Published in final edited form as:

FEBS J. 2009 February ; 276(3): 660–668. doi:10.1111/j.1742-4658.2008.06815.x.

Autocatalytic processing of procathepsin B is triggered by proenzyme activity

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Summary

Cathepsin B and other cysteine proteases are synthesized as zymogens, which are processed to their mature forms autocatalytically or by other proteases. Autocatalytic processing was suggested to be a bimolecular process, whereas initiation of the processing has not been clarified as yet. Procathepsin B was shown by zymography to hydrolyze the synthetic substrate Z-Arg-Arg-AMC, suggesting that procathepsin B is catalytically active. The activity based probe DCG-04, which is an E-64 type inhibitor, was found to label both mature cathepsin B and its zymogen, confirming zymography data. Mutation analyses in the linker region between the propeptide and the mature part revealed that autocatalytic processing of procathepsin B is largely unaffected by mutations in this region, including mutations to prolines. Based on these results a model for autocatalytic activation of cysteine cathepsins is suggested, involving propeptide dissociation from the active-site cleft as the first step during zymogen activation. This unimolecular conformational change is followed by a bimolecular proteolytic removal of the propeptide, which can be accomplished in one or more steps. Such activation, which can be also facilitated by glycosaminoglycans or by binding to negatively charged surfaces, may have important physiological consequences, as cathepsin zymogens were often found secreted in various pathological states.

Keywords

procathepsin B; lysosomal cysteine protease; processing; autoactivation; DCG-04

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Introduction

Cysteine cathepsins comprise a group of papain-like cysteine proteases, found predominantly in lysosomes. Cathepsin B (EC 3.4.22.1) is one of the most abundant and thoroughly studied. It plays an important role in nonselective protein degradation inside lysosomes, and is involved in the processing of other proteins and hormones, such as trypsinogen and thyroglobulin (reviewed in [1–3]). Secreted cathepsin B is often associated with pathological conditions, such as cancer progression [3–5], rheumatoid arthritis and osteoarthritis [3, 6].

Cysteine cathepsins, including cathepsin B, are synthesized as inactive proenzymes which are activated by other proteases or by autocatalytic processing in the acidic environment of late endosomes and lysosomes (reviewed in [1, 2]). From the crystal structures of procathepsins B, and L it is evident that the propeptide, which is removed during activation, blocks access to the active site that is already formed in the proenzyme [7–10]. The propeptide forms a predominantly α -helical domain, which is positioned as a “hook” at the top of the catalytic site, where it interacts with the mature part strengthening the interaction [9]. The propeptide chain then continues in an extended conformation across the active-site cleft and towards the N-terminus of the mature enzyme in the direction opposite to that of substrate binding, thereby serving as a linker between the “hook” domain and the N-terminus of the mature enzyme. This N-terminal-linker- “hook” arrangement with its reverse orientation compared to substrate binding strongly resembles the “sinker”-linker-“hook” arrangement in XIAP (X-inhibitor of apoptosis protein), which is known to block the executioner caspases [11].

The pH optimum for *in vitro* autocatalytic processing of procathepsin B, as well as of some other cathepsins, is around 4.5 [12–14]. At lower pH, the interaction between the propeptide and the mature part is weakened [15–17], resulting in a looser conformation of the proenzyme. This is followed by intermolecular cleavage of the procathepsin B propeptide [14]. However, initiation of the activation process remained an unsolved question, although it has been suggested that proenzymes may exhibit minor catalytic activity, which could potentially initiate the chain reaction [14, 18–20]. Although processing can be very rapid at higher concentrations of the proenzyme [14], it is not clear whether propeptide removal is accomplished in a single step or through one or more intermediates, as has been suggested [21].

In order to address these questions, we have studied the autocatalytic activation of recombinant human procathepsin B in the presence and absence of various small molecules under different conditions, and by performing mutation analysis. Procathepsin B was shown to exhibit low catalytic activity, which is sufficient to trigger autocatalytic activation of the zymogen. In addition, autocatalytic activation of procathepsin B was found to be largely insensitive to mutations in the cleavage-site region and could proceed at neutral pH when bound to heparin and other negatively bound surfaces, which could account for an extracellular physiological role of cathepsins.

