The *early* and *late* processing of lysosomal enzymes: Proteolysis and compartmentation

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Abstract. Lysosomal enzymes are subjected to a number of modifications including carbohydrate restructuring and proteolytic maturation. Some of these reactions support lysosomal targeting, others are necessary for activation or keeping the enzyme inactive before being segregated, while still others may be adventitious. The non-segregated fraction of the enzyme is secreted and can be isolated from the medium. It is considered that the secreted lysosomal enzymes fulfill certain physiological and pathophysiological roles. By comparing the secreted and the intracellular enzymes it is possible to distinguish between the reactions that occur before and after the segregation. In this review the reactions that may influence the segregation are referred to as the early processing and those characteristic for the enzymes isolated from lysosomal compartments as the late processing. The early processing is characterized mainly by modifications of carbohydrate side chains. In the late processing, proteolytic fragmentation represents the most conspicuous changes. The review focuses on the compartmentation of the reactions and the proteolytic fragmentation of lysosomal enzyme precursors. While a plethora of proteolytic reactions are involved, our knowledge of the proteinases responsible for the particular maturation reactions remains very limited. The review points also to work with cells from patients affected with lysosomal storage disorders, which contributed to our understanding of the lysosomal apparatus.

Key words. Lysosomes; lysosomal enzymes; maturation; proteinases; proteolysis; secretion.

Introduction

Lysosomes were discovered by Christian de Duve and collaborators nearly forty years ago [13, 57]. These studies were a landmark of the 'grind-and-find' era of biochemistry [237], and a cornerstone of cell biology, biochemistry and physiology studies concerned with secretion and endocytosis. The organelles were isolated by differential sedimentation, and characterized as particles containing acid hydrolase activities such as acid phosphatase, β-glucuronidase and cathepsin D. An important criterion is the latency of the enzymatic activity, which is 'released in soluble form and simultaneously activated when the granules are disrupted' [58].

Because of their latency, lysosomes have been referred to as 'sac-like granules'. In fact, lysosomes are enclosed by a single membrane, and this was demonstrated when pictures of lysosomes became available as a result of rapid progress in cytochemistry and in the application of electron microscopy in the second half of the 1950s. Special thanks to the late Alex B. Novikoff, who used to say 'seeing is believing', and with admirable devotion taught others to profit from using the methods of cell biology in biochemistry, lysosomes have become synonymous with a whole group of dynamic organelles, comprising the classic residual or dense bodies, pinocytic and phagocytic vacuoles, and a few other membrane-enclosed structures derived from the Golgi apparatus [237]. There is a great variability in the appearance of the dense bodies alone. According to de Duve, these organelles can be identified under the electron microscope by their 'messy' contents [57].

The Golgi apparatus has been described as the 'center stage' in the processing and distribution of proteins with different destinations [80]. In studies by Palade and colleagues on the itinerary of secretory proteins in the exocrine pancreas, the organelles involved have been ordered into a sequence of unique pre- and post-Golgi compartments, beginning in the rough endoplasmic reticulum and culminating with mature zymogen granules. The dynamics of the labelling of intracellular organelles with radioactive leucine has also indicated that newly-synthesized protein flows from the rough endoplasmic reticulum through the Golgi complex to lysosomes [46, 80].

If various proteins with many destinations are synthesized in one organelle, how are they sorted? What is the mechanism of targeting of the various membrane-associated, secretory and lysosomal proteins? In the 1970s three developments have had a major impact on our understanding of the history of the lysosomal enzymes. Firstly, there was the elucidation of enzyme defects in many lysosomal storage diseases, which comprise a large and clinically heterogeneous group of inherited disorders of metabolism [301]. Secondly, we have witnessed a refinement of various preparatory and immunological techniques and their subsequent application in studies on the biosynthesis of individual enzymes. And finally, there were discoveries of recognition phenomena in receptor-mediated endocytosis: clearance by the liver of plasma-protein with terminal galactose residues [10], regulation of cholesterol metabolism [111], uptake of vitamin B_{12} in fi-

broblasts[380], and correction of mucopolysaccharide degradation in cultured cells by adding into the medium lysosomal enzymes that were produced by normal cells[224]. Further studies on the uptake[222,310] and on the biosynthesis of lysosomal enzymes in fibroblasts[133] and in macrophages[306] have provided the basis for our current understanding of the processing and segregation of lysosomal enzymes. Recognition of lysosomal enzymes via oligosaccharides containing mannose-6-phosphate, and its role in the biogenesis of lysosomes, have been discussed in detail in several recent reviews[51,53,110,184,260,354].

It has also become apparent that targeting mechanisms that do not depend on phosphorylation of lysosomal enzymes add to the complexity of the biogenesis of lysosomes[183,354]. In I-cell disease, which is characterized by the lack of phosphorylation of lysosomal enzymes, liver parenchyma and several other cell types contain roughly normal amounts of lysosomal enzymes. Further, in normal cells a phosphorylation-independent targeting to lysosomes occurs with glucocerebrosidase[5,215], acid phosphatase[114] and various integral lysosomal membrane proteins[186] including the *lamp* proteins[293].

The segregation of acid phosphatase, and of the great number of soluble lysosomal enzyme precursors studied so far, is accompanied and/or followed by a proteolytic cleavage. This processing allows the distinction between 'new' and 'old', in some cases between zymogen und active forms, and usually also between secreted and lysosomal forms of the enzymes. This review is an attempt to discuss data on the maturation of lysosomal enzymes along with those on the biogenesis of lysosomes in normal and pathological situations. In clinics, approximately fifty different lysosomal storage diseases have been described[301]. New methods of molecular genetics have been vigorously applied in studies of these diseases[12,240]. As a result, the primary structures of many lysosomal enzymes and their precursors have been determined. These data are very useful in studies on transport, recognition and maturation of the precursors.

I apologize for any omissions of relevant papers which may make this review incomplete. Besides the general references given above, a wealth of information on lysosomes may be found in books[109,144], including the outstanding series *Lysosomes in Biology and Pathology* edited by Fell and Dingle and later by Dingle, Dean and Sly (Elsevier, Amsterdam).

The synthesis and the early processing of lysosomal proteins

The processing of lysosomal proteins begins during their synthesis in the rough endoplasmic reticulum, continues through their transport into the lumen of Golgi cisternae, and is completed after their arrival in the acidic prelysosomal and lysosomal compartments. For simplicity I suggest that the modifications occurring prior to the segregation should be referred to as the *early* processing of lysosomal enzymes. The segregation takes place after the passage through the Golgi apparatus, usually within one hour of the synthesis. This has been demonstrated for cathepsin D[198]. The segregation may be delayed if the precursor has to be incorporated into an oligomeric complex before leaving the endoplasmic reticulum, as was observed for the α-subunit of β-hexosaminidase[56]. During the early processing the only proteolytic modification of lysosomal proteins is the cleavage of the signal peptide. The *late* processing is suggested to comprise the proteolytic fragmentation and trimming of the C and N termini of the polypeptides and of the carbohydrate residues from their side chains. In particular cases the late reactions bring about an activation of zymogens; sometimes they may prelude the eventual degradation of the lysosomal proteins. The late processing comprises the maturation which is usually recognized as a fragmentation of the lysosomal proteins. It will be discussed in another section of this review.

By in vitro translation of mRNA it has been shown that porcine[74] and mouse[283] cathepsin D, mouse β-glucuronidase[283], rat β-glucuronidase[236] and cathepsin H[228], yeast carboxypeptidase Y[214] and other lysosomal enzymes are synthesized in rough microsomes, and that the nascent polypeptides are co-translationally translocated into and glycosylated in the lumen of the rough microsomes. In rat liver, early biosynthetic forms of cathepsin D[227], cathepsin B[229] and cathepsin B and L[233] have been found to fractionate with microsomes; this finding also indicates that lysosomal enzymes are synthesized in the rough endoplasmic reticulum. This localization of the biosynthesis can be postulated for all lysosomal proteins bearing Asn-linked oligosaccharides. It should be kept in mind, however, that a transport of cytosolic proteins into lysosomes cannot be ruled out[213]. A model system for this kind of transport has been developed by Dice and co-workers. In ribonuclease A the sub-N-terminal sequence Lys-Phe-Glu-Arg-Gln[41,61] seems to mediate a heat-shock protein-dependent[43,61] transfer into lysosomes. Furthermore, Holzer and co-workers have shown that proteolysis is involved in glucose-induced inactivation of fructose 1,6-bisphosphatase[295]. According to Chiang and Scheckman this process involves a regulated transfer of the 1,6-bisphosphatase directly from the cytosol into the vacuole[42]. Recent experiments have indicated that in antigen-presenting cells in the endoplasmic reticulum a transport of peptide fragments takes place from the cytosol into the lumen of the organelle[72,338]. In principle, a similar pathway can also be envisaged for the transport of proteins synthesized in the cytosol into lysosomes. A paradigm for a post-translational transport into lysosomes seems to be yeast vacuolar α-mannosidase, a non-glycosylated protein which is delivered to lysosomes without being translocated into the lumen of the endoplasmic reticulum. Yoshihisa and Anraku placed the cDNA encoding this protein in front

of invertase cDNA, and observed that the construct directed the synthesis of a fusion protein that had been targeted into the vacuole, and lacked carbohydrate even in the invertase portion of the molecule[379]. α-Mannosidase has also been delivered to the vacuole in several *sec* mutants which are defective in the secretory pathway. Its precursor seems to enter an extracytosolic lumen within or distal to the Golgi apparatus, perhaps directly through the vacuolar membrane, the tonoplast. Currently, the endoplasmic reticulum, the bacterial cell membrane, mitochondrial and chloroplast membranes are known to possess receptor/translocator systems for proteins[258, 259, 275, 350]. Peroxisomal[344], vacuolar and lysosomal membranes are likely to be added to this list.

