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ACTION OF PROTEOLYTIC ENZYMES ON LIPOTROPINS AND ENDORPHINS: BIOSYNTHESIS, BIOTRANSFORMATION AND FATE

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1. INTRODUCTION

β-Lipotropin is a polypeptide hormone with lipolytic activity which was first identified in the anterior lobe of the pituitary gland (Li, 1964; Li *et al.*, 1965; Chrétien and Li, 1967). β-Endorphin, which constitutes the 31 amino acids containing COOH-terminal portion of β-lipotropin, was discovered later to exist as an individual peptide with potent opioid activities (Li and Chung, 1976; Bradbury *et al.*, 1976b; Gráf *et al.*, 1976a). The structural relationship between the two peptides made clear that β-lipotropin could serve as a precursor of biologically active peptides and pointed to the significance of the proteolytic enzymes responsible for the conversion process. Now it has been established that β-lipotropin and β-endorphin, together with adrenocorticotropin (ACTH), α-melanotropin (α-melanocyte stimulating hormone, α-MSH) and other biologically active peptides, are derived from a single precursor protein, termed pro-opiomelanocortin by specific proteolytic processing (Mains *et al.*, 1977; Chrétien *et al.*, 1979; Herbert *et al.*, 1980; Eipper and Mains, 1980).

Differences in proteolytic processing of pro-opiomelanocortin underly the existence of distinct sets of biologically active peptides derived from pro-opiomelanocortin. For instance, β-lipotropin, together with ACTH and a large peptide of the NH_2-terminal portion is the major biosynthetic end product in the anterior pituitary (Mains and Eipper, 1978a,b; Herbert *et al.*, 1980). However, in the intermediate lobe of the pituitary, in brain and presumably also in placenta and pancreas, main products are β-endorphin and γ-lipotropin, α-melanotropin, corticotropin-like intermediate lobe peptide (CLIP) and several shorter NH_2 terminal pro-opiomelanocortin fragments (Fig. 1) (Mains and Eipper, 1978a,b, 1979; Roberts *et al.*, 1978; Crine *et al.*, 1978, 1979; Eipper and Mains, 1981; Liotta and Krieger, 1980; Liotta *et al.*, 1981; Smyth and Zakarian, 1982). These peptides all arise during a discrete proteolytic cleavage mechanism which is directed by paired basic amino acids. This type of proteolytic cleavage constitutes a universal mechanism in the biosynthesis of many biologically active peptides and proteins (Steiner *et al.*, 1974, 1975, 1980) and is indicated as biosynthetic processing; the resulting fragments are generally considered as biosynthetic end products.

In addition to biosynthetic processing, proteolytic enzymes with different specificity act on biosynthetic end products and can generate shorter peptides with biological activities (e.g. Peach, 1977). The behaviorally active β-endorphin fragments α-endorphin and γ-endorphin are examples of pro-opiomelanocortin peptides formed by proteolytic conversion of the endproduct β-endorphin (Austen *et al.*, 1978a; Burbach *et al.*, 1980a, 1981). Such proteolytic events, cleaving biosynthetic end products and producing biologically active peptides, may be indicated by biotransformation. Biotransformation does not employ a single type of proteolytic cleavage but different mechanisms leading to production or modification of biological activity can be distinguished. Finally, peptidases

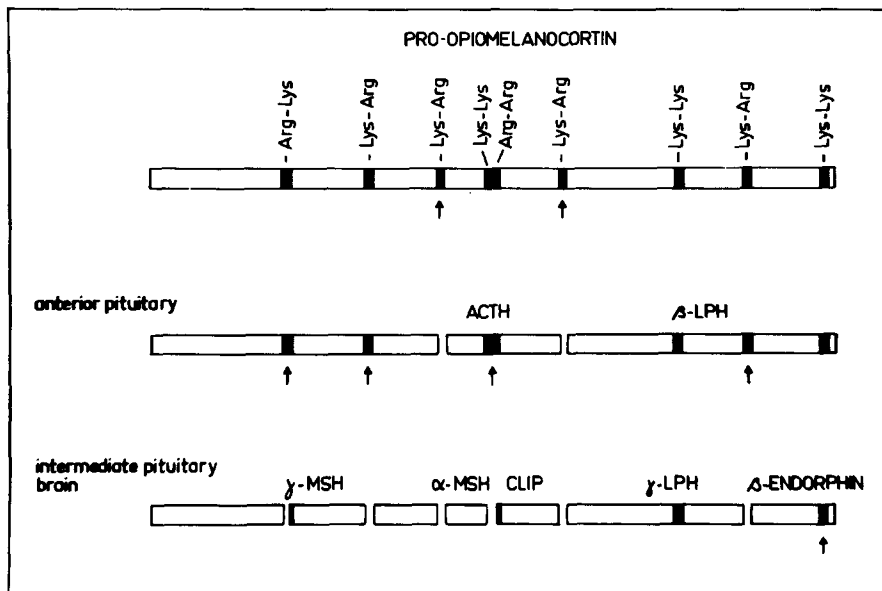

Fig. 1. The structural organization of pro-opiomelanocortin and its products of processing in the anterior and neurointermediate lobes of the pituitary gland and the brain. The arrows indicate the cleavages of paired basic amino acid residues (dark areas).

of various tissues and cellular compartments can mediate degradation and inactivation of peptides, while peptidases in blood and peripheral organs contribute to the disappearance of peptides in the circulation.

This article reviews the actions of proteolytic enzymes on β-lipotropin, β-endorphin and related peptides. The proteolytic processes are discussed from the viewpoint of production of peptides (i.e. biosynthesis and biotransformation) as well as inactivation and elimination of peptides (degradation and fate).

2. STRUCTURE AND FORMS OF LIPOTROPINS AND ENDORPHINS

The lipotropins and endorphins comprise a family of several biologically active peptides. The molecular forms by which they occur and display their biological activities are determined by the proteolytic cleavage of their precursors. Often the molecular forms of peptides by themselves are indicative for the types of proteolytic cleavage taking place. Structure and forms of lipotropins and endorphins are briefly reviewed below.

Table 1. *Comparison of β-Lipotropins of Various Mammalian Species*

Species	Number of Residues	Percentage of Sequence Homologous with the Human Sequence	Sequence Numbering of *β-Endorphin-(1–31)*	Reference
human	89		β-LPH-(59–89)	Hsi *et al.*, 1981; Spiess *et al.*, 1982; Seidah *et al.*, 1982; Takahashi *et al.*, 1981; Whitfield *et al.*, 1982.
porcine	91	68	β-LPH-(61–91)	Gilardeau and Chrétien, 1972; Gráf *et al.*, 1974
ovine	91	78	β-LPH-(61–91)	Li *et al.*, 1965; Gráf and Li, 1973
whale	91	73	β-LPH-(61–91)	Kawauchi *et al.*, 1980a.
bovine	93	73	β-LPH-(63–93)	Lohmar and Li, 1967, Pankov, 1973; Li *et al.*, 1977; Nakanishi *et al.*, 1979.
rat	71	79	β-LPH-(41–71)	Drouin and Goodman, 1980.
mouse	71	77	β-LPH-(41–71)	Uhler and Herbert, 1983; Notake *et al.*, 1983.

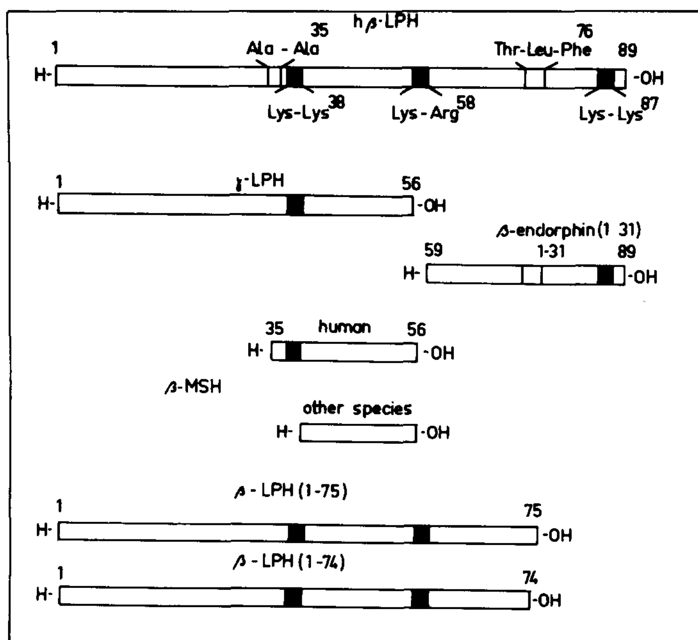

FIG. 2. β-Lipotropin and derived peptides. The structure of human β-lipotropin, hβ-LPH-(1–89), is shown as representative for the β-lipotropins of mammalian species. The dark boxes represent pairs of basic amino acids; the open boxes indicate other characteristic sequences subject to proteolytic cleavage. For details of peptides related to β-endorphin-(1–31) see Fig. 3. The sequence of human β-lipotropin is:

H–Glu[1]–Leu–Thr–Gly–Gln–Arg–Leu–Arg–Glu–Gly[10]–Asp–Gly–Pro–Asp–Gly–Pro–Ala–Asp–Asp–

Gly[20]–Ala–Gly–Ala–Gln–Ala–Asp–Leu–Glu–His–Ser[30]–Leu–Leu–Val–Ala–Ala–Glu–Lys–Lys–Asp–

Glu[40]–Gly–Pro–Tyr–Arg–Met–Glu–His–Phe–Arg–Trp[50]–Gly–Ser–Pro–Pro–Lys–Asp–Lys–Arg–

Tyr–Gly[60]–Gly–Phe–Met–Thr–Ser–Glu–Lys–Ser–Gln–Thr[70]–Pro–Leu–Val–Thr–Leu–Phe–Lys–Asn–

Ala–Ile–Ile–Lys–Asn–Ala–Tyr–Lys–Lys–Gly–Glu[89]–OH

2.1. LIPOTROPINS

2.1.1. *β-Lipotropin*

β-Lipotropin was first discovered in extracts of ovine pituitary glands (Li, 1964; Birk and Li, 1964). In subsequent years the β-lipotropin of several other species has been purified and sequenced. Recently, amino acid sequences have also been deduced from nucleotide sequences of cloned genes or cDNAs encoding pro-opiomelanocortin. A comparison of β-lipotropins is presented in Table 1. Not until recently was consensus reached concerning the NH_2-terminal sequence of human lipotropin (Fig. 2); now data of amino acid and nucleotide sequence agree (Hsi *et al.*, 1981; Spies *et al.*, 1982; Seidah *et al.*, 1982; Takahashi *et al.*, 1981; Whitfield *et al.*, 1982).

The various β-lipotropins vary in length from 71 residues (mouse, rat) to 93 residues (bovine) (Table 1), mainly due to differences in the NH_2-terminal region. In general, this region is highly heterologous, while a high sequence homology is present in the 50 residues of the COOH-terminal region of β-lipotropins.

2.1.2. *β-Lipotropin-(1–77)/-(1–75)*

The COOH-terminally truncated form of porcine β-lipotropin (β-lipotropin-(1–77)) has been identified as product of β-lipotropin cleavage by pituitary and brain homogenates (Gráf and Kenessey, 1976; Gráf *et al.*, 1979a,b). Dorsa *et al.* (1982) detected in rat pituitary a large peptide cross-reacting with an antiserum for the COOH-terminus of γ-endorphin

(β-endorphin-(1–17)) and suggested that it was related to this β-lipotropin-(1–77) (rat β-lipotropin-(1–56)). Recently, Burbach and Wiegant (1984) detected in human pituitaries a similar peptide in considerable amounts cross-reacting with a COOH-terminal γ-endorphin antiserum (Loeber *et al.*, 1979; Loeber and Verhoef, 1981). This peptide was only slightly smaller than β-lipotropin. By isolation and determination of amino acid composition it was shown that the COOH-terminal tryptic fragment was β-endorphin-(10–17). These data indicated that this peptide was human β-lipotropin-(1–75). These studies suggest that human β-lipotropin-(1–75), porcine β-lipotropin-(1–77) and rat β-lipotropin-(1–56) are endogenously present in anterior pituitary tissue and point to the significance of proteolytic enzymes cleaving the (human) Leu^{75}–Phe^{76} bond of β-lipotropin.

