Demonstration by electrospray mass spectrometry that the peptidyl dipeptidase activity of cathepsin B is capable of rat cathepsin B C-terminal processing

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Electrospray mass spectrometric techniques were used to demonstrate that mature (single-chain) recombinant rat cathepsin B is capable of sequentially removing the three dipeptides which comprise the C-terminal extension of the proenzyme. A pepsin-cleaved form of a non-active mutant recombinant rat procathepsin B (Cys-29-Ser) was used as a 'substrate' to study C-terminal processing by mature cathepsin B. The results indicate that the first two residues (Arg-Phe) are removed efficiently,

while the remaining four (Gln-Tyr-Trp-Gly), particularly the final two, are much more resistant to proteolysis. These cleavages were pronounced at pH 5.0 compared with pH 6.0, in agreement with the lower pH optimum for cathepsin B exopeptidase activity reported previously. From this example of the peptidyl dipeptidase activity of cathepsin B we conclude that removal of the C-terminal extension may occur in any intracellular compartment where active cathepsin B is found.

INTRODUCTION

Cathepsin B (EC 3.4.22.1) is a unique cysteine proteinase of the papain superfamily in that, in addition to its well-characterized endopeptidase activity (Barrett and Kirschke, 1981), it also exhibits exopeptidase (peptidyl dipeptidase) activity with protein substrates (Aronson and Barrett, 1978; Bond and Barrett, 1980; Pohl et al., 1987). This property has been suggested to provide an easy means of distinguishing cathepsin B from the closely related lysosomal cysteine proteinases, cathepsins H, L and S (Pohl et al., 1987). While it has also been proposed that cathepsin B is only an exopeptidase (Takahashi et al., 1986a), the interaction of cathepsin B isolated from several species with α_2 -macroglobulin clearly indicates that this enzyme is indeed an endopeptidase (Mason, 1989).

The sequences of preprocathepsin B from rat, mouse and human sources, determined from cDNA cloning, indicate that the enzyme is synthesized as a 339-residue protein (San Segundo et al., 1985; Chan et al., 1986). The precursor proteins possess an homologous six-residue C-terminal extension which had not been observed by conventional protein sequencing of the mature lysosomal enzyme (Takio et al., 1983; Ritonja et al., 1985), although from a recent cDNA study a slightly different situation appears to exist in the bovine case (Béchet et al., 1991). The function of the C-terminal extension is not yet known, but a role as a sorting sequence has been proposed (Erickson and Blobel, 1983). This suggestion is supported by the finding that it appears to be unimportant for protein folding, and does not seem to influence or modulate the activity of the recombinant enzyme (Hasnain et al., 1992).

By means of a recombinant expression system in yeast, a non-active rat procathepsin B has been expressed and purified (Rowan et al., 1992). Recently, we proposed a model for the processing of procathepsin B to a mature single-chain form

based on studies *in vitro* using the recombinant rat procathepsin B. In this model procathepsin B can be cleaved by cathepsins B or L to yield a single-chain form of the enzyme with a six- or four-residue N-terminal extension respectively. These extensions are then removed via the action of dipeptidylpeptidase I (cathepsin C) to give the commonly found sequence (Rowan et al., 1992). While N-terminal processing can be monitored by conventional automated protein sequencing techniques using Edman degradation, no such method has been available for the study of C-terminal processing. In this paper we demonstrate the application of electrospray m.s. (Covey et al., 1988; Fenn et al., 1989; Smith et al., 1990) for such a study. We have used a pepsin-cleaved form of a non-active recombinant precursor protein as a 'substrate' for exopeptidase processing by mature cathepsin B (Figure 1). Treatment of procathepsin B with pepsin removes the proregion which carries an N-linked oligosaccharide and leaves an homogeneous protein with a five-residue N-terminal extension relative to the mature single-chain form (Rowan et al., 1992). M.s. analyses demonstrate that the C-terminal extension of procathepsin B is indeed a substrate for the action of mature cathepsin B.

MATERIALS AND METHODS

Materials

Porcine pepsin was obtained from Worthington Biochemicals Corporation (Freehold, NJ, U.S.A.). L-3-Carboxy-2,3-*trans*-epoxypropionyl-leucylamido(4-guanidino)butane (E-64) and pepstatin A were from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Plasmid construction and purification of recombinant rat procathepsin B (C29S:S115A) were as described (Rowan et al., 1992). The single-letter code for amino acids [IUPAC-IUB Joint Commission on Biochemical Nomenclature (1985)] is used when referring to mutations within the procathepsin B sequence.

