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Autocatalytic processing of recombinant human procathepsin B is a bimolecular process[1]

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Abstract Cathepsin B and other lysosomal cysteine proteinases are synthesized as inactive zymogens, which are converted to their mature forms by other proteases or by autocatalytic processing. Procathepsin B autoactivation was shown in vitro at pH 4.5 to be a bimolecular process with K_s and k_{cat} values of 2.1 ± 0.9 μM and 0.12 ± 0.02 s^{-1}, respectively. Autoactivation is substantially accelerated in the presence of active cathepsin B molecules, indicating that mature cathepsin B is the catalytic species in the process. Proenzyme is cleaved without significant conformational changes as judged by circular dichroism, suggesting that propeptide unfolding occurs only after the cleavage. Procathepsin B autoactivation is pH-dependent with a pH optimum at 4.5 and with no processing observed at pH > 6.0. However, in the presence of 0.5 μg/ml of dextran sulfate, relatively rapid processing is observed even at pH 6.5 ($t_{1/2} \sim 90$ min), suggesting that glycosaminoglycans are involved in in vivo processing of lysosomal cysteine proteases.

Key words: Procathepsin B; Processing; Autoactivation

1. Introduction

Cathepsin B is the most abundant lysosomal cysteine protease and is involved in a number of cellular processes, including protein degradation and turnover [1,2]. The enzyme was also found extracellularly in some pathological states such as arthritis [3], Alzheimer's disease [4], cancer invasion and metastasis [5,6].

Like other related proteases, cathepsin B is synthesized as a preproenzyme [7]. The prepeptide is removed during the passage to the endoplasmic reticulum and procathepsin B undergoes proteolytic processing to active, mature enzyme in late endosomes or lysosomes [8,9]. Propeptide, which is removed in the last step, serves several important functions: (i) it regulates enzymatic activity in vitro by acting as a tight binding inhibitor of mature cathepsin B [10], (ii) it is responsible for proper targeting of the enzyme [11] and (iii) it might be important for the stability and proper folding of the enzyme, as shown for cathepsin L [12]. In vitro experiments showed that procathepsin B could be activated by the action of different proteinases, such as pepsin and cathepsin D [13], and by autocatalytic processing at acidic pH, which was suggested to be a unimolecular process [13–15]. Later studies on cathepsins L and K, however, suggested that the reaction mechanism involves intermolecular as well as intramolecular steps [16,17]. Further evidence that an intramolecular activation mechanism is highly unlikely came from structural studies on procathepsins B, L and K [18–23]. The structure of the catalytic part was shown to be formed in the proenzyme and the propeptide was shown to be bound in the active site cleft in the direction opposite to the normal substrate. This would therefore require a large movement of the propeptide to bind into the active site in the proper orientation for cleavage [19].

In this work, we provide unambiguous evidence that procathepsin B autoprocessing is a bimolecular process, which can be substantially accelerated in the presence of charged surfaces. On the basis of these results, a model has been suggested for autoprocessing of cathepsin B and related proteinases.

2. Materials and methods

2.1. Materials

Z-Arg-Arg-AMC and Z-Arg-Arg-pNA were purchased from Bachem (Bubendorf, Switzerland). Dimethylsulfoxide was obtained from Merck (Darmstadt, Germany). E-64 was from Peptide Research Institute (Osaka, Japan).

Recombinant human procathepsin B and human cathepsin B were produced and purified [24]. In order to produce mutant procathepsin B (Cys-29-Ser), mutagenesis was performed as described [25]. The oligonucleotide 5′-GCTCCTCCTGGGCCTT-3′ (the altered base is underlined) was used for the introduction of the Cys-29-Ser substitution. Refolding and purification of mutant procathepsin B were carried out as described previously [24].

Protein concentrations were determined by absorbance using a Perkin Elmer λ-18 spectrophotometer (USA) according to Pace et al. [26]. The concentration of active cathepsin B was determined by active site titration with E-64 [27].

