Potent Slow-Binding Inhibition of Cathepsin B by Its Propeptide†

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ABSTRACT: A peptide (PCB1) corresponding to the proregion of the rat cysteine protease cathepsin B was synthesized and its ability to inhibit cathepsin B activity investigated. PCB1 was found to be a potent inhibitor of mature cathepsin B at pH 6.0, yielding a K_i = 0.4 nM. This inhibition obeyed slow-binding kinetics and occurred as a one-step process with a $k_1 = 5.2 \times 10^5$ M^{-1} s^{-1} and a $k_2 = 2.2 \times 10^{-4}$ s^{-1}. On dropping from pH 6.0 to 4.7, K_i increased markedly, and whereas k_1 remained essentially unchanged, k_2 increased to 4.5×10^{-3} s^{-1}. Thus, the increase in K_i at lower pH is due primarily to an increased dissociation rate for the cathepsin B/PCB1 complex. At pH 4.0, the inhibition was 160-fold weaker (K_i = 64 nM) than at pH 6.0, and the propeptide appeared to behave as a classical competitive inhibitor rather than a slow-binding inhibitor. Incubation of cathepsin B with a 10-fold excess of PCB1 overnight at pH 4.0 resulted in extensive cleavage of the propeptide whereas no cleavage occurred at pH 6.0, consistent with the formation of a tight complex between cathepsin B and PCB1 at the higher pH. The synthetic propeptide of cathepsin B was found to be a much weaker inhibitor of papain, a structurally similar cysteine protease, and no pH dependence was observed. Inhibition constants of 2.8 and 5.6 μM were obtained for papain inhibition by PCB1 at pH 4.0 and 6.0, respectively. Incubation with a 10-fold excess of the propeptide resulted in its cleavage by papain at both pH values. The results presented here complement the data from similar investigations with serine and aspartic proteases and with metalloproteases indicating that the proregion of all four classes of protease plays an important physiological role in inactivating their corresponding enzyme.

Most proteases are synthesized as inactive precursors with N-terminal peptide chain extensions termed proregions, and active enzyme is formed as the result of proteolytic cleavage within these proregions. Proteases have been assigned to four classes on the basis of their active centers: metalloproteases and aspartic, serine, and cysteine proteases. The best characterized class, the serine proteases, includes enzymes such as trypsin and chymotrypsin which have very short proregions of <10 residues and others such as subtilisin E (Wong & Doi, 1986) and α-lytic protease (Silen et al., 1988) which have proregions of 77 and 166 amino acid residues, respectively. With the exception of trypsin-like proteases, proteases from all four classes generally have long proregions which are thought to play important roles in protein folding as well as in inhibiting the proteases prior to maturation (Zhu et al., 1989; Ohta et al., 1991; Baker et al., 1992a,b). Thus, once formed, the mature proteases do not necessarily contain all of the information required for their correct folding, as demonstrated for subtilisin E (Zhu et al., 1989).

Recent studies on metalloproteases (carboxypeptidase A) and serine (carboxypeptidase Y, subtilisin E, and α-lytic protease) and aspartic (cathepsin D, renin, and pepsin) proteases have shown that their corresponding propeptides inhibit the mature enzymes, often with K_i's in the nanomolar range. The propeptide of each protease appears to be specific in its inhibition since they generally only inhibit related proteases. This is illustrated for the propeptide of α-lytic protease, which is a potent inhibitor of that enzyme and the homologous *Streptomyces griseus* protease B but does not inhibit the more distantly related enzyme elastase (Baker et al., 1992a). Similar results were obtained for the propeptides of the aspartic proteases chicken pepsin and cathepsin D (Fusek et al., 1991). Evidence for the involvement of the proregion in protein folding is extensive for the serine proteases. For example, the propeptides of subtilisin E (Ohta et al., 1991) and α-lytic protease (Baker et al., 1992b) play an essential role in the correct refolding of denatured protein to re-form active enzyme.