Results

Procathepsin B is active on small synthetic substrate

In a previous study a low catalytic activity against the substrate Z-Arg-Arg-AMC was detected during the early stages of autocatalytic activation of procathepsin B, although it was never clarified whether this activity belonged to the zymogen [14]. In order to address this question, the possible activity of procathepsin B on this substrate was investigated by zymography. Recombinant human procathepsin B and cathepsin B were produced in *E. coli* and thus represented non-glycosylated enzymes. Initially, procathepsin B, cathepsin B, and inactive cathepsin B obtained by 2-hour incubation at pH 7.6 and 37 °C [22], were applied to native PAGE. Electrophoresis was performed at pH 7.4, where procathepsin B retained its stability and cannot autoactivate [14], whereas prolonged exposure to this pH results in inactivation and unfolding of mature cathepsin B [22]. Therefore, inactive unfolded cathepsin B was used as a negative control. As expected, procathepsin B migrated as a single band excluding the processing during electrophoresis (Figure 1). In addition, cathepsin B migrated as a single band with a completely different mobility from unfolded cathepsin B excluding unfolding of the enzyme during electrophoresis. In the next step, zymography was performed at pH 6.0, i.e. a condition where no autoactivation of procathepsin B can be detected [14]. Both cathepsin B and procathepsin B exhibited catalytic activity (Figure 1), suggesting that procathepsin B is catalytically active. In contrast, inactivated unfolded cathepsin B did not show any activity against the fluorogenic substrate (Figure 1). In another experiment, procathepsin B was found to hydrolyze the synthetic substrate Z-Arg-Arg-AMC *in vitro* under the same conditions (i.e. pH 7.6), consistent with the zymography results. However, the hydrolysis rate was ~100-fold lower as compared with the mature enzyme. In contrast, under these conditions procathepsin B was not able to hydrolyze denatured collagen type I, which was efficiently hydrolyzed by mature cathepsin B (data not shown). This is in agreement with the general idea that procathepsin B and other procathepsins cannot autocatalytically process at neutral pH due to the inhibitory role of the propeptide, although the active site is already formed and capable of hydrolyzing the substrates.

Autocatalytic processing of procathepsin B is delayed in the presence of small molecule inhibitors

In order to further understand the initial steps of procathepsin B autocatalytic processing, we tried to inhibit procathepsin B processing by addition of E-64, a broad spectrum inhibitor of cysteine proteases. The inhibitor concentrations were varied between 5 and 20 % of the molar concentration of procathepsin B. As processing of procathepsin B is typically around 45–50 % efficient, a higher inhibitor concentration would completely abolish any catalytic activity of the enzyme, thereby preventing detection of cathepsin B activity. All processing curves were sigmoid, showing a bimolecular process with negligible procathepsin B activity as compared to the activity of the mature cathepsin B (Figure 2). As seen, autocatalytic processing of procathepsin B was significantly delayed in the presence of E-64, suggesting that E-64 primarily inhibited the mature enzyme. However, from this experiment it was not possible to conclude whether E-64 could bind also to procathepsin B. In order to address this question, E-64 was replaced with the radioactively labelled analog DCG-04 (¹²⁵I-DCG-04)

[23]. The major advantage of this inhibitor is the possibility to detect the radioactively labelled proteins by autoradiography. Samples of procathepsin B and cathepsin B were incubated in the presence of ^{125}I -DCG-04 at pH 5.8, since processing was not expected to occur at this pH [14]. As can be seen in Figure 3B (lower panel) both the proform and the mature form of cathepsin B were found to bind ^{125}I -DCG-04, suggesting that both species are catalytically active. However, labelling of the zymogen was much weaker, suggesting substantially slower binding of the probe to the zymogen as compared to the mature enzyme.

To confirm the specific nature of interaction between DCG-04 and cathepsin B species, the enzyme samples were incubated with E-64 prior to labelling with DCG-04. Five μM E-64 completely abolished binding of ^{125}I -DCG-04 to both cathepsin B species (Figure 3, lanes 2 and 5), confirming the specific binding of the activity-based probe to the enzyme. In an additional experiment, the inactive procathepsin B Cys29Ser mutant did not label with the probe, thereby excluding non-specific binding of the probe to the enzyme (Figure 3, lanes 7–9). This is in agreement with specific labelling of cathepsin B and procathepsin B as the two active cathepsin species (Figure 3, lanes 1 and 4). In the last control experiment, preheated cathepsin B samples incubated with ^{125}I -DCG-04, did not label with the probe, consistent with its binding being specific.

Identification of cleavage sites during procathepsin B autocatalytic processing

After demonstrating that the zymogen can exhibit catalytic activity, we next wanted to validate the zymogen activity on other substrates. Therefore we performed a mutation analysis of the cleavage region between the propeptide and the mature enzyme around Met56-Phe57 (Figure 4), which is a conserved cleavage site during processing [13, 24]. All the mutants (Table 1) except the C29S variant contain a common R54N replacement in the putative P3 position, which was designed on the basis of E-64 binding to cathepsin B, where the positively charged agmatine group, structurally related to arginine, binds into the S3 substrate binding site [25]. The other mutations were focused on the P1 Met56 residue and/or on the P1'-P4' residues (Phe₅₇Thr₅₈Glu₅₉Asp₆₀). Whereas the deletion mutants were expected to increase tension in the flexible C-terminal propeptide region and thus prevent cleavage in this segment, the other mutants were expected to prevent or delay cleavage due to diminished affinity [26].