2.2. Kinetic measurements

All measurements were performed at 37°C using plastic tubes to avoid the effect of glass surfaces on the autoprocessing rate of procathepsin B [28]. The processing buffer was Tris-HCl pH 7.6, 3 mM EDTA, 5 mM cysteine and was acidified to the required pH with 1 M Na-acetate pH 4.0. When not specified, kinetics of cathepsin B autoprocessing were studied at pH 4.5, shown to be optimal for this reaction [29]. Procathepsin B was processed in 5 ml (0.22–1.42 μM final concentration) or 1 ml (at 3.4 and 6.5 μM) of processing buffer. Aliquots of 20, 10 or 5 μl were then withdrawn at appropriate times and added to 2 ml substrate solution (10 μM Z-Arg-Arg-AMC concentration) in 0.1 M phosphate buffer pH 6.0, containing 1 mM EDTA and 0.1% (w/v) polyethyleneglycol 6000 (Serva) to prevent adsorption to the cuvette walls. Such a buffer was chosen as optimal for synthetic substrate hydrolysis by cathepsin B [1], as well as to prevent further processing of the enzyme (see Section 3). Fluorescence of the released product was monitored continuously for 1 min in a Perkin Elmer LS-50 spectrofluorimeter (USA) at excitation and emission wavelengths of 370 and 460 nm, respectively. The dimethylsulfoxide concentration was less than 3% throughout.

Fast reactions (i.e. formation and dissociation of the procathepsin B-cathepsin B complex) were monitored by a DX 17MV stopped-flow apparatus (Applied Photophysics, UK) essentially as described previ-

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[1] Dedicated to Prof. Pavao Mildner on the occasion of his 80th birthday.

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ously [30,31]. Briefly, in the first experiment (complex formation), one syringe was filled with pre-warmed 50 nM cathepsin B in processing buffer and the other with 500 μM Z-Arg-Arg-AMC and 10 μM procathepsin B in the processing buffer at pH 6.0 in order to prevent spontaneous procathepsin B autoactivation. A 10:1 (v/v) mixing ratio resulting in a constant final pH of 4.5 was used. In the second experiment, one syringe was filled with pre-warmed 50 μM Z-Arg-Arg-AMC in the processing buffer and the other with pre-warmed 10 μM cathepsin B and 20 μM procathepsin B in the processing buffer. The enzyme and proenzyme were mixed just prior to the measurement (< 45 s). A 10:1 (v/v) mixing ratio was used to induce dissociation of the complex. The release of AMC product in both experiments was monitored at an excitation wavelength of 360 nm with a cutoff filter with ~50% transmission at 400 nm. For control, the experiments were repeated with cathepsin B or procathepsin B alone.

2.3. Circular dichroism (CD) measurements

Fifty μl of procathepsin B, cathepsin B or mutant procathepsin B (Cys-29-Ser) was diluted into 240 μl of 0.1 M Tris-HCl buffer, pH 7.6, containing 3 mM EDTA. After addition of 10 μl 0.01 M DTT and 45 μl of 1 M acetate buffer, pH 4.0, to adjust the pH to 4.3, reaction mixtures were centrifuged in order to remove precipitated protein and incubated at 37°C. CD spectra were measured in an AVIV 62A DS CD spectrometer (Lakewood, NJ, USA) immediately and after 5 h, when autoprocessing was completed at this pH. Cells with pathlengths of 1 cm in the near UV region (340–250 nm) and 1 mm in the far UV region (250–210 nm) were used. Protein concentrations were 0.31 mg/ml for mature cathepsin B and 0.29 mg/ml for Cys-29-Ser procathepsin B and wild-type procathepsin B, respectively, in both near and far UV regions.

2.4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Procathepsin B activation was analyzed by SDS-PAGE under reducing conditions on 12% gels (7×8 cm) according to Laemmli [32]. DTT (final concentration 0.2 μM) was added to procathepsin B solution (57 or 7.6 μM procathepsin B in processing buffer). The reaction mixture was incubated at pH 4.5 and 37°C. Twelve μl aliquots were taken at different time intervals and processing was stopped by the addition of 4 μl of 4×sample buffer, containing 4% (v/v) of 2-mercaptoethanol, followed by 5 min boiling.

