SPECIFIC CLEAVAGE OF LIPOTROPIN C-FRAGMENT BY ENDOPEPTIDASES; EVIDENCE FOR A PREFERRED CONFORMATION

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Received May 9,1977

SUMMARY

Four soluble endopeptidases catalysed the cleavage of lipotropin C-Fragment specifically in the central section, suggesting that the peptide has a preferred conformation. The conformational properties direct the course of degradation of C-Fragment by membrane-bound proteases from brain. γ-Endorphin and methionine enkephalin were identified as the principal products.

INTRODUCTION

The C-Fragment of lipotropin, lipotropin 61-91, which was originally isolated from pituitary (1,2) and later found in brain (3), possesses strong and long-lasting analgesic properties (4). Shorter peptides related in sequence, γ-endorphin 61-77, α-endorphin 61-76 (5) and methionine enkephalin 61-65 (6) have relatively weak and transient actions (7,8). Previous studies have shown that the NH_2-terminal tyrosine of C-Fragment resists the action of aminopeptidases (9) and the COOH-terminal sequence is highly resistant to attack by carboxypeptidases (10). Thus it is likely that an endopeptidase is responsible for the termination of the action of C-Fragment. Here we show that

ISSN 0006-291X

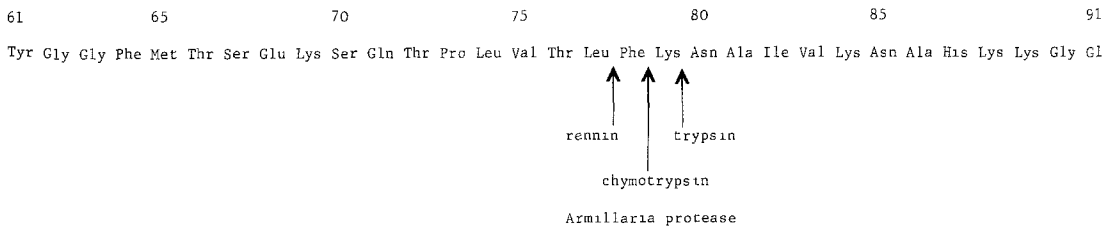

Fig. 1. Sites of cleavage of C-Fragment under mild conditions of endopeptidase digestion.

the preferred conformation of C-Fragment protects the NH_2-terminal region against proteolytic attack, leaving the central section of the peptide susceptible to cleavage by endopeptidases (Fig.1). In the presence of membrane-bound proteases from brain, C-Fragment undergoes stepwise degradation to form γ-endorphin, α-endorphin and methionine enkephalin.

METHODS

C-Fragment was isolated from pig pituitary (1). The heptapeptide 61-67 and the pentapeptide 61-65 were synthesised by solid phase methods. Peptide 61-73 was obtained by carboxypeptidase A digestion of 61-77, followed by purification by gel chromatography on Sephadex G-50 in 50% (v/v) acetic acid and preparative chromatography on paper in n-butanol-pyridine - acetic acid - water (15:10:3:12). C-Fragment was citraconylated according to Dixon and Perham (11). Iodinated [^{125}I]-peptides were prepared by treating the peptide (100 μg) at pH 7.4 in 0.05 M sodium phosphate (100 μl) with Na [^{125}I] (100 μCi) in 0.3 M sodium phosphate (10 μl), followed by addition of chloramine-T (50 μg). After 2 min sodium metabisulphite (0.6 mg) in 0.05 M sodium phosphate (100 μl) was added and the peptide was desalted on a column (0.5 x 5 cm) of Sephadex G-10 in 50% acetic acid. The specific activity of the products was in the region of 2 Ci/mmole.

C-Fragment (0.5 mg/ml), to which [^{125}I]C-Fragment (100,000 dpm) had been added, was incubated with TPCK-treated trypsin, α-chymotrypsin (Worthington Ltd.), staphylococcal protease (Miles Laboratories) or Armillaria protease (a gift from Dr. H. Gregory of I.C.I., Alderley Park) in 1.2 ml of

Table 1. Action of endopeptidases on lipotropin C-Fragment (LPH 61-91)

Peptides were isolated and characterized as described in the text. Yields were calculated from the radioactivity associated with each product after gel chromatography.