2.1.3. *γ-Lipotropin and β-Melanotropin*

Several NH_2-terminal forms of lipotropins have been identified. The complete NH_2-terminal portion resulting from cleavage of β-lipotropin at the (human) paired basic residues Lys^{57}–Arg^{58} has been termed γ-lipotropin (Fig. 2). In most species having a intermediate pituitary lobe γ-lipotropin seems to be the most abundant form of NH_2-terminal peptides derived from β-lipotropin. In addition, in several species peptides comprising the midportion of β-lipotropin have been detected. These peptides stimulate the pigmentation of amphibian skin and have been termed β-melanotropins (β-MSH) (Lerner *et al.*, 1954; Li, 1957) (Fig. 2). In the structure of β-lipotropin of several species, except mouse and rat, β-melanotropin is enclosed by two pairs of basic amino acid residues which can be cleaved during biosynthetic processing. A longer form of β-melanotropin, having NH_2-terminally a tetrapeptide extension (Fig. 2) has been isolated from human pituitary glands (Harris and Lerner, 1957; Pickering and Li, 1963). However, it has been demonstrated that this form of human β-melanotropin is an extraction artifact (Scott and Lowry, 1974; Bloomfield *et al.*, 1974; Bachelot *et al.*, 1977; Tanaka *et al.*, 1978a) possibly produced by cleavage by cathepsin D (Barát *et al.*, 1979).

2.2. ENDORPHINS

2.2.1. *β-Endorphin*

β-Endorphin is the 31 amino acid containing COOH-terminal portion of β-lipotropin. The peptide was found to have potent opiate-like activities (Bradbury *et al.*, 1976b; Li and Chung, 1976; Loh *et al.*, 1976; Cox *et al.*, 1976; Van Ree *et al.*, 1976). Since then β-endorphin has been subject to extensive research.

The structure of β-endorphin of a variety of species has been determined by isolation and amino acid sequencing of the peptide or via deduction from the nucleotide sequence of cloned cDNAs or a genomic fragment coding its precursor protein pro-opiomelanocortin. The structures are presented in Table 2.

The various mammalian β-endorphins display strong homology. Differences occur only in position 23 (Ile or Val), 27 (Tyr or His) and 31 (Gln or Glu). A discrepancy in data on the structure of rat β-endorphin exists with respect to the amino acid in position 26 (Val or Ala) (Table 2), which may be due to a polymorphism, rat strain differences, or modification of nucleotides during cloning procedures (J. Drouin, personal communication).

Multiple forms of β-endorphin exist due to two types of modification. Firstly, the NH_2-terminus of β-endorphin can be acetylated (Smyth and Zakarian, 1978, 1979; Zakarian and Smyth, 1979; Smyth *et al.*, 1979). This acetylation inactivates the opiate-like properties of the peptide (Deakin *et al.*, 1980). Acetyltransferase activities capable of acetylating the NH_2-terminus of β-endorphin have been detected (Chappell *et al.*, 1982; Glembotski, 1982b; O'Donohue, 1983). Secondly, the COOH-terminus of β-endorphin can be trimmed by proteolytic cleavage of the Lys^{28}–Lys^{29} residues followed by carboxypeptidase B like removal of these basic amino acids (Mains and Eipper, 1982; Eipper and Mains, 1981). This cleavage is an extension of biosynthetic processing. β-Endorphins having His at position 27, lose this residue due to proceeding carboxypeptidase action

TABLE 2. *Structure of β-Endorphin from Various Species**

Species	Ref.‡	Structure
human	A	H–^{1}Tyr–Gly–Gly–Phe–Met–Thr–Ser–Glu–Lys–Ser–^{11}Gln–Thr–Pro–Leu–Val–Thr–Leu–Phe–Lys–Asn–^{21}Ala–Ile–Ile–Lys–Asn–Ala–Tyr–Lys–Lys–Gly–^{31}Glu–OH
bovine	B	
ovine	C	
camel	D	H– ———— ^{27}His ———— ^{31}Gln–OH
whale	E	
mouse	F	
rat	G	H– ———— ^{26}(Val)†–^{27}His ———— ^{31}Gln–OH
porcine	H	H– ———— ^{23}Val ———— ^{27}His ———— ^{31}Gln–OH
equine	I	H– ———— ^{6}Ser ———— ^{27}His ———— ^{31}Gln–OH
turkey	J	H– ———— ^{9}His ———— ^{12}Met ———— ^{15}Leu ———— ^{23}Val ———— ^{25}Ser ———— ^{31}Gln–OH
ostrich	K	H– ———— ^{6}Ser ———— ^{9}Arg–Gly–Arg–Ala ———— ^{23}Val ———— ^{25}Ser ———— ^{31}Gln–OH
salmon-I	L	H– ———— ^{6}Lys–Pro–Tyr–Thr–Lys–^{11}Gln–Ser–His–Lys–Pro–Leu–Ile–Thr–Leu–Leu–^{21}Lys–His–Ile–Thr–Leu–Lys–Asn–Glu–^{29}Gln–OH
salmon-II	M	H– ———— Lys–Ser–Trp–Asn–Glu–Arg–Ser–Gln–Lys–Pro–Leu–Leu–Thr–Leu–Phe–^{21}Lys–Asn–Val–Ile–Ile–Lys–Asp–Gly–Gln–^{30}Gln–OH

(In the printed table, a brace groups refs B–F, bovine to mouse, and they share the single structure line shown in the camel row.)

*The amino acid sequences are presented in comparison with the human β-endorphin sequence: only amino acid residues different from those in human β-endorphin are shown. Structures are shown as non-acetylated peptides.

†In the sequence of rat β-endorphin a Val^{26} residue has been deduced from the nucleotide sequence of an isolated rat pro-opiomelanocortin gene (Drouin and Goodman, 1980). However, recent data derived from the nucleotide sequence of rat pro-opiomelanocortin cDNA (Drouin, personal communication) and amino acid composition of isolated peptides (e.g. Rubinstein *et al.*, 1977; Bennet *et al.*, 1983) shows that an Ala^{26} residue is present.

‡References: (A) Chrétien *et al.*, 1976b; (B) Li *et al.*, 1977; (C) Chrétien *et al.*, 1976b; Seidah *et al.*, 1977b; (D) Li and Chung, 1976; (E) Kawauchi *et al.*, 1980a; (F) Uhler and Herbert, 1983; (G) Rubinstein *et al.*, 1977; Drouin and Goodman, 1980; (H) Bradbury *et al.*, 1976a; Gráf *et al.*, 1976a; (I) Li *et al.*, 1981; (J) Chang *et al.*, 1980; (K) Naudé *et al.*, 1981a,b; (L) Kawauchi *et al.*, 1979; (M) Kawauchi *et al.*, 1980b.

(Eipper and Mains, 1981; Evans *et al.*, 1981; Smyth *et al.*, 1982; Zakarian and Smyth, 1982a,b; Bennett *et al.*, 1983). Consequently, at least six forms of β-endorphin exist, i.e. acetylated and nonacetylated β-endorphin-(1–31), -(1–27) and -(1–26).

In the intermediate lobe of the pituitary the predominant forms are the acetylated, truncated forms N^{α}-acetyl-β-endorphin-(1–31), N^{α}-acetyl-β-endorphin-(1–27) and N^{α}-acetyl-β-endorphin-(1–26), due to the processing pathway which involves a more rapid acetylation than proteolytic cleavage (Eipper and Mains, 1981; Glembotski, 1982a; Liotta *et al.*, 1981; Zakarian and Smyth, 1982a,b). In the anterior lobe acetylation and proteolytic trimming does not take place and β-endorphin-(1–31) is the predominantly occurring form (Mains and Eipper, 1981; Zakarian and Smyth, 1982a,b; Smyth and Zakarian, 1982).

In brain a more complex profile of multiple forms of β-endorphin is obtained. In the hypothalamus which contains the pro-opiomelanocortin neuronal cell bodies proteolytic processing but virtually no acetylation occurs; predominant forms are β-endorphin-(1–31) and -(1–27) (Zakarian and Smyth, 1982a,b; Weber *et al.*, 1981; Ng *et al.*, 1982). Acetylation of β-endorphin seems to take place in extrahypothalamic areas containing terminals of the pro-opiomelanocortin neurons, in particular hippocampus, septum, colliculae, amygdala and brainstem (Zakarian and Smyth, 1979; Zakarian and Smyth, 1982a,b, Wiegant *et al.*, 1983a). Other regions are rich in both acetylated and non-acetylated forms, like the thalamus and midbrain. Thus, two different sets of modified β-endorphins are found in different brain regions. It has been proposed that they represent two different processing pathways of β-endorphin in the brain (Zakarian and Smyth, 1982a,b): one mainly producing the opiate active form β-endorphin-(1–31), the other producing the N^{α}-acetylated optiate inactive forms. These peptides, however, may still possess other central activities. For instance, the opiate inactive β-endorphin fragment N^{α}-acetyl-γ-endorphin is fully active in behavioural paradigms (Wiegant *et al.*, 1983a).

2.2.2. *β-Endorphin Fragments: α- and γ-Endorphins*

Two peptides being the 1–16 and 1–17 sequence of β-endorphin were initially identified from ovine hypothalamic-hypophyseal extracts by the group of Guillemin (Guillemin *et*

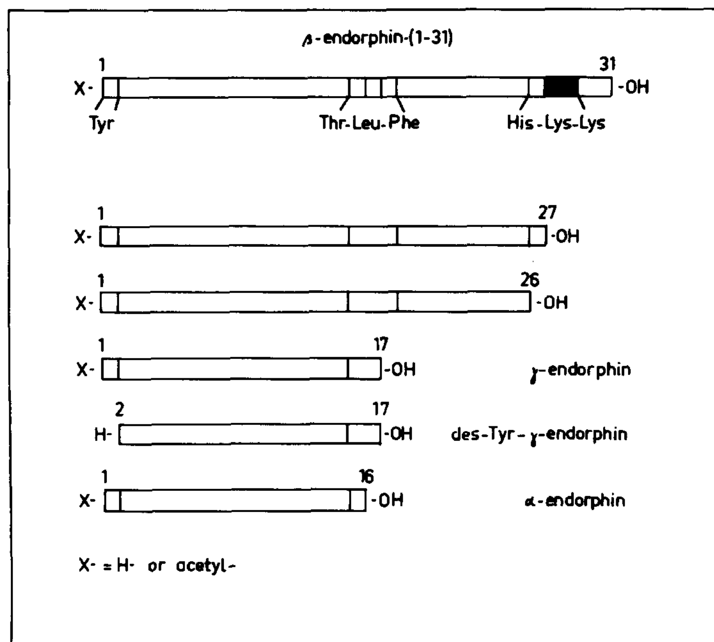

FIG. 3. β-Endorphin and derived peptides. Amino acid sequences of portions where proteolysis takes place are shown. These sequences are representative for most mammalian species, except human. Human β-endorphin has a Tyr^{26} residue (see Table 2); as a consequence the peptide β-endorphin-(1–26) does not occur in human tissues.

al., 1976; Ling *et al.*, 1976). These peptides have been designated α-endorphin (β-endorphin-(1–16)) and γ-endorphin (β-endorphin-(1–17)) (Fig. 3). Subsequently, several reports have indicated indirectly the presence of α- and γ-endorphins in pituitary tissue by detecting peptides with opiate-like properties of the size of these β-endorphin fragments (Giagnoni *et al.*, 1977; Sabol, 1978; 1980; Lissitsky *et al.*, 1978). Peptides related to α- and γ-endorphin have been immunologically and/or chromatographically characterized in extracts of rat anterior and intermediate pituitary (Jegou *et al.*, 1978, 1979; Fukata *et al.*, 1979; Verhoef *et al.*, 1980a,b, 1982; Dorsa *et al.*, 1982; Wiegant *et al.*, 1983a,b), in rat brain (Verhoef *et al.*, 1980, 1982a,b; Dorsa *et al.*, 1981; Wiegant *et al.*, 1983a,b), in human pituitary and brain (Burbach and Wiegant, 1983; Wiegant *et al.*, unpublished observations), in guinea pig myenteric plexus (Opmeer *et al.*, 1980), and in rat testicular tissue (Tsong *et al.*, 1982; Shu-Dong *et al.*, 1982; Margioris *et al.*, 1983). Immunohistochemical localization studies have shown the storage of α-endorphin and γ-endorphins in pituitary and rat brain (Bégeot *et al.*, 1978; Vaudry *et al.*, 1980). Recently, α- and γ-endorphins have been isolated from human pituitaries and chemically characterized (Burbach and Wiegant, 1983).

N^{α}-Acetylated-α- and -γ-endorphins have been identified in the rat neurointermediate lobe and extrahypothalamic brain (Wiegant *et al.*, 1983a,b; Sykes *et al.*, 1983). The distribution of α- and γ-endorphins parallels that of β-endorphin although their levels are 5–10 fold lower (Verhoef *et al.*, 1980; 1982a,b).

Formation of α- and γ-endorphins requires proteolytic cleavage in the -Thr^{16}-Leu^{17}-Phe^{18} sequence of β-endorphin. Such cleavage is distinctly different from the classical biosynthetic cleavage of paired basic amino acids which underly the formation of the COOH-terminally truncated forms of β-endorphin, β-endorphin-(1–27) and -(1–26) (Fig. 3). Proteolytic mechanisms able to form α- and γ-endorphins from β-endorphin have been indicated (see Section 4.1.).

