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BIOSYNTHESIS OF THE ENKEPHALINS AND ENKEPHALIN-CONTAINING POLYPEPTIDES

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INTRODUCTION

In the past decade, major advances have been made in understanding how opiate drugs exert their extraordinary effects upon pain and pleasure through the brain. This research has culminated in the discovery of naturally occurring opiate-like (opioid) substances in the brain and in various peripheral tissues. The first endogenous opiate peptides to have been identified were the pentapeptides, methionine-enkephalin or Met-enkephalin (Tyr-Gly-Gly-Phe-Met), and leucine-enkephalin or Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu), which differ only in their carboxy terminal amino acid (1). Met-enkephalin was found to be part of a previously discovered pituitary peptide, β-lipotropin (β-LPH), 91 amino acids long (2). The sequence from residues 61 to 65 of β-LPH is identical to Met-enkephalin. Although the 91-residue peptide has no opioid activity, very potent activity appears if the bond between residues 60 and 61 is cleaved. The resulting peptide, β-LPH (61–91), named β-endorphin, is a very potent opioid compound.

Research on the opioid peptides has become so wide-reaching and has touched so many different branches of science that an exhaustive coverage of the topic is impossible. This review concentrates on the isolation of

precursors to the enkephalins, some of which may have physiological functions of their own. We refer the reader to more general scientific reviews in this field (3–5) and a nontechnical review (6) for a broader survey.

BACKGROUND STUDIES

Pharmacological properties of the opiates suggested that these compounds must act at specific receptor sites. For example, the existence of potent opiate antagonists such as naloxone (which elicits no analgesic action itself but which can block the actions of opiate agonists, such as morphine) favored the receptor concept. Also favoring this idea was the finding that opiate actions are stereospecific, being produced almost exclusively by the (–)-isomer. The early efforts to identify opiate receptors by biochemical techniques utilized these specific pharmacological properties to screen for the binding of radioactive drugs to brain cell membranes (7–9). A direct correlation was demonstrated between opiate binding and pharmacological action (10, 11). Studies by Kosterlitz & Waterfield (12) had shown that opiate agonists inhibited the electrically induced contractions of the guinea pig ileum and that opiate antagonists specifically reversed this effect. This preparation provided a useful assay with which to determine whether the binding sites involved a pharmacologically relevant opiate receptor. A close correlation was observed between the affinity of a large number of opiate agonists and antagonists for binding sites and their pharmacological potencies in the same strips of guinea pig intestine (13). These reports first established the experimental importance of in vitro opiate receptor binding studies and suggested that the physiological roles of opiates are mediated by interactions with cellular receptors.

The discovery of the opiate receptors suggested the existence of naturally occurring opiates. Presumptive evidence for the presence of an endogenous opiate-like activity came from the discovery that electrical stimulation of the brainstem produces analgesia (14, 15). Furthermore, this stimulation analgesia could be reversed by naloxone (16), suggesting that stimulation could be releasing endogenous analgesic substances in the brain whose effects were blocked by opiate antagonists.

To identify these substances, Hughes (17) showed that brain extracts can imitate the effect of morphine upon electrically induced contractions of the mouse vas deferens and guinea pig ileum and that the inhibitory effect can be blocked by naloxone. Hughes et al (1) isolated the active agents from pig brain and showed that it consists of the two pentapeptides, Met-enkephalin and Leu-enkephalin. The Met-enkephalin-containing polypeptide, β-endorphin, was also shown to be a potent opioid peptide in receptor binding assays and in bioassays using guinea pig ileum and mouse vas deferens (18–22).

The discovery of these opioid peptides indicated that perhaps receptors could be categorized as either enkephalin or endorphin receptors. Indeed, multiple receptors were hypothesized to be present in brain by Martin (23). Opiate receptors have subsequently been classified as: μ-receptors, which have high affinity for morphine; κ-receptors, which have high affinity for the mixed agonist-antagonist ketocyclazocine; and δ-receptors, which have high affinity for another mixed function opiate, SKF-10,047. Tested in various bioassays and radioreceptor assays, enkephalin analogs, opiate drugs, and opiate peptides have different activity profiles (24), suggesting that the enkephalins are involved in neurotransmitter (neuromodulator) processes whereas β-endorphin may be released under severe stress and may act for prolonged periods.

The identification of Met-enkephalin as residues 61–65 of β-LPH and of β-endorphin [as β-LPH (61–91)] led to the hypothesis that the β-LPH is the precursor of both peptides. Although this is the case for β-endorphin, an in vivo precursor relationship for Met-enkephalin could not be demonstrated. Early studies showed that incubations of β-LPH with pituitary extracts resulted in the formation of opioid activity (25). The pituitary opioid peptide's precursor was identified in a series of elegant experiments (26–28). This large molecular weight (31,000) protein is known as proopiocortin (29) or proopiomelanocortin (30) because it contains the sequence of both β-LPH and adrenocorticotropic hormone (ACTH). Within the ACTH molecule is the sequence of α-melanotropin (α-MSH); the sequence of β-melanotropin (β-MSH) is contained within the structure of β-LPH. The total sequence of proopiomelanocortin is now known from the work of Nakanishi et al (31, 32), who isolated the DNA that codes for this multihormone protein. Most importantly, the amino acid sequence revealed that each active peptide is bracketed by pairs of basic amino acid residues whose occurrence at strategic sites of cleavage is typical of prohormones (33). Such pairs are extremely susceptible to the actions of trypsin-like enzymes present in pituitary (19) and may activate the peptides.

