Autocatalytic Processing of Recombinant Human Procathepsin L

CONTRIBUTION OF BOTH INTERMOLECULAR AND UNIMOLECULAR EVENTS IN THE PROCESSING OF PROCATHEPSIN L IN VITRO

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The autocatalytic processing of procathepsin L was investigated in vitro using purified recombinant proenzyme expressed in Pichia pastoris. Pure intermolecular processing was studied by incubating the mutant procathepsin L (C258), which cannot autoactivate with a small amount of mature active cathepsin L. The results clearly establish that, contrary to recent reports, intermolecular processing of procathepsin L is possible. The main cleavage sites are located at or near the N terminus of the mature enzyme, in an accessible portion of the proregion, which contains sequences corresponding to the known substrate specificity of cathepsin L. Contrary to procathepsins B, K, and S, autocatalytic processing of procathepsin L can generate the natural mature form of the enzyme. A continuous assay using the substrate benzoyl-Arg-4-methylcoumarinyl-7-amide hydrochloride has also been used to obtain information on the nature of the steps involved in the autocatalytic processing of wild-type procathepsin L. Processing is initiated by decreasing the pH from 8.0 to 5.3. The influence of proenzyme concentration on the rate of processing indicates the existence of both unimolecular and bimolecular steps in the mechanism of processing. The nature of the unimolecular event that triggers processing remains elusive. Circular dichroism and fluorescence measurements indicate the absence of large scale conformational change in the structure of procathepsin L on reduction of pH. However, the bimolecular reaction can be attributed to intermolecular processing of thezymogen.

Cathepsin L is a lysosomal cysteine protease that plays a major role in intracellular protein degradation (1, 2). Like many mammalian proteases, cathepsin L is synthesized as an inactive proenzyme, which is subsequently processed to the mature form (3, 4). Cleavage of the 96-residue proregion, which is located between the signal sequence and the N terminus of the mature enzyme, is necessary to generate the fully active 221-residue mature enzyme (5); therefore, the proregion serves as a regulator of catalytic activity. Accordingly, the propeptide of cathepsin L has been shown to be a potent inhibitor of the mature protease (6). The proregion is also required for the proper folding of the protein (7). Procathepsin L is stable at high pH, and the proregion protects the protein from the denaturing effect of neutral to alkaline pH (8). The proregion also mediates the pH-dependent membrane association of procathepsin L, which may play a role in transport to the lysosome or activation of the proenzyme (9). In addition to its role in protein degradation, evidence has accumulated for the participation of cathepsin L in various physiological and pathological processes, e.g. tumor invasion and metastasis (10, 11), bone resorption (12), spermatogenesis (13), and arthritis (14, 15). In some cases, extracellular events involve thezymogen form (e.g. Refs. 16 and 17), and the role for the proregion in regulation of the catalytic activity and stabilization of procathepsin L could be very important.

The recently determined three-dimensional structure of human procathepsin L has demonstrated the molecular basis for the inhibition of this cysteine protease by its propeptide (18). However, details of the mechanism for conversion of thezymogen to the mature enzyme remain obscure. Cleavage of the proregion to yield active mature cathepsin L can occur autocatalytically under acidic conditions (16), but it is not yet clear whether processing occurs by intramolecular and/or intermolecular events. Procathepsin L was recently reported to process exclusively by an intramolecular reaction mechanism (8, 19). This work was supported by grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil (to E. C.), NATO, and the Ministério de la recherche et de l'espace, France (to E. D.). This is NRCC Publication 41400. The Biotechnology Research Institute and McGill University are members of the Protein Engineering Network of Excellence, sponsored by the government of Canada. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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procathepsin L where the critical catalytic residue Cys\textsuperscript{25} is replaced by a serine, therefore preventing intramolecular cleavage of the proenzyme. The intermolecular processing was carried out by exogenously added active cathepsin L. The recombinant activatable procathepsin L as well as the C25S mutant have been expressed in *Pichia pastoris* and purified to homogeneity. The two variants of recombinant procathepsin L have also been used to set up a continuous assay for kinetic studies and to investigate possible conformational changes that could be linked to the triggering of processing. Based on the results of these studies, structural aspects of the autocatalytic processing of procathepsin L are discussed.

