PREPARATION AND PROPERTIES OF LIPOTROPIN C-FRAGMENT WITH ANIONIC SUBSTITUENTS ATTACHED TO THE LYSINE RESIDUES

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1. Introduction

C-Fragment was first isolated from porcine pituitary glands and shown to comprise residues 61–91 of lipotropin [1]. The 31-residue peptide acts as a potent opiate agonist in the central nervous system; it binds specifically to morphine receptors in brain [2] and produces long-lasting analgesia when administered cerebroventricularly [3,4]. It seems certain that the NH_2-terminal pentapeptide of C-Fragment forms a binding site which is necessary for the expression of opiate activity [2]. Although this section is present in a number of peptides derived from C-Fragment (methionine enkephalin, 61–65; γ-endorphin, 61–77, and C′-Fragment, 61–87) these molecules are comparatively weak agonists. A question thus arises concerning the role of the COOH-terminal residues of C-Fragment. One possibility is that these residues form direct binding interactions with brain opiate receptors; another is that they might induce a specific conformation of the NH_2-terminal region of the molecule by an intramolecular effect. It is anticipated that studies with synthetic or chemically modified derivatives of C-Fragment should clarify the function of the COOH-terminal region.

A characteristic feature of the C-Fragment sequence is its high content of lysine residues. Of these, the paired residues in the COOH-terminal tetrapeptide appear to be important for the agonist activity since their absence from C-Fragment, as in C′-Fragment, leads to severe loss of analgesic potency [5]. In order to investigate the role of the lysine residues, modification of the ε-NH_2 groups was undertaken by a route which did not involve reaction at the essential NH_2-group of the terminal tyrosine. This was achieved by trypsin digestion of citraconylated lipotropin, which allowed the isolation of ε-*N*-pentacitraconyl C-Fragment (CT_5 C-Fragment) together with arginine-containing peptides derived from the NH_2-terminal region of lipotropin.

A preparation of C-Fragment from ovine lipotropin has been described which involved the formation of the citraconylated peptide as an intermediate [6] but the product was not isolated. In this report we describe the preparation of CT_5 C-Fragment in homogeneous form and in high yield; a study of its binding properties to brain opiate receptors is presented.

2. Materials and methods

2.1. *Preparation of citraconylated lipotropin*

Lipotropin (1.3 μmol), obtained from porcine pituitary [1], was dissolved in 2 ml 0.5 M Na_2HPO_4, at pH 8.5 and a 300-fold molar excess of citraconic anhydride (Aldrich) as a 30% v/v solution in 1,4 dioxan was added with vigorous mixing. At intervals during the addition the pH of the reaction mixture was adjusted to 8.5 with 1 M NaOH and the solution was incubated for 1 h. Complete substitution of amino groups was demonstrated by dansylation of 1 nmol peptide, with glycine added as an internal standard; after hydrolysis no ε-*N*-dansyllysine or dansylglutamic acid could be detected.

2.2. *Tryptic digestion of citraconylated lipotropin*

Digestion was performed with TPCK-treated

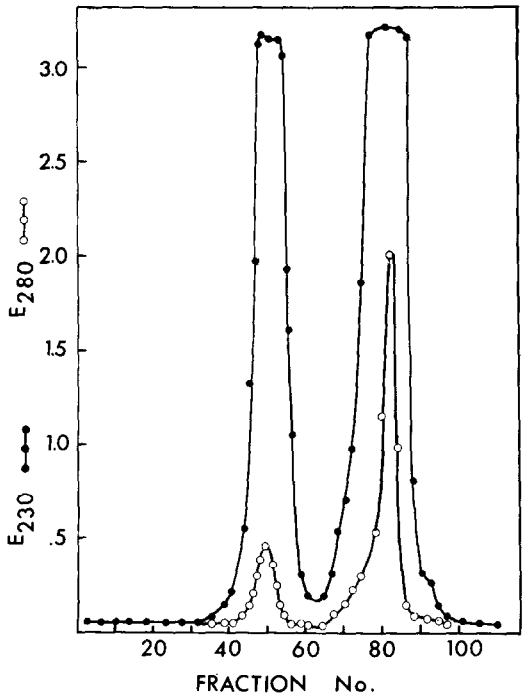

Fig.1. Gel filtration of tryptic peptides derived from citraconylated lipotropin (CT_{11} lipotropin) on Sephadex G-50 in 0.02 M Tris–HCl, pH 8.5. Fraction volumes were 4.0 ml.

trypsin (Worthington) at an enzyme/peptide ratio of 1:400, w/w, at pH 8.5 and 37°C, using the same solution employed in the citraconylation reaction. After 2 h the digestion was terminated by the addition of a four-fold molar excess of ovomucoid trypsin inhibitor (Sigma), which was allowed to react for 5 min at 37°C.

