Journal of Cellular Biochemistry 24:121–130 (1984)
Protein Transport and Secretion 173–182

Golgi/Granule Processing of Peptide Hormone and Neuropeptide Precursors: A Minireview

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Proteolytic processing of precursor proteins is a phylogenetically ancient and widely used mechanism for producing biologically active peptides. Proteolytic cleavage of proproteins begins only after transport to the Golgi apparatus has been completed and in most systems may continue for many hours within newly formed secretory vesicles as these are stored in the cytosol or transported along axons to more peripheral sites of release. Paired basic residues are required for efficient proteolysis in most precursors, suggesting that a small number of specialized tryptic proteases exist that have great site selectivity but can process many sites within the same precursor or in different precursors within the same cell, or in different cells or tissues. Cleavage-site choice may be strongly influenced by other factors, such as secondary and tertiary structure, but definitive structural information on precursor proteins is lacking. Modifications such as glycosylation, phosphorylation, and sulfation also are Golgi associated but are not known to influence proteolytic processing patterns. Golgi/granule processing also rarely occurs at sites other than pairs of basic amino acids, including single basic residues (trypsinlike), Leu-Ala, Leu-Ser, or Tyr-Ala bonds (chymotrysinlike) as well as other specialized nontryptic cleavages, suggesting that mixtures of proteases coexist in the Golgi/granule system. Cathepsin B-like thiol proteases, or their precursors, have been implicated as the major processing endopeptidases in several systems. Carboxypeptidase B-like enzymes also have been identified in secretion granules in several tissues and appear to be metalloenzymes similar in mechanism to the pancreatic carboxypeptidases, but with a lower pH optimum. The role of the Golgi apparatus in sorting newly formed secreted products from lysosomal hydrolases may have permitted the development in evolution of an intimate relationship between certain of the lysosomal degradative enzymes, such as cathepsin B or its precursors, and the Golgi/granule processing systems. The sequestration of the proteolytic products of precursors within secretion granules leads to the coordinate discharge of highly complex mixtures of peptides having related or overlapping biological activities. The cosecretion of nonfunctional peptide "leftovers," such as the proinsulin C-peptide, can serve as useful markers of secretion or cellular localization, as well as of evolutionary relationships. Errors in cleavage due to point mutations in precursors have been identified in several systems, leading to the accumulation of incorrectly processed materials in the

Received August 30, 1983; accepted November 1, 1983.

circulation. These and/or defects in converting proteases per se represent interesting areas for study in the search for disturbances in the production of neuroendocrine substances.

Key words: proinsulin, converting enzymes, thiol proteases, Islets of Langerhans, carboxyperhdases

It is now widely recognized that many small bioactive peptides as well as peptide neurotransmitters are cleaved from larger precursor proteins that are processed during their migration through the cell into storage or transport vesicles to yield a variety of active products. Proinsulin was the first of these proteins to be isolated and studied in detail [1–3]. The subsequent discovery of proparathyroid hormone in 1972 [4], provided the first evidence that peptides lacking disulfide bonds might also be derived from larger precursors. In the last decade precursors of many other peptide hormones and neurotransmitters as well as of many cellular and viral proteins have emerged almost continuously [5,6]. The discovery of the multifunctional or polyprotein type of hormone precursor, as exemplified by proopiomelanocortin (PMOC) [7] was a significant finding, which has transformed concepts in the field of neurobiology. As new precursors are found their structures are now being rapidly elucidated by means of gene cloning technology. These techniques are providing an avalanche of new information to be correlated with the tissue distribution, processing and physiology of these peptides.

