Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 2096–2100, March 1982
Neurobiology

Adrenal opioid proteins of 8600 and 12,600 daltons: Intermediates in proenkephalin processing

(enkephalin/opioid peptide/adrenal medulla/tryptic mapping/microsequence analysis)

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Contributed by Sidney Udenfriend, December 16, 1981

ABSTRACT [Met]Enkephalin-containing proteins of 8600 and 12,600 daltons have been isolated from acid extracts of bovine adrenal medulla and purified to homogeneity, and their sequences have been determined by a combination of automated Edman degradation, tryptic mapping, and enzymatic time-course hydrolysis. The 8600-dalton protein contains one copy of the [Met]enkephalin sequence at the COOH terminus and the 12,600-dalton protein contains three copies of [Met]enkephalin, of which two are internal and the third is at the COOH terminus. They possess identical NH_2-terminal amino acid sequences, suggesting that the 8600-dalton protein is derived from the 12,600-dalton protein by intracellular proteolytic processing. This is supported by results from tryptic maps of both proteins. Furthermore, chemical analysis of the tryptic peptides obtained from the 12,600-dalton protein indicates that it also contains the amino acid sequence that corresponds to a previously characterized enkephalin-containing polypeptide of 3800 daltons (peptide F) [Jones *et al.* (1980) *Arch. Biochem. Biophys.* 204, 392–395]. All three polypeptides appear to be intermediates in posttranslational processing of a still larger polyenkephalin precursor molecule, proenkephalin, and part of a biosynthetic pathway leading to smaller enkephalin-containing polypeptides and free enkephalins.

Many enkephalin-containing polypeptides (ECPs) have been isolated from bovine adrenal medulla (1–5). Most of the smaller [0.5- to 1-kilodalton (kDal)] and intermediate-sized (2- to 5-kDal) ECPs have been purified to homogeneity and some have had their amino acid sequence completely determined (6–13). This report presents structural data on two of the larger ECPs, those of masses 8.6 and 12.6 kDal. The approach chosen for the study of these two proteins was to determine their NH_2- and COOH-terminal amino acid sequences. The amino acid sequence of the central portion of each protein was not determined but was characterized by the tryptic peptides obtained from this region. Sequence information was also expected to come from cloning the cDNA obtained from proenkephalin mRNA, which was being carried on in our laboratories concurrently (see *Discussion*). The structural data obtained by this strategy show a direct precursor–product relationship between the two ECPs as well as to a previously characterized 3.8-kDal ECP (8). These observations provide additional details of the precursors and intermediates in the proenkephalin biosynthetic pathway (9).

MATERIALS AND METHODS

Purification of the 8.6- and 12.6-kDal ECPs from chromaffin granules of bovine adrenal medulla has been described (7). Homogeneity of the polypeptides was established by $NaDodSO_4$ gel electrophoresis (14) and manual Edman degradation (6). Both proteins were reduced and carboxymethylated prior to chemical characterization, tryptic mapping, and microsequence analysis. This was accomplished by dissolving the protein (5 nmol) in 500 μl of 0.5 M ammonium acetate, pH 8/6 M guanidine hydrochloride containing 2 μmol of dithiothreitol and incubating the mixture at room temperature for 1 hr. After reductive cleavage of the disulfide bonds, sodium monoiodoacetate (4 μmol) was added, and the resulting solution was incubated in the dark at room temperature for 2 hr. The reaction was terminated by addition of 2-mercaptoethanol, the pH was decreased to 4.0 with acetic acid, and the mixture was applied to a Zorbax trimethylsilane column (DuPont; 6 μm; pore size, 10 nm) that had been equilibrated with 0.9 M pyridine acetate buffer (pH 4.0). The column was then washed with 20 ml of the same buffer to remove excess reagents, buffer salts, and denaturing agent. The carboxymethylated protein was removed from the HPLC column by eluting with 40% (vol/vol) 1-propanol/0.9 M pyridine acetate, pH 4.0. The column effluent was monitored by a fluorescamine detection system (15).

