Review–Hypothesis

The processing of peptide precursors

'Proline-directed arginyl cleavage' and other monobasic processing mechanisms

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The classical conversion site in precursors of regulatory peptides is a sequence of two basic amino acids. During recent years, however, a group of monobasic cleavage sites has emerged. In certain cell systems it has been shown that the monobasic cleavage mechanism is both a specific mechanism which only attacks a particular basic residue, and a distinct mechanism which can be separated from the dibasic cleaving mechanism within the same cell. The vast majority of monobasic cleavages occur at single arginines although cleavage after a lysine residue has also been demonstrated. There is no 'consensus sequence' of amino acids surrounding the single basic residue which is the apparent signal for proteolytic processing. However, in approximately one third of the cases, a proline residue is found either just before or just after the basic residue. On the basis of this 'proline-directed arginyl cleavage' it is discussed how the conformation of the peptide backbone might be important for this type of cleavage. Finally, it is suggested that tissue-specific expression of different processing enzymes, e.g. dibasic and monobasic specific forms, might explain the tissue-specific processing of precursors like the pro-opiomelanocortin and the CKK and somatostatin precursor.

Post-translational modification Hormone Neuropeptide Growth factor cDNA deduced precursor Tissue-specific processing

1. INTRODUCTION

Regulatory peptides are synthesized as part of relatively larger precursors which normally include the well-known signal peptide sequence. After the signal peptide has ensured that the primary translation product is translocated into the cisternae of the endoplasmic reticulum, it is cleaved from the precursor. With the exception of a few hormones like growth hormone and prolactin [1,2], further proteolysis is required before the active regulatory peptide is generated. This secondary proteolytic event is generally performed by endopeptidases, although in a few cases, e.g. mellitin, a dipeptidylpeptidase can be the activating enzyme [3]. The classical cleavage site is a sequence of two basic amino acids, as described in the pioneering work of Donald Steiner and Ronald Chance on proinsulin and Tager and Steiner on proglucagon [4–6]. Such dibasic cleavage sites have been found in almost all precursors described since then. In higher eukaryotes the enzyme has as yet escaped final purification and characterization, mainly due to the fact that endoproteinases of similar specificity are found in large amounts in lysosomes. These are difficult to exclude during the preparation of secretory granules where the true processing enzymes are believed to be found in small amounts. In yeast, however, Thorner and coworkers [7,8] recently characterized the structural

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gene for a similar processing enzyme with specificity for pairs of basic residues. This elegant work was based on a strain of yeast which, due to a mutation in the enzyme, was unable to produce the active regulatory peptides α-factor and killer-toxin [7].

Although the enzyme as such has not been isolated from mammals, we do know about its specificity in some detail. The cleavage seems to occur on the COOH-terminal side of the two basic residues, i.e. after the last one. The processing scheme, originally proposed by Steiner, consisted of such a tryptic-like cleavage followed by removal of the basic residues by a carboxypeptidase B-like enzyme [4,9]. The isolation and characterization of the carboxypeptidase B-like enzyme is likely to be imminent [10–12]. All possible combinations of Lys and Arg are found in dibasic cleavage sites. However, the sequence Lys-Arg is most generally used [13]. It is not known if there are one or more different dibasic specific processing enzymes. In case there is only one enzyme responsible for all the dibasic cleavages known in precursors, this enzyme can only recognize the two basic residues as there is no other common sequence of amino acids on either side. However, pairs of basic residues do occur in precursors without being cleaved. Thus, it appears that either the two basic residues have to be presented to the enzyme in a particular conformation or there may exist several enzymes which in addition recognize some of the flanking, non-basic residues of the cleavage site.

The fact that precursors are proteolytically cleaved after pairs of basic residues, as discussed above, is well established. Such sequences are inevitably indicated when the cDNA deduced structures of precursors are presented. However, within the last few years it has become clear that processing after single basic residues can also occur. As indicated in table 1, the list of precursors in which such a monobasic processing is found at present includes precursors for hormones, growth factors, neuropeptides, an interleukin, frog skin peptides, and a yeast toxin. In the following, the monobasic cleavage mechanism is discussed in general and certain characteristics from specific cell systems are presented. Finally, it is discussed how the existence of such a monobasic endopeptidase on the one hand makes it more difficult to predict which part of a cDNA deduced precursor will result in secretory peptides and, on the other, how it might help to explain tissue-specific processing of precursors.