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant procathepsin L (C25S) was expressed and purified as described previously (6). In addition to the active site muta- tion, the proenzyme also lacks the glycosylation site (Thr\textsubscript{110} in Asn\textsubscript{108}-Asp\textsubscript{109}-Thr\textsubscript{110} replaced by Ala). The vector (pPic9) and *P. pastoris* strain GS115 were purchased from Invitrogen Corp. (San Diego, CA). The substrate benzyloxycarbonyl-l-phenylalanyl-l-arginine 4-methylcoumarinyl-7-amide hydrochloride (Cbz-Phe-Arg-MCA)\textsuperscript{3} and the irreversible inhibitor E-64 (1-[\{(L-trans-epoxy succinyl)-l-leucyl]-aminol-4-gua-Nidino(butane) were purchased from IAF Biochem International Inc. (Laval, Canada). The cathepsin L propeptide (4p-90p)\textsuperscript{3} was used for fluorescence and circular dichroism measurements was obtained as described previously (6).

**Expression and Purification of Recombinant Human Procathepsin L and Cathepsin L**—Human procathepsin L was expressed in *P. pastoris* as an α-factor fusion construct as described previously for procathepsin L (C25S) (6). The culture medium (pH 6.5–7.0) was centrifuged (4000 g (C25S) (6)). The culture medium (pH 6.5–7.0) was centrifuged (4000 g as an enzyme and circular dichroism measurements was obtained as described previously (6).

**Circular Dichroism and Fluorescence Measurements**—Circular dichroism measurements were performed on a Jobin Yvon CD6 dichrograph as described previously (6). Spectra were recorded at 25 °C in 20 mM buffer (sodium acetate, pH 4.0; sodium phosphate, pH 6.0 and 8.0) and in the presence of 10% CH\textsubscript{3}CN. Samples were preincubated for several hours in each buffer before recording the spectra. The concentration product was monitored using excitation and emission wavelengths of 287 nm and 332 nm, respectively. The reaction mixture was stirred continuously in the cuvette during the reaction. The product versus time curves were fitted to the equation,

![Equation](http://www.jbc.org/)

where \(v_{PE}\) represents the initial rate of product release (which should reflect activity of the proenzyme if any), \(v_E\) corresponds to the rate for mature cathepsin L, and \(k_{on}\) is a first order rate constant.

**RESULTS AND DISCUSSION**

**Identification of Cleavage Sites in the Autocatalytic Intermolecular Processing of Procathepsin L**—Recombinant procathepsin L (C25S) has been expressed in *P. pastoris* at a yield, after purification, of approximately 10 mg/liter of buffered minimal glycerol complex culture medium. Autocatalytic intermolecular processing was investigated by incubating procathepsin L (C25S) with a small amount of mature active cathepsin L at pH 5.1. Purified procathepsin L (C25S) was detected as a single band that migrates at an apparent molecular mass of 39 kDa on SDS-PAGE (Fig. 1). Upon incubation with cathepsin L, two new bands with apparent molecular masses of 30 and 10 kDa were observed. These bands result from the conversion of the proenzyme to the single chain mature form of cathepsin L and propeptide fragment(s). It must be noted that the molecular weights of the precursor (proenzyme) and mature forms of cathepsin L are 35.8 and 24.2 kDa, respectively, but that these proteins migrate with higher apparent molecular masses on SDS-PAGE (23). The conversion results from inter- molecular processing of procathepsin L (C25S) was purified by ion exchange chromatography (SP-Sepharose), while the propeptide fragments were isolated by HPLC. The protein and peptides were subjected to N-terminal amino acid sequencing analysis. In addition, the propeptide fragments were also char-
characterized by ion spray mass spectrometry. The results are given in Table I.