The co-translational transport of the lysosomal enzymes into the lumen of the endoplasmic reticulum is accompanied by the proteolytic cleavage of the signal peptide. Thus, the translocated proteins are likely to be subjected to a very early proteolytic processing, i.e. the cleavage of the signal peptide. Except for yeast alkaline phosphatase, which is an integral membrane protein[167, 172, 173] in all established cDNAs of lysosomal proteins N-terminal sequences have been found that fulfill the characteristics of cleavable signal peptides[105]. The loss of the signal sequence can be deduced from the difference in the size of the proteins that are synthesized in the absence of microsomal membranes (and contain the signal sequence but no carbohydrate) and those prepared in the presence of the membranes and subjected to deglycosylation[76], or those which are synthesized in cells during a brief metabolic labelling in the presence of tunicamycin[283], an inhibitor of glycosylation. With porcine cathepsin D, Erickson et al. have demonstrated an interaction of the signal sequence in the preprotein with the signal recognition particle[79], and shown the cleavage of the signal peptide by determining the radioactivity in the N-terminal sequences of cathepsin D that has been synthesized in vitro in the presence and absence of membranes with a radioactive amino acid[76].

The export of lysosomal proteins from the endoplasmic reticulum probably proceeds similarly to that of other proteins. This process has been reviewed by Rose and Doms[282]. In the endoplasmic reticulum and in the Golgi apparatus, lysosomal proteins are subjected to processing of the carbohydrate. In the soluble proteins the carbohydrate becomes phosphorylated (reviewed in references 53, 110, 134, 182, 183 and 354) and in a few cases also sulphated[28]. The phosphorlyation (i.e. the transfer of N-acetylglucosaminylphosphate to C-6 hydroxyl groups of one or two mannose residues) is an early modification. It occurs on the *cis* side of the Golgi apparatus. Unlike the uncovering (i.e. the removal of the N-acetylglucosamine that covers the mannose 6-phosphate residues) the phosphorylation can be demonstrated even in the presence of brefeldin A[274]. This phosphorylation prevents the trimming of high-mannose oligosaccharides, and thus largely suppresses the formation of complex oligosaccharides in the soluble lysosomal proteins. In the absence of the phosphorylation (I-cell disease) fibroblasts secrete a large amount of lysosomal enzymes and these proteins contain predominantly complex oligosaccharides[132, 206, 352]. The phosphorylation of the β-chain of β-hexosaminidase proceeds with a distinct efficiency at each of its four glycosylated sites[313].

In human fibroblasts the secreted procathepsin D, i.e. the non-targeted product of the early processing, represents a mixture of three forms, each bearing two oligosaccharides, such that either none, one or both are sensitive to endo-β-N-acetylglucosaminidase H[132]. As compared to normal human fibroblasts, in I-cell disease the proportion of lysosomal enzyme molecules bearing the resistant oligosaccharides is increased[132, 206, 352], whereas in transfected BHK cells it is decreased[147]. Thus, the heterogeneity in the early processing is related to the glycosylation system of the cell. A variation in the glycosylation has also been reported for arylsulphatase A in secretions from different human cell lines[363]. However, this difference seems to be accounted for by both the carbohydrate processing, and by the expression of two alleles which encode arylsulphatase A bearing either one or two asparagine-linked oligosaccharides[108, 187, 360, 362].

The role of the above-mentioned sulphation in lysosomal proteins is not known. It is one of the modifications occurring in complex oligosaccharides and is likely to be expressed in the *mid* or *trans* Golgi apparatus. The phosphorylation is necessary for the segregation of the soluble lysosomal enzymes in fibroblasts and several other cell types. This segregation occurs predominantly after transport of the proteins through the Golgi apparatus. The transport[261, 282] and segregation[51, 53, 134, 182–184, 260, 354] have been the subject of several recent reviews.

The compartmentation of the early- and late-processed forms

During the transport that takes place after the cleavage of the signal peptide, and before the segregation from the secretory pathway, probably no proteolytic cleavage takes place in normal lysosomal enzymes. The segregation is not complete, and in all cells examined a portion of the early-processed forms of the soluble lysosomal proteins is secreted. In fact, in most cell types the early-processed species are the only forms of lysosomal proteins that are released into the medium[132, 306]. The early-processed forms are usually referred to as precursors. The precursors that are targeted to lysosomes are subjected to the late processing.

Body fluids such as serum and urine contain both the early-processed (non-fragmented) and the late-processed (fragmented) forms of lysosomal proteins[382]. The actual sources of the early- and late-processed forms of lysosomal enzymes in the body fluids, however, are not known.

Nearly all metabolizing nucleate cells can release the early forms in the course of biosynthesis [133, 306]. In addition, stimulated platelets have been shown to release the precursor form of at least one lysosomal protein, prosaposin [141]. The late forms of the extracellular lysosomal enzymes most probably originate from osteoclasts, and from dead cells, cytotoxic cells, degranulating neutrophils [169, 180, 367], macrophages [305] and perhaps also from platelets [143]. Osteoclasts are specialized in a localized degradation of the intercellular matrix. These cells release acid and lysosomal enzymes into a sealed pericellular compartment [305]. A similar localized release of lysosomal enzymes is observed in cytotoxic lymphocytes [257, 341] and natural killer cells [33]. A distinct mechanism appears to be used for the transfer of lysosomal enzymes between activated lymphocytes and fibroblasts [1, 246]. Here lysosomal proteins are passed from cell to cell in the precursor form [245].

For a long time it has been known that in various pathological situations such as nephrotic syndrome, hepatitis, fibrosis of the liver, kidney transplant rejection, and renal disorders, the activity of β-hexosaminidase in urine or serum is elevated [165, 366, 368, 382]. It has also been shown that, for example, in diabetes [248, 368], liver fibrosis [267] and cirrhosis [154] the isozyme pattern of β-hexosaminidase in serum is changed. In the context of this review I should point to the fact that the elevation of activity may be due to a characteristic change in either the early- or late-processed forms of a lysosomal enzyme. Thus, in the sera of patients with hepatitis, only the activity of the precursor forms of β-hexosaminidase and cathepsin D is elevated [382]. It is not surprising that the high level of lysosomal enzymes in serum that is characteristic of I-cell disease patients [370] is accounted for by the precursor forms of the enzymes [354, 382]. In contrast, the increase in the level of these enzymes in the urine of patients with nephrotic syndrome concerns both the early- and late-processed forms [382]. Mature and precursor forms of cathepsin B or a related proteinase have been found in blood plasma of patients with septic shock or malignant tumors [11], and in malignant ascitic fluid [168].

The distribution of the early (precursor) and the late (mature) processed forms of a lysosomal protein between the cell and the medium is shown in figure 1. In most cells a small amount of the precursor is secreted [126, 133, 306]. The secreted precursor is enriched in molecules bearing larger, usually complex, oligosaccharides [132], although it also contains phosphorylated oligosaccharides [127]. These molecules have escaped the receptor-mediated intracellular targeting to lysosomes via the secretory route A (fig. 1) and are available for receptor-mediated endocytosis (route C). The endocytosed precursors end up in the lysosomes. This has been demonstrated in the early studies of so-called *corrective factors* in lysosomal storage diseases [222, 224]. Although the extracellular route for the transport of lysosomal proteins, known as *secretion-recapture* [222], is a minor pathway of the lysosomal targeting, in certain situations it may be of physiological importance [354]. In cultured human fibroblasts it accounts for the delivery of up to 12% of total lysosomal enzymes to lysosomes [353, 371]. The uptake, route C, is responsible for the clearance of lysosomal proteins from the body fluids.

