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THE COMPLETE AMINO ACID SEQUENCE OF α-NEO-ENDORPHIN

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SUMMARY: After re-purification by reverse phase high performance liquid chromatography, α-neo-endorphin was submitted to structural analyses, performed by dansyl-Edman degradation, as well as by C-terminal analysis by ^{3}H-labeling. The full sequence of α-neo-endorphin has been determined to be : Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro-Lys, in which the 8th residue previously reported as Arg is found to be Tyr. A synthetic decapeptide with the above sequence was verified to be identical with natural α-neo-endorphin. For further structural confirmation, tryptic and chymotryptic peptides were also identified. Thus, the complete sequence of α-neo-endorphin has been definitely established. Its potent opioid activity in the guinea-pig ileum assay is also discussed.

The present paper will report the complete amino acid sequence of α-neo-endorphin. In 1979, we isolated α-neo-endorphin as the first form of "big" Leu-enkephalin from 30,000 porcine hypothalami in a yield of ca. 50 μg (1). Although the peptide was considered to be one of the key compounds for solving the biosynthetic problem of Leu-enkephalin, only the following partial sequence was proposed : Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Arg-Pro-(Gly, Tyr_2,Lys_2,Arg) (1,2). In spite of our attempts to elucidate the residual C-terminal sequence, ambiguity was inevitably involved in the data obtained from direct Edman degradation, especially as the reaction proceeded to the carboxyl end.

During the course of our recent isolation from brain of β-neo-endorphin and PH-8P, conditions for high performance liquid chromatography (HPLC) on a reverse phase column have been developed for separating strongly basic peptides, such as the above "big" Leu-enkephalins (3,4). Accordingly, the remaining sample of α-neo-endorphin in the amount of ca. 10 μg was submitted to reverse phase HPLC. α-Neo-endorphin thus repurified was subjected to the present structural analyses. Amino acid composition of the specimen newly determined indicated a decapeptide structure, shorter by 5 residues than

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anticipated from the previous data, probably due to removal by HPLC of impurities that were not detected in our previous purification.

With the repurified sample, dansyl-Edman degradation, combined with the C-terminal analysis by tritium-labeling (5), has been successfully performed to determine the full sequence of α-neo-endorphin to be : Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro-Lys, where the 8th residue previously reported as Arg is revised to Tyr. A decapeptide with the above sequence has been synthesized by the solution method. The synthetic peptide has been verified to be identical with natural α-neo-endorphin by their comparison on HPLC. Furthermore, tryptic and chymotryptic peptides have also been identified to confirm the structure determined above. Thus, the complete amino acid sequence has been unambiguously established.

The present paper also describes the opioid activity in the guinea-pig ileum assay of α-neo-endorphin, compared with β-neo-endorphin (2), which has been identified as C-terminal deleted peptide from α-neo-endorphin.

MATERIALS AND METHODS

Repurification of α-neo-endorphin by HPLC : α-Neo-endorphin had been isolated previously in a yield of ca. 50 μg from 30,000 porcine hypothalami (1). Our previous purification consisted of successive gel filtrations and ion exchange chromatographies, but HPLC was not involved. Purity and homogeneity of the previous preparation were confirmed by TLC and electrophoresis on a cellulose plate.

About 10 μg of α-neo-endorphin, left after past analyses, was dissolved in 1M acetic acid and applied on a μ-Bondapak C-18 column (3.9 x 300 mm : Waters Associates). The column was eluted with 10 mM ammonium formate (pH 4.0), using a linear gradient elution of CH_3CN from 10% to 50%. Elution profile was monitored by U.V. measurements at 210 and 280 nm, as well as by the radioimmunoassay after trypsinization of the aliquots, as follows; aliquots of all fractions (0.005 μl equivalent) were taken, lyophilized and then trypsinized. After termination of the digestion by adding soy-bean trypsin inhibitor, the radioimmunoassay was performed by using anti-[Arg^6]-Leu-enkephalin antiserum, as reported previously (6). To each assay tube was added 2 μg of bacitracin to prevent adsorption of the sample to the tube surface.