2.3. *Chromatography of the tryptic peptides*

The digestion mixture was applied to a 122 X 1.5 cm column of Sephadex G-50 equilibrated with 0.02 M Tris–HCl, at pH 8.5. The first fraction contained three peptides, accounting for residues 1–23, 24–51 and 61–91 of lipotropin; the digestion conditions did not lead to cleavage of the Arg–Asp bond in peptide 1–23. The second fraction contained the nonapeptide 52–60 and citraconic acid. The combined fractions from the first peak were applied to a 40 X 0.9 cm column of DEAE-Sephadex A 25 equilibrated with 0.02 M Tris–HCl, at pH 8.5. Peptides were eluted with a linear salt gradient formed from 200 ml 0.1 M NaCl and 200 ml 0.5 M NaCl in the equilibration buffer (fig.2).

The compositions and yields of the peptides obtained are listed in table 1. Single fraction amino acid analysis of material in the shoulder on the CT_5-C-Fragment peak was identical in composition to the main peak. As this retarded material is more acidic, it seems likely to contain citraconylated serine and threonine residues, a side reaction known to be involved in the citraconylation of proteins [7]. This material was not present in the peptide solutions used in the binding studies. The peptides 1–23 and 24–51, which contain no serine and threonine, were eluted in symmetrical peaks.

2.4. *Removal of the citraconyl groups*

Peptides were incubated in 0.1 M pyridine-acetate, at pH 4, for 24 h, 37°C and the solutions were concentrated in vacuo. The peptides were desalted on columns of Sephadex G-25 in 50% (v/v) acetic acid and the solvent was removed by rotary evaporation at below 25°C. The 61–91 peptide thus obtained had an absorption spectrum and composition indistinguishable from that of C-Fragment isolated from pituitary.

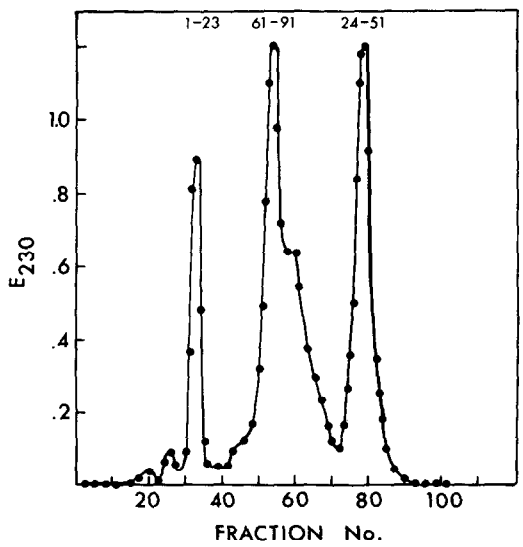

Fig.2. Elution profile of tryptic peptides from CT_{11} lipotropin from DEAE-Sephadex A 25. Fraction volumes were 4.5 ml.

Table 1
Composition of tryptic peptides obtained from citraconylated lipotropin

Peptide (residue number)	1–23		61–91		24–51	
Yield (μmol)	1.01		1.02		0.8	
Recovery (%)	79		80		63	
Composition	Obs.	Theor.	Obs.	Theor.	Obs.	Theor.
Lys	0.0	0	5.0	5	3.0	3
His	0.0	0	1.0	1	1.0	1
Arg	1.9	2	0.0	0	1.0	1
Asp	0.9	1	2.0	2	1.0	1
Thr	0.0	0	2.8	3	0.0	0
Ser	0.0	0	1.9	2	0.0	0
Glu	4.0	4	3.0	3	7.2	7
Pro	5.3	5	1.0	1	1.0	1
Gly	2.2	2	3.0	3	1.8	2
Ala	8.4	8	1.95	2	4.6	5
Val	0.0	0	1.4[a]	2	1.0	1
Met	0.0	0	0.8	1	0.94	1
Ile	0.0	0	0.4[a]	1	0.0	0
Leu	1.1	1	2.4	2	2.0	2
Tyr	0.0	0	0.8	1	1.6	2
Phe	0.0	0	1.8	2	0.92	1