Table 1

Precursors for regulatory peptides that are cleaved by a monobasic specific endopeptidase (in most cases the major peptide product is stated)

Hormones
Pancreatic polypeptide
Vasopressin
Atrial natriuretic polypeptide
Somatostatin
Relaxin
Gastrin releasing peptide
Growth factors
Interleukin-3
Insulin-like growth factor-I
Insulin-like growth factor-II
Neural growth factor
Epidermal growth factor
Neuropeptides
Substance P
Cholecystokinin
FMRF-amide
VIP
Proenkephalin-A
Proenkephalin-B
Frog skin peptides
Xenopsin
PGLa
Other peptides
Yeast killer toxin
Honey bee secapin
Aplysia A and B peptides

2. 'PROLINE-DIRECTED ARGINYL CLEAVAGE' – A SUBSET OF MONOBASIC PROCESSING INDICATING THE IMPORTANCE OF PEPTIDE CONFORMATION

The only pattern which emerges when the amino acids surrounding monobasic cleavage sites are lined up is the frequent occurrence of proline residues either immediately before or after the arginine (fig.1). If the sequence is Pro-Arg, the

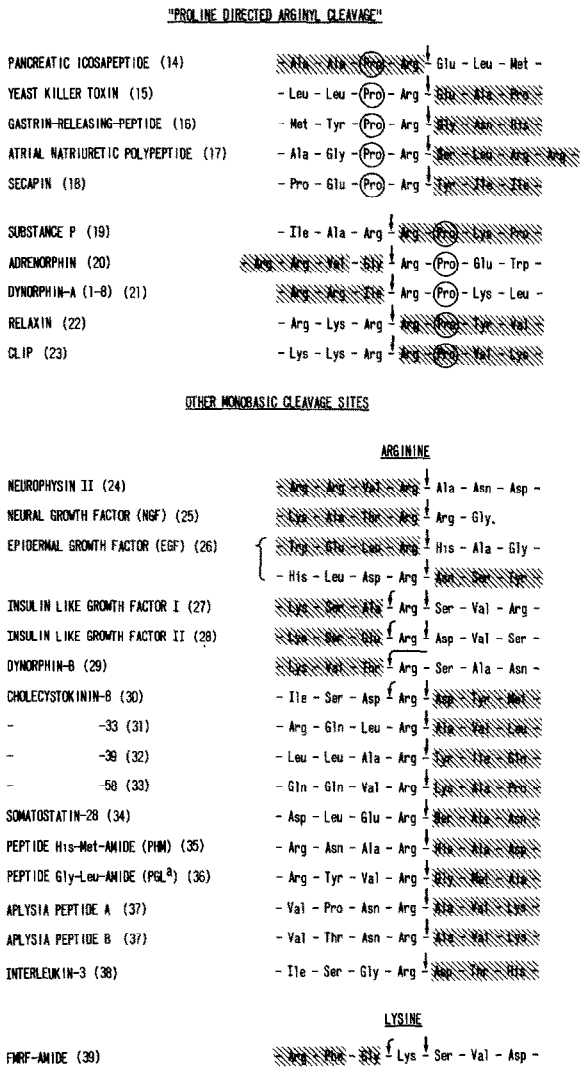

Fig.1. Proline-directed arginyl cleavage and other monobasic cleavage sites in precursors of regulatory peptides. The relevant peptide product is indicated by a hatched bar and the name is written to the left followed by the number of a reference concerning either the precursor structure or the specific cleavage site. Prolines situated adjacent to target arginines are encircled. Vertical arrows point to the peptide bond which is suggested initially to be attacked by an endopeptidase (except in the case of dynorphin-B where the cleavage point suggested by Devi and Goldstein [29] is indicated by a long curved arrow). Short curved arrows indicate the following action of the carboxypeptidase B-like enzyme. Glycine residues, which subsequently will serve as the nitrogen donor during the formation of an α-carboxyamide group on the final bioactive peptide [40], have been put in separate hatched bars.