Initially, processing of procathepsin B mutants was analyzed by SDS-PAGE. Proenzymes were clearly present on the gel as 36 kDa bands (data not shown). After 3-hour incubation of procathepsin B mutants in the presence or absence of dextran sulfate prior to electrophoresis, 29 kDa bands corresponding to mature cathepsin B were observed (data not shown). The cleavage sites were determined by N-terminal sequencing of the mature enzymes after processing (Table 1). Most of the mutants were cleaved after Met56 (Ala56), with some additional cleavages occurring in the mutated regions with several Ala residues. However, introducing Pro in the P1 or P1' position abolished cleavage at Met56 and resulted in alternative cleavages upstream and/or downstream from the original cleavage site, thereby precluding us from making a noncleavable procathepsin B mutant.

Next, we evaluated the activity of the mature forms resulting from the processing of procathepsin B mutants. All these forms of cathepsin B with different N-terminal extensions

exhibited similar activity against Z-Arg-Arg-AMC (not shown), in agreement with the idea that the neo N-terminus of mature cathepsin B is not important for its catalytic activity. Finally, the processing rates of the procathepsin B mutants were compared. To ensure equal starting concentrations, the procathepsin B variants were subjected to processing in the presence of dextran sulfate to complete the process reasonably quickly (approximately 1 hour) and to prevent possible inactivation. Mature cathepsin B generated was then active-site titrated by E-64 directly in the processing mixture to determine the processing efficiency. The processing rates of procathepsin B mutants and native procathepsin B (equal concentrations) were then determined in the presence and absence of dextran sulfate (Table 1). The R54N procathepsin B variant, which served as a basis for all other mutations, was processed at a rate almost 3-fold lower than the wild-type procathepsin B, supporting the suggested important role of Arg54 in substrate recognition. Most of the other mutants were processed somewhat faster than the R54N variant. The exceptions were the T58A ED and E59A/D60A mutants, which were processed ~5-fold faster than the wild-type zymogen, and the F57A and F57A/T58A/E59A/D60A mutants, which were processed ~2-fold slower. Surprisingly, the F57P mutant was processed substantially faster than the F57A mutant, probably due to different cleavage sites, which could result from stepwise processing.

Since Quraishi and Storer [21] detected a processing intermediate starting with L41, R40A and K39A/R40A mutants on the wild-type background were generated. However, the processing of these mutants, which appear to have a role in GAG binding, was up to 2-fold faster than the processing of the wild-type variant ($t_{1/2} = 28$ vs. 55 min, respectively; ref. [27]). This suggests that the Arg40-Leu41 cleavage may not be essential for processing as Arg is the preferred residue in the S1 position of cysteine cathepsins [26].

Discussion

Zymogen activation is one of the crucial steps in regulating the activity of proteases [28, 29]. Although there have been a number of attempts to explain the mechanism of autocatalytic activation of cysteine cathepsins (reviewed in [1, 30]), none have succeeded in explaining the initial activity of the proteases, which was observed at the very beginning of processing [14, 18–20, 27]. In addition, it has been suggested that processing may proceed through several intermediate steps, but their importance for the actual processing was not evaluated [21]. The results presented in this study demonstrate that the initial activity observed during processing belongs to the activity of the cathepsin B zymogen, as detected by a small synthetic substrate and affinity labelling by the activity-based probe ^{125}I -DCG-04. As seen in the crystal structure of the cathepsin zymogens [7–10] the propeptide binds in the active site in a direction opposite to that of the substrate, thereby preventing substrate hydrolysis. The data thus suggest that substrate hydrolysis can be explained by the flexibility of the propeptide, which is presumably greatly increased at acidic pH. This is supported by *in vitro* studies of the interaction between the propeptide and mature enzymes, which demonstrated a substantially weaker affinity of the propeptides at acidic than at neutral pH [15–17].

The major outcome of the mutagenesis studies was that cathepsin B is not a very specific enzyme and is capable of cleaving procathepsin B at different sites, which is in agreement with the general broad specificity of the cathepsins [26]. Although the preferential cleavage

site appears to be at the Met56-Phe57 bond, mutating Met56 or Phe57 to Pro leads to new N-terminal variants (Figure 4). This precluded us from making a catalytically active, nonprocessed or partially processed zymogen, suggesting that the same probably holds true for processing of other cysteine cathepsins.

Based on these and previous results [14, 17, 21, 27] a common mechanism for the autocatalytic processing of papain-like cysteine endoproteases is proposed. Initially, the pH change facilitates propeptide movement from its normal position within the active-site cleft in the zymogen, thereby converting the latter into an active form. This appears to be a dynamic equilibrium, which is shifted towards the inactive form at neutral pH and towards the active form at acidic pH, consistent with the inability of procathepsin B to cleave a macromolecular proteinaceous substrate at neutral pH. Moreover, this conformational change, which is the only unimolecular step of the mechanism, is not accompanied by any larger structural changes, such as unfolding of the “hook” domain, as demonstrated previously using the catalytic Cys29Ser procathepsin B mutant [14].

