanilide (Sigma). The concentrations of proteolytic enzymes were determined from initial rates using the following kinetic values: subtilisin BPN′, $k_{cat} = 55\ s^{-1}$ and $K_m = 0.15$ mM (Russell, 1987); subtilisin Carlsberg, $k_{cat} = 950\ s^{-1}$ and $K_m = 0.23$ mM (Russell, 1987); chymotrypsin, $k_{cat} = 45\ s^{-1}$ and $K_m = 0.04$ mM (DelMar et al., 1979); pancreatic elastase, $k_{cat} = 16.6\ s^{-1}$ and $K_m = 1.15$ mM (Beith & Wermuth, 1973).

Kinetic Measurements. Characterization of k_{on}, k_{off}, and K_i for both the cyclic peptide and CI-2 was performed es-

* To whom correspondence should be addressed.

‡ Present address: Department of Chemical and Biochemical Engineering, University College, London WC1E 7JE, U.K.

[1] Subtilisin BPN′ is also known as subtilisin Novo.

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FIGURE 1: Structure of the complex between CI-2 and subtilisin BPN′ [coordinates from McPhalen and James (1988)]. The peptide chain of each protein is shown, with CI-2 emboldened. Only the loop region of the inhibitor is involved in the interaction.

sentially as described by Longstaff et al. (1990). All measurements were in 0.144 M Tris-HCl, pH 7.8, and were at 25 °C. Reduction of the disulfide bond was achieved using 1 mM DTT. Data analysis was performed by nonlinear regression (Leatherbarrow, 1990a) using the GraFit computer program (Leatherbarrow, 1990b).

Synthetic Peptide. The 18-residue cyclic peptide described in Figure 3 was synthesized by Cambridge Research Biochemicals, Cambridge, U.K., and was generously donated by Drs. Paul Sheppard and Ian M. Varndell of Cambridge Research Biochemicals. The material was supplied in purified form (bioassay grade, >95%) and was used without further purification.

Molecular Modeling. The cyclic peptide was modeled into the coordinates of CI-2 (generously provided by Dr. M. N. G. James and Dr. C. A. McPhalen) using the program FRODO (Jones, 1978). Preliminary coordinates were then transferred to the program MacroModel (Still, 1989) and energy-minimized to ensure that the modeled disulfide adopted an energetically feasible conformation.

RESULTS

Design of the Synthetic Inhibitor. The X-ray structures of CI-2, and of the complex between CI-2 and subtilisin BPN′, show that inhibition occurs by the formation of a tight noncovalent complex (McPhalen & James, 1987, 1988). No large conformational changes occur in either protein on association, although the relatively flexible reactive site loop of CI-2 and subtilisin loop 96–105 become much better ordered in the complex. The structure of this complex is shown in Figure 1. Very few residues from CI-2 are involved in the interaction, which is limited to the extended loop region between residues ~54 and 63. The conformation of this loop is maintained by various interactions involving side chains extending from the core of the protein, particularly Arg65, Arg67, and Phe69. We have recently shown that replacement of Arg67 with alanine causes a significant 1.5 kcal mol^{-1} destabilization of CI-2 (Jandu et al., 1990). The mutation Arg65→Ala results in no recoverable expressed protein (unpublished observations), which suggests that removal of this interaction greatly destabilizes the protein. A minimum requirement for inhibition by CI-2 could therefore be the inhibitory loop region itself, plus the residues that maintain the loop structure, residues 65–69. Examination of the CI-2 structure suggests that this requirement is met by a single peptide segment, extending between residues ~53 and 70. In CI-2, the ends of this region are in close proximity. Modeling studies indicated that it would be possible to form a disulfide bond between the ends of this structure, if residues 53 and 70 were replaced by Cys