Although it has also been demonstrated that the propeptides of carboxypeptidase A and numerous aspartic proteases are potent inhibitors of the corresponding mature enzymes, there has yet been no report of the inhibitory properties of the propeptides of cysteine proteases. However, the proregion of papain has been shown to play an important role in expression of the active enzyme since deletion of the portion of the gene coding for the proregion of papain prevents the heterologous expression of the enzyme (Vernet et al., 1991). Cathepsin L has been expressed in *Escherichia coli*, and deletions in the proregion resulted in improper protein folding and/or processing of the renatured enzyme (Smith & Gottesman, 1989). The proregions of cysteine proteases are generally >90 amino acid residues in length. For example, those of papain (Cohen et al., 1986) and cathepsins H (Ishidoh et al., 1987), L (Portnoy et al., 1986), and S (Shi et al., 1992) are 107, 92, 96, and 98 amino acid residues long, respectively. In contrast, cathepsin B has a relatively short proregion of only 62 amino acid residues (Chan et al., 1986). Lysosomal cysteine proteases such as cathepsin B are thought to play an important role in intracellular protein degradation, and cathepsin B is of great interest since extracellularly it has been implicated in the pathogenesis of rheumatoid arthritis (Mort et al., 1984;

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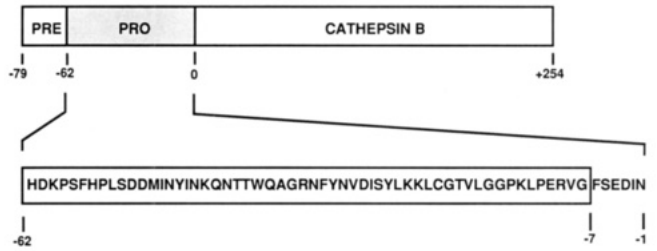

FIGURE 1: Schematic diagram of rat pre-procathepsin B showing the primary sequence of the proregion which was chemically synthesized (residues –62 to –7).

Trabandt et al., 1991), muscular dystrophy (Katunuma & Kominami, 1987), and tumor metastasis (Sloane, 1990). Due to its physiological relevance and the shorter length of its prosequence compared to those of other cysteine proteases, we chose to study the role of the propeptide of cathepsin B.

Cathepsin B is synthesized as a glycosylated preproenzyme (Chan et al., 1986), and processing to a 29-kDa single-chain active form occurs on route to the lysosome. Recently, rat procathepsin B has been expressed in yeast. In this system, autoprocessing occurs to yield fully active enzyme (Hasnain et al., 1992). Maturation of the recombinant precursor has been studied in vitro using an inactive mutant where the active-site cysteine was changed to a serine (Rowan et al., 1992). It was shown that intermolecular processing of procathepsin B occurs by cleavage of the propeptide, leaving a short N-terminal extension which can be trimmed by the action of exopeptidases. Autoprocessing by the recombinant cathepsin B leaves a six-residue N-terminal extension corresponding to the first six C-terminal residues of the propeptide. We have chemically synthesized a peptide representing the first 56 residues from the N-terminus (PCB1) (denoted as residues –62 to –7 in Figure 1). The inhibitory properties of the synthetic propeptide toward recombinant rat liver cathepsin B were investigated to gain insight into possible physiological roles of the proregion. To explore the specificity of the propeptide, we have also studied its inhibition of a structurally similar cysteine protease, papain.

MATERIALS AND METHODS

Papain, DTT, and 2,2′-dipyridyl disulfide (PDS)[1] were purchased from Sigma. HPLC-grade DMSO and acetonitrile were used throughout. Agarose–Ahx-Gly-Phe-Gly-semicarbazone was obtained from Aminotech, Ottawa, Canada. The substrate Z-Phe-Arg-MCA and the inhibitor E-64 were purchased from IAF Biochem International Inc., Laval, Quebec.