Amino acid analysis : One tenth of α-neo-endorphin purified as above was hydrolyzed in 4M methanesulfonic acid containing 0.2% tryptamine (Pierce) at 110°C for 20 hr. After neutralization, the hydrolysate was analyzed on a Hitachi #835 amino acid analyzer.

Dansyl-Edman degradation : Sequence analyses were carried out with about half of the purified α-neo-endorphin by the dansyl-Edman procedure previously employed (3,4). Special care was taken to avoid the loss of sample on extraction with butyl acetate. The dansyl amino acids were identified by TLC on a polyamide sheet.

Carboxy-terminal analysis : About one tenth of the purified α-neo-endorphin, after lyophilization, was submitted to the selective C-terminal tritiation in the described manner (5). The specific radioactivity of ^3H_2O used was 1 Ci/ml. The C-terminal tritiated peptide thus obtained was hydrolyzed with 6N HCl. After repeated lyophilizations, the hydrolysate was submitted to

dansylation. Each fluorescent spot of dansyl amino acid, separeted by TLC on a polyamide sheet, was cut out of the sheet, extracted with 1M NH_3-acetone (1:1, v/v) and submitted to radioactivity counting in 10 ml of Aquasol II (NEN) by the aid of liquid scintillation counter.

Syntheses of the peptides : α-Neo-endorphin with the newly established sequence and one of its tryptic peptides (T-II : Lys-Tyr-Pro-Lys) were synthesized by the solution method for the purpose of structural identification. Details in the syntheses will be reported in a separate paper. Physical constants of the peptides were determined as follows;

α-neo-endorphin : $[\alpha]_D^{20.5}$=-37.1° (c: 0.51, H_2O); Rf^1=0.55, Rf^2=0.68

Lys-Tyr-Pro-Lys(T-II) : $[\alpha]_D^{20.0}$=-27.3° (c: 0.40, H_2O); Rf^1=0.23, Rf^2=0.35

Rf values were determined by TLC on a cellulose plate (Merck), developed by the following two solvent systems;

Rf^1 : 1-butanol:CH_3CO_2H:H_2O: = 4:1:5 (v/v) (upper phase)

Rf^2 : 1-butanol:CH_3CO_2H:H_2O:pyridine = 15:3:12:10 (v/v)

Homogeneity of the peptides was confirmed by HPLC as well as by TLC. Purity of the peptides was further verified by elemental analyses and amino acid analyses, both of which showed good agreement with theoretical values. Another tryptic peptide (T-I) and other peptides used in the present study were also synthesized in our laboratory, as previously reported (2,3,7).

Enzymatic digestion of α-neo-endorphin : Natural and synthetic α-neo-endorphin (1.0 μg each) were digested with 40 ng of γ-chymotrypsin (Sigma) in 10 μl of 1% ammonium bicarbonate (pH 8.0) at 37°C for 2 hr. After addition of 1M acetic acid to terminate the reaction, the digests were applied on a μ-Bondapak C-18 column and eluted with 50 mM phosphate buffer (pH 2.0), using a linear gradient elution of CH_3CN from 0% to 50% at a flow rate of 2.0 ml/min. Tryptic digestion was also performed with TPCK-trypsin (Worthington) and the resulting digests were analyzed by HPLC under the same conditions described above. Chymotryptic peptides (Ch-I, Ch-II and Ch-IV), prepared from synthetic α-neo-endorphin, were separated by HPLC and identified respectively, on the basis of their amino acid analysis data. These peptides were used for identifying chymotryptic peptides of natural α-neo-endorphin by their chromatographic comparison.

Opioid activity : Electrically stimulated myenteric plexus-longitudinal muscle from guinea-pig ileum was used according to the reported manner (8). Opioid activities of α- and β-neo-endorphin were determined and compared with those of Met- and Leu-enkephalin.

