The Occluding Loop in Cathepsin B Defines the pH Dependence of Inhibition by Its Propeptide†

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ABSTRACT: Papain-like proenzymes are prone to autoprocess under acidic pH conditions. Similarly, peptides derived from the proregion of cathepsin B are potent pH-dependent inhibitors of that enzyme; i.e., at pH 6.0 the inhibition of human cathepsin B by its propeptide is defined by slow binding kinetics with a K_i of 3.7 nM and at pH 4.0 by classical kinetics with a K_i of 82 nM. This pH dependency is essentially eliminated either by the removal of a portion of the enzyme's occluding loop through deletion mutagenesis or by the mutation of either residue Asp22 or His110 to alanine; e.g., the mutant enzyme His110Ala is inhibited by its propeptide with K_i's of 2.0 ± 0.3 nM at pH 4.0 and 1.1 ± 0.2 nM at pH 6.0. For the His110Ala mutant the inhibition also displays slow binding kinetics at both pH 4.0 and pH 6.0. As shown by the crystal structure of mature cathepsin B [Musil, D., et al. (1991) *EMBO J. 10*, 2321−2330] Asp22 and His110 form a salt bridge in the mature enzyme, and it has been shown that this bridge stabilizes the occluding loop in its closed position [Nägler, D. K., et al. (1997) *Biochemistry 36*, 12608−12615]. Thus the pH dependency of propeptide binding can be explained on the basis of a competitive binding between the occluding loop and the propeptide. At low pH, when the Asp22−His110 pair forms a salt bridge stabilizing the occluding loop in its closed conformation, the loop more effectively competes with the propeptide than at higher pH where deprotonation of His110 and the concomitant destruction of the Asp22−His110 salt bridge results in a destabilization of the closed form of the loop. The rate of autocatalytic processing of procathepsin B to cathepsin B correlates with the affinity of the enzyme for its propeptide rather than with its catalytic activity, thus suggesting a possible influence of occluding loop stability on the rate of processing.

Cathepsin B (EC 3.4.22.1) is the most abundant lysosomal cysteine protease and is a unique member of the papain superfamily (*1*) in that it exhibits both endopeptidase and dipeptidyl carboxypeptidase (exopeptidase) activity. The X-ray crystal structure of mature cathepsin B reveals the molecular basis for this duality (*2*). Unlike other papain-like proteases, cathepsin B possesses an extra structural element referred to as the "occluding loop" which contributes to the primed subsites of the substrate binding cleft. Two critical residues of the occluding loop, His110 and His111, are strategically positioned to "occlude" extended substrates from binding to the enzyme and to accept the negatively charged carboxylate of the P_2' residue at the C-terminus of the substrates. Removal of this exposed disulfide loop (residues Cys108−Cys119) in cathepsin B was shown to increase affinity for the protein inhibitor cystatin C and the cathepsin B propeptide due to unrestricted access of these inhibitors to the active site (*3*). In addition, this variant of procathepsin B was shown to autoprocess much more slowly than the wild-type enzyme (*3*). Comparison of the recently determined three-dimensional structures of rat (*4*) and human (*5*, *6*) procathepsin B with mature cathepsin B (*2*) reveals that the occluding loop is a highly flexible segment of the

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[1] Residue numbering relates to mature human cathepsin B.

[2] Abbreviations: Z-Phe-Arg-MCA, benzyloxycarbonyl-L-phenylalanyl-L-arginine 4-methylcoumarinyl-7-amide; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; DMSO, dimethyl sulfoxide; DTT, dithiothreitol. Δ(Cys108−Cys119) corresponds to the occluding loop deletion mutant as reported in Illy et al. (*3*).

[3] In the text, the words "proregion" and "prosegment" refer to the polypeptide stretch located N-terminal to the mature enzyme in the proenzyme, while the word "propeptide" refers to the chemically synthesized polypeptide corresponding to the proregion sequence but without the mature enzyme.

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RAT      HDK    V   DMI  I  R        R        I   K      V       L E  G
HUMAN    RSRPSFHPLS  DELVNYVNKQ  NTTWQAGHNF  YNVDMSYLKR  LCGTFLGGPK  PPQRVM
         1p      10p         20p         30p         40p         50p

                                Region 3    Region 2        Region 1
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FIGURE 1: Prosequences of rat and human cathepsin B showing the primary sequence of the proregions which were chemically synthesized (residues 1p−56p). Consensus of these two sequences is 86% within the 21p−50p segment.