Tryptic digestion of the carboxymethylated proteins and isolation of the tryptic peptides by HPLC on an Ultrasphere octadecylsilane column (Altex; 5 μm; pore size, 10 nm) were carried out as reported (5, 16) with the following modifications. Prior to HPLC analysis, the pH of the digestion mixture was adjusted to 4.0 with acetic acid, 2-mercaptoethanol (20 μl) was added, and the mixture was heated for 20 min at 80°C. This procedure reduced any methionine residues that oxidized during the digestion period. At this point, ^{125}I-labeled [Leu]enkephalin (≈2000 cpm) was added to the digestion mixture to act as an internal standard for the chromatography runs. The tryptic peptides obtained were assayed for opioid activity after treatment with carboxypeptidase B (5). Opioid activity was measured by a radioreceptor assay that used neuroblastoma glioma hybrid cells with tritiated [Leu]enkephalin as the competing ligand (17). Amino acid analyses were carried out at the picomole level with a fluorescamine amino acid analyzer (18). Carboxypeptidase Y and aminopeptidase M time-course hydrolyses were carried out according to the procedure of Jones *et al.* (19). Identification and quantitation of free amino acids in the digestion mixtures were carried out by HPLC analysis using precolumn fluorescence derivatization with *o*-phthaldialdehyde (19). Automated Edman degradations were carried out in a modified Beckman 890C sequencer (20, 21) using 2 nmol of the 8.6-kDal ECP or 1 nmol of the 12.6-kDal ECP. Phenylthiohydantoin derivatives of amino acids were analyzed by HPLC on Zorbax octadecylsilane or cyanopropylsilane columns (DuPont) with

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Abbreviations: ECP, enkephalin-containing protein or polypeptide; kDal, kilodalton.

absorbance monitored at 254 and 313 nm on a Waters Associates chromatograph (22, 23).

RESULTS

Both purified proteins gave single bands on $NaDodSO_4$ slab gel electrophoresis. As further evidence of homogeneity, each protein was subjected to manual Edman degradation, and glutamic acid was established as the NH_2-terminal residue of both.

Since earlier studies had indicated the presence of cysteine residues (2, 7), both proteins were subjected to carboxymethylation. After this modification, their amino acid compositions were determined (Table 1). These analyses differ somewhat from those previously published for the two proteins (2, 7); however, the differences are significant only for tryptophan, proline, and cysteine. The first two can present special problems for quantitation by the fluorescamine amino acid analyzer (18). As for cysteine, in the earlier studies the ECPs were not reduced and carboxymethylated as was done here. This procedure yields more reliable data. Because of the differences in amino acid analyses, the calculated molecular weights of the two ECPs differ from those reported previously (2, 7).

The 8.6-kDal carboxymethylated ECP was digested with trypsin and subjected to HPLC. The resulting tryptic map is shown in Fig. 1*A*. Two of the tryptic peptides exhibited opioid receptor-binding activity. One (T9-7) was obtained in a yield of 40% and was chromatographically identical to and had the same amino acid composition as synthetic [Met]enkephalin (Tyr-Gly-Gly-Phe-Met). Since this peptide contained neither lysine nor arginine, it is most likely derived from the COOH terminus of the protein. The other tryptic peptide (T9-8) exhibited less opioid-binding activity (Fig. 1*A*) than peptide T9-7 even though it was isolated in higher yield (49%). The amino acid composition of the peptide was identical to that of peptide T9-7 except for the presence of an additional lysine residue. Since both peptides had been treated with carboxypeptidase B prior to the binding assay, the structural difference between the two peptides must be restricted to the NH_2 terminus. This was confirmed by carboxypeptidase Y and aminopeptidase M time-course hydrolyses, which established the structure of peptide T9-8 as Lys-Tyr-Gly-Gly-Phe-Met. This unexpected peptide must also have been derived from the COOH terminus of the 8.6-kDal ECP because it does not possess a COOH-terminal lysine or arginine residue. Thus, both peptides (T9-7 and T9-8) appear to have been derived from the same [Met]enkephalin sequence by differential trypsin action. If this is the case, then tryptic digestion should have released free lysine in a yield comparable with that of peptide T9-7. Amino acid analysis confirmed the presence of free lysine (T9-1) in the digestion mixture. The yield was 44%. Since no other tryptic peptides exhibited opioid binding activity, the 8.6-kDal ECP must contain a single [Met]enkephalin sequence.

