the FEBS Journal

# Autocatalytic processing of procathepsin B is triggered by proenzyme activity

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

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(Received 17 September 2008, revised 13 November 2008, accepted 24 November 2008)

doi:10.1111/j.1742-4658.2008.06815.x

Cathepsin B (EC 3.4.22.1) 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 yet been clarified. Procathepsin B was shown by zymography to hydrolyze the synthetic substrate 7-*N*-benzyloxycarbonyl-L-arginyl-L-arginylamide-4-methylcoumarin (Z-Arg-Arg-NH-MEC), 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 the 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. On the basis of these results, a model for autocatalytic activation of cysteine cathepsins is proposed, 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 because cathepsin zymogens were often found secreted in various pathological states.

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 [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 [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

**Abbreviations**
Z-Arg-Arg-NH-MEC, 7-*N*-benzyloxycarbonyl-L-arginyl-L-arginylamide-4-methylcoumarin.

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 the 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 approximately 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 has 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 elsewhere [21].

To address these questions, we 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 charged surfaces, which may account for an extracellular physiological role of cathepsins.

## Results

### Procathepsin B is active on a small synthetic substrate

In a previous study, a low catalytic activity against the substrate 7-*N*-benzyloxycarbonyl-L-arginyl-L-arginylamide-4-methylcoumarin (Z-Arg-Arg-NH-MEC) was detected during the early stages of autocatalytic activation of procathepsin B, although it was not clear whether this activity belonged to the zymogen [14]. 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 *Escherichia coli* and thus represented nonglycosylated enzymes. Initially, procathepsin B, cathepsin B and inactive cathepsin B, obtained by a 2 h incubation at pH 7.6 and 37 °C [22], were applied to native PAGE. Electrophoresis was performed at pH 7.4, where procathepsin B retains 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 (Fig. 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 (Fig. 1), suggesting that procathepsin B is catalytically active. By contrast, inactivated unfolded cathepsin B did not show any activity against the fluorogenic substrate (Fig. 1). In another experiment, procathepsin B was found to hydrolyze the synthetic substrate Z-Arg-Arg-NH-MEC *in vitro* under the same conditions (i.e. pH 7.6), consistent with the zymography results. However, the hydrolysis rate was approximately 100-fold lower compared to the mature enzyme. By contrast, under these conditions, procathepsin B was unable to hydrolyze denatured

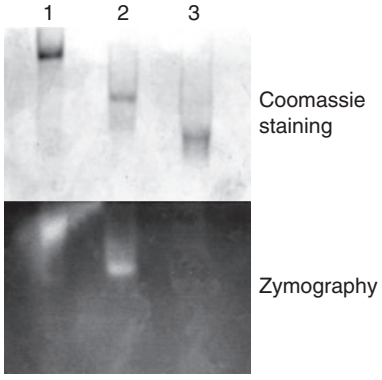


**Fig. 1.** Analysis of procathepsin B activity on Z-Arg-Arg-NH-MEC with zymography (bottom) and native PAGE (top) at pH 7.4: (1) procathepsin B; (2) cathepsin B; and (3) cathepsin B, previously inactivated by a 2 h incubation at pH 7.6 and 37 °C. Further details are provided in the Experimental procedures.

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

To further understand the initial steps of procathepsin B autocatalytic processing, we attempted to inhibit procathepsin B processing by addition of E-64, a broad spectrum inhibitor of cysteine proteases. The inhibitor concentrations were varied over a range that was 5–20% of the molar concentration of procathepsin B. Because processing of procathepsin B is typically 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 compared to the activity of the mature cathepsin B (Fig. 2). As demonstrated, 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. Thus, to address this question, E-64 was replaced with the radioactively labelled analogue DCG-04 ($^{125}$I-DCG-04) [23]. The major advantage of this inhibitor is the possibility of detecting 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 because processing was not expected to occur at this pH [14]. As shown in Fig. 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 a substantially slower binding of the probe to the zymogen 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. E-64 at a concentration of 5 μM completely abolished binding of $^{125}$I-DCG-04 to both cathepsin B species (Fig. 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 nonspecific binding of the probe to the enzyme (Fig. 3, lanes 7–9). This is in agreement with specific labelling of cathepsin B and procathepsin B as the two active cathepsin species (Fig. 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 (Fig. 3, lanes 3, 6 and 9).