FIGURE 2: Superimposition of the conformations adopted by the occluding loop in procathepsin B (blue) and mature cathepsin B (brown). Note the positions of both His110 and His111 with respect to the main body of the enzyme and the orientations adopted by the disulfide bridge linking Cys108 to Cys119 (green). Single letter codes are used.

protein. The backbone of the occluding loop is able to undergo a conformational transition in which the tip of the loop moves by as much as 10 Å. The largest movement is that of the side chain of His111, which is displaced by over 14 Å with a simultaneous large movement of His110. The X-ray crystal structure of procathepsin B also reveals that the 62-residue proregion adopts an extended conformation along the surface of cathepsin B with the majority of close contacts provided by residues 21p−47p of the prosegment (Figure 1).

Cathepsin B residues in contact with the proregion are located within three major areas. The first major contact region is the substrate binding cleft of the enzyme, which accommodates residues 41p−47p of the prosegment (region 1). In this region, the prosegment adopts an extended conformation and binds in a direction opposite to that expected for natural substrates. The second major site is the primed subsite of the active site cleft, termed the occluding loop crevice, which becomes exposed upon movement of the occluding loop. Exposure of this surface on cathepsin B is required for residues 29p−40p of the proregion to bind (region 2). The third major contact region is the hydrophobic prosegment binding loop (exosite) which interacts with residues 21p−26p of the prosegment (region 3). The ability of the proregion to utilize all possible interactions with the surface of cathepsin B, namely, with the active site, the occluding loop crevice, and the exosite, is therefore highly dependent on the conformational mobility of the occluding loop. Analysis of the observed conformations which are adopted by the occluding loop (Figure 2) shows that specific contacts between His110 and His111 of the occluding loop and the rest of the protein change as the occluding loop shifts from the "open" conformation found in the proenzyme structure (*4*−*6*) to the "closed" one found in the structure of the mature enzyme (*2*). When the loop is in the open conformation, the side chains of residues His110 and Asn222 as well as His111 and Asp224 are in close proximity to one another. Following the disposal of the proregion to form the mature enzyme, these contacts are destroyed and new ones are formed. In mature cathepsin B, the side chain of His110 stacks against Trp221, and its ϵN forms a salt bridge with Asp22, located in the primed subsite of the active site cleft. The side chain of His111 is closest to Leu181, Val112, and the backbone of Asp224 and Trp225, but no salt bridges are present. Furthermore, Arg116 forms a hydrogen bond with Asp224.

It has been suggested that the expected effect of pH on the stability of the occluding loop; i.e., due to protonation/deprotonation of the stabilizing salt bridges, could in part define the pH dependencies observed for the exo- and endopeptidase activities of cathepsin B (*7*). In addition, it has been observed that the processing of procathepsin B and the inhibition of mature cathepsin B by its corresponding propeptide exhibit similar pH dependencies; i.e., at neutral pH, which can be expected to favor the open conformation of the loop, procathepsin B is less prone to autoprocessing (*8*) and the propeptide is a tight binding inhibitor (*9*), whereas at acidic pH, where the closed conformation of the loop is expected to be favored, the propeptide binds less tightly and the proenzyme autoprocesses more rapidly. Thus it is the goal of this present study to explore the possible links between the pH dependency of the cathepsin B occluding loop conformational flexibility, the propeptide binding affinity, and procathepsin B autoprocessing.

MATERIALS AND METHODS

The substrate Z-Phe-Arg-MCA was purchased from IAF Biochem International Inc., Laval, Québec. The synthesis and purification of the rat cathepsin B propeptides were as described previously (*9*). The pepsin−agarose resin was purchased from the Sigma Chemical Co. Recombinant human cystatin C was a generous gift from Dr. Irena Ekiel (Biotechnology Research Institute).

Synthesis and Purification of Human PCB1. Peptide synthesis of the human cathepsin B propeptide (residues 1p−56p) was carried out using standard Fmoc chemistry on an Advanced Chemtech MPS 396 solid-phase synthesizer. Crude human peptide (hPCB1) was partially purified by HPLC on a Vydac C4 (300 Å) column (5 × 25 cm) using a linear 20−60% acetonitrile gradient at a flow rate of 33 mL/min for 120 min (A = 0.1% TFA in HPLC grade water; B = 0.1% TFA in HPLC grade acetonitrile). Human PCB1 was rechromatographed with a Vydac C18 column (0.46 × 25 cm) using a linear 10−70% acetonitrile gradient (1%/min) at a flow rate of 1 mL/min and stored lyophilized at 4 °C. Amino acid analysis was performed using a Beckman Model 6300 amino acid analyzer. Electrospray mass spectral analysis using a Perkin-Elmer SCIEX API III spectrometer

operated in the positive mode for detection of protonated species confirmed the expected molecular mass of 6512 Da.

