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Conformation limited proteolysis in the common neurophysin-copeptin precursor shown by trypsin-Sepharose chromatographic proteolysis

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The guinea pig two-domain precursor of MSEL-neurophysin and copeptin has been passed through a trypsin-Sepharose column in order to mimic the enzyme processing by a membrane-bound endopeptidase. Only two cleavages were observed located in the inter-domain sequence (at Arg-94 and Arg-98), in contrast to several additional cleavages found when free neurophysin or copeptin is subjected to soluble trypsin. Because the physiological maturation involves a single cleavage at Arg-94, both local accessibility in the precursor and narrow specificity of the enzyme are implied in the processing.

Neurophysin-copeptin precursor; Prohormone processing enzyme; Conformation-limited proteolysis; Enzyme specificity; Affinity chromatography

1. INTRODUCTION

In contrast to most mammals investigated in which the vasopressin MSEL-neurophysin-copeptin precursor is fully processed into three fragments during axonal transport from hypothalamus to neurohypophysis, in guinea pig at least 20% of the precursor is split into two parts, active vasopressin on the one hand and MSEL-neurophysin-copeptin on the other [1–3]. The complete amino acid sequence of the partial precursor has been determined and the link between the 93-residue MSEL-neurophysin and the 38-residue copeptin has been shown to be made by an arginine residue (Arg-94) [1]. The cause of incomplete processing is not clear; this could be due to a peculiar variation in the guinea pig precursor amino acid sequence or to a slight change in the specificity of the guinea pig processing trypsin-like endopeptidase. During normal processing, cleavage at the carboxyl group of Arg-94 is followed by removal of this residue through a carboxypeptidase B-like enzyme since C-terminal arginine is not found in the 93-residue MSEL-neurophysin directly isolated from neurohypophysis (fig.1) [1].

Prohormone processing enzymes are assumed to be membrane-bound enzymes acting either at the Golgi complex level or within secretory granules [4,5]. Because prohormones are not usually attached to membranes, processing should occur by interactions between soluble precursor proteins and luminal catalytic domains of immobilized enzymes, perhaps by passage of the protein substrates on successive ordered proteolytic enzymes. Cleavages of the precursor should occur in amino acid sequences accessible to enzymes, namely inter-domain sequences. Furthermore, processing enzymes have a specificity more restricted than those of digestive enzymes. To study the peculiar accessibility of some regions of the precursor, we have designed a procedure that could mimic the ordered proteolysis of precursors by membrane-

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bound enzymes. Guinea pig MSEL-neurophysin-copeptin precursor has been passed through a column of immobilized trypsin for a short and determined time as could occur physiologically with the processing enzymes. Products of the filtrate have been examined for locating the bonds cleaved during this limited proteolysis.

2. MATERIALS AND METHODS

2.1. *MSEL-neurophysin, copeptin and MSEL-neurophysin-copeptin precursor*

MSEL-neurophysin, copeptin and MSEL-neurophysin-copeptin precursor have been purified, after 0.1 M HCl extraction of frozen guinea pig posterior pituitaries, by molecular sieving on Sephadex G-75 and Bio-Gel P_4 as described [1,2]. Homogeneity has been checked by N-terminal amino acid sequence analysis through an Applied Biosystems 470 A gas-phase microsequencer under conditions described [6]. Because complete amino acid sequences of the three components have been established [1,2], peptide bonds enzymatically cleaved in the precursor could be located by determining new N-terminal sequences appearing after contact with the proteolytic enzyme.

2.2. *Immobilized trypsin*

Bovine trypsin has been immobilized onto Sepharose 4B, according to Axen et al. [7]. 20 ml of settled Sepharose 4B (Pharmacia), washed with deionized water on a sintered glass, have been diluted up to 40 ml with water and the pH has been adjusted to 11 with 4 N NaOH. Cyanogen bromide (0.5 g) has been added and the pH maintained with NaOH for 10 min at 4°C. Activated Sepharose has been washed with water (500 ml) on a sintered glass funnel, then with a 0.05 M borate buffer, pH 9.0, for 5 min at 4°C. Activated Sepharose has then been suspended in borate buffer (60 ml) and 300 mg of bovine trypsin (Choay CB 526), dissolved in the same borate buffer containing 0.5 M NaCl, have been added. The mixture has been stirred at 4°C for 18 h. Trypsin-Sepharose has been washed on sintered glass with 0.05 M acetate buffer, pH 4.0, containing 0.3 M NaCl and 0.01 M $CaCl_2$.

The capacity of trypsin-Sepharose has been evaluated with Kunitz pancreatic trypsin inhibitor (Iniprol Choay) which gives a stoichiometric complex with trypsin [8,9]. 2.5 ml of trypsin-Sepharose suspended in 0.1 M Tris buffer, pH 7,0, containing 0.05 M $CaCl_2$ have been mixed with 1500 nmol of inhibitor (3 ml) and stirred for 2 h at 4°C. From the amount of inhibitor remaining in the supernatant, it could be deduced that 150 nmol of inhibitor have been fixed per ml of trypsin-Sepharose. Before affinity proteolysis, trypsin-Sepharose has been diluted 30 times with Sepharose so that the final titre of the immobilized enzyme was 5 nmol trypsin per ml.