Table 1. Amino acid compositions of the 3.8-kDal, 8.6-kDal, and 12.6-kDal ECPs

Amino acid	3.8-kDal peptide (peptide F)	8.6-kDal protein	12.6-kDal protein
Asx	2	4.06 (4)	5.90 (6)
Thr	0	6.87 (7)	6.77 (7)
Ser	0	5.65 (6)	5.60 (6)
Glx	6	10.98 (11)	16.50 (17)
Pro	1	5.25 (5)	6.38 (6)
Gly	7	2.75 (3)	9.58 (10)
Ala	1	6.10 (6)	6.61 (7)
CMC	0	5.65 (6)	5.69 (6)
Val	2	0.00 (0)	2.01 (2)
Met	3	0.93 (1)	3.75 (4)
Ile	0	0.00 (0)	0.07 (0)
Leu	3	14.09 (14)	17.25 (17)
Tyr	3	1.95 (2)	4.93 (5)
Phe	2	1.05 (1)	3.10 (3)
His	0	1.05 (1)	1.12 (1)
Lys	3	6.50 (7)	11.04 (11)
Arg	1	2.12 (2)	4.19 (4)
Trp	0	0.75 (1)	0.71 (1)
Total	34	77	113

Samples (100–200 pmol) were hydrolyzed at 110°C for 22 hr in 200 μl of constant boiling HCl. Tryptophan was determined by hydrolysis in the presence of constant boiling HCl and 4% (vol/vol) thioglycolic acid. Results for the 8.6-kDal and 12.6-kDal proteins represent means of 10 analyses. The amino acid composition of the 3.8-kDal polypeptide was obtained from its reported amino acid sequence (8). Values in parentheses represent theoretical amino acid composition. CMC, carboxymethylcysteine.

The COOH-terminal sequence of the 8.6-kDal ECP was determined by carboxypeptidase Y time-course hydrolysis. The release data established the first seven residues as Lys-Lys-Tyr-Gly-Gly-Phe-Met-COOH. This sequence is in complete agreement with the results obtained by analysis of the tryptic peptides. The NH_2-terminal sequence of the protein was determined by automated Edman degradation. Unambiguous results were obtained for the first 36 cycles of degradation (Fig. 2).

Tryptic digestion of the carboxymethylated 12.6-kDal ECP produced five peptides having opioid receptor-binding activity (Fig. 1*B*). After amino acid analysis and enzymatic time-course peptidase procedures, one tryptic peptide (T13-10) was found to be free [Met]enkephalin and another (T13-11) was found to be [Met]enkephalin with an NH_2-terminal arginine residue. The yields of these peptides were 92% and 8%, respectively. Since neither possessed a COOH-terminal lysine or arginine, both must have been derived from the [Met]enkephalin sequence at the COOH terminus of the 12.6-kDal ECP. This was confirmed by carboxypeptidase Y time-course hydrolysis of the intact protein, which established the COOH-terminal sequence of the 12.6-kDal ECP as Lys-Arg-Tyr-Gly-Gly-Phe-Met-COOH. Sequence analysis of the other tryptic peptides having opioid receptor-binding activity yielded the structures Tyr-Gly-Gly-Phe-Met-Lys (T13-7), Lys-Tyr-Gly-Gly-Phe-Met-Lys (T13-8), and Lys-Tyr-Gly-Gly-Phe-Met-Lys-Arg (T13-9). Since each enkephalin sequence is followed by a lysine residue, all of them must be located internally in the 12.6-kDal ECP. Furthermore, the total yield of the three was 170%, indicating the presence of more than one internal enkephalin sequence. From these data, it was concluded that the 12.6-kDal ECP must contain two internal copies of the [Met]enkephalin sequence in addition to the [Met]enkephalin located at its COOH terminus.