Expression of Wild-Type and Occluding Loop Variants of Human Procathepsin B. In vitro site-directed mutagenesis was performed as described previously (*3, 7*). The oligonucleotide 5′-TGT GAG CAC GCT GTG AAC GGC GCC C-3′ (mutated bases are underlined) was used for the His111Ala mutation which introduced a new *Dra*III site. These cDNA constructs, consisting of wild-type and occluding loop variants of human procathepsin B as a fusion with the preproregion of yeast α-factor, were digested with *Xho*I and *Not*I, and the proenzyme fragments were subcloned into the pPIC9 vector (Invitrogen Inc., San Diego, CA) and expressed in the yeast *Pichia pastoris*. For integration into the *Pichia* genome, the pPIC9-based constructs were linearized by cleavage with *Bgl*II and purified. The *P. pastoris* host strain GS115 (Invitrogen Inc.) was then transformed with the linearized constructs by electroporation. Positive transformants were grown for 2 days in medium containing glycerol as the carbon source followed by incubation in the presence of methanol for a further 3 days to induce expression of recombinant protein. The consensus sequence for oligosaccharide substitution in the mature protein had been removed by the substitution Ser115Ala for all variants of cathepsin B. The site for oligosaccharide substitution within the proregion (Asn21p) was left unaltered. For the purpose of clarity on SDS−PAGE (12% gels), recombinant proenzymes were deglycosylated using endoglycosidase H prior to their purification.

Purification of Procathepsin B. The recombinant proenzymes were purified from the culture supernatant using a hydrophobic resin under nonacidic conditions. The culture supernatant (250 mL) was concentrated to 40 mL using an Amicon stirred cell (YM-10 membrane). During concentration, the buffer was exchanged to 50 mM Tris (pH 7.4) containing 1.2 M $(NH_4)_2SO_4$. Concentrated proenzyme samples were then applied to a 10 mL column of butyl-Sepharose (Pharmacia Inc.) resin. Proenzyme fractions eluted from the column by applying a linear gradient of decreasing ammonium sulfate concentration. Procathepsin B (wild type and mutants) eluted at 0.6−0.8 $(NH_4)_2SO_4$, and the samples were stored at 4 °C.

Procathepsin B Activation. Both wild-type and His111Ala procathepsin B autoprocessed efficiently against 50 mM sodium acetate (pH 5.0) to form mature enzyme. The occluding loop deletion mutant, Δ(Cys108−Cys119), and variants carrying the mutation Asp22Ala or His110Ala autoprocessed slowly under these conditions. For this reason, these variants of procathepsin B were readily converted to mature enzyme at pH 4.7 using 50 units/mL pepsin immobilized on agarose resin. Immobilized pepsin was removed by filtration following 2 h of incubation with proenzyme. The processed enzymes were purified, and their k_{cat}/K_M values for Z-Phe-Arg-MCA were obtained as described previously (*3, 7*).

Procathepsin B Autoprocessing. Autoprocessing of purified procathepsin B to form mature enzyme was monitored using SDS−PAGE (12% gels). Wild-type (8 μM), His111Ala (3 μM), His110Ala (7 μM), and Asp22Ala (6 μM) procathepsin B were subjected to acidic pH conditions, i.e., at 50 mM sodium acetate buffer (pH 5.0) with 1 mM DTT.

Kinetic Measurements. Kinetic fluorescence measurements were carried out using a Perkin-Elmer LS-5B luminescence spectrometer which monitored MCA formation using an excitation wavelength of 380 nm and a detection wavelength of 440 nm. Since the K_M of wild-type human cathepsin B for the substrate Z-Phe-Arg-MCA was estimated to be 0.100 mM under these conditions (*3*), a substrate concentration of 10 μM was used for slow binding kinetics ($[S] \ll K_M$). Z-Phe-Arg-MCA concentrations of 10, 20, 40, and 80 μM were used for plots of $1/v$ vs [inhibitor] (*10*). The final concentration of cathepsin B mutants was 0.1 nM for each assay, except for the Δ(Cys108−Cys119) mutant at pH 4.0 where a final concentration of 1.5 nM was used due to its low activity under these conditions (*3*). Unless otherwise stated, assays were performed at 25 °C, and conditions were 50 mM phosphate (pH 6.0) or acetate (pH 4.0−5.0) buffer containing 0.2 mM EDTA, 1 mM DTT, and 3% DMSO. The enzymes studied were sufficiently stable under the assay conditions used for the time required.

