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An Unusual Specificity in the Activation of Neutrophil Serine Proteinase Zymogens†

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ABSTRACT: The majority of proteinases exist as zymogens whose activation usually results from a single proteolytic event. Two notable exceptions to this generalization are the serine proteinases neutrophil elastase (HNE) and cathepsin G (cat G), proteolytic enzymes of human neutrophils that are apparently fully active in their storage granules. On the basis of amino acid sequences inferred from the gene and cDNAs encoding these enzymes, it is likely that both are synthesized as precursors containing unusual C-terminal and N-terminal peptide extensions absent from the mature proteins. We have used biosynthetic radiolabeling and radiosequencing techniques to identify the kinetics of activation of both proteinases in the promonocyte-like cell line U937. We find that both N- and C-terminal extensions are removed about 90 min after the onset of synthesis, resulting in the activation of the proteinases. HNE and cat G are, therefore, transiently present as zymogens, presumably to protect the biosynthetic machinery of the cell from adventitious proteolysis. Activation results from cleavage following a glutamic acid residue to give an activation specificity opposite to those of almost all other serine proteinase zymogens, but shared, possibly, by the "granzyme" group of related serine proteinases present in the killer granules of cytotoxic T-lymphocytes and rat mast cell proteinase II.

Most proteolytic enzymes are stored as inactive precursors (zymogens), presumably to protect the biosynthetic and transport machinery of the body from adventitious proteolysis. Upon reaching their target locations, zymogens await activation that occurs following limited proteolysis by specific activator proteases. This process, reviewed recently by Neurath (1989), occurs in most members of the chymotrypsin superfamily of serine proteinases, the most numerous proteinase superfamily, whose members include the coagulation, fibrinolytic, and pancreatic proteinases.

The specificity and chemical principles of zymogen activation are best understood for the pancreatic proteinases chymotrypsin and trypsin. A single proteolytic event (cleavage of the Arg-15/Ile-16 bond in chymotrypsinogen or the equivalent Lys-6/Ile-7 bond in trypsinogen) generates an α-amino group in a location that enables the proteins to adopt their catalytic conformations (Kraut, 1977). Other cleavages may occur (Miller et al., 1971), but these do not result in zymogen activation. Whereas the structural changes associated with zymogen activation of chymotrypsin superfamily members are only known for the pancreatic enzymes, the specificity of activation is conserved for almost all members, with activation following cleavage after a basic amino acid (Lys or Arg).

The neutrophil proteinases elastase (HNE)[1] and cathepsin G (cat G), members of the chymotrypsin superfamily (Sinha et al., 1987; Salvesen et al., 1987), exist as catalytically competent forms in their storage granule (Starkey, 1978; Senior

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[1] Abbreviations: HNE, human neutrophil elastase (EC 3.4.21.37); cat G, cathepsin G (EC 3.4.21.20); E-64, *N*-[4-[[*N*-[(L-3-*trans*-carboxyoxiran-2-yl)carbonyl]-L-leucyl]amino]butyl]guanidine; DCI, 3,4-dichloroisocoumarin; ATZ, anilinothiazolinone; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate.

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& Campbell, 1984). On the basis of amino acid sequences inferred from the genes and cDNAs encoding these enzymes, however, it is likely that both are synthesized as precursors containing unusual N-terminal extensions not found in the mature enzymes (Salvesen et al., 1987; Takahashi et al., 1988; Farley et al., 1989). We have hypothesized that the extensions may serve as zymogen activation peptides that prevent premature activation during transport of the enzymes to their storage location in neutrophils. Moreover, activation of the neutrophil proteinase zymogens, should they exist, would result from cleavage after an acidic amino acid (Glu), giving a specificity "opposite" to other chymotrypsin superfamily members.

To test this hypothesis, we have examined the kintics of proteolytic processing and activation of HNE and cat G in the promonocyte-like cell line U937 by biosynthetic radiolabeling and radiosequencing techniques. This has enabled us to determine the existence of zymogens of the neutrophil proteinases and the specificity of their activation. Our results imply that similar events occur during the synthesis of related proteinases from mast cells (Woodbury et al., 1981) and cytotoxic T-lymphocytes (Tschopp & Jongeneel, 1988).