However, a series of studies indicated that it was doubtful that β-endorphin serves as a precursor for enkephalin in the brain. First of all, brain levels of β-endorphin are only 5 to 10 percent those of the enkephalins (34, 35). Furthermore, the regional localizations of β-endorphin and the enkephalins differ considerably throughout the brain. Although the hypothalamus may contain high levels of both peptides, other areas, such as caudate and the globus pallidus, contain much more enkephalin than β-endorphin (34, 36, 37). In fact, even in the hypothalamus, enkephalin and β-endorphin have different distributions. This was demonstrated by immunohistochemical staining of serial sections from rat hypothalamus, which showed that although β-endorphin, β-LPH, and ACTH were present in the same cells, enkephalin staining did not overlap any of the β-endor-

phin cells (38). These experiments clearly pointed out that, despite their structural similarities, enkephalin and β-endorphin represent two separate neuronal systems. Finally, it is clear that Leu-enkephalin must be synthesized separately from β-endorphin as its amino acid sequence is not present in proopiocortin (32). Therefore it was essential to determine the precursors and biosynthetic pathway leading to the enkephalins.

METHODS

Although we do not describe in detail the many methods used to achieve the results described below, there were three major improvements in methodology that led to the rapid advance from the discovery of enkephalin-containing polypeptides (ECPs) to their complete structural determination. These three areas were: (*a*) assays, (*b*) starting tissue and extraction procedures, and (*c*) high performance liquid chromatography.

As discussed earlier, inhibition of guinea pig ileum or mouse vas deferens contractions had been used to assay for the enkephalins. Although these provided a physiologically relevant assay they were too time consuming and insensitive to be of practical use during rapid purification procedures. Two assays proved to be extremely useful in the purification of ECPs. The first was radioimmunoassay (RIA), which several laboratories have used (39–41). The other was the radioligand binding assay, which our laboratory employed extensively (42). The latter assay utilized the NG 108–15 cell line developed by Klee & Nirenberg (43), which contains opiate receptors. Both the RIA and radioligand binding assay could accomodate a large number of samples and provide rapid results. The sensitivity of these assays ranges from the low picomole level to femtomole levels for some RIAs. Thus very little sample was consumed in the assay.

Another factor that greatly enhanced assay sensitivity was the use of trypsin (44), and later trypsin combined with carboxypeptidase B (45), to digest the enkephalin-containing polypeptides prior to assay. These enzyme treatments released free enkephalin, which is 10 to 50 times more active in both types of assays. Although it was only later that the magnitude of the insensitivity of these assays to most of the ECPs was found, it was, in fact, anticipated that increased sensitivity would result when the enzyme treatments were initiated.

The choice of starting tissue also greatly influenced the progress of ECP isolation and characterization. Early work with striatal tissue proved to be unproductive. Although high in enkephalin content, this tissue contains relatively low levels of ECPs and is composed of numerous different cell types. The use of the adrenal medulla and, in particular, chromaffin granules isolated from the adrenal medulla, provided a tremendously enriched

source of ECPs. The reasons for choosing the adrenal medulla are described below.

Another element that allowed for the ultimate enkephalin-containing polypeptide purifications was the method of extraction. We found that an acidic extraction buffer (pH 2) significantly increased the recovery of ECPs compared to pH 5.5 and pH 7.5 extraction buffers. At higher pH substantial proteolysis occurred, which degraded the ECPs. Other laboratories have used different extraction buffers but their common feature is a low pH. The protease inhibitors pepstatin A and phenylmethane sulfonyl fluoride were also included in our extraction buffers to further limit proteolysis.

Although the methods described above played a vital role in the discovery and characterization of ECPs, the major factor that led to the rapid advances in this area was the use of high performance liquid chromatography (HPLC) for purification. Several laboratories made use of HPLC, but Udenfriend's laboratory provided the major examples of the advantages of this approach (46–48) when combined with an ultrasensitive peptide detection system. There were several reasons for the use of HPLC in these studies: (*a*) the ability to put large volumes, with low levels of peptides, onto the HPLC column (trace enrichment) and still obtain excellent resolution; (*b*) very high recoveries of peptides, usually 85% or better; (*c*) short chromatography times, generally under 2 h; (*d*) extremely high resolution, especially with reverse-phase columns; (*e*) volatile buffer systems that allowed vacuum removal of solvent for further HPLC steps or for chemical characterization; and (*f*) availability of several detection systems. We chose a post column detection system using fluorescamine (49) because of its high sensitivity and compatability with a wide variety of buffer systems.