Analysis of Data. Under the experimental conditions used, progress curves for the inhibition of the occluding loop mutants by propeptides at pH 6.0 (and at pH <4.7 for Δ(Cys108−Cys119), His110Ala, and Asp22Ala) followed typical one-step slow-binding kinetics as defined by the equations (*11*−*15*)

$$E + I \underset{k_{off}}{\overset{k_{on}}{\Leftrightarrow}} EI \tag{1}$$

$$[P] = v_s t + \frac{(v_i - v_s)[1 - \exp(-k_{obs} t)]}{k_{obs}} \tag{2}$$

$$k_{obs} = k_{on}[I] + k_{off} \tag{3}$$

where [P] is the concentration of free MCA formed, v_i and v_s are the initial and steady-state velocities, respectively, t is the time, and k_{obs} is 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. For each data set, the enzyme−inhibitor dissociation constant (K_i) values were obtained from the relationship $v_i/v_s - 1 = [I]/K_i$ (*16*), where v_i represents the initial rate for substrate hydrolysis in the absence of inhibitor. A plot of k_{obs} vs [inhibitor] remains linear over the range of inhibitor concentrations studied (4−40 nM), confirming that inhibition of the occluding loop mutants by both human and rat propeptides occurs by a one-step process (*9*). Due to the near-zero intercept, k_{off} values were calculated using the relationship $K_i = k_{off}/k_{on}$. Inhibition of wild-type and His111Ala cathepsin B at pH 4.0−4.5 gave linear plots of [P] vs time, and K_i values were obtained from plots of $1/v$ vs [inhibitor] (*10*) at the four substrate concentrations.

RESULTS AND DISCUSSION

As shown in Table 1, the affinity of cathepsin B for a peptide derived from its proregion is increased approximately 40-fold at pH 6.0 and 200-fold at pH 4.0 by the partial deletion of the occluding loop. In addition, the affinity of cathepsin B for the same propeptide is increased significantly at both pH 4.0 and pH 6.0, although to a lesser degree, by

Table 1: Equilibrium and Kinetic Data for the Inhibition of Human Cathepsin B Mutants by both Human and Rat Cathepsin B Propeptides at pH 4.0 and 6.0

		pH 4.0			pH 6.0			
propeptide[a]	enzyme	K_i[b] (nM)	k_{on} (M^{-1} s^{-1}) × 10^{-5}	k_{off} (s^{-1}) × 10^4	K_i[b] (nM)	k_{on} (M^{-1} s^{-1}) × 10^{-5}	k_{off} (s^{-1}) × 10^4	K_i (pH 4.0)/K_i (pH 6.0)
human	wild type	82.0 ± 8[c]			3.7 ± 0.3	0.9	3.4	22.0
human	Δ(C108−C119)	0.3 ± 0.1	7.2	2.2	0.1 ± 0.02	18.0	1.9	3.0
human	Asp22Ala	5.5 ± 0.5	0.9	4.8	2.3 ± 0.4	0.9	2.1	2.4
human	His110Ala	2.0 ± 0.3	1.7	3.4	1.1 ± 0.2	1.5	1.7	1.8
human	Asp22Ala/His110Ala	1.4 ± 0.2	2.1	2.9	2.0 ± 0.3	1.0	2.0	0.7
human	His111Ala	86.0 ± 7[c]			3.4 ± 0.4	1.1	3.7	25.0
rat	wild type	85.0 ± 6.0[c]			5.4 ± 0.6	0.5	2.7	16.0
rat	Δ(C108−C119)	0.6 ± 0.1	4.2	2.5	0.1 ± 0.03	20.2	2.0	6.0
rat	Asp22Ala	5.1 ± 0.5	0.5	2.6	3.4 ± 0.4	1.4	4.8	1.5
rat	His110Ala	4.1 ± 0.3	0.4	1.6	1.1 ± 0.3	1.9	2.1	3.7
rat	Asp22Ala/His110Ala	1.8 ± 0.2	1.1	2.0	2.8 ± 0.4	0.8	2.2	0.6
rat	His111Ala	87.0 ± 8.0[c]			6.0 ± 0.4	0.6	3.6	14.5

[a] Propeptides are comprised of residues 1p−56p of the corresponding cathepsin B proregion. [b] The K_i values are given as the averages of four determinations with the calculated standard deviations. [c] Classical inhibition observed; K_i's were determined from plots of $1/v$ vs [I] (*10*).