Materials and Methods

Poly(vinylidene difluoride) membranes (Immobilon) were obtained from Millipore Corp. *Staphylococcus aureus* fixed cell membranes (Pansorbin) were from Calbiochem. [^{35}S]-Methionine (800 Ci/mmol) and [^{3}H]isoleucine (100 Ci/mmol) were from New England Nuclear. The rabbit antiserum to HNE was from Athens Reserach and Technology, Athens, GA. The goat antiserum to cat G and the origin of the U937 cell line have been described previously (Salvesen et al., 1987). 1,10-Phenanthroline, 3,4-dichloroisocoumarin (DCI), *N*-[4-[[*N*-[(L-3-*trans*-carboxyoxiran-2-yl)carbonyl]-L-leucyl]-amino]butyl]guanidine (E-64), and aprotinin–Sepharose were from Sigma. Endoglycosidases F and H were from Boehringer. All reagents for protein sequencing and peptide synthesis were from Applied Biosystems Inc.

Standard Biosynthetic Radiolabeling. U937 cells were maintained in RPMI medium containing 10% fetal bovine serum at 37 °C in a 5% CO_2 atmosphere. For standard biosynthetic radiolabeling, cells were harvested and washed in RPMI salts solution by centrifugation (500*g*, 4 min) and resuspended to a density of 2×10^6/mL in RPMI medium lacking Met. Following a 30-min starvation period, [^{35}S]Met was added to 100 μCi/mL and incubation continued for 30 min. Cells were washed by centrifugation in full RPMI medium and resuspended to 2×10^6/mL in full (chase) medium. Samples (1 mL) were withdrawn at timed intervals.

Biosynthetic Radiolabeling for Radiosequencing. Cells prepared for radiosequence analysis were starved for 30 min in medium without Met or Ile, followed by addition of [^{35}S]Met and [^{3}H]Ile to a concentration of 200 μCi/mL each. The [^{3}H]Ile was first concentrated 10-fold by freeze-drying (Waters et al., 1988). Labeling was allowed to proceed for 30 min after which cells were treated as described above.

Lysis and Immunoprecipitation. Harvested cells were washed twice in ice-cold RPMI salts solution (13000*g*, 15 s) and stored for up to 3 days at −70 °C. Cells were lysed by three freeze/thaw cycles (dry ice–ethanol/37 °C) in 0.5 mL of 50 mM Tris and 1 M NaCl, pH 8.0, containing 0.5% Triton X-100 and a protease inhibitor cocktail (Salvesen & Nagase, 1989) containing 1 mM 1,10-phenanthroline, 50 μM DCI, and 10 μM E-64. Ten microliters of a 20% suspension of *S. aureus* cell membranes was incubated with agitation for 1 h at room temperature with the lysates. The supernatant was recovered by centrifugation (13000*g*, 2 min) and added to 10 μL of an antiserum raised against cat G or HNE. Following incubation at 4 °C overnight, immune complexes were recovered by the addition of *S. aureus* membranes and washed four times by resuspension in lysis buffer without inhibitors. The pellet was washed a final time in 10 mM Tris and 0.1 mM EDTA pH 8.0, aspirated, and treated for 2 min at 90 °C in SDS sample buffer containing 1% mercaptoethanol.

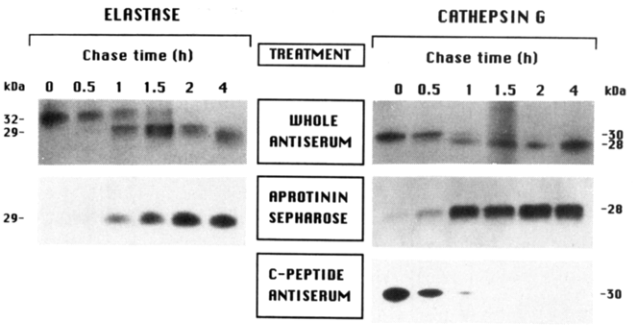

FIGURE 1: Posttranslational processing of elastase and cathepsin G. Biosynthetically radiolabeled U937 cells were lysed and treated with specific antisera to the whole proteins (top panels), with aprotinin–Sepharose followed by specific antisera (middle panels), or with an antiserum raised against a synthetic peptide to the putative C-peptide extension of cathepsin G (bottom panel).