The two main propeptide fragments obtained indicate the presence of cleavage sites between the proregion and the mature enzyme at the Phe<sup>89p</sup>-Gln<sup>90p</sup> and Gln<sup>90p</sup>-Glu<sup>91p</sup> bonds. The location of these two processing sites in procathepsin L is shown in Fig. 2 based on the recently determined three-dimensional structure of the proenzyme (18). The proregion consists of a globular domain formed by the first 75 residues and an extended 21-residue segment at the C-terminal portion. The globular domain contains three α-helices and is highly structured. The C-terminal portion, however, is less structured, and in particular, residues 80p–96p of the proregion make few contacts with cathepsin L (18). It can be seen that the Phe<sup>89p</sup>-Gln<sup>90p</sup> and Gln<sup>90p</sup>-Glu<sup>91p</sup> cleavage sites are localized in that portion of the proregion (Fig. 2B). The temperature factors indicate that the mobility of these residues is higher than the rest of the molecule. In addition, using truncated propeptides, it was found that the 15 C-terminal residues of the propeptide contribute little to overall inhibition (6), in agreement with the observation that few interactions exist between this region and the enzyme (18). Cleavage therefore occurs in a region that is relatively accessible to proteolysis. Cathepsin L and cysteine proteases in general have a relatively broad substrate specificity, yet they can specifically autoprocess. The accessibility of the C-terminal propeptide region to proteolysis is certainly a predominant factor determining the processing specificity of cysteine protease proenzymes. The accessible region, however, must contain acceptable residues for the enzyme, as recently demonstrated in the study of propapaya proteinase IV (24). Propapaya proteinase IV does not autoprocess due to its own very restricted substrate specificity. Autocatalytic processing could be achieved by creating a mutation that converts the C-terminal portion of the proregion into a propapaya proteinase IV substrate (24). Considering the substrate specificity of cathepsin L, the two cleavage sites identified in the present study contain well accepted residues for the P<sub>2</sub>-P<sub>1</sub>-P<sub>1</sub> positions, i.e. Lys-Val-Phe and Val-Phe-Gln, consistent with the predominant preference for a hydrophobic residue in P<sub>2</sub> (25).

Characterization by the mature enzyme forms produced by intermolecular processing indicates that cleavage also occurs after positions 95p or 96p to yield the mature enzyme with a one-amino acid N-terminal extension or with the correct N terminus (Table I). Again, the P<sub>2</sub>-P<sub>1</sub>-P<sub>1</sub> residues (Leu-Phe-Tyr and Phe-Tyr-Glu) correspond to the known substrate specificity of cathepsin L. From our study, we cannot clearly establish whether initial cleavage can at least in part occur directly at position 95p or 96p, with the resulting propeptide fragment being cleaved after positions 89p or 90p, or if an initial proteolytic step after residue 89p or 90p is followed by further trimming on the N terminus to generate mature cathepsin L. It must be noted, however, that all four cleavage sites are in a fairly accessible region of the proenzyme. Previous studies on processing of procathepsin L from v-Ha-ras-transformed NIH3T3 cells showed the production of two different forms of mature enzyme, with N-terminal extensions of 2 and 6 amino acids.

### Table I

<table>
<thead>
<tr>
<th>Assignment</th>
<th>Mass&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Assignment</th>
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<tbody>
<tr>
<td>Propeptide fragments</td>
<td></td>
<td>Mature enzyme</td>
</tr>
<tr>
<td>FDHSLEAQWT&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10,598 Phe&lt;sup&gt;89p&lt;/sup&gt;-Gln&lt;sup&gt;90p&lt;/sup&gt;</td>
<td>EAPXSVDWXEK ND Cathepsin L + 1-amino acid</td>
</tr>
<tr>
<td>FDHSLEAQT&lt;sup&gt;c&lt;/sup&gt;x</td>
<td>10,469 Phe&lt;sup&gt;89p&lt;/sup&gt;-Phe&lt;sup&gt;90p&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>EPLFY ND</td>
<td>Glu&lt;sup&gt;90p&lt;/sup&gt;-Tyr&lt;sup&gt;91p&lt;/sup&gt;</td>
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<tr>
<td>Mature enzyme</td>
<td>Cathepsin L</td>
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<td>EPLFY ND</td>
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<tr>
<td>EAPXSVDWXEK ND</td>
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<sup>a</sup> Masses differ from calculated values based on sequence of human procathepsin L due to the fact that procathepsin L (C25S) contains a Leu residue instead of Phe at position 78p (18).