ENKEPHALIN PRECURSOR DISCOVERY

Although the precursor relationship between proopiocortin, β-lipotropin, and β-endorphin was well established, the enkephalins had not been shown to be included in this pathway. Several pieces of evidence led us to believe that a separate enkephalin biosynthesis pathway existed. Studies in the laboratories of Hughes & Kosterlitz (50–52) showed that the enkephalins were biosynthesized via a ribosomal process and that a lag period existed between labeling and the appearance of the enkephalins, suggestive of proteolytic processing. Immunological studies demonstrated that the enkephalins and β-endorphin were found in separate neurons (37, 38). In addition, the striatum had high levels of the enkephalins but virtually no β-endorphin or its precursors (53). Finally, no β-endorphin containing the Leu-enkephalin sequence had been detected.

With this background we began our search for enkephalin precursors in the bovine striatum. Our initial results strongly suggested the presence of

a non-β-endorphin precursor (53). These results were confirmed by Hughes's laboratory (54) with guinea pig, rat, and mouse striatum. As discussed above, further work with the striatum was extremely difficult.

Immunohistochemical data from Hökfelt's laboratory (55, 56) showing enkephalin-like immunoreactivity in the adrenal medulla provided the impetus to examine that tissue as a source of enkephalin precursors. We (44) and others (39, 40) demonstrated that the enkephalins and larger enkephalin-containing polypeptides were present in the adrenal medula. These studies further showed the ECPs were stored with catecholamines in the chromaffin granules (40). The ECPs ranged in molecular weight from ~ 1000 to 50,000 daltons. The largest precursor was shown to have several enkephalin sequences with a ratio of six Met-enkephalin sequences to one Leu-enkephalin sequence (45).

Using a cell-free translation system with adrenal medullary mRNA it was shown that this translation protein had a molecular weight of 31,000 (57). This was a smaller molecular weight than that estimated by size exclusion chromatography (45), but it was consistent with later SDS-polyacrylamide gel electrophoresis. Another group has suggested a Met-enkephalin precursor of greater than 70,000 daltons on the basis of cell-free translation of mRNA from a human pheochromocytoma (58). Whether this represents another source of Met-enkephalin has not been determined, but this protein does not appear to be the precursor of the ECPs isolated from chromaffin granules.

Possible enkephalin precursors with molecular weights of 90, 60, and 40 kilodaltons (kDal) have been observed in striatal tissue by Hughes's group (59). Whether these represent a separate pathway in the brain also remains to be determined. Data from two other laboratories, in contrast, has suggested that the enkephalin biosynthesis pathway is the same in the adrenal and striatum. In vitro translation of mRNA isolated from bovine striatum produced a 31,000 dalton protein containing both Met- and Leu-enkephalin (60). Udenfriend's laboratory has noted similarities in the ECPs not only in these tissues but in other species as well (61). Their data consists of size exclusion chromatography showing similarities in molecular weight among ECPs from the adrenal, myenteric plexis, and striatum from guinea pig, and HPLC data showing similar elution of the 8600 and 12,600 dalton ECPs from guinea pig striatum and beef adrenal. In addition, Met-enkephalin-Arg^6-Phe^7, which seems characteristic of the adrenal precursor, has been identified in most enkephalin-containing tissues (61).

ENKEPHALIN-CONTAINING POLYPEPTIDES

The earliest studies on the bovine adrenal medulla demonstrated the presence of a series of enkephalin-containing polypeptides ranging in size from

20,000 to 1,000 daltons (44). In order to demonstrate precursor/product relationships among these ECPs, purification to homogeneity and sequence analysis was necessary.

Initially, in the course of these studies, the chemical characterization of those peptides from the medulla that are within the molecular weight range of the enkephalins and that bind to the opiate receptor was undertaken. Not only were Met- and Leu-enkephalin isolated, as expected, but also a series of enkephalin peptides extended by one to three amino acids at the carboxyl end of the pentapeptide sequence were found (62, 63). The first of these unknown peptides to be purified to homogeneity was shown to have a sequence of Tyr-Gly-Gly-Phe-Met-Arg-Phe, and became known as Met-enkephalin-Arg^6-Phe^7 (64). This opioid heptapeptide was also found in bovine (64), human, and rat (65) striatum in concentrations comparable to Leu-enkephalin. Distribution of the heptapeptide in the brain followed that of Met-enkephalin, with highest concentrations in the globus pallidus, intermediate levels in caudate-putamen and hypothalamus, and low levels in cortex and cerebellum (66). Although the heptapeptide was equipotent to the enkephalins in the isolated organ bioassays, it was eight times more potent than Met-enkephalin when administered directly into cerebral ventricles of mice and antinociceptive activity measured (67). Reports of Met-enkephalin-Arg^6-Phe^7 as the first endogenous peptide able to act at the κ-receptor (68) are provocative but have yet to be substantiated (69).