Synthesis and Purification of PCB1. Peptide synthesis was carried out using standard Fmoc chemistry on an Applied Biosystems 431A solid-phase synthesizer. Crude peptide (PCB1) was purified to homogeneity by HPLC on a Vydak C4 (300 Å) column (1 × 25 cm) using a linear 10–70% acetonitrile gradient (1%/min) at a flow rate of 2 mL/min (A = 0.1% TFA in water; B = 0.1% TFA in acetonitrile). PCB1 was rechromatographed using the same conditions and stored in solution at 4 °C. The HPLC elution profile of PCB1 stored under these conditions remained unchanged for at least 7 days. Peptide purity and concentration were determined by amino acid analysis on a Beckman Model 6300 amino acid analyzer. Protein sequencing using an Applied Biosystems 473A pulsed-liquid peptide sequencer confirmed the amino acid sequence of PCB1, and electrospray mass spectral analysis of an SCIEX API III spectrometer operated in the positive ion mode for detection of protonated species confirmed the expected molecular mass of 6378 daltons.

Purification of Recombinant Cathepsin B and Commercial Papain. Recombinant cathepsin B expressed in yeast (Rowan et al., 1992) was purified from the culture supernatant. The supernatant was concentrated to 20 mL using an Amicon spiral concentrator (S1Y10 cartridge) followed by an Amicon stirred-cell (YM-10 membrane). During concentration, the buffer was exchanged to 50 mM phosphate (pH 6.0) containing 1 mM EDTA. Concentrated supernatant containing cathepsin B was mixed with 5 mL of agarose–Ahx-Gly-Phe-Gly-semicarbazone resin, and the enzyme was purified as previously described (Rich et al., 1986) with the following modifications: The mixture was stirred in the presence of 1 mM DDT overnight at 4 °C on a labquake shaker. Fractions eluted from the affinity column which contained cathepsin B activity were pooled and concentrated in an Amicon stirred-cell (YM-10 membrane). The concentrated cathepsin B was washed 3 times with 100 mL of 50 mM phosphate (pH 6.0) containing 1 mM EDTA and reconcentrated to remove any unbound PDS, and was stored (ca. 1 mL) in silanized vials at 4 °C. Cathepsin B stored as the PDS-inactivated form was activated for 15 min on ice, by diluting stock enzyme into buffer containing 1 mM DDT. Commercial papain (Sigma) was purified on a mercurial agarose affinity column (Sluyterman & Wijdenes, 1970), and was activated with β-mercaptoethanol and passed over a G-25 column prior to use. For both enzymes, concentrations were determined by active-site titration with the inhibitor E-64 (Barrett & Kirschke, 1981), and protein purity was checked by running SDS–PAGE gels and Western blots.

Kinetic Measurements. Kinetic fluorescence measurements were carried out using a Cary 2200 spectrometer equipped with a fluorescence attachment to monitor MCA formation using an excitation wavelength of 380 nm and a 440 nm cutoff filter. A substrate (Z-Phe-Arg-MCA) concentration of 20 μM was used for slow-binding kinetics ($[S] \ll K_M$), i.e., since for this substrate K_M was estimated to be 0.188 mM under the experimental conditions used. Z-Phe-Arg-MCA concentrations of 20, 40, 80, and 120 μM were used for $1/v$ vs [I] plots (Dixon, 1953). All measurements for cathepsin B were performed at 25 °C, and assay conditions were 0.1 M phosphate (pH 5.0–6.0) or acetate (pH 4.0–5.0) buffer containing 1 mM EDTA, 1 mM DDT, 0.025% Brij-35, and 3% DMSO. The slow-binding kinetic properties measured at pH 5.0 using acetate or phosphate buffers were the same, indicating that the results are not due to the different assay buffers. Measurements for papain were carried out as described for cathepsin B except that Brij-35 was not used and DMSO was replaced with 5% acetonitrile.