The structures of several precursors are summarized schematically in Figure 1. Most of these are as large or larger than proinsulin, and contain one or more biologically active regions within them dispersed along their polypeptide chains in what appears to be a random pattern. Tandemly repeated active regions appear in some precursors and occasionally more than one bioactivity may be contained within a single polypeptide, as in proopiomelanocortin [7] and proenkephalin [12]. The three-dimensional organization of these multifunctional precursor proteins is not yet known, and it thus remains unclear as to whether these proteins are made up of more than one folded domain, defined as a unit of organized secondary and tertiary structure, so that they might correctly be called polyproteins [6], as has become the current useage. The intracellular proteolytic mechanisms for the processing of precursor proteins is currently a subject of great interest, since these mechanisms can regulate the size and biological properties of the products derived from any given precursor, giving rise to mixtures of differing potency and/or half-life at different sites. An excellent example is the differential processing of POMC in the anterior versus middle lobes of the pituitary giving rise to either adrenocorticotropin (ACTH) or its smaller derivative, β-melanocyte stimulating hormone (MSH) [15]. The first step of cellular processing in almost all cases is the removal of the signal peptide or prepeptide from the N-terminally extended presecretory form of the precursor as it is being translated on the ribosomes or very shortly thereafter [8,16–19]. The signal peptidase has been localized to the inner membrane of cysternae of the rough endoplasmic reticulum (RER) [18,20]. Almost all secreted proteins in both prokaryotes and eukaryotes have been found to utilize the signal peptide mechanism in their initial segregation across the RER membrane.

This brief review will focus in greater detail on *pro*protein processing mechanisms and will briefly review the current state of knowledge regarding the enzymes involved, their intracellular localization, and their role(s) in generating a wide range of secreted products from many endocrine or neural cells. In view of the rather