Table 2: Role of Rat Propeptide Aspartic Acid Residues in the Inhibition of Rat Cathepsin B by Its Propeptide at pH 4.0 and 6.0[a]

Propeptide[b]	K_i (nM) at pH 6.0	k_{on} ($M^{-1}s^{-1}$) x 10^{-5}	k_{off} (s^{-1}) x 10^4	K_i (nM) at pH 4.0[c]	K_i (pH 4.0) / K_i (pH 6.0)
FHPLSDDMINYINKQNTTWQAGRNFYNVDISYLKKLCGTVLGGPKLPERVG	0.2 ± 0.09	27.0 ± 13.0	4.7 ± 0.8	90.4 ± 0.1	450
FHPLSDDMINYINKQNTTWQAGRNFYNV**N**ISYLKKLCGTVLGGPKLPERVG	1.7 ± 0.4	3.0 ± 0.7	5.2 ± 2.5	460 ± 16	270
FHPLSD**N**MINYINKQNTTWQAGRNFYNV**N**ISYLKKLCGTVLGGPKLPERVG	2.4 ± 0.2	6.9 ± 2.0	16.2 ± 3.2	620 ± 36	260
FHPLS**NN**MINYINKQNTTWQAGRNFYNV**N**ISYLKKLCGTVLGGPKLPERVG	7.0 ± 2.3	4.3 ± 1.6	28.0 ± 10	1,800 ± 240	250

[a] Assay conditions were 0.1 M phosphate (pH 6.0) or acetate (pH 4.0) buffer containing 1 mM EDTA, 1 mM DTT, 0.025% Brij-35, and 3% DMSO. [b] Substituted propeptide residues are underlined and in boldface. [c] Classical inhibition observed; K_i's determined from plots of $1/v$ vs [I] (Dixon, 1953).

the mutation of either or both residues His110 and Asp22 to alanines. As shown by Nägler et al. (*7*) these mutations result in the destabilization of the closed conformation of the occluding loop found in the mature enzyme, thus decreasing its ability to compete with the propeptide for binding to the active site cleft.

It has been previously shown that a synthetic peptide with a sequence identical to that of the proregion of rat cathepsin B is a much weaker inhibitor (160-fold) of that enzyme at pH 4.0 than at pH 6.0 (*9*). At pH 6.0 the rat cathepsin B propeptide displays slow binding inhibition kinetics whereas at the lower pH it behaves as a classical competitive inhibitor. This pH-dependent switch in inhibition type can be accounted for by the lower affinity of the enzyme for the propeptide at pH 4.0 coupled with a more rapid rate of dissociation at the lower pH. As can be seen from Table 1, the human cathepsin B−propeptide interaction displays a similar though smaller (22-fold) pH dependency. The weaker binding and faster off rates observed for both the rat and human propeptides correlate with the observation that the processing of procathepsin B is considerably faster at pH 4.0 than at pH 6.0. Fox et al. (*9*) reported that the pH dependency of the K_i for the inhibition of rat cathepsin B by its propeptide is consistent with the influence of multiple ionizations in the pH range 4.0−6.0, and it is suggested that the ionization of one or more carboxylic groups is important for binding. However, the question of whether these carboxylic acid residues reside on the mature enzyme, the propeptide, or both was not addressed. From the present study, on the basis of the results discussed below, it is evident that it is residues within the mature enzyme that are responsible for this effect.

On comparing the sequences of the rat and human propeptides (Figure 1), it can be seen that only three acidic side chains are conserved, i.e., aspartic acid residues at positions 11p and 34p and aspartic acid (rat) and glutamic acid (human) at position 12p. To explore the possibility that one or more of these acid groups contributes to the pH dependency of propeptide binding, peptides with a five-residue deletion at the N-terminus were synthesized in which each of the three aspartic acid residues found in the rat propeptide was sequentially substituted by asparagine residues [the deletion of five residues from the N-terminus results in a 3-fold increase in K_i (pH 4.0)/K_i (pH 6.0) versus the full-length propeptide but does not adversely affect the overall pH dependency of binding, whereas it facilitates synthesis]. The dissociation constants obtained for each of the modified peptides at pH 4.0 and 6.0 are given in Table 2. It can be concluded from Table 2 that the carboxylic acid side chains found within the propeptides do not contribute significantly to the pH dependency of propeptide binding. In addition, the presence of negative charges within the propeptide does not have a large influence on the overall strength of peptide binding. A recent study (*7*) indicates that pH-dependent conformational changes occur in the occluding loop of cathepsin B in the pH range 3.0−8.0. It was suggested that at the higher pH the loop becomes more