Adsorption to Aprotinin–Sepharose. Cells destined for treatment with aprotinin–Sepharose were lysed in the absence of DCI and allowed to react for 60 min at room temperature with 50 μL of a 50% suspension of aprotinin–Sepharose. The Sepharose was washed three times with 1 mL of lysis buffer, and bound protein was eluted by incubating the Sepharose for 15 min in 1 mL of 50 mM sodium acetate buffer, pH 4.5, containing 300 mM NaCl. The eluate was recovered by centrifugation, adjusted to pH 7.5 by the addition of 1 M Tris-HCl, pH 7.5, and made 50 μM in DCI, and incubation with specific antisera was performed as described above.

Gel Electrophoresis. The supernatant from SDS-treated immunoprecipitates was recovered by centrifugation and run in SDS gel electrophoresis (Bury, 1981) in 5–15% linear gradient gels. Gels were stained, destained, equilibrated in 1 M sodium salicylate (Chamberlain, 1979), dried, and fluorographed for 1–3 days at −70 °C. Immunoprecipitates for radiosequencing were treated with sample buffer containing recrystallized SDS and run in SDS–PAGE with upper reservoir buffer containing recrystallized SDS and 0.1 mM thioglycolic acid (Matsudiara, 1987). Gels were electroblotted (Matsudiara, 1987) to poly(vinylidene difluoride) membranes which were dried and exposed directly to X-ray film overnight at −70 °C.

Peptide Antiserum. The peptide FKLLDQMETPL, corresponding to the putative C-peptide extension of cathepsin G, was synthesized on an Applied Biosystems 430A peptide synthesizer, coupled to ovalbumin by using glutaraldehyde (Kagen & Glick, 1979), and used to raise a rabbit antiserum.

Radiosequence Analysis. Radioactive bands were excised from poly(vinylidene difluoride) membranes for radiosequence analysis using an Applied Biosystems 477A protein sequencer. Unconverted anilinothiazolinone (ATZ) derivatives were collected directly into scintillation vials for radioactive counting in a Beckman LS5000TD liquid scintillation counter with windows set at 0–250 for ^{3}H and 400–700 for ^{35}S. All samples were counted for 10 min, giving a 95% confidence level of errors around the mean of 2–12%, but usually less than 5%.

Glycosidase Digestions. Materials from [^{35}S]Met-labeled U937 cell lysates was immunoprecipitated with specific antisera and eluted from *S. aureus* cell membranes by incubation