The December 1979 issue of *Proceedings of the National Academy of Sciences of the USA* contained not only our report of the isolation of the Met-enkephalin-Arg^6-Phe^7 molecule (64) but also an article on the isolation from porcine hypothalami of what was termed "pro-methionine-enkephalin," i.e. Met-enkephalin-Arg^6 (70), which was also identified in adrenal medulla (62). In addition, this issue contained the report of the presence of dynorphin-(1–13), a pituitary opioid heptadecapeptide that was shown to be approximately 700 times more potent than Leu-enkephalin in the guinea pig ileum muscle preparation (71). Recently the entire sequence of dynorphin (1–17) was determined (72, 73) as Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln. These three novel opioid peptides and the isolation of a "big" Leu-enkephalin termed α-neoendorphin (74, 75) provided the first structural evidence for enkephalin-containing peptides other than the β-endorphin and its precursors.

Four additional small Met-enkephalin-containing peptides were isolated from chromaffin granules of the adrenal medulla and their structures established as Met-enkephalin-Arg^6-Arg^7, Met-enkephalin-Lys^6, Met-enkephalin-Arg^6 (62), and Met-enkephalin-Arg^6-Gly^7-Leu^8 (63). These hexa-, hepta-, and octapeptides are also present in bovine and guinea pig brain and myenteric plexus (61, 62).

The characterization of the ECPs of intermediate size (3000–6000 daltons) from adrenal medulla was undertaken next. It soon became clear that the isolated enkephalins that were extended at their carboxy termini by one to three amino acids were contained within these larger peptides (47, 76–79). For example, sequence analysis of what was termed peptide B (3600 daltons) (47) indicated that the carboxy terminus was Met-enkephalin-Arg^6-Phe^7 (77). Similarly, sequence analysis of a 5300 dalton ECP revealed that its carboxy terminus consisted of the octapeptide, Met-enkephalin-Arg^6-Gly^7-Leu^8 (79).

Multienkephalin sequence polypeptides were also characterized (47). Peptide F (3800 daltons) was shown to contain two copies of the Met-enkephalin sequence, whereas peptide I (4900 daltons) contains a Leu-enkephalin sequence and a Met-enkephalin sequence (76). This chemical data was the first demonstration of a common precursor for both Met- and Leu-enkephalin.

In addition, an opioid peptide of molecular weight 3200, designated as peptide E (47), had been purified to homogeneity (78). The sequence of this peptide was found within peptide I (residues 15 to 39) preceded by the typical (33) double basic amino acid cleavage site (i.e. -Lys-Arg-). A series of fragments of peptide I were isolated by Mizuno et al (80, 81) and designated as BAM-12P (peptide I 15–27), BAM-20P (peptide I 15–35), BAM-22P (peptide I 15–37). However, the enzymatic production of these molecules in vivo is unlikely since unusual cleavages (e.g. at a Glu-Trp sequence) would have to occur in order for peptide I to be considered a physiological precursor of those peptides. The fact that these peptides were isolated from the whole adrenal medulla and not from isolated chromaffin granules may explain these unusual proteolytic cleavages.

Of the adrenal peptides isolated, peptide E was unique in that it was about 30 times more potent in the guinea pig ileum assay than Met-enkephalin and β-endorphin, although only about half as active as dynorphin (1–13), and its effects were completely reversed by naloxone (78). Like dynorphin (1–13), the twitch inhibition produced by peptide E was reversed very slowly by washing the preparation. Removal of the carboxy-terminal residue Leu^{25} from peptide E did not alter its potency in the guinea pig ileum assay (78). Similar treatment of Leu-enkephalin is known to reduce its opiate receptor affinity by more than 90%. Therefore it was concluded that the carboxy-terminal Leu-enkephalin moiety of peptide E does not contribute significantly to its opioid activity and that the amino-terminal Met-enkephalin must be the major determinant of the peptide's potency (78). Similarly, dynorphin (1–13), native dynorphin (1–17), and α-neoendorphin, peptides with potent opioid activity in the guinea pig ileum assay, all contain an amino-terminal Leu-enkephalin sequence. The lower potency of

peptide I, the precursor of peptide E, undoubtedly was due to the lack of such an exposed enkephalin sequence at the amino terminus. However, the presence of an amino-terminal enkephalin sequence does not ensure high opiate activity in the ileum assay. For example, peptide F, which contains an amino-terminal Met-enkephalin sequence, was shown to be a much weaker opiate agonist than even Met-enkephalin itself (78).

The length of peptide E and the sequence of the residues following the Met-enkephalin sequence play an important role in determining biological activity. As was observed by Mizuno et al (80), the activity of BAM-12P [peptide E (1–12)] in the guinea pig ileum assay was quite low. At least three residues can be removed from the carboxyl terminus of peptide E without a significant loss of opioid activity in the guinea pig ileum assay (81). However, removal of five residues from the carboxyl terminus reduces the activity of peptide E substantially (78, 81). From these data there seems to be reason to believe that these peptides can and do serve a physiologic role beyond that of just enkephalin precursors.

The larger ECPs of 8600, 12,600, and 18,200 molecular weight (46, 48, 82) were also purified to homogeneity and contained one, three, and four Met-enkephalin sequences respectively. Once again, the enkephalin sequences were found to be bracketed by pairs of basic amino acids with the exception of the octapeptide, Met-enkephalin-Arg^6-Gly^7-Leu^8. Its sequence is found at the carboxyl terminal of the 18,200 dalton enkephalin-containing polypeptide but is preceded by a Lys-Arg sequence, which implies that the opioid octapeptide may indeed function as more than just a Met-enkephalin precursor.