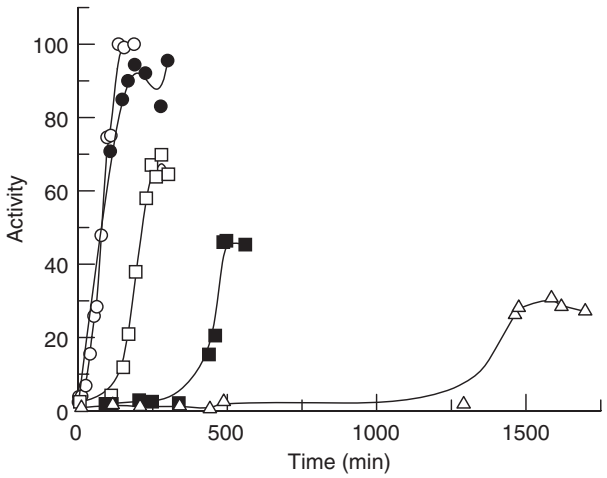


**Fig. 2.** Autocatalytic processing of 0.17 μM procathepsin B in the presence of 0 (○), 1.7 (●), 8.5 (□), 17 (■) and 34 (Δ) nM E-64 at pH 4.5 and 37 °C. Aliquots were taken from the reaction mixtures and added to 10 μM Z-Arg-Arg-NH-MEC substrate solution. Fluorescence of the released 7-amino-4-methylcoumarin was followed continuously with a spectrofluorimeter at the excitation and emission wavelengths of 370 nm and 460 nm, respectively. Further details are provided in the Experimental procedures.

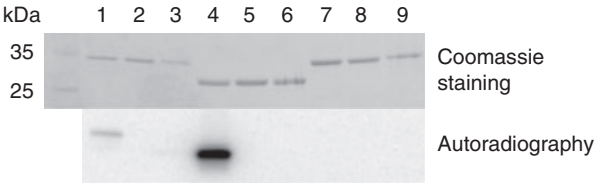


**Fig. 3.** Labelling of procathepsin B by $^{125}$I-DCG-O4. Five micrograms of recombinant protein (*pCatB*, procathepsin B; *CatB*, cathepsin B; *pCatB C29S*, catalytic procathepsin B mutant) were diluted into acetate buffer (pH 5.6) and incubated in the absence or presence of 5 μM E-64 (*E-64*) for 40 min at 25 °C followed by the addition of $^{125}$I-DCG-04. In the control experiment, procathepsin B was pre-heated to 95 °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 analysed by autoradiography (lower panel). Lanes: 1, pCatB; 2, pCatB + E-64; 3, pCatB P.H.; 4, CatB; 5, CatB + E-64; 6, CatB P.H.; 7, pCatB C29S; 8, pCatB C29S + E-64; 9, pCatB C29S P.H.

### Identification of cleavage sites during procathepsin B autocatalytic processing

After demonstrating that the zymogen can exhibit catalytic activity, we next aimed 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, 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 (Phe57Thr58-Glu59Asp60). Although 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 analysed by SDS/PAGE. Proenzymes were clearly present on the gel as 36 kDa bands (data not shown). After a 3 h 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 preventing the formation of 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-NH-MEC (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 h) 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 threefold lower than the wild-type procathepsin B, supporting the proposed important role of Arg54 in substrate recognition. Most of the other mutants were processed somewhat faster than the R54N variant. The exceptions were the T58AΔED and E59A/D60A mutants, which were processed approximately five-fold faster than the wild-type zymogen, and the F57A and F57A/T58A/E59A/D60A mutants, which were processed approximately two-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.

Because 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 two-fold faster than the processing of the wild-type variant ($t_{1/2}$ = 28 versus 55 min, respectively) [27]. This suggests that the Arg40-Leu41 cleavage may not be essential for processing because 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 [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, although their importance for the actual processing was not evaluated [21]. The results obtained in the present 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

**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$^{-1}$ dextran sulfate, obtained by a discontinuous method, are given together with the respective SEM. The proenzyme concentration was 0.37 μM in all the experiments. The cleavage sites determined by N-terminal amino acid sequencing after autocatalytic processing of proenzyme variants in the absence of DS are marked with arrows. Partial propeptide sequence (residues 46 to 62 of the propeptide) is given in the first line. Further details are given in the Experimental procedures.

| 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 | $^{62}$K | | | | |
| 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; T58AΔED) | 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 | ↓$^{46}$L | G | G | P | | | | | | | | | | | | | | | | | |
| | | | | ↓ $^{48}$G | P | K | P | P | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | ↓ $^{59}$E | D | L | K | L | P | | |
| | | | | | | | | | | | | | | | | | ↓ $^{60}$D | L | K | L | P | | |
| pcathB (R54N, T58AΔED, M56P) | 65 ± 5 | 41 ± 3 | | | | | | | | | | | | | | | | ↓ $^{61}$L | K | L | P | A | |
| pcathB (R54N, T58AΔED, M56P) | 50 ± 20 | 50 ± 3 | | | | | | | | | | | | | | | | ↓ $^{61}$L | K | L | P | A | |

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 (Table 1). This prevented us from making a catalytically active, nonprocessed or partially processed zymogen, suggesting that the same probably holds true for processing of other cysteine cathepsins.

On the basis of the results obtained in the present study, as well as those of previous studies [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 Cys29-Ser 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, with the first one comprising the 'hook' removal, as 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 the 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 is possible that, at least initially, intermediate forms and intact zymogens are also cleaved by activated intact and partially processed zymogens. This is in agreement with the findings of a study [31] demonstrating 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. 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 because the 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, with the only difference being that glycosaminoglycans and other negatively charged surfaces are much more efficient and can facilitate processing also at a higher pH. Therefore, it is proposed 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 the secretion of various cathepsin forms from lysosomes and subsequent processing at the membranes, but also likely results from differential trafficking and synthesis because different splice variants of cathepsins are 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, poses a persistent threat to the system, which cannot be so easily eliminated because 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 the 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 the development of several diseases such as cancer and arthritis.