Examination of the tryptic maps of the two ECPs showed a clear correlation between the nonenkephalin tryptic peptides produced (those that did not have receptor-binding activity) from each protein; i.e., all of the nonenkephalin peptides produced by the 8.6-kDal ECP (Fig. 1*A*) were also produced by the 12.6-kDal ECP (Fig. 1*B*). This correlation was based not only on elution times, peak shapes, and peak areas but also on the amino acid compositions of the individual tryptic peptides (Table 2). It therefore seemed likely that the 8.6-kDal ECP is derived from the 12.6-kDal ECP. This was verified by automated Edman degradation of the 12.6-kDal ECP, which established the NH_2-terminal amino acid sequence of the molecule to be identical to that of the 8.6-kDal ECP. Thus, the 8.6-kDal

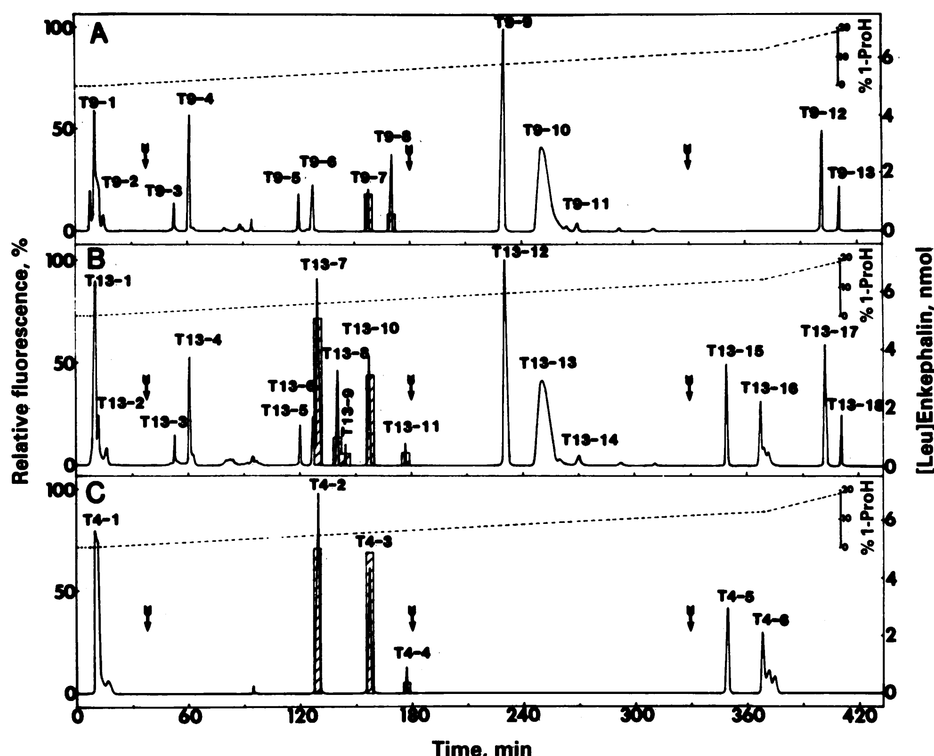

FIG. 1. Tryptic maps of 8.6-kDal carboxymethylated ECP (*A*), 12.6-kDal carboxymethylated ECP (*B*), and 3.8-kDal ECP (*C*). In each case, 4 nmol of purified ECP in 200 μl of 0.2 M phosphate buffer (pH 8.2) was digested with 1 μg of trypsin for 18 hr at 37°C. After digestion, the pH of the mixture was adjusted to 4.0 with acetic acid and 2-mercaptoethanol was added to reduce any methionine residues oxidized. Then, ^{125}I-labeled [Leu]enkephalin ($\approx$2000 cpm) was added as an internal standard; this material produced three reproducible peaks of radioactivity (↕). The peptides were separated by HPLC on an Ultrasphere octadecylsilyl column (particle size, 5 μm; 4.6 × 250 mm) using a linear gradient of 1-propanol (1-PrOH) (----) in 0.9 M pyridine acetate (pH 4.0) at a flow rate of 20 ml/hr. Five percent of the column effluent was diverted to the detection system. Aliquots of each fraction were digested with carboxypeptidase B and assayed for receptor-binding activity. Tryptic peptides are numbered according to their elution position; T9-x, those from the 8.6-kDal ECP; T13-x, those from the 12.6-kDal ECP; T4-x, those from the 3.8-kDal ECP. ▨, [Leu]Enkephalin equivalents; ——, relative fluorescence.

ECP must represent the first 77 residues of the 12.6-kDal ECP (see Table 1 and Fig. 2).