RESULTS AND DISCUSSION

Purification of the previous preparation of α-neo-endorphin by reverse phase HPLC is shown in Fig. 1. There was a single main peak and no others were detected in appreciable amounts. As marked by the black bar, immunoreactivity against anti-[Arg^6]-Leu-enkephalin detected after trypsinization was located only in the main peak. The single immunoreactive peak was collected. Figure 2A shows the HPLC pattern of α-neo-endorphin thus repurified, which was then subjected to the present analyses. Amino acid analysis of the purified specimen indicated a decapeptide compositon, consisting of $Pro_{0.9}$,$Gly_{2.2}$,$Leu_{1.1}$,$Tyr_{2.0}$,$Phe_{1.0}$,$Lys_{1.9}$,$Arg_{1.0}$. Compared with our previous data, the content of Gly, Tyr, Lys and Arg decreased considerably.

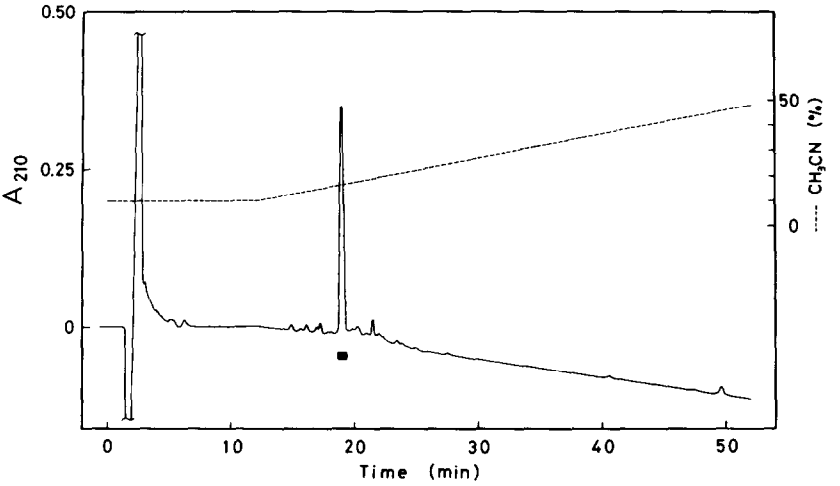

Fig. 1. Repurification of α-neo-endorphin by reverse phase HPLC.

Sample loaded : 10 μg of previous preparation of α-neo-endorphin.
Column : μ-Bondapak C-18 (3.9 x 300 mm)
Elution : A linear gradient of 10 mM ammonium formate (pH 4.0) containing CH_3CN, from 10% to 50% CH_3CN.
Flow rate : 2 ml/min
Black bar indicates the immunoreactive fraction (after trypsinization).

With half of the purified α-neo-endorphin, dansyl-Edman degradation successfully proceeded to the carboxyl end, in contrast with our previous analyses, to reveal the decapeptide sequence, as shown in Fig. 3. With respect to the 8th residue, which was reported to be Arg (1), only Tyr was identified as dansyl derivative by TLC on a polyamide sheet in the present analysis. It is likely that erroneous assignment in the previous work was derived from possible interference by Arg-rich impurities. On C-terminal analysis by the selective tritium-labeling, only Lys was found as the radioactive amino acid, which was identified as its dansyl derivative. Thus, all the data mentioned above indicate the decapeptide structure of α-neo-endorphin, with Lys-extension at the C-terminus of β-neo-endorphin, which has recently been identified in porcine hypothalamus (3).

In order to verify the proposed structure, synthesis of the decapeptide with the sequence determined above was carried out by the solution method. Figure 2 shows chromatographic identity of natural α-neo-endorphin with synthetic decapeptide. Both peptides eluted at identical positions in HPLC on the same column of μ-Bondapak C-18. In another solvent system, using ammonium formate-CH_3CN, the identity of both peptides was also confirmed (data not shown). In addition, when TLC on a cellulose plate was performed by developing with the solvent system of 1-butanol:acetic acid:water:pyridine = 15:3:12:10 (v/v), both peptides also showed identical Rf value (0.68), which