flexible and as such less able to compete with extended endopeptidase substrates for the S′ subsites. Conversely, at lower pH values the more rigid loop binds more tightly to those sites, thus occluding them and giving rise to a higher level of exopeptidase activity. That this pH dependence of the conformation of the occluding loop also plays a major role in the pH dependency of the propeptide binding is evidenced by the effect of partial deletion of the loop. In Table 1 it can be seen that for this mutant, Δ(Cys108−Cys119), the effect of pH on the K_i of the propeptide is essentially eliminated. Also from Table 1 it can be seen that removal of the ionic interactions between residues Asp22 and His110 through the mutation of either or both residues to alanine also largely eliminates the pH dependency of the inhibition of cathepsin B by its propeptide. Essentially, as with the occluding loop deletion mutant, the high affinity of the propeptide is maintained through the pH range 4.0−6.0. As discussed above, the salt bridge between residues Asp22 and His110 is important for maintaining the loop in a closed/rigid conformation. These results again support the view that at low pH (4.0) as opposed to pH 6.0 the occluding loop is able to compete more effectively with the propeptide for the binding site on the enzyme. Clearly, any change to the overall charge of the Asp22−His110 ion pair can be expected to influence the conformational stability of the occluding loop and, as a consequence, the measured K_i of the propeptide. Thus the deprotonation of His110 or the protonation of Asp22 can be expected to influence propeptide binding. Despite the fact that His110 and His111 of the occluding loop reside adjacent to one another, there is significant selectivity observed for the His110 residue to regulating the pH dependence of propeptide binding. This selectivity can be accounted for by their difference in chemical environment since the His110 residue anchors the occluding loop to the rest of the enzyme (Figure 2). In order for the Asp22−His110 interaction to influence the propeptide binding, it is necessary that the pK_a of either of these two residues be within or very close to the pH range 4.0−6.0. Given that the stabilization effect decreases at higher pH, it is necessary to conclude that it is the deprotonation of the ϵN of His110 that is responsible for the observed pH dependency.

Since the pH dependency of propeptide binding in the pH range of 4.0−6.0 appears to be determined by the integrity of the occluding loop and since mutation of either residue Asp22 or His110 (but not His111) to alanine eliminates the pH dependency (Table 1), as discussed above, it is possible to speculate that it is the protonation state of the Asp22−His110 ion pair that directly determines the pH dependency of K_i. In this case the relationship of measured K_i [K_i(meas)] to the intrinsic K_i and pH is given by the equation

$$K_i(\text{meas}) = K_i(1 + \text{H}/K_3)/(1 + \text{H}/K_4)$$

where K_i = dissociation constant for the propeptide binding to the enzyme when His110 is deprotonated and K_3 and K_4 are the pK_a's of His110 in the free enzyme and enzyme−propeptide complex, respectively. It is interesting to note (Table 1) that the effect of the aspartic acid and histidine mutations to alanines is not additive; i.e., the double mutation, Asp22Ala/His110Ala, has the same effect as the individual mutations. This implies that the two single mutations have equivalent effects; i.e., the end result of the two mutations is the same and does not reflect interactions of these side chains with other groups. For example, in the open state of the occluding loop His110 interacts with residue Asn222. If the effect of the His110Ala mutation was due, in part, to a loss of the His110−Asn222 interaction, it is expected that the effect of the individual mutations His110Ala and Asp22Ala should show a significant degree of additivity for the double mutant.

In the study of Fox et al. (*9*) it was reported that the pH dependency of K_i is due to the influence of pH on k_{off} rather than k_{on}. Since both k_{off} and k_{on} for the mutants Asp22Ala and His110Ala are largely pH independent (Table 1), it follows that for the wild-type enzyme the pH dependency of k_{off} is a reflection of the pH dependency of the aspartate−histidine interaction. How is this possible if, as discussed above and as demonstrated by the crystal structure of procathepsin B, the aspartate−histidine interaction is broken when the proregion and presumably the propeptide is bound? One possible explanation is that for the enzyme−propeptide complex protonation of His110 serves to actively displace the propeptide; i.e., rather than the sequence, dissociation of the propeptide followed by formation of the aspartate−histidine ion pair, the ion pair is either partially or fully formed prior to the dissociation of the propeptide. Thus displacement of the propeptide would take place as a two-step process: an initial displacement from the occluding loop binding site as a result of the closing of the occluding loop and formation of the Asp22−His110 ion pair, followed by dissociation of the propeptide from the active site cleft. Clearly, competing mechanisms could involve initial displacement from the active site followed by dissociation from the occluding loop binding site and the direct one-step simultaneous displacement from both sites. The pH dependency results would suggest a significant role for the two-step process involving the earlier displacement from the occluding loop site. Conversely, the lower pH sensitivity of k_{on} implies that, for the binding process, the binding of propeptide to the open form of the enzyme (Asp22−His110 ion pair broken) predominates.