2.5. Theoretical

Autocatalytic processing can be represented by Scheme 1:

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} 2E$$

Scheme 1.

where E, S and ES represent cathepsin B, procathepsin B and the reversible complex, respectively. In Scheme 1, the Michaelis constant is given by $(k_{-1}+k_{cat})/k_{+1}$ and the catalytic constant by k_{cat}. Assuming that the complex is formed under quasi-equilibrium conditions ($k_{-1} \gg k_{cat}$), K_m can be substituted by the equilibrium dissociation constant, K_s, defined by:

$$K_s = \frac{[E]\cdot[S]}{[ES]} \quad (1)$$

In the later stages of an autocatalytic reaction, [ES] is not negligible compared to [S] and Eq. 1 needs to be modified:

$$K_s = \frac{([E]-[ES])([S]-[ES])}{[ES]} \quad (2)$$

Rearranging,

$$[ES]^2-([E]+[S]+K_s)[ES]+[E][S]=0 \quad (3)$$

which has the following solution for [ES]:

$$[ES] = \frac{([E]+[S]+K_s)-\sqrt{([E]+[S]+K_s)^2-4[E]\cdot[S]}}{2} \quad (4)$$

The following conservation law is valid under equilibrium conditions [33]:

$$X = E + ES \quad (5)$$

where X represents the total concentration of enzyme at any time t during the reaction. Scheme 1 can therefore be reduced to Scheme 2:

$$X \xrightarrow{k_{cat}} 2E$$

Scheme 2.

The solution for [ES], given by Eq. 4, was introduced into the system of differential equations, describing the time course of autocatalytic activation of procathepsin B according to the mechanism represented by:

$$\frac{d(X)}{dt} = -k_{cat}(ES) + 2k_{cat}(ES) = k_{cat}(ES) \quad (6)$$

$$\frac{d(ES)}{dt} = -k_{cat}(ES) \quad (7)$$

$$\frac{d(S)}{dt} = -k_{cat}(ES) \quad (8)$$

Unfortunately, such a system of differential equations has no analytical solution. Therefore, the evaluation of kinetic parameters from differential Eqs. 6–8 was done by simultaneous fitting of the numerically solved system to all experimental data using a non-linear least square analysis [34]. Since the aliquot was diluted 100–400-fold in the substrate solution, resulting in a rapid establishment of the new equilibrium $E+S \rightleftarrows ES$, prior to measurement, the measured quantity $[E] \approx [X]$, i.e. $[E]+[ES]$.

3. Results

3.1. Kinetics of procathepsin B autoprocessing

The kinetics of procathepsin B autoprocessing were studied at pH 4.5 by a discontinuous method. Fig. 1 shows a sigmoidal increase of cathepsin B activity showing the appearance of newly processed cathepsin B during the reaction. All data (24 data sets) were simultaneously analyzed as described under Section 2 (Section 2.5) and a good fit to the experimental data (Fig. 1, for clarity, only six data sets are shown) indicated that the proposed model (Scheme 1) satisfactorily described the process, giving the best estimates of 2.1 ± 0.9 μM for K_s and 0.12 ± 0.02 s^{-1} for k_{cat}. In agreement with the proposed mechanism, the rate of processing was concentration-dependent with half-lives of ~100 and ~15 min at a 0.22 and 6.5 μM procathepsin B concentration, respectively (Fig. 1).

To verify the bimolecularity of the process, autoactivation of procathepsin B was followed in the presence of 10 or 20% (v/v) glycerol. Autoprocessing of procathepsin B (0.68 μM) in the absence of glycerol was completed in ≈140 min ($t_{1/2} = 75 \pm 5$ min; Fig. 1), but was ~2-fold slower in the presence of 10% glycerol ($t_{1/2} = 160 \pm 10$ min) and ~4-fold slower in the presence of 20% glycerol ($t_{1/2} = 320 \pm 20$ min) (data not shown). In another experiment, a catalytic amount of active cathepsin B (0.057 μM final concentration) was added to procathepsin B (0.68 μM final concentration), which substantially accelerated the processing ($t_{1/2} = 25 \pm 5$ min vs. $t_{1/2} = 75 \pm 5$; data not shown).