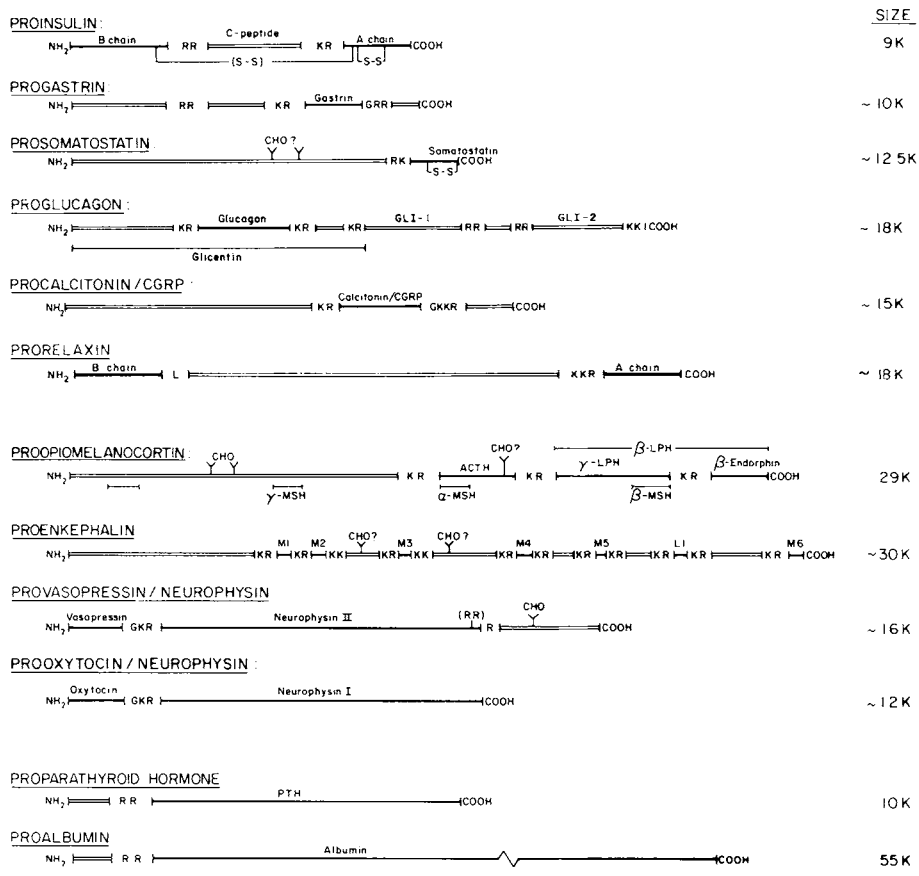

Fig. 1. Schematic structures of some of the known proproteins showing residues at cleavage sites using the single letter amino acid designations. Heavy single lines indicate regions which appear as biologically active products. Data sources are as follows: proinsulin [8], progastrin [9], prosomatostatin [8], proglucagon [10], procalcitonin/CGRP [11], prorelaxin [45], POMC [7,8], proenkephalin [12], provasopressin/neurophysin [13], prooxytocin/neurophysin [14], proparathyroid hormone [4], proalbumin [8].

consistent use of paired basic residues at cleavage sites in a wide variety of precursors, it seem likely that a single enzyme or a rather limited repertoire of similar cellular proteases may carry out processing in many different tissues.

SUBCELLULAR LOCALIZATION OF PROPROTEIN PROCESSING SYSTEMS

In contrast to prepeptide processing discussed earlier, which occurs very rapidly during synthesis or shortly after peptide chain completion, proprotein processing begins only 10–20 min after synthesis and folding of the peptide chain has been completed in the RER and the peptides have been transferred to the Golgi area, where they are concentrated into secretory granules [21,22]. The movement from RER to the Golgi is an energy-requiring process mediated by small transport vesicles, which appear to bud from smooth regions of the RER and migrate to the Golgi area, where they fuse with the cis elements of the Golgi complex [21,23,24]. Biochemical and

TABLE I. Golgi Functions

1. Secretory product packaging
2. Formation of lysosomes
3. Maturation of membrane proteins
4. Proteolytic processing of proproteins
5. Glycosylation (N-linked terminal, O-linked)
6. Amidation
7. Acetylation
8. Sulfation
9. Phosphorylation

radioautographic studies [24–26] have confirmed that proteolytic processing begins within the Golgi for a number of different proproteins, it then continues for many hours as these products progressively mature within newly formed secretion granules developed from the trans Golgi elements [27,28]. These secretion granules can be isolated after their contents have been prelabeled biosynthetically for an appropriate period (eg, 15-min pulse, 30-min chase) and can be used to study processing events under appropriate conditions in vitro [28]. Table I lists some of the known functions of the Golgi apparatus [23].

In vivo, as conversion nears completion, the storage granules may undergo morphological changes associated with condensation of the products, but these products are usually retained in the granules and released coordinately during exocytosis. In the cells of the pancreas this results in the release of essentially equimolar amounts of insulin and C-peptide into the circulation [3,29]. In neurosecretory cells a similar sequence of cellular events may occur during the long period of axonal transport, providing ample time for the proteolytic maturation of precursors [30]. Only low levels of proteolytic enzymes are probably required, eg, for the β-cell if it is assumed that the converting enzyme is as active as pancreatic trypsin a molar ratio of about one molecule protease per 10^4-10^5 substrate molecules should suffice to achieve the observed in vivo rates of conversion [31]. This fact, and the inherent technical difficulties of efficiently isolating and recovering intact Golgi and secretion granule fractions free of other contaminating cellular organelles (such as lysosomes) from small amounts of endocrine tissues complicates the task of identifying converting protease(s) [32].

CLEAVAGE SITES

The most frequent sites of cleavage in all precursor proteins is at pairs of basic amino acids such as Arg-Arg or Lys-Arg, and it has been shown that trypsinlike and carboxypeptidase B-like enzymes working in concert can generate the correct natural products from some of these precursors, as for example with proinsulin [3,31] (see Fig. 2). Scrutiny of the amino acid sequences surrounding these cleavage sites in a number of precursors reveals great variability except for the two dibasic amino acids. No clearly defined secondary structural features emerge on analysis by the Chou-Fasman method, suggesting that the main requirements for cleavage may be that these sites be well exposed on the surface of the protein. Not all pairs of basic residues are