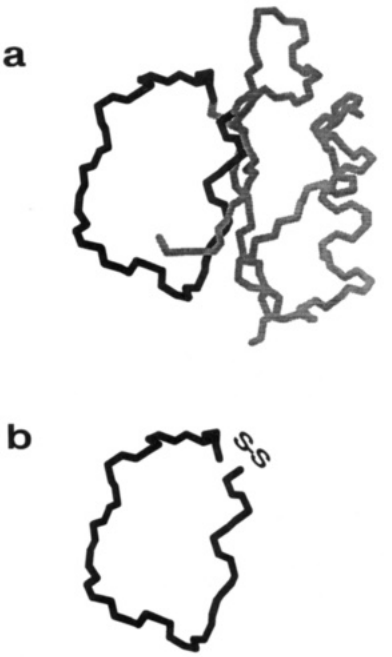

FIGURE 2: Design of the peptide inhibitor. The backbone structures of (a) CI-2 and (b) the peptide inhibitor are shown. The residues that are incorporated into the peptide are shown in black; the remainder of the CI-2 residues are shaded. The region that is chosen to create the peptide consists of the binding loop, together with most of the residues that are responsible for maintaining the structure of this loop. A disulfide bridge is introduced into the peptide to create a closed cyclic molecule.

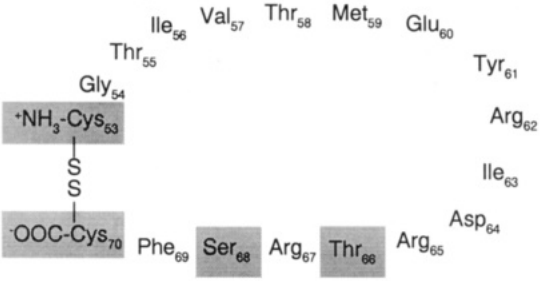

FIGURE 3: Sequence of the cyclic peptide. Numbering is based on that for CI-2 (McPhalen & James, 1987). Residues that differ from those found in CI-2 are shaded, and are Cys53 (Val), Thr66 (Val), Ser68 (Leu), and Cys70 (Val) (sequence in CI-2 is given in parentheses).

residues. The formation of a closed cyclic molecule will restrict the conformational space of the peptide and should force the molecule to adopt a structure similar to the equivalent region in the parent protein. The strategy is outlined in Figure 2.

Two further alterations were incorporated into the peptide. The side chains of residues Val66 and Leu68 are buried in the core of the CI-2 protein, but in the proposed peptide structure will be exposed to solvent. Exposed hydrophobic residues could destabilize the structure (Dill, 1990), and so these side chains were replaced by the hydrophilic Thr and Ser, respectively. The final sequence is shown in Figure 3. On the basis of the X-ray structure of CI-2, the cyclic peptide structure was built using MacroModel (Still, 1989). Energy minimization of the modeled structure using the AMBER force field (Weiner et al., 1986) suggests that the disulfide can be formed with minimal disruption to the rest of the molecule. The energy-minimized structure of the cyclic peptide is shown in Figure 4.

Stoichiometry of Inhibition by the Cyclic Peptide. The addition of a proteinase inhibitor such as CI-2 to a solution of proteinase results in progressive loss of activity due to the formation of a 1:1 complex. This gives a titration curve

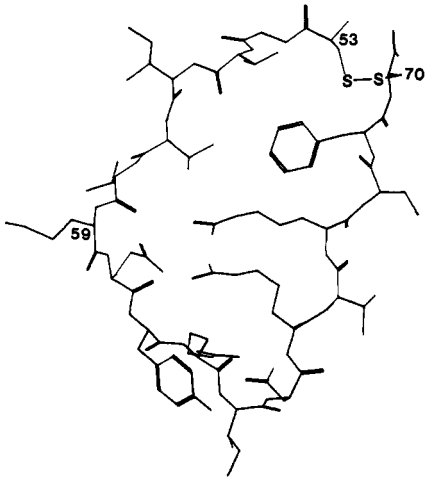

FIGURE 4: Modeled structure of the cyclic peptide. The coordinates of CI-2 (McPhalen & James, 1987) were used, and the sequence shown in Figure 3 was modeled in using the program FRODO (Jones, 1978). The structure was then energy-minimized with the AMBER force field (Weiner et al., 1986) using the program MacroModel (Still, 1989).