<sup>b</sup> A second fragment was found in the same peak in very small amount (5%), starting at His<sup>6p</sup> (N-terminal sequence, FDHSLEAQWXT).

<sup>c</sup> This fragment was not detected by mass spectrometry.

<sup>d</sup> ND, not determined.
acids (26). The later form was proposed to be proteolytically inactive. We did not detect any enzyme intermediate containing a 5- or 6-amino acid N-terminal extension. This could be due to the fact that such intermediates are further processed rapidly to the mature enzyme forms. It must be noted that a 5-amino acid peptide corresponding to Glu91p-Tyr95p was detected as one of the propeptide fragments generated by intermolecular processing. An additional cleavage site was detected between residues Thr3p and Phe4p (as well as a minor site between Asp5p and His6p) at the N-terminal portion of the proregion (Table I). Since no electron density was found for the first four residues in the crystal structure of procathepsin L, indicating that these residues are disordered in the crystal, this region should be susceptible to cleavage. Similar cleavages at the N terminus of the proregion were observed in the processing of propapaya proteinase IV and procathepsin K (24, 27).

It must be noted in Fig. 1 that there is very little difference in the extent of processing between 1 and 2 h of incubation with cathepsin L, i.e. the bands for the trace amount of nonprocessed procathepsin L (C25S) and the propeptide fragments are identical for the two incubation times. This is attributed to the fact that the propeptide generated by intermolecular processing cannot inhibit the small amount of active cathepsin L used for the experiment and also explains reports that intermolecular processing of nonactivable variants of procathepsins B and S by catalytic amounts of the active proteases is relatively slow compared with the autocatalytic, intramolecular processing (22, 28). With more active enzyme present, as in the case of processing of fully "activable" procathepsin L, the initial events are followed by further degradation of the propeptide. For this reason, the propeptide fragments can be more difficult to isolate. However, propeptide fragments with significant binding affinity to cathepsin L can remain present transiently, since late. However, propeptide fragments with significant binding affinity to cathepsin L can remain present transiently, since late. However, propeptide fragments with significant binding affinity to cathepsin L can remain present transiently, since late. However, propeptide fragments with significant binding affinity to cathepsin L can remain present transiently, since late. However, propeptide fragments with significant binding affinity to cathepsin L can remain present transiently, since late. However, propeptide fragments with significant binding affinity to cathepsin L can remain present transiently, since late. However, propeptide fragments with significant binding affinity to cathepsin L can remain present transiently, since late. However, propeptide fragments with significant binding affinity to cathepsin L can remain present transiently, since late.

Continuous Monitoring of Wild-type Procathepsin L Autocatalytic Processing—To obtain information on the nature of the steps involved in the autocatalytic processing of wild-type procathepsin L, we have used a continuous assay based on the hydrolysis of the substrate Cbz-Phe-Arg-MCA by the active enzyme generated in the process. Typical progress curves obtained at varying concentrations of the proenzyme are shown in Fig. 3A. The rate of substrate hydrolysis increases with time due to time-dependent release of cathepsin L from procathepsin L until a constant rate is obtained that corresponds to the activity of fully processed cathepsin L. The reaction was initiated by diluting the stock proenzyme solution (kept at pH 8.0) into the assay mixture (pH 5.3). The reaction was carried out in presence of 10 μM substrate (Cbz-Phe-Arg-MCA). Concentrations of proenzyme indicated are in μM, typical curve fitting of a progress curve (0.24 μM proenzyme) by nonlinear regression of the data to Equation 1. The curve corresponding to the best fit is represented as a dark line through the data points. To better visualize the quality of the fit, the inset represents the residuals (differences between experimental and calculated data) as a function of time. It can be seen that the data are in good agreement with a first order rate of processing. C, replot of the first order rates of processing (kobs) obtained by nonlinear regression of the data to Equation 1 as a function of procathepsin L concentration.