In the analysis, we used a mixed equilibrium and steady-state assumption, which assumes essentially instantaneous complex formation during the entire course of the reaction.

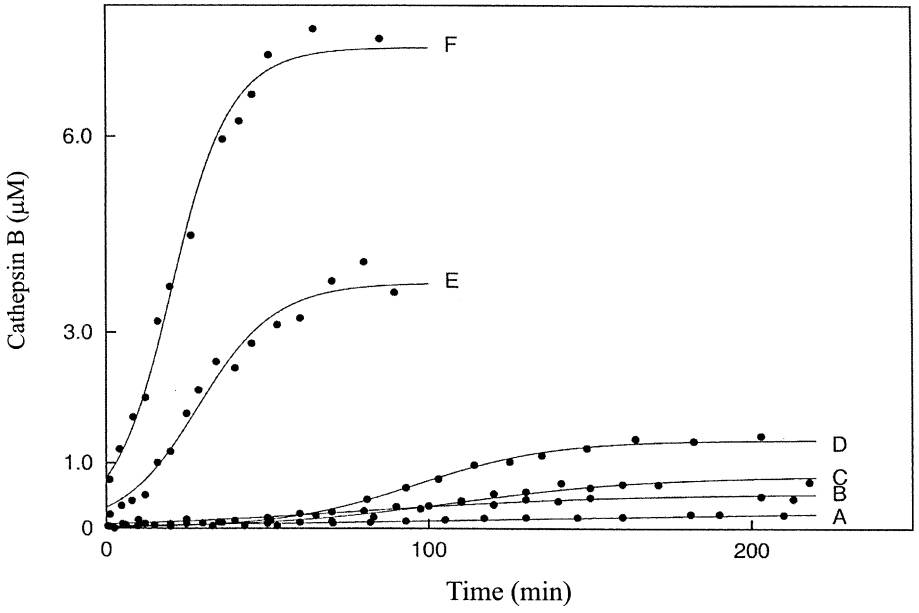

Fig. 1. Time course of procathepsin B autoprocessing at pH 4.5 and 37°C. Starting concentration of procathepsin B: (A) 0.24 μM, (B) 0.78 μM, (C) 0.47 μM, (D) 1.34 μM, (E) 3.44 μM and (F) 6.58 μM. The solid lines are the theoretical curves corresponding to the best estimates of K_s (2.1 μM) and k_{cat} (0.12 s^{-1}) obtained by simultaneous non-linear regression analysis as described under Section 2.5. All experimental details are given in Section 2.

Kinetic data obtained under the experimental conditions used, however, did not provide any information on the time course of complex formation and, therefore, prevented us from treating the reaction as a non-equilibrium system. To obtain additional information on the rapid steps involved, i.e. complex formation and dissociation, we used stopped-flow mixing. We tried to follow the complex formation, but the time course of AMC formation was linear, indicating that association was complete within the dead time of the apparatus (~2 ms), thus justifying the initial assumption. In control experiments performed using cathepsin B or procathepsin B alone, AMC formation was also linear, although the slope was ~20-fold steeper in the presence of cathepsin B, indicating that during the stopped-flow measurement (0–60 s), no significant amount of procathepsin B was processed. In an additional attempt, we tried to dissociate the complex by 10-fold dilution. However, complete processing of procathepsin B was observed in less than 45 s under the conditions used (10 μM cathepsin B and 20 μM procathepsin B concentrations).

To confirm the enzyme assay studies, autoprocessing of procathepsin B was followed by SDS-PAGE as outlined in Section 2. At both procathepsin B concentrations (57 and 7.6 μM, not shown), processing was virtually complete within 20–25 min (Fig. 2). This time is in reasonable agreement with the results obtained at a high procathepsin B concentration (6.5 μM) by the enzyme assay method. Although no concentration-dependence of the processing rate could have been observed at high procathepsin B concentrations (>5 μM) by either of the two methods used, this can be explained by the very high procathepsin B concentration, which was higher than the K_s value of the process (2.1 μM).