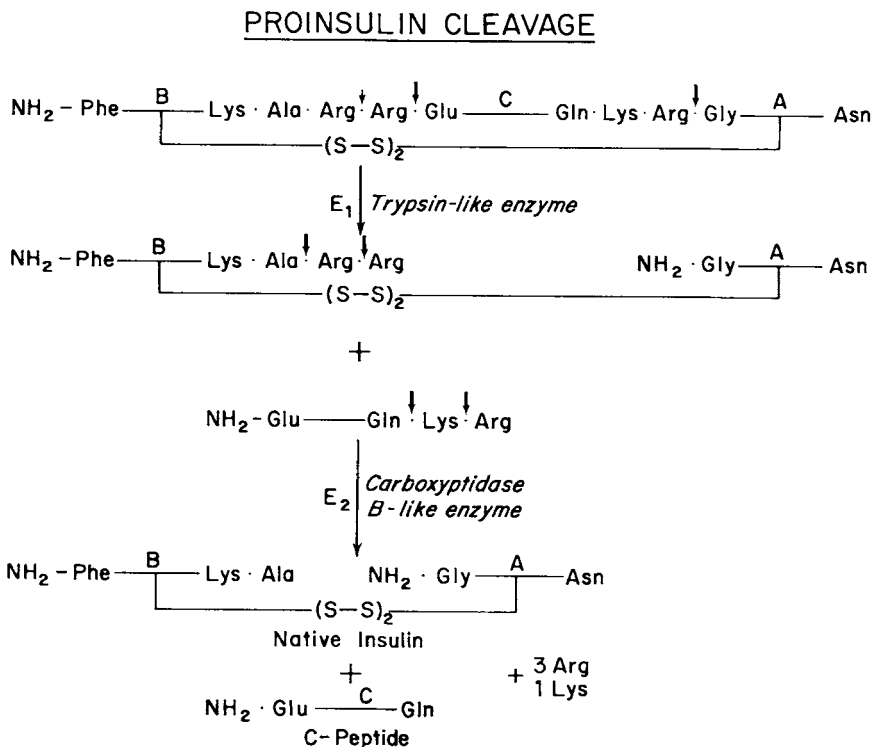

Fig. 2. Cleavage of prohormones at pairs of basic amino acids by a dual-enzyme processing system, as exemplified by proinsulin [33].

cleaved and this may be due to their location in less accessible segments such as helical or "buried" regions. Unfortunately, as mentioned earlier, no reliable three-dimensional structural information on any of these proteins is currently available.

Abnormal proinsulins and proalbumins have been described in which mutations in the basic residue pairs at cleavage sites have led to the substitution of the basic residues, ie, Arg to a neutral residue in an abnormal proinsulin [34], Arg to Gln in the Christchurch proalbumin [35], and Arg to His in proalbumin Lille [36]. In all cases studied thus far processing is prevented or greatly retarded by this change. Experimental replacement of the Arg or Lys residues of anglerfish islet proinsulin, proglucagon, and prosomatostatin by incorporation of the analogs conavanine or thialysine during biosynthesis also prevents cleavage of these prohormones [37], providing further evidence for the essential role of dibasic amino acids. On the other hand, cleavage may not always require pairs of basic amino acids, as exemplified by chicken proalbumin, which has the sequence Ala-Arg on the C-terminal side of the propeptide and is readily processed [38].

Several other precursors have been identified that undergo cleavage at a single basic amino acid (usually arginine). These include dog proinsulin [39], which contains a processed single arginine in its C-peptide at position 8, the vasopressin-neurophysin

precursor (see Fig. 1) and the precursor of canine pancreatic polypeptide [40]. Cholecystokinin (CCK) also is processed at several single basic residue sites [41]. What is unclear is whether these sites are processed by the same protease(s) that normally recognizes the dibasic pairs or by other trypsinlike proteases associated with the endoplasmic reticulum (ER) or storage granules. In studies on the biosynthesis of canine pancreatic polypeptide in cultured dog pancreatic F cells, it has been observed that cleavage at a single basic residue site is lost during prolonged culture, while dibasic processive activity is retained [40].