Analysis of Data. Under the experimental conditions used, progress curves for the inhibition of cathepsin B by PCB1 at pH 4.7–6.0 followed typical slow-binding kinetics as defined by the equation:

$$[P] = v_s t + \frac{(v_i - v_s)[1 - \exp(-k_{obs} t)]}{k_{obs}} \quad (1)$$

where P is the product formed, v_i and v_s are the initial and steady-state velocities, respectively, t is the time, and k_{obs} is

[1] Abbreviations: Z-Phe-Arg-MCA, carbobenzoxy-L-phenylalanyl-L-arginine 4-methylcoumarinyl-7-amide hydrochloride; E-64, 1-(L-*trans*-epoxysuccinyl-L-leucylamino)-4-guanidinobutane; PCB1, chemically synthesized pro-region of cathepsin B (residues –62 to –7); PDS, 2,2′-dipyridyl disulfide; Ahx, aminohexyl.

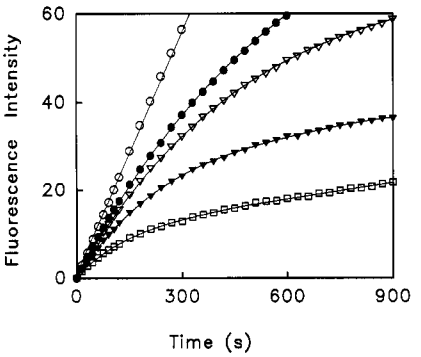

FIGURE 2: Progress curves for the slow-binding inhibition of 0.38 nM cathepsin B by PCB1 at pH 6.0 in the presence of 20 μM Z-Phe-Arg-MCA. PCB1 concentrations (in nanomolar) were as follows: (open circles) 0; (closed circles) 5; (open triangles) 10; (closed triangles) 15; (open squares) 20. Data points were collected every 3 s; however, for clarity of the figure, only every tenth point is displayed. The solid lines through the points represent the fit of all the data points collected for each progress curve to eq 1. From these fits, a value of k_{obs} was obtained for each PCB1 concentration.

the rate constant for inhibition. Nonlinear regression using the program Enzfitter (published by Elsevier-Biosoft, Cambridge, U.K.) gave the individual parameters (v_i, v_s, and k_{obs}) for each progress curve. Inhibition of cathepsin B at pH 4.0–4.5 and of papain at pH 4.0 and 6.0 gave linear plots of [P] vs time, and enzyme–inhibitor dissociation constant (K_i) values were obtained from plots of $1/v$ vs [I] (Dixon, 1953) at the four substrate concentrations.

Reactivation of PCB1-Inhibited Cathepsin B. Cathepsin B (71 nM) was incubated with PCB1 (200 nM) for 30 min at 25 °C in the pH 6.0 phosphate buffer described above. The reaction mixture was diluted 800-fold, yielding final concentrations of 0.089 nM cathepsin B and 0.25 nM PCB1 in the cuvette. Substrate hydrolysis was initiated by addition of Z-FR-MCA (20 μM final concentration) to the cuvette.

HPLC Analysis of PCB1 Incubated with Cathepsin B and Papain. PCB1 was incubated with either cathepsin B or papain at pH 4.0 or pH 6.0 overnight at 25 °C. PCB1 and either enzyme are well resolved by HPLC on a Vydak C18 column using a 10–70% acetonitrile gradient in 0.1% TFA. Samples were analyzed by HPLC at t = 0 h and t = 24 h.

RESULTS

Inhibition of Cathepsin B at pH 4.7–6.0. PCB1 exhibited slow-binding inhibition of cathepsin B activity in the range pH 4.7–6.0 (Figure 2) when the enzyme was added to a mixture containing the propeptide and Z-Phe-Arg-MCA. The rate of substrate hydrolysis decreased from an initial rate (v_i) to a much slower steady-state rate (v_s) according to the first-order rate constant (k_{obs}).

