THE NH_2-TERMINUS OF C-FRAGMENT IS RESISTANT TO THE ACTION OF AMINOPEPTIDASES

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SUMMARY

The NH_2-terminal residue of C-Fragment (Lipotropin 61-91) was resistant to cleavage by aminopeptidase M or a membrane bound aminopeptidase from brain. Peptides representing residues 61-65, 61-67, 61-68 and 61-73 of lipotropin, each containing the NH_2-terminal sequence of C-Fragment, were degraded readily by the same enzymes. γ-Endorphin (61-77) and the octadecapeptide (61-78) were more resistant than the shorter peptides but less than C-Fragment. The results indicate that C-Fragment, C'-Fragment and to a lesser degree γ-endorphin possess conformational properties which protect the NH_2-terminus from enzymic degradation. The significance of the NH_2-terminal stability is discussed in relation to the duration of the analgesic actions of C-Fragment and related peptides.

INTRODUCTION

The analgesic action of C-Fragment is profound and long lasting (1) whereas the effects of shorter peptides related to C-Fragment are slight (2). Numerous studies have shown that methionine enkephalin (61-65), for example, produces only weak analgesia (3-6) and it has been suggested that the transient nature of its action is due to rapid degradation by aminopeptidases in brain (7). Since the sequence of methionine enkephalin is the same as the sequence at the NH_2-terminus of C-Fragment, an aminopeptidase capable of digesting the pentapeptide should be equally effective at removing residues from the NH_2-terminus of C-Fragment.

The present study was carried out to compare the susceptibilities of C-Fragment, methionine enkephalin and peptides of intermediate length to digestion by aminopeptidase M, and parallel studies were performed using a membrane bound aminopeptidase purified from brain homogenates. The results show that C-Fragment, though having a potentially sensitive NH_2-terminal

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sequence, is highly resistant to degradation by aminopeptidase enzymes. The stability of this 31 residue peptide indicates that it may possess conformational properties.

MATERIALS AND METHODS

C-Fragment (61-91) and C'-Fragment (61-87) were isolated from porcine pituitary as described previously (8). The ε-pentacitraconyl derivative of C-Fragment was obtained by trypsin digestion of citraconylated lipotropin (9); it was isolated by chromatography on a column of Sephadex A-25 at pH 9.0. The octadecapeptide containing residues 61-78 of lipotropin was isolated from a chymotryptic digest of C-Fragment and the heptadecapeptide γ-endorphin (61-77) was obtained from a rennin digest (10). The tridecapeptide (61-73) was obtained from γ-endorphin by digestion with carboxypeptidase A and the octapeptide (61-68) by digestion of C-Fragment with staphylococcal protease. The heptapeptide Tyr-Gly-Gly-Phe-Met-Thr-Ser (61-67) and the pentapeptide Tyr-Gly-Gly-Phe-Met (61-65, methionine enkephalin) were prepared by solid phase synthesis (11); they were purified by ion exchange chromatography on columns of Sephadex C-25 and Sephadex A-25 and by gel filtration on Sephadex G-10. Homogeneity was confirmed by thin layer chromatography on plates of Silica Gel 60F 254 (Merck Ltd.) developed with butanol-acetic acid-H_2O (4:1:5 upper phase) and by amino acid analysis after total enzymic digestion or hydrolysis in 6N-hydrochloric acid at 110°C for 16 hours. Aminopeptidase M was obtained from Röhm Ltd., Darmstadt and carboxypeptidase A from Worthington Ltd. Bacitracin was from Sigma Ltd.

Purification of a membrane bound aminopeptidase from rat brain: Brain was removed from freshly killed Sprague-Dawley rats and after excision of the cerebellum and pituitary stalk the tissue was homogenized in 0.32M sucrose (7 ml/g wet weight) using a glass-teflon homogenizer. The suspension was centrifuged at 1000 g for 10 min, then the supernatant was removed and centrifuged at 17,000 g for 20 min. Membranes containing aminopeptidase activity were prepared either by extensive washing in 50 mM Tris-0.1 M sodium chloride (pH 7.4) or by lysis in H_2O, followed by separation of synaptosomal membrane fragments by centrifugation on discontinuous sucrose density gradients (12). Protein estimation was performed by the method of Lowry et al. (13).