In addition to α- and γ-endorphins other β-endorphin fragments have been detected. Peptides with chromatographic properties of acetylated and non-acetylated β-endorphin-(1–21) have been detected in rat pituitary and brain tissue (Sykes *et al.*, in preparation) and β-endorphin-(1–18) has been isolated from human autopsy pituitaries (Vuolteenako and Leppäluoto, 1983).

Important biological functions of α- and γ-endorphins have been discovered by de Wied and co-workers (de Wied, 1978; de Wied *et al.*, 1980). It has been demonstrated that α-endorphin and γ-endorphin each represent a separate class of neuropeptides with distinct behavioral activities (de Wied, 1978; Van Ree *et al.*, 1980; Van Ree and de Wied, 1982; de Wied and Jolles, 1982). The behavioral effects of these peptides are independent from the opiate-like activities of α- and γ-endorphin. For example, N^{α}-acetylated-γ-endorphin and fragments of γ-endorphin such as des-Tyr^{1}-γ-endorphin (β-endorphin-(2–17) and β-endorphin-(6–17) which are devoid of opiate-like effects display full behavioral activities (de Wied, 1978; de Wied *et al.*, 1978, 1980; Wiegant *et al.*, 1983a). These data demonstrate the significance of α- and γ-endorphins as neuropeptides in the brain and point to a functional role of the proteolytic enzymes responsible for their formation (Burbach and de Wied, 1980, 1982; Burbach and de Kloet, 1982).

3. ACTION OF PROTEOLYTIC ENZYMES ON β-LIPOTROPIN

The structural relationship of β-lipotropin with γ-lipotropin, β-melanotropin and β-endorphin (Fig. 2) has prompted several investigators to indicate β-lipotropin as a precursor peptide (Chrétien and Li, 1967; Bradbury *et al.*, 1975, 1976a). Subsequent biosynthetic studies have supported this concept (Chrétien *et al.*, 1976a, 1979; Herbert *et al.*, 1980; Eipper and Mains, 1980). The primary function of β-lipotropin as precursor is also emphasized by the absence of an outspoken biological, e.g. hormonal, activity of β-lipotropin: although β-lipotropin possesses lipolytic activity on rabbit adipose tissue, it has very little lipolytic activity on rat and human fat cells (Chrétien and Lis, 1978; Lis, 1982). As a consequence of this precursor function of β-lipotropin studies on the

proteolytic mechanisms underlying the processing of β-lipotropins into its endproducts have been undertaken. In particular two tissues have been subject to these studies. Firstly, the pituitary gland, which is most rich in β-lipotropin (anterior lobe) and its conversion products β-endorphins and γ-lipotropins (intermediate lobe) and secondly, the brain, in which the biological significance of the β-lipotropin conversion product β-endorphin as opioid neuropeptide is outspoken.

3.1. PROTEOLYSIS OF β-LIPOTROPIN BY PITUITARY ENZYMES

3.1.1. *Processing Enzymes: Cleavage at Paired Basic Amino Acids*

Much experimental work has been focused on the formation of β-endorphin from β-lipotropin. It involves cleavage of peptide bonds of the paired basic amino acid residues in the (porcine) β-lipotropin sequence Asp^{58}-Lys-Arg-Tyr^{61}. Different approaches have been chosen to track down the proteolytic enzyme involved in this cleavage in the pituitary gland.

Bradbury *et al.* (1976a) using the synthetic hexapeptide H-Lys-Asp-Lys-Arg-Tyr-Gly-OH, observed that the Arg–Tyr bond was rapidly cleaved by an enzyme activity isolated from secretory granules of the pituitary. This proteolytic activity had a molecular weight of approximately 100,000 dalton and had a pH optimum of 8.5. It was not inhibited by soya bean trypsin inhibitor, but was inactivated by phenylmethylsulphonyl fluoride. These properties distinguish the pituitary enzyme from pancreatic trypsin (Bradbury *et al.*, 1976a; Austen and Smyth, 1978; Smyth *et al.*, 1978).

At the same time Gráf and co-workers observed that by exposing porcine β-lipotropin to a pituitary homogenate β-lipotropin-(1–60) was formed (Gráf and Kenesey, 1976; Gráf *et al.*, 1977). Other products present in the digests were β-lipotropin-(1–46) and -(1–79). In parallel with the formation of β-lipotropin-(1–60) peptides with opiate activity in the mouse vas deferens and guinea pig ileum bioassays were generated. The potency ratio's in these bioassays, however, indicated that the peptide was not β-endorphin-(1–31), but rather a shorter peptide like β-endorphin-(1–9) (Gráf *et al.*, 1977). The proteolytic activity generating β-lipotropin-(1–60) was diminished in anterior pituitary homogenates of animals treated with dexamethasone or ACTH and was increased in the pituitary of adrenalectomized animals. The activity of the enzyme could also be determined by employing the synthetic substrate Z-Lys-Pro-Arg-p-nitroanilide and showed similar changes after experimental manipulation (Kenessey *et al.*, 1979; Gráf *et al.*, 1978a,b).

Further studies of Gráf and coworkers on the proteolytic activity responsible for β-endorphin formation has involved subcellular fractionation studies and partial purification of the enzyme from porcine anterior pituitaries (Kenessey *et al.*, 1978; Gráf and Kenessey, 1981). The enzyme activity generating β-lipotropin-(1–60) co-purified with small and large secretory granule fractions (Kenessey *et al.*, 1978). These subcellular fractions of anterior pituitary were similarly enriched in enzyme activity cleaving the synthetic substrate Z-Lys-Pro-Arg-p-nitroanilide. During subcellular fractionation the Leu^{77}–Phe^{78} cleaving activity was dissociated from the Arg^{60}–Tyr^{61} cleaving enzyme (Gráf *et al.*, 1978a). The processing enzyme activity remained associated with secretory granule membranes after repeated freezing and thawing and Triton-X-100 treatment. However, after prolonged incubation of granule fractions at 37°C, pH 8, considerable activity was released. Further purification of solubilized enzyme activity involved ammonium sulfate precipitation and gel filtration, or affinity chromatography on immobilized Kunitz trypsin inhibitor (Gráf *et al.*, 1979a; Gráf and Kenessey, 1981). A purification of at least 25 times was achieved. Further detailed biochemical studies on the enzyme activity were hampered by the extreme instability of the enzyme (Gráf and Kenessey, 1981).

Austen (1982) using the synthetic substrate Ac-Arg-Arg-naphthylamide, purified a serine protease-like enzyme from porcine anterior pituitaries that released β-endorphin from β-lipotropin. The enzyme had a molecular weight of 60,000 dalton and a pH optimum of 8–8.5. Other observations of processing enzymes with basic pH optima in the pituitary

have been made by Lee and Lee (1982) using [^{125}I]pro-opiomelanocortin and H-Arg-Leu-OH and by Heinrich and Todd (1979) using pro-insulin as substrate. Using adrenal enkephalin precursors as substrate, Lindberg *et al.* (1982a,b) found similar proteolytic activity in adrenal chromaffin granules. These basic enzyme activities may be members of the kallikreins, a group of serine proteases with regulatory action (Schachter, 1980). Indeed, a recent report demonstrated the presence of a kininogenase resembling glandular kallikrein in the rat pars intermedia (Powers and Nasjletti, 1983). The pH optimum of 8, the resistance of soya bean trypsin inhibitor and subcellular distribution indicate similarity of this kallikrein with activities described by Gráf and Austen.

In other studies biosynthetically labeled pro-opiomelanocortin has been employed to define processing enzyme activity with apparently different properties (Loh and Chang, 1982). Loh and co-workers recognized an enzyme activity which met the criteria for processing enzymes such as production of β-lipotropin, a β-endorphin-like peptide, a large form of ACTH and α-MSH (Loh and Chang, 1982; Loh and Gainer, 1982a; Chang *et al.*, 1982; Loh *et al.*, 1982). The activity was detected in purified secretory granule fractions of rat and bovine neurointermediate lobes (Loh and Chang, 1982; Chang *et al.*, 1982) and rat anterior lobe (Chang and Loh, 1983). The processing enzyme activity has been characterized by the use of inhibitors as an acid thiol, arginyl endopeptidase with a pH optimum of approximately pH 5 (Loh and Gainer, 1982a; Chang *et al.*, 1982). This is close to the internal pH of 5.5 in secretory granules (Johnson and Scarpa, 1976; Russel and Holz, 1981).

This enzyme activity may be related or similar to other processing enzymes with acidic pH optima identified in pancreatic islet secretory granules of angler fish and rat pancreas (Fletcher *et al.*, 1980, 1981; Docherty *et al.*, 1982; 1983) and adrenal chromaffin granules (Mizuno *et al.*, 1982; Evangelista *et al.*, 1982; Troy and Musacchio, 1982). These enzymes have been defined on the basis of cleavage of pro-insulin, pro-glucagon, pro-somatostatin and/or enkephalin precursors. Several of these enzyme activities have been identified as cathepsin B (MacGregor *et al.*, 1979a,b; Docherty *et al.*, 1983). Properties of processing enzymes are listed in Table 3.

It has not been established as yet which of the two kinds of processing enzymes is physiologically involved in precursor processing or whether both have separate functions. The two types of enzymes differ markedly (Table 3), but fulfill several criteria for processing enzymes. However, the studies which describe purification and properties of the enzymes differ in tissue, species, substrates, assay conditions, and approach of purification. These aspects have recently been reviewed (Docherty and Steiner, 1982; Lazure *et al.*, 1983).

3.1.2. *Chymotryptic-Like Enzymes*

Seidah *et al.* (1983) recently described properties of an enzyme activity from a pig anterior pituitary secretory granule lysate. The enzyme had specificity towards some Phe–X and Tyr-X bonds in rat pro-opiomelanocortin, suggesting similarity with chymotrypsin. In the β-lipotropin region of pro-opiomelanocortin it cleaved the Phe^{168}–Arg^{169} (rat β-lipotropin-(30–31)) bond. The pH optimum was 8. Chymotryptic-like activity has been reported to be present in anterior pituitary tissue previously (Horsthemke and Bauer, 1981). Noteworthy, enzymes with chymotryptic-like specificities have been implicated in the processing of several hormones including gastric inhibitory polypeptide, relaxin, pro-insulin and the COOH-terminal glycopeptide of the vasopressin precursors (Docherty and Steiner, 1982; Jörnvall *et al.*, 1981; Hudson *et al.*, 1981; Tager *et al.*, 1973; Smyth and Massey, 1979, Land *et al.*, 1982).

3.1.3. *Cathepsin D*

The observation of Gráf and Kenessey (1976) that digestion of porcine β-lipotropin by anterior pituitary homogenates at pH 6.5 resulted predominantly in cleavage of the

TABLE 3. *Putative Processing Enzymes Cleaving at Paired Basic Amino Acids*

Authors (ref.)	Substrate	Source of Enzyme Activity	pH optimum	Classification of Enzyme Activity
Gráf *et al.* (1979a) Gráf and Kenessey (1981)	porcine β-lipotropin Z-Lys-Pro-Arg-*p*-nitroanilide	anterior pituitary granules (porcine)	8.0	trypsin-like (not inhibited by soybean trypsin inhibitor)
Austen (1982)	porcine β-lipotropin Ac-Arg-Arg-naphthylamide	anterior pituitary granules (porcine)	8.0–8.5	serine protease
Lee and Lee (1982)	[^{125}I]pro-opiomelanocortin H-Arg-Leu-OH	pituitary microsomes	8.0-9.0	metallo(?) protease (requires Mn^{2+} or Mg^{2+})
Heinrich and Todd (1979)	pro-insulin H-Arg-Arg-β-naphthylamide	anterior pituitary fraction	8	(complete inhibition by *p*-chloromercuribenzoate, DTT and *o*-phenanthroline)
Lindberg *et al.* (1982a,b)	chromaffin granule lysate (pro-enkephalins) peptide F	adrenal chromaffin granules	8.0	serine protease
Chang *et al.* (1982)	[^{3}H]pro-opiomelanocortin (toad)	posterior pituitary granules (bovine)	4–5	arginyl-thiol protease
Loh and Gainer (1982a)	[^{3}H]pro-opiomelanocortin (toad)	posterior pituitary granules (rat)	5	arginyl-thiol protease
Chang and Loh (1983)	[^{3}H]pro-opiomelanocortin	anterior pituitary granules (rat)	5	arginyl-thiol protease
Mizuno *et al.* (1982)	chromaffin granule lysate (pro-enkephalins)	adrenal chromaffin granules	5.5	arginyl-thiol protease
Fletcher *et al.* (1980, 1981)	pro-insulin, pro-glucagon, pro-somatostatin	pancreatic islet granules	4.5–5.5	arginyl-thiol protease
Troy and Masacchio (1982)	chromaffin granule lysate peptide E	adrenal chromaffin granules	5.7	thiol protease
Evangelista *et al.* (1982)	Tosyl-Arg-methylester Bam-12, Leu-8	adrenal chromaffin granules	5	(PMSF insensitive)
Docherty *et al.* (1982, 1983)	pro-insulin	pancreatic islet granules	5–6	thiol protease, cathepsin B
MacGregor *et al.* (1979a,b)	pro-parathyroid hormone	parathyroid fraction	6–7	thiol protease
Habener *et al.* (1977)	pro-parathyroid hormone	parathyroid extract	—	(inhibited by EDTA, not by tosyl-Lys-chloromethylketone)

Leu^{77}–Phe^{78} bond, thus in formation of β-lipotropin-(1–77), has prompted further studies on the proteolytic enzyme involved. The pH optimum of approximately 4, the inhibition by 10^{-6} M pepstatin, which is a carboxyl-proteinase inhibitor (Umezawa and Aoyagi, 1977), and its subcellular localization suggested that the enzyme activity might be lysosomal cathepsin D (Gráf *et al.*, 1978a, 1979a). Indeed, cathepsin D purified from human pituitaries cleaved β-lipotropin into a product co-migrating with β-lipotropin-(1–77) (Benuck *et al.*, 1978b) (see Section 3.3.1).