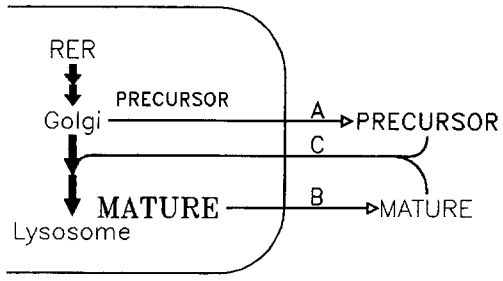

Figure 1. Basic compartmentalization of precursor and mature forms of lysosomal enzymes. A, B and C refer to secretory, exocytic and endocytic pathways in the itinerary of lysosomal enzymes.

The clearance is dependent on various receptors and not only on the recognition of mannose 6-phosphate residues. In experiments with rat liver cells it has been shown that in parenchymal cells the endocytosis of lysosomal enzymes can be mediated by both mannose 6-phosphate and galactose-binding receptors [345]. In non-parenchymal cells endocytosis is also mediated by receptors that recognize mannose and N-acetylglucosamine residues [3, 346]. Mannose and N-acetylglucosamine-mediated clearance of lysosomal enzymes has been demonstrated also in eviscerated animals, indicating that extrahepatic tissues contribute to carbohydrate-mediated clearance of lysosomal enzymes [2, 296]. Probably the major contribution to this uptake is provided by macrophages which bind the lysosomal enzymes through mannose or N-acetylglucosamine residues [315]. The corresponding lectin is expressed in macrophages and endothelial cells [120]. It has been isolated and characterized from rat [120] and rabbit alveolar macrophages [192] and human placenta [191]. This receptor has been suggested to participate in the intracellular segregation of lysosomal hydrolases in macrophages [316]. Indeed, mannose-containing ligands inhibit the intracellular segregation of lysosomal enzymes in rabbit alveolar macrophages [244], and an increase in the expression of the mannose receptor results in a decrease in the secretion of lysosomal enzymes in either unstimulated or stimulated macrophages [302]. The mannose receptor is expressed in alveolar, peritoneal, bone marrow and monocyte-derived macrophages, but not in monocytes. The receptor is likely to regulate the extracellular level of lysosomal enzymes [244, 302], i.e. to participate mainly in endocytosis (route C in fig. 1).

In several cell types derived from bone marrow, e.g. neutrophils and macrophages, lysosomal enzymes are also packaged into granules which are distinct from the normal lysosomes. They have a characteristic biogenesis [14, 16] and enzymatic constituents or 'armory'. Upon

binding of certain mono- or polyvalent ligands or particles such as fMet-Leu-Phe or bacteria to the cell surface [180, 197, 311, 367] the granules can fuse with the plasma membrane or phagocytic vacuoles. Thus, in neutrophils, azurophil granules contain typical lysosomal enzymes [15, 169] besides their characteristic components such as myeloperoxidase [16] and bactericidal factors [97, 189]. From several pieces of evidence it may be concluded that lysosomal enzymes are subjected to similar late processing in both lysosomes and granules. Yet, the packaging of the precursors into the granules is different from that into the lysosomes and so is the fate of their contents. The granules in bone marrow-derived cells fuse with phagosomes or with the plasma membrane after an endocytosis or binding of target cells or of inflammatory agents [169, 300, 367]. The discharge resembles the regulated secretion in gland cells and, at least after some stimuli, it is preceded by a rise in the intracellular Ca^{2+} concentration [180, 311]. These granules are likely to be a major source of the late-processed forms of lysosomal enzymes in the extracellular fluid. It should also be mentioned that cathepsin B is present in secretions from endocrine cells of heart atrium [365] and pancreatic islets [69]. The available data indicate that these cells may be a source of a partially processed cathepsin B in the extracellular fluid. In promyelocates HL-60, in which myeloperoxidase is synthesized as a larger precursor and subjected to a proteolytic maturation during the late processing [7, 129, 175, 220, 247, 375], both myeloperoxidase and lysosomal enzymes are localized in azurophilic granules [15]. This co-localization is observed also with cathepsin D and myeloperoxidase (H. Robenek and A. Hasilik, unpublished). From the kinetics of the maturation of cathepsin D in promyelocytes [129] it may be concluded that azurophilic granules contain mature cathepsin D, and that also during an exocytosis of these granules mature cathepsin D is released from the cells.

The lysosomal apparatus is affected in a characteristic way by weak bases to which membranes are permeable. These agents destroy the proton gradients in intracellular compartments, and thus interfere with the pH-dependent segregation of various ligands, including lysosomal enzymes [112]. In the pathways shown in figure 1, weak bases would stimulate route A, i.e. the secretion of the early-processed forms of lysosomal enzymes. This had been shown initially with cultured human fibroblasts [126]. In HL-60 cells, perturbation of proton gradients in cells has different effects on the fate and packaging of myeloperoxidase and cathepsin D. Firstly, weak bases partially inhibit both the maturation and the packaging of cathepsin D. In the presence of NH_4Cl procathepsin-D is only partially converted into the mature form of the enzyme, and the secretion of the former is stimulated [129]. Under these conditions, however, the maturation of myeloperoxidase is nearly normal and its secretion is not enhanced. In other studies it has also been shown that the maturation of promyeloperoxidase is merely retarded, if it is changed at all, when the cells are subjected to a metabolic labelling in the presence of NH_4Cl [221, 322]. Akin and Kinkade have demonstrated the maturation of the myeloperoxidase precursor in isolated granules and shown that it can take place even at neutral pH [6]. Thus it appears that procathepsin D is segregated in at least two distinct ways, and possibly to two different compartments. Firstly, in a weak base-sensitive manner into a lysosome-like compartment where its late processing is inhibited in the presence of the drug. Secondly, in a weak base-insensitive manner to a granula-like compartment where its late processing proceeds even at an elevated pH, in a similar way to that of the myeloperoxidase precursor.

It has been reported that in stimulated mouse macrophages the enhanced secretion is accounted for by the late-processed forms alone [306–308]. In contrast, in peritoneal macrophages from rats treated with sodium caseinate secretion of precursors has been found [178], and in adherent human macrophages, secretion of both precursor and processed forms has been observed [153] either in the presence or in the absence of a stimulation in vitro. The differences between these observations seem to be of a quantitative nature, and may be explained by a varying sensitivity in the detection of the precursors, and in the degree of differentiation and/or stimulation of the isolated macrophages, rather than by differences in the behavior of macrophages between species.

In our study it has been shown that human monocytes and macrophages stimulated by Percoll and zymosan particles, respectively, secrete a large proportion of lysosomal enzymes in both early- and late-processed forms. In the absence of zymosan, macrophages prepared from either peripheral blood or spleen secreted solely the early forms. Non-elicited mouse peritoneal macrophages are also likely to secrete newly synthesized lysosomal enzymes, because the secretion is abolished in the presence of cycloheximide [297]. Furthermore, we have examined the effect of NH_4Cl on the secretion of lysosomal enzymes in monocyte-derived macrophages and observed that the drug stimulates the secretion of only the early forms of lysosomal enzymes. Previously, Riches and Stanworth have demonstrated that weak bases enhance the secretion of lysosomal enzymes in macrophages [279]. We can assume that in macrophages, as in promyelocytes, early-processed forms of lysosomal enzymes are packaged by at least two dissimilar mechanisms: a lysosomal-type packaging that is sensitive to weak bases and a granule-type packaging that is not.

In this scheme it is not possible to distinguish between two alternatives in which both pathways feed the precursors into one or two populations of organelles. The stimulation of the secretion of the granular contents, e.g. by zymosan, results in the release from the cells of both early- and late-processed forms and this may indicate that the granules are forced to fuse with the plasma membrane before completion of the late processing of the

precursors. The proportion of the early-processed forms in the secretion will be smaller in cells in which the late processing proceeds faster.

The late processing: Trimming of the oligosaccharide side chains

Let us consider the processing in a mammalian cell. At the end of the early processing, lysosomal enzymes are present in the Golgi apparatus in their precursor forms. In molecular terms these precursors are not homogeneous. The inhomogeneity of the molecules at this stage concerns predominantly their carbohydrate side chains. During their exit from the Golgi apparatus the proteins are subjected to a selection. With the help of mannose 6-phosphate, and maybe other receptors, the proteins are retrieved from the constitutive secretory pathway. By default the non-segregated molecules are subjected to secretion.

Because of the carbohydrate recognition during segregation the processing status of the side chains in the secreted fraction is not representative of the situation at the end of the early processing in the Golgi apparatus. However, the protein moieties of lysosomal enzymes secreted by the default pathway are represented solely by their precursor forms. The proteolytic processing of the late phase is confined to compartments that receive lysosomal proteins after the recognition events. Operationally these compartments are referred to as prelysosomal and lysosomal.