Slow-binding inhibition generally fits one or two mechanisms (Cha, 1975). A single-step process is represented by mechanism A:

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \qquad (2)$$

for which

$$k_{obs} = k_2 + k_1[I] \qquad (3)$$

Mechanism B involves a two-step process:

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EI^* \qquad (4)$$

for which

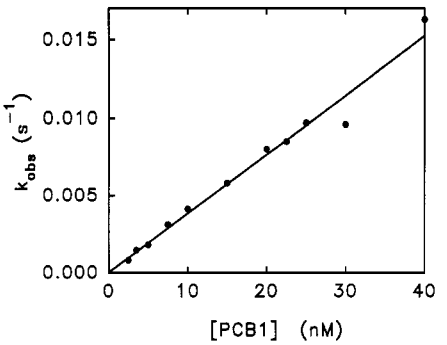

FIGURE 3: Plot of k_{obs} vs [PCB1] concentration for data collected using cathepsin B at pH 6.0. From a regression analysis of the data, a value of k_1 can be obtained (see text).

$$k_{obs} = k_4 + \frac{k_3[I]}{[I] + K_i} \qquad (5)$$

where $K_i = k_2/k_1$. It is possible to differentiate between the two mechanisms since a plot of k_{obs} vs [I] remains linear for mechanism A but saturates in [I] for mechanism B. A plot of initial velocity (v_i) vs [I] will also distinguish between the two mechanisms since v_i is unchanged for mechanism A but decreased with increasing [I] for mechanism B (Cha, 1975). The progress curves in Figure 2 show little or no change in v_i during the early stages of the reaction, and a plot of k_{obs} vs [I] (Figure 3) remains linear over the range of inhibitor concentrations studied (5–40 nM), suggesting that inhibition of cathepsin B by PCB1 occurs by a one-step process (mechanism A). Progress curves for substrate hydrolysis on dilution of a preincubated mixture of cathepsin B and PCB1 showed a lag phase, during which little substrate was hydrolyzed, followed by an increase in substrate hydrolysis until the steady-state velocity (v_s) was reached. The magnitude of v_s was similar either when the reaction was initiated by addition of enzyme or when enzyme was preincubated with PCB1, consistent with slow-binding kinetics (Morrison, 1969; Cha, 1975; Morrison & Walsh, 1988; Morrison & Stone, 1985).

For each data set, the values of v_i, v_s, and k_{obs}, obtained as described under Materials and Methods, can be replotted using eq 3 and the relationship $v_i/v_s - 1 = [I]/K_i$ (Izquierdo-Martin & Stein, 1992) to obtain values for the parameters defined by mechanism A, i.e., k_1, k_2, and K_i. For example, from such plots, the inhibition of cathepsin B by PCB1 at pH 6.0 yielded a K_i = 0.4 nM and a $k_1 = 5.2 \times 10^5$ M^{-1} s^{-1}. Unfortunately, from the plot of eq 3 (Figure 3), the near-zero intercept prevents an accurate determination of k_2. However, using the relationship $K_i = k_2/k_1$, a $k_2 = 2.2 \times 10^{-4}$ s^{-1} was calculated. Similar calculations for data collected at pH 4.7 and 5.0 show that k_1 remained unchanged (5.2×10^5 M^{-1} s^{-1}) while k_2 increased to a value of 4.5×10^{-3} s^{-1} at pH 4.7. The inhibition at pH 6.0 appeared to be reversible since an 800-fold dilution of a preincubated mixture of E + PCB1 (1:3) into an assay mixture resulted in a gradual increase in enzymatic activity such that after 45 min approximately 7% of the enzymatic activity had been regained.

Inhibition of Cathepsin B at pH 4.0–4.5. Although slow-binding kinetics were observed for cathepsin B inhibition by PCB1 over the pH range 4.7–6.0, below pH 4.7, the progress curves became more linear, and typical competitive inhibition was observed; i.e., over the pH 4.0–4.5 range, the rate of substrate hydrolysis appeared to be linear, and $1/v$ vs [I] plots (Dixon, 1953) yielded intersecting lines. At pH 4.0, a value of 64 nM was obtained for K_i (Table I). Above pH 4.0 and

Table I: Enzyme–Inhibitor Dissociation Constants for the Inhibition of Cathepsin B and Papain by PCB1 at pH 4.0 and 6.0

enzyme	K_i (nM) pH 4.0	K_i (nM) pH 6.0
cathepsin B	64 ± 9[a]	0.4 ± 0.1[b]
papain	2800 ± 1100[a]	5600 ± 3500[a]

[a] Calculated from $1/v$ vs [I] plots (Dixon, 1953). [b] Calculated from v_i and v_s values (see Analysis of Data).

