LIPOTROPIN C-FRAGMENT HAS A COOH-TERMINAL SEQUENCE WITH HIGH INTRINSIC RESISTANCE TO THE ACTION OF EXOPEPTIDASES

Michael J. Geisow and Derek G. Smyth

National Institute for Medical Research,
Mill Hill, London NW7 1AA

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SUMMARY

The resistance of C-Fragment (lipotropin 61-91) to the action of carboxypeptidase enzymes is shown to be due to the presence of consecutive lysine residues in the COOH-terminal sequence -Lys-Lys-Gly-Gln. A synthetic hexapeptide Ala-Tyr-Lys-Lys-Gly-Gln exhibited the same resistance, whereas the corresponding peptide with a single lysine was digested rapidly. The COOH-terminal stability of C-Fragment is thus a property of the primary structure and does not depend upon a specific molecular conformation. The significance of the stability is discussed in relation to the duration of biological activities exhibited by C-Fragment in the presence of brain exopeptidases.

INTRODUCTION

C-Fragment (lipotropin 61-91) was isolated from porcine pituitary glands (1) and shown to have long-lasting analgesic properties (2,3). This 31-residue peptide is by far the most potent of a number of peptides with opiate activity which have been reported to occur in the central nervous system. During attempts to remove the two COOH-terminal residues of C-Fragment (glutamine and glycine) it became apparent that the COOH-terminus is highly resistant to the action of carboxypeptidase A. This was an unexpected finding since glutamine, and to a lesser extent glycine, should be readily removed by the enzyme (4). The molecular basis for the COOH-terminal stability of C-Fragment forms the subject of this report.

MATERIALS AND METHODS

Carboxypeptidase A, 43 units/mg treated with diisopropylfluorophosphate, was obtained from Worthington Biochemical Corporation. Aminopeptidase M was obtained from Röhm. C-Fragment was isolated as previously described (1). The synthetic peptides were produced by the Merrifield solid-phase method and purified by ion-exchange chromatography; homogeneity was established by amino acid analysis and by total enzyme digestion with aminopeptidase M.

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Carboxypeptidase A solutions were prepared as described by Ambler (4). Stock solutions of the enzyme were 30 µM with a specific activity of 100 µmole hippuryl-L-phenylalanine hydrolysed/min/mg protein. Digestions were carried out at 37°C in 2 ml of 0.15 M Tris HCl at the indicated pH values.

The enzyme to peptide ratio was 1:200 mole/mole. Enzyme digestions were terminated by the addition of portions of the reaction mixture to 2 ml of 0.2 M citrate buffer at pH 2.2. Samples were analysed immediately, or after storage at -20°C, on a Beckman 120c amino acid analyser.

Synaptosomes from rat brain were prepared as described by Gray and Whittaker (5) and extensively washed in 0.05 M Tris HCl, pH 7.4, containing 0.1 M NaCl. The membranes were purified on discontinuous sucrose density gradients as described by Jones and Matas (6) and resuspended in the same buffer. Membrane protein was estimated by the method of Lowry et al. (7).

RESULTS AND DISCUSSION

C-Fragment lost less than 1% of COOH-terminal glutamine when treated with carboxypeptidase A (1:200 mole enzyme/mole peptide) for four hours under physiological conditions of pH and ionic strength; at pH 8.0, less than 20% was removed in the same time. To define the basis of this unusual stability, peptides I and II which contain the COOH-terminal sequence -Lys-Gly-Gln of porcine C-Fragment were synthesised and their resistance to digestion by carboxypeptidase was investigated. The peptides were prepared with tyrosine

	26	27	28	29	30	31
I	Ala-	Tyr-	Lys-	Lys-	Gly-	Gln
II	Ala-	Tyr-	Lys-		Gly-	Gln

in place of histidine at position 27, a replacement which occurs naturally in the human sequence, to facilitate synthesis.

Peptides I and II were incubated with carboxypeptidase A and the rates of release of glutamine and glycine were determined. The results are shown in Figure 1. With the hexapeptide I at pH 8.0 the same very slow release of COOH-terminal residues that had been observed with C-Fragment took place; at pH 7.4 there was negligible release. This finding shows that the resistance of COOH-terminal glutamine and glycine in C-Fragment is a property of the sequence and is unlikely to be related to a specific conformation of the molecule which could make these residues less accessible to the enzyme. The fifty-fold increase in the rate of release of