When two procathepsin B molecules come into close contact, one active zymogen molecule cleaves the propeptide from the second molecule. It is very likely that propeptide removal occurs in at least two consecutive steps, the first one being the “hook” removal, since Quraishi and Storer [21] detected several intermediate forms starting downstream of the “hook” region (Leu41 and Cys43 from the propeptide). These shortened zymogen forms, with presumably higher enzymatic activity, facilitate removal of the rest of the propeptide from the interacting procathepsin B molecules. Fully active mature cathepsin B molecules then enter the cycle and process the majority of the intact or partially processed zymogen molecules. It can be suggested that, at least at the beginning, intermediate forms and intact zymogens are also cleaved by activated intact and partially processed zymogens. This is in agreement with the findings that the truncated procathepsin B zymogens, resulting from a gene lacking exons 2 and 3 and with a propeptide shortened by 34 residues, possess substantial catalytic activity [31]. Recently, glycosaminoglycans, which can facilitate autocatalytic activation of cysteine cathepsins, were shown to induce a conformational change in procathepsin B upon binding, resulting in propeptide removal from the active site cleft and conversion of the zymogen into a better substrate for mature cathepsin B [27]. Moreover, such procathepsin B processing was observed during a purification step on heparin Sepharose even at pH 7.6, demonstrating their extreme efficiency (data not shown). In addition to glycosaminoglycans, other charged surfaces were found to enable autocatalytic processing at neutral pH, as processing of procathepsin B during filtration through microcon cellulose membrane at pH 7.6 was also observed (data not shown). The molecular mechanism of cathepsin activation induced by pH lowering and/or by glycosaminoglycans is probably similar in both cases, the only difference being that glycosaminoglycans and other negatively charged surfaces are much more efficient and can facilitate processing also at higher pH. Therefore, it can be suggested that this unimolecular conformational change has a dual role: first, it converts the zymogen into an active form, and second, it converts the zymogen into a better substrate, although the latter may be more applicable to glycosaminoglycans [27].

In vivo processing of cysteine cathepsins is probably more complex. The relative insensitivity of procathepsin B processing to mutations in the linker region suggests that cathepsins are well adapted to the cellular environment, and explains why they can be activated by multiple proteases [1, 30, 32]. All these different pathways of activation may thus account for the presence of active cathepsin or procathepsin species outside lysosomes, which under normal conditions are held under the control of endogenous inhibitors such as cystatins and serpins [33]. However, the existence of extralysosomal and extracellular cathepsins in disease is not only linked to secretion of various cathepsin forms from lysosomes and subsequent processing at the membranes, but probably also results from differential trafficking and synthesis, as different splice variants of cathepsins were found primarily in cancer [3, 5, 31]. Moreover, the fact that cathepsin zymogens are very resistant towards pH-induced inactivation combined with their ability to be readily activated even under unfavourable conditions, pose a persistent threat to the system, which cannot be so easily eliminated as zymogens are resistant to inhibition by endogenous inhibitors.

In conclusion, procathepsin B was found to be an active species, suggesting that autocatalytic activation of cysteine cathepsins is a multi-step process, starting with a unimolecular conformational change of the zymogen, which unmasks the active site and in the presence of negatively charged molecules/surfaces also converts the zymogen into a better substrate. This is followed by a bimolecular proteolytic removal of the propeptide, which can be accomplished in one or more steps. Such active cathepsin species could have important roles in physiology, including development of several diseases such as cancer and arthritis.

Experimental procedures

Materials

Restriction enzymes were obtained from MBI Fermentas (Canada) and New England Biolabs (GB), T4 DNA ligase was from Roche (Switzerland), polynucleotide T4 kinase from MBI Fermentas (Canada), and *Vent* DNA polymerase from New England Biolabs (GB). Oligonucleotides were from MWG-Biotech (Germany). Z-Arg-Arg-AMC from Bachem (Switzerland), E-64 from Peptide research Institute (Japan) and dextran sulfate from Sigma (USA). DCG-04 was prepared as described [23].

Procathepsin B and its mutants were synthesized in *E. coli* and purified as described [12]. The recombinant proteins were nonglycosylated as a consequence of the expression system. However, all the potential glycosylation sites are located on the surface of the protein pointing towards the solvent and thus do not interfere with glycosaminoglycan binding, autocatalytic activation of the zymogen or activity of the mature enzyme [9, 13, 27]. All proteins were verified by SDS-PAGE and N-terminal amino acid sequence analysis. Protein concentrations were determined from absorption spectra according to Pace *et al.* [34]. The active proenzyme concentrations were determined by activation and active-site titration of the resulting enzyme with E-64 [35].