Thus, a number of enkephalin-containing polypeptides, ranging in size from hexapeptides to proteins of approximately 18,000 daltons, were purified. The presence of all these polypeptides and the structural relationships among them suggested that they were all intermediates in a common biosynthetic pathway and that they were derived from a larger common proenkephalin molecule (45).

PROENKEPHALIN STRUCTURE

Although, as described in the preceding section, numerous adrenal ECPs and ECPs from other tissues were isolated, the structure of the complete proenkephalin protein was unknown. A partial structure could be deduced from the sequences of the various ECPs and a probable structure was suggested on this basis (83). The isolation of the mRNA proenkephalin translation product appeared to be progressing slowly; thus several groups turned to nucleic acid cloning techniques to isolate proenkephalin DNA.

The first reports used a cDNA probe constructed from a unique Trp-Trp-

Met-Asp-Tyr sequence in the 3200 dalton ECP (Peptide E) (84), or a probe from the Met-enkephalin sequence in β-endorphin (85). These reports both demonstrated that the mRNA coding for proenkephalin was about 1500 nucleotides long. Thus the proenkephalin protein could be no larger than 50,000 daltons and was probably smaller because of the poly A tail on the mRNA. The cDNA probe was extended with reverse transcriptase and the DNA sequence shown to correspond precisely with the expected sequence in the 3200 dalton ECP (84).

Early in 1982 the complete sequence of bovine proenkephalin was described simultaneously by two groups (86, 87). The DNA and amino acid sequences were the same and confirmed the enkephalin-containing polypeptide arrangement proposed earlier (83). The protein has a molecular weight of 30,000 daltons. Shortly thereafter the structure of human proenkephalin was reported (88). The structure of human proenkephalin was remarkably similar to that of bovine. The complete structure of both bovine and human proenkephalin is shown in Figure 1.

The first item to note from this figure is that the amino acid sequences corresponding to dynorphin and α-neoendorphin are not present in proenkephalin. These peptides must therefore be derived from another precursor. There are six Met-enkephalin sequences and one Leu-enkephalin sequence, which agrees with data from the protein characterization (45). The enkephalin sequences, with two exceptions, are bracketed by pairs of basic amino acids, which are proteolytic processing sites (89). These two exceptions are at amino acids 191–192 and 266–267, which have only a single Arg residue. It appears that these sites are not cleaved in vivo, accounting for the presence of Met-enkephalin-Arg^6-Gly^7-Leu^8 (65) and Met-enkephalin-Arg^6-Phe^7 (66). It is interesting to note that both of these larger enkephalins have been conserved in the proenkephalin sequences between beef and human. Whether they represent peptides with different physiological functions or are possibly physiologically more stable remains to be seen.

The human sequence has a three amino acid insert (residues 85–87) and a two for one insert (residues 178–179); thus the human proenkephalin is four amino acids longer than the bovine. The first twenty-four residues are presumably a leader sequence, as the sequence of these first amino acids is consistent with that function and all the isolated ECPs from this region have Glu-Lys-Ser at the amino terminal (48, 82). Following the leader sequence is a stretch of 75 residues containing six Cys residues, presumably forming three disulfide bonds. This feature is similar to that seen at the amino terminus of proopiocortin. There are just 35 amino acid differences between the two species, with only 19 of them being nonconservative.

The major feature to notice in these two proenkephalins is their remarkable similarity in sequence despite the evolutionary distance between them. This similarity is particularly noticeable in two of the ECPs, the 3200 and