The rat and human cathepsin B propeptides (residues 1p−56p) share an overall sequence homology of 71% (Figure 1). Furthermore, fragment analysis and alanine scanning studies revealed that the segment of polypeptide between the NTTW motif (21p−24p), which binds to the hydrophobic prosegment binding loop (exosite) on the enzyme, and the CGT (42p−44p) motif, which binds through the active site cleft of cathepsin B, is crucial for the binding affinity of the free peptide to cathepsin B (*17*, *18*). The homology between these polypeptides within the segment 21p−50p increases to 86%. Therefore, it is not surprising that both the rat and human propeptides display similar inhibitory activity toward mature human cathepsin B. In fact, the K_i values for the human propeptide did not show any significant differences over those of the rat (Table 1).

Procathepsin B, in vivo, is synthesized as a glycoprotein consisting of two solvent-exposed sites of N-linked oligosaccharide substitution, one at Asn21p of the prosegment located near the hydrophobic prosegment binding loop (exosite) and a second at Asn113 located on the occluding loop of the enzyme. Due to the fact that Asn21p is not in direct contact with the surface of the enzyme (*4*−*6*), it can reasonably be concluded that the absence of glycosylation on Asn21p of

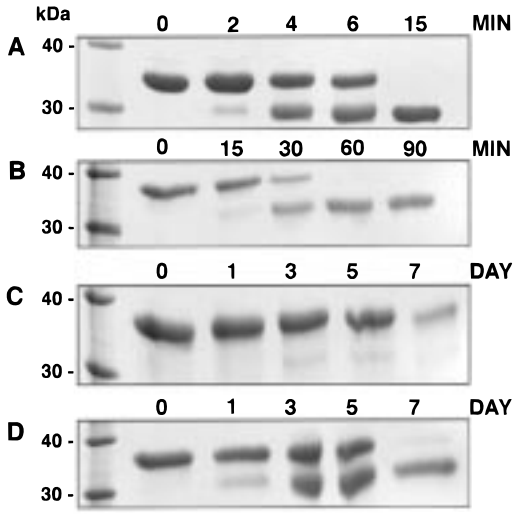

FIGURE 3: Monitoring procathepsin B (36 kDa) autoprocessing to mature cathepsin B (30 kDa) in 50 mM acetate buffer, pH 5.0, with 1 mM DTT using SDS−PAGE (12% gels). Gels A, B, C, and D correspond to wild-type (8 μM), His111Ala (3 μM), His110Ala (7 μM), and Asp22Ala (6 μM) procathepsin B, respectively. Initial proenzyme concentrations are indicated in brackets. Note the incubation times.

the chemically synthesized propeptides will not significantly affect their affinity for the enzyme. This is supported by alanine scanning studies (*17*) which did not link any unique importance of Asn21p to propeptide binding. Conversely, the absence of glycosylation on Asn113 could affect the conformational stability of the occluding loop and, as a consequence, the values of the inhibition constants obtained for the propeptides. However, similar pH dependencies of autoprocessing of the glycosylated and nonglycosylated proenzymes are observed (data not shown), implying that the influence of glycosylation may be small.