Site-directed mutagenesis

Site-directed mutagenesis was performed using PCR as described by Michael [36]. The plasmid and outer primer oligonucleotides used were constructed by Kuhelj *et al.* [12]. The mutagenic oligonucleotides 5'-CCACCCAGAACGTTATGTTTACCG-3' and 5'-GCTCCTCCTGGGCCTT-3' were used to introduce the R54N and C29S¹ substitutions, respectively. Additional mutants were prepared using a vector with cDNA for pcatB(R54N) as a template and the following mutagenic oligonucleotides: 5'-CCAGAACGTTATGTTTGCAGCTGAAGCTGCCTGC-3' (for the T58A ED mutant: R54N, T58A, deletion of E59 and D60), 5'-GAACGTTATGTTTACCGCAGCTCTGAAGCTGCCTGC-3' (for the ED59AA mutant: R54N, E59A, D60A), 5'-CCAGAACGTTATGTTTGCAGCTGCAGCTGAAGCTGCCTGC-3' (for the TED58AAA mutant: R54N, T58A, E59A, D60A), 5'-CCCAGAACGTTATGGCTGCAGCTGCAGCTGAAGCTGCCTG-3' (for the FTED57AAAA mutant: R54N, F57A, T58A, E59A, D60A), 5'-CCAGAACGTTATGGCTACCGAGGACCTGAAGC-3' (for the F57A mutant: R54N, F57A), 5'-CCAGAACGTT(GC)CGTTTACCGAGG-3' (a degenerate primer for M56A and M56P mutants; R54N, M56A and R54N, M56P, respectively) and 5'-GAACGTTATGCCGACCGAGGACC-3' (for F57P mutant: R54N, F57P). The second set of mutants were prepared using the vector with cDNA for pcatB (R54N, T58A, deletion of E59 and D60) as a template and mutagenic primers as follows: 5'-CCAGAACGTTCCGTTTGCAGTGA-3' (for T58A ED_M56P mutant: R54N, M56P, T58A, deletion of E59 and D60) and 5'-GAACGTTATGCCGGCACTGAAGCT-3' (for T58A ED_F57P mutant: R54N, F57P, T58A, deletion of E59 and D60). Mutagenic oligonucleotides were phosphorylated by T4 polynucleotide kinase prior to the mutagenesis reaction. Each PCR mixture (100 µl) contained 500 ng of a plasmid template, 50 pmol of each of the three oligonucleotides (the two outer and a mutagenic one), 20 nmol of each of the four deoxynucleoside triphosphates, Taq DNA ligase buffer, 5 U of Vent DNA polymerase and 5 U of Taq DNA ligase. After 35 cycles of PCR amplification (94 °C, 60 s; 50 °C, 60 s; 65 °C 240 s) the PCR products were cleaned by QIAEX II extraction kit (Qiagen, USA) and cloning was carried out as described previously [12]. The propeptide numbering is used throughout, unless stated otherwise.

Kinetic measurements

Processing of procathepsin B and its mutants was examined at 37 °C and pH 4.5 (0.1 M acetate buffer, containing 1 mM EDTA and 5 mM DTT) as described by Rozman *et al.* [14]. 0.17 – 1.33 µM proenzyme was incubated in 1 ml of the processing buffer. Aliquots of 5, 10 or 20 µl were taken from the reaction mixtures at appropriate times and added to 2.495 - 2.48 ml of 10 µM Z-Arg-Arg-AMC substrate solution in 0.1 M phosphate buffer, pH 6.0, containing 1 mM EDTA and 0.1% (w/v) polyethylene glycol 6000 (Serva). Fluorescence of the released AMC was followed continuously with a C-61 spectrofluorimeter (Photon Technology International, USA) at the excitation and emission wavelengths of 370 nm and

¹C29S mutation substituted active-site Cys29 on the mature part of the enzyme to a serine residue.

460 nm, respectively. When specified, processing was accelerated by the addition of dextran sulfate (25 µg/ml) or decelerated by the addition of E-64 in the processing buffer. The final concentration of procathepsin B variants in the processing buffer was 0.37 µM throughout.

Detection of ¹²⁵I-DCG-04-labeled proteins

Proteins (1.7 µg) were incubated in 50 mM sodium acetate, pH 5.8, containing 5 mM DTT, 150 mM NaCl and 1 mM EDTA in the presence or absence of 5 µM E-64 for 40 min at 25 °C, followed by the addition of small amounts of radioactive probe ¹²⁵I-DCG-04 and additional 40 min incubation under the same conditions. In a control experiment, protein sample was incubated for 5 min at 95 °C prior to the addition of ¹²⁵I-DCG-04. The samples were then separated by SDS-PAGE and stained with Coomassie Brilliant Blue R250 or visualized by autoradiography using a Typhoon Trio (GE, USA) as described previously [23].

N-terminal amino acid analysis

Procathepsin B variants (1.0 – 3.15 µg) were incubated in the processing buffer at 37 °C for 3 hours. The products were separated by SDS-PAGE under reducing conditions on 12.5 % gels and electroblotted to PVDF membrane (Bio-Rad, USA). The protein bands were subjected to Edman degradation on an Applied Biosystems Procise 492A protein sequencer.