3.2. Structural studies of procathepsin B processing

Since lowering the pH was suggested to trigger autocatalytic processing of procathepsin B [19], possible conformational changes accompanying processing were monitored by CD in the near and far UV region using procathepsin B, mature cathepsin B and inactive Cys-29-Ser mutant of procathepsin B. Near UV CD spectra were similar for all three proteins and did not change significantly even after incubation at pH 4.3 for several hours (data not shown). Far UV CD spectra for the three proteins were also similar at the beginning of processing. Whereas the Cys-29-Ser procathepsin B spectrum remained almost the same after prolonged exposure to pH 4.3, small changes were observed in the procathepsin B spectrum (Fig. 3). Changes were most pronounced below 222 nm, indicating a difference in the α-helix content.

3.3. Effect of pH and dextran sulfate on the autoprocessing of procathepsin B

Autocatalytic processing of procathepsin B was studied as a function of pH in the absence and presence of dextran sulfate. In a preliminary experiment, processing at pH 4.5 was monitored in the presence of 5 and 20 μg/ml dextran sulfate. Since no difference in the processing rate was observed, all subse-

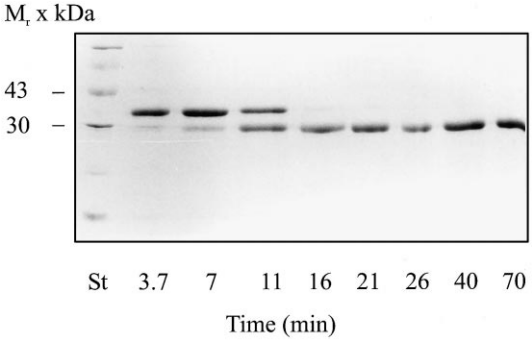

Fig. 2. SDS-PAGE analysis of procathepsin B activation at pH 4.5 and 37°C. Procathepsin B (57 μM) was incubated in the processing buffer from 0 to 70 min as described in Section 2. Low M_r standards are shown in lane 1 (St).

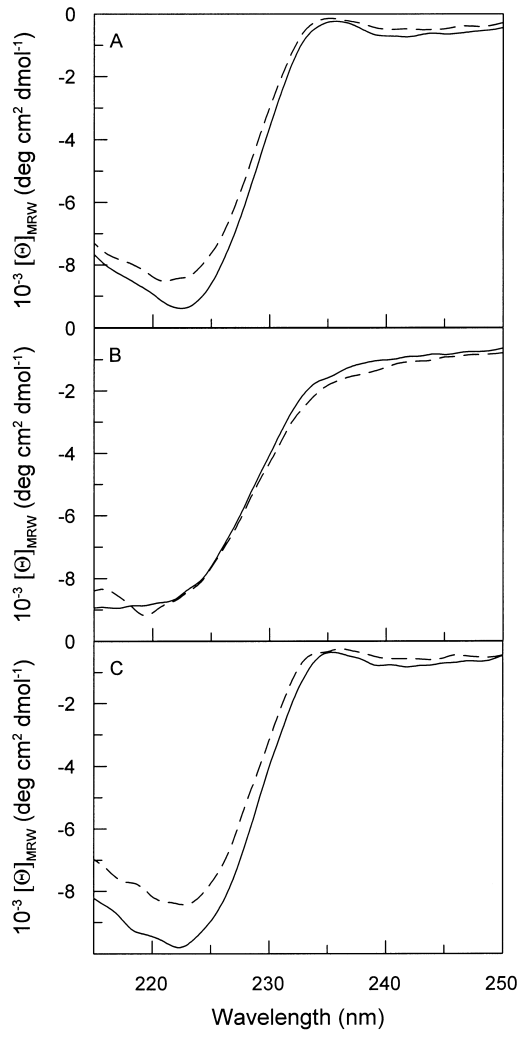

Fig. 3. Far UV CD spectra of mature cathepsin B (A), Cys-29-Ser procathepsin B (B) and procathepsin B (C) at pH 4.3 and 37°C. Solid lines correspond to $t = 1$ min after mixing and dashed lines to $t = 5$ h. Protein concentrations were 0.31 mg/ml in A, 0.29 mg/ml in B and 0.29 mg/ml in C. Other experimental details are given in Section 2.

quent experiments were performed in the presence of 5 μg/ml dextran sulfate, a concentration similar to those used in studies of procathepsin L [28] and factor XII [35] activation. In the absence of dextran sulfate, processing was the fastest at pH 4.5 (Table 1) and the rate decreased towards lower or higher pH, in agreement with previous results [15]. In the presence of dextran sulfate, processing was substantially faster (15–40-fold, Table 1). Furthermore, a reasonably rapid processing was observed even at pH 6.5, in contrast to the results in the absence of dextran sulfate where no processing was observed above pH 6.0.

