TWO GLYCOSYLATION SITES ON THE N-TERMINAL SEGMENT OF PORCINE PARS DISTALIS PRO-OPIOMELANOCORTIN

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1. Introduction

Comparison of the N-terminal sequence of the ACTH/LPH precursor pro-opiomelanocortin [1] deduced from the bovine cDNA [2] with the sequence determined for rat pars intermedia precursor [1] established the presence of an N-terminal 26 residues signal peptide [1]. After its excision by a signal peptidase, the remaining portion of the molecule was shown to contain an N-terminal segment composed of 103 amino acids [2] separated from corticotropin and carboxy-terminal β-lipotropin by a pair of basic residues [2]. The N-terminal segment of pro-opiomelanocortin contains a structure which bears marked sequence homology with α- and β-melanotropin thus it was termed γ-melanotropin [2]. Pulse-chase experiments in rat pars intermedia [3] as well as in mouse AtT-20 tumor cells [4] showed that the N-terminal fragment of pro-opiomelanocortin does not mature appreciably [5] into smaller fragments during the first 4 h of chase incubation. Such an observation could mean that it would be possible to isolate from pituitary extracts this N-terminal fragment in sufficient amounts to be completely characterized. Results obtained from crude extracts of ox pituitaries [6] indicated that virtually no maturation of the N-terminal peptide is observed in anterior pituitaries whereas ~50% of this peptide matures into smaller fragments in pars intermedia cells.

Utilizing the observation that the sequence of the N-terminal fragment of ox and rat [1,2] had identical positions of cysteine residues, a microsequencing method was used to monitor the complete purification of such a peptide from whole human pituitaries [7,8]. In [9], the N-terminal fragment of pro-opiomelanocortin from whole pig pituitaries was isolated and partially characterized. We report here the purification of a N-terminal peptide of 103 amino acids from pig anterior pituitaries extracts. It is shown that its γ-melanotropin segment is identical in composition to both ox [2] and man [7,8] homologues. Furthermore, primary structure determination revealed the presence of 2 glycosylation sites similar to the results obtained from rat pars intermedia biosynthetic studies [10].

2. Materials and methods

2.1. *Isolation of the N-terminal fragment of pro-opiomelanocortin from porcine anterior pituitaries*

Porcine pituitary glands obtained within 30 min of death were dissected into anterior and neurointermediate lobes and immediately frozen on dry ice. Extractions were performed at 4°C in 0.2 M HCl. Following acetone precipitation and centrifugation, the precipitate obtained was subsequently chromatographed on CM-cellulose [11]. The unretained material was resolved by high-performance liquid chromatography (HPLC) on a Waters Associates liquid chromatograph using a μ-C_{18} column eluted with a triethylamine phosphate/acetonitrile gradient, as in [12].

2.2. *Amino acid analysis and microsequencing*

Microsequencing of the N-terminal fragment purified was done on the reduced and carboxymethylated material with iodo-[^{14}C]acetamide as in [5]. Amino acid analysis of the reduced and carboxymethylated peptide was done in triplicate on a Beckman 121MB amino acid analyser 24, 48 and 72 h hydrolysis. Glucosamine and galactosamine are well separated in this system [5].

2.3. *Tryptic peptide mapping*

Carboxymethylated [5] N-terminal fragment of pro-opiomelanocortin (2 mg) was digested for 7 h at 37°C with DCC-treated trypsin. This was followed by HPLC purification on a μ-C_{18} column [12]. The various peptides collected (see fig.1) were individually repurified under the same conditions, hydrolysed and subjected to amino acid analysis (see table 1).

3. Results and discussion

After CM-cellulose chromatography, the unretained material was purified by HPLC and the various peptides obtained screened by radioactive microsequencing for the presence of Cys 2, 8, 20 and 24. A peptide was thus purified possessing such a sequence. Identical cysteine positions have been described for the N-terminal portion of pro-opiomelanocortin from ox [2], mouse [13] and man [4,5]. A computer data bank search indicated that the probability of finding an identical partial sequence in other known proteins or segments of proteins was <0.0004% (we thank Dr M. Dayhoff for this search) [14].

The characterization of such isolated peptide was thus undertaken. Amino acid analysis gave the following results:

Lys (3), His (1), Arg (8), SCMC (4), Asp (9), Thr (4), Ser (10), Glu (12), Pro (8), Gly (17), Ala (7), Val (3), Met (1), Ile (1), Leu (8), Tyr (1), Phe (4), Trp (2).

Glucosamine and galactosamine were shown to be present at ⩾2:1. These results show that this peptide is glycosylated and is 103 amino acids long like the bovine [2], murine [4] and human [7,8] homologues.