Digestion of peptides with aminopeptidase M: The peptide (0.34 mM) was incubated at 37°C with the enzyme (0.02 mg/ml) in 0.1 M ammonium bicarbonate at pH 7.4 and DL-norleucine was included as an internal standard. At intervals aliquots of the solution were removed and added to 1 ml of 0.2 M citrate buffer at pH 2.2. The released amino acids were determined on a Beckman 120 C amino acid analyser.

Digestion of peptides with the brain aminopeptidase: Peptides (0.34 mM) were agitated gently at 37°C in a suspension of membranes (2.4 mg protein/ml) in 0.05 M sodium chloride containing 0.1 M sodium phosphate at pH 7.4. At intervals aliquots of the solution were removed and subjected to amino acid analysis. The specific activity of the aminopeptidase in washed membranes was 2.8×10^{-3} μmole tyrosine released from peptide 61-67 per min per mg protein and 3.4×10^{-3} μmole tyrosine released per min per mg protein in Fraction F of the sucrose density gradient of Whittaker et al. (12). Comparable results were obtained on digestion of the peptides with the different membrane preparations.

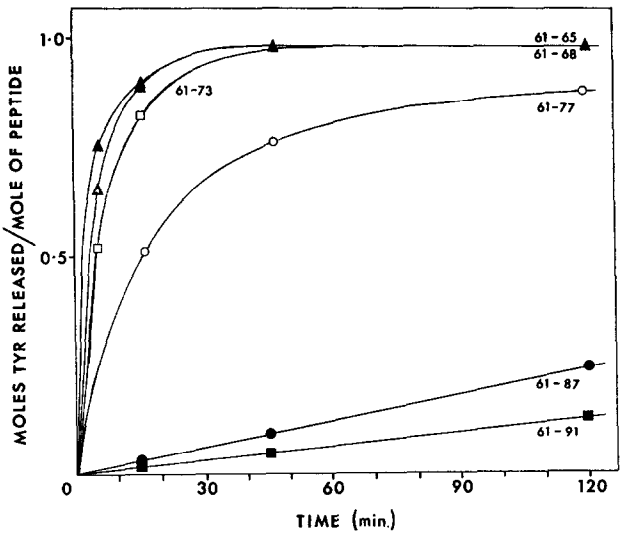

Fig. 1. Rate of release of tyrosine from C-Fragment and related peptides by aminopeptidase M. Incubations were carried out at pH 7.4, 37°C, as described in the text.

Symbols:
(▲) Tyr-Gly-Gly-Phe-Met (61-65);
(△) Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu (61-68);
(□) tridecapeptide representing residues 61-73 of lipotropin;
(○) γ-endorphin (61-77); (●) C'-Fragment (61-87); (■) C-Fragment (61-91).

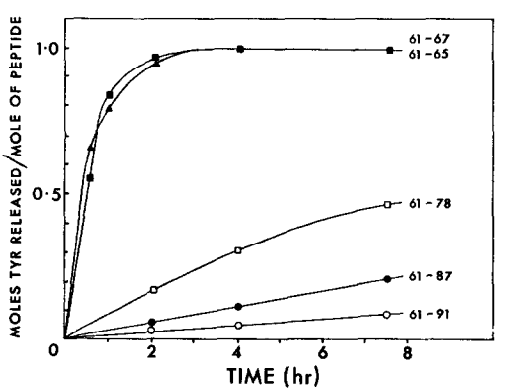

Fig. 2. Rate of release of tyrosine from C-Fragment and related peptides by a membrane bound aminopeptidase from brain. Incubations were performed at pH 7.4, 37°C, as described in the text.