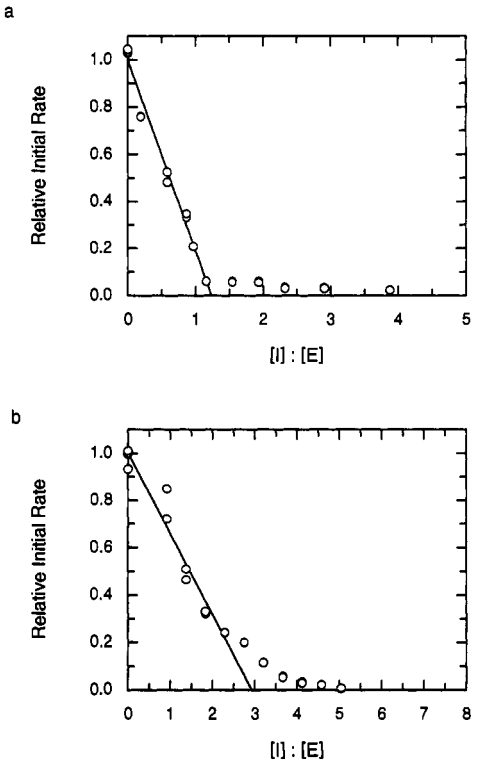

FIGURE 5: Inhibition of subtilisin BPN′ by (a) CI-2 and (b) the cyclic peptide. Enzyme and inhibitor were preincubated for 2 h prior to measuring the rate of substrate hydrolysis, to allow time for formation of the EI complex. The substrate concentration was 0.093 mM and the enzyme concentration 5.2×10^{-8} M. Initial rates are scaled relative to the rate at [I] = 0. The concentration of enzyme was calculated from the rate at [I] = 0 and that of the inhibitors is based on the dry weight.

characteristic of tight binding inhibition, as shown in Figure 5a for the inhibition of subtilisin BPN′ by CI-2. Such curves allow the concentration of active inhibitor to be determined from the x-axis intercept provided the concentration of proteinase is known. The inhibition curve given by the cyclic peptide is shown in Figure 5b. The result clearly demonstrates that the peptide retains inhibitory activity. The concentration values displayed on the x axis, which are based on the dry weight of the supplied peptide, suggest that only around a third of the material is active. The reason for this discrepancy is unclear and could represent solid impurities (e.g., salt) in the

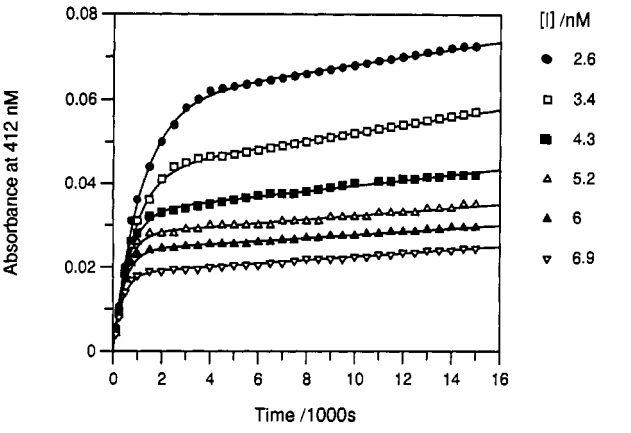

FIGURE 6: Slow binding kinetics of subtilisin BPN′ by the cyclic peptide. The time course of inhibition is shown at varying concentrations of peptide. The substrate concentration was 0.106 mM and enzyme concentration 2.5×10^{-10} M.

supplied sample, or inactive forms of the peptide itself. It could also be due to incomplete cyclization, as the linear form is inactive (see later). Whatever the cause, we have chosen to use the active concentration of peptide in the subsequent experiments.