Enzyme	Incubation conditions			NH_2-terminal peptide released	Yield per cent
	Enzyme:Peptide w/w	Time h	Temp °C		
Rennin	1:200	8	37	61 - 77	78
	1:20	48	37	61 - 64	87
Chymotrypsin	1:500	1	20	61 - 78	90
	1:100	16	20	61 - 64	95
Trypsin	1:5000	2.5	37	61 - 79	67
	1:100	16	37	61 - 69	80
Armillaria protease	1:5000	1	37	61 - 78	81
	1:100	16	37	61 - 68	75
Staphylococcal protease	1:1000	2.5	37	61 - 68	4
	1:10	24	37	61 - 68	100

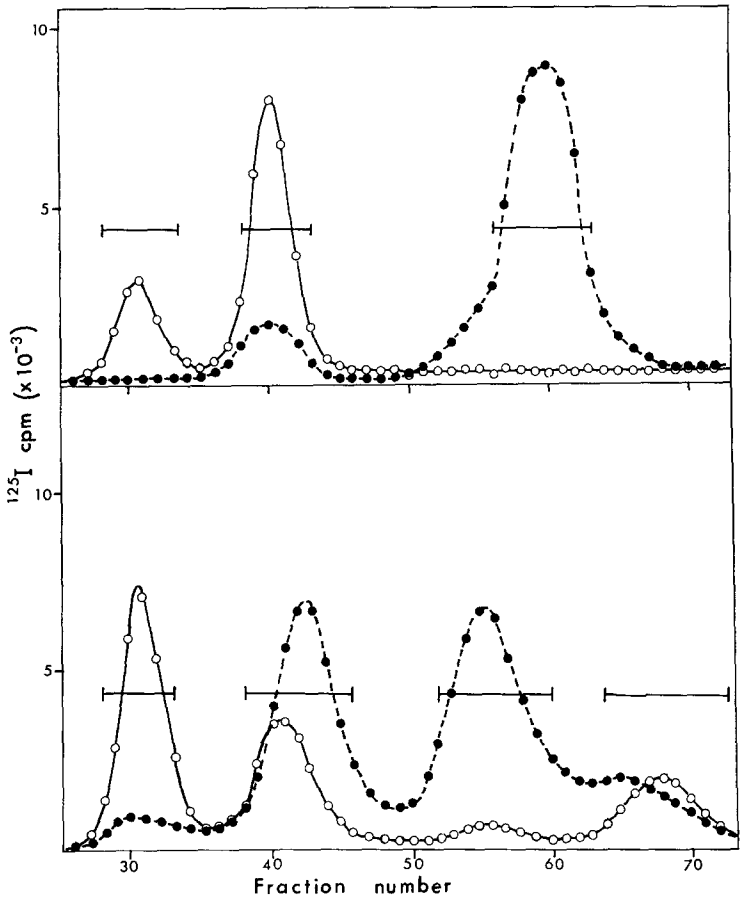

Fig. 2. Gel filtration of enzymic digests of [^{125}I]C-Fragment on Sephadex G-50. In the upper Figure, the heavy line shows the elution profile obtained after digestion with rennin under mild conditions (enzyme substrate ratio 1:200, 8 h, 37°C) and the dashed line that obtained after protracted digestion (enzyme substrate 1:20, 48 h, 37°C). Fractions (1.8 ml) 28-33 contained residual C-Fragment, fractions 38-43 contained γ-endorphin, and fractions 56-63 the peptide comprising residues 61-64. In the lower Figure, the heavy line shows the results obtained by digestion with membrane bound proteases at pH 7.4 in the presence of bacitracin and the dashed line the results obtained at pH 5.0. Fractions 28-33 contained residual C-Fragment, fractions 38-46 contained γ-endorphin (pH 7.4) or α-endorphin together with 61-73 (pH 5), fractions 52-60 contained methionine enkephalin (pH 7.4) and 61-68 (pH 5) and fractions 64-73 contained tyrosine.

0.05 M NaCl containing 0.1 M sodium phosphate at pH 7.4. Digestion with rennin was carried out at pH 4 in 0.15 M pyridine

Table II. R_F values of [^{125}I] peptides related to the NH_2-section of C-Fragment on thin-layer chromatography on cellulose MN 400

Peptide	solvent system	
	n-Butanol-pyridine-acetic acid-water (15:10:3:12 by vol)	n-Propanol-0.2M NH_4OH (3:1 v/v)
61-79	0.79	0.52
61-78	0.87	0.63
61-77	0.83	0.70
61-76	0.74	0.73
61-73	0.62	0.12
61-68	0.75	-
61-67	0.77	-
61-65	0.83	-

acetate. Other conditions are described in Table 1. After incubation at 37°C, the reactions were terminated by the addition of an equal volume of acetic acid, the solutions were concentrated *in vacuo* and the products resolved on a column (1 x 150 cm) of Sephadex G-50 in 50% acetic acid (Fig.2). Peptides were located in the effluent fractions by measurement of radioactivity and by electrophoresis at pH 1.9. In some cases further purification was obtained by ion-exchange chromatography or by preparative electrophoresis on paper at pH 1.9. Products were characterised by amino acid analysis.