Symbols:
(▲) Tyr-Gly-Gly-Phe-Met (61-65); (■) Tyr-Gly-Gly-Phe-Met-Thr-Ser (61-67);
(□) octadecapeptide (61-78); (●) C'-Fragment (61-87);
(○) C-Fragment (61-91).

RESULTS AND DISCUSSION

The rates of enzymic release of tyrosine from a series of peptides with the NH_2-terminal sequence Tyr-Gly-Gly-Phe- are shown in Figures 1 and 2. From C-Fragment, tyrosine was released by aminopeptidase M only with great difficulty; at an enzyme to substrate ratio of 1:60 (w/w) less than 10 per cent of the NH_2-terminal residue was removed in 2 hours. From C'-Fragment the NH_2-terminal tyrosine was again released slowly under the same conditions. In contrast, the release of tyrosine from the pentapeptide 61-65, heptapeptide 61-67, octapeptide 61-68 and tridecapeptide 61-73 was rapid. Tyrosine was released at an intermediate rate from γ-endorphin (61-77) and from the octadecapeptide (61-78). Similar results were obtained with a membrane bound aminopeptidase from brain (Figure 2).

It seems likely that the unusual stability of C-Fragment is due to a folding of the peptide chain which renders the NH_2-terminal residue less accessible. This is consistent with conclusions drawn from a study of the chiroptic properties of C-Fragment and methionine enkephalin which suggested that the NH_2-terminal tyrosines of the two peptides are present in different environments (14). Additional evidence for conformational restraints in C-Fragment has been reported in a study involving proteolysis by endopeptidases (10); it was observed that cleavage takes place readily in the central region of the peptide but the NH_2-terminal region resists degradation. The present results indicate that the NH_2-terminal tyrosine of C-Fragment, and also that of C'-Fragment, is concealed within the structure of the molecule.

The conformation of C-Fragment does not appear to be stabilized by electrostatic linkages involving ε-NH_2 groups because the ε-pentacitraconyl derivative, which differs markedly in polarity from C-Fragment, was resistant to aminopeptidase digestion. The large difference in the NH_2-terminal stability of the heptadecapeptide (61-77) and the heptadodecapeptide (61-87) suggests that the longer peptide has a preferred conformation which affects the disposition of the NH_2-terminal tyrosine. It may be relevant that

predictions of secondary structure have indicated that residues 71-77 of C-Fragment are present predominantly in the β-configuration and residues 81-89 in helical form (14).

The high degree of stability exhibited by the NH_2-terminus of C-Fragment, together with the known resistance of the COOH-terminal residues to attack by carboxypeptidase enzymes (15) provides an explanation for the long duration of the analgesic action of C-Fragment compared with the short lasting effects of the small peptides. It is notable that C'-Fragment, which lacks only the four COOH-terminal residues of C-Fragment, has a high NH_2-terminal stability. Its analgesic potency is low but the effects are long lasting (6, 16). In contrast the 61-77 peptide, which also has a low potency, is less stable and its duration of action is shorter than that of C-Fragment (2, 6). The analgesic potencies of the peptides are clearly related to their affinities for brain opiate receptors (17); the duration of the analgesia they produce is a reflection of *in vivo* stability.

The ease of degradation of the peptides that contain up to thirteen residues is consistent with an inability to form stable elements of secondary structure and it underlies the transient nature of their actions. Because of their susceptibility to proteolysis during biological studies *in vitro*, attempts have been made to stabilize the enkephalins by the addition of bacitracin (18). Our experiments show that this substance strongly inhibits the brain aminopeptidase; consequently bacitracin can exert a stabilizing influence on the enkephalins but it would have little effect on the degradation of C-Fragment.

The stability of C-Fragment against attack by exopeptidases suggests that the degradation of this peptide *in vivo*, with concomitant disappearance of the biological effects, is catalysed by the action of an endopeptidase.

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