Two adrenal opioid polypeptides: Proposed intermediates in the processing of proenkephalin

(encephalin/opioid peptide/adrenal medulla/microsequence determination)

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ABSTRACT Two enkephalin-containing polypeptides of 3600 and 4900 daltons have been isolated from extracts of bovine adrenal medulla, purified to homogeneity, and analyzed by a combination of automated Edman degradation and enzymatic time course hydrolysis. The 4900-dalton polypeptide contains two copies of enkephalin, one an internal [Met]enkephalin sequence, the other a [Leu]enkephalin sequence at the carboxyl terminus. Sequence analysis of the 3600-dalton polypeptide has not been completed, but the polypeptide has been shown to contain a single [Met]enkephalin sequence followed by an -Arg-Phe linkage that forms the carboxyl terminus of the molecule. On the basis of these and other findings, we propose that the above enkephalin-containing polypeptides are intermediates in the biosynthesis of the enkephalins and that they are generated by posttranslational processing from a large multivalent enkephalin precursor molecule, proenkephalin. The term "multivalent" is used to indicate a polypeptide with many identical functional sequences.

Recent studies on the biosynthetic pathway of the opioid penta-peptides, [Met]enkephalin and [Leu]enkephalin, have led to the isolation of a number of enkephalin-containing polypeptides from bovine adrenal chromaffin granules (1, 2). These range in size from hexapeptides to polypeptides of approximately 50 kilodaltons (kDal). All but a few have been purified to homogeneity and some have had their amino acid sequences completely determined (3–8). The presence of this series of enkephalin-containing polypeptides of decreasing size suggests that we are dealing with precursors and intermediates in a biosynthetic pathway leading to the enkephalins. This pathway may be common to enkephalin-containing tissues such as adrenal medulla, brain (9, 10), and intestine (10). Direct evidence for such a pathway comes from pulse-chase experiments (11) and from the order of appearance of the enkephalin-containing intermediates in the rat adrenal gland after denervation (12). This report presents structural data on two enkephalin-containing polypeptides of mass 3.6 and 4.9 kDal, obtained from adrenal chromaffin granules. These two polypeptides are proposed intermediates in the formation of free enkephalin from a large multivalent precursor, proenkephalin. The term "multivalent" is used to indicate a polypeptide with many identical functional sequences.

EXPERIMENTAL PROCEDURES

Isolation, from chromaffin granules of bovine adrenal medulla, of the 4.9-kDal enkephalin-containing polypeptide has been described (4). The 3.6-kDal enkephalin-containing polypeptide (shown as peptide B in ref. 4) was purified by the same procedures except that a diphenyl reverse-phase high-performance liquid chromatography (HPLC) column (10 μm, 10 nm pore size) (13, 14) was employed for final purification. Tryptic digestion of the purified 3.6-kDal polypeptide and isolation of the resulting tryptic peptides by HPLC were performed as reported (4). The tryptic peptides were treated with carboxypeptidase B as described (14), and opioid activity was measured by a radioreceptor binding assay (15). Cyanogen bromide cleavage of the purified 4.9-kDal polypeptide was performed according to the procedure of Jones and Gurd (16). Amino acid analyses were performed with fluorescamine detection (17). The time courses of hydrolyses by aminopeptidase M and carboxypeptidase Y were studied according to the procedures of Light (18) and Hayashi (19), respectively. Identification and quantitation of free amino acids in the digestion mixtures were accomplished by HPLC analysis using precolumn fluorescence derivatization with o-phthaldialdehyde (20). Automated Edman degradations were performed in a modified Beckman 890C sequencer (21, 22) on 2–5 nmol samples. Phenylthiohydantoin derivatives of amino acids were analyzed by HPLC on Du Pont Zorbax octadeylsiline and cyanopropsilsline columns, using a Waters Associates chromatography system (23, 24). Complete details of the sequence determination of these and other enkephalin-containing polypeptides will be presented elsewhere.