Abbreviations used: E-64, L-3-carboxy-2,3-*trans*-epoxypropionyl-leucylamido(4-guanidino)butane; Ter, termination codon.

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Figure 1 Diagrammatic representation of recombinant rat cathepsin B used in the C-terminal processing studies and the predicted molecular masses (in Da) of the C-terminally processed forms

(a) Y represents an asparagine-linked oligosaccharide. The mutation C29S (the active-site cysteine) prevents autoprocessing of the expressed proenzyme and the replacement S115A removes the consensus sequence for N-linked oligosaccharide substitution in the mature enzyme. The portion of the proregion and associated carbohydrate removed by pepsin cleavage is indicated as the stippled area. (b) The masses are calculated for the non-glycosylated pepsin-generated single-chain form (C29S:S115A) which has a five-residue N-terminal extension.

In the numbering system used, the N-terminus of mature cathepsin B is designated as residue 1. Mature single-chain cathepsin B (S115A) was purified essentially as described previously (Lee et al., 1990). All the cathepsin B constructs used in this work contained the mutation S115A to remove the consensus sequence (Asn-Gly-Ser) for N-linked oligosaccharide substitution in the mature protein (Figure 1).

Preparation of cathepsin B for C-terminal processing studies

Procathepsin B (C29S:S115A) (3 mg) was resuspended in 1 ml of 0.3% (v/v) acetic acid. Porcine pepsin (6 µg) was then added and the mixture incubated at 40 °C for 2 h, after which time pepstatin A was added to a final concentration of 5 µM. A small aliquot was removed for SDS/PAGE analysis (King and Laemmli, 1971) and the remainder dried in eight equal aliquots using a Savant speed vac concentrator.

C-terminal processing

Pepsin-treated procathepsin B (C29S:S115A) (0.35 mg) was resuspended in 100 µl of water, and an equal volume of 50 mM Mes buffer, pH 5.0, containing 2 mM EDTA and 10 mM cysteine (Buffer A) was added. E-64 (1 µM final concentration) was added to one tube. Purified recombinant cathepsin B (0.1, 1.0 or

5.0 µg) was added and all tubes were incubated at 37 °C for various times up to 24 h. The reactions were stopped by the addition of E-64 to 1 µM. Samples were then adjusted to a volume of 2 ml with water and dialysed against 100 vol. of water followed by two changes of 5% (v/v) acetic acid. After dialysis, the samples were concentrated using Centricon-10 micro-concentrators (Amicon, Danvers, MA, U.S.A.) to 200–300 µl, and then kept at 4 °C until m.s. analyses were performed.

Expression of cathepsin B mutants lacking the C-terminal extension

Using single-stranded DNA from the procathepsin B constructs (S115A) and (C29S:S115A), further mutants of rat procathepsin B were constructed by site-directed mutagenesis (Kunkel et al., 1987) using the oligonucleotide 5'-CCACGCACTCCAGTAA-TACTGGGGAAG-3' to introduce a termination codon (Ter) at residue 255 of the mature cathepsin B sequence, thereby eliminating the six-residue C-terminal extension in the expressed proteins (altered bases are underlined). Positive mutants were screened for by the loss of a *ScaI* restriction site, and used to transform *Saccharomyces cerevisiae* strain BJ3501 (Ito et al., 1983). Yeast was cultured as described previously (Rowan et al., 1992). The mutant (S115A:Q255Ter) was purified to homogeneity according to Lee et al. (1990).