Requirement for a Disulfide Bond in the Peptide. To test whether the disulfide bond is necessary for inhibition, the above assay was repeated in the presence of DTT, which would act to reduce the -S-S- to -SH + HS-. No detectable inhibition was found under these conditions if the peptide was preincubated with DTT. This shows that disulfide bond formation is required for tight association of the peptide with the proteinase. However, if the peptide was incubated with subtilisin prior to addition of DTT, inhibition was not reversed by the reductant. This was not caused by the subtilisin protecting the peptide from reduction, since the presence of free thiols on the reduced subtilisin–peptide complex could be demonstrated by titration with Ellmans reagent (Deakin et al., 1963) (results not shown). It is therefore concluded that once the proteinase–peptide complex is formed, the disulfide is no longer required for inhibition.

Kinetic Properties of Inhibition. The interaction of subtilisin with CI-2 is characteristic of slow binding inhibition. The formation of the EI complex is slow at low [I], and it is possible to determine values for the association rate constant, k_{on}, by analyzing the time course of inhibition at varying inhibitor concentrations. The inhibition of subtilisin BPN′ by CI-2 demonstrates a typical slow attainment of equilibrium, with the initial rate of substrate hydrolysis being independent of inhibitor concentration. Very similar kinetic profiles are found for the cyclic peptide (Figure 6). These inhibition curves can be analyzed by assuming a simple model for the inhibition, as shown below (Longstaff et al., 1990).

$$E + I \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} EI \qquad (1)$$

The curves in Figure 6 are described by the integrated rate equation:

$$A = v_s t + (v_0 - v_s)(1 - e^{-kt})/k \qquad (2)$$

where A is the absorbance of the product, v_0 is the initial noninhibited and v_s the final inhibited rates of reaction, respectively, k is the rate constant for obtaining inhibition, and t is time. The relationship between the rate constant, k, from this equation, and the on and off rate constants, k_{on} and k_{off}, is

$$k = k_{off} + k_{on}[I]/(1 + [S]/K_m) \qquad (3)$$

Table I: Inhibition of Various Proteinases by CI-2 and the Synthetic Cyclic Peptide

	CI-2			cyclic peptide		
proteinase	K_i (M)	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_i (M)	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})
subtilisin BPN′	2.9×10^{-11}	1.8×10^{5}	5.4×10^{-6} [a]	2.8×10^{-11}	6.5×10^{5}	1.8×10^{-5} [a]
subtilisin Carlsberg	1.3×10^{-10}	1.1×10^{5}	1.3×10^{-5} [a]	1.1×10^{-10}	2.4×10^{5}	2.6×10^{-5} [a]
chymotrypsin	1.6×10^{-8}	3.9×10^{5} [b]	6.1×10^{-3}	1.6×10^{-8}	3.9×10^{5} [b]	6.2×10^{-3}
pancreatic elastase	3.0×10^{-10}	(–) [c]	(–) [c]	3.9×10^{-10}	(–) [c]	(–) [c]

[a] Calculated from K_i and k_{on} using eq 5. [b] Calculated from K_i and k_{off} using eq 5. [c] Binding data did not conform to a single slow binding step; only K_i is calculated.

In principle, the variation of k with [I] could allow both k_{on} and k_{off} to be determined. In practice, k_{off} is too small to allow accurate determination from such plots. The final rate, v_s, in eq 2 allows K_i to be determined as

$$\frac{v_0 - v_s}{v_s} = \frac{[I]}{K_i(1 + [S]/K_m)} \quad (4)$$

k_{off} can be calculated from K_i and k_{on} using the relationship

$$K_i = k_{off}/k_{on} \quad (5)$$

A plot of $(v_0 - v_s)/v_s$ versus [I] for inhibition of subtilisin BPN′ by the cyclic peptide is linear (not shown), consistent with the kinetic scheme shown above (eq 1). The values for the kinetic constants obtained for CI-2 and for the peptide are summarized in Table I. The values found here for CI-2 differ slightly from those given by Longstaff et al. (1990).