scissile bond appears to be the one involving the carboxyl group of the Arg, but that involving the amino group when the sequence is Arg-Pro. In the case where the peptide fragment formed by the endopeptidergic cleavage ends in Pro-Arg, the carboxypeptidase B-like enzyme cannot readily remove the arginine, and only around 10–15% of the molecules are trimmed in this way (unpublished).

The fact that prolines are found in the penultimate position to the single basic residue in about one third of the cases may be important for the cleavage mechanism as proline will influence the three-dimensional orientation of the peptide backbone. The α-nitrogen atom in proline is part of the rigid pyrrolidine ring and no rotation of either the N–C bond in the ring or the peptide C–N bond is possible. Thus, a rigid kink or bend of the peptide chain is found at a proline residue. Proline can also introduce structural heterogeneity since unlike other peptide bonds, the X–Pro bond can be either of the stereoisomeric *cis* or *trans* conformation [41]. It is possible that a particular X–Pro bond in a precursor will take up a certain isomeric form while the precursor is intact in the Golgi cisternae and the budding secretory granule. It should be noted in this context that the interior composition of the cell, in particular the secretory granule, is very complex and quite different from a test tube. In the first instance, the amount of water present is so low that there is barely enough for hydration of the proteins. It can be expected therefore that the proteins will be in a state rather similar to crystals [42]. In the second, if the precursor is first cleaved, for example by another endoprotease, then it is likely that *cis-trans* isomerisation may occur since the energy barrier for isomerisation of the X–Pro bond is rather low [41]. Such conformational changes could very well be of importance in the processing scheme as it is known that enzymes can have a requirement for a particular conformation of the X–Pro bond [43].

In general, the special conformational constraint induced by proline residues will influence the rate at which proteolytic enzymes cleave peptides. Typically, prolines slow down the proteolysis. A classical example is the slow cleavage by trypsin between a basic residue and a proline. On the other hand, a proline residue can also make the peptide chain more susceptible to proteolysis; for example,

it is known that the best substrates for thrombin have a proline situated just before the arginine [44]. Thus, it is suggested that the prolines located around monobasic cleavage sites in peptide precursors are important in presenting the arginine in an optimal way to the processing enzyme.

In a few of the examples listed in fig.1, it could be argued that the processing enzyme instead of being a distinct enzyme, directed by the following Arg-Pro sequence, is the well-known dibasic specific endopeptidase which in this case will not cleave between the Arg and the Pro but instead cleaves just before the Arg, which is still after a basic sequence. Generally, this argument has been used to explain the mechanism for processing of ACTH to α-MSH and CLIP in the intermediary lobe of the pituitary. However, in the case of adrenorphin and dynorphin-A (1–8), there are no basic residues preceding the Arg-Pro sequence. It is suggested that these newly identified peptides indicate that a distinct type of enzyme is also involved in the biogenesis of more established hormones like α-MSH. This furthermore offers a simple explanation for the tissue-specific processing of the pro-opiomelanocortin (see later).

3. OTHER MONOBASIC CLEAVAGE SITES – A HETEROGENEOUS GROUP

Apart from the role of a proline residue in directing cleavage at a monobasic site, there is no obvious 'consensus sequence' which indicates that a particular basic residue is at a processing site. The only chemical feature which might be noted is that polar and charged amino acids, usually acidic ones, are dominating residues among those surrounding the monobasic cleavage site. Also, basic residues are frequently found three residues prior to the site (fig.1).