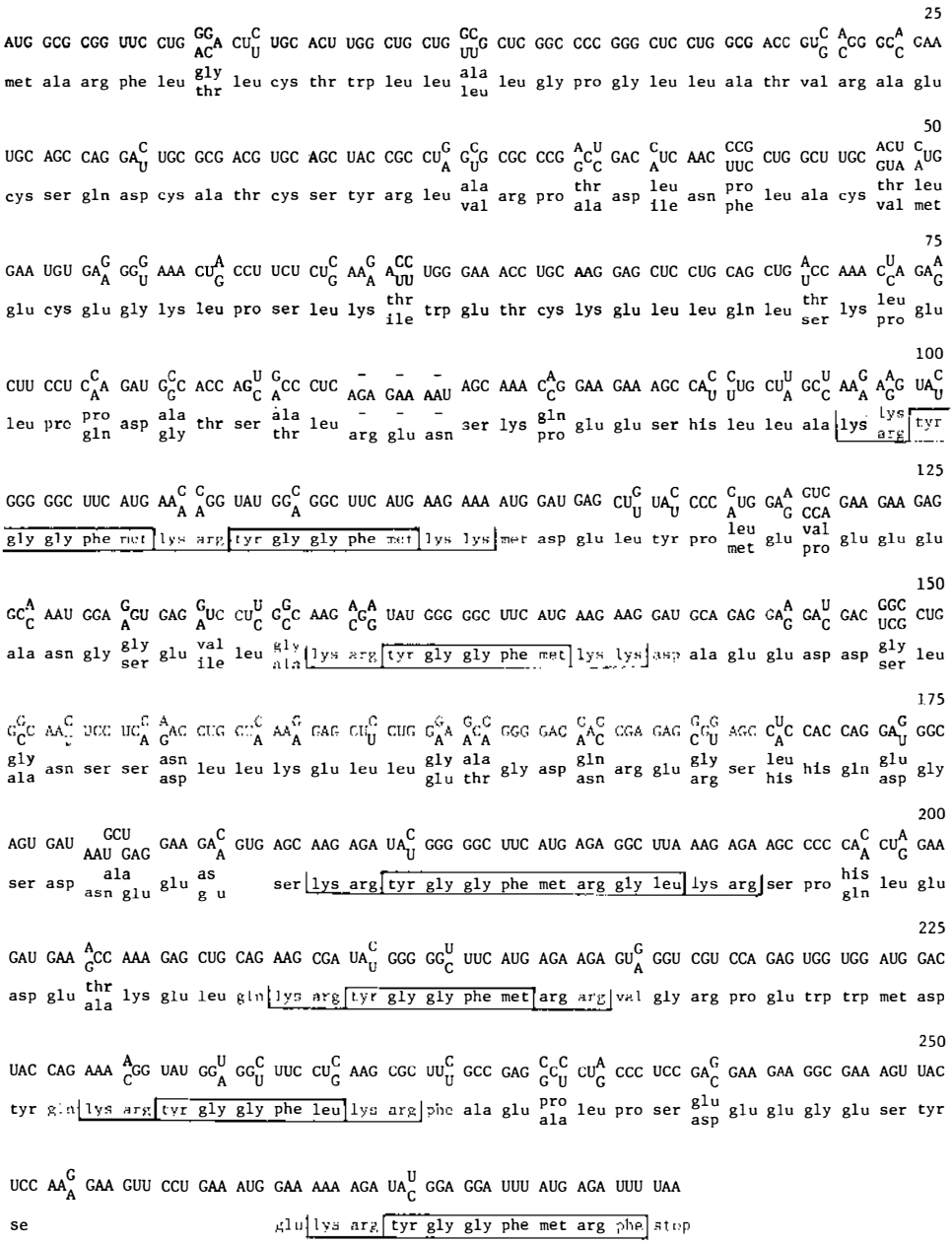

Figure 1 **Human and bovine proenkephalin DNA and amino acid sequence. The DNA and amino acid sequences from human (88) and bovine proenkephalin (86, 87) are shown. Where there are differences or insertions the human sequence is shown below and the bovine above. The enkephalin sequences (100–104, 107–111, 146–150, 210–214, and 230–234) are boxed in as are the extended enkephalin sequences (186–193 and 261–267). The basic amino acid pairs are also shown.**

3600 dalton peptides (residues 210–234 and 237–267 respectively). The 3200 dalton peptide is identical in both species and the 3600 dalton peptide has only one nonconservative amino acid change. In addition to these peptides, there are at least six stretches of 10 to 20 invariant amino acids. Of even greater importance is the identical sequence and location of the enkephalin sequences and their bracketed basic amino acid pairs. Another conserved region is the site of possible glycosylation (residues 152–154). Although glycosylation at this site has not been demonstrated, it was suggested (79) as a site because of the very high molecular weight observed for the 5300 dalton peptide when electrophoresed on SDS polyacrylamide gels.

The similarities between these two species coupled with the evidence that other species and tissues have similar proenkephalins implies several things about the biosynthesis of the enkephalins and enkephalin-containing polypeptides. The first is that the paired basic amino acids are of great importance in the processing of proenkephalin. There is only one change in these amino acids, a conservative Lys-Arg change at residue 99. Second, the larger enkephalins, Met-enkephalin-Arg^6-Phe^7 and Met-enkephalin Arg^6-Leu^7-Gly^8, are conserved, which suggests a possible physiologic role for these two peptides. The identical sequence for the 3200 dalton peptide and the single nonconservative change in the 3600 dalton peptide also strongly indicate a physiologic role for these peptides. The nature of these roles remains to be pursued.

Following preparation of this review, Numa's group published the DNA and corresponding amino acid sequences of the neoendorphin/dynorphin precursor from porcine hypothalmus (100). Their strategy was similar to that described above for the isolation of adrenal proenkephalin. They prepared all possible DNA probes for the sequence Lys-Trp-Asp-Asn-Gln from dynorphin. Using these probes they isolated clones that allowed for sequencing of the dynorphin precursor.