4. Discussion

The results show that autoactivation of procathepsin B is a bimolecular process. The sigmoidal increase of cathepsin B activity with time (Fig. 1) indicated that processing is an intermolecular rather than an intramolecular process. In the latter case, an exponential increase of activity with time would have been observed. Furthermore, the rate of processing was concentration-dependent (Fig. 1) and not independent, as would be expected for an intramolecular process. Additional evidence for the bimolecularity of autoprocessing was provided by the experiments in the presence of glycerol, which is known to slow down diffusion of the molecules in solution by increasing the solution viscosity. In the presence of 20% (v/v) glycerol, a ~4-fold deceleration of autoprocessing was observed, whereas in the case of an intramolecular (unimolecular) process, glycerol should not have affected the processing rate. Moreover, addition of a small amount of mature active cathepsin B accelerated the process ~3-fold, indicating that mature cathepsin B is the catalytic species in the process. Finally, the only apparent deviation from the bimolecularity, virtual concentration-independence of the processing rate at a high procathepsin B concentration, could well be explained by the saturation conditions, i.e. $[So] \gg K_m$.

An important question concerns the origin of the initial cleavage reaction. Based on the crystal structure of procathepsin B [19], it was suggested that an early conformational change in the propeptide induced by the pH drop may trigger processing of the proteinases. This was supported by another study, where pH-induced conformational changes were observed on the propeptide of cathepsin L [36]. However, when autoactivation of cathepsin L was studied, no conformational changes were observed during processing [17], in contrast with the above suggestion. In agreement with the latter study, we observed no conformational change when Cys-29-Ser procathepsin B was exposed to pH 4.3 for as long as 5 h (Fig. 3), which is considerably longer than the time needed for complete processing of the enzyme. Also mature cathepsin B proved to be completely stable and active during this period (Fig. 3, J. Rozman, unpublished results). However, conformational changes in the α-helices were observed by the same treatment of procathepsin B (Fig. 3). Moreover, after 5 h, the far UV CD spectra of procathepsin B and mature cathepsin B were similar, consistent with procathepsin B being completely processed. This further suggests that propeptide, which is mainly α-helical [18,19], was unfolded after the cleavage, explaining also the results of Jerala et al. [36].

Although a major conformational change in the propeptide, triggered by the pH drop, is not the initial step in the activation of papain-like proteinases, interactions between propeptide and the catalytic part are substantially weaker after the pH change, as judged on the basis of interaction between

Table 1
Effect of pH and dextran sulfate on the rate of procathepsin B processing at 37°C

pH	−DS $t_{1/2}$ (min)	+DS $t_{1/2}$ (min)
4.0	140 ± 10	n.d.
4.5	75 ± 5	5 ± 1
4.9	130 ± 10	7 ± 1
5.1	160 ± 10	n.d.
5.8	800 ± 50	18 ± 2
6.25	n.d.	25 ± 2
6.55	n.d.	90 ± 5

Estimates of procathepsin B processing half-times in the absence (−DS) and in the presence (+DS) of 5 μg/ml dextran sulfate, obtained by the discontinuous method, are given together with their S.E.M.s. The procathepsin B concentration was 0.68 μM in all experiments. Other experimental details were as described in Section 2. n.d. is not determined.