The most likely processing mechanism is an initial cleavage on the carboxyl side of the arginine followed by the removal of the exposed basic residue by the carboxypeptidase B-like enzyme. Apparently, the basic residues are also removed from 'non-biologically active' spacer peptides, which has been shown with fragments of the CCK precursor [45]. In a few cases, for example neurophysin II, the arginine is not removed, although the primary structure of the peptide does not point to any obvious reason for this and the secretory granules do have carboxypeptidase activity to remove basic residues from other fragments of the precursor, in this case from the vasopressin intermediate [24]. This could indicate that the single arginine is held in a particular steric configuration, in this case not determined by a neighbouring proline residue but probably by the structure of the rest of the neurophysin.

Recently, an aminopeptidase with specificity for basic residues was reported in secretory granules from endocrine cells [46]. Thus, the possibility does exist that the endopeptidergic cleavage initially could happen on the NH_2-terminal side of the arginine, whereafter this residue if needed can be removed by the aminopeptidase. In the case of dynorphin-B a preliminary report indicates that an endopeptidase in fact does cleave on the NH_2-terminal side of the arginine [29].

The processing sites for neural growth factor (NGF) and epidermal growth factor (EGF) have been included in fig.1, although the biogenesis of

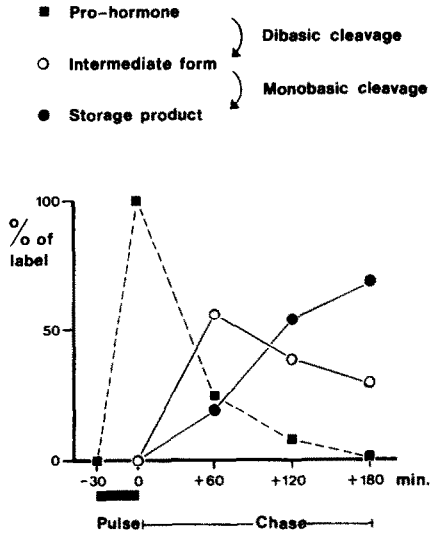

Fig.2. Different kinetics of dibasic and monobasic processing in pancreatic polypeptide cells. The prohormone (○—○) which contains both a dibasic and a monobasic conversion site is initially labelled with radioactive amino acids during a 30 min pulse period. The dibasic conversion of the precursor during the chase period follows a time course which is relatively rapid and similar to that of, e.g. proinsulin conversion. The monobasic cleavage happens later, apparently first after the precursor has been cleaved to an intermediate form (○—○) ([52,53] and unpublished).

these peptides could very well be rather different from that of the neuropeptides for example. The processing enzymes for NGF and EGF are synthesized in parallel with the precursor in a molar ratio of 1:1 [47,48], whereas the elusive processing enzymes for neuropeptides are believed to be found only in minute amounts as compared to their substrates, the peptide precursors. The processing enzyme for NGF, the so-called γ-subunit of 7S-NGF complex, has recently been cloned and shown to be a member of the glandular kallikrein multigene family which seems to comprise at least 23 other non-allelic genes besides the NGF converting enzyme [49].

Processing after a single lysine residue has been quite well documented in only two cases. In the polyvalent precursor for FMRF-amide, which contains at least 19 copies of this peptide, it is assumed that monobasic cleavage takes place in at least 15 places, followed by removal of the Lys and transformation of the exposed COOH-terminal glycine to the amide group [39]. The cholecystokinin precursor may also be cleaved after a single Lys, giving rise to cholecystokinin-22 [45,50].

4. THE MONOBASIC CLEAVAGE CAN BE A SPECIFIC AND DISTINCT PROCESSING MECHANISM

Several lines of evidence indicate that the monobasic cleavage mechanism can be distinguished from the dibasic converting enzyme within the cell. Prohormone conversion through dibasic cleavage has been thoroughly studied in, for example, insulin-producing cells. Here it has been shown to occur just as the secretory granule buds off from the so-called 'trans' part of the Golgi complex, when the vesicles are still coated with clathrin [51]. During pulse-chase experiments the radioactivity, which is incorporated into the prohormone form initially, is almost all converted into fragments after a 60–90 min chase period. In only a few cases are the kinetics of the monobasic conversion known. However, studies on the biogenesis of pancreatic icosapeptide from the pancreatic polypeptide precursor indicate that the monobasic cleavage occurs at quite a different time and place within the cell from the dibasic one. The initial cleavage of the precursor, at a dibasic site, has a time course very similar to that described for proinsulin conversion [52], whereas the generation of the pancreatic icosapeptide from its intermediate form, through a monobasic cleavage, happens later (fig.2) [53]. In the same way, the generation of neurophysin II from its intermediary form is also a rather slow process [54]. Whether the late occurrence of monobasic cleavage is a general phenomenon remains to be seen.