3.2. Proteolysis of β-Lipotropin by Brain Enzymes

The proteolytic conversion of β-lipotropin by enzymes in brain tissue has been investigated in order to define the processes responsible for the production of the opioid peptide β-endorphin. Kenessey *et al.* (1977) investigated the proteolysis of porcine β-lipotropin by brain homogenates at pH 6.5 and pH 8.0. The predominant product formed at pH 6.5 had electrophoretic properties of β-lipotropin-(1–77). No regional differences in the profiles of products were observed in brain. The electrophoretic pattern was similar as the one produced by homogenates of pituitary pars distalis, pars intermedia and pars nervosa. The cleavage of β-lipotropin was stimulated by dithiothreitol and Versene (Kenessey *et al.*, 1977). Further work of this group confirmed these data at pH 4.0 and demonstrated that the formation of β-lipotropin-(1–77) by brain homogenates was due to cathepsin D activity (Gráf *et al.*, 1979a). Cathepsin D isolated from bovine brain by affinity chromatography (Benuck *et al.*, 1978a) cleaved the Leu–Phe bond of porcine and human β-lipotropin (Benuck *et al.*, 1978b; Gráf *et al.*, 1979b; Barát *et al.*, 1979). At pH 8 porcine β-lipotropin was degraded only very slowly by brain homogenates. No degradation products have been identified under these conditions. However, at pH 8 in the presence of bacitracin opiate activity was generated during incubation of β-lipotropin with a mitochondrial-synaptosomal (P2) fraction of rat brain as tested on the mouse vas deference preparation (Gráf *et al.*, 1979a) indicating that the Arg^{60}–Tyr^{61} bond of porcine β-lipotropin was cleaved. However, accumulation of opiate activity was much less than of an analogous fraction of the pituitary.

Austen and Smyth (1978) found in brain only low enzyme activity cleaving the synthetic substrate H-Lys-Asp-Arg-Tyr-Gly-OH (porcine β-lipotropin-(57–62)) between Arg and Tyr. The enzyme activity was most concentrated in the pituitary gland.

3.3. Action of Purified Enzymes on β-Lipotropin

3.3.1. *Cathepsin D*

Above mentioned action of cathepsin D on β-lipotropin (see Section 3.1.3) has been investigated in more detail by using the purified enzyme. Cathepsin D purified from calf brain by affinity chromatography on pepstatin–Sepharose (Benuck *et al.*, 1978a) split initially the Leu–Phe bond of human (residues 75–76) and porcine β-lipotropin (residues 77–78), forming β-lipotropin-(1–75) and -(1–77) respectively, and β-endorphin-(18–31) (Gráf *et al.*, 1979b; Barát *et al.*, 1979). During longer incubation of porcine β-lipotropin the Ala^{32}–Glu^{33} peptide bond was cleaved as well by the enzyme preparation. The secondary cleavage site in human β-lipotropin was the Ala^{34}–Ala^{35} bond (Barát *et al.*, 1979) (Fig. 2). This cleavage could be responsible for artifactual formation of human β-melanotropin. Based on these data Barát *et al.*, (1979) pointed to a peculiar specificity of cathepsin D, which rather seems to have a preference for the hydrophobic residues Leu-Val at positions 2 or 3 before the cleavage site than for the amino acids forming the cleaved bond. This suggestion is supported by observations of Lebouille and Burbach (unpublished) that the action of cathepsin D purified from bovine spleen on small synthetic peptides derived from β-endorphin is prevented when the Leu-Val sequence is modified (Table 4).

In contrast to above mentioned studies seven to eight cleavage sites in porcine β-lipotropin have been reported by Akopyan and colleagues who used a cathepsin D

TABLE 4. *Cleavage of β-Lipotropin and Related Peptides by Cathepsin D: Significance of the Leu–Val Sequence to Direct Cleavage*

Peptide	Sequence Surrounding the Cleavage Site		Reference
porcine β-lipotropin	H–Glu^{1}– — –Gly^{29}–[Leu–Val]–Ala↓Glu^{33}– —		(A)
	–Pro^{73}–[Leu–Val]–Thr–Leu↓Phe^{78}–Lys– — –Gln^{91}–OH		
human β-lipotropin	H–Glu^{1}– — –Leu^{31}–[Leu–Val]–Ala↓Ala^{35}– —		(B)
	– — –Pro^{71}–[Leu–Val]–Thr–Leu↓Phe–Lys– — –Glu^{89}–OH		
β-endorphin	H–Tyr^{1}– — –Pro^{13}–[Leu–Val]–Thr–Leu↓Phe^{18}–Lys– — –Glu^{31}–OH		(C)
[Ac–Glu^{13},Glu^{22}–$NHCH_3$]-β-endorphin-(13–22)	Ac–Glu^{13}–[Leu–Val]–Thr–Leu↓Phe–Lys– — –Glu^{22}–$NHCH_3$		(D)
[D–Pro^{13},Glu^{22}–$NHCH_3$]-β-endorphin-(13–22)	D–Pro^{13}–[Leu–Val]–Thr–Leu↓Phe–Lys– — –Glu^{22}–$NHCH_3$		(D)
[Ac–Val^{15},Lys^{19}–$NHCH_3$]-β-endorphin-(15–19)	Ac–[Val^{15}]–Thr–Leu–Phe–Lys^{19}–$NHCH_3$	NO CLEAVAGE	(D)
insulin β-chain	–His^{10}–[Leu–Val]–Glu↓Ala^{14}–		(E)

(A) Gráf *et al.* (1979b); (B) Barát *et al.* (1979), the data have been reconsidered in view of the revised human β-lipotropin structure (see Fig. 2); (C) Gráf *et al.* (1979b), Benuck *et al.* (1978b), Burbach *et al.* (1980b); (D) Lebouille and Burbach (unpublished); (E) Press *et al.* (1960).

preparation from bovine hypothalamus (Arytyunyan *et al.*, 1980, 1981; Akopyan *et al.*, 1982). These sites include the 14–15, 25–26, 26–27, 29–30, 31–32, 49–50, 63–64 and 77–78 peptide bonds of porcine β-lipotropin (for structure see references in Table 1). These results differ from those of Gráf *et al.*, (1979b) and Barát *et al.*, (1979) who found only two cleavage sites. Differences may be due to the purity of the cathepsin D preparations and/or the incubation conditions used.

3.3.2. *Plasmin*

Studies on the fragmentation of porcine β-lipotropin by plasmin showed that the Lys^{79}–Asn^{80} bond was most susceptible to the enzyme; the fragment β-lipotropin-(1–79) appeared as initial product (Gráf, 1976). Secondary cleavage sites were the Arg^{51}–Trp^{52} and Arg^{60}–Tyr^{61} peptide bonds. The Lys^{46}–Met^{47}, Lys^{69}–Ser^{70} and Lys^{84}–Asn^{85} bonds were hydrolyzed only very slowly. Further lysyl and arginyl bonds of porcine β-lipotropin were not cleaved under the experimental conditions (Gráf, 1976; Gráf and Li, 1973). Although plasmin displayed some preference for hydrophobic residues in the surrounding region of the cleavage site adjacent to lysyl or arginyl residues, no clear-cut preference for particular residues was obtained, possibly due to conformational properties of β-lipotropin.

3.3.3. *Trypsin*

The influence of β-lipotropin conformation on proteolytic enzyme action has been studied using immobilized trypsin (Gráf and Hollósi, 1980). Mild digestion of porcine β-lipotropin by trypsin–Sepharose in aqueous solution resulted in cleavage of the Arg^{51}–Trp^{52} and Arg^{60}–Tyr^{61} bonds. In 20% trifluoroethanol, a secondary structure promoting solvent, the Arg^{60}–Tyr^{61} bond was exclusively split. The Arg^{51}-Trp^{52} sequence lies in a region with high α-helix potential and may therefore have a more rigid conformation as compared to the Arg^{60}–Tyr^{61} sequence which may be sterically exposed for enzyme attack since it is neighboured by two β-turns (Gráf and Hollósi, 1980). These data have been considered as support for the views of Geisow (1978) and Loh and Gainer (1978, 1979) that specificity of proteolytic processing is partly directed by the conformation of the substrate. However, later work of Loh and Gainer (1982b) seem to contradict this view, since it was shown that glycosylated and non-glycosylated pro-opiomelanocortin was qualitatively and quantitatively similarly processed by a pro-opiomelanocortin converting enzyme activity.

Trypsin has also been used to prepare authentic opiate-active β-endorphin from citraconylated β-lipotropin (Seidah *et al.*, 1977a; Geisow and Smyth, 1977b).

3.3.4. *Tonin*

Tonin, a serine protease converting angiotensinogen directly into angiotensin II, occurs in high concentrations in the submaxillary gland, and is also present in the pituitary (Boucher *et al.*, 1974; Demassieux *et al.*, 1976; Seidah *et al.*, 1979). At pH 6.8 ovine β-lipotropin was split by tonin purified from rat submaxillary glands between residues 50–51, 51–52, 60–61 and 78–79 (Seidah *et al.*, 1979). At pH 5.0 main cleavage sites were 23–24, 51–52, 60–61, 78–79 and 79–80 (Chan *et al.*, 1981). Amongst fragments released during digestion were β-lipotropin-(61–78), -(61–79) and -(61–91), which all possess opioid properties. Optimal accumulation of opioid activity was reached by prolonged tonin digestion at pH 5.0 (Chan *et al.*, 1981). Using the release of opioid activity after tonin digestion, an assay to detect β-lipotropin was constructed (Chan *et al.*, 1981).

3.3.5. *Cathepsin B*

Cathepsin B purified from calf brain has been shown to split β-lipotropin into several products (Suhar and Marks, 1979; Marks *et al.*, 1980). The products and cleavage sites have not been determined, however.

4. ACTION OF PROTEOLYTIC ENZYMES ON β-ENDORPHIN

β-Endorphin is a product of the intracellular processing of pro-opiomelanocortin in the pituitary gland (Crine *et al.*, 1979; Mains and Eipper, 1979; Roberts *et al.*, 1978) as well as in the brain (Liotta *et al.*, 1980; Gramsch *et al.*, 1980). The potent opioid properties of β-endorphin indicated an important physiological function of the peptide in the central nervous system (Bradbury *et al.*, 1976b; Cox *et al.*, 1976; Li and Chung, 1976; Loh *et al.*, 1976; Van Ree *et al.*, 1976; Seidah *et al.*, 1977). Consequently, most studies on the proteolysis of β-endorphin have been concerned with brain tissue. Many of them were carried out from the viewpoint of inactivation of opioid activity or mere degradation of β-endorphin. Others, however, have considered proteolytic cleavage of β-endorphin as a process necessary for formation of peptides with biological function.

Firstly, these studies have focused on α- and γ-endorphins. The β-endorphin fragments α- and γ-endorphins and their N^{α}-acetylated forms are endogenous peptides in the pituitary and the brain and they possess separate biological activities indicating a function of β-endorphin as precursor peptide (see Section 2.2.2.). The biotransformation of β-endorphin into these peptides has been investigated.

Secondly, prompted by the identification of the enkephalins by Hughes *et al.* (1975), β-endorphin has initially been considered as precursor or biosynthetic intermediate of methionine-enkephalin. A few reports deal with enzyme activities able to release the methionine-enkephalin sequence (β-endorphin-(1–5)) from β-endorphin. Later research, however, has clearly demonstrated that methionine-enkephalin is not derived from β-endorphin and that a separate precursor for methionine-enkephalin exists (Comb *et al.*, 1982; Gubler *et al.*, 1982; Legon *et al.*, 1982; Noda *et al.*, 1982a,b).