There is an interesting correlation between the rate of autoprocessing of purified full-length variants of procathepsin B (Figure 3) and the decrease in the mature enzyme's affinity for the propeptides at pH 4.0 (Table 1). For example, the affinities of the propeptides toward mature wild-type and His111Ala cathepsin B are similarly affected by pH; i.e., there is a marked decrease in affinity at low pH. Similarly, exposure of the His111Ala procathepsin B to acidic pH conditions leads to a rate of maturation comparable to wild-type procathepsin B; i.e., under the conditions given for Figure 3 processing of these enzymes is complete within minutes rather than days. Conversely, there is a maintenance of potent inhibition of the Δ(Cys108−Cys119), Asp22Ala, and His110Ala forms of the enzyme by the propeptides at low pH, and the proenzymes of these three variants process much slower than either wild-type or His111Ala procathepsin B (Figure 3). The Δ(Cys108−Cys119) proenzyme requires 5 days of incubation at pH 5.0 (*3*), and both Asp22Ala and His110Ala procathepsin B require over 7 days under the same conditions. Full-length procathepsin B is stable in neutral pH environments when the occluding loop favors an open state and is prone to autoprocess under acidic pH to form mature cathepsin B where the occluding loop favors a closed state. It is interesting to note, therefore, that the gating equilibrium of the occluding loop and autoprocessing of procathepsin B are both pH dependent. The Asp22Ala and His110Ala procathepsin B mutants shared much slower rates

Table 3: Activity of Cathepsin B toward Z-Phe-Arg-MCA

	k_{cat}/K_M ($M^{-1}s^{-1}$)		
	pH 4.0	pH 5.0	pH 6.0
wild type	180 000	380 000	430 000
His111Ala	680 000	1 150 000	1 225 000
His110Ala	190 000	320 000	400 000
Asp22Ala	550 000	900 000	1 200 000
Δ(Cys108-Cys119)	75 000	330 000	261 000

of autoactivation at pH 5.0 compared to wild-type and His111Ala procathepsin B (Figure 3). Once the occluding loop mutants have been matured using immobilized pepsin, however, they are still able to cleave small synthetic substrates (Table 3). Hence, the catalytic capability of these mutants has not been disrupted but the autoprocessing machinery has been greatly perturbed. It is possible, therefore, that the closure of the occluding loop plays an important and unique step in the elimination of the proregion in procathepsin B. Such a processing mechanism would explain the observation that perturbation of the pH-dependent gating equilibrium of the occluding loop has a profound effect on the affinity of the free propeptide binding at low pH (Tables 1 and 2) as well as the rates of procathepsin B autoprocessing (Figure 3).

The absence of an occluding loop and the presence of longer proregions in other papain-like proenzymes (>90 residues versus 62 residues in procathepsin B) would suggest that the pH-triggering mechanism of autoprocessing in procathepsin B is as unique as the enzyme's dual exopeptidase and endopeptidase character. Sequence alignment reveals that Asp22 in cathepsin B is not entirely conserved throughout the papain superfamily (*1*). It is replaced by an asparagine residue in many other cysteine proteases, such as papain, cathepsin L, and cathepsin H, and substituted by a tyrosine residue in cathepsin S. Furthermore, structural alignment indicates that the residue located in this position is not in close proximity to the bound prosegment (*19*). Therefore, other enzymes would not share the same pH dependence of prosegment binding as that observed for cathepsin B. In addition, the negative charge of a highly conserved aspartate residue in the proregion of propapain (Asp65p, papain numbering) was shown to be important in maintaining the papain precursor in a latent form and to participate in an electrostatic triggering mechanism of propapain processing (*20*). Despite the difficulty in aligning the prosegments of propapain and that of procathepsin B, the closest match to Asp65p in propapain is Asp34p in procathepsin B (cathepsin B numbering, Figure 1). As noted earlier, replacement of Asp34p in the cathepsin B propeptide to an asparagine did not alter the pH dependence of cathepsin B inhibition. Curiously, structural alignment reveals that the conserved Asp65p resides within an area of propapain which is homologous to the position of the occluding loop in procathepsin B (*19*). Furthermore, only the k_{off} value of the propeptide in cathepsin B was found to be pH dependent in the pH range 4.7−6.0 (*9*) and not that of k_{on}, as is the case with the cathepsin L propeptide (*21*). Interestingly, the modest increase in K_i values of the propeptides for the Δ(Cys108−Cys119) mutant of cathepsin B as the pH is dropped from 6.0 to 4.0 corresponds to a similarly modest decrease in the k_{on} value only. This suggests that the presence of the occluding loop in wild-type cathepsin B is responsible

for the observed increase in the off-rate of the propeptide upon exposure to low pH. It has already been established that maturation of procathepsin B proceeds by an autoactivation mechanism (*22*, *23*) and that the main role of cathepsin B in its natural environment, the acidified lysosome, is to act as an exopeptidase (*3*) which relies on the closed configuration of the occluding loop.

In summary, this study confirms a link between the pH dependencies of the cathepsin B occluding loop conformation and the propeptide binding affinity and supports a direct correlation of this link with the in vitro rates of procathepsin B autoprocessing.

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