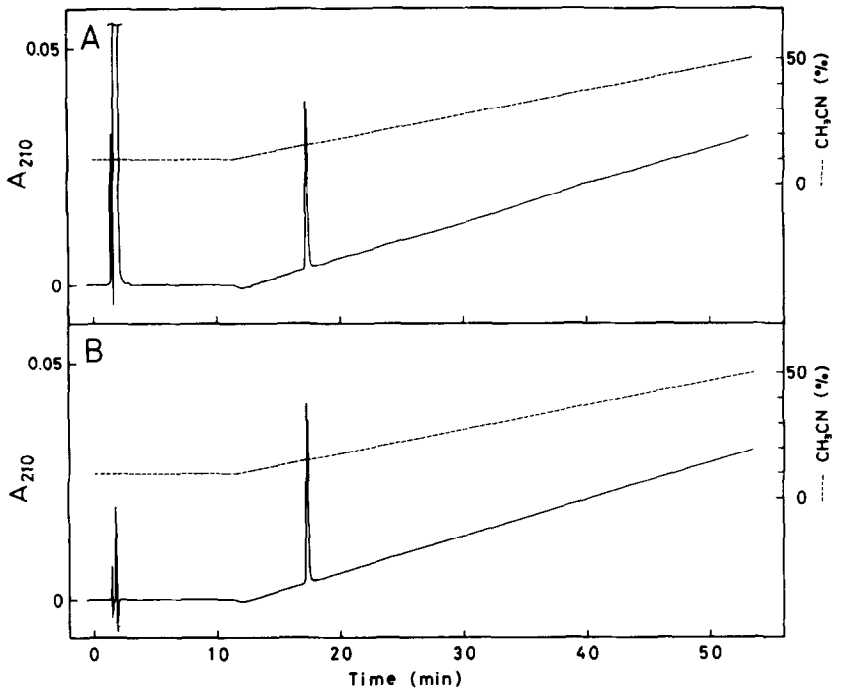

Fig. 2. Reverse phase HPLC of α-neo-endorphin.
(A : natural, B : synthetic)

Sample loaded : (A) : 1 nmole of natural α-neo-endorphin repurified.
(B) : 1 nmole of synthetic α-neo-endorphin.
Column : μ-Bondapak C-18 (3.9 x 300 mm)
Elution : A linear gradient of 50 mM KH_2PO_4-H_3PO_4 (pH 2.0) containing CH_3CN, from 10% to 50% CH_3CN.
Flow rate : 2 ml/min
Both peptides were chromatographed on the same column under exactly the same conditions for their identification.

was also in good agreement with the reported Rf (0.68) of the previous specimen of α-neo-endorphin, determined under the same conditions as above (1). This fact verified that the specimen of natural α-neo-endorphin did not undergo any change or decomposition during storage and repurification. As will be described, opioid activity of the synthetic α-neo-endorphin is also in accordance with that of the old specimen.

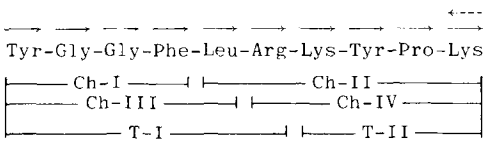

Fig. 3 . The complete amino acid sequence of α-neo-endorphin.
(⟶) : Determined by the dansyl-Edman procedure.
(⟵) : Determined by the tritium-labeling method.
T : Tryptic peptide, Ch : Chymotryptic peptide.