In an attempt to determine the nature of the remaining residues in the 12.6-kDal ECP, previously characterized adrenal enkephalin-containing polypeptides (20–23) were examined. One of these polypeptides (peptide F; ref. 8) was selected as possibly representing the COOH-terminal region of the 12.6-kDal ECP. The selection of this polypeptide was based on its mass of 3.8 kDal and the fact that it contains two copies of [Met]enkephalin, one at the NH_2 terminus and the other at the

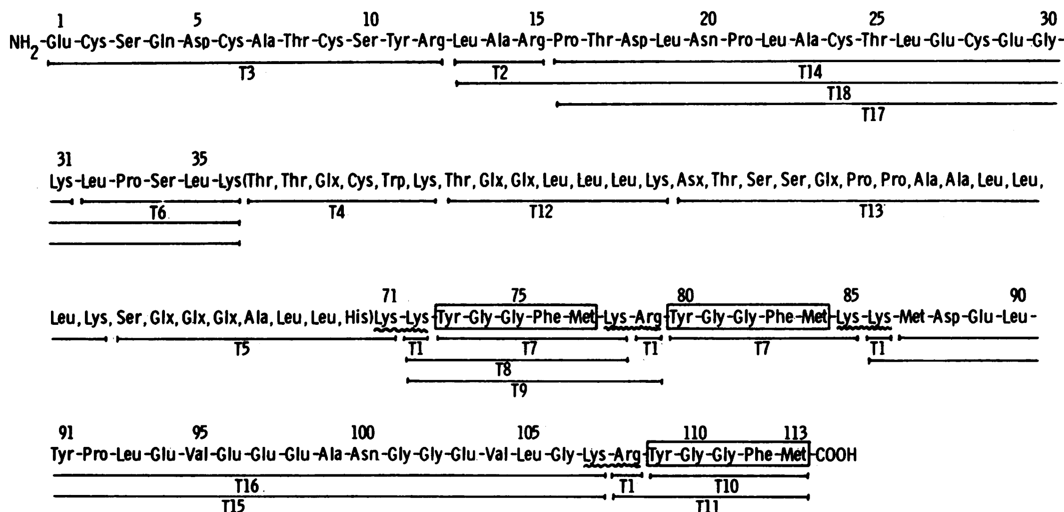

FIG. 2. Tentative structure of the 12.6-kDal ECP and alignment of its tryptic peptides (the order of peptides T4, T12, T13, and T5 is arbitrary because their exact relationships to each other cannot be deduced from the present data). Residues 1–77 represent the tentative structure of the 8.6-kDal ECP and residues 80–113 represent the reported amino acid sequence of the 3.8-kDal ECP (8). [Met]Enkephalin sequences are boxed. 〰, Possible sites for intracellular proteolytic processing of the 12.6-kDal ECP.

Table 2. Summary of nonenkephalin tryptic peptides isolated from the 12.6-kDal, 8.6-kDal, and 3.8-kDal polypeptides

Shared residues	12.6-kDal ECP		8.6-kDal ECP		3.8-kDal ECP	
	No.	Yield, %	No.	Yield, %	No.	Yield, %
Ala,Leu,Arg	T13-2	(65)	T9-2	(60)	NP	—
Asx,Thr,Ser_2,Glx_2,Ala,CMC_3,Tyr,Arg	T13-3	(76)	T9-3	(88)	NP	—
Thr_2,Glx,CMC,Trp,Lys	T13-4	(61)	T9-4	(74)	NP	—
Ser,Glx_3,Ala,Leu_2,His,Lys	T13-5	(76)	T9-5	(76)	NP	—
Ser,Pro,Leu_2,Lys	T13-6	(11)	T9-6	(10)	NP	—
Thr,Glx_2,Leu_3,Lys	T13-12	(95)	T9-9	(96)	NP	—
Asx,Thr,Ser_2,Glx,Pro_2,Ala_2,Leu_3,Lys	T13-13	(73)	T9-10	(82)	NP	—
Asx_2,Thr_2,Glx_2,Pro_2,Gly,Ala,CMC_2,Leu_3,Lys	T13-14	(8)	T9-11	(10)	NP	—
Asx_2,Glx_6,Pro,Gly_3,Ala,Tyr,Met,Val_2,Leu_3,Lys_2	T13-15	(45)	NP	—	T4-5	(42)
Asx_2,Glx_6,Pro,Gly_3,Ala,Tyr,Met,Val_2,Leu_3,Lys	T13-16	(30)	NP	—	T4-6	(34)
Asx_2,Thr_2,Ser,Glx_2,Pro_3,Gly,Ala,CMC_2,Leu_5,Lys_2	T13-17	(50)	T9-12	(44)	NP	—
Asx_2,Thr_2,Ser,Glx_2,Pro_3,Gly,Ala_2,CMC_2,Leu_6,Lys_2,Arg	T13-18	(5)	T9-13	(8)	NP	—

Designations for the tryptic peptides are as in Fig. 1. NP, not present; CMC, carboxymethylcysteine.