Following trypsin digestion and peptide mapping by HPLC (fig.1), nine major tryptic fragments (T_1–T_9) were purified and their amino acid composition determined (table 1). Fig.2 shows the proposed alignment of amino acids in this molecule compared to the reported bovine sequence [2]. The sum of tryptic fragments T_5–T_7 have an identical amino acid composition to the reported γ-melanotropin segment in ox [2] and man [7,8] indicating conservation of this structure between species. However, a number of amino acid substitutions were observed in fragments T_1, T_3, T_4, T_8 and T_9 (fig.2). In agreement with [3],

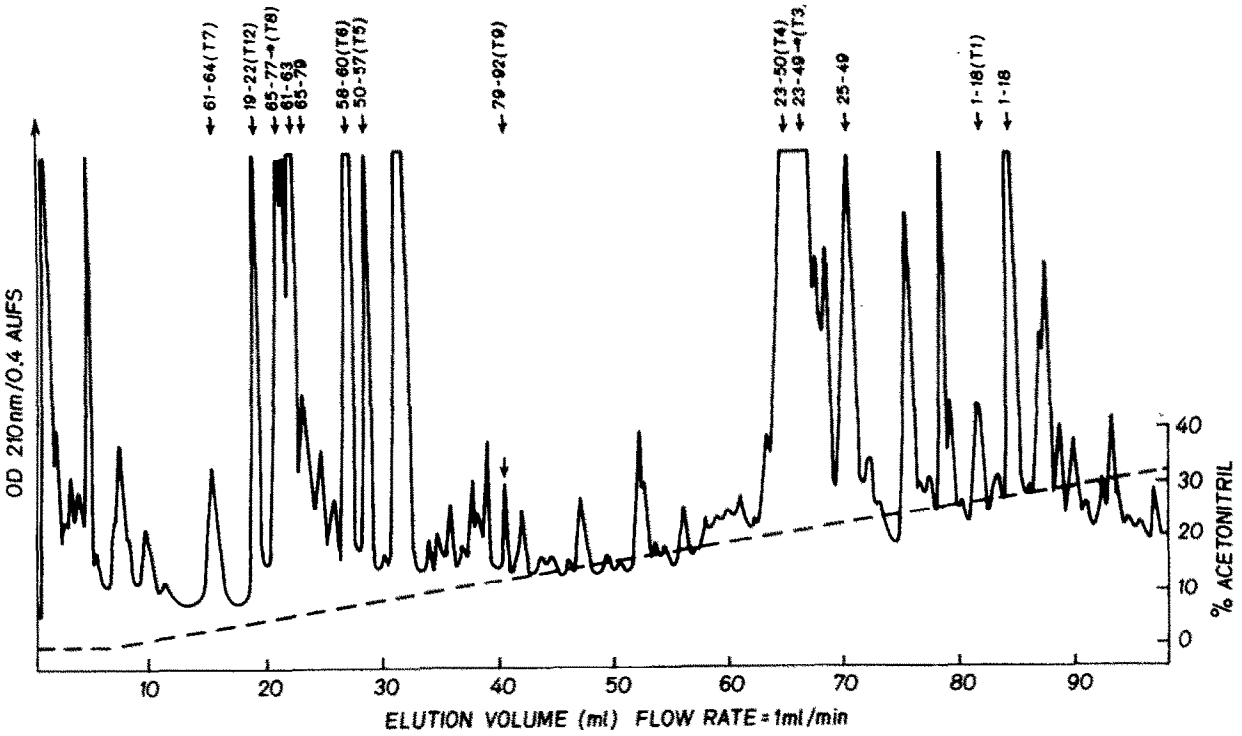

Fig. 1. HPLC purification on a μ-C_{18} column of the tryptic digest of the NH_2-terminal ACTH/LPH precursor, pro-opiomelanocortin. The position of the arrows denotes the elution volume of the isolated peptides.

Table 1
Amino acid composition of tryptic fragments of porcine N-terminal segment of pro-opiomelanocortin