3. RESULTS AND DISCUSSION

0.18 mg of precursor (about 10 nmol) have been passed through a trypsin-Sepharose column (0.3 × 2.5 cm) equilibrated at pH 7.4, at room temperature for 3 min. The filtrate has been collected and concentrated. An aliquot has been taken for electrophoretic control and the material (corresponding to 0.16 mg of the initial precursor) has been subjected to Edman degradation in the gas-phase sequencer. Table 1 gives the results of the degradation. Three amino acid sequences can be recognized. The first (6 residues) corresponds to the N-terminal sequence of MSEL-neurophysin (which is the same as the N-terminal sequence of the precursor). The second sequence (3 residues) corresponds to Ala-95–Gly-96–Asp-97 and shows that the bond Arg-94–Ala-95 has been split. The third sequence (6 residues) corresponds to Ser-99–(Asn)-100–Val-101 and indicates that the bond Arg-98–Ser-99 has also been cleaved (fig.1). Of the 9 arginyl bonds present in the precursor and susceptible to trypsin when the polypeptide is unfolded by oxidation or reduction-alkylation [1], only two have apparently been split by trypsin-Sepharose.

These results show that the inter-domain sequence comprising the C-terminal sequence of MSEL-neurophysin and the N-terminal sequence of copeptin is particularly accessible to proteolytic enzymes. Under physiological conditions, the processing enzyme apparently splits only after Arg-94 and not after Arg-98 so that the accessibility of the inter-domain sequence is completed by the narrower specificity of the processing endopeptidase.

In contrast to the physiological processing, MSEL-neurophysin 1–94 keeps its C-terminal arginine as shown by the more basic position in

Table 1

Amino acid sequences of the precursor fragments given by trypsin-Sepharose

Sequencing cycle	1st sequence			2nd sequence			3rd sequence		
	No. residue in the precursor	Nature of the residue	pmol	No. residue in the precursor	Nature of the residue	pmol	No. residue in the precursor	Nature of the residue	pmol
1	1	Ala	(2430)[a]	95	Ala	(2430)[a]	99	Ser[b]	240
2	2	Leu	680	96	Gly	580	100[c]	-	-
3	3	Ser[b]	100	97	Asp	110	101	Val	620
4	4	Asp	395				102	Thr[b]	55
5	5	Thr[b]	40				103	Gln	415
6	6	Glu	160				104	Leu	580

[a] Ala being N-terminal in two sequences (fig.1), the value given is cumulative
[b] Ser and Thr derivatives are unstable and values are low
[c] Asn-100 of the precursor is *N*-glycosylated (fig.1) so that it cannot be identified as is the usual Asn derivative

polyacrylamide gel electrophoresis. In HPLC, however, the positions of MSEL-neurophysin 1–93 (directly isolated from tissue) and MSEL-neurophysin 1–94 (from precursor trypsin processing) are not very different.

Trypsin-Sepharose has not split at the level of Arg-86 or Arg-114, cleavages that occur when soluble trypsin acts on free MSEL-neurophysin or free copeptin. Although it is hard to evaluate the yields of the cleavages at Arg-94 and Arg-98, values for Leu-2 (680 pmol), Gly-96 (580 pmol) and Val-101 (620 pmol) in the three respective sequences suggest a rough stoichiometry between the three components and a similar yield for the two cleavages that are apparently complete.

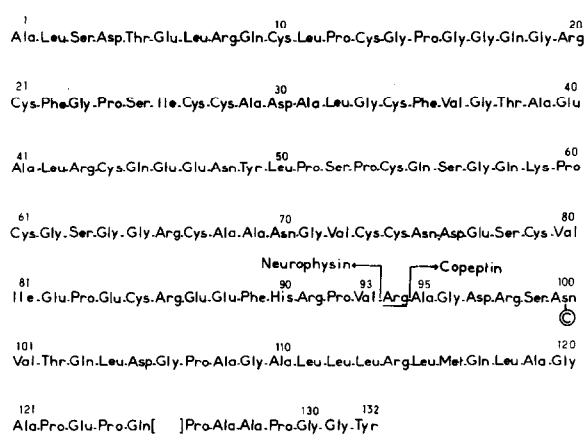

Fig.1. Amino acid sequence of the guinea pig neurophysin–copeptin precursor. Arrows show the bounds of neurophysin and copeptin. [ ] Amino acid deletion assumed by comparison with other known mammalian copeptins. C, carbohydrate.

Because trypsin-Sepharose has not split arginyl bonds that are accessible for soluble trypsin in free neurophysin or copeptin, we can assume that the two domains shield one another from enzymic attack. Trypsin is usually unable to split native proteins but can cleave locally unfolded amino acid sequences [10] such as those linking two domains. Sequences flanking the susceptible bond must probably also be inserted into the catalytic cleft. The two bonds split by trypsin-Sepharose are neighbours (Arg-94 and Arg-98) and might be simultaneously inserted and consecutively cleaved. Although the mechanism of action of the processing endopeptidase is unknown, we can state that the enzymic accessibility in the precursor conformation is one of the factors determining the specific cleavage and it must be programmed in the corresponding gene.

The effect of the microenvironment on the mode of action of immobilized enzymes is certainly important [11]. In view of this point, Sepharose matrix could be regarded as neutral, but the luminal domain of membrane-bound enzymes might be conformationally modified by interactions with neighbouring intrinsic proteins.

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