COOH terminus (8). Also, when its amino acid composition is added to that of the 8.6-kDal ECP, the composition of the 12.6-kDal ECP (with the exception of lysine and arginine) is obtained (Table 1). That the 3.8-kDal polypeptide is indeed derived from the 12.6-kDal protein is shown by comparison of their tryptic maps (Fig. 1 *B* and *C*) and by the amino acid composition of their shared tryptic peptides (Table 2). Confirmation that the 3.8-kDal polypeptide represents the COOH terminus of the 12.6-kDal protein was established by carboxypeptidase Y digestion of the intact protein. The order of amino acids released by carboxypeptidase action was consistent with the reported COOH-terminal sequence of the 3.8-kDal ECP, Glu-Val-Leu-Gly-Lys-Arg-Tyr-Gly-Gly-Phe-Met-COOH (8).

A final observation can be made about the structure of the 12.6-kDal ECP based on all of the above data. The two internal [Met]enkephalin sequences in the 12.6-kDal ECP must be linked in tandem by a Lys-Arg sequence. Furthermore, the first [Met]enkephalin sequence in the series represents the COOH terminus of the 8.6-kDal ECP and the second [Met]enkephalin sequence represents the NH_2 terminus of the 3.8-kDal ECP. Thus, the 8.6-kDal ECP is connected to the 3.8-kDal ECP via a Lys-Arg linkage to form the 12.6-kDal ECP. The tentative structure for the 12.6-kDal ECP presented in Fig. 2 is consistent with the available data from amino acid analyses, tryptic mapping, and microsequence analyses.

DISCUSSION

We have reported previously the presence of free enkephalins and a number of ECPs in extracts of bovine adrenal chromaffin granules (2, 3). Many of the ECPs contain more than one copy of enkephalin and a few contain both [Met]- and [Leu]enkephalin. The smaller ECPs include hexa-, hepta-, and octapeptides and ECPs of 3.2, 3.6, 3.8, 4.9, 5.3, 8.6, and 12.6 kDal (1, 7–11, 24). Larger ECPs, 20–30 kDal, have been isolated and partially characterized (unpublished observations). Evidence has also been presented for an even larger ECP (40–50 kDal) that contains both [Met]- and [Leu]enkephalin in a ratio of ≈6:1 (5). These findings suggest that an enkephalin biosynthetic pathway