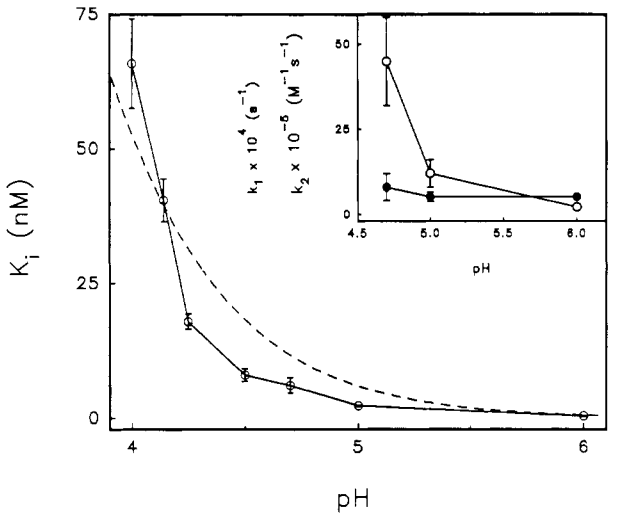

FIGURE 4: pH dependence of the K_i for cathepsin B inhibition by PCB1. K_i values were calculated as described in the text. Data points are connected by a solid line, and the dashed line represents the attempted fit of the data points to an equation assuming a single pK_a. Inset: pH dependence of k_1 (solid circles) and k_2 (open circles).

below pH 4.7, some curvature was visible during the first few seconds of the reaction due to a rapid approach to the steady-state velocity at low pH. In addition to the lack of apparent slow binding at low pH, the K_i also increased dramatically (Figure 4), indicating that the binding between cathepsin B and PCB1 is weaker. Attempts to fit the data in Figure 4 to an equation assuming a single pK_a proved unsuccessful (dashed line in Figure 4), implying that the dependency of K_i on pH must be due to the ionization of more than one group on either the enzyme or PCB1.

Inhibition of Papain at pH 4.0 and 6.0. The kinetics of inhibition of papain by PCB1 at both pH 4.0 and pH 6.0 were similar to those seen for cathepsin B at pH 4.0 in that substrate hydrolysis appeared to be linear for the duration of the experiment. Papain was inhibited weakly at both pH 4.0 and pH 6.0 (K_i = 2.8 and 5.6 μM, respectively) (Table I), indicating that there is little or no difference in selectivity in papain inhibition.

PCB1 Cleavage by Cathepsin B and Papain. A 10-fold excess of PCB1 was incubated for 24 h with either cathepsin B or papain at pH 4.0 or 6.0. PCB1 incubated with cathepsin B at pH 6.0 showed no change in the HPLC profile over the 24 h, indicating that both the propeptide and cathepsin B remained intact. When PCB1 was incubated with cathepsin B at pH 4.0 or with papain at pH 4.0 and 6.0, there was extensive loss of the peaks corresponding to both the peptide and the enzyme which were both present at t = 0 h. Furthermore, a number of peaks corresponding to new peptides were eluted in the early stages of the chromatogram, suggesting that over the 24-h period, cathepsin B (pH 4.0) and papain (pH 4.0 and 6.0) in addition to cleaving PCB1, also autodigest, thus accounting for the loss of peaks corresponding to the propeptide and enzyme and for the appearance of many new peptides (not shown).

DISCUSSION

PCB1 is a potent inhibitor of cathepsin B with a dissociation constant (K_i) of 0.4 nM at pH 6.0. This dissociation constant is comparable with values obtained for the inhibition of the other classes of proteases by their corresponding propeptide (Baker et al., 1992a; San Segundo et al., 1982; Fusek et al., 1991). Under the experimental conditions used, the inhibition of cathepsin B by PCB1 at pH 6.0 is consistent with a slow-binding mechanism as described by Morrison (1969). Slow-binding inhibitors have been identified for a variety of enzymes, e.g., hirudin inhibition of thrombin (Stone & Hofsteenge, 1986), Z-prolinylprolinal inhibition of prolyl carboxypeptidase (Bakker et al., 1990), and avidin inhibition of pyruvate carboxylase (Duggleby et al., 1982). In the present study, it is shown that the inhibition of cathepsin B by PCB1 is best described by mechanism A, a one-step process involving the slow binding of inhibitor to the enzyme with no subsequent isomerization of the enzyme–inhibitor complex.