The dibasic specific processing enzyme cannot, in fact, cleave at single basic residues, which has been shown in naturally occurring cases of site-directed mutagenesis. If mutations happen at one of the basic residues in a cleavage site, in e.g. proinsulin or proalbumin, the normal cleavage process is prevented or greatly impaired [55,56]. Further evidence for the notion that monobasic and dibasic processing can be distinct mechanisms is found in primary cultures of pancreatic polypeptide cells, in which the monobasic cleavage mechanism disappears after 24–48 h, whereas the dibasic processing mechanism functions perfectly well after 10 days of culture [13,53]. This finding indicates that the monobasic-specific enzyme apparently needs some type of co-factor which is lost in time. The amidating enzyme also loses its activity due to the lack of a co-factor, conceivably ascorbate [57,58].

Generally, the monobasic processing enzyme must be highly specific as the precursors are not cleaved at the other single arginines found in them. This is true even when the possible miscleaved peptides are looked for specifically. Again, in the case of the precursor for pancreatic polypeptide, two of the other arginines are found in sequences similar to the major monobasic cleavage site shown in fig.1. However, although peptides which are generated through miscleavage can be isolated and sequenced, the amount corresponds to less than 1% of the major storage product, pancreatic icosapeptide (unpublished).

5. PROBLEMS IN PREDICTING POSTTRANSLATIONAL MODIFICATIONS FROM cDNA DEDUCED PRECURSOR STRUCTURE

Today almost all information concerning the structure of precursors is deduced from cDNA sequences. Often, very little information, if any, is available concerning the peptide structure. Thus,

one is forced to guess which posttranslational modifications will occur and thereby which peptides will be the products. There are some quite reliable methods of predicting the cleavage site between the signal peptide and the propeptide [59]. However, as yet there is no way to identify which pairs of basic residues are conversion sites. Such pairs are therefore usually all pointed out as putative cleavage sites when precursor structures are published. In the same way, all the putative glycosylation sites (Asn-X-Thr/Ser) are indicated, although we do not know if these will in fact be derivatized. This problem is obviously even greater when monobasic cleavage sites are considered, but usually they are not. Monobasic cleavage sites can only be identified on the basis of firm peptide chemistry support.

Some of the difficulties in predicting posttranslational events are shown in fig.3. Had no peptide chemistry information been available when the complete structure for the pancreatic polypeptide precursor was deduced from the cDNA structure, a glycosylation site and a dibasic cleavage site would have been pointed out. However, neither of these is normally used (fig.3a) and, furthermore, the monobasic processing site, which in fact is used, would not have been noticed [14]. Recently, the introns in that gene have been located at both the monobasic and dibasic sites which in fact are used for proteolytic processing [60]. PGL[a] is an actual example of how a dibasic site was initially guessed to be the processing site in a precursor which was deduced from a cDNA sequence [61]. When the peptide was eventually isolated and sequenced, it turned out that the conversion took place at a monobasic site three residues further down the precursor (fig.1) [36].

Occasionally, some indirect evidence may indicate that an unnoticed monobasic site is used for conversion. The precursor for xenopsin is such an example (fig.3b). When the cDNA deduced precursor for this peptide was first published, a pair of basic residues was indicated as the major endopeptidergic cleavage site [62]. However, no known processing enzyme is able to produce the final xenopsin molecule from the suggested intermediary form. If instead the precursor is cleaved at a monobasic site three amino acids downstream, an attack by dipeptidylpeptidase IV, as shown by Kreil and co-workers [3] for pro-