Amino acid	T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8	T_9
Lys			1.17 (1)	1.77 (2)					
His					0.78 (1)				
Arg		1.09 (1)	1.02 (1)	0.70 (1)	0.83 (1)	1.00 (1)	1.67 (2)		1.72 (2)
SCMC	2.21 (2)	1.22 (1)	0.60 (1)	1.22 (1)					
Asp	2.21 (1)		4.22 (4)	4.26 (4)		0.89 (1)		0.85 (1)	
Thr	1.03 (2)		1.75 (2)	1.91 (2)					
Ser	3.50 (3)		1.25 (1)	1.22 (1)				1.94 (4)	
Glu	4.24 (4)		2.89 (4)	3.05 (4)				0.77 (1)	4.20 (4)
Pro			4.73 (5)	4.70 (5)					1.56 (2)
Gly			2.26 (2)	2.09 (2)	1.15 (1)		1.23 (1)	7.71 (4)	3.46 (3)
Ala		1.06 (1)	2.80 (2)	2.53 (2)				1.04 (2)	1.52 (1)
Met			0.87 (1)	0.96 (1)	1.15 (1)				0.84 (2)
Ile		1.00 (1)							
Leu	4.21 (4)		1.86 (2)	2.09 (2)					
Tyr					0.78 (1)				
Phe			0.91 (1)	1.04 (1)	0.90 (1)		1.00 (1)		
Trp	1.00 (1)					1.00 (1)			
Glucosamine			0.08	0.13				1.00	
Galactosamine			0.26	0.21				0.12	
Fragment	1–18	19–22	23–49	23–50	51–57	58–60	61–64	65–77	79–82

(x) Expected from bovine cDNA sequence [2]

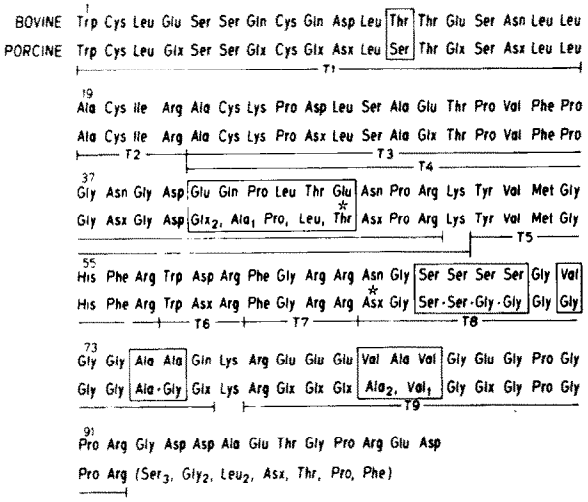

Fig.2. Proposed sequence of the N-terminal fragment of anterior pituitary porcine pro-opiomelanocortin. The sequence deduced from residues 1–92 was based on comparison of amino acid composition of peptides T_1–T_9, to their homologues bovine peptides [2] assuming maximum sequence homology and barring sequence inversions. The carboxy-terminal 11 amino acid peptide composition was deduced by the difference between the total amino acid composition of the native N-terminal peptide and that obtained from the sum of compositions of peptides T_1–T_9. (*) Proposed glycosylation sites.

T_1 shows that in porcine Ser_{12} replaces Thr_{12} in the bovine homologue. Also the res. 15 was shown to be Ser. However, the finding of Glu_4 is in disagreement with the Asp_4 reported [9]. This conclusion was reconfirmed by direct sequencing (not shown).

Hexosamine analysis of the tryptic fragments revealed the presence of a glucosamine-rich moiety in the peptide T_8 and a galactosamine-rich moiety in fragments T_3 and T_4. On the basis of the suggestion that Asn-linked sugars are rich in glucosamine and generally occur within an Asn–X–Ser or Thr segment, where X could be any amino acid [15], it is proposed that the glycosylation site in T_8 is Asn_{65} (fig.2). The presence of large amount of galactosamine compared to glucosamine in peptides T_3 and T_4 would argue against an Asn glycosylation site in these peptides [16]. Furthermore, amino-terminal sequence determination of the first 35 residues of a similar peptide extracted from whole porcine pituitaries revealed the presence of Asp_{27}, Ser_{29} and Thr_{32} [9], therefore eliminating the possibility of glycosylation at these residues. We suggest that the only other possible site of glycosylation would be Thr_{45}. In accordance with such a hypothesis, *O*-glycosylation on either Ser or Thr usually occurs in a Pro-rich region and contains galactosamine as the major hexosamine [15]. This is

exactly what we found in peptide T_3 and T_4.

That bovine pituitary peptides are immunoreactive for the γ-melanotropin sequence and bind to concanavalin A [6] is indicative that the N-terminal segment of pro-opiomelanocortin is glycosylated, but no direct experimental clues as to the site(s) of glycosylations can be deduced. Based on our findings of 2 glycosylation sites on both sides of the γ-melanotropin sequence, the immunoreactive glycopeptide isolated [6] can be either a γ-melanotropin with an N-terminal extension and/or a C-terminal extension, thus making it more difficult to assess the site of glycosylation on their isolated material.

Our data, based on chemical characterization, demonstrate not only that the N-terminal fragment of pro-opiomelanocortin is a major product of maturation and is definitely a glycopeptide, but also that its sequence is quite homologous to similar peptides in ox [2] and man [7,8]. Moreover, it has two sites of glycosylation probably at Thr_{45} and Asn_{65}.

Acknowledgements

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