Another less frequently used site of processing is between Leu-Ala or Leu-Ser residues, presumably requiring an enzyme having chymotrypsin-like specificity. Cleavage at a Leu-Ala bond occurs in the human, pork, and rat proinsulin C-peptides, but usually only 20–30% of the total C-peptide is processed at this position, even after a prolonged chase, and it has accordingly been viewed as an incidental or minor cleavage [42–44]. Recently Hudson et al [45] have observed that prorelaxin, a homologue of proinsulin, is processed at a Leu-Ser bond on the N-terminal side of its 109 residue connecting peptide segment (see Fig. 1). Their findings imply that chymotrypsinlike cleavage may be a major processing mechanism in some cases. On the other hand the cleavage site between the connecting segment and the A chain is made up of three basic amino acids, indicating that trypsinlike processing is also involved. In summary, these variations on the theme suggest that a number of different proteolytic enzymes may normally be present int he Golgi/granule system and participate to varying degrees in prohormone processing. The selection of cleavage sites thus may be dictated both by the nature and relative activities of these enzymes as well as by the primary, secondary, and tertiary structure of the precursors.

CHARACTERIZATION OF CONVERTING PROTEASES

Endopeptidases

Attempts have often been made to implicate various trypsinlike serine proteases in the cleavage of hormone precursors. Among these are the kallikreins [46] and plasmin or plasminogen activator [48]. However, it has not been clearly established that any of these proteases can correctly process proinsulin to generate both native insulin and C-peptide. In the case of plasmin it has been shown that cleavage of proinsulin generates almost equal amounts of insulin and a form of insulin with a residual arginine residue before the glycine at the N-terminus of the A chain [47]. Removal of this arginine would require a novel amino peptidase that has not yet been demonstrated. Trypsin cleavage of proinsulin also generates small amounts ($<5\%$) of Arg-(Ao) insulin, but such a form is present only at very low levels in native insulin (less than 0.5%). In the absence of carboxypeptidase B, both plasmin and trypsin tend to cleave the Lys-Ala or Lys-Thr bond between B_{29} and B_{30} when proinsulin is the substrate (this bond is not readily attacked in insulin), giving rise to a partially degraded form of insulin that does not occur naturally [31]. It therefore seems more reasonable to view these serine proteases as possible candidates for carrying out those rarely occurring cleavages at single basic-residue sites discussed above.

Much of the presently available evidence in several endocrine granule systems indicates that thiol proteases having properties similar to cathepsin B may well be the main processing enzymes [49–51]. Several thiol-containing proteases have recently

been identified in studies on partially purified islet granule fractions [37,50,51]. These have been labeled selectively using a sensitive site-specific probe, ^{125}I-Tyr-Ala-Lys-Arg chloromethyl ketone, and include a major 31.5-kilodalton component that is probably identical with cathepsin B, as well as a larger component of about 39 kilodaltons [52]. The 39-kilodalton component also appears to be related to cathepsin B, inasmuch as treatment with pepsin converts it to a form that is similar in size to cathepsin B and can be immunoprecipitated by an antiserum to cathepsin B [Docherty, K. unpublished data]. Using more highly purified insulin secretory granules from an islet cell tumor [53], it has been possible to show that both the higher and lower molecular weight forms of this cathepsin B-like material are present in both lysosomes and secretion granules while cathepsin H appears only in the lysosomes. Moreover, both of these cathepsin B-like components are labeled more readily with the dibasic chloromethylketone than with a comparable probe containing only a single arginine residue [Docherty, K., Hutton J., and Steiner D.F. unpublished results].

In our experience purified cathepsin B (from rat liver lysosomes) does not correctly cleave proinsulin [52]. Other workers have reported conversion of proinsulin by cathepsin B preparations [54], but in their experiments the cleavage products were not thoroughly characterized. A considerable amount of additional evidence has accumulated in recent years that indicates that ordinary lysosomal cathepsin B is unlikely to be the converting protease for proinsulin or other prohormones because of its broad specificity [for a review see 32].