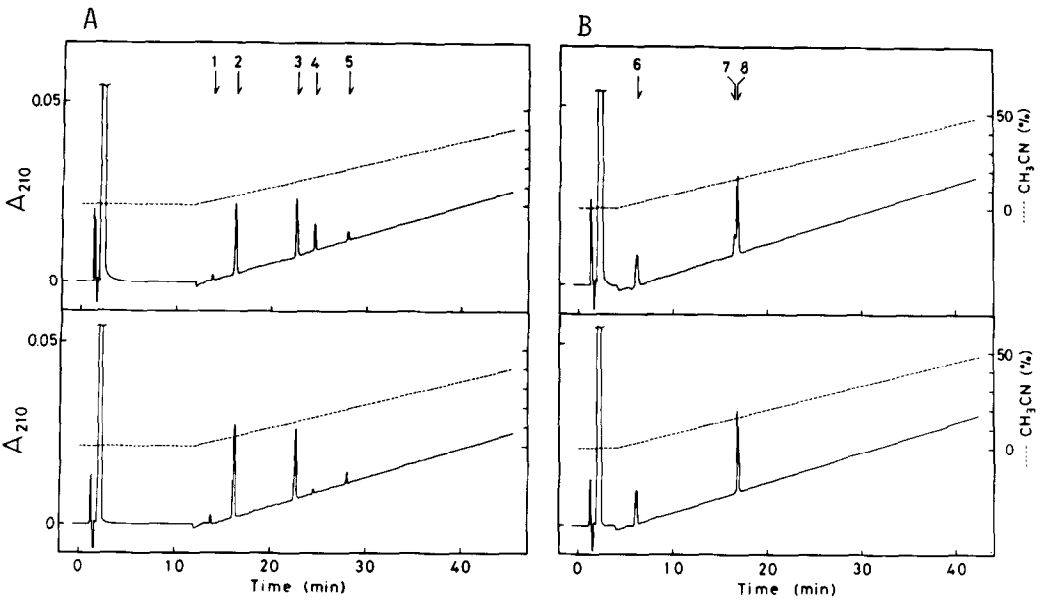

Fig. 4. Reverse phase HPLC of (A) chymotryptic and (B) tryptic digests of α-neo-endorphin. (upper : natural, lower : synthetic)

Sample loaded : (A) : chymotryptic digests of α-neo-endorphin (1 μg)
(B) : tryptic digests of α-neo-endorphin (1 μg)
Column : μ-Bondapak C-18 (3.9 x 300 mm)
Elution : A linear gradient of 50 mM KH_2PO_4-H_3PO_4 (pH 2.0) containing CH_3CN, from 0% to 50% CH_3CN.
Flow rate : 2 ml/min
Arrows indicate elution positions of the following peptides;
1) Ch-IV, 2) Ch-II, 3) Ch-I, 5) Ch-III, 6) T-II, 8) T-I,
4, 7) undigested α-neo-endorphin (see Fig. 3).

Further structural confirmation is provided by enzymatic digestions of natural and synthetic α-neo-endorphin. Figures 4A and 4B represent HPLC patterns of chymotryptic and tryptic digests of α-neo-endorphin, respectively. It is obvious that natural α-neo-endorphin (upper) undergoes enzymatic cleavage in the identical manner with synthetic decapeptide (lower). As seen from Fig. 4A, chymotryptic digestion of α-neo-endorphin resulted in the formation of four fragments (peaks #1,2,3 and 5), along with a small amount of unreacted starting peptide (peak #4). All peptide peaks were identified on HPLC with the respective peptides, which isolated from the chymotryptic digests of the synthetic α-neo-endorphin and identified on the basis of their amino acid compositions. Thus, peaks #1,2,3,4 and 5 were proved to correspond to peptides, Ch-IV, Ch-II, Ch-I, α-neo-endorphin and Ch-III, respectively. (Sequences of the above peptides are shown in Fig. 3.) Thus, it is proved that chymotrypsin cleaved the linkage of Phe^4-Leu^5 predominantly, but the linkage of Leu^5-Arg^6 to a lesser extent.

Table I. Opioid Activity of α- and β-Neo-endorphin

(Guinea-Pig Ileum Assay)

Peptides	IC_{50} (nM)	Relative Potency*
Leu-enkephalin	117	100
Met-enkephalin	33	360
α-Neo-endorphin	5.6	2100
β-Neo-endorphin	15.4	760

*) Leu-enkephalin was taken as 100.