cathepsin B and the propeptide [10]. As a consequence, proenzyme probably adopts a looser conformation, in which propeptide is bound less tightly into the active site without loss of the secondary structure. Initiation of the chain reaction of peptide cleavage, however, remains an intriguing part of the whole process. Although we and others [16,37,38] have observed that proenzymes exhibit a very small catalytic activity, which may be sufficient to initiate the chain reaction, presence of catalytic amounts of other proteinases, either from tissue or resulting from various expression systems, cannot be totally excluded. In the next step, processing is rapidly accomplished by the increasing number of catalytically active enzyme molecules. Such a transactivation was observed for both cathepsin B (see Section 3) and also cathepsin K [16]. Cleaved propeptides were suggested to delay the final processing step by inhibiting mature proteinases [10,15,39]. However, quantitative processing over the whole concentration range, which was considerably faster at higher procathepsin B concentrations (Fig. 1), was observed, suggesting that such a mechanism is not very likely. It is more likely that the propeptide, after serving its role to prevent inappropriate protease activity and being cleaved, dissociates from the protease. This is followed by unfolding and degradation of the propeptide by proteases. The major pH-induced conformational changes of cathepsin S propeptide and its rapid cleavage by cathepsin L below pH 5.5 are in line with our suggestion [40]. Alternatively, propeptide can be cleaved at several sites already during activation, thus eliminating or diminishing its inhibitory function, as observed for propeptides of cathepsins L and K [16,17].

Procathepsin B autoactivation in the presence of dextran sulfate was shown to be substantially accelerated (15–40-fold) and significant up to pH 6.5, whereas in the absence of dextran sulfate, it is very slow at $pH > 5.5$. Although no major acceleration of procathepsin B activation was observed in the presence of heparin or chondroitin sulfate [15], rapid processing of procathepsin L was observed in the presence of dextran sulfate [28] and other glycosaminoglycans [41]. Dextran sulfate and related glycosaminoglycans can thus substantially accelerate in vitro autoactivation of lysosomal cysteine proteases by weakening the interaction between propeptide and the catalytic part. Since glycosaminoglycans can be found in lysosomes as a result of proteoglycan recycling, it is possible that they are also involved in in vivo processing of cysteine proteinases, although there is no experimental evidence yet.