4.1. PROTEOLYSIS OF β-ENDORPHIN IN BRAIN TISSUE

4.1.1. *Homogenates and Soluble Fractions*

β-Endorphin is rapidly degraded by proteolytic enzymes present in homogenates and soluble fractions of brain tissue. This process has mainly been investigated by following the release of amino acids during exposure of β-endorphin to soluble fractions of brain tissue. Marks *et al.*, (1977) observed that after 5 min of incubation at pH 7.6 3 to 7% of several amino acid residues from different positions of the β-endorphin sequence appeared, while other amino acids were absent. However, after 30 min, 60 min and 180 min of incubation (Marks *et al.*, 1977; Marks *et al.*, 1978; Marks, 1978) all amino acid residues were detected in approximately equal amounts, with some preference for tyrosin, methionine, glutamine and leucine (for the primary structure of β-endorphin see Table 2). Using a similar approach Patthy *et al.* (1977) also observed a time dependent release of all the constituent amino acids, proportionally to the data reported by Marks *et al.* (1977) except for methionine which was considerably lower in the experiment of Patthy and co-workers and phenylalanine and lysine which were released more rapidly. These studies indicated: (1) that initially a limited number of peptide bonds are cleaved and (2) that multiple types of enzyme activities cleaving the β-endorphin sequence at different sites are involved in the degradation. The authors suggested the action of trypsin-like enzyme activity.

The preferential release of lysine, leucine and phenylalanine suggested peptidase action on the mid position of β-endorphin, which was in accord with earlier findings of Gráf and co-workers (Gráf and Kenessey, 1976; Kenessey *et al.*, 1977) of an enzyme activity cleaving the Leu^{17}–Phe^{18} bond of β-endorphin. The high degree of release of tyrosine from the NH_2-terminus of β-endorphin indicated the presence of aminopeptidase activities.

Additional studies with peptidase inhibitors and D-amino acid substituted analogs provided more insight in the mechanism of proteolysis of β-endorphin by brain soluble enzymes. The inhibition of tyrosine release by bacitracin, an antibiotic peptidase inhibitor (Desbuquois *et al.*, 1974), provided more evidence for the involvement of aminopeptidases (Patthy *et al.*, 1977). Moreover it appeared that also the release of phenylalanine, leucine,

isoleucine and valine was markedly inhibited by bacitracin which led the authors to suggest that also brain carboxypeptidases were inhibited by bacitracin. Indeed, it was observed that bacitracin not only effectively protects β-endorphin fragments against aminopeptidase degradation, but it also inhibits carboxypeptidases as well as endopeptidases in a rather non-specific fashion (Burbach, unpublished). The effect of bacitracin in the presence of Boc-D-Phe-Pro-Arg-OH, a selective serine protease inhibitor (Bajusz *et al.*, 1975, 1978), was even more outspoken (Gráf *et al.*, 1979a) and showed the different inhibitory mechanisms of these compounds. Pepstatin, an inhibitor of carboxyl proteases such as cathepsin D (Umezawa and Aoyagi, 1977), had only a minor effect. The most significant effect of inhibition of β-endorphin degradation by pepstatin was the slower release of leucine and phenylalanine, which is in agreement with cleavage of the Leu^{17}–Phe^{18} bond by cathepsin D (Benuck *et al.*, 1978b; Gráf *et al.*, 1979b, see also Section 4.1.3).

Marks *et al.* (Marks *et al.*, 1978; Grynbaum *et al.*, 1977) provided additional arguments for the action of exo- and endopeptidase activities on β-endorphin by studying the degradation of β-endorphin analogs. The replacement of $glycine^2$ by D-alanine significantly reduced NH_2-terminal breakdown as reflected by the relatively low release of tyrosine (Grynbaum *et al.*, 1977). The yields of all amino acids released from [D-Ala^2]-β-endorphin was reduced as compared to β-endorphin, indicating that the substitution also affected other cleavage sites in the peptide. Incubation of the [D-Leu^{17},D-Lys^{19}]-analog of β-endorphin with a mouse brain extract led to rapid release of tyrosine and relatively rapid release of alanine and isoleucine. The overall degradation of the analog was slower than that of native β-endorphin (Marks *et al.*, 1978). The triple substituted analog [D-Thr^6, D-Leu^{17}, D-Lys^{19}]-β-endorphin was even more resistant to degradation by soluble peptidases. Again the release of tyrosine, isoleucine, and alanine was predominant.

These experiments, based on the release of free amino acids indicated the involvement of a number of different peptidases in the proteolysis. Firstly, aminopeptidases released rapidly the NH_2-terminal tyrosine, but were less active in removing the contiguous glycine residues. However, tyrosine release seemed to be preceded by internal bond cleavages (Gráf *et al.*, 1979a). Secondly, endopeptidases cleaved β-endorphin at different sites, primarily in the midportion of the peptide around residues 15 to 22. Thirdly, a role for carboxypeptidases in the degradation of initially formed β-endorphin fragments was also suggested (Patthy *et al.*, 1977). Indeed, the COOH-terminal amino acids of [D-Ala^2]-γ-endorphin and [D-Ala^2]-α-endorphin were rapidly released upon incubation with mouse brain extracts (Grynbaum *et al.*, 1977). The experimental approach, which is not suitable for identification of the products of proteolysis, did not enable one to define the precise cleavage sites nor the sequence of proteolytic events. In general, the observations made indicate that the mechanism of β-endorphin proteolysis by soluble fractions of brain is comparable with that by brain membrane associated peptidases (see Section 4.1.2).

Experiments aimed to identify the products of β-endorphin proteolysis by soluble peptidase are limited. Rossier *et al.* (1977) showed by radioimmunoassay that α-endorphin was one of the intermediates in the proteolysis of β-endorphin by rat brain extracts. Under the conditions used β-endorphin had a half-life of 47 min and α-endorphin represented approximately 5% of the initial amount of β-endorphin after 2 hr of incubation. Palmour *et al.*, (1978) incubated [^{125}I]-iodinated β-endorphin and [Leu^5]-β-endorphin with dialyzed supernatants from rat brain and compared radioactive products by thin-layer electrophoresis with standard peptides. A very rapid degradation of the β-endorphins was observed accompanied by the formation of free tyrosine as the main radioactive product. The half-life of β-endorphin was 12 min and that of [Leu^5]-β-endorphin 9 min. The main peptide fragments found were β-endorphin-(1–4) and [Leu^5]-β-endorphin-(1–5). Small quantities of γ-endorphin were found. The production of peptides with chromatographic properties of α- and γ-endorphin by whole brain homogenates has been reported (Davies *et al.*, 1982). Experiments of our group showed that rat brain cytosol converted β-endorphin into a number of peptides including α- and γ-endorphin as characterised by high-pressure liquid chromatography. Homogenates and various subcellular fractions including mitochondria, microsomes, synaptosomes and soluble fractions also generated

α- and γ-endorphins, suggesting a broad cellular distribution of proteolytic enzymes able to produce these β-endorphin fragments (Burbach, unpublished).

Enzyme activities cleaving the Met^5–Thr^6 bond of β-endorphin, thus forming methionine-enkephalin, have been detected in brain soluble fractions (Aono *et al.*, 1978; Koida *et al.*, 1979; Austen *et al.*, 1977a; Austen and Smyth, 1978; Knight and Klee, 1979; Palmour *et al.*, 1978; Orlowski *et al.*, 1980). Knight and Klee (1979) described properties of this enzyme activity, while others have purified enzymes cleaving the Met^5–Thr^6 bond (Koida *et al.*, 1979; Orlowski *et al.*, 1980). Initially these enzymes were thought to have a role in the biosynthesis of methionine-enkephalin. However, since the discovery of enkephalin biosynthetic pathways independent of β-endorphin, these enzymes have received little attention.

4.1.2. *Slice Preparations, Synaptosomes and Synaptic Membranes*

Experiments on the proteolysis of peptides employing homogenates and soluble extracts are likely to expose a bulk of degradative peptidases to the substrate which are not functionally involved in the conversion and degradation of the peptides under physiological conditions. In several studies on the proteolysis of endorphins, it has been attempted to restrict the interference of 'contaminating' peptidases by using brain slices, synaptosomal preparations or isolated synaptic membranes. The assumption that functional proteolytic events in the conversion or degradation of endorphins take place extracellularly, in or near the synaptic environment, the sites of storage, release and action of endorphins, underly the choice of these preparations. In addition, a push–pull perfusion technique has been employed to study the conversion of locally applied peptide in a restricted brain area (Burbach, 1983).

Smyth and Snell (1977a,b) incubated [^{125}I-Tyr^1]-β-endorphin or [^{125}I-Tyr^1]-γ-endorphin with slices of the rat striatum. Under the experimental conditions [^{125}I]-β-endorphin had a half-life of 3.2 hr; the half-life of [^{125}I]-γ-endorphin was notably shorter, i.e. 1.1 hr. Bacitracin, a bacterial substance with aminopeptidase inhibiting properties (Desbuquois *et al.*, 1974), did not affect the proteolysis of [^{125}I]-β-endorphin, but it increased the half-life of [^{125}I]-γ-endorphin to 3.6 hr. These results indicated firstly that the NH_2-terminus of β-endorphin is markedly more resistant to proteolytic attack than γ-endorphin and secondly that cleavage of β-endorphin is not initiated by aminopeptidases, but by other types of peptidases presumably endopeptidases. Protecting the NH_2-terminal [^{125}I]tyrosine residue of products by including bacitracin in the incubation medium, Smyth and Snell (1977a,b) demonstrated that the major product of conversion of [^{125}I]-β-endorphin was [^{125}I]-γ-endorphin. Low quantities of [^{125}I]-α-endorphin, methionine-enkephalin, β-endorphin-(1–6), and β-endorphin-(1–7) were reported (Smyth and Snell, 1977b). These investigators pointed out that these products were formed extracellularly in the slice preparation. No active uptake of [^{125}I]peptides by the tissue was observed.

Similar results were obtained when washed synaptosomes were used (Austen *et al.*, 1977a,b; Austen and Smyth, 1977a,b; Smyth *et al.*, 1977). At pH 7.4 [^{125}I]-β-endorphin was principally converted into [^{125}I]-γ-endorphin and there was a small amount of [^{125}I]-methionine-enkephalin. The accumulation of these peptides was strongly favoured by the presence of bacitracin or by acid pH due to inhibition of aminopeptidase activity. At pH 5.0 the proteolysis was more rapid and the products characterized were [^{125}I]-α-endorphin, [^{125}I]-β-endorphin-(1–13), -(1–8) and [^{125}I]methionine-enkephalin. Austen *et al.* (1977; Austen and Smyth 1977a,b) also observed that synaptosomes contained an endopeptidase cleaving the peptide β-endorphin-(1–7) into methionine-enkephalin and the dipeptide Thr-Ser. This endopeptidase activity was associated with membranes, but did not co-purify with the membrane components carrying the opiate receptors. A similar endopeptidase activity has been found by Knight and Klee (1979) and by Koida and co-workers (Koida *et al.*, 1979; Aono *et al.*, 1978).

Main events in the proteolytic conversion of β-endorphin by brain synaptic membrane associated peptidases have been detected by chemical characterization of β-endorphin

fragments which accumulated during incubation of β-endorphin-(1–31) with brain synaptic membrane preparations (Burbach *et al.*, 1980a; 1981; Burbach and de Kloet, 1982). Incubation of β-endorphin under different pH conditions resulted in formation of β-endorphin-(1–21) and -(2–21), β-endorphin-(1–17) and -(2–17) together with -(18–31), and shorter NH_2-terminal peptides as β-endorphin-(1–16), -(1–14), and -(1–13) (Burbach *et al.*, 1980a, 1981; Burbach and de Kloet, 1982). In addition, a small amount of β-endorphin-(1–18) was detected (Burbach, 1980). From time course studies it appeared that β-endorphin-(1–17) and -(1–21) were the initially formed products.

The proteolytic conversion of β-endorphin-(1–17) (γ-endorphin) and -(2–17) have been further investigated in detail. γ-Endorphin is susceptible to amino- and carboxy- and endopeptidases in brain synaptic membranes. At pH 6.7 β-endorphin-(2–17), -(5–17), -(6–17), -(7–17), -(8–17) and -(1–16) are found, while at pH 5.0 exclusively the carboxypeptidase products β-endorphin-(1–16), -(1–15), -(1–14) and -(1–13) are formed (Burbach *et al.*, 1981). In view of its non-opioid, neuroleptic-like activities and its clinical application as antipsychotic neuropeptide (de Wied, 1978; de Wied *et al.*, 1978; Verhoeven *et al.*, 1979, 1981; Van Ree *et al.*, 1983) studies have been concerned with the proteolytic cleavage of des-tyrosine-γ-endorphin (β-endorphin-(2–17)). The main metabolites which accumulated during *in vitro* incubation of des-tyrosine-γ-endorphin with a synaptic membrane preparation, were isolated in two high-pressure liquid chromatography steps and chemically characterized (Burbach *et al.*, 1980c). Two sets of fragments were identified. Firstly the COOH-terminally truncated fragments β-endorphin-(2–16), -(2–14) and -(2–13) were found; β-endorphin-(2–13) was present in highest amounts. Secondly, peptides with intact COOH-terminus were formed *in vitro* by exposure of des-tyrosine-γ-endorphin with synaptic membranes. They included β-endorphin-(5–17), -(6–17), -(7–17) and -(8–17). The major products were β-endorphin-(5–17) and -(6–17). The time dependency of their formation indicated that β-endorphin-(5–17) was initially formed, while β-endorphin-(6–17) appeared later. Possibly β-endorphin-(5–17) was formed by endopeptidase action on des-tyrosine-γ-endorphin (Burbach *et al.*, 1980c).