The late processing events add to the molecular heterogeneity of lysosomal enzymes, which may be further aggravated during purification. Within lysosomal compartments the carbohydrate side chains are subjected to trimming, which may proceed at very different rates in different lysosomal proteins. A large number of different oligosaccharide structures have been found in lysosomal enzymes including high mannose, complex and hybrid types. For example, structural studies have been performed with β-glucuronidase[35,148,207], cathepsin D[218,326], cathepsin B[327,335], and cathepsin H[335], α-fucosidase[9], α-glucosidase[216], β-glucosidase[330] and β-hexosaminidase[242,251]. In all these enzymes heterogeneous carbohydrate structures have been found. Trimming of oligosaccharides by sialidase and other lysosomal glycosidases has been shown to be rather inefficient in I-cell fibroblasts[351,352].

The heterogeneity of the oligosaccharides is generated in part during the synthesis, i.e. the early processing (a well-studied example is β-hexosaminidase[313], see above) and in part during the late processing. Thus, within a 20-h incubation a 56-kDa intermediate form of the α chain of human β-hexosaminidase is converted to the mature 54-kDa form by a trimming of the carbohydrate moiety[194]. In cathepsins B and H, and in a small fraction of β-glucuronidase an unusually short oligosaccharide has been found. Its structure, Manα1-6Manβ1-4GlcNacβ1-4[Fucα1-6]GlcNAc, indicates that in particular locations the oligosaccharides are exposed to hydrolysis. Thus, in porcine cathepsin D the oligosaccharide linked to Asn-67 in subjected to a more extensive trimming than that linked to Asn-183[303,326].

Studies by Montreuil and co-workers[209] and Carver and Brisson[39] show that carbohydrate side chains assume unique structures.

Therefore, a particular carbohydrate structure is likely to interact with or cover a defined area on the surface of the protein it is attached to. Such an interaction may provide a mutual protection against hydrolases for both protein and carbohydrate. This may explain differences in the trimming of the carbohydrate in different enzymes at the individual glycosylated sites. This view is compatible with current observations on the heterogeneity of carbohydrate side chains at the individual glycosylation sites in different proteins. Thus, O'Dowd et al., who examined the structure of the carbohydrate in the different glycosylation sites in β-chain of human β-hexosaminidase[242], reported that each oligosaccharide site was characterized by a distinct pattern of trimming of the peripheral sugar residues.

The late processing: Fragmentation of the protein

Scope. Many precursors are subjected to fragmentation and trimming of termini. Some details of these reactions became apparent after sequences of the mature proteins and of their precursors had been determined. The power of this approach has been nicely demonstrated in a recent study on human iduronate-2-sulphatase. The results show that the mature enzyme contains two subunits, 42 kDa and 14 kDa, that the larger one originates from the N-terminal portion of the precursor, and that the fragmentation is accompanied by an N-terminal trimming which removes 8–10 residues from the large subunit[23,373].

Organisms. Biosynthesis of a larger molecular weight precursor of a lysosomal enzyme was orginally described in yeast. Later, synthesis of lysosomal enzyme precursors was described in various mammalian and other cells. Precursors are known for β-hexosaminidase in *Tetrahymena thermophila*[151], α-mannosidase[36,37,205,252], β-glucosidase[36,37] and acid phosphatase[34] in *Dictyostelium discoideum*. Similarly, in plants the storage proteins vicilin[44,45,103,104], legumin[44,45,52,70,162], glycinin[22] and sporamin[136], and also lectins[195], are synthesized as larger precursors. In this case the processing of the precursors seems to depend, at least in part, on their autophagic sequestration[139].

Compartments and kinetics. Except for the distinct behavior of the lysosomal proteins in macrophages and monocytes (see above) it is generally observed that the fragmentation of the precursors of lysosomal proteins occurs after their segregation from the secretory pathway. Retention of the proteins in the endoplasmic reticu-

lum inhibits the maturation of the precursors by retarding their transport into the prelysosomal compartment. This has been observed for β-glucuronidase in kidney cells of mice expressing egasyn [30] and cathepsin D bearing the retention signal KDEL at the C-terminus [254, 255], in the transitional elements in cells treated with brefeldin A [274], and in the Golgi apparatus in the presence of monensin [7, 263, 329] or cyanate [128]. The latter two substances may also interfere with the late processing by perturbing proton gradients.

The late processing is initiated in a prelysosomal compartment [31, 107] after segregation in the *trans*-Golgi apparatus [116, 117, 184, 261, 310, 354] and packaging into clathrin-coated vesicles [198]. Similarly to the mannose 6-phosphate-dependent segregation, the late processing is inhibited in the presence of weak bases. However, in macrophages [153], promonocytes [119] and promyelocytes [129, 221, 322] the late processing is rather insensitive to these agents. The fragmentation usually takes a few hours. The fragmentation of β-galactosidase in mouse peritoneal macrophages proceeds with a $t/_2$ of 1 h [308], and that of cathepsin D in human fibroblasts proceeds in two steps and is completed in 5–8 h [107, 126]. In yeast the cleavage of the lysosomal (vacuolar) enzyme precursors and their intracellular transport is much faster than in mammalian cells: carboxypeptidase Y [131] and proteinase A [171] are cleaved with a half-life of 6 minutes. Some enzymes like α-glucosidase are subjected to a sequential fragmentation that goes on for several days [126, 250, 278].

As mentioned above, lysosomal enzyme precursors can be delivered into lysosomes via the secretory, extracellular and endocytic compartments. After endocytosis, normal fragmentation of the precursors is observed [88, 119, 122, 250, 318]. The fragmentation is probably initiated in endosomes which contain proteinases that have been shown to process endocytosed proteins [25, 64, 200]. An endosome membrane-associated form of cathepsin D has been suggested to be involved in this process [63]. Proteolysis in endosomes may be responsible for the processing of antigens [54, 118, 125]. It remains a matter of debate, however, whether the endosomal or lysosomal compartments or both are involved in this process [124]. Autophagosomes are likely to receive most substrate proteins by a non-selective engulfment of cytoplasm [179]. Phagocytic and endocytic targeting has been proposed to converge in a prelysosomal compartment [113], which seems to receive sequentially structural (*lamp* proteins) and functional (e.g. the acidifying ATPase) components of the lysosomal membrane and then lysosomal enzymes along with the mannose 6-phosphate receptor [71]. Thus it is likely that maturation of lysosomal enzyme precursors also takes place in autophagosomes.

Species specificity. A large range of species-dependent velocities has been reported for the fragmentation of cathepsin D. This can be explained by the presence of a characteristically long stretch of amino acid residues at the surface of cathepsin D in some species, which is distinctly prone to proteolysis (see below). For example the fragmentation of the intermediate form of human cathepsin D proceeds much faster than that of bovine or hamster cathepsin D. The latter is only partially cleaved within 1 day of synthesis. As can be expected, hamster cells that have been transfected with human cathepsin D cDNA cleave the human enzyme rapidly and their own slowly [158]. A rapid cleavage of the human enzyme has also been observed in other recipient cells [50].

Membrane proteins. Lysosomal membranes contain, besides structural proteins, a number of transport proteins, which as yet have been rather poorly characterized. The exceptions are: a small group of so-called *lamp* or *lgp* proteins and arylsulphatase C (steroid sulphatase) which are integral membrane proteins, β-glucosidase (glucocerebrosidase), which is associated with the luminal aspect of the membrane, and acid phosphatase which is soluble though derived from an integral membrane precursor. The targeting of all these proteins is independent of mannose 6-phosphate residues. Several *lamp* proteins from various species including human have been characterized [38, 197] and cloned [94]. They comprise a group of homologous, highly glycosylated proteins with a short C-terminal cytosolic domain that has been implicated in targeting of these proteins [372]. An extensive literature is available on *lamp* proteins from several species, describing a high content of carbohydrate and the presence of polylactosamine side chains [38, 67], on a protective role of the carbohydrate [21], on characteristic cell type-dependent distribution in different subcellular membranes [21, 96, 193, 197], and on the translocation of the *lamp* proteins to the plasma membrane in thrombin-activated platelets [82]. Targeting of human *lamp*-1 protein to lysosomes depends on a short tyrosine-containing signal in its C-terminal cytosolic tail [372]. The *lamp* proteins are subjected to an extensive and differentation-dependent processing of the oligosaccharides but not a proteolytic processing.

β-Glucosidase contains four carbohydrate chains [78, 342], but none of the latter are phosphorylated [5, 215]. The nature of the interaction of this enzyme with the lysosomal membrane and the mechanism of the targeting are not established. It has been suggested that the enzyme is bound to acidic phospholipids [152]. It is not subjected to any late proteolytic processing; it is neither fragmented nor N-terminally [78, 314, 342] and C-terminally trimmed [342].

The lysosomal tartrate-sensitive acid phosphatase has been cloned [140, 262], and several steps in its processing have been elucidated. It is synthesized as a glycosylated transmembrane protein with a luminally oriented N-terminus. Without receiving any mannose 6-phosphate residues it is transported to prelysosomal compartments [114, 333], the plasma membrane and eventually to lysosomes where it is released into the lumen of the organelle by proteolysis [115, 359]. After cleavage of the signal peptide the N-terminus is not subjected to any further

processing. The transport to the plasma-membrane has been observed in BHK cells expressing the human enzyme[29], but not in rat liver[331]. The later fragmentation affects both the cytosolic and luminal domains. It is an irony that, in many respects (such as resistance of its targeting to the I-cell mutation), this classical marker of lysosomes behaves differently from other enzymes that are present in the lumen of lysosomes. The mechanism of its targeting has not been elucidated yet, although it is known to depend on the cytosolic domain that contains a functional tyrosine residue[256].