On the other hand, trypsinization of α-neo-endorphin yielded two fragments, peaks #6 and 8, which were identified with T-II (Lys-Tyr-Pro-Lys) and T-I ([Arg^6]-Leu-enkephalin), by comparing with synthetic peptides, respectively. In the case of natural specimen, a small amount of unreacted peptide was recovered (peak #7). Thus, it is concluded that α-neo-endorphin was cleaved by trypsin specifically at the linkage of Arg^6-Lys^7 into two fragments. Consequently, the identity of natural α-neo-endorphin with the synthetic decapeptide has been definitely confirmed.

In addition, the opioid activity in the guinea-pig ileum assay of the synthetic α-neo-endorphin was also found to show good agreement with the potency of natural preparation (IC_{50} : 5 nM), previously determined (2). Table I summarizes opioid activities of α- and β-neo-endorphin, compared with those of Leu- and Met-enkephalin. α-Neo-endorphin is about 3 times more potent than β-neo-endorphin. Remarkable enhancement of the potency by Lys-extension at C-terminus of β-neo-endorphin suggests that C-terminal Lys of α-neo-endorphin may be preferable for its tighter binding to the receptor.

Finally, "big" Leu-enkephalins identified in brain so far are to be classified into two families, as seen from Table II. The first family con-

Table II. Amino Acid Sequences of Leu-Enkephalin Related Peptides.

Leu-enkephalin	Tyr-Gly-Gly-Phe-Leu
[Arg^6]-Leu-enkephalin	Tyr-Gly-Gly-Phe-Leu-Arg
α-Neo-endorphin	Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro-Lys
β-Neo-endorphin	Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro
Dynorphin	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro---
PH-8P (Dynorphin[1-8])	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile

sists of α- and β-neo-endorphin, whose N-terminal Leu-enkephalin sequence is followed by a characteristic sequence of Arg^{6}-Lys^{7}, while members of another family, dynorphin (9) and PH-8P (4), have Arg^{6}-Arg^{7} sequence succeeding to Leu-enkephalin moiety. The presence of such a basic amino acid pair as above in the molecule, strongly suggests a possibility that these "big" Leu-enkephalins may serve as probable intermediates of Leu-enkepahlin . Moreover, there may exist two different pathways to Leu-enkephalin in brain, although a question as to whether neo-endorphin and dynorphin sequences are involved both in a common precursor or separately in the respective precursors, remains to be investigated. In addition, potent opioid activities of these "big" Leu-enkephalins suggest that they may have their own physiological functions.

REFERENCES

1. Kangawa, K., Matsuo, H., and Igarashi, M. (1979) Biochem. Biophys. Res. Commun., 86, 153-160.
2. Matsuo, H., Kangawa, K., Nakagawa, Y., Chino, N., Sakakibara, S., and Igarashi, M. (1979) in "Peptide: Structure and Biological Function", Ed. by Gross, E., and Meienhofer, J. (Pierce Chem. Co., Ill.) pp. 873-876.
3. Minamino, N., Kangawa, K., Chino, N., Sakakibara, S., and Matsuo, H. Biochem. Biophys. Res. Commun., submitted.
4. Minamino, N., Kangawa, K., Fukuda, A., and Matsuo, H. (1980) Biochem. Biophys. Res. Commun., 95, 1475-1481.
5. Matsuo, H., Baba, Y., Nair, R.M.G., Arimura, A., and Schally, A.V. (1971) Biochem. Biophys Res. Commun., 43, 1334-1339.
6. Kangawa, K., Mizuno, K., Minamino, N., and Matsuo, H. (1980) Biochem. Biophys. Res. Commun., 95, 1467-1474.
7. Chino, N., Nakagawa, Y., Sakakibara, S., Hayashi, Y., Kangawa, K., and Matsuo, H. (1980) in "Peptide Chemistry: 1979", Ed. by Yonehara, H. (Protein Research Foundation, Osaka) pp. 215-218.
8. Paton, W.D.H., and Aboozar, M. (1968) J. Physiol., 194, 13-33.
9. Goldstein, A., Tachibana, S., Lowney, L.I., Hunkapillar, M., and Hood, L. (1979) Proc. Natl. Acad. Sci. U.S.A., 76, 6666-6670.