In agreement with above described findings is the observation that [^{3}H]-β-endorphin-(2–13) was the only detectable product after push–pull perfusion of [^{3}H-Phe^4]-des-tyrosine-γ-endorphin in the rat nucleus caudatus (Burbach, 1983). Due to the position of the label, peptides like β-endorphin-(5–17) and shorter fragments escaped detection. This finding suggested that the product was formed during contact of des-tyrosine-γ-endorphin with extracellular tissue compartments locally in the brain.

Evidence for formation of a similar set of metabolites of des-tyrosine-γ-endorphin by peptidases in whole rat brain homogenates and striatal slices was provided by Schoemaker *et al.* (1982). Based on co-elution with synthetic peptides in HPLC these investigators reported the presence of β-endorphin-(6–17) as principal fragment and smaller quantities of β-endorphin-(4–17), -(5–17), -(10–17), -(12–17) and -(2–16) after exposure of des-tyrosine-γ-endorphin to homogenates and slices. The studies of Burbach *et al.* (1980c) and of Schoemaker *et al.* (1982) demonstrate that a combination of carboxypeptidase, aminopeptidase and endopeptidase activities are involved in the proteolytic conversion of des-tyrosine-γ-endorphin. The significance of production of β-endorphin-(6–17) is based on its property to retain the full biological activity of des-tyrosine-γ-endorphin (de Wied *et al.*, 1980). Thus, des-tyrosine-γ-endorphin has been proposed as an active metabolite of β-endorphin (Burbach *et al.*, 1980c; Pedigo *et al.*, 1981; Schoemaker *et al.*, 1982).

Above data are indicative for a preferred pathway of β-endorphin conversion (Fig. 4) (Burbach, 1980; Burbach *et al.*, 1981; Burbach and de Kloet, 1982). The initial cleavage of β-endorphin takes place exclusively at two internal bonds, directed by the conformational properties of β-endorphin (see Section 4.4). An endopeptidase cleaves the Leu^{17}–Phe^{18} bond and forms γ-endorphin (β-endorphin-(1–17) together with β-endorphin-(18–31). This endopeptidase (γ-endorphin generating endopeptidase, γEGE) is distinctly different from lysosomal cathepsin D. The endopeptidase was active on β-endorphin at neutral pH and above and was not inhibited by 10^{-4} M pepstatin (Burbach *et al.*, 1980b). Lebouille and Burbach (1983, in preparation) developed a sensitive,

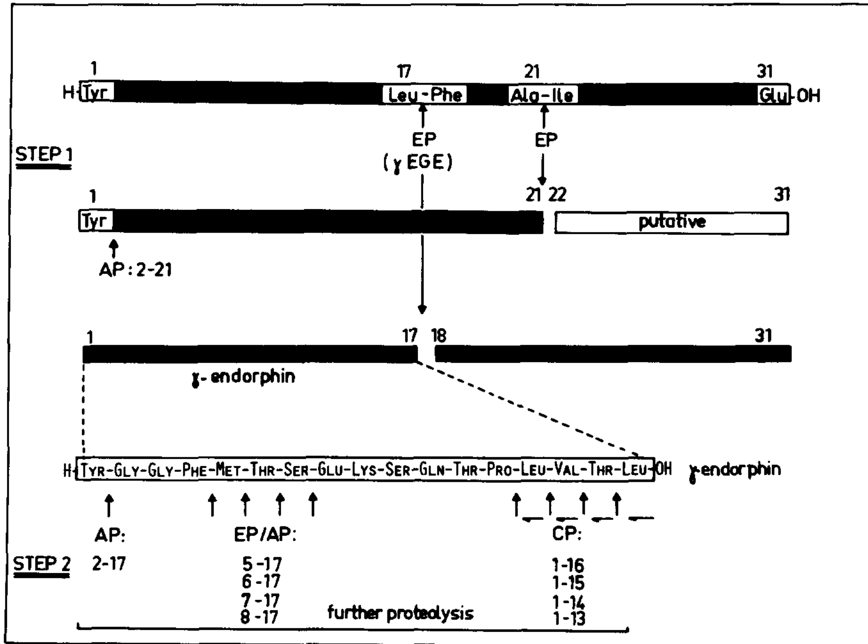

FIG. 4. Proteolytic conversion of β-endorphin by brain synaptic membranes. Proteolysis occurs in a step-wise fashion. STEP 1: initial cleavage of β-endorphin by endopeptidases (EP). Cleavage sites are located at the Leu^{17}–Phe^{18} and Ala^{21}–Ile^{22} bonds. Identified initial products are β-endorphin-(1–17) (γ-endorphin) together with β-endorphin-(18–31), and β-endorphin-(1–21). The formation of β-endorphin-(22–31) is presumed. The endopeptidase activity responsible for the Leu^{17}–Phe^{18} cleavage has been termed γ-endorphin generating endopeptidase (γEGE). STEP 2: secondary cleavage of products by exopeptidases. Aminopeptidase (AP) activity removes readily residue Tyr^1, but does not seem to cleave the Gly^2–Gly^3 bond. Identified products are β-endorphin-(2–17) (des-tyrosine-γ-endorphin, DTγE), β-endorphin-(2–16) (des-tyrosine-α-endorphin) and β-endorphin-(2–21). Carboxypeptidase activity (CP) removes sequentially COOH-terminal amino acids from γ-endorphin, but stops at the Pro^{13} residue. Identified products are β-endorphin-(1–16) (α-endorphin), β-endorphin-(1–15), -(1–14) and -(1–13). In addition, the peptides β-endorphin-(5–17), -(6–17), -(7–17) and -(8–17) have been identified. Likely, they have been formed by putative endopeptidase action on γ-endorphin followed by aminopeptidase cleavage. All products are subject to further proteolysis and are therefore transient metabolites. The figure summarizes data taken from Burbach *et al.*, 1980, 1981; Burbach and de Kloet, 1982.

quantitative assay for this endopeptidase γEGE by using a synthetic peptide substrate insensitive to cathepsin D. The γEGE measured by this assay had a pH optimum of 8.5 and was pepstatin-insensitive.

The identification of β-endorphin-(1–21) indicated that the Ala^{21}–Ile^{22} in human β-endorphin was also cleaved (Fig. 4). Recently, a peptide with properties of β-endorphin-(1–21) has been detected in rat pituitary and brain tissue (unpublished).

While the conformation of β-endorphin is highly protective against exopeptidases, γ-endorphin is readily cleaved by amino- and carboxypeptidases (Burbach *et al.*, 1981). Aminopeptidase activity forms des-tyrosine-γ-endorphin, but seems to stop at the Gly^2–Gly^3 sequence (Hersch *et al.*, 1980). Carboxypeptidase activity removes sequentially the COOH-terminal residues of γ-endorphin, but stops at the Pro^{13} residue, a known stop for carboxypeptidases (Hartsuck and Lipscomb, 1971). In addition, endopeptidases may act on γ-endorphin (Burbach *et al.*, 1980b, 1981). These enzyme activities contribute to the eventual accumulation of fragments generated from β-endorphin. It was observed that the amounts of accumulated α- and γ-endorphins were highly dependent on small pH changes of the incubation medium. The ratio of α- to γ-type peptides shifted from 0.35 to 3.5 when pH changed from 6.7 to 5.9 (Burbach *et al.*, 1980a; Burbach and de Wied, 1981, 1982). This observation is explained by the differences in pH dependencies of peptidases involved. In particular, the carboxypeptidase converting γ-endorphin into α-endorphin has an optimum of pH 5 or below. It resembles a peptidase activity cleaving the COOH-terminus of adrenocorticotropin (Wang *et al.*, 1983). Aminopeptidase activity

releasing the NH_2-terminal tyrosine has a pH optimum of 7 (Burbach *et al.*, 1979a). In view of the outspoken central activities of α- and γ-endorphins, the various proteolytic activities involved in the conversion of β-endorphin may have a regulatory role in the formation of β-endorphin fragments. These findings of a highly pH sensitive enzyme system may indicate that endogenous factors controlling the activity of individual peptidases contribute to regulation of levels of α- and γ-endorphins *in situ*.

4.1.3. *Cerebrospinal Fluid*

The susceptibility of β-endorphin and methionine-enkephalin (β-endorphin-(1–5)) to proteolytic activity in human cerebrospinal fluid *in vitro* has been investigated (Burbach *et al.*, 1979a; Burbach, 1982). Samples of cerebrospinal fluid were incubated with human β-endorphin-(1–31) and β-endorphin-(1–5) for 5 hr at 37°C and the amounts of peptides were assessed by radioimmunoassay. No decrease in immunoreactivity was found for the two peptides when human cerebrospinal fluid of non-psychiatric patients or schizophrenic patients was used (Burbach *et al.*, 1979a). It was concluded that endorphins are not significantly degraded by proteolytic activities in cerebrospinal fluid. The studies suggested that altered levels of endorphins in cerebrospinal fluid are a reflection of changes in cellular metabolism and release of endorphins and not due to proteolytic enzymes in the cerebrospinal fluid.

4.1.4. *Purified Enzymes from Brain Tissue*

The action of several proteolytic enzymes purified from brain tissue on β-endorphin and related peptides has been investigated. These enzymes can be grouped in three classes on the basis of the site of cleavage in the β-endorphin sequence.

The Leu^{17}–Phe^{18} bond of β-endorphin is a site sensitive to the action of the purified acid proteinase cathepsin D. Cathepsin D purified from calf brain by affinity chromatography on pepstatin–Sepharose (Benuck *et al.*, 1978a) exclusively cleaved β-endorphin at the Leu^{17}–Phe^{18} bond forming γ-endorphin and β-endorphin-(18–31) (Gráf *et al.*, 1979b). The enzyme displayed activity in the pH range 3.0–7.0 and was completely inhibited by 10^{-6} M pepstatin. Cathepsin D purified from other tissues, such as human pituitaries (Benuck *et al.*, 1978a; Barát *et al.*, 1979) and bovine spleen, obtained as a commercial preparation (Burbach *et al.*, 1980b), cleaved the Leu–Phe bond of β-endorphin or β-LPH similarly (see also section 3.1.2.).

The cleavage of the Met^5–Thr^6 bond of NH_2-terminal β-endorphin sequences by purified brain enzymes has been described. Koida and coworkers (1979; Aono *et al.*, 1978) purified such an enzyme by affinity chromatography using immobilized β-endorphin-(2–7). This enzyme selectively cleaved the Met^5–Thr^6 bond. It may resemble an enzyme activity which was detected in crude fractions of brain tissue cleaving the same peptide bond of β-endorphin or derivatives (Knight and Klee, 1979; Austen *et al.*, 1977a; Austen and Smyth, 1978; Smyth and Snell, 1977b; Palmour *et al.*, 1978; Orlowski *et al.*, 1980; Burbach *et al.*, 1981).

Hersch *et al.* (1980, 1981) purified an aminopeptidase from bovine brain which removed the NH_2-terminal tyrosine from endorphins. The hydrolysis of the Tyr^1–Gly^2 bond was most rapid from β-endorphin-(1–5). The rate of hydrolysis of β-endorphin-(1–16) and -(1–17) was approximately 30% of that of β-endorphin-(1–5), whereas β-endorphin-(1–31) was cleaved at a rate of only 0.5% as compared to β-endorphin-(1–5) (Hersch *et al.*, 1980). The relative resistance of β-endorphin is also observed with aminopeptidase activity from other sources (Austen and Smyth, 1977c; Burbach *et al.*, 1979b) and is related to conformational restraints of the peptide for proteolytic attack (Austen and Smyth, 1977a,b; Geisow and Smyth, 1977; Nicolas *et al.*, 1981). Interestingly, an aminopeptidase purified from human brain removing the NH_2-terminal tyrosine from β-endorphin-(1–5) is unable to cleave the NH_2-terminus of β-endorphin-(1–16) (Traficante *et al.*, 1980).

Cathepsin B purified from bovine brain has been reported to cleave β-endorphin (Suhar and Marks, 1979). However, the site of cleavage has not been determined as yet.