RESULTS

Isolation and Purification of Opioid Peptides from Adrenal Medullary Granules. Chromatography of extracts of adrenal medullary chromaffin granules on Sephadex G-75 separates enkephalin-containing polypeptides according to size (1, 3). The fraction corresponding to 2–5 kDal contains several enkephalin-containing polypeptides as determined by HPLC (Fig. 1A). Purification of the 3.8- and 4.9-kDal polypeptides (peptides F and I, Fig. 1A) has been reported as well as the complete amino acid sequence of the 3.8-kDal polypeptide (4, 8). Another enkephalin-containing polypeptide obtained by RP-18 chromatography (peptide B, Fig. 1A) was further purified on an Ultrasphere octadeylsiline column (Fig. 1B). Chromatography of the most active fractions on a diphenyl reverse-phase column yielded a symmetrical peak coinciding with opioid activity (Fig. 1C). Amino acid analysis was performed on each of the fractions within the peak area to confirm the homogeneity of the peptide. A single peak was again obtained when the polypeptide was rechromatographed on the same diphenyl reverse-phase column. End group analysis revealed a single amino acid (phenylalanine), confirming homogeneity. The molecular weights of 3600 and 4900, calculated from amino acid analysis of the two polypeptides (data not shown), were in accord with their elution positions on gel chromatography.

Abbreviations: kDal, kilodalton(s); HPLC, high-performance liquid chromatography.
and phenylalanine in approximately equimolar amounts. The 3.6-kDal polypeptide was also subjected to carboxypeptidase Y time course hydrolysis, which established the carboxyl-terminal amino acid sequence as -Gly-Phe-Met-Arg-Phe-COOH. These findings indicate that the 3.6-kDal enkephalin-containing polypeptide contains a [Met]enkephalin-Arg\textsuperscript{6} sequence near the carboxy terminus of the molecule and that the hexapeptide is followed by phenylalanine. On the basis of the amino acid composition (i.e., 2 Arg and 2 Lys) and the release of free arginine by trypsin, the [Met]enkephalin-Arg\textsuperscript{6}-Phe\textsuperscript{16} sequence is most likely preceded by Lys-Arg. When the amino-terminal amino acid sequence of this polypeptide was determined with automated Edman degradation, unambiguous results were obtained for the first 11 cycles. On the basis of all the above data, a tentative partial sequence for the 3.6-kDal enkephalin-containing polypeptide was deduced (Fig. 3).

Sequence Analysis of the 4.9-kDal Enkephalin-Containing Polypeptide. Separation of the tryptic peptides from the 4.9-kDal polypeptide and its partial analysis were reported previously (4, 8). Results from those studies indicated that the peptide contains a [Leu]enkephalin sequence located at the carboxy terminus that is preceded by lysine or arginine and a [Met]enkephalin sequence in the internal portion of the peptide preceded by lysine or arginine and followed by arginine. The remaining primary structure of the polypeptide has now been elucidated from tryptic and cyanogen bromide fragments prepared and purified by HPLC (data not shown). Examination of the amino acid compositions of the resulting fragments revealed the presence of tryptophan, which had not been reported for the intact polypeptide (4). When the amino acid composition of the 4.9-kDal polypeptide was redetermined utilizing hydrolysis conditions more favorable to the analysis of tryptophan, the findings verified the presence of two tryptophan residues.

From the amino acid composition, as well as carboxypeptidase Y and aminopeptidase M time course hydrolyses of the cyanogen bromide fragments and their chemical sequencing (data not shown), the remainder of the primary structure of the polypeptide was obtained. The complete primary structure of the 4.9-kDal enkephalin-containing polypeptide is presented in Fig. 3.

DISCUSSION

It is now apparent that the two opioid peptides [Met]enkephalin and [Leu]enkephalin, discovered in 1975 by Hughes et al. (25), are produced via a pathway that does not include \(\beta\)-endorphin (1, 26). The enkephalin pathway apparently begins with a large enkephalin-containing polypeptide (ca. 50 kDal) that has been reported previously (3). Evidence for an enkephalin-containing protein of this size has also been reported by other laboratories (9, 27). This protein, which we have thus far purified several-fold, may well be the primary product of translation of the en-

![Figure 1](image1.png)