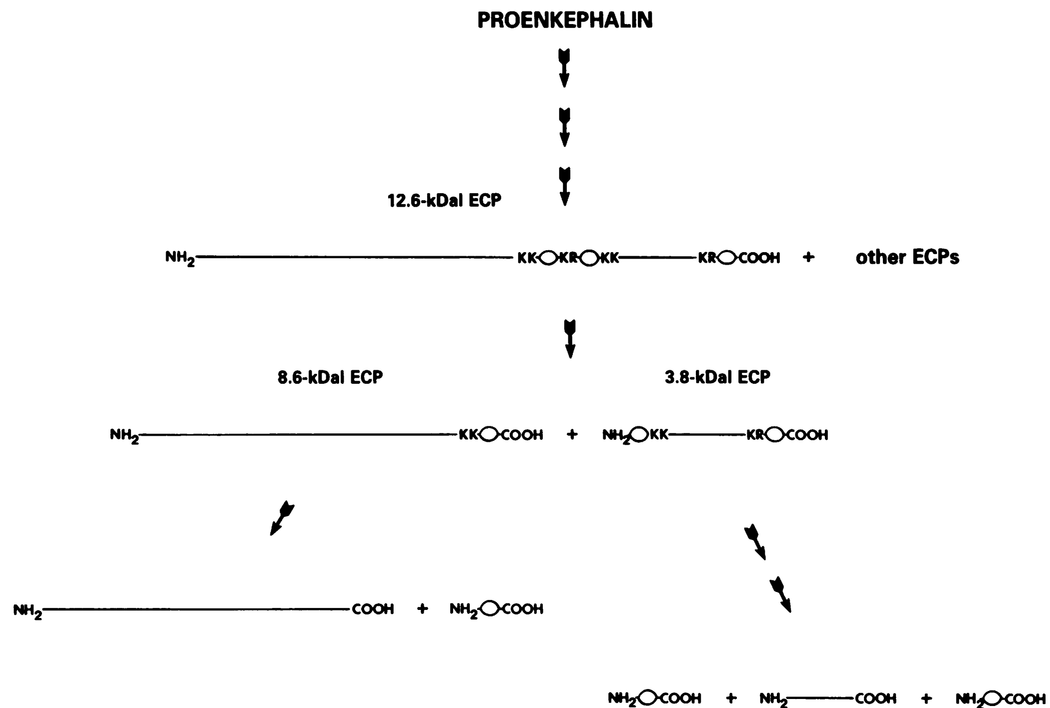

FIG. 3. Possible steps in the enkephalin biosynthetic pathway. O, [Met]Enkephalin; K, lysine; R, arginine.

exists in the adrenal medulla that begins with a single gene product, a precursor protein, proenkephalin, containing both [Met]- and [Leu]enkephalin. More direct support for a gene product of the size of proenkephalin comes from the recent characterization of mRNA from bovine adrenal medulla that hybridizes to a synthetic decahexanucleotide probe based on a nonenkephalin sequence in the 4.9-kDal ECP (25). This proenkephalin mRNA contained ≈1500 bases, consistent with a gene product of 40–50 kDal.

Posttranslational processing of proenkephalin would yield the enkephalin-containing intermediates described above as well as free enkephalins. Other evidence for such a pathway comes from pulse–chase experiments (26) and from the order of appearance of the ECPs in the rat adrenal gland after denervation (27).

In an earlier report, evidence was presented that the 3.2-kDal ECP was derived from the 4.9-kDal ECP (10); however, little else has been known concerning the exact structural relationships among the various enkephalin-containing intermediates. In this report, structural evidence from the 8.6-kDal and 12.6-kDal ECPs demonstrates their relationship to one another as well as to the 3.8-kDal ECP. From these results, some additional steps in the enkephalin biosynthetic pathway can be postulated (Fig. 3); as shown, the proenkephalin molecule is initially processed through several steps to form the 12.6-kDal ECP and other ECPs of intermediate size. The 12.6-kDal ECP undergoes further processing to yield the 8.6-kDal ECP and the 3.8-kDal ECP. The 8.6-kDal ECP can be processed to yield one copy of free [Met]enkephalin; the 3.8-kDal ECP can yield two copies of free [Met]enkephalin.

For such a pathway to function, the processing mechanism must proceed by specific proteolytic cleavages at strategic sites. These sites can be identified as paired basic residues (lysine and/or arginine) in the 12.6-kDal ECP (Figs. 2 and 3). Structural data from the other ECPs also supports this conclusion. Additional data from the DNA sequence of the proenkephalin gene (28) corroborate the sequences of the ECPs that we have characterized and show the order in which they occur in the proenkephalin molecule. As might be expected, the various ECPs are separated from one another by Lys-Lys, Arg-Arg or Lys-Arg linkages, which are recognized processing sites of other prohormones (29).

We thank Dr. S. Stein for his helpful discussions. We also thank Ms. L. D. Gerber, Mr. L. Brink, Ms. D. Woehle, and Mr. D. Hawke for their excellent technical assistance and Mrs. C. Wright for preparation of this manuscript.