FIG. 3. Continuous assay for the autocatalytic processing of wild-type procathepsin L. A, fluorescence versus time progress curves obtained for the autocatalytic processing of procathepsin L. The reaction was initiated by diluting the stock proenzyme solution (kept at pH 8.0) into the assay mixture (pH 5.3). The reaction was carried out in presence of 10 μM substrate (Cbz-Phe-Arg-MCA). Concentrations of proenzyme indicated are in μM. B, typical curve fitting of a progress curve obtained for the autocatalytic processing of procathepsin L. The reaction was initiated by diluting the stock proenzyme solution (kept at pH 8.0) into the assay mixture (pH 5.3). The reaction was carried out in presence of 10 μM substrate (Cbz-Phe-Arg-MCA). Concentrations of proenzyme indicated are in μM. C, replot of the first order rates of processing (kobs) obtained by nonlinear regression of the data to Equation 1 as a function of procathepsin L concentration.

In addition, as the concentration of proenzyme tends toward 0, the extrapolated rate constant is equal to 0.9 × 10−3 s−1, indicating also the presence of a unimolecular event, independent of the concentration of the proenzyme, in the processing of procathepsin L.

Procathepsin L has recently been reported to process exclusively by an intramolecular mechanism (8, 19). In the experiments carried out by Nomura and Fujisawa (8), it was found that cathepsin L could not process the inactive C25S mutant form of procathepsin L at pH 4.0. Therefore, it was concluded
that intermolecular processing does not take place and that the autocatalytic processing of procathepsin L occurs by an intramolecular mechanism. However, when essentially the same experiment was repeated in the present study, intermolecular processing was definitively detected (see Fig. 1). The stability of mature cathepsin L might be responsible for this apparent contradiction. From a close examination of the gel reported in Ref. 8, it seems that under the experimental conditions used by Nomura and Fujisawa for processing (30-min incubation at pH 4.0, 37 °C) the mature cathepsin L generated would be unstable and go undetected. In the work performed by McDonald and Emerick (19), the absence of a sigmoidal activation profile in the rate of formation of mature cathepsin L was interpreted as evidence for an intramolecular reaction mechanism. Such an activation mechanism would be reflected by a lag period in the initial portion of the progress curves in Fig. 3, which was not detected in the present study. Rather, the processing appears to be a first-order process, based on our individual progress curves. However, the replots of the rate constants \(k_{\text{cat}}\) obtained from these progress curves presented in Fig. 3C suggests the existence of an intermolecular process. This kinetic behavior is a reflection of the complexity of the mechanism for processing of procathepsin L. It appears that processing involves both unimolecular and bimolecular (intermolecular) components. During this process, the propeptide is released from the proenzyme in an almost intact form, at least transiently. This propeptide has been shown to possess considerable inhibitory activity (6) and could slow down the rate of processing by inhibiting the intermolecular cleavage step. Therefore, it is difficult to develop a model that would take into account all of these factors and predict what the progress curve should look like. In addition it has been shown that interaction on glass or plastic surfaces can activate processing of procathepsin L (29, 30) and complicates the in vitro measurements. For all of these reasons, although the quality of the fits obtained for the data presented in Fig. 3 seems excellent, the model only represents a simplified view, and the results of the continuous assay (or any other assay of this type) should be interpreted in a qualitative manner.