Native polyacrylamide gel electrophoresis and zymography

Native PAGE was performed on a 7 % gel at pH 7.4 as described by McLellan [37]. After electrophoresis, the gel was incubated for 5 min in 0.1 M phosphate buffer, pH 6.0, containing 10 mM DTT, 1 mM EDTA, and 0.1 % (w/v) polyethylene glycol 6000, and covered by 5 ml of 40 µM substrate Z-Arg-Arg-AMC in the same buffer. Fluorescence of the released product was monitored under the UV-lamp. The gel was stained with Coomassie Brilliant Blue R250 afterwards.

Acknowledgments

We thank Adrijana Leonardi for N-terminal amino acid sequencing and Prof. Roger H. Pain for critical reading of the manuscript. The work was supported by grants (P0140 and J1-6488) to B.T. and V.T. from the Slovenian Ministry of Higher Education, Science and Technology and by the Human Frontier Science Project Grant RGP0024/2006-C to B.T. and M.B. The work was further supported by National Institutes of Health National Technology Center for Networks and Pathways grant U54 RR02084 to M.B. and Sandler Family Supporting Foundation grant to M.S.

References

1. Turk B, Turk D, Turk V. Lysosomal cysteine proteases: more than scavengers. *Biochim Biophys Acta*. 2000; 1477:98–111. [PubMed: 10708852]
2. Turk V, Turk B, Turk D. Lysosomal cysteine proteases: facts and opportunities. *EMBO J*. 2001; 20:4629–4633. [PubMed: 11532926]
3. Vasiljeva O, Reinheckel T, Peters C, Turk D, Turk V, Turk B. Emerging roles of cysteine cathepsins in disease and their potential as drug targets. *Curr Pharm Des*. 2007; 13:387–403. [PubMed: 17311556]
4. Kos J, Lah TT. Cysteine proteinases and their endogenous inhibitors: target proteins for prognosis, diagnosis and therapy in cancer (review). *Oncol Rep*. 1998; 5:1349–1361. [PubMed: 9769367]

5. Mohamed MM, Sloane BF. Cysteine cathepsins: multifunctional enzymes in cancer. *Nat Rev Cancer*. 2006; 6:764–775. [PubMed: 16990854]
6. Baici A, Lang A, Horler D, Kissling R, Merlin C. Cathepsin B in osteoarthritis: cytochemical and histochemical analysis of human femoral head cartilage. *Ann Rheum Dis*. 1995; 54:289–297. [PubMed: 7763107]
7. Coulombe R, Grochulski P, Sivaraman J, Menard R, Mort JS, Cygler M. Structure of human procathepsin L reveals the molecular basis of inhibition by the prosegment. *Embo J*. 1996; 15:5492–5503. [PubMed: 8896443]
8. Cygler M, Sivaraman J, Grochulski P, Coulombe R, Storer AC, Mort JS. Structure of rat procathepsin B: model for inhibition of cysteine protease activity by the proregion. *Structure*. 1996; 4:405–416. [PubMed: 8740363]
9. Podobnik M, Kuhelj R, Turk V, Turk D. Crystal structure of the wild-type human procathepsin B at 2.5 Å resolution reveals the native active site of a papain-like cysteine protease zymogen. *J Mol Biol*. 1997; 271:774–788. [PubMed: 9299326]
10. Turk D, Podobnik M, Kuhelj R, Dolinar M, Turk V. Crystal structures of human procathepsin B at 3.2 and 3.3 Å resolution reveal an interaction motif between a papain-like cysteine protease and its propeptide. *FEBS Lett*. 1996; 384:211–214. [PubMed: 8617355]
11. Riedl SJ, Renatus M, Schwarzenbacher R, Zhou Q, Sun C, Fesik SW, Liddington RC, Salvesen GS. Structural basis for the inhibition of caspase-3 by XIAP. *Cell*. 2001; 104:791–800. [PubMed: 11257232]
12. Kuhelj R, Dolinar M, Punger ar J, Turk V. The preparation of catalytically active human cathepsin B from its precursor expressed in *Escherichia coli* in the form of inclusion bodies. *Eur J Biochem*. 1995; 229:533–539. [PubMed: 7744077]
13. Mach L, Mort JS, Glossl J. Maturation of human procathepsin B. Proenzyme activation and proteolytic processing of the precursor to the mature proteinase, in vitro, are primarily unimolecular processes. *J Biol Chem*. 1994; 269:13030–13035. [PubMed: 8175723]
14. Rozman J, Stojan J, Kuhelj R, Turk V, Turk B. Autocatalytic processing of recombinant human procathepsin B is a bimolecular process. *FEBS Lett*. 1999; 459:358–362. [PubMed: 10526165]
15. Carmona E, Dufour E, Plouffe C, Takebe S, Mason P, Mort JS, Menard R. Potency and selectivity of the cathepsin L propeptide as an inhibitor of cysteine proteases. *Biochemistry*. 1996; 35:8149–8157. [PubMed: 8679567]
16. Fox T, de Miguel E, Mort JS, Storer AC. Potent slow-binding inhibition of cathepsin B by its propeptide. *Biochemistry*. 1992; 31:12571–12576. [PubMed: 1472493]
17. Quraishi O, Nägler DK, Fox T, Sivaraman J, Cygler M, Mort JS, Storer AC. The occluding loop in cathepsin B defines the pH dependence of inhibition by its propeptide. *Biochemistry*. 1999; 38:5017–5023. [PubMed: 10213604]
18. Baker KC, Taylor MA, Cummings NJ, Tunon MA, Worboys KA, Connerton IF. Autocatalytic processing of pro-papaya proteinase IV is prevented by crowding of the active-site cleft. *Protein Eng*. 1996; 9:525–529. [PubMed: 8862553]
19. Mason RW, Gal S, Gottesman MM. The identification of the major excreted protein (MEP) from a transformed mouse fibroblast cell line as a catalytically active precursor form of cathepsin L. *Biochem J*. 1987; 248:449–454. [PubMed: 3435459]
20. McQueney MS, Amegadzie BY, D'Alessio K, Hanning CR, McLaughlin MM, McNulty D, Carr SA, Ijames C, Kurdyla J, Jones CS. Autocatalytic activation of human cathepsin K. *J Biol Chem*. 1997; 272:13955–13960. [PubMed: 9153258]
21. Quraishi O, Storer AC. Identification of internal autoproteolytic cleavage sites within the prosegments of recombinant procathepsin B and procathepsin S. Contribution of a plausible unimolecular autoproteolytic event for the processing of zymogens belonging to the papain family. *J Biol Chem*. 2001; 276:8118–8124. [PubMed: 11115496]
22. Turk B, Dolenc I, Žerovnik E, Turk D, Gubenšek F, Turk V. Human cathepsin B is a metastable enzyme stabilized by specific ionic interactions associated with the active site. *Biochemistry*. 1994; 33:14800–14806. [PubMed: 7993907]