It is interesting to note from the literature that slow-binding inhibitors generally possess k_1 values of 10^5–10^7 M^{-1} s^{-1} (Duggleby et al., 1982; Nicklin & Barrett, 1984; Björk et al., 1989) and that no correlation appears to exist with regard to inhibitor size. The k_1 of 5.2×10^5 M^{-1} s^{-1} measured in this study for the binding of PCB1 to cathepsin B between pH 4.7 and 6.0 is in the range reported for other systems exhibiting slow-binding kinetics (Duggleby et al., 1982; Nicklin & Barrett, 1984; Björk et al., 1989). In contrast to the relatively narrow range of k_1 values measured for slow-binding inhibitors, the values of k_2, and consequently K_i, vary over 5-orders of magnitude for different enzyme–inhibitor complexes. For example, the chicken cystatin/papain complex has a $k_2 = 5.7 \times 10^{-7}$ s^{-1} (Björk et al., 1989), and the chicken cystatin/ficin complex has a $k_2 = 1.1 \times 10^{-2}$ s^{-1} (Björk & Ylinenjärvi, 1990), whereas the value obtained in this study for cathepsin B with PCB1 at pH 6.0 is 2.2×10^{-4} s^{-1}. Furthermore, the 20-fold increase in k_2 observed at pH 4.7, suggests that the decrease in affinity of PCB1 for cathepsin B is due primarily to an increase in the dissociation rate for the complex. This may be due to the protonation of one or more groups on the enzyme, PCB1 or both, which are important for binding. Since the effect is largest below pH 5.0, it is possible that the ionization of one or more carboxylate groups is important for binding.

Cystatins and stefins are potent protein inhibitors of cysteine proteases which usually show slow-binding inhibition kinetics (Björk et al., 1989; Brömme et al., 1991). The inhibition of papain, ficin, chymopapain A, dipeptidyl peptidase, and cathepsin B and S by cystatins and stefins (Nicklin & Barrett, 1984; Björk et al., 1989; Björk & Ylinenjärvi, 1990; Brömme et al., 1991; Lindahl et al., 1992) exhibits k_1 values of 10^6–10^7 M^{-1} s^{-1} and k_2 values of 10^{-2}–10^{-7}, yielding K_i's ranging from 5 nM for actinidin/chicken cystatin (Björk & Ylinenjärvi, 1990) to 11 fM for papain/cystatin C (Lindahl et al., 1992). The k_1 values for the binding of the cystatins and stefins to the cysteine proteases are 4–19 times higher than that observed for PCB1 binding to cathepsin B. This difference could be explained by the cystatins and stefins possessing a more rigid and hence defined structure (Bode et al., 1988; Stubbs et al., 1990) in solution than the smaller and hence potentially more flexible PCB1. The induction of additional structure in the propeptide on binding to the enzyme would clearly result in a slower k_1 rate.