**Circular Dichroism and Fluorescence Measurements**—Processing of procathepsin L occurs by an autocatalytic mechanism at low pH while the proenzyme is stable at pH 8.0. To probe for possible conformational changes at low pH required for triggering of processing, we have used circular dichroism and fluorescence measurements of procathepsin L (C25S). The near-UV CD spectrum is sensitive to a asymmetry of aromatic side chains, particularly for tryptophan residues, and can give a qualitative evaluation of changes in tertiary structure. A strong increase in circular dichroism was observed previously when the cathepsin L propeptide bound to cathepsin L (6). This was attributed to the presence of hydrophobic interactions involving tryptophan residues in the cathepsin L-propeptide complex, which are absent in the propeptide or cathepsin L alone. From examination of the procathepsin L crystal structure, it can be seen that three tryptophan residues (Trp12p, Trp15p, and Trp35p) form part of the hydrophobic core of the N-terminal globular domain of the proregion (18). In addition, tryptophan residues are also found at the binding site of the proregion on the enzyme (Trp189, Trp193). If conformational changes occur involving these portions of the proregion, we would expect the near-UV CD spectrum to be affected. As shown in Fig. 4A, there is virtually no difference in the near-UV circular dichroism signals for pH 4.0, 6.0, and 8.0. The far-UV CD spectrum gives a measure of the protein secondary structure. The signal observed for procathepsin L (C25S) with a maximum at 190 nm and two minima at 210 and 220 nm is characteristic of an \(\alpha/\beta\) protein. Again very little difference is observed between the CD spectra obtained at various pH values (Fig. 4B). These results indicate that there is no large scale conformational change in the structure of procathepsin L when the pH is lowered from 8.0 to 4.0. If conformational changes do occur, they must be very localized and minimal or involve only a small fraction of the molecules in solution at any one time and thus go undetected by CD.

The fluorescence of a tryptophan residue is particularly sensitive to its microenvironment and can also be used to probe conformational changes in proteins. Procathepsin L contains nine tryptophan residues, four of them found in the proregion and buried in hydrophobic regions. If large scale conformational changes occur in the proregion, the fluorescence properties of these tryptophan residues would most likely be affected. From fluorescence measurements, it was found that the emission maximum for procathepsin L(C25S) at pH 7.0 is at 326 nm and that the fluorescence anisotropy of tryptophan residues is 0.080. This is in agreement with the fact that the tryptophan residues in the proenzyme are buried in hydrophobic environments. By comparison, the fluorescence maximum and fluorescence anisotropy for tryptophan in the cathepsin L propeptide are 348 nm and 0.035, respectively, which indicates that free...
Glycine in P1. A mutant of propapaya proteinase IV containing autoprocess, presumably due to its own restricted specificity for propapaya proteinase IV (24). Propapaya proteinase IV cannot sight into the nature of such an intramolecular cleavage was suggested for the processing of recombinant propapain, rapidly degraded) or present in such small amounts as to be study. However, such intermediates might be unstable (11). Sheahan, K., Shuja, S., and Murnane, M. J. (1989) Cancer Res. 49, 3809–3814.

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12. Kakegawa, H., Nikawa, T., Tagami, K., Kamioka, H., Stumiani, K., Kawa, propapaya in solution adopts a mainly random structure. Lowering the pH to 3.7 has no effect on either the emission maximum or anisotropy for the proenzyme, which were measured at 325 nm and 0.081, respectively, again indicating the absence of major conformational changes at low pH.

Nature of the Steps Involved in the Autocatalytic Processing of Procathepsin L—The results of the present study clearly establish that intermolecular processing of procathepsin L occurs and can generate the correct mature form of the enzyme. Cleavage takes place mainly in a portion of the proregion located close to or at the N terminus of the mature enzyme. The intermolecular cleavage sites are accessible and contain sequences corresponding to the known substrate specificity of cathepsin L. By comparison, procathepsins B, K, and S yield mature enzyme forms containing N-terminal extensions of several amino acids (20, 27, 28). In a recent study published while this manuscript was in preparation, evidence was obtained that also supports the involvement of an intermolecular process in the activation of procathepsin K (27). Cleavages were observed close to the C-terminal portion of the proregion, as observed in the present study. The preferred cleavage sites in procathepsin K occurred one or two amino acids after each proline residue in the proregion. These sites are located in regions that are predicted to be fairly accessible for proteolysis based on the structure of procathepsin L.