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References

[1] Kirschke, H., Barrett, A.J. and Rawlings, N.D. (1995) Protein Profile 2, 1581–1643.
[2] Turk, B., Turk, V. and Turk, D. (1997) Biol. Chem. 378, 141–150.
[3] Mort, J.S., Recklies, A.D. and Poole, A.R. (1984) Arthritis Rheum. 27, 509–515.
[4] Cataldo, A.M. and Nixon, R.A. (1990) Proc. Natl. Acad. Sci. USA 87, 3861–3865.
[5] Sloane, B.F., Moin, K. and Lah, T. (1994) in: Biochemical and Molecular Aspects of Selected Cancers (Pretlow, G. and Pretlow, T.P., Eds.), Vol. 2.T, pp. 411–466, Academic Press, San Diego, CA.
[6] Kos, J. and Lah, T.T. (1998) Oncol. Rep. 5, 1349–1361.
[7] Chan, S.J., San Segundo, B., McCormick, M.B. and Steiner, D.F. (1986) Proc. Natl. Acad. Sci. USA 83, 7721–7725.
[8] Nishimura, Y., Kawabata, T. and Kato, K. (1988) Arch. Biochem. Biophys. 261, 64–71.
[9] Kominami, E., Tsukahara, T., Hara, K. and Katunuma, N. (1988) FEBS Lett. 231, 225–228.
[10] Fox, T., de Miguel, E., Mort, J.S. and Storer, A.C. (1992) Biochemistry 31, 12571–12576.
[11] Hanewinkel, H., Glössl, J. and Kresse, H. (1987) J. Biol. Chem. 262, 12351–12355.
[12] Tao, K., Stearns, N.A., Dong, J., Wu, Q.L. and Sahagian, G.G. (1994) Arch. Biochem. Biophys. 311, 19–27.
[13] Rowan, A.D., Mason, P., Mach, L. and Mort, J.S. (1992) J. Biol. Chem. 267, 15993–15999.
[14] Mach, L., Schwihla, H., Stüwe, K., Rowan, A.D., Mort, J.S. and Glössl, J. (1993) Biochem. J. 293, 437–442.
[15] Mach, L., Mort, J.S. and Glössl, J. (1994) J. Biol. Chem. 269, 13030–13035.
[16] McQueney, M.S., Amegadzie, B.Y., D'Alessio, K., Hanning, C.R., McLaughlin, M.M., McNulty, D., Carr, S.A., Ijames, C., Kurdyla, J. and Jones, C.S. (1997) J. Biol. Chem. 272, 13955–13960.
[17] Ménard, R., Carmona, E., Takebe, S., Dufour, E., Plouffe, C., Mason, P. and Mort, J.S. (1998) J. Biol. Chem. 273, 4478–4484.
[18] Turk, D., Podobnik, M., Kuhelj, R., Dolinar, M. and Turk, V. (1996) FEBS Lett. 384, 211–214.
[19] Podobnik, M., Kuhelj, R., Turk, V. and Turk, D. (1997) J. Mol. Biol. 271, 774–788.
[20] Cygler, M., Sivaraman, J., Grochulski, P., Coulombe, R., Storer, A.C. and Mort, J.S. (1996) Structure 4, 405–416.
[21] Coulombe, R., Grochulski, P., Sivaraman, J., Ménard, R., Mort, J.S. and Cygler, M. (1996) EMBO J. 15, 5492–5503.
[22] La Londe, J.M., Zhao, B., Janson, C.A., D'Alessio, K.J., McQueney, M.S., Orsini, M.J., Debouck, C.M. and Smith, W.W. (1999) Biochemistry 38, 862–869.
[23] Sivaraman, J., Lalumière, M., Ménard, R. and Cygler, M. (1999) Protein Sci. 8, 283–290.
[24] Kuhelj, R., Dolinar, M., Pungercar, J. and Turk, V. (1995) Eur. J. Biochem. 229, 533–539.
[25] Michael, S.F. (1994) Biotechniques 16, 410–412.
[26] Pace, C.N., Vajdos, F., Fee, L., Grimsley, G. and Gray, T. (1995) Protein Sci. 4, 2411–2423.
[27] Turk, B., Krizaj, I., Kralj, B., Dolenc, I., Popovic, T., Bieth, J.G. and Turk, V. (1993) J. Biol. Chem. 268, 7323–7329.
[28] Mason, R.W. and Massey, S.D. (1992) Biochem. Biophys. Res. Commun. 189, 1659–1666.
[29] Kuhelj, R. (1998) Ph.D. Thesis, University of Ljubljana.
[30] Turk, B., Dolenc, I., Turk, V. and Bieth, J.G. (1993) Biochemistry 32, 375–380.
[31] Turk, B., Stoka, V., Björk, I., Boudier, C., Johansson, G., Dolenc, I., Colic, A., Bieth, J.G. and Turk, V. (1995) Protein Sci. 4, 1874–1880.
[32] Laemmli, U.K. (1970) Nature 227, 680–685.
[33] Cha, S. (1968) J. Biol. Chem. 243, 820–825.
[34] Stojan, J. (1997) J. Chem. Inf. Comput. Sci. 37, 1025–1027.
[35] Tankersley, D.L. and Finlayson, J.S. (1984) Biochemistry 23, 273–279.
[36] Jerala, R., Zerovnik, E., Kidric, J. and Turk, V. (1998) J. Biol. Chem. 273, 11498–11504.
[37] Mason, R.W., Gal, S. and Gottesman, M.M. (1987) Biochem. J. 248, 449–454.
[38] Baker, K.C., Taylor, M.A.J., Cummings, N.J., Tuñón, M., Worboys, K.A. and Connerton, I.F. (1996) Protein Eng. 9, 525–529.
[39] Carmona, E., Dufour, E., Plouffe, C., Takebe, S., Mason, P., Mort, J.S. and Ménard, R. (1996) Biochemistry 35, 8149–8157.
[40] Maubach, G., Schilling, K., Rommerskirch, W., Schultz, J.E., Weber, E. and Wiederanders, B. (1997) Eur. J. Biochem. 250, 745–750.
[41] Ishidoh, K. and Kominami, E. (1995) Biochem. Biophys. Res. Commun. 217, 624–631.