The putative protein deduced from the DNA sequence is 256 amino acids long and contains a hydrophobic leader sequence like the adrenal proenkephalin. The six cysteine residues in the dynorphin precursor are located in almost exactly equivalent positions to those in proenkephalin. The neoendorphin sequence is located at amino acids 175–206; dynorphin immediately follows that sequence, separated by a Lys-Arg cleavage site (residues 209–225). Another Leu-enkephalin-containing peptide (residues 228–256) follows the dynorphin sequence also preceded by a Lys-Arg cleavage site. The isolation of a portion of this new enkephalin-containing peptide from bovine pituitary has also recently been reported (101). The isolated peptide was composed of only the first thirteen amino acids and was apparently cleaved at a single Arg residue site. If the bovine and porcine sequences are identical then proteolytic cleavage probably occurred during isolation, as all

other cleavages in both the dynorphin precursor and proenkephalin occur at double basic amino acid sites.

With the identification of this hypothalmic enkephalin precursor all the presently known enkephalin-containing polypeptides are accounted for. This final precursor follows the pattern of the previously identified precursors, proopiocortin and proenkephalin, in being composed of probable bioactive peptides that are proteolytically cleaved from the precursor protein by cleaving at double basic amino acid sites.

PROENKEPHALIN PROCESSING

As described above, the proenkephalin translation protein appears to have a signal peptide (residues 1–24) that is probably utilized for entry into the chromaffin granule during granule formation. This peptide ends in a small neutral amino acid (ala) where cleavage occurs. Support for cleavage at this site comes from the fact that none of the enkephalin-containing polypeptides isolated to date contain that sequence, whereas three of them, the 8600, 12,600, and 18,200 dalton peptides, all start at amino acid 25 (48, 82). There are pairs of basic amino acids, predominantly Lys-Arg, at cleavage sites identified from the isolated ECPs. The single Arg residues, as mentioned above, do not appear to represent cleavage sites. The proenkephalin processing pathway is shown schematically in Figure 2. All the ECPs shown in this figure have been purified and characterized.

There does not appear to be any further processing of the enkephalin-containing polypeptides upon release (exocytosis) from the isolated perfused bovine adrenal gland (90). The pattern of ECPs released in the perfused adrenal was the same as that seen in isolated chromaffin granules. The ratio of catecholamine to ECPs was also the same in the perfusate and in the isolated granules. An exception to this was noted in a human pheochromocytoma in primary culture (91), but whether this is generally true for pheochromocytomas has not been shown. The processing of the ECPs in the blood has not been studied to any extent but may play a role in the physiologic function of the enkephalin-containing polypeptides.

It was suggested that a trypsin-like enzyme and a carboxypeptidase B-like enzyme would be required to generate the enkephalin and enkephalin-containing polypeptides from the precursor (45). Evidence has been presented that a trypsin-like enzyme is present in the adrenal medulla (92). We have recently identified an enzyme in bovine adrenal chromaffin granules that cleaves proenkephalin peptides at basic amino acids (93). The enzyme cleaves on the carboxy side of the basic amino acid. This enzyme has a pH optimum of about 5 with the artificial substrate, tosyl arginine methyl ester. This pH optimum is consistent with the chromaffin intragranular pH of 5.2–5.6 (94). Others have shown an enzyme present in the granules that can