Evidence supporting the involvement of a unimolecular step in the processing of procathepsin L was also obtained in the present study using a continuous assay. The existence of a unimolecular event in the processing mechanism of cysteine protease zymogens has been suggested previously based on similar experiments with procathepsin B and propapain (22, 31). Although that process has frequently been ascribed to intramolecular processing of the proenzyme, direct evidence for intramolecular cleavage has been difficult to obtain, and the nature of this event remains elusive. Whether or not the unimolecular step corresponds to an intramolecular process cannot be ruled out or directly supported by the present work. By comparing the three-dimensional structures of cathepsin L (32) and procathepsin L (18), no rearrangement of the N terminus of the mature enzyme is observed following processing; therefore, intramolecular cleavage cannot occur directly at the N-terminal position of cathepsin L unless major conformational changes are involved. The initial intramolecular proteolytic cleavage must occur elsewhere in the proregion, generating a catalytically active processing intermediate that could be further processed to yield mature cathepsin L. No processing intermediates other than those resulting from cleavage near the C terminus of the proregion have been observed in this study. However, such intermediates might be unstable (i.e. rapidly degraded) or present in such small amounts as to be undetectable. The existence of a processing intermediate has been suggested for the processing of recombinant propapain, but the intermediate could not be identified (31). Possible insight into the nature of such an intramolecular cleavage was provided recently by a very interesting study using a mutant of propapaya protease IV (24). Propapaya protease IV cannot autoprocess, presumably due to its own restricted specificity for glycine in P1. A mutant of propapaya proteinase IV containing a glycine residue at the proregion-mature enzyme boundary was shown to process autocatalytically. Interestingly, a partially processed proenzyme was observed, resulting from cleavage after a glycine residue located at a position corresponding to Gly<sup>77</sup>P in procathepsin L, and it was proposed that this cleavage could correspond to the initial proteolytic event. In the crystal structure of procathepsin L, it can be seen that Gly<sup>77</sup>P is located at the active site, in proximity to the catalytic cysteine residue. From a sequence alignment of 39 cysteine protease precursors (18), this residue is usually found to be a small amino acid, allowing the proregion segment to pack in close proximity to the active site cysteine residue of the proenzyme. It must be noted, however, that the proregion binds in the opposite direction to that of a normal substrate and cannot be cleaved by the normal catalytic mechanism (33).

Alternatively, the unimolecular step could represent a process other than an intramolecular cleavage reaction. For example, a rate-limiting conformational change that exposes the active site and renders the proenzyme active (e.g. dissociation of the proregion from the active site) would correspond to a unimolecular process. An “activated” proenzyme could then cleave another procathepsin L molecule by an intermolecular mechanism. In that regard, it is interesting to note that the rate constant for the unimolecular process obtained from the intercept in Fig. 3B (9 × 10<sup>-4</sup> s<sup>-1</sup>) is very similar to the rate of dissociation of the complex formed between cathepsin L and its propeptide (2.4 × 10<sup>-4</sup> s<sup>-1</sup>) determined in a previous study (6).

Circular dichroism and fluorescence experiments rule out the existence of a major or large scale conformational change. However, such structural variations could be limited to a small fraction of molecules in solution that trigger processing. It is also possible that a relatively localized and minimal conformational change might be sufficient to expose the active site of procathepsin L. The observation that small synthetic inhibitors can be incorporated at the active site of proenzymes (29, 31) suggests that the proregion does not remain in constant intimate contact with the active site. There has been an increasing number of studies reporting the activity of cysteine proteasezymogens (16, 19, 24, 27, 34). In the case of procathepsin K, the activity of the zymogen against Cbz-Phe-Arg-MCA was estimated to be 2000-fold lower than that of the mature enzyme (27). No evidence of procathepsin L activity against Cbz-Phe-Arg-MCA was observed within experimental error from the continuous assay used in the present study, indicating that zymogen activity, if any, must be much lower than that of mature cathepsin L. However, one must consider the possibility that the increase in rate observed in the continuous assays might not be due to the generation of mature enzyme but reflect conformational changes that confer activity to the proenzyme. This would be consistent with previous reports that a proenzyme activation step might precede proteolytic cleavages in the mechanism of procathepsin B processing (22). More work is required to clarify this point and establish the nature of all individual steps in the processing mechanism of cysteine proteases.
Processing of Procathepsin L


Autocatalytic Processing of Recombinant Human Procathepsin L: CONTRIBUTION OF BOTH INTERMOLECULAR AND UNIMOLECULAR EVENTS IN THE PROCESSING OF PROCATHEPSIN L IN VITRO
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