1. Stern, A. S., Lewis, R. V., Kimura, S., Rossier, J., Stein, S. & Udenfriend, S. (1980) *Arch. Biochem. Biophys.* **205**, 606–613.
2. Stern, A. S., Lewis, R. V., Kimura, S., Kilpatrick, D. L., Jones, B. N., Kojima, K., Stein, S. & Udenfriend, S. (1980) in *Biosynthesis, Modification, and Processing of Cellular and Viral Polypeptides*, eds. Koch, G., and Richter, D. (Academic, New York), pp. 99–110.
3. Lewis, R. V., Stern, A. S., Rossier, J., Stein, S. & Udenfriend, S. (1979) *Biochem. Biophys. Res. Commun.* **89**, 822–829.
4. Clement-Jones, V., Corder, R. & Lowry, P. J. (1980) *Biochem. Biophys. Res. Commun.* **95**, 665–673.
5. Lewis, R. V., Stern, A. S., Kimura, S., Rossier, J., Stein, S. & Udenfriend, S. (1980) *Science* **208**, 1459–1461.
6. Kimura, S., Lewis, R. V., Stern, A. S., Rossier, J., Stein, S. & Udenfriend, S. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 1681–1685.
7. Lewis, R. V., Stern, A. S., Kimura, S., Stein, S. & Udenfriend, S. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 5018–5020.
8. Jones, B. N., Stern, A. S., Lewis, R. V., Kimura, S., Stein, S., Udenfriend, S. & Shively, J. E. (1980) *Arch. Biochem. Biophys.* **204**, 392–395.
9. Stern, A. S., Jones, B. N., Shively, J. E., Stein, S. & Udenfriend, S. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 1962–1966.
10. Kilpatrick, D. L., Taniguchi, T., Jones, B. N., Stern, A. S., Shively, J. E., Hullihan, J., Kimura, S., Stein, S. & Udenfriend, S. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 3265–3268.
11. Jones, B. N., Shively, J. E., Kilpatrick, D. L., Kojima, K. & Udenfriend, S. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 1313–1315.
12. Mizuno, K., Minamino, N., Kangawa, K. & Matsuo, H. (1980) *Biochem. Biophys. Res. Commun.* **97**, 1283–1290.
13. Mizuno, K., Minamino, N., Kangawa, K. & Matsuo, H. (1980) *Biochem. Biophys. Res. Commun.* **95**, 1482–1488.
14. Kimura, S., Lewis, R. V., Gerber, L. D., Brink, L., Rubinstein, M., Stein, S. & Udenfriend, S. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 1756–1759.
15. Böhlen, P., Stein, S., Stone, J. & Udenfriend, S. (1975) *Anal. Biochem.* **67**, 438–445.
16. Rubinstein, M., Chen-Kiang, S., Stein, S. & Udenfriend, S. (1979) *Anal. Biochem.* **95**, 117–121.
17. Gerber, L. D., Stein, S., Rubinstein, M., Wideman, J. & Udenfriend, S. (1978) *Brain Res.* **151**, 117–126.
18. Stein, S., Böhlen, P., Stone, J., Dairman, W. & Udenfriend, S. (1973) *Arch. Biochem. Biophys.* **155**, 203–212.
19. Jones, B. N., Pääbo, S. & Stein, S. (1981) *J. Liq. Chromatogr.* **4**, 565–586.
20. Wittman-Liebold, B. (1973) *Hoppe-Seyler's Z. Physiol. Chem.* **354**, 1415–1431.
21. Hunkapiller, M. W. & Hood, L. E. (1980) *Science* **207**, 523–525.
22. Zimmerman, C. L., Appella, E. & Pisano, J. J. (1976) *Anal. Biochem.* **75**, 77–85.
23. Johnson, N. D., Hunkapiller, M. W. & Hood, L. E. (1979) *Anal. Biochem.* **100**, 335–338.
24. Kilpatrick, D. L., Jones, B. N., Kojima, K. & Udenfriend, S. (1981) *Biochem. Biophys. Res. Commun.* **103**, 698–705.
25. Gubler, U., Kilpatrick, D. L., Seeburg, P. H., Gage, L. P. & Udenfriend, S. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 5484–5487.
26. Rossier, J., Trifaro, J. M., Lewis, R. V., Lee, R. W. H., Stern, A. S., Kimura, S., Stein, S. & Udenfriend, S. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 6889–6891.
27. Lewis, R. V., Stern, A. S., Kilpatrick, D. L., Gerber, L. D., Rossier, J., Stein, S. & Udenfriend, S. (1981) *J. Neurosci.* **1**, 80–82.
28. Gubler, U., Seeburg, P. H., Gage, L. P. & Udenfriend, S. (1982) *Nature (London)* **295**, 206–208.
29. Steiner, D. F., Quinn, P. S., Chan, S. J., Marsh, J. & Tager, H. S. (1980) *Ann. N.Y. Acad. Sci.* **343**, 1–16.