4.2. PROTEOLYSIS OF β-ENDORPHIN BY PITUITARY ENZYMES

The β-endorphin fragments α- and γ-endorphin (β-endorphin-(1–16), -(1–17), respectively) occur in the pituitary pointing to the existence of proteolytic enzymes in the pituitary cleaving the Thr^{16}–Leu^{17}–Phe^{18} region of β-endorphin. Studies performed on porcine β-lipotropin-(1–91) revealed the presence of endopeptidases cleaving the Leu^{77}–Phe^{78} and Lys^{79}–Asn^{80} bonds of β-lipotropin (Gráf and Kenessey, 1976; Gráf *et al.*, 1977; Benuck *et al.*, 1978b; see also Section 3.1.2). These data suggest that these enzyme activities also cleave the same bonds of β-endorphin. Indeed, cathepsin D purified from human pituitary tissue cleaved the Leu^{17}–Phe^{18} of β-endorphin (Gráf *et al.*, 1979b; Benuck *et al.*, 1978b). In preliminary experiments Lebouille and Burbach detected soluble and particulate pepstatin-insensitive neutral peptidase activities cleaving the Leu–Phe bond (unpublished). In addition a cation-sensitive endopeptidase that splits the Met^5–Thr^6 bond of β-endorphin-(1–16) has been purified from bovine pituitaries (Orlowski *et al.*, 1980).

4.3. PROTEOLYSIS OF β-ENDORPHIN BY ENZYMES OF PERIPHERAL ORGANS

Proteolysis of β-endorphin by subcellular fractions of peripheral organs has been observed. $[^{125}I]$-β-Endorphin and its analog $[^{125}I]$-$[Leu^5]$-β-endorphin were degraded by supernatant and membranes of kidney (Palmour *et al.*, 1978). A rapid formation of free $[^{125}I]$tyrosine was observed and small amounts of $[^{125}I]$-β-endorphin-(1–17), -(1–8), -(1–5), and -(1–4) were detected. The degradation rate was highest in the supernatant fraction. Activities in supernatant and membranes were to some extent sensitive to bacitracin.

The presence of β-endorphin and fragments in a myenteric plexus preparation of guinea-pig ileum, which is sensitive to the opioid activities of β-endorphin, prompted Opmeer and colleagues to investigate the proteolysis of β-endorphin by membranes from this tissue (Opmeer *et al.*, 1980, 1982). β-Endorphin was converted by these membranes *in vitro* into β-endorphin-(1–17), -(1–16), -(2–17) and -(2–16). These peptides appeared as transient metabolites during incubation. β-Endorphin-(1–17) accumulated in largest quantities. These data suggest that β-endorphin is converted in the myenteric plexus by similar proteolytic mechanisms as in the brain (Fig. 4). The time course of accumulation of β-endorphin fragments during proteolysis by myenteric plexus membranes was changed after exposure of the tissue to morphine (Opmeer *et al.*, 1982). Combined with the observation of altered endogenous levels of β-endorphin fragments in the myenteric plexus after morphine treatment (Opmeer *et al.*, 1980), it was suggested that chronic morphine exposure increased the turnover of β-endorphin-(1–17).

It has been reported that homogenates of the whole gut, mucosa and myenteric plexus regions convert β-endorphin similarly into α-and γ-endorphins and related fragments. However, quantitative differences between these tissues have been observed (Davis *et al.*, 1983).

4.4. ACTION OF PURIFIED ENZYMES ON β-ENDORPHIN: CONFORMATIONAL RESTRAINTS FOR PROTEOLYSIS

The remarkable conformational properties of β-endorphin were first recognized by Smyth and co-workers investigating the susceptibility of the peptide to exo- and endopeptidases (Geisow and Smyth, 1977a; Austen and Smyth, 1977a,b,c). The conformation protects both NH_2 and COOH termini of β-endorphin against exopeptidases and leaves the mid-portion of the peptide open for proteolytic attack. This conformational property appears to direct the proteolytic cleavage of the peptide by purified enzymes as well as by proteolytic activities in tissue fractions. The stepwise formation of neuroactive β-endorphin fragments such as γ- and α- endorphins seems to be partly due to the

preferred conformation of β-endorphin. The evidence for this conformation based on the action of purified proteolytic enzymes is briefly reviewed here.

It was noticed that β-endorphin-(1–31) was unusually stable during treatment with carboxypeptidase A (Geisow and Smyth, 1977a) and aminopeptidase M (Austen and Smyth, 1977c). On the other hand β-endorphin is easily cleaved by various endopeptidases. These endopeptidases initially cleave peptide bonds in the 16–22 region of β-endorphin, while only at more stringent incubation conditions additional bonds are cleaved. Rennin, chymotrypsin, trypsin and Armillaria protease initially cleave the Leu^{17}–Phe^{18}, Phe^{18}–Lys^{19}, Lys^{19}–Asn^{20}, and Phe^{18}–Lys^{19} bonds of β-endorphin respectively. Secondary cleavage sites are the Phe^{4}–Met^{5} (rennin, chymotrypsin), Lys^{9}–Ser^{10} (trypsin) and Glu^{8}–Lys^{9} bonds (Armillaria protease) (Austen and Smyth, 1977a,b). Thermolysin, a metalloendopeptidase with broad specificity, initially cleaves the Ala^{21}–Ile^{22} bond at very short incubation time. Longer incubation results in cleavage of many bonds (Nicolas *et al.*, 1980).

5. FATE OF LIPOTROPINS AND ENDORPHINS IN THE PERIPHERAL CIRCULATION

In the peripheral circulation endogenous lipotropins and endorphins, secreted from the pituitary gland or administered exogenously, are subject to distribution and elimination. Studies on the metabolic fate of lipotropins and endorphins have mainly focused on the half-lifes ($t_{1/2}$) of endogenous and exogenous peptides in the blood and on the stability of lipotropins and endorphins in plasma *in vitro*.

5.1. Half-Life

The plasma half-lifes of exogenous lipotropins and β-endorphin have been assessed after intravenous injection of the peptides, usually by measuring the immunoreactivity of the peptide in a radioimmunoassay system. To control for possible crossreaction of degradation products of the administered peptides in these assay systems gel filtration chromatography has been employed by several investigators. Two studies have employed radioactively labeled peptides (Houghton *et al.*, 1980; Verhoef *et al.*, 1983).

In general the decay of peptide concentrations in the plasma shows a biphasic pattern. The initial decline in lipotropin and β-endorphin levels is representative for the distribution of the peptides over different extravascular spaces, while the secondary, slower decline is due to the elimination of the peptides from the circulation mainly by metabolic processes. Most studies recognize the biphasic disappearance rates of lipotropins and β-endorphin, and often the pharmacokinetic parameters associated with each separate phase, are derived mathematically (e.g. Gibaldi and Perrier, 1975). In other cases an 'overall' half-life is given, which mostly approximates the plasma half-life associated with the secondary or elimination phase.

5.1.1. *Endogenous Peptides*

The disappearance rates of endogenous lipotropins and endorphins from the peripheral circulation can only be determined in subjects with elevated plasma levels of these peptides in which the secretion of these peptides is abruptly suppressed by infusion of corticosteroids. After suppression of the pituitary hypersecretion by cortisol in a patient with Addison's disease, Bertagna *et al.* (1981) observed a rapid fall in both immunoreactive γ-lipotropin and immunoreactive β-lipotropin including β-endorphin. The initial and secondary half-lifes ($t_{1/2}$) of γ-lipotropin were 80 and 170 min, and for β-lipotropin/β-endorphin 100 and 180 min. Data on the individual peptides were subsequently obtained after separation by gel filtration showing similar values for γ-lipotropin and β-lipotropin, whereas β-endorphin disappeared much more rapidly with a half-life of approximately 40 min in the initial phase. In the study of Tanaka *et al.* (1978b) the disappearance of immunoreactive β-melanotropin was followed with time in three female

patients with Addison's disease during infusion of cortisol. It was shown that the immunoreactivity detected by the β-MSH radioimmunoassay in human plasma represented the lipotropins γ-lipotropin and β-lipotropin (Tanaka *et al.*, 1978a,b; Bloomfield *et al.*, 1974; Bachelot *et al.*, 1977). Tanaka *et al.*, (1978b) found that in two of the three patients investigated the disappearance of lipotropins was monophasic, with $t_{1/2}$-values of 133 and 72 min respectively. In the third patient the disappearance was biphasic, with an initial $t_{1/2}$ of 44 min and $t_{1/2}$ of 100 min in the second part of the disappearance curve. The average overall $t_{1/2}$ for disappearance of endogenous lipotropins in these patients was approximately 80 min, while that of immunoreactive ACTH was 40 min (Tanaka *et al.*, 1978a,b).

5.1.2. *Exogenous Peptides*

A number of reports have presented experiments on the disappearance of β-endorphin and β-lipotropin in plasma after systemic injection. In these studies largely different doses of administered peptides have been employed and experiments have been carried out in different species.

In human subjects the disappearance of intravenously administered β-endorphin and β-lipotropin was assessed by specific radioimmunoassay procedures. Liotta and co-workers, administering intravenously boli of 250 μg to 370 μg of highly purified human β-lipotropin found that the disappearance of β-lipotropin was biphasic with overall $t_{1/2}$-values ranging from 33 to 69 min (Liotta *et al.*, 1978; Aronin *et al.*, 1981). This $t_{1/2}$ is based on calculated values of the apparent volume of distribution (V_d) and metabolic clearance rate (MCR), which were generated by integration of multi-exponential equations (Liotta *et al.*, 1978). In these experiments the MCR for human β-lipotropin was between 0.57 and 0.23 l/min.

After intravenous administration of 100 μg β-endorphin to three normal human subjects Aronin *et al.* (1981) found a biphasic disappearance of the peptide with $t_{1/2}$ of 32, 51 and 50 min and a MCR of 0.49, 0.47, and 0.45 l/min, respectively. From comparison with the pharmacokinetic data obtained with β-lipotropin in the same series of experiments, the authors suggest that β-endorphin has both a more rapid MCR and a more rapid initial disappearance than β-lipotropin (Aronin *et al.*, 1981). At 30 min after injection the average percentage of peptide remaining was 1.53%/l for β-endorphin and 2.25%/l for β-lipotropin.

Foley *et al.* (1979) studied the disappearance of intravenously administered high doses (5 mg and 10 mg) of synthetic human β-endorphin to patients suffering from chronic pain due to cancer. The $t_{1/2}$ values of the distribution and elimination phases were between 2.3 and 7.4 min, and 27 and 132 min, respectively. The MCR's obtained by Foley *et al.* (1979) (average 5.6 ml min^{-1} kg^{-1}, $n = 4$) were comparable to those of Aronin *et al.* (1981) (average 7.8 ml min^{-1} kg^{-1}). The two studies, however, are difficult to compare for pharmacokinetic data due to large differences in dose (5 or 10 mg vs 100 μg) and in methods used for calculation of the pharmacokinetic data.

After intravenous administration of β-endorphin to psychiatric patients in doses ranging from 3 to 9 mg, Lehman *et al.* (1979) reported a monophasic disappearance rate of the peptide with a half-life varying between 12 and 35 min. Berger *et al.* (1980) found a biphasic disappearance with $t_{1/2}$-values of 15 (α-phase) and 39 (β-phase) min after an intravenous bolus injection of 20 mg of β-endorphin to schizophrenic patients. These values approximate the data of Reid *et al.* (1981)), who investigated the plasma disappearance rate of β-endorphin after a bolus injection of 2.5 mg. These authors derived three disappearance constants from their experimental data: the initial half-life was 4 min, the midrange component had a half-life of 13 min and the slow component had a half-life of 46 min.