**Fig. 1.** Purification of the 3.6-kDal enkephalin-containing polypeptide. (A) Pooled fractions corresponding to 2–5 kDal from a Sephadex G-75 chromatography of an acid extract of 9.5 g of chromaffin granules were applied to a LiChrosorb RP-18 column (10 \(\mu\)m, 4.6 \(\times\) 250 mm) at a rate of 80 ml/hr. The column was washed with 20 ml of starting buffer (0.5 M formic acid/0.4 M pyridine, pH 4.0) and peptides were eluted with a gradient of 1-propanol (—-—) in the same buffer at a rate of 30 ml/hr. Four percent of the column effluent was diverted to the detection system. Aliquots of each fraction (3 min) were digested with trypsin and carboxypeptidase B and assayed for opioid activity. B, peptide B; F, peptide F (3.8 kDal); I, peptide I (4.9 kDal). (B) The fractions corresponding to the 3.6-kDal enkephalin-containing polypeptide (shown as peptide B) were pooled and lyophilized. The peptides were redissolved in the above starting buffer and were then injected onto an Ultrasphere octadecylsilane column (4.6 \(\times\) 250 mm). Peptides were eluted at a flow rate of 20 ml/hr and assayed as in A. (C) The fractions containing the major activity were pooled, lyophilized, and chromatographed on a diphenyl reverse-phase column (10 \(\mu\)m, 4.6 \(\times\) 250 mm) as in B. Hatched bars, [Leu]enkephalin equivalents; ——, relative fluorescence.

![Figure 2](image2.png)

**Fig. 2.** Tryptic peptides derived from the 3.6-kDal enkephalin-containing polypeptide. After treatment with trypsin, the digests were applied to a LiChrosorb RP-18 column (10 \(\mu\)m, 4.6 \(\times\) 250 mm). The column was eluted at 30 ml/hr with 0.5 M formic acid/0.4 M pyridine, pH 4.0, using a gradient of 1-propanol (—-—). Aliquots of each fraction were digested with carboxypeptidase B and assayed for opioid-receptor binding activity. Hatched bars, [Leu]enkephalin equivalents; ——, relative fluorescence; arrows, synthetic standards (Enk, enkephalin).
3.6 kDal Enkephalin-Containing Polypeptide:

1  5  10
Phe-Ala-Glu-Pro-Leu-Pro-Ser-Glu-Glu-Glu-Gly(Ser,Glx,Glx,Glx,Glx,Pro,Val,Met,Tyr,
25  30
Lys)Lys-Arg-Tyr-Gly-Gly-Phe-Met-Arg-Phe

4.9 kDal Enkephalin-Containing Polypeptide:

1  5  10  15  20
Ser-Pro-Thr-Leu-Glu-Asp-Glu-His-Lys-Glu-Leu-Gln-Lys-Arg-Tyr-Gly-Gly-Phe-Met-Arg-
25  30  35  39

FIG. 3. Primary structures of the two polypeptides.

Enkephalin gene. The protein contains as many as seven [Met]enkephalin sequences per [Leu]enkephalin sequence (3). Because the ratio was obtained on material that had not been purified substantially, the actual amounts may be as high as 14 [Met]enkephalins and 2 [Leu]enkephalins per molecule. Processing of such a multiple enkephalin-containing (multivalent) proenkephalin by proteases and peptidases would be expected to produce numerous enkephalin-containing intermediates of decreasing size and ultimately free enkephalins. Many such enkephalin-containing compounds have been isolated and characterized (3).

The data obtained thus far from sequence analysis and trypsin mapping of the enkephalin-containing polypeptides isolated from adrenal extracts confirm the presence of typical recognition sites for precursor processing (28). Each internal enkephalin sequence that we have found is bracketed by residues that require first a trypsin-like cleavage for the release of the enkephalin-containing intermediates. This renders them susceptible to further processing by a carboxypeptidase B-like protease to yield either smaller enkephalin-containing polypeptides or free [Met]enkephalin and [Leu]enkephalin. The recognition sites for the internal [Met]enkephalin sequences that we have observed may be summarized as follows:

-X-X-Tyr-Gly-Gly-Phe-Met-X-Y,

in which X is always arginine or lysine and Y is, except for one instance, arginine or lysine. Thus, as previously reported (8), the 3.8-kDal polypeptide contains an amino-terminal [Met]enkephalin sequence followed by a -Lys-Lys- linkage and a carboxyl-terminal [Met]enkephalin sequence that is preceded by a -Lys-Arg- linkage. A similar situation exists for the 4.9-kDal polypeptide (Fig. 3), which has an internal [Met]enkephalin sequence preceded by a -Lys-Arg- linkage and followed by an -Arg-Arg- linkage, and also a carboxyl-terminal [Leu]enkephalin sequence preceded by a -Lys-Arg- linkage. The occurrence of these paired basic residues at strategic sites of cleavage is typical of prohormones (28).