Membranes from rat brain were prepared by extensive washing of the synaptosomal fraction (P2) described by Whittaker *et al.* (12). Incubations of [^{125}I]C-Fragment (100,000 dpm; 2 Ci/mmole) with membrane preparations (0.42 mg protein/ml) were performed in 10^{-3}M bacitracin for 16h at 37°C in 1 ml of 0.05 M sodium chloride and either 0.1 M sodium phosphate at pH 7.4 or 0.1 M ammonium acetate at pH 5, and the suspensions were gently agitated. The reactions were terminated by addition of acetic acid, membranes were removed by centrifugation and the products after concentration *in vacuo* were resolved by gel filtration on Sephadex G-50 (Fig.2) and identified by chromatography on thin-layers of cellulose MN 400 (Camlab, Cambridge) together with [^{125}I] or

[^{131}I] labelled reference peptides (Table 2). The peptides were visualised by autoradiography. The heptapeptide 61-67 (0.75 μmoles) was incubated in 4 ml of 0.1 M sodium phosphate at pH 7.4 with membranes (1.9 mg protein) and 10^{-3}M bacitracin for 1h at 37°C and the products after concentration were resolved on a column (1.5 x 150 cm) of Sephadex G-25 in 50% acetic acid and by preparative electrophoresis at pH 1.9.

RESULTS AND DISCUSSION

Rennin, chymotrypsin, Armillaria mellea protease and trypsin catalysed cleavage of C-Fragment at positions 77-78, 78-79, 78-79 and 79-80 respectively and the corresponding NH_2 terminal peptides were isolated in high yield (Table 1). Under mild conditions of digestion, the proteases failed to cleave the NH_2-terminal section of C-Fragment at sites that would be expected to be susceptible. Chymotrypsin did not attack Phe-Met in positions 64 and 65, Armillaria protease and staphylococcal protease gave negligible cleavage at Glu-Lys at positions 68 and 69, trypsin exhibited a strong preference for Lys-Asn at positions 79 and 80 rather than Lys-Ser in positions 69-70, and rennin did not attack the Phe-Met bond at positions 64 and 65 or the Leu-Val bond at positions 74 and 75. Thus the resistant section of C-Fragment appears to extend from position 61 up to position 75 (Fig.1). In addition, the corresponding COOH-terminal fragments, 78-91 (66% yield) from the rennin digest, 80-91 (42% yield) from the tryptic digest and 79-91 (85% yield) from the chymotryptic digest were isolated. The products formed under the mild conditions of digestion retain a degree of resistance to enzymic attack

for they were isolated in high yield from the reaction mixtures.

Cleavage could be made to take place at the less reactive sites in the NH_2-terminal section of C-Fragment by performing the digestions under more vigorous conditions (Table 1) or by using a hexacitraconyl derivative as substrate. It seems likely, therefore, that C-Fragment adopts a preferred conformation which places residues 77-80 in a relatively exposed environment and renders other residues less accessible. The unusual resistance of the NH_2-terminal tyrosine to aminopeptidases (9) and studies of chiroptical properties (13) have already shown that C-Fragment is subject to conformational restraints.

It is of particular interest that mild digestion of C-Fragment with rennin led to the formation of γ-endorphin, as this peptide is present in hypothalamus (5). It was anticipated that brain would contain an enzyme with the ability to catalyse the same cleavage and an enzyme with the required property was found in extensively-washed membranes prepared from rat brain. The results of digestion of [^{125}I]C-Fragment are shown in Fig.2. The main product formed at pH 7.4 in the presence of bacitracin was γ-endorphin and there was a small amount of methionine enkephalin. At pH 5 C-Fragment was degraded more rapidly, giving rise to α-endorphin and methionine enkephalin together with small amounts of 61-73 and 61-68.

The heptapeptide 61-67 no longer exhibits the protected

conformation of C-Fragment. Quantitative cleavage was obtained at the Phe-Met bond by chymotrypsin using conditions under which no cleavage took place at this position with C-Fragment. Moreover, in contrast to the protracted exposure of C-Fragment to brain membranes in the presence of bacitracin required to yield methionine enkephalin, the heptapeptide rapidly generated methionine enkephalin and the dipeptide Thr-Ser in amounts sufficient for characterisation by amino acid analysis. The enzyme that forms methionine enkephalin had a pH optimum between 7.5 and 9.0.

It is clear that the conformation of C-Fragment directs the route by which the peptide is inactivated. The conformational properties impart stability to the NH_2-terminal region and this is retained to a degree in the corresponding region of the endorphins. Thus the formation of γ-endorphin *in vivo* does not require the action of an enzyme with a unique specificity nor does the survival of this peptide in brain depend on packaging within the protected environment of a vesicle. The formation of methionine enkephalin appears to be catalysed by an endopeptidase with a higher pH optimum than that which forms γ-endorphin from C-Fragment but the pentapeptide is formed slowly and being vulnerable to exopeptidases (9,14) has only a transient existence.

The results demonstrate that γ-endorphin, α-endorphin and methionine enkephalin can be produced *in vitro* by sequential proteolysis of C-Fragment. It remains to be seen

whether *in vivo* these shorter peptides are elaborated to perform a specific function or whether they are simply intermediates which form transiently during the extracellular degradation of C-Fragment.

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