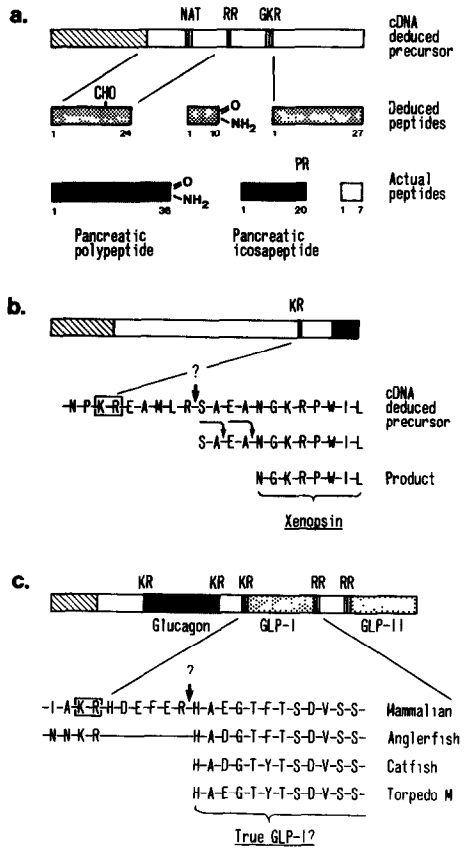

Fig.3. Problems in predicting posttranslational modifications from cDNA deduced precursor structures. (a) The precursor for pancreatic polypeptide – two easily recognized processing sites are not used, a glycosylation, CHO, site, NAT (Asn-Ala-Thr), and a dibasic cleavage site RR (Arg-Arg); whereas a non-obvious monobasic cleavage site, PR (Pro-Arg), is used [14]. (b) The precursor for the frogskin peptide, xenopsin – the boxed sequence, KR, is the dibasic processing site initially suggested [62]. The vertical arrow points to a monobasic cleavage site which instead would result in an intermediary form, which can readily be turned into the final peptide product through the action of the dipeptidylpeptidase IV [3]. (c) The glucagon-like peptide-I, GLP-I, part of the mammalian precursor for glucagon – the boxed sequence, KR, is the dibasic conversion site initially suggested [63]. The arrow points to a monobasic cleavage site which instead would give rise to a glucagon-like peptide which has been preserved during evolution. The long line in the anglerfish structure indicates that these amino acids are not coded for in the gene [64]. The structure of the catfish peptide [65] and the *Torpedo marmorata* peptide (Conlon et al., unpublished) derived through peptide chemistry work are shown below the cDNA deduced precursor structures.

mellitin, will easily activate the intermediary form (fig.3c).

It is important which processing sites are indicated in a cDNA deduced precursor, since this almost always determines which peptides will be synthesized by pharmaceutical firms and eventually tested for biological activity by physiologists. For obvious reasons, pairs of basic residues attract most attention. However, as pointed out this might be misleading. The precursor for glucagon is an example of a precursor which has been the template for the design of several peptides. It has generally been assumed that all the dibasic cleavage sites would be used to give rise to two glucagon-like peptides, GLP-I and GLP-II, in addition to some uninteresting fragments and of course glucagon itself [63]. In fact, it is a truncated form of GLP-I which has been preserved during evolution [64,65]. This peptide, starting with the histidine residue which is important for glucagon-like activity, can be cleaved from the mammalian precursor by a monobasic specific cleaving enzyme (fig.3c). Thus, it could be argued that the truncated peptide is the true GLP-I.

6. TISSUE-SPECIFIC EXPRESSION OF MONOBASIC SPECIFIC CONVERTING ENZYMES CAN EXPLAIN TISSUE-SPECIFIC PROCESSING OF CERTAIN PRECURSORS

It is well known that the same primary translation product can be processed differently in different tissues. The most recognized example is the pro-opiomelanocortin which is processed to ACTH in the anterior lobe of the pituitary and to MSH in the intermediary lobe. The current most appealing explanation of this phenomenon is that proteolytic enzymes with different substrate specificity may exist and that they are expressed in a tissue-specific manner. This is already known to be the case for the acetylating enzyme of secretory granules which is found only in MSH cells [66]. Tissue-specific expression of monobasic specific converting enzymes could explain the selective processing of some precursors in different tissues.