## Experimental procedures

### Materials

Restriction enzymes were obtained from MBI Fermentas (Burlington, Canada) and New England Biolabs (Stevenage, UK); T4 DNA ligase was obtained from Roche (Basel, Switzerland); polynucleotide T4 kinase was obtained from MBI Fermentas; and *Vent* DNA polymerase was obtained from New England Biolabs. Oligonucleotides were obtained from MWG-Biotech (Ebersberg, Germany). Z-Arg-Arg-NH-MEC was obtained from Bachem (Bubendorf, Switzerland); E-64 was obtained from the Peptide Research Institute (Osaka, Japan); and dextran sulfate was obtained from Sigma (St Louis, MO, USA). DCG-04 was prepared as described previously [23].

Procathepsin B and its mutants were synthesized in *E. coli* and purified as described previously [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 interefere 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 aminoacid 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′-CCACCCCAGAACGT TATGTTTACCG-3′ and 5′-GCTCCTCCTGGGCCTT-3′) were used to introduce the R54N and C29S substitutions, respectively (where the C29S mutation substituted active-site Cys29 on the mature part of the enzyme to a serine residue). Additional mutants were prepared using a vector with cDNA for pcatB(R54N) as a template and the following mutagenic oligonucleotides: 5′-CCAGAACGTTA TGTTTGCACTGAAGCTGCCTGC-3′ (for the T58AΔED mutant: R54N, T58A, deletion of E59 and D60); 5′-GAAC GTTATGTTTACCGCAGCTCTGAAGCTGCCTGC-3′ (for the ED59AA mutant: R54N, E59A, D60A); 5′-CCAG AACGTTATGTTTGCAGCTGCACTGAAGCTGCCTGC-3′ (for the TED58AAA mutant: R54N, T58A, E59A, D60A); 5′-CCCAGAACGTTATGGCTGCAGCTGCACTGAAGC TGCCTG-3′ (for the FTED57AAAA mutant: R54N, F57A, T58A, E59A, D60A); 5′-CCAGAACGTTATGGC TACCGAGGACCTGAAGC-3′ (for the F57A mutant: R54N, F57A); 5′-CCAGAACGTT(GC)CGTTTACCGA GG-3′ (a degenerate primer for M56A and M56P mutants; R54N, M56A and R54N, M56P, respectively); and 5′-GAA CGTTATGCCGACCGAGGACC-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: 5′-CCAGAACGTTCCGTTTGCACTGAA-3′ (for T58AΔED_M56P mutant: R54N, M56P, T58A, deletion of E59 and D60) and 5′-GAACGTTATGCCGGCACTGA AGCT-3′ (for T58AΔED_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 for 60 s; 50 °C for 60 s; 65 °C for 240 s), the PCR products were cleaned by the QIAEX II extraction kit (Qiagen, Valencia, CA, USA) and cloning was carried out as described previously [12]. 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 dithiothreitol) as described by Rozman *et al*. [14]. Proenzyme (0.17–1.33 μM) 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-NH-MEC substrate solution in 0.1 M phosphate buffer (pH 6.0) containing 1 mM EDTA and 0.1% (w/v) polyethylene glycol 6000 (Serva, Wichita Falls, TX, USA). Fluorescence of the released 7-amino-4-methylcoumarin was followed continuously with a C-61 spectrofluorimeter (Photon Technology International, Birmingham, NJ, USA) at the excitation

and emission wavelengths of 370 and 460 nm, respectively. When specified, processing was accelerated by the addition of dextran sulfate (25 $\mu g \cdot mL^{-1}$) 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 $^{125}$I-DCG-04-labelled proteins

Proteins (1.7 μg) were incubated in 50 mM sodium acetate (pH 5.8) containing 5 mM dithiothreitol, 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 $^{125}$I-DCG-04 and an additional 40 min of incubation under the same conditions. In a control experiment, protein sample was incubated for 5 min at 95 °C prior to the addition of $^{125}$I-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 Healthcare, Milwaukee, WI, 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 h. The products were separated by SDS/PAGE under reducing conditions on 12.5% gels and electroblotted to poly(vinylidene difluoride) membrane (Bio-Rad, Hercules, CA, USA). The protein bands were subjected to Edman degradation on an Procise 492A protein sequencer (Applied Biosystems, Foster City, CA, USA).

### 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 dithiothreitol, 1 mM EDTA and 0.1% (w/v) polyethylene glycol 6000, and covered by 5 mL of 40 μM substrate Z-Arg-Arg-NH-MEC in the same buffer. Fluorescence of the released product was monitored under an UV lamp. The gel was stained subsequently with Coomassie brilliant blue R250.

## Acknowledgements

We thank Adrijana Leonardi for N-terminal amino acid sequencing and Professor 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.

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