Cathepsin B treatment of procathepsin B (C29S:S115A:Q255Ter)

Medium (4 litres) from a culture expressing the mutant procathepsin B (C29S:S115A:Q255Ter) was clarified by centrifugation (5000 g, 5 min) and the supernatant concentrated, using an Amicon Spiral Concentrator fitted with an S1Y10 membrane, to approx. 120 ml. After further concentration to 20 ml using an Amicon ultrafiltration chamber (YM10 membrane), the pH value of the concentrate was adjusted to pH 4.0 by the addition of 0.5 M sodium formate buffer, pH 3.0. Pepsin (2 µg/ml final concentration) was then added and the mixture incubated at 40 °C for 40 h after which time pepstatin A was added to 2 µM. The sample was dialysed against two changes of 50 vol. of water and then applied to a column of Sephadex G-25 medium (2.5 cm × 40 cm), equilibrated in 0.3% (v/v) acetic acid, at a flow rate of 5 ml/min. The fractions comprising the major protein peak were pooled and lyophilized in two equal aliquots. These aliquots were resuspended in 400 µl of water, and then an equal volume of Buffer A was added. Insoluble material was removed by centrifugation. Cathepsin B (5 µg) was added to one aliquot and after incubation for 24 h at 37 °C, E-64 was added to a concentration of 1 µM. The samples were then dialysed and concentrated as described above.

Protein determination

Procathepsin B was estimated using an $A_{280}^{1\%}$ of 20 determined for mature cathepsin B (Barrett, 1973). The mature region of procathepsin B comprises 80% of the total protein. This value was used to estimate the amount of protein remaining following pepsin cleavage. The incubations therefore were performed with an active enzyme: 'substrate' ratio of approx. 1:3000 (unless stated otherwise).

M.s. analysis

A triple-quadrupole mass spectrometer (the API III LC/MS/MS system, Sciex, Thornhill, Ontario, Canada) was used, a detailed description of which has appeared elsewhere (Covey et al., 1991;

Figure 2 Full m.s. scan of the pepsin-cleaved procathepsin B (C29S:S115A)

Each labelled peak represents a different charge state of the protonated protein. Protein concentration was approx. 45 μ M in a 15% (v/v) acetic acid solution. Flow rate was 1.0 μ l/min with a sprayer voltage of +4 to 6 kV. The inset highlights a single charge state (21⁺) showing the native (peak 1), oxidized (peak 2) and sulphate (peak 3) adducts. The experimentally derived molecular masses of the three peaks are 29076 $\pm$ 1.3 Da, 29092 $\pm$ 1.1 Da, 29175 $\pm$ 1.8 Da respectively.

Feng et al., 1991). In brief, the mass spectrometer has a mass-to-charge ratio (m/z) range of 0–2400 and is fitted with a pneumatically assisted electrospray (ionspray) interface (Bruins et al., 1987). Multiply charged protein ions were generated by spraying the sample solution through a stainless-steel capillary held at a high potential (+4–6 kV). The sample solution [adjusted to 15% (v/v) acetic acid] was delivered to the sprayer by a syringe infusion pump (model 22, Harvard Apparatus, South Natick, MA, U.S.A.) through a fused silica capillary of 100 μ m internal diameter. Liquid flow rate was 1.0 μ l/min for sample introduction. The potential on the sampling orifice of the instrument was set at +35 V during calibration and was raised to +100 V for proteins to enhance the ion signals. The instrument m/z scale was calibrated with the ammonium adduct ions of poly-(propylene glycols). Average mass values of the proteins were calculated from the m/z peaks in the charge distribution profiles of the multiply charged ions (Covey et al., 1988; Loo et al., 1989; Feng et al., 1991).

RESULTS

The theoretical molecular mass for the pepsin-cleaved procathepsin B, possessing the six-residue C-terminal extension, was calculated to be 29077 Da. The expected masses of all of the potential C-terminally processed forms are presented in Figure 1. M.s. analysis (a full scan is shown in Figure 2) clearly indicated that the starting material possessed only the pepsin-cleaved, six-residue extended form of the enzyme (Table 1), although oxidized and sulphate adducts of the protein were also present (Figure 2). The pepsin-cleaved form of the mutant procathepsin B lacking the C-terminal extension was found to have a mass of 28237 $\pm$ 1.9 Da.

After incubation of the pepsin-cleaved six-residue C-terminal extended protein with active cathepsin B at an enzyme:substrate ratio of 1:3000, almost all of the starting material had disappeared by 1 h (at pH 5.0) with the concomitant appearance of a 28774 Da protein (Figure 3). In comparison, the same extent of conversion took four times longer at pH 6.0. At 2 h there was

Table 1 Experimentally determined molecular masses from m.s. analyses of the cathepsin B digestion of the pepsin-cleaved procathepsin B (C39S:S115A) (1:3000) at pH 5.0 and pH 6.0

The bar (–) indicates that no species was detected in the sample. The assignment refers to the extra C-terminal residues compared with normally processed enzyme. Abbreviation: ND, not determined.