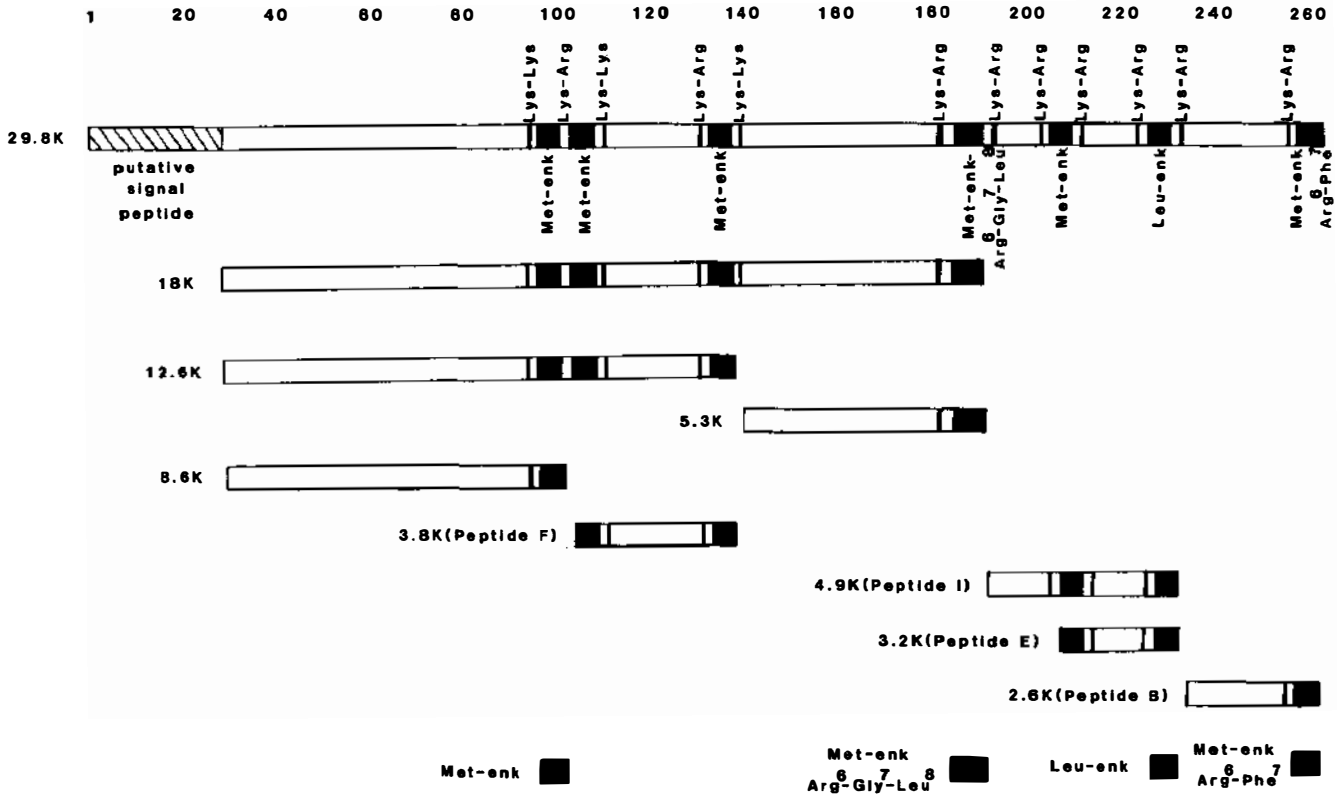

Figure 2 Proenkephalin processing in bovine adrenal medulla. The peptides isolated from chromaffin granules suggest the following sequence of proteolytic cleavages of proenkephalin. The signal peptide is presumably removed upon entry of the protein through the membrane into the granule. The subsequent trypsin-like cleavages then yield the ECPs observed in the granules. It is clear which peptides would yield Leu-enkephalin (4900 dalton or 3200 dalton), Met-enkephalin-Arg^6-Phe^7 (2600 dalton), and Met-enkephalin-Arg^6-Gly^7-Leu^8 (5300 dalton), but whether one peptide or several of them yield Met-enkephalin is not clear.

release Met-enkephalin from dialyzed granule extracts (95). Since there are a number of ECPs that contain carboxy-terminal Met-enkephalin sequences, this enzyme is probably the same as the enzyme we have identified. By enzyme cleavage on the carboxy side of the basic amino acid preceding the pentapeptide, Met-enkephalin would be released from those peptides.

The presence of a carboxypeptidase B activity in crude extracts of adrenal chromaffin granules has been demonstrated (96). A more highly purified preparation of this enzyme has recently been reported (97). This enzyme also has a pH optimum consistent with the low intragranular pH and has a unique feature of being activated by Co^{2+}. The K_i for the substrates Met- and Leu-enkephalin-Arg^6 are in the 50–80 μM range, whereas the Lys^6 analogs are two to three times higher. These were measured against an artificial substrate Dansyl-Phe-Leu-Arg and therefore do not represent a true K_m, but they indicate that the enzyme may have some specificity. The presence of both of these enzymes within the chromaffin granules supports the processing mechanisms proposed by Steiner (33) for prohormones. In addition, the report on the carboxypeptidase
presence of a Co^{2+}-activated enzyme in brain whose distribution paralleled that of the enkephalins in various brain regions, which suggests that the same enzyme is present in both types of enkephalin-containing tissues.

Indirect evidence indicates that precursor processing by the trypsin-like enzyme is regulated in some manner. This evidence includes: 1. the majority of possible sites in the ECPs are not cleaved in isolated chromaffin granules but homogenization of the granules at pH 5.5 causes a greatly decreased level of ECPs (unpublished data); 2. in vivo "pulse-chase" studies indicate that cleavages stop after 90–110 h (98) in the denervated adrenal gland; 3. time course studies show the partially purified trypsin-like enzyme does not cleave all the available substrate (93); and 4. more enzyme activity is observed after affinity chromatography, which suggests activation of the enzyme (93). Whether this regulation is due to the sequence specificity of the enzyme or occurs by inhibition of the enzyme must still be determined.

Another level of regulation has been suggested by the report that enkephalin-like immunoreactivity is found only in epinephrine-containing cells in the hamster adrenal medulla (99). Whether this will be true for all species remains to be seen, but if so, then either the synthesis of proenkephalin or its processing is under regulation. All of these indications of regulation of ECP biosynthesis further emphasize their possible roles as hormones.

SUMMARY

The biochemical characterization of the enkephalins and the enkephalin-containing polypeptides and their biosynthesis has reached an advanced

state. From the structural data showing great similarities between human and beef proenkephalin there is strong evidence for physiological roles for some of the extended enkephalins and for at least two of the ECPs. If, like the different hormones contained within proopiocortin, the different ECPs in proenkephalin perform different coordinate functions, their cosecretion with the catecholamines from the adrenal gland suggests that these functions play a role in the response to stress. It appears that proenkephalin represents another multi-hormone precursor, like proopiocortin, that is processed by proteolysis to yield a variety of peptides active in both the endocrine and nervous systems. The research effort must now turn toward resolving the nature of the physiologic functions these peptides perform.

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