In yeast alkaline phosphatase (the *PHO8* gene product), which is anchored in the vacuolar membrane, the cytosolic tail also participates in the targeting[172,173]. It should be noticed that this enzyme is oriented towards the cytosol with its N-terminus, unlike the precursor of human acid phosphatase, where the C-terminus is towards the cytosol. Yeast alkaline phosphatase is synthesized as an inactive integral membrane precursor without a cleavable signal sequence. The precursor undergoes a proteinase A-dependant cleavage. The truncation removes a fragment from the C-terminus of the precursor, and the mature enzyme remains anchored in the membrane. Yeast acid phosphatase (the *PHO5* gene product) is also synthesized as a membrane-bound precursor. This precursor is cleaved off the membrane in the Golgi apparatus and the solubilized enzyme is secreted[298].

Arylsulphatase C is a membrane protein that is localized in several compartments, including the endoplasmic reticulum and lysosomes. Several groups have isolated and characterized the cDNA of this enzyme[17,26,320,376]. Its C- and N-terminal domains are luminally oriented and glycosylated; its carbohydrate is not phosphorylated[320]. No difference in the size of the lysosomal and microsomal forms has been detected in SDS-PAGE which indicates that the enzyme is not subjected to a major fragmentation.

Soluble proteins. Most soluble lysosomal proteins are synthesized as larger precursors and subjected to a distinct fragmentation (see below). However, after a large number of soluble lysosomal proteins had been examined it became apparent that not all of them are subjected to an endoproteolytic cleavage. During the late processing, rather small changes in the size have been reported for human arylsulphatase A[360], α-fucosidase[62,161,190], α-N-acetylgalactosaminidase[149], and porcine[75], human[249], mouse[98,307] and rat[268] β-glucuronidases. Small changes in size can be explained by C- or N-terminal processing or by a modification of the carbohydrate moiety. If a change in size persists after treatment with glycopeptidase F, it may be assumed that one or both termini of the protein backbone have been cleaved. For example, this technique has been used to indicate a proteolytic processing of rat β-glucuronidase[268]. In an earlier study on porcine β-glucuronidase and cathepsin D, endo-β-N-acetylglucosaminidase H was used for this purpose[75]. However, this endoglycosidase does not cleave complex and extensively trimmed oligosaccharides, and this property may complicate the interpretation of results that have been elaborated with it. The processing of several proteins has been studied extensively. As examples, cathepsin D and β-hexosaminidase will be discussed in detail below.

In the case of those lysosomal enzymes that have been cloned, and for which, in addition, the N-terminal sequence of the mature form has been determined experimentally, it is possible to detect any N-terminal processing. It can be concluded that there is little if any N-terminal processing beyond the cleavage of the signal sequence in human β-glucuronidase[249], human arylsulphatase A[319], human acid phosphatase[115,262], human α-N-acetylgalactosaminidase[149,343,364], human[241] and rat[84] α-L-fucosidase (noticing that the N-terminus of the human enzyme is probably blocked[95]), and human α-galactosidase A[24,185]. An interesting group of lysosomal proteins is represented by activator proteins which participate in the degradation of glycolipids. Burg et al. have reported that G_{M2}-activator protein (SAP-3) is synthesized as a larger precursor[32]. From a comparison of the primary sequence of G_{M2}-activator protein[91] and of the structure that has been deduced from the nucleotide sequence of cloned cDNA[299], it can be concluded that the precursor is subjected to an N-terminal processing. Other SAP proteins are formed by a fragmentation of a polyprotein, which will be discussed separately.

Fragmentation and activation. The proteolytic maturation of lysosomal enzyme precursors, which may involve major changes in the size of the polypeptides, has been pointed out in earlier reviews[133,306]. In several cases only a few amino acid residues are cleaved, in a process that may involve an endoproteolytic fragmentation or an exoproteolytic trimming. As an increasing number of the proteins have been studied it has become clear that the fragmentation is not a universal event in the history of lysosomal enzymes, and that in only a few cases does the fragmentation process involve the activation of a zymogen. In mammalian cells this has been shown for cathepsin D[48,135,211,272], which perhaps activates itself in unimolecular reaction[48]. Several closely related non-lysosomal aspartic proteinases, including pepsins, renin and cathepsin E[377] are synthesized as zymogens. Among these, pepsinogens can self-activate in an acidic milieu[85,166]. Similarly to cathepsin D, cathepsin L[99,226,233,235], cathepsin H[231,235] and also cathepsin B[83,233] are synthesized as inactive zymogens, but their activation probably depends on the action of other enzymes. With cathepsin D as a catalyst, activation of procathepsin L can be demonstrated in vitro[232,369]. It has been suggested that cathepsin B[83] and L[288] participate in the cleavage of their own precursors. The mechanisms of the fragmentation will be discussed below.

Barrett has purified human cathepsin D and shown that it contains two non-covalently-linked polypeptide chains[19]. This author has suggested that the two chains

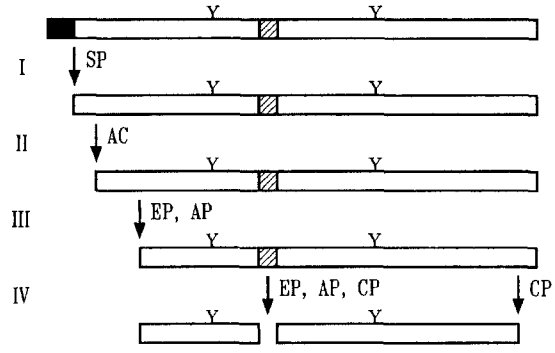

Figure 2. Proteolytic processing of cathepsin D. The abbreviations refer to signal peptidase (SP), autocatalysis (AC), endo- (EP), amino- (AP) and carboxypeptidase (CP) catalyzed processing. The fragmentation of cathepsin D from different species into the heavy and light chains may proceed at different speeds or be absent, depending on the particular primary structure (shaded) and the presence of a susceptible loop in the folded protein. The signal sequence is shown in black and the location of asparagine-linked carbohydrate in both chains is indicated by the letter Y. In human fibroblasts reaction I (cleavage of the signal sequence) takes place in the endoplasmic reticulum. Reactions II (the activation) and III (at least a part of the N-terminal trimming of the single/light chain form) are initiated in a prelysosomal compartment or low density lysosomes. Reaction IV (a thiol proteinase-dependent fragmentation, and trimming of the termini) takes place in dense lysosomes. It is possible that the C-terminal trimming is initiated already at the level of the single chain cathepsin D in low-density lysosomes.

are formed from a larger precursor. In the initial studies on the biosynthesis of cathepsin D in human[126], porcine[76] and mouse[283] cells a fragmentation of a larger precursor has been described. Tang and co-workers pointed out that procathepsin D from different species contains in its sequence a stretch of amino acids that is absent in the closely related sequence of pepsinogens[334, 378]. This stretch is likely to form a loop at the surface of the folded protein that is prone to proteolysis. A cleavage at this loop results in the formation of the 'light' and 'heavy' chains in mature cathepsin D. The cleavage is followed by further trimming, which will be discussed below. A scheme illustrating the fragmentation of human cathepsin D is shown in figure 2.

Exoproteolytic maturation. Upon fragmentation additional N- and C-termini are created, and depending on their accessibility and sensitivity these may be subjected to further endo- or exoproteolytic cleavages. Processing of porcine cathepsin D results in a release of 5 residues and that of bovine cathepsin D in the release of 2 residues from the backbone of the precursors[73, 334, 378]. It has been suggested that the fragmentation of porcine cathepsin D and rat cathepsin B is followed by an aminopeptidolytic trimming catalyzed by cathepsins C and H[378].

The original observation by Barrett[19] that purified cathepsin D contains two polypeptides raised the question of their origin. The answer, namely that the two polypeptides are formed from a common precursor, has been elaborated in two ways. One of them demonstrated the kinetics of the formation of the polypeptides from the precursor in metabolically labeled cells[74, 126, 283], and the other elucidated the primary structure of the two polypeptides and showed that the polypeptides can be ordered in tandem along the known sequence of pepsin[303, 304, 328] or of human cathepsin D, which has been deduced from the sequence of cDNA isolated first by Faust et al.[81]. A slow aminopeptidolytic cleavage of the N-termini in both fragments of human cathepsin D is the probable cause of their microheterogeneity. It has been shown by microsequencing of cathepsin D from human placenta and cultured cells that the fragments represent mixtures of molecules starting with any residue from Val^{-3} through Gly^{+1} and from Ala^{+104} through Gly^{+107} (reference 147 and E. Conner, personal communication). A similar heterogeneity has been observed in the light chain of myeloperoxidase[159, 336] and in the large fragment of mature α- and β-chains of β-hexosaminidase[196] (see below), and is likely to occur in many other lysosomal proteins.