With few exceptions, processing of a precursor molecule leads to the release of only one biologically active species. One such exception is pro-opiocortin, in which case several species are released, each a distinct biological entity (28). However, the proenkephalin molecule contains multiple copies of a single species, [Met]enkephalin, and at least one copy of [Leu]enkephalin. The latter could have evolved from [Met]enkephalin by a single base mutation. Thus, by posttranslational processing, a single gene product (proenkephalin) has the potential to yield many identical copies of a small biologically active segment. This type of mechanism represents a means for the production and high fidelity amplification of a small peptide sequence, one that is inherent in the DNA, but is made evident only by posttranslational processing. It remains to be seen whether other small bioactive peptides are formed by a similar mechanism.

As seen in Fig. 3, the 3.6-kDal polypeptide contains only one enkephalin sequence. This is the only internal enkephalin sequence isolated thus far from an enkephalin-containing polypeptide that is not bracketed on both sides by a pair of basic residues. The terminal heptapeptide sequence of the 3.6-kDal polypeptide, [Met]enkephalin-Arg6-Phe', and the presence of this same heptapeptide and the corresponding hexapeptide, [Met]enkephalin-Arg5, in free form in adrenal and brain extracts (6, 14) suggest that the 3.6-kDal polypeptide may be the immediate precursor of these small enkephalin-containing peptides. Possible steps in the biosynthetic pathway involved in the generation of these small peptides are outlined in Fig. 4. The presence of free [Met]enkephalin-Arg6-Arg2 and [Met]enkephalin-Arg6 in tissue extracts (14) suggest that they may be derived from the 4.9-kDal enkephalin-containing polypeptide by an analogous series of steps. Complete sequence analysis of the primary gene product and the intermediate polypeptides, as well as characterization of the postulated proteases and peptidases required for processing, will establish unequivocally the overall enkephalin biosynthetic pathway.

The presence of a phenylalanine residue at the carboxyl terminal position of the 3.6-kDal polypeptide has additional significance. If this polypeptide represents an internal sequence of the ≈50-kDal gene product, its formation would require a chymotrypsin-like cleavage. Cleavage at chymotrypsin-sensitive peptide bonds is not common but has been observed in the processing of rat proinsulin (29). An alternative explanation is that the 3.6-kDal polypeptide represents the carboxyl terminus of the entire ≈50-kDal proenkephalin molecule and that the
[Met]enkephalin and [Leu]enkephalin sequences representing all the other reported enkephalin-containing polypeptides (3) occur internally in proenkephalin, as shown in Fig. 5.

In addition to the intermediates characterized in our laboratory, three other enkephalin-containing polypeptides have been reported. One of these is a 12-membered peptide, termed BAM-12P, recently isolated from bovine adrenal medulla (6). It contains a [Met]enkephalin sequence at its amino terminus, and its sequence corresponds exactly to residues 13–26 of the 4.9-kDal polypeptide described here (Fig. 3). If BAM-12P is a fragment of the 4.9-kDal polypeptide, as is apparent, it must have been generated by cleavage of a Glu-Trp peptide bond. This is an unexpected cleavage site for hormone processing (28). The other two enkephalin-containing polypeptides that have been reported, α-neo-endorphin (30) and dynorphin (31), were isolated from porcine hypothalamus and pituitary gland, respectively. Both contain an amino-terminal [Leu]enkephalin sequence. However, in the case of α-neo-endorphin, the pentapeptide sequence is followed by Arg-Lys, whereas in dynorphin it is followed by Arg-Arg. The remaining portions of their known structures are totally unrelated. Only one of these peptides could be contiguous with the 4.9-kDal enkephalin-containing polypeptide (Fig. 5). It remains to be seen what relationship, if any, exists between these two reported peptides and the proenkephalin that we believe initiates the enkephalin biosynthetic pathway.

A final point to consider is the possible physiological significance of the enkephalin-containing polypeptides. Although the biosynthetic pathway ultimately leads to free enkephalins, some of the intermediate polypeptides may possess biological activities of their own. It should be noted that the heptapeptide [Met]enkephalin-Arg⁶-Phe⁷ has appreciably more analgesic activity than [Met]enkephalin when administered directly into the cerebral ventricles of mice (32). A larger enkephalin-containing polypeptide, which has been purified and completely characterized, has been found to be unusually potent on the guinea pig ileum (33). Thus, it is conceivable that the amino acid residues that surround the enkephalin sequences in these enkephalin-containing polypeptides may yield derivatives with unique properties by conferring additional specificity for cell recognition and additional stability in vivo, or by amplifying the interaction between these peptides and cell surface receptors.

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