In the rat 150 μg of intravenously injected β-lipotropin had a plasma half-life of 4.2 min; β-endorphin, in the same dose, had a half-life of 9.2 min as determined by Chang *et al.* (1978). In contrast, Pezalla *et al.* (1978) found that in the rabbit purified ovine β-endorphin had a shorter plasma half-life than ovine β-lipotropin when administered in equimolar

TABLE 5. *Pharmacokinetics of Intravenously Administered β-endorphin and β-lipotropin*

Peptide	Approximate i.v. Dose (μg)	Species/Subject	Disappearance Curve	$t_{1/2}$ (min)	Reference
β-endorphin	100	human/normal	biphasic	32 overall	Aronin *et al.*, 1981
β-endorphin	100	human/normal	biphasic	51 overall	Aronin *et al.*, 1981
β-endorphin	100	human/normal	biphasic	50 overall	Aronin *et al.*, 1981
β-endorphin	5,000	human/chronic pain	biphasic	α: 7.4; β: 27	Foley *et al.*, 1979
β-endorphin	10,000	human/chronic pain	biphasic	α: 6.7; β: 39	Foley *et al.*, 1979
β-endorphin	10,000	human/chronic pain	biphasic	α: 4.9; β: 45	Foley *et al.*, 1979
β-endorphin	3,000	human/psychiatric	monophasic	12	Lehman *et al.*, 1979
β-endorphin	9,000	human/psychiatric	monophasic	35	Lehman *et al.*, 1979
β-endorphin	20,000	human/schizophrenic	biphasic	α: 15; β: 39	Berger *et al.*, 1980
β-endorphin	2,500	human/normal ($n = 3$)	triphasic	α: 4.1; β: 13.1; γ: 46.2	Reid *et al.*, 1981
β-endorphin-(2–17)	5	rat	biphasic	α: 0.7; β: 5.5; biological: 0.9	Verhoef *et al.*, 1983
β-endorphin-(6–17)	3	rat	biphasic	biological: 0.6	Verhoef *et al.*, 1983
β-endorphin	150	rat	biphasic	9.2 overall	Chang *et al.*, 1978
β-endorphin	0.3	rat	biphasic	2.0 overall	Houghten *et al.*, 1980
β-endorphin	1.5	rabbit	biphasic	4.0 overall	Houghten *et al.*, 1980
β-endorphin	70	rabbit	biphasic	4.8 overall	Pezalla *et al.*, 1978
β-endorphin	125	rabbit	biphasic	14 overall	Merin *et al.*, 1980
β-lipotropin	270	human/normal ($n = 3$)	biphasic	37.3 overall	Liotta *et al.*, 1978
β-lipotropin	250 or 370	human/normal	biphasic	45 overall	Aronin *et al.*, 1981
β-lipotropin	250 or 370	human/normal	biphasic	69 overall	Aronin *et al.*, 1981
β-lipotropin	250 or 370	human/normal	biphasic	33 overall	Aronin *et al.*, 1981
β-lipotropin	150	rat	biphasic	4.2 overall	Chang *et al.*, 1978
β-lipotropin	250	rabbit	biphasic	13.7 overall	Pezalla *et al.*, 1978
β-melanotropin	100	rabbit	biphasic	5.1 overall	Pezalla *et al.*, 1978

amounts (approximately 70 μg of β-endorphin, $t_{1/2} = 4.8$ min; 250 μg of β-LPH, $t_{1/2} = 13.7$ min). Porcine β-melanotropin had a half-life of 5.1 min. The plasma half-life of β-endorphin in the rabbit was reported to be approximately 14 min by Merin *et al.* (1980). Using synthetic tritiated β-endorphin Houghton *et al.* (1980) found that in the rat the initial plasma half-life of 0.3 μg β-endorphin was approximately 2 min, while in the rabbit the initial half-life of 1.5 μg β-endorphin was 5 min. These values are lower than those of Chang *et al.* (1978), Pezalla *et al.* (1978), and Merin *et al.* (1980) possibly due to the methodology measuring intact peptide instead of immunoreactivity. Verhoef *et al.* (1983) investigated the plasma disappearance of the β-endorphin fragments β-endorphin-(2–17) (des-tyrosine-γ-endorphin) and β-endorphin-(6–17) (des-enkephalin-γ-endorphin) after intravenous administration in the rat. The peptides carried a $[^3H]$-label in residue Phe^4 and Lys^9, respectively. Separation of intact peptide from radioactive products recovered from plasma were obtained by high-pressure liquid chromatography. β-Endorphin-(2–17) had a biphasic disappearance curve; the calculated biological half-life was 0.9 min. The biological half-life of β-endorphin-(6–17) was 0.6 min (Table 5).

The data on the disappearance of β-endorphin and β-lipotropin after exogenous administration are summarized in Table 5. In humans the disappearance rate of these peptides appears to follow a biphasic pattern with a rapid initial decline in plasma concentration, followed by a slower elimination phase. Although in the various studies a variety of doses has been used to assess the pharmacokinetic data, generally the half-life of β-endorphin found is in the order of 20–50 min. There is a tendency that the half-life of β-lipotropin is somewhat longer than that of β-endorphin as indicated by studies on endogenous as well as exogenous β-lipotropin and endorphins (Aronin *et al.*, 1981; Bertagna *et al.*, 1981). Slower disappearance rates are found after suppressing the pituitary secretion by cortisol. These values, however, may be influenced by incomplete suppression during the experiments.

In rodents, rats and rabbits, the half-life of β-endorphin and β-lipotropin is shorter than in humans varying between 2 and 14 min. Such differences between large and small species

also exist for ACTH (Reith and Neidle, 1981). There is no general agreement on a difference in disappearance rates of β-endorphin and β-lipotropin in rabbits (Pezalla *et al.*, 1978; Chang *et al.*, 1978).

5.2. Proteolysis by Enzymes in the Peripheral Circulation

Several studies have been concerned with the proteolytic cleavage of β-lipotropin and β-endorphin in the peripheral circulation. Liotta and co-workers (Liotta *et al.*, 1978; Aronin *et al.*, 1981) showed that after intravenous injection of β-lipotropin in humans immunoreactivity measured in radioimmunoassay systems for β-lipotropin represented intact β-lipotropin. Immunoreactivity eluted in a single peak with [^{125}I]-β-lipotropin during gel filtration while no conversion to β-endorphin or NH_2-terminal sequences could be detected. In contrast with these data Pezalla *et al.* (1978) reported that in the rabbit β-lipotropin was cleaved into peptides tentatively identified as γ-lipotropin and β-endorphin. Characterization of these peptides was based on separation of plasma samples by gel filtration on Sephadex G-50 and detection by radioimmunoassay systems directed against different portions of the β-lipotropin sequence.

The proteolytic cleavage of endorphins *in vivo* circulating in the blood or *in vitro* has been investigated in more detail. Merin *et al.* (1980) did not detect any degradation products of β-endorphin in the plasma after an intravenous bolus injection in rabbits. These authors employed gel filtration and a radioimmunoassay system. The absence of metabolites may be due to the rather strict specificity of the β-endorphin antiserum used. From the injecting of tritiated β-endorphin in the rabbit, Houghten *et al.* (1980) found that after 45 min 70% of the total radioactivity in plasma represented intact β-endorphin. In the plasma of the rat at least 50% of the total radioactivity represented intact β-endorphin as shown in different chromatographic systems. After subcutaneous injection of the β-endorphin fragment [^{3}H-Lys^{9}]-β-endorphin-(6–17) in the rat Verhoef *et al.* (1983) using high-pressure liquid chromatography detected three radioactive metabolites in the plasma. The major product was [^{3}H]lysine; minor components had chromatographic properties of β-endorphin-(6–15) and -(6–14). After intravenous administration only very small quantities of β-endorphin-(6–15) were found in addition to the free [^{3}H]amino acids.

Houghten *et al.* (1980) found by radioimmunoassay determination that during *in vitro* incubation of β-endorphin with rat plasma the peptide was degraded slowly; after an incubation period of 3 hr at 37°C 60% of β-endorphin remained intact. In human serum and plasma the levels of immunoreactive β-endorphin are fluctuating depending on the time and temperature of storage, indicating the presence of enzyme activities generating β-endorphin, likely from β-lipotropin, as well as degrading the peptide (Wiedemann *et al.*, 1979). Burbach *et al.* (1979b) observed a high resistance of β-endorphin to proteolytic activity in human plasma. In diluted plasma no significant decrease in immunoreactive β-endorphin was detected during incubation of 2 hr at 37°C. Similarly, Rossier *et al.* (1977) did not observe any degradation of β-endorphin in diluted rat serum after 30 min of incubation at 37°C. However, during incubation of human [^{125}I]-β-endorphin-(1–31) in diluted plasma a slow release of [^{125}I]tyrosine was observed, indicating that a portion of the peptide was cleaved to a minor extent by plasma proteinases (Burbach *et al.*, 1979b). The immunoreactivity of the β-endorphin fragments α- and γ-endorphin, and des-tyrosine-γ-endorphin decreased detectably with 80% remaining after 2 hr at 37°C in diluted human plasma. Since the antisera for α- and γ-endorphin used recognized the COOH-terminal sequences of the peptides (Loeber *et al.*, 1979; Loeber and Verhoef, 1981) these data indicated that the COOH-termini of these peptides were rather resistant to attack by plasma enzymes. By following the release of the NH_2-terminal tyrosine from iodinated β-endorphin fragments, it appeared that NH_2-terminus of β-endorphin-(1–9) was cleaved rapidly (half maximal release at 8.5 min), while α- and γ-endorphin were more resistant to attack of the NH_2-terminus (half maximal release of [^{125}I]tyrosine at 64 min). These data indicate that the main enzyme activity cleaving short endorphin fragments in serum is an aminopeptidase-like activity. β-Endorphin is resistant to this type of cleavage, while the

susceptibility of the β-endorphin fragments to aminopeptidase cleavage is a function of their chain length (Austen and Smyth, 1977a; Burbach *et al.*, 1979b; Hersch *et al.*, 1980).

6. CONCLUDING REMARKS

This review has focused on the proteolysis of lipotropins and endorphins. Three fundamentally different functional aspects of the proteolytic enzyme systems are considered. One enzyme system is part of the chain of intracellular events that process precursor polypeptides into the products of biosynthesis. It is responsible for biosynthesis of β-endorphin by cleaving its precursor pro-opiomelanocortin. The other uses the biosynthetic end products as substrates in order to generate a variety of peptides with modified activities, as apparent from the biotransformation of β-endorphin into smaller fragments with distinct central activities. A third enzyme system inactivates biological active peptides. The family of peptides related to lipotropins and endorphins provide outstanding examples of these three different proteolytic systems.

The proteolytic processing of the multifunctional precursor pro-opiomelanocortin is a well defined example of the mechanism of peptide biosynthesis and illustrates the determining role of the processing enzymes. These processing enzymes virtually all recognize pairs of basic amino acids and determine the nature of the biologically active peptides produced by a certain cell type. For instance, β-lipotropin is one of the end products of the processing of pro-opiomelanocortin in the anterior lobe of the pituitary gland, while enzymes of the intermediate lobe and the brain use β-lipotropin as substrate to produce β-endorphin. Consequently β-lipotropin has been adopted as substrate in studies on these processing enzymes. As yet, two types of proteolytic enzymes have been recognized that fulfill several criteria for processing enzymes: firstly, serine proteases with a basic pH optimum and with similarity to the kalikreins, and secondly, thiol argynyl proteases with acidic working range. Direct evidence of the physiological involvement of either of these enzymes in the processing of prohormones is still lacking.

In the early stages of endorphin research the proteolytic cleavage of β-endorphin was considered to be part of a degradative mechanism. However, the findings of unique sets of β-endorphin fragments endogenously in brain and pituitary tissue (e.g. α- and γ-endorphins) and the discovery of their separate and distinct biological activities has substantiated the concept that biotransformation of β-endorphin is a functional mechanism to generate and regulate active peptides. By now we have begun to learn enough about the properties of this type of proteolysis that criteria for enzymes involved in biotransformation can be indicated. These criteria should take into account the localization of enzyme and substrate, the biological significance of the products of enzyme action, and a regulatory role of the enzyme.

(1) If biotransformation is secondary to the intracellular biosynthetic processing, the enzymes should use end products of biosynthesis as substrates and be localized strategically towards these end products, e.g. linked with sites of storage or release. With respect to the compartmentalization intra- as well as extracellular sites of biotransformation can be considered. For example, α- and γ-endorphins are at one hand present in the same cells as their precursor peptide β-endorphin and are co-released upon stimulation, e.g. from the pars intermedia of the pituitary gland, suggesting a intracellular site of biotransformation, possibly intragranular. At the other hand, formation of α- and γ-endorphins can be mediated by enzymes in synaptic membranes and soluble fractions, suggesting a post-secretional site of biotransformation of β-endorphin.

(2) If biotransformation serves to generate novel biological activities, the products of enzyme action should display activities unique to them and distinct from those of their precursors, and be endogenously present.

(3) If biotransformation has a regulatory role, the enzyme activity should be able to accommodate the availability of its products to changed situations. This implicates that a functional relationship between activity of the enzymes and the endogenous levels of the products exists.

In contrast to biosynthetic processing and biotransformation, the inactivation of biologically active peptides can be accomplished by many if not all tissues. In general tissues display a high degradative capacity towards peptides. In the peripheral circulation this leads to short biological half-lives of peptides.

Future research that aims to define functional aspects of proteolytic enzymes involved in the metabolism of biologically active peptides will largely depend on knowledge of the properties of these proteolytic enzymes. Studies in this direction will be greatly assisted by the development of synthetic substrates that allow specific and sensitive assessment of enzyme activity. Also, studies employing specific inhibitors or antisera can be used to probe the enzymes and to modulate enzyme activity. Such studies can be informative for defining a regulatory role of the enzymes. In addition it will be necessary to extend our knowledge of the forms and biological activities of endogenous peptides and their metabolites. The family of peptides related to the lipotropins and endorphins provides an outstanding biological system to define the roles of proteolytic enzymes in biosynthesis, biotransformation and inactivation.

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