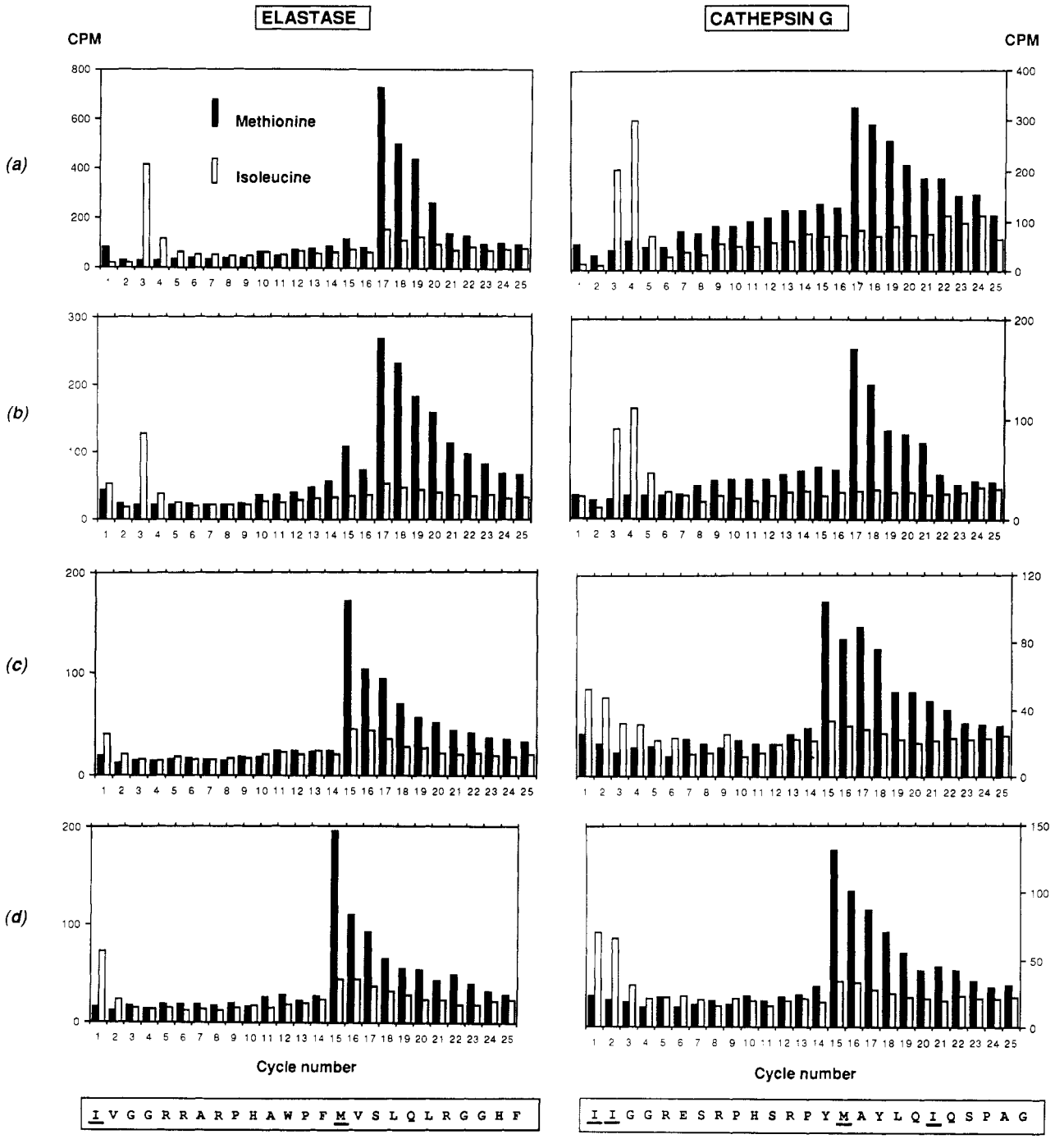

FIGURE 2: Radiosequence analyses of biosynthetically radiolabeled elastase and cathepsin G. Pulse–chase experiments were performed on U937 cells, followed by immunoprecipitation using specific antisera to elastase or cathepsin G. Each panel contains sequence analysis of radiolabeled material corresponding to bands seen in the top panels of Figure 1. Radioactive counts per minute (cpm) associated with each seequence cycle is plotted for [^{35}S]Met (closed bars) and [^{3}H]Ile (open bars). (a) 30-min pulse, no chase; (b) 30-min pulse, 60-min chase, upper band; (c) same as (b), but lower band from gel; (d) 30-min phase, 120-min chase. The bottom of each set of panels shows the N-terminal sequence of the mature proteins, with residues used for radiolabeling underlined.

for 15 min in 10 mM NaOH. The eluates were collected by centrifugation, neutralized by addition of appropriate buffer for treatment with endoglycosidase F or H (Keesey, 1987), and incubated overnight at 37 °C in the presence of the proteinase inhibitor cocktail described above. Samples were run in SDS gel electrophoresis followed by fluorography as described above.

Results and Discussion

Neutrophil elastase and cathepsin G undergo processing events between 60 and 90 min after the onset of synthesis in U937 cells that result in a decrease in size of 3 kDa for the former and 2 kDa for the latter (Figure 1). The timing of these events correlates with development of an ability of each protein to bind to aprotinin–Sepharose (Figure 1). Since many serine proteinases, including HNE and cat G, are inhibited by aprotinin and are adsorbed by aprotinin–Sepharose (Barrett & McDonald, 1980; Baugh & Travis, 1976), this result implies that the enzymes are synthesized as zymogens that become catalytically competent as the result of a processing event during their transit through the cell. An appropriate analogy would be the pancreatic serine proteinases trypsin and chymotrypsin which are strongly adsorbed to aprotinin–Sepharose, but whose zymogens fail to bind (Johnson & Travis, 1976).