Incubation time (h)	Experimental mass (Da)		Assignment
	pH 5.0	pH 6.0	
0	29076.3 $\pm$ 1.3	29079.4 $\pm$ 0.9	+6
0.1	29078.1 $\pm$ 1.1	29077.2 $\pm$ 2.1	+6
	28774.2 $\pm$ 1.8	–	+4
	28717.5 $\pm$ 1.2	–	+3
1	–	29079.1 $\pm$ 2.5	+6
	28926.5 $\pm$ 2.7	–	+5
	28774.1 $\pm$ 0.9	28774.6 $\pm$ 1.6	+4
	28716.7 $\pm$ 1.5	–	+3
	28530.5 $\pm$ 0.8	28530.6 $\pm$ 2.0	+2
2	–	29077.6 $\pm$ 2.0	+6
	28774.8 $\pm$ 0.8	28775.2 $\pm$ 2.1	+4
	28531.7 $\pm$ 0.9	28530.1 $\pm$ 2.9	+2
4	ND	28529.9 $\pm$ 0.9	+4
	ND	28774.1 $\pm$ 1.5	+2
24	28774.7 $\pm$ 1.5	ND	+4
	28531.7 $\pm$ 0.5	ND	+2
	28367.3 $\pm$ 2.4	ND	+1
	28239.7 $\pm$ 2.3	ND	0
24*	–	28774.1 $\pm$ 1.5	+4
	28531.7 $\pm$ 0.5	28532.4 $\pm$ 1.8	+2
	28367.7 $\pm$ 1.3	28367.6 $\pm$ 2.4	+1
	28240.2 $\pm$ 0.4	–	0
24†	28531.6 $\pm$ 1.3	ND	+2
	28366.4 $\pm$ 2.2	ND	+1
	28240.9 $\pm$ 0.8	ND	0

* Cathepsin B: 'substrate' ratio of 1:300.

† Cathepsin B: 'substrate' ratio of 1:60.

Figure 3 Expanded m.s. profiles from the cathepsin B digestion of the pepsin-cleaved procathepsin B (1:3000) at pH 5.0

Representative m/z spectra from the 21^+ charge state for the various time points are presented. Where no protein species were present in the m/z spectra (1340–1400), data from the initial full-scans was used. The position of forms of the 'substrate' with six, four, two or zero residues of the C-terminal extension are indicated. ^a Cathepsin B: 'substrate' ratio of 1:300; ^b Cathepsin B: 'substrate' ratio of 1:60.

little difference, whereas by 24 h the predominant form was a 28531 Da protein; this same extent of conversion at pH 6.0 occurred only after a similar digestion with 10 times the amount of exogenous cathepsin B. Increasing the amount of cathepsin B activity in the digests 10- and 50-fold led to the appearance of a

28239 Da protein species (Figure 3). No such protein was detected in any of the pH 6.0 incubations (Table 1). Some minor protein peaks were also detected with corresponding masses of 28367 Da, 28717 Da and 28930 Da respectively (Table 1; see also Figure 1). No forms lacking more than the six residues were observed and cathepsin B digestion of the pepsin product of the procathepsin B mutant lacking the C-terminal extension also showed no further C-terminal processing.

Analysis of active recombinant cathepsin B indicated that C-terminal processing occurs during yeast culture. While the mutant lacking the C-terminal extension (S115A:Q255Ter) gave a single protein of the expected molecular mass, the forms detected for the enzyme synthesized with the extension (S115A) were found to represent a mixture of forms with the removal of either four residues or the entire C-terminal extension.

DISCUSSION

The observation that cathepsin B purified from yeast cultures appeared to lack most, if not all, of its encoded C-terminal extension led us to consider that cathepsin B itself was involved in the removal of this extension. In order to produce a protein possessing the full C-terminal extension, a non-active cathepsin B mutant (C29S:S115A) was constructed. Since electrospray m.s. allows very accurate determinations of molecular mass, it was possible to use this technique to study the C-terminal processing of this protein.