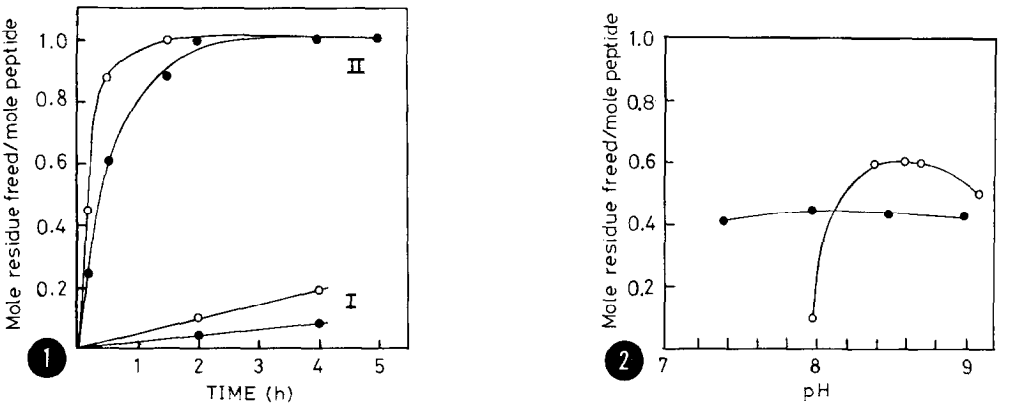

Fig. 1. Rate of release of C-terminal amino acids from the synthetic peptides I and II. Peptides were digested with carboxypeptidase A (1:200 mole enzyme/mole peptide, pH 8.0, 37°C).
-o-o-, glutamine and -●-●-, glycine.

Fig. 2. The pH dependence of the release of C-terminal glutamine from the synthetic peptides I and II. Peptide I (-o-o-) was incubated for 4 h and peptide II (-●-●) for 10 min with carboxypeptidase A in 0.15 M Tris HCl, at each pH value shown.

COOH-terminal residues from the pentapeptide II confirms that it is the primary structure of C-Fragment and peptide I which accounts for their stability; in particular the property appears to be related to the presence of paired lysine residues.

It is probable that the concerted effect of the adjacent positive charges may be implicated in the stability of petide I. To investigate this possibility, digestions of I and II were carried out in the pH range 7 - 9 (Figure 2), since at the higher pH values a greater proportion of the amino groups of the peptides are present in their un-ionized form. It was found that at pH values above 7.4 there was a marked increase in the rate of release of COOH-terminal residues from I, with an optimum at pH 8.7; the rate of release of residues from II was little affected by pH. When the digestions of I and II were performed at their pH optima, the rate of release of glutamine and glycine was still much faster from the pentapeptide II than from the hexapeptide I.

A similar increase in the susceptibility of I to carboxypeptidase was observed when the basic groups of the peptide were blocked by acetylation; in this case the neutral ε-N-acetyllysine residues were released. Citraconylation of the basic groups of I (or of C-Fragment) also led to an increase in the rate of release of COOH-terminal residues at pH 8.0, approaching the values obtained with the unblocked peptides at their optimum pH; the acidic ε-N-citraconyllysine residues were not released.

It appears that the paired lysine residues at the COOH-terminus of C-Fragment confer stability upon the terminal glutamine and glycine. The stability may be due in part to the positive charges on the lysines; however, additional factors such as combined residue size may also be involved.

Carboxypeptidase activity was found to be present in extensively-washed rat brain synaptosomes, in association with the opiate receptor activity. In one preparation the activity corresponded to 0.07 μmole of hippuryl-L-phenylalanine hydrolysed/min/mg protein. When the synaptosomes were lysed in hypotonic buffer and fractionated on a discontinuous sucrose density gradient, carboxypeptidase activity was found in the synaptic membrane fraction and not in the mitochondrial or myelin fractions. This suggests that the activity may be an integral property of synaptic membranes. However, as expected from the model experiments, C-Fragment exposed to the membrane fractions containing carboxypeptidase A-like activity showed no release of glutamine or glycine when incubated for at least two hours in the membrane buffer at 37°C.

The stability of C-Fragment in the presence of carboxypeptidase enzymes was a surprising finding. It correlates well with the ability of the peptide to inhibit [^{3}H]-dihydromorphine binding to synaptosomes *in vitro* even when pre-incubated for several hours with the membrane preparations (8). In this context, parallel studies have demonstrated the stability of the NH_2-terminal tyrosine residue of C-Fragment under similar conditions (9).

In the binding assay, C-Fragment (lipotropin 61-91) is about 20 times more potent than C'-Fragment (lipotropin 61-87) or the shorter peptides 61-78

and 61-65. The COOH-terminal residues of C-Fragment therefore appear to be important for affinity and hence for the potency of the peptide as an analgesic agent. Moreover, the analgesia produced by intraventricular injection of C-Fragment persists for several hours. The present results show that the COOH-terminal residues of C-Fragment render the peptide resistant to carboxypeptidase action in brain and thus play a role in maintaining the duration of the biological action. The resistance of C-Fragment to both aminopeptidase and carboxypeptidase enzymes implies that the termination of its biological effects is initiated by an endopeptidase.

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