From a comparison of the primary structure of rat cathepsin B[331] and of the corresponding cDNA sequence[291] it is apparent that the maturation results in a removal of glycine and arginine residues from the fragmentation area of the precursor. Thus, the maturation involves both an endo- and an exoproteolytic cleavage. Studies relevant to the fragmentation and subsequent trimming of the polypeptide backbones of cathepsins B[40, 68, 86, 204, 225, 229, 329], H[89, 90, 155, 225, 230, 231, 280] and L[100, 156, 157, 164, 199, 226, 264, 276, 280, 340, 358] will not be reviewed further in detail, because they have recently been discussed and compared to those on cathepsin D by Erickson[73]. For a summary on thiol proteinases the reader is referred to papers by Kominami and Katunuma[176] and Barrett et al.[20].

An involvement of a carboxypeptidase(s) in the late processing was shown first for porcine cathepsin D and β-glucuronidase. These enzymes were isolated after a pulse-chase metabolic labeling of cultured cells, and the kinetics of the release by carboxypeptidase Y of radioactive methionine appeared to be different for the early and late processing forms[75]. A C-terminal processing also occurs in myeloperoxidase, in which a single serine residue is cleaved from the C-terminus of the large subunit[159]. As mentioned below, the pro-α (but not the pro-β) chain of human β-hexosaminidase[196] and, also both of the precursor peptides that are formed during the maturation of the α and β chains of β-hexosaminidase[150], are subjected to C-terminal processing. In the case of more extensive cleavages at the C-terminus, such as that reported for α-glucosidase[142], in which the C-terminal maturation results in a reduction of the molecular mass by approximately 20 kDa, it is not directly apparent whether endo- or exoproteolytic cleavages are involved. Thus there are many examples of C-terminal processing in maturing lysosomal proteins. The enzymes responsible for this trimming, however, have not been identified. Besides carboxypeptidases, such as the *kexl*-related β-galactosidase-protective protein[101, 160, 266, 339], cathepsin B may be involved. An

exopeptidase activity of this cathepsin has been demonstrated by Tang and co-workers[325,329]. The trimming of the N-termini is probably catalyzed by dipeptidyl aminopeptidases such as cathepsin C and aminopeptidases such as cathepsin H[324].

Fragmentation and trimming of β-hexosaminidase. A well-studied example of precursor fragmentation is human β-hexosaminidase. Both chains of the enzyme are synthesized as precursors[126,223]. Further analysis of their processing has been supported by DNA cloning and hence elucidation of the primary structure of both the α[181,217,271] and β[243,269] chains. The pro-α chain has been sequenced at different stages of maturation and the results indicate that 67 residues are removed from the N-terminus of this precursor in several steps[150,194]. Initially a fragment with 64 residues is cleaved off the precursor endoproteolytically, while residues No 87–89 are cleaved from the remaining main chain aminopeptidolytically.

N-terminal sequencing of the α chain from the purified enzyme has indicated that it is a mixture of species terminating with Thr-89 and Leu-90[150,196]. The cleaved precursor peptide remains associated with the enzyme and undergoes a C-terminal processing[150]. Similar details have been elaborated in studies on the maturation of the β chain of β-hexosaminidase. The N-terminus of the purified precursor has been determined[321]. After the segregation the precursor loses two residues from the N-terminus[273] and subsequently is cleaved into the three fragments of the mature protein which are interconnected by disulphide bridges[150,196,273]. The smallest fragment, the 'precursor peptide', is derived from the proximal portion of the precursor and is subjected to a trimming which removes two residues from its N-terminus[273] and a few others from its C-terminus[150]. At the N-terminus of the larger (C-terminal) fragment of the β chains from isoenzymes A and B Leu-316 and Lys-315 have been found, respectively. The fragmentation occurs between Ser-311 and Lys-315 and is followed by an exoproteolytic processing. It appears that the N-terminus of the larger fragment of the β-chain is more protected from the aminopeptidolytic cleavage within isoenzyme A than within isoenzyme B[196]. The proteolytic processing of α and β chains of β-hexosaminidase is shown schematically in figure 3.

Attempts to cleave the precursors of β-hexosaminidase in vitro using a lysosomal preparation from human fibroblasts have been only partially successful. They indicated that a thiol proteinase may be involved[88].

Fragmentation of a polyprotein. The isolation of the cDNA[47,60,239,277] encoding sphingolipid activator SAP or saposin[210] proteins pointed to a novel role for the fragmentation of proteins in lysosomes. SAP-1 (saposin B) accelerates the breakdown of cerebroside sulphate, ganglioside G_{M1} and globotriaosylceramide. SAP-2, which is also called A_1 activator and saposin C, stimulates sphingomyelinase, α-galactosyl- and β-glucosylceramidase. SAP-3 stimulates the hydrolysis of the G_{M2}-ganglioside by β-hexosaminidase A (see above). The primary sequences of SAP-1[91], SAP-2[170,294], a related protein called component C[92] and SAP-3[91] are available. It is certainly a surprise to observe that the same cDNA is isolated when either SAP-1[60] or SAP-2[239,277] are cloned. Fürst et al.[92] pointed out that the fragmentation of the protein encoded by this cDNA may yield at least three related, biologically active proteins, SAP-1, SAP-2 and component C. The isolated cDNA encodes a polyprotein with four regions of internal homology[92,239]. The three distal regions encode SAP-1, SAP-2 and component C, and the proximal region encodes another saposin with activator properties similar to SAP-2[238]. In an earlier study, Fujibayashi and Wenger[93] showed that SAP-2, a small protein with a molecular mass of 7.6 kDa, is synthesized from a relatively large precursor of 50 kDa (both numbers refer to the protein moiety). The results of the later cloning work have explained why the precursor is so much bigger than the mature activator.

Prosaposin has been shown to be present in various kinds of human secreted fluids such as cerebrospinal fluid, semen, milk, pancreatic juice and bile[141]. Furthermore, this or a similar protein (referred to as sulphated glycoprotein 1) is secreted in a large amount by rat Sertoli cells into the tubular fluid[47]. Therefore, it has been speculated that the polyprotein is involved in a transport of glyco-

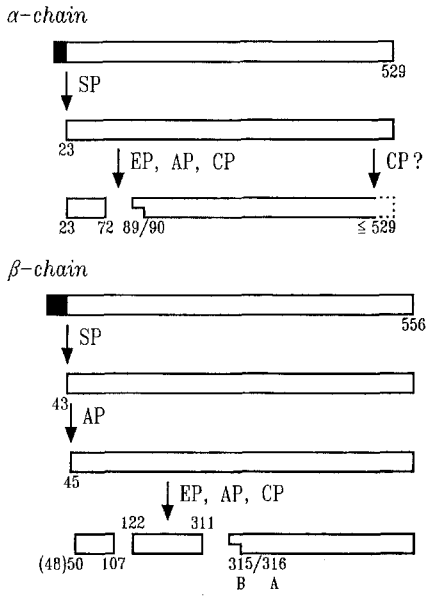

Figure 3. Compilation of the proteolytic maturation of α- and β-chains in human β-hexosaminidases A and B. The numbering of the residues in the α-chain[307] and β-chain[42a,44] refers to the literature cited; SP, EP, AP and CP refer to processing by the signal peptidase and endo-, amino- and carboxypeptidase, respectively. The existence of alternative and intermediate processing forms is indicated by parentheses and slashes. The dotted line indicates a portion of the molecule in which the extent of the processing is not well defined. A and B indicate the difference in the N-terminal processing of the larger fragment of the β-chain in the isoenzymes A and B, respectively. For the sake of simplicity, disulphide bridges between and within the subunits are not shown in the figure.

lipids. It is worth noting that these proteins show a sequence homology with the pulmonary surfactant-associated protein SP-B[253]. It has been suggested that a partially processed form (60 kDa) of prosaposin possesses neuraminidase activity[265]. This possibility should be subjected to further examination.

Changed patterns of fragmentation. In a few cases an inhibition or absence of the fragmentation of one or more lysosomal proteins have been observed. For example, in I-cell fibroblasts the residual intracellular precursor of cathepsin D is processed to give a large fragment which has a molecular weight of 33 kDa, 2 kDa larger than in normal cells[126]. It is likely that the enzyme(s) responsible for the C-terminal processing are more affected by the mutation than those involved in the fragmentation.