Inhibition Kinetics with Subtilisin Carlsberg. Subtilisin Carlsberg and subtilisin BPN′ differ by 82 residues in sequence, but have very similar tertiary structures (McPhalen & James, 1988). CI-2 also inhibits subtilisin Carlsberg strongly, but the K_i is greater than that found with subtilisin BPN′ (Longstaff et al., 1990). The cyclic peptide was also found to inhibit subtilisin Carlsberg, with kinetic behavior similar to that observed with subtilisin BPN′. The kinetic constants for these interactions are listed in Table I.

Inhibition Kinetics with Chymotrypsin. Although originally described as a chymotrypsin inhibitor, CI-2 inhibits chymotrypsin relatively poorly. Due to a higher k_{off}, the inhibition is no longer slow binding under the assay conditions used (Longstaff et al., 1990). K_i can therefore be determined using standard kinetic assays, but k_{on} is too fast to obtain in the manner described above. However, dilution of EI complex results in dissociation at an appreciable rate, and so k_{off} can be calculated using eq 6. The value for k_{on} is obtained by

$$A = \frac{k_{cat}[E_0](k_{off}t + e^{-k_{off}t} - 1)}{k_{off}} \quad (6)$$

application of eq 5. The cyclic peptide was found to inhibit chymotrypsin in a similar manner to the CI-2 protein. Inhibition was much weaker than for subtilisin, and slow binding was not observed. Dissociation of the complex was observed in a similar manner to that found for CI-2, allowing k_{off} to be determined, and showing that inhibition is reversible. The calculated kinetic parameters are given in Table I.

Effect of Disulfide Reduction on the k_{off} with Chymotrypsin. Earlier, it was shown that reduction of the disulfide bond abolished association of the peptide with subtilisin BPN′ but that reduction after binding did not result in subsequent dissociation of the complex. The dissociation of the peptide from subtilisin BPN′ is too slow to measure, but for chymotrypsin, it is possible to determine the effect of disulfide bond reduction on the k_{off}. The k_{off} values found in the presence and absence of DTT as a reducing agent are, respectively, 3.9×10^{-3} and 6.2×10^{-3} s^{-1}. It is clear that reduction of the disulfide has only a small effect on the off rate constant, confirming our earlier qualitative observations that reduction does not cause dissociation of the enzyme–peptide complex.

Inhibition Kinetics with Pancreatic Elastase. CI-2 also inhibits pancreatic elastase (Longstaff et al., 1990), although the simple inhibition scheme found for subtilisin does not apply. In contrast to the results of Longstaff et al., we find that the inhibition by pancreatic elastase results in progress curves in which the initial rate varies with the concentration of inhibitor. Such results are characterized by a mechanism involving formation of an intermediate, as in eq 7 (Shapiro & Riordan,

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EI' \quad (7)$$

1984). This mechanism is found to apply to both CI-2 and the cyclic peptide. Values for K_i can be obtained by application of eq 4 once the EI complex has formed, and are given in Table I.

Discussion

The aim of this study was to determine whether the region of the CI-2 molecule that is responsible for inhibition could be reproduced using a much smaller synthetic peptide. If the approach was successful, peptide synthesis would offer many advantages for inhibitor design including the incorporation of unnatural amino acids, nonscissile linkages, and reactive groups around the active-site region. Although serine proteinase inhibitors show great variability in these regions (Laskowski et al., 1987), a synthetic analogue could allow the inhibitory specificity to be tailored in more subtle ways than are available using the natural repertoire of amino acids alone.