[a] Resistant Ile–Val bond

Analysis was carried out after 16 h hydrolysis in 6 N HCl at 110°C

2.5. *Measurement of the affinity of CT_5 C-Fragment for brain opiate receptors*

The ability of peptides to reduce the specific binding of [G-^{3}H]naloxone (New England Nuclear, 20 Ci; mmol^{-1}, 1 X 10^{-9} M) and [1,7,8-^{3}H]dihydromorphine (Radiochemical Centre, Amersham, 70 Ci; mmol^{-1}, 1 X 10^{-9} M) was measured essentially as previously described [2]. Incubation of tritiated opiates, peptides and membranes was carried out for 15 min at 30°C in 1 ml 0.05 M Tris–HCl, pH 7.4, containing 100 mM NaCl.

3. Results and discussion

The ability of CT_5 C-Fragment to reduce the specific binding of [^{3}H]naloxone and [^{3}H] dihydromorphine to brain membranes was compared with that of C-Fragment (fig.3). The binding curves show that modification of the five lysine residues leads to a large decrease in affinity. The product was three orders of magnitude less effective than C-Fragment in dis-

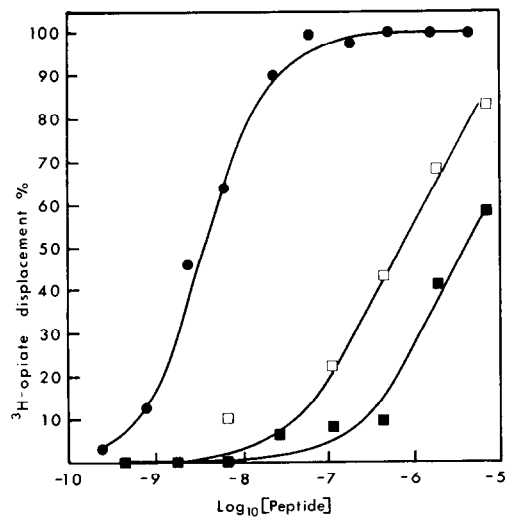

Fig.3. The curves show the displacement of specifically-bound opiates from rat brain synaptosomal membranes. I_{50} is the peptide concentration required for 50% displacement of specifically-bound opiate. (-●--●-) Displacement of [G-^{3}H] naloxone by acid-treated CT_5 C-Fragment, I_{50} 3 nM; (□–□) Displacement of [1,7,8-^{3}H]dihydromorphine by CT_5 C-Fragment, I_{50} 0.7 μM; (■–■) Displacement of [G-^{3}H]naloxone by CT_5 C-Fragment, I_{50} 4.0 μM.

placing naloxone from the brain receptors. It was forty-times less potent than the pentapeptide methionine enkephalin in the same assay. Removal of the substituents led to full recovery of the C-Fragment potency, assessed by binding and by analgesic activity.

It should be noted that the NH_2-terminal octapeptide of C-Fragment (61–68), which does not contain a lysine residue, is not affected by the citraconylation reaction. The decrease in the binding properties of C-Fragment accompanying citraconylation must therefore be the consequence of the modification of residues which are distant from the NH_2-terminus. The low affinity cannot be due to rapid degradation of the peptide during the binding assay since the pentacitraconyl derivative exhibited the same high resistance as C-Fragment to the action of brain exopeptidases [8]; nor does it seem likely that the low affinity should be due to a disturbance of conformation in the NH_2-terminal region because C′-Fragment, which lacks the lysine residues at positions 88 and 89, has conformational properties similar to those of C-Fragment. These properties protect the NH_2-terminus from proteolytic attack, a phenomenon exhibited by C-Fragment, C′-Fragment and CT_5 C-Fragment [8].

The low affinity of the CT_5 C-Fragment for brain opiate receptors may be attributed to the inversion of charge on the lysine residues. It seems likely that receptors possess anionic groups proximal to the ϵ-NH_3^+ groups of C-Fragment; the anionic nature of CT_5 C-Fragment would tend to exclude this derivative from the receptor site. The same effect could result if the substituents interfered sterically with the formation of the peptide–receptor complex. In either case the results emphasise the importance of the region of the peptide chain contiguous with the NH_2-terminal pentapeptide for receptor affinity. In particular the basic residues must be considered as candidates for receptor interaction. Further experiments involving modification of individual lysine residues will clarify their molecular role in the central activity of C-Fragment.

Acknowledgements

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