An indirect effect on the processing of the α chain of β-hexosaminidase has been observed in infantile generalized N-acetylneuraminic acid storage disease[121]. The protein backbone of the α chain formed in the affected cells is approximately 2 kDa larger than in control cells, indicating that the accumulation of sialic acid results in an inhibition of the exoproteolytic trimming. A few other lysosomal storage diseases are known in which the processing of an enzyme is impaired. In cells from a rather large group of Tay-Sachs disease patients the mutations were shown to result in a partial or complete block of the maturation of the α chain of β-hexosaminidase[56, 87, 188, 270]. The precursor α chain is not exported from the endoplasmic reticulum[188, 270] and is rather rapidly degraded[188, 270]. These and other defects in the biosynthesis of β-hexosaminidase have been discussed in detail by Neufeld in a recent review[223]. In certain cases of glycogenosis II[278], Sandhoff disease[66] and G_{M1}-gangliosidosis[146, 219], α-N-acetylgalactosaminidase deficiency[149] and fucosidosis[62, 161], mutations have been described that impair the maturation of the precursors, most probably by affecting their transport competence. The impaired transport also precludes the phosphorylation of the precursors[149, 270, 278]. Conditions that interfere with lysosomal acidification or with the intracellular transport also affect the processing of lysosomal enzymes. This was shown initially for β-hexosaminidase and α-glucosidase in fibroblasts that had been treated with NH_4Cl or chloroquine[126].

In human promocytes U937 an incomplete processing of the β chain of β hexosaminidase has been observed[119]. The precursor is fragmented to a 52-kDa glycopeptide which resembles a processing intermediate that is formed in other cells through the cleavage of the N-terminal precursor peptide. The block in the subsequent processing indicates that this cleavage may depend on a particular enzyme that is missing in U937 cells.

Fragmentation of yeast vacuolar proteins. Studies on yeast proteinases and their inhibitors in the laboratory of Holzer[145, 287] in the early 1970s greatly stimulated the interest of many other laboratories in the biogenesis of these proteins and in their targeting to the yeast vacuole. As mentioned above, carboxypeptidase Y[65, 131, 132, 171, 203, 214], and then a number of other yeast vacuolar proteins, were shown to be synthesized via larger precursors (reviewed in reference 174). For example, yeast proteinase B is synthesized as a 73-kDa precursor, which is processed to the mature 33-kDa protein via a partially active 42-kDa intermediate[201, 202, 208]. The processing of this intermediate is catalyzed by proteinase A, probably with the participation of proteinase B[201]. Proteinase A is an aspartic proteinase with a structural similarity to pepsin[8, 374]. Like pepsin, proteinase A is synthesized as an inactive precursor. This 52-kDa precursor is activated by cleavage between Glu-66 and Gly-77[8, 374]. The process is probably autocatalytic. It results in the formation of the mature 42-kDa polypeptide[202], and occurs after the delivery of the precursor into the vacuole. Proteinase A has been shown to be responsible for the activation of carboxypeptidase Y[65, 137, 203] and of several other vacuolar enzyme precursors[174, 381], though not all those that are known. Proteinase B and possibly other proteinases are also involved in the maturation of the precursors. However, the phenotype of the *pep*4-3 mutation[137], which affects the structural gene of proteinase A, suggests a germinal role of this particular proteinase in the processing of other vacuolar proteins[8, 172, 284, 374].

In the N-terminal portion of the precursor of proteinase A a protein signal for the targeting of the precursor to vacuoles has been found[171, 286]. In recent years, much progress has been made in work on yeast mutants that are defective in various stages of the intracellular protein transport[173, 284–286], including the export from the Golgi-apparatus[4] and targeting to vacuoles[281, 285]. At an increased temperature these latter mutants secrete soluble lysosomal enzymes in their precursor forms[281, 285]. In some of them the vacuolar membrane marker α-mannosidase is missorted[281]. For further details on this exciting field the reader is referred to reviews by Rothman et al.[286] and Deshaies et al.[59] that have been published recently.

Mechanisms of fragmentation. The proteolytic processing of lysosomal enzyme precursors is performed by a concerted action of endo- and exoproteinases present in prelysosomal and lysosomal compartments. Attempts to find the enzymes responsible for the fragmentation have used both genetic and biochemical methods. As mentioned above, mainly thanks to extensive genetic experimentation, the key role of proteinase A in the activation of other enzymes has been elucidated in yeast. In mammalian cells cathepsin D is the homolog of proteinase A. It may be a self-activable aspartic proteinase, but it is not clear whether it serves a similar function in the processing of other precursors. A potent inhibitor of cathepsin D and other aspartic proteinases (pepstatin) is available. Pepstatin can be applied to cultured cells to inhibit the intracellular cathepsin D. However, with a few excep-

tions little or no change in the processing of lysosomal proteins has been observed in cells treated with this inhibitor. Since cathepsin D preferentially cleaves peptide bonds in the vicinity of hydrophobic amino acids, it is likely to contribute to the hydrolysis of denatured proteins or their fragments. Furthermore, cathepsin D may fulfil the role of an activating proteinase, similar to proteinase A in yeast, by specifically cleaving and activating the precursor forms of other lysosomal proteins. However, the specificity of the other activated proteinases may overlap with that of cathepsin D. Therefore, not all functions of this proteinase are likely to be revealed in short-term experiments with pepstatin. Kato and coworkers have demonstrated that a treatment of rat hepatocytes with pepstatin impairs the activation of cathepsin L[232] and cathepsin H[231] precursors. The processing of acid phosphatase in human cells is also sensitive to pepstatin[115]. Wiederanders and Kirschke have demonstrated activation of the precursor of cathepsin L with cathepsin D in vitro[369]. Leupeptin and other inhibitors of thiol proteinases impair the processing of cathepsin D in human[138] and rabbit[289] fibroblasts. In rat macrophages the processing of the precursors of cathepsins B, H and L to their single-chain forms has been shown to depend on metalloproteinases, and the subsequent fragmentation into their two-chain forms on thiol proteinases[123]. The proteolytic processing of human cathepsin B also proceeds in several steps, and the final conversion of a 33-kDa to a 27-kDa form is catalyzed by a thiol proteinase[122].

The processing of storage proteins in seeds is likely to be initiated at paired basic residues[52] and may be analogous to that in the regulated secretory pathway in mammalian cells.

It is desirable to understand the proteolytic events in the processing of normal and mutant enzymes in human cells, because one of the possible approaches to the therapy of lysosomal storage diseases may be based on inhibition of the degradation of unstable mutant enzymes. It has been pointed out[182, 354, 355] that there is a class of defects in which unstable mutant enzymes are subjected to a rapid degradation and that inhibition of proteinases initiating this degradation might be therapeutically useful. A particular case of lysosomal enzyme instability is observed in galactosialidosis, in which a defect in a protective component of an enzyme complex causes a loss of β-galactosidase activity, due to an enhanced proteolytic degradation[323, 348, 349]. An unstable form of β-galactosidase has also been observed in G_{M1}-gangliosidosis[146, 348]. A theoretical consideration of the storage problem[292] reveals that a slight increase in the steady state level of the affected enzymatic activity may prevent the storage if a critical threshold value of the enzymatic activity is restored. In cultured cells from some patients with lysosomal disorders such as galactosialidosis[323, 348], metachromatic leucodystrophy[355–357], multiple sulphatidosis[318], and Gaucher disease[163], proteinase inhibitors have enhanced the activity of the affected enzymes. In metachromatic leucodystrophy fibroblasts, the treatment has enhanced utilization of the sulphatide that has been added to cultured cells[356]. Unfortunately, in therapy of lysosomal storage diseases an application of proteinase inhibitors has not been successful yet. As a model for an enhanced rate of degradation, modified human cathepsin D, that had been synthesized in the presence of the threonine analog β-hydroxynorvaline, was shown to be degraded by a concerted action of aspartic and thiol proteinases[138].

As mentioned above, the proteolysis can be inhibited by lysosomotropic weak bases which raise the intralysosomal pH. The proteolysis can also be prevented by inhibiting the intracellular transport, e.g. by brefeldin A[274], or by chilling the cells during the incubation[27, 234]. At temperatures below 37 °C the transport of lysosomal precursors through the prelysosomal and proximal compartments is retarded. Thus, at 20–23 °C an accumulation of the processing intermediate of 53 kDa is observed, indicating that the processing of the precursor to the single-chain form of human cathepsin D proceeds in an early lysosomal compartment[27]. Using subcellular fractionation Gieselmann et al.[107] have shown that the fragmentation of the 53-kDa cathepsin D precursor to the 47-kDa intermediate is initiated in lower density organelles, whereas the formation of the mature chains (31-kDa and a smaller fragment) is accomplished in the dense lysosomes[106]. This latter reaction is one which is sensitive to inhibitors of thiol proteinases, as mentioned above[138].