The specificity of the processing events that result in activation of the HNE and cat G zymogens was investigated by

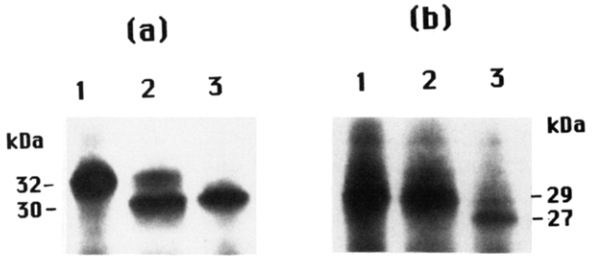

FIGURE 3: Deglycosylation of biosynthetically radiolabeled elastase. Deglycosylations were performed on material that had been biosynthetically radiolabeled for 30 min (a) and material that had been labeled and chased for 4 h (b). Lanes 1, no glycosidase; lanes 2, 5 milliunits of endoglycosidase H; lanes 3, 100 milliunits of endoglycosidase F.

radiosequence analysis (Figure 2). In the earliest biosynthetic forms of both proteins, ATZ-[^{35}S]Met appeared in cycle 17 and tailed off gradually (Figure 2a). An equivalent tailing off in Met was also observed, but with a peak in cycle 15, when purified unlabeled HNE and cat G were sequenced from poly(vinylidene difluoride) membranes. The net yield (pmol) of phenylthiohydantoin-Met in cycles 15–20 was 9.7, 8.5, 4.2, 2.5, 1.1, and 0.8 for cat G and 6.4, 4.5, 2.9, 0.9, 0.5, and 0.3 for HNE. It is likely that this tailing effect is a consequence of sequencing from these membranes following transfer from SDS gels. The positions of Met and Ile in the earliest biosynthetic form of the proteins are consistent with the presence of two amino acid residues preceding the N-terminal residue of the mature proteins, Ser-Glu for HNE and Gly-Glu for cat G.

After 2 h of chase, the distribution of Met and Ile is identical with that of the mature proteins (Figure 2d), indicating complete removal of the N-terminal extensions. Removal of the peptides take place around 1 h of chase, i.e., 60–90 min after the onset of synthesis, as shown by the radiosequence data in Figure 2, panels b and c. At this 1-h chase period, material is fairly evenly divided between two forms (see Figure 1). For each protein, the upper band contained primarily the immature N-terminus (Figure 2b) and the lower band the mature one (Figure 2c). We were unable to detect sequence corresponding to the proposed signal peptides of either of these proteins, but this is not surprising if signal peptide removal is cotranslational as in most other proteins. These results thus confirm recent predictions of the likely location of signal peptidase cleavage sites based on recognition consensus (Salvesen et al., 1987; Takahashi et al., 1988; Farley et al., 1989). Removal of the two-residue extensions is complete by 1 h after the onset of synthesis, and no further proteolytic trimming at the N-terminal region of the proteins occurs.

Coincident with the removal of the N-terminal residue is a decrease in molecular mass of 2–3 kDa for both proteins (Figure 1). Since both proteins are known to contain Asn-linked carbohydrate chains (Baugh & Travis, 1976; Watorek et al., 1988), we speculated that this decrease in molecular weight may represent heavy trimming of carbohydrate following core glycosylation. Indeed, treatment of early biosynthetic forms with endoglycosidases H or F resulted in a decrease in molecular mass of 2 kDa for HNE (Figure 3a). However, treatment with endo F of material that had been chased for 4 h also resulted in the same decrease in molecular weight. This later material was resistant to endo H and is likely composed of complex carbohydrate side chains. Consequently, since early and late biosynthetic forms of the proteins contain approximately the same amount of Asn-linked carbohydrate, we suspect that the decrease in molecular weight that occurs about 1 h after the onset of synthesis is not due to carbohydrate trimming. Rather, we reason that this change represents the loss of the C-terminal regions of cat G and HNE that are predicted by cDNA and gene sequencing (Salvesen et al., 1987; Nakamura et al., 1988; Takahashi et al., 1988; Farley et al., 1989) but are absent from the mature proteins isolated from neutrophils (Sinha et al., 1987; Watorek et al., 1988).

Moreover, an antiserum raised against a synthetic peptide corresponding to the putative C-terminal extension of cat G recognizes the 30-kDa early biosynthetic form of the protein, but not the later 28 kDa, forms (Figure 1). This confirms the existence of the extension as well as its removal between 60 and 90 min after onset of synthesis. We do not know the location in the cell of these processing events, although it is likely that they occur during, or just after, granule packaging as is the case with lysosomal enzymes (Portnoy et al., 1986).