In fig.1 it is suggested that the cleavage of ACTH which gives rise to α-MSH and CLIP is performed by a proline-directed processing enzyme rather than the dibasic specific type of enzyme as generally believed. The selective expression of such a conformation-dependent enzyme in only the melanocytes in the intermediary lobe of the pituitary could explain the occurrence of MSH only in these cells. Recently, a converting enzyme with specificity for pairs of basic residues has been isolated from the pituitary. This enzyme cleaved all Lys-Arg sequences in pro-opiomelanocortin except the one at the MSH-CLIP junction, indicating that the structure at this site is different from normal dibasic sites [67] (this can be due either to the preceding Gly-Lys sequence or to the following Arg-Pro).

The somatostatin precursor gives rise mainly to somatostatin-14 in for example the pancreas, but somatostatin-28 in the stomach [68,69]. In this case a tissue-specific expression of a dibasic- and a monobasic-specific processing enzyme can easily explain the different peptide patterns (fig.4).

The proteolytic processing of the CCK precursor is mainly performed at monobasic sites (fig.1). The only exception is the combined cleavage and amidation site just after CCK-8 [30] (fig.4). Certain differences in the proteolysis of the CCK precursor have been described in different tissues [70]. In the guinea pig a very clear-cut separation of dibasic and monobasic processing is found. In the brain, the dominating CCK form is amidated CCK-8, which reflects both dibasic and monobasic processing, whereas in the small intestine the dominating CCK form is a large amidated form indicating that monobasic cleavages do not occur to any major extent (Hilsted, Rehfeld and Schwartz, unpublished).

As the structure of many precursors has now been revealed, the concept of the cellular processing mechanism has changed somewhat. At present, the generalisation must be that cleavage of precursors occurs mainly at basic residues, more often at pairs but also at single basic residues. The distinction between monobasic and dibasic cleavage mechanisms can be very clear in certain cells. It is possible, however, that families of converting enzymes will be isolated, like the kallikrein family which already includes a monobasic specific converting enzyme [49]. Whether the monobasic and dibasic specific enzymes are related proteins remains to be seen. The frequent occurrence of proline residues, especially around monobasic cleavage sites, seems to indicate that besides the

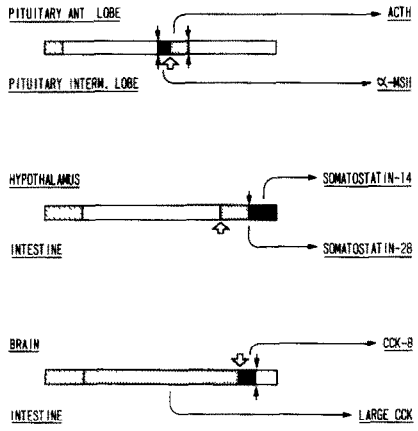

Fig.4. Tissue-specific processing of precursors for regulatory peptides. (a) Pro-opiomelanocortin is processed to ACTH, in the anterior lobe of the pituitary, but is further processed to α-MSH in the intermediary lobe. In the text it is discussed how tissue-specific expression of an ACTH-cleaving enzyme, maybe a proline-directing arginyl cleaving one, can explain this phenomenon. (b) The somatostatin precursor is processed mainly to somatostatin-14 in the hypothalamus, but to somatostatin-28 in the intestine. (c) The cholecystokinin precursor in the guinea pig brain is processed mainly to cholecystokinin-8, but to a large amidated form of cholecystokinin in the small intestine of that animal (Hilsted et al., unpublished). In the latter two cases tissue-specific expression of dibasic specific (closed arrows) and monobasic specific (open arrows) processing enzymes can explain the occurrence of different peptides from the same precursor in different tissues. This scheme is a simplification since there is some processing to somatostatin-14 and to smaller forms of CCK in the tissues where the larger forms of these peptides are the dominating forms.

amino acid sequence also the three-dimensional structure of the precursor is important for the processing mechanism.

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