23. Greenbaum D, Medzihradszky KF, Burlingame A, Bogyo M. Epoxide electrophiles as activity-dependent cysteine protease profiling and discovery tools. *Chem Biol.* 2000; 7:569–581. [PubMed: 11048948]
24. Rowan AD, Mason P, Mach L, Mort JS. Rat procathepsin B. Proteolytic processing to the mature form in vitro. *J Biol Chem.* 1992; 267:15993–15999. [PubMed: 1639824]
25. Turk D, Podobnik M, Popovi T, Katunuma N, Bode W, Huber R, Turk V. Crystal structure of cathepsin B inhibited with CA030 at 2.0-Å resolution: A basis for the design of specific epoxysuccinyl inhibitors. *Biochemistry.* 1995; 34:4791–4797. [PubMed: 7718586]
26. Turk D, Gun ar G, Podobnik M, Turk B. Revised definition of substrate binding sites of papain-like cysteine proteases. *Biol Chem.* 1998; 379:137–147. [PubMed: 9524065]
27. Cagli D, Punger ar JR, Pejler G, Turk V, Turk B. Glycosaminoglycans facilitate procathepsin B activation through disruption of propeptide-mature enzyme interactions. *J Biol Chem.* 2007; 282:33076–33085. [PubMed: 17726009]
28. Neurath H. Evolution of proteolytic enzymes. *Science.* 1984; 224:350–357. [PubMed: 6369538]
29. Turk B. Targeting proteases: successes, failures and future prospects. *Nat Rev Drug Discov.* 2006; 5:785–799. [PubMed: 16955069]
30. Bromme D, Nallaseeth FS, Turk B. Production and activation of recombinant papain-like cysteine proteases. *Methods.* 2004; 32:199–206. [PubMed: 14698633]
31. Mehtani S, Gong Q, Panella J, Subbiah S, Peffley DM, Frankfater A. In vivo expression of an alternatively spliced human tumor message that encodes a truncated form of cathepsin B. Subcellular distribution of the truncated enzyme in COS cells. *J Biol Chem.* 1998; 273:13236–13244. [PubMed: 9582368]
32. Dalet-Fumeron V, Boudjennah L, Pagano M. Competition between plasminogen and procathepsin B as a probe to demonstrate the in vitro activation of procathepsin B by the tissue plasminogen activator. *Arch Biochem Biophys.* 1996; 335:351–357. [PubMed: 8914932]
33. Turk B, Turk D, Salvesen GS. Regulating cysteine protease activity: essential role of protease inhibitors as guardians and regulators. *Curr Pharm Des.* 2002; 8:1623–1637. [PubMed: 12132995]
34. Pace CN, Vajdos F, Fee L, Grimsley G, Gray T. How to measure and predict the molar absorption coefficient of a protein. *Protein Sci.* 1995; 4:2411–2423. [PubMed: 8563639]
35. Turk B, Križaj I, Kralj B, Dolenc I, Popovi T, Bieth JG, Turk V. Bovine stefin C, a new member of the stefin family. *J Biol Chem.* 1993; 268:7323–7329. [PubMed: 8463267]
36. Michael SF. Mutagenesis by incorporation of a phosphorylated oligo during PCR amplification. *Biotechniques.* 1994; 16:410–412. [PubMed: 8185910]
37. McLellan T. Electrophoresis buffers for polyacrylamide gels at various pH. *Anal Biochem.* 1982; 126:94–99. [PubMed: 7181120]