The general mechanism of serine proteinase zymogen activation involves removal of an N-terminal activation peptide (Neurath, 1989). However, since this cleavage in pro HNE and pro cat G coincided with C-peptide removal, we could not be certain that the neutrophil proteinase zymogens followed the general mechanism. We occasionally noticed a small amount of anti-HNE-precipitable material migrating with the 32-kDa band following adsorption to aprotinin–Sepharose. This would imply that N-peptide removal, and not C-peptide removal, was responsible for generating active enzyme, but this result was not sufficiently reproducible for us to rule out participation of the C-peptides in zymogen activation. However, we think it most likely that HNE and cat G zymogens follow the general mechanism and that the C-peptides serve some other function. They may, for example, be involved in cell-sorting mechanisms, acting as signals for azurophil granule packaging.

Serine proteinase zymogens have been studied most intensively by using trypsinogen and chymotrypsinogen, which serve as models for other systems. Their activation results from the ability of the α-amino group of Ile-16 (equivalent to the first Ile in the sequences of mature HNE and cat G), generated by proteolytic cleavage of the precursor, to form an ion pair with Asp-194. This interaction forms the "oxyanion hole" that enables the enzyme to stabilize the tetrahedral intermediate during the hydrolysis of substrate, thereby increasing catalytic

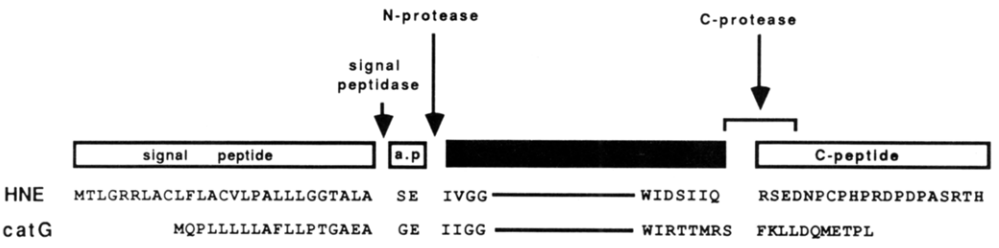

FIGURE 4: Proteolytic processing of precursors of elastase and cathepsin G. Arrows indicate positions of proteolytic cleavages. The solid bar represents the mature, active, proteinases, the majority of whose sequence is abbreviated. a.p. = putative activation peptide. The exact location of the cleavage resulting in C-peptide removal is unknown but is probably within the region bracketed.

efficiency for substrates and binding strength for inhibitors several orders of magnitude over the zymogen (Huber & Bode, 1978; Bode, 1979). This mechanism is thought to be shared by most members of the chymotrypsinogen superfamily (Neurath, 1989). We show that it is likely to hold for HNE and cat G since affinity for aprotinin correlates with catalytic competence in model serine proteinases (Bode, 1979). However, unlike all vertebrate members of the chymotrypsinogen superfamily analyzed to date, activation of pro HNE and pro cat G does not follow cleavage of a basic amino acid (Figure 4). The activation peptides of HNE and cat G are the shortest so far documented. Although one could envisage a zymogen containing a single-residue activation piece that prevented formation of the characteristic Ile–Asp ion pair, such a candidate has yet to be identified.

The novel specificity of zymogen activation demonstrated here may be shared by several of the "granzyme" group of putative serine proteinases found in cytotoxic T-lymphocytes (Lobe et al., 1986; Tschopp & Jongeneel, 1988) and the zymogen of mast cell proteinase II (Benfey et al., 1987) that have homologous potential activation dipeptides. These proteinases, together with HNE and cat G, share common features: (i) they are all stored in granules that await mobilization into a secretory compartment; (ii) they are all thought to be stored in catalytically competent forms; (iii) although it has only been shown for HNE and cat G, activation of all but granzyme A should follow cleavage of a Glu residue; (iv) they are all found in white blood cells or their bone marrow progenitors. We suspect that a single protease, expressed in most white blood cell progenitors, could be responsible for their activation, but we have no data on this yet.

We think that double-label radiosequencing should prove valuable in determining the specificity and kinetics of proteolytic processing of many proteins. We are fortunate that Met is close enough to the N-terminals of HNE and cat G for it to be used as an identifier. This will not always be the case in other proteins, but the main advantage of using [^{35}S]Met is for speed of data aquisition. For example, it is our experience that when a radioactive band is not seen on a radioautograph after 12 h under the specified conditions, then there will not be enough radioactivity incorporated into the sample for sequence analysis. We envisage that, with the judicious use of a ^{3}H-labeled amino acid and [^{35}S]Met or [^{35}S]Cys, many proteins could be radiosequenced according to the protocol described here.

Acknowledgments

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