We recently have found that cathepsin B is derived in biosynthesis from a larger precursor of approximately 44 kilodaltons, as shown in Figure 3 [Steiner and Carroll, unpublished data]. A faint band of this size is also detected by ^{125}I-Tyr-Ala-Lys-Arg chloromethylketone in the islet secretion granule fractions [52], raising the interesting possibility that an active precursor form or processive intermediate of procathepsin B, or a related thiol protease, may be cosegregated into secretion granules along with proinsulin and participate in its conversion, while the bulk of mature cathepsin B [56] may be directed mainly to the lysosomes. Further studies are now in progress on the nature of procathepsin B and its mode of processing to generate mature cathepsin B and/or intermediate active forms.

Exopeptidases

In addition to the endoproteolytic processing necessary to generate active fragments, a carboxypeptidase B-like activity is required to remove the C-terminal basic amino acids left behind on these peptides by the trypsinlike enzymes [31]. In earlier studies we demonstrated that proinsulin labeled biosynthetically with ^{3}H-arginine was processed in isolated secretion granules to insulin and C-peptide, accompanied by the liberation of free ^{3}H-arginine [28]. No evidence was found for the release of dipeptides of basic amino acids such as Arg-Arg or Lys-Arg. We also demonstrated that lysed granule preparations could release C-terminal arginine residues from ^{3}H-Arg-labeled proinsulin nicked by trypsin prior to addition to soluble granule extracts. In subsequent studies using a variety of assay methods, we showed that islets contain a soluble carboxypeptidase B requiring a metal ion such as zinc but having a pH optimum of 5.5, which is considerably lower than that for pancreatic carboxypeptidase B [57]. This activity was inhibited by EDTA, β-mercaptoethanol and o-phenanthroline, but not by diisopropyl-fluorophosphate (DFP) or iodoacetamide. Recently two groups have described similar carboxypeptidase B-like activities in either adrenal