The K_i of 0.4 nM for PCB1 inhibition of cathepsin B at pH 6.0 is comparable with the corresponding value of 0.1 nM obtained for the inhibition of α-lytic protease by its propeptide (Baker et al., 1992a), but it is lower than the K_i values obtained for propeptide inhibition of cathepsin D (K_i = 30 nM) and chicken pepsin (K_i < 10 nM) (Fusek et al., 1991). A number of small peptides corresponding to parts of the proregion of renin have been synthesized, and they exhibit K_i values in the micromolar range for that enzyme (Kumar & Kasell, 1977; Cumin et al., 1985). However, no data are available for inhibition of renin by its full-length propeptide. Carboxypeptidase A, a metalloprotease, is inhibited by its propeptide with a K_i = 1.9 nM (San Segundo et al., 1982), which is 5-fold weaker than cathepsin B inhibition by PCB1. Inhibition of the serine protease subtilisin E by its propeptide with a K_i of 540 nM (Ohta et al., 1991) is >5000-fold weaker than the inhibition of α-lytic protease by its propeptide (Baker et al., 1992a). This is particularly interesting because it has been shown that the proregions of subtilisin E (Zhu et al., 1989; Ohta et al., 1991) and α-lytic protease (Baker et al., 1992b) are essential for the refolding of denatured enzyme to yield active enzyme. Clearly, despite the different affinities the proregions have for mature enzyme, varying over 3 orders of magnitude, effective protein folding still takes place for both subtilisin E and α-lytic protease. The apparent essential nature of the propeptide for refolding of subtilisin E is in contrast to the situation observed by Bryan et al. (1992) with subtilisin BPN′. They have recently shown that subtilisin BPN′ can be refolded in the absence of its propeptide. The refolding process was found to be strongly dependent on the buffer ionic strength, being more effective at high ionic strength (I = 0.5). It was suggested that when present, the propeptide, although not essential for folding, may help overcome the high kinetic barrier between folded and unfolded states.

At pH 4.0, PCB1 is a 160-fold weaker inhibitor of cathepsin B when compared to its action at pH 6.0. At the lower pH, PCB1 behaves as a classical competitive inhibitor where the establishment of an equilibrium between E, I, and EI occurs rapidly. This is in contrast to the slow-binding kinetics observed at pH 6.0. The weaker inhibition and faster inhibitor on–off rates at pH 4.0 are both consistent with a proposed mechanism for the processing of cysteine proteases as shown for procathepsin B (Rowan et al., 1992) and propapain (Vernet et al., 1991). These proenzymes are both processed more effectively at acidic pH. In addition, renatured procathepsin L is processed efficiently at pH 3.0–5.0 (Smith & Gottesman, 1989). At pH 4.0, the weaker inhibition of cathepsin B by PCB1 and the consequential cleavage of the propeptide by the enzyme are in accord with a processing mechanism that is triggered by a drop in pH; i.e., at pH 6.0, a tight complex is formed between the proenzyme prodomain and the mature protease. On changing to acidic pH, the interaction between the pro and mature domain becomes weaker, and the propeptide in this looser complex becomes more susceptible to proteolysis. Such a processing mechanism would explain the observation that procathepsin B does not undergo processing before being transported to the acidic environment of the lysosome (Nishimura & Kato, 1987).

At pH 6.0, papain is inhibited by PCB1 with a K_i = 5.6 μM, i.e., 14 000 times less effectively than cathepsin B under the same conditions. Inhibition of papain activity by the propeptide is equally poor at pH 4.0, yielding a K_i of 2.8 μM, suggesting that there is little or no effect of pH on papain inhibition by PCB1. This selective inhibition of cathepsin B by PCB1 may be physiologically important since mammalian cells contain many proteases, all with different proregions. A lack of selectivity in protease inhibition by the proregions could result in indiscriminate protease inhibition and thus interfere with an otherwise effective mechanism for controlling the activity of individual proteases.

Data obtained for four serine proteases reveal the importance of the proregion in ensuring that protein folding results in the production of correctly assembled active enzyme as well as inhibition of enzymatic activity (Ohta et al., 1991; Winther & Sorenen, 1991; Lee et al., 1991; Baker et al., 1992b). Evidence for inhibition of aspartic proteases (Kumar & Kasell, 1977; Cumin et al., 1985; Fusek et al., 1991) and metalloproteases (San Segundo et al., 1982) by their propeptides is consistent with a mechanism of protein folding which may be similar for all four classes of proteases, and it would not be surprising if the propeptides have similar functions in these enzymes. We are currently synthesizing smaller fragments of the propeptide of cathepsin B to study their inhibitory properties and to investigate the role the propeptide plays in protein folding.

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