The production of various protonation states of the protein of interest is fundamental to electrospray m.s. The pepsin-cleaved procathepsin B substrate used in this study can have a maximum of 29 positive charges (ten Lys, ten Arg, eight His plus a free N-terminus) when fully protonated. From Figure 2 it is evident that the starting material showed a charge distribution with forms close to the maximum charge number. It is also worth noting that on removal of the first dipeptide (Arg-Phe) a protonated group is lost, and this is reflected in a shift of the spectral charge-state profile towards a higher m/z value. Full m.s. scans were made for each sample, and charge states from 13^+ to 26^+ were observed. In the starting material, the predominant form was 22^+ (Figure 2), whereas after removal of the first dipeptide, the 21^+ form became the most abundant charge state. For simplicity, only the expanded 21^+ charge-state regions of the various m/z spectra are shown (Figure 3). All of the other charge-state regions had the same spectral features and helped to serve as reproducibility checks. The initial full-scans indicated that no protein species were present in other areas of the m/z spectrum.

The finding of both the oxidized (R. Feng and Y. Konishi, unpublished work) and sulphate (Chowdhury et al., 1990) adducts of a protein species is not uncommon. Our data clearly indicate for example, that the three peaks observed for the starting material were of the expected masses for the native, oxidized and sulphate adducts of the protein (see inset in Figure 2). Calculation of the molecular masses of various species in a given profile can be hampered by the presence of these adducts. In particular, the small separation between the principle peak and that bearing an additional oxygen atom can lead to convolution of the peak, thus giving inaccurate mass determinations (Feng et al., 1991). Once identified, however, accurate calculation of all the protein species is possible.

Treatment of the non-active recombinant rat procathepsin B enzyme with pepsin has previously been shown to produce a mature single-chain form of cathepsin B which possesses a five-residue N-terminal extension (Rowan et al., 1992). It was envisaged that this cleaved protein would also possess the six-residue C-terminal extension, giving it an expected molecular

mass of 29077 Da (Figure 1) based on the predicted amino-acid sequence deduced from DNA-sequence analysis (San Segundo et al., 1985). Electrospray m.s. gave exact agreement with the predicted mass (Table 1). This indicated that O-linked glycosylation, a common feature of recombinant proteins expressed in yeast, is not present in the mature enzyme sequence. Furthermore, since the experimentally obtained molecular masses could be determined to within the accuracy of 1 Da, this supports the assumption that all but two of the cysteine residues of rat cathepsin B are present as disulphide bonds as in the bovine enzyme (Baudyš et al., 1990). Using this homogeneous material as a 'substrate' for exogenously added active cathepsin B, three products of its peptidyl dipeptidase activity were anticipated. These three forms were detected, and the results indicate that the sequential removal of the three dipeptides was both pH- and time-dependent. Small amounts of the products of carboxypeptidase activity, indicating removal of single amino-acid residues, were also detected in some of the digests as shown by the minor peaks in Figure 3. This type of activity has been observed previously with cathepsin B acting as an exopeptidase (Takahashi et al., 1986b). The finding of increased peptidyl dipeptidase activity at pH 5.0, compared with that at pH 6.0, is in agreement with the reported lower pH optimum for this type of cathepsin B activity (Pohl et al., 1987). The exopeptidase activity would therefore be optimal in an acidic compartment such as the lysosome.

The X-ray crystallographic structure of fully processed human lysosomal cathepsin B has recently been deduced (Musil et al., 1991). From this structure it is evident that the final two C-terminal residues are not well-resolved, suggesting that they, and by extrapolation the six-residue C-terminal extension, are exposed to solvent. During the course of C-terminal processing, removal of the extension would be possible since the last two residues of what will become the mature enzyme would be bound in the S_1 and S_2 subsites [in the terminology of Schechter and Berger (1967)] thus enabling cleavage of the final two residues of the C-terminal extension present as the P'_1 and P'_2 residues. No further proteolysis would then be possible, since earlier residues should be inaccessible.

In our study the ratio of active enzyme to 'substrate' was very low unlike that present in the lysosome. We therefore conclude that, once active, cathepsin B rapidly removes the C-terminal extension from other cathepsin B molecules (i.e. most probably in the lysosome). Since the lack of the C-terminal extension does not appear to affect the expression and folding of procathepsin B (Hasnain et al., 1992) it may be that this extension is important for targeting the latent (inactive) proenzyme in mammalian cells.

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