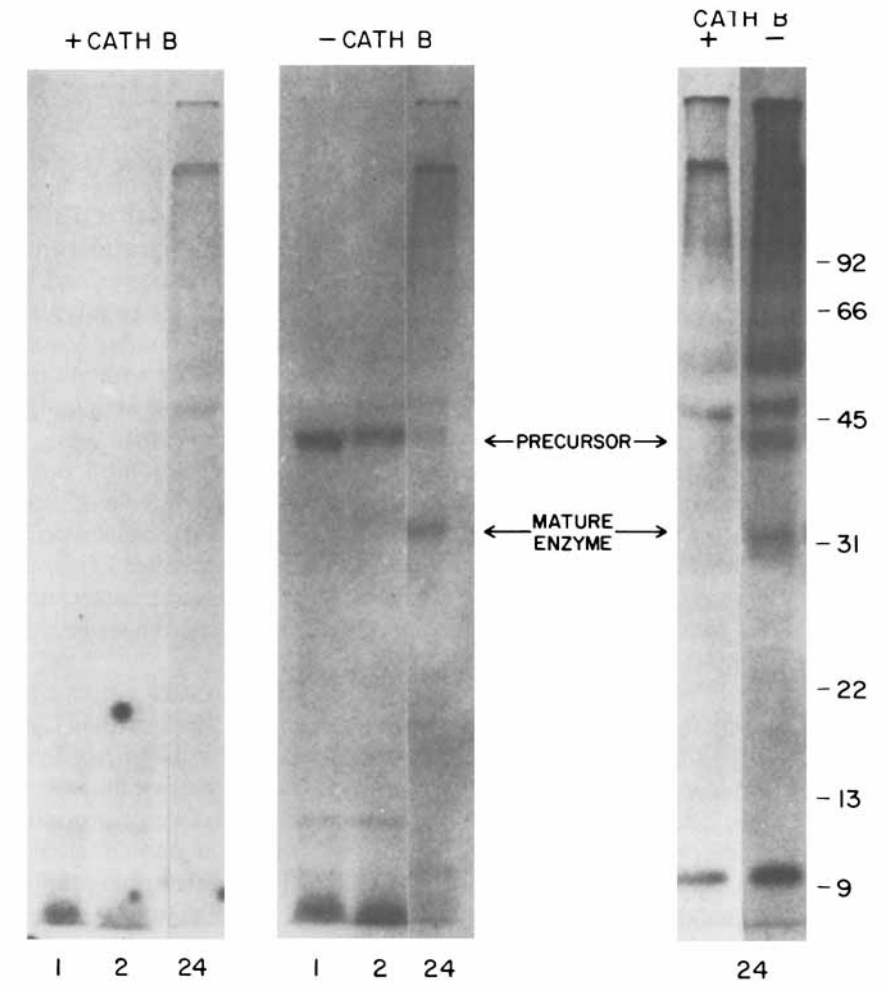

Fig. 3. Demonstration of a precursor of cathepsin B in isolated rat islets. Groups of approximately 1,000 islets were incubated at 37°C for 1 hr, 500 islets for 2 hr, or 200 islets for 24 hr with 100 μCi each of ^{3}H-phenylalanine and ^{3}H leucine as described [55]. After incubation islets were washed twice, sonicated briefly, and immunoprecipitation of cathepsin B was carried out as described [52]. Slab gel electrophoresis and fluorography were performed as described [55]. Lanes designated "+ CATH B" were samples in which 10 μg of unlabeled rat liver cathepsin B were added during immunoprecipitation. Experiment shown in two right-hand lanes was a separate group of islets (approximately 500), which were first extracted with acid ethanol before immunoprecipitation. The acid insoluble protein residue was redissolved in 0.1 M NaOH prior to immunoprecipitation. Molecular weight markers are indicated on the right margin.

homogenates [58] or purified chromaffin granules [59]. Approximately 60% of the chromaffin granule enzyme is soluble and is inhibited by EDTA or o-phenanthroline and activated strongly by $CoCl_2$ [59]. On the other hand, the lysosomal carboxypeptidase was not stimulated by $CoCl_2$ and differed in its distribution and properties from the chromaffin granule enzyme. Similar properties have been described recently for the islet enzyme, as well [63]. Thus the available evidence suggests that a distinct

carboxypeptidase B-like enzyme is utilized in secretory granules for prohormone processing. The question as to whether the same enzyme is active in all tissues remains to be settled, as does its possible relationship to the acidic lysosomal carboxypeptidases.

EVOLUTIONARY ASPECTS OF PRECURSOR PROCESSING

Evidence now accumulating indicates that proteolytic processing is an ancient and widespread mechanism for producing small biologically active peptides in cells. Processing of precursors at pairs of basic residues has been found to occur in primitive vertebrates [60] as well as in invertebrates such as Aplysia [40] and recently also in yeast. It has been shown that the yeast α-mating factor, a small secreted peptide, is derived in biosynthesis from a larger precursor protein. This prohormonelike protein contains a long nonrepetitive N-terminal segment followed by four tandemly arranged copies of a larger peptide that contains the α-mating factor sequence separated by pairs of basic residues that serve as processing sites [61]. The nature of the enzyme in which yeast processes dibasic pairs also is not yet known but is obviously of considerable interest, in view of ongoing attempts to express a number of eukaryotic hormone precursors in this organism. In addition, both the α-mating factor precursor and the precursor of mellitin, a toxic peptide in bee venom, illustrate yet another processive mechanism, an amino dipeptidase which cleaves dipeptides, such as Pro-Glu, stepwise from the N-terminal side [62].

ACKNOWLEDGMENTS

Studies in the author's laboratory have been supported by PHS grants AM13914 and AM20595. We are endebted to Dr H. Kirschke (Halle, GDR) for generously supplying an antiserum to cathepsin B and to Ms Lisa Fuller for expert assistance in preparing this manuscript.

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