Figure 1. Analysis of procathepsin B activity on Z-Arg-Arg-AMC with zymography (bottom) and native PAGE (top) at pH 7.4: (1) procathepsin B, (2), cathepsin B, (3) cathepsin B, previously inactivated by a 2-hour incubation at pH 7.6 and 37 °C. Other experimental details are in Experimental procedures section.

Figure 2.

Autocatalytic processing of 0.17 μM procathepsin B in the presence of 0 (○), 1.7 (●), 8.5 (□), 17 (■), 34 (△) nM E-64 at pH 4.5 and 37 °C. Aliquots were taken from the reaction mixtures and added to 10 μM Z-Arg-Arg-AMC substrate solution. Fluorescence of the released AMC was followed continuously with a spectrofluorimeter at the excitation and emission wavelengths of 370 nm and 460 nm. Other experimental details are in Experimental procedures section.

Figure 3.

Labeling of procathepsin B by ^{125}I -DCG-04. Five μg of recombinant protein (*pCatB*, procathepsin B; *CatB*, cathepsin B; *pCatB C29S*, catalytic procathepsin B mutant) were diluted into acetate buffer, pH 5.6, and incubated in the absence or presence of 5 μM E-64 (*E-64*) for 40 min at 25 $^{\circ}\text{C}$ followed by the addition of ^{125}I -DCG-04. In the control experiment procathepsin B was pre-heated to 95 $^{\circ}\text{C}$ for 5 min (*P.H.*). Samples were resolved by SDS-PAGE (10–20 % gradient gel). Gels were subsequently stained with Coomassie Brilliant Blue R250 (upper panel) or analyzed by autoradiography (lower panel).

Table 1

Processing of procathepsin B variants

Estimates of processing half-times of procathepsin B variants in the absence (–DS) and presence (+DS) of 25 µg/ml dextran sulfate, obtained by discontinuous method, are given together with their SEMs. Proenzyme concentration was 0.37 µM in all the experiments. The cleavage sites determined by N-terminal amino acid sequencing following autocatalytic processing of proenzyme variants in the absence of DS are marked with arrows. All other experimental details are given in Experimental procedures section.

procathepsin B variant		−DS t _{1/2} (min)	+DS t _{1/2} (min)	cleavage site															
partial propeptide sequence		⁴⁶ L G G P P K P P Q R V M F T E D L K ⁶²																	
wild-type		60 ± 5	15 ± 1	↓ ⁵⁷ F T E D L															
pcathB R54N		155 ± 20	16 ± 1	↓ ⁵⁷ F T E D L															
pcathB (R54N; T58A ED)		37 ± 5	40 ± 3	↓ ⁵⁷ F A L K L															
				↓ ⁵⁸ A L K L															
pcathB (R54N; ED59AA)		28 ± 5	15 ± 1	↓ ⁵⁷ F T A A L															
pcathB (R54N; TED58AAA)		85 ± 10	17 ± 1	↓ ⁵⁷ F A A A L															
pcathB (R54N; F57A)		300 ± 50	33 ± 3	↓ ⁵⁷ A T E D L															
				↓ ⁵⁸ T E D L K															
				↓ ⁵⁵ V M A T E															
pcathB (R54N; FTED56AAAA)		300 ± 50	18 ± 1	↓ ⁵⁷ A A A A L															
				↓ ⁵⁸ A A A L															
				↓ ⁵⁹ A A L															
pcathB (R54N, M56A)		90 ± 10	16 ± 1	↓ ⁵⁷ F T E D L															
pcathB (R54N, M56P)		125 ± 40	90 ± 5	↓ ⁶⁰ D L K L P															
pcathB (R54N, F57P)		80 ± 30	118 ± 5	↓ ⁴⁸ G P K P P															
				↓ ⁴⁶ L G G P															
				↓ ⁵⁹ E D L K L P															

procathepsin B variant	-DS t _{1/2} (min)	+DS t _{1/2} (min)	cleavage site																		
partial propeptide sequence			⁴⁶ L	G	G	P	K	P	P	Q	R	V	M	F	T	E	D	L	K ⁶²		
															↓	⁶⁰ D	L	K	L	P	
pcaB (R54N, T58A ED, M56P)	65 ± 5	41 ± 3														↓	⁶¹ L	K	L	P	A
pcaB (R54N, T58A ED, M56P)	50 ± 20	50 ± 3														↓	⁶¹ L	K	L	P	A