The inhibition constants for the synthetic peptide are found to be similar to those for CI-2 itself. The cyclic peptide is an extremely potent proteinase inhibitor, with values for K_i in the picomolar range when inhibiting subtilisins. Moreover, the specificity of the CI-2 protein is retained in the cyclic peptide. For both CI-2 and the cyclic peptide, the potency of inhibition is subtilisin BPN′ > subtilisin Carlsberg > pancreatic elastase > chymotrypsin. This strongly suggests that the interactions involving CI-2 and the cyclic peptide are broadly the same and implies that the cyclic peptide has retained the structural features of the active-site loop from CI-2. No loss of activity was observed during the period of the experiments. This suggests that, in common with CI-2, proteolysis of the peptide occurs very slowly. The mechanism of inhibition is, for each of the proteinases studied, the same for CI-2 and for the cyclic peptide. Classical inhibition kinetics are observed with chymotrypsin; slow binding, single-step kinetics (eq 1) are found for binding to subtilisin BPN′ and subtilisin Carlsberg. Binding to pancreatic elastase, with both CI-2 and the cyclic peptide, shows evidence for an intermediate step, i.e., the mechanism is as in eq 7. We also observe this mechanism with the subtilisins when using much higher concentrations of CI-2 or of the cyclic inhibitor (results not shown), implying that an initial weak "docking" of inhibitor to the proteinase is a general feature of the inhibition.

When designing a peptide based upon part of the structure of CI-2, we considered it necessary to introduce a disulfide

bond in order to force the peptide into a defined orientation. The disulfide was essential to tight binding by the peptide, and the reduced peptide had no detectable inhibitory activity. However, if the disulfide was reduced after the proteinase–peptide complex had formed, the k_{off} value was not greatly affected. The effect of reduction is therefore on association, the most probable reason being that little of the reduced peptide has the correct structure for binding. This could either be because the inherent flexibility of a linear peptide results in a very low proportion of molecules having the correct geometry for binding (i.e., there is an entropic advantage in forming a cyclic molecule) or be that the reduced peptide adopts a different conformation that does not bind to the proteinase. It is also possible that the linear molecule is cleaved by the proteinase, although we did not test this. However, once the peptide has bound to the proteinase, it is no longer important to maintain the disulfide bond in order to retain binding. Okada et al. (1989) have compared the binding to chymotrypsin and cathepsin G of eglin C and a linear octapeptide comprising the P_4–P_4' residues of eglin C. The peptide was found to bind 5–6 orders of magnitude more weakly. Large reductions in binding constant are also found for an octapeptide based on α_1-proteinase inhibitor (McRae et al., 1980). It is possible that a similar difference in binding affinity applies for our reduced peptide—under the conditions used, however, such a loss of binding activity would have resulted in no observable inhibition.

Cyclic peptides have been used by Powers et al. (1981) to inhibit human leukocyte elastase. The best inhibitor they found, which was based on the sequence of α_1-proteinase inhibitor, had K_i = 0.39 mM. Disulfide-linked nonapeptides based on the sequence of soybean Bowman–Birk inhibitor have been used with greater success by Terada et al. (1978), although the tighest binding found was 3 orders of magnitude less than the natural inhibitor. Our cyclic peptide, while larger than those used in these studies, was a far more effective inhibitor and had binding comparable to the protein from which it was designed. We feel that the reason for our success is because our peptide was based on the X-ray structure of the inhibitor. Designing peptides using sequence alone is clearly not sufficient, and it is also necessary to have the correct structure for good interaction with the enzyme.

In summary, our results show that it is possible to use information from X-ray crystallography together with peptide synthesis to create a small peptide mimic that retains the activity of the original protein. Although CI-2 is an ideal system for production of such analogues, having only a limited area of contact with the proteinase that it inhibits, it should also be possible to apply peptide synthesis techniques to produce analogues for other systems. In this regard, we note that very interesting results have recently been obtained by reproducing the catalytic triad of serine proteinases in order to generate enzyme activity (Hahn et al., 1990). As peptide synthesis becomes increasingly routine, we expect such techniques to be of great importance in studies on the processes of molecular recognition.

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