In a few cases (see above) processing of lysosomal enzyme precursors has been examined in vitro. Apart from the self-activation of cathepsin D, however, these studies did not reveal which proteinases are involved in the reactions. Rabbit cathepsin D is a 48-kDa single chain enzyme. It is synthesized as a 53-kDa precursor that can be trimmed in vitro to a 51-kDa intermediate if mature cathepsin D is added, as shown by Samarel et al.[290]. A fragmentation to a 48-kDa product is observed if the incubation is performed in the presence of cathepsin B[290]. In lysosomes that had been isolated from human fibroblasts, fragmentation of the 47-kDa single-chain intermediate of this enzyme was observed to depend on the integrity of the organelles and on their internal acidification[106]. Similarly to the processing in the intact cells[138], this fragmentation depends on the activity of thiol proteinases. In a study of in vitro processing of human β-hexosaminidase, Frisch and Neufeld have also observed that lysosomal thiol proteinases contribute to the fragmentation[88]. However, the processing with a lysosomal fraction is not complete and the 63-kDa precursor of the β-chain is not processed beyond an intermediate of 53 kDa. It is worth mentioning the observation that incubation of the precursor chains with trypsin results in the formation of smaller fragments. These fragments have similar sizes to the polypeptides of the mature enzyme[88], which suggests that the precursors contain loops that are

prone to proteolysis and can be cleaved by several proteinases.

Neufeld and co-workers pointed out that in precursors of lysosomal enzymes leucine residues occur at the N-terminus that is formed after cleavage of the signal peptide at a much higher frequency than in other proteins which are synthesized in the endoplasmic reticulum[194]. It may be speculated that such residues represent a specificity signal for compartmentation or cleavage. Another observation on the common occurrence of a particular amino acid residue, asparagine, within sequences that are subjected to proteolytic processing has been presented by Tang and co-workers. Asparagine is found in the cleaved area in cathepsin D from several species[334, 378], and also in β chain of human β-hexosaminidase[196, 273]. Fragmentation of the single-chain rat cathepsin B is followed by a removal of two residues[291], as can be concluded from a comparison of the amino acid sequence of the mature two-chain enzyme[331] with the sequence of the precursor as deduced from the nucleotide sequence of the cDNA[291]. One possibility is that a thiol proteinase[122, 123] cleaves the precursor between Asn-47 and Gly-48 residues[291], and an aminopeptidase removes two residues from the heavy chain.

What is the role of the proteolytic processing of lysosomal proteins? It is rather unlikely that the additional sequences that are present in the precursor forms of lysosomal enzymes have no function and are only removed as superfluous material. The signal sequence directs the transport of the nascent protein into the luminal space and the remainder of the sequence somehow participates in the folding. Recently, Conner has demonstrated that the precursor domain in human cathepsin D is indispensable for the formation of stable procathepsin D molecules[49]. Further, precursor-specific domains are involved in targeting. Thus, the N-terminal portion of proteinases A[171] and the C-terminal domain of alkaline phosphatase[173] in yeast 'address' the precursors to the vacuole. In precursors of soluble lysosomal enzymes in mammalian cells the precursor-specific domains may contribute to the phosphorylation signal; however, in human cathepsin D it has been shown that this signal resides in other parts of the molecule[18]. Furthermore, it has been shown that in vitro mature forms of lysosomal enzymes can also be phosphorylated[361].

An expression of a lysosomal hydrolytic activity during the transport from the rough endoplasmic reticulum to lysosomes might be harmful. At least partial control is achieved by limiting the concentration of protons in various compartments. This kind of control seems to be sufficient for most glycosidases. Precursors of these enzymes are enzymatically active; the activity can be conveniently demonstrated using secretions of cultured fibroblasts which contain the precursors[135, 321]. Sonderfeld-Fresko and Proia have synthesized β-hexosaminidase B in vitro and demonstrated that in the presence of microsomes the precursors assemble into enzymatically active dimers within 15 minutes of the synthesis[312].

As discussed above, cathepsin D and several other lysosomal enzymes are synthesized as inactive precursors, and fragmentation of these zymogens is important in the regulation of their biological activity. However, many of these proteins, including cathepsin D are subjected to further proteolytic processing and the reason for this modification is unknown. One simple possibility is that in lysosomes all exposed proteolysis-prone areas that can be deleted without loss of function are subjected to cleavage. Some of the cleavages may result in a labilization of the proteins. Even the proteinases themselves are subjected to eventual degradation[177]. The protection of vulnerable protein is indeed an important phenomenon in the physiology of the lysosomal apparatus. A β-galactosidase, sialidase and a carboxypeptidase form a large complex with mutually dependant components. Defects in the structure or in the processing of the carboxypeptidase moiety cause an impairment of the whole complex, with clinical signs of galactosialidosis[55, 101, 102, 347]. Before the enzymatic activity of the carboxypeptidase subunit was established this subunit had been referred to as the protective protein. Defects of this subunit have been discussed above.

In several lysosomal proteins a heterogeneity in the trimming of N-termini has been observed. This trimming is likely to progress in time, to correlate with the age of the protein, and to initiate its degradation. For example, the leupeptin-sensitive fragmentation of human cathepsin B from its 33-kDa to its 27-kDa form can predetermine the degradation, if the smaller chain is more prone to proteolysis than the larger.

Discoveries of novel functions of the modifications are imminent. Various fragments of the saposin precursor that are larger than the mature saposins have been found in distinct amounts in different human tissues[238]. Thus, it is possible that the biological functions of saposins are modulated by their release from the polyprotein precursor, and perhaps also by an alternative fragmentation of this precursor.

In the extracellular milieu, in foci of infection and inflammation, lysosomal enzyme precursors may become fragmented, and also activated. Outside such foci, however, the hydrolases should remain inactive. This control could be provided by shifts in pH in the vicinity of the inflammatory cells. Further, α_2-macroglobulin has been shown to bind both thiol[309, 317] and aspartic[309, 337] lysosomal proteinases. Thus, complexing with α_2-macroglobulin may be a prelude to the clearance of the active lysosomal proteinases. The latent precursors do not bind to α_2-macroglobulin[212]. They may provide for a reservoir of proteinases that can be locally activated, participate in lytic reactions and finally be cleared.

Many questions remain to be clarified on the proteolytic processing of lysosomal enzymes, on the contribution of particular proteinases to this processing, and on their

compartmentation and the regulation of their expression and activity. First of all, it will be helpful to understand the processing of lysosomal enzymes at the molecular level. The overwhelming progress in molecular biology that we are witnessing at present shows that these questions can be answered.

Addendum

Recently M. Hiraiwa and co-authors (Biochem. biophys. Res. Commun. *177* (1991) 1211–1216) have shown that the 60-kDa protein that had been proposed to be a fragment of prosaposin with a sialidase activity[265] is in fact an immunoglobulin G heavy chain that apparently had contaminated the preparation.

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Proteases and proteolysis in the lysosome

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Abstract. Proteins sequestered by a non-selective bulk process within the lysosomes turn over with an apparent half-life of about 8 minutes and this rapid lysosomal proteolysis is initiated by endopeptidases, in particular by the cathepsins D and L. We describe also the cathepsins B and H which show mainly exopeptidase and only low endopeptidase activity. Especially cathepsin H is most probably the only lysosomal aminopeptidase in many cell types. Additionally, the properties of other mammalian lysosomal endo- and exopeptidases are compared.
Finally, we discuss some of the conditions for the action of lysosomal proteases as the low intralysosomal pH, the high part of lysosomal thiol groups and the absence of intralysosomal proteinase inhibitors.
Key words. Lysosome; protein degradation; proteinase; cathepsin.

Introduction

The autophagic-lysosomal pathway of intracellular protein degradation is a non-selective bulk process[44] that accounts for the main part of total protein breakdown. It has been estimated that more than 90% of all long-lived protein and a large fraction of the short-lived protein are degraded in lysosomes[2]. Protein sequestered within the lysosomes turns over with an apparent half-life of about 8 minutes[54], a value in excellent agreement with data on the functional morphology of lysosomes[58].

Such rapid proteolysis needs a highly effective proteolytic environment, and it is the aim of this review to describe briefly some of the lysosomal proteases. For earlier reviews and more detailed reports compare[1,3–6,11–17,19,35,41,42,51,52,62,68].

We assume that as a rule lysosomal proteolysis is initiated by endopeptidases (proteinases), which are rate-limiting, and that this process is immediately continued by the lysosomal exopeptidases. The free amino acids, and also some dipeptides, can diffuse through the lysosomal membrane. Cytosolic exopeptidases are able to split the remaining peptides rapidly. The resulting free amino acids can be used as substrates for the synthesis of new proteins or for degradation as energy sources.

Lysosomal proteases

An old name for cellular proteases is cathepsin. This name was introduced in 1929 and is derived from the Greek term meaning 'to digest'[74].

Some of the lysosomal cathepsins are now recognized as exopeptidases, such as cathepsin A, which is a carboxypeptidase, or cathepsin C, which is a dipeptidylpeptidase[16,52]. Other cathepsins act as endo- as well as exo-