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α,N-ACETYL β-ENDORPHIN [1-8] IS THE TERMINAL PRODUCT OF PROCESSING OF ENDORPHINS IN THE MELANOTROPE CELLS OF *XENOPUS LAEVIS*, AS DEMONSTRATED BY FAB TANDEM MASS SPECTROMETRY

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Summary: The major N-terminal acetylated endorphin of the pars intermedia of *Xenopus laevis* was purified and submitted to fast-atom bombardment tandem mass spectroscopy. The collisionally induced dissociation MS/MS spectrum of the $[M+H]^+$ ion revealed sufficient fragment ions to determine unambiguously the identity of the peptide as α,N-acetyl β-endorphin [1-8], the sequence of which was predicted on the basis of the nucleotide sequence of *Xenopus* POMC cDNA. The determination was confirmed by showing that the synthetic peptide of this structure had identical FAB tandem mass spectrometric characteristics as the endogenous endorphin. We conclude that α,N-acetyl β-endorphin [1-8] is the terminal product of processing of endorphins in the melanotrope cell of *Xenopus laevis*.

Proopiomelanocortin (POMC) is a 35-kiloDalton precursor protein that, in melanotrope cells of the pituitary gland, is processed to produce peptides such as melanophore stimulating hormone (α-MSH) and C-terminal truncated forms of β-endorphin (1). In melanotrope cells of mammals both α-MSH and the endorphins are N-terminally acetylated. The melanotropic activity of α-MSH is potentiated by this event while the opioid activity of the endorphins is lost (1,2). The major tissue forms are α-MSH and acetylated endorphin [1-26], [1-27] and [1-31] (3). It is generally accepted that the same acetyl transferase is responsible for the acetylation of both α-MSH and the endorphins (4). In the pars intermedia of the South African toad *Xenopus laevis*, however, acetylation of these peptides appears to be regulated differentally. Acetylation of α-MSH occurs just prior to or during the process of secretion and therefore the major tissue form

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of the peptide is desacetyl α-MSH (5-8). For the endorphins, acetylation takes place intracellularly and the major tissue forms of the peptide are C-terminally truncated, N-terminally acetylated endorphins (7). While α-MSH has been structurally characterized in this amphibian species (8), definitive identification of the endorphins is still lacking. Dores *et al.* (9) have suggested that the major form of acetylated endorphin in melanotropes of *X. laevis* consists of 8 to 12 amino acid residues from the N-terminal of β-endorphin. More recently, on the basis of a coelution study with synthetic endorphins, Maruthainar *et al.* (10) proposed that the major form is the 8 amino acid peptide.

In this study the main acetylated endorphin end product in the pars intermedia of *X. laevis* has been purified and its sequence determined using fast-atom bombardment (FAB) tandem mass spectrometry. To confirm the deduced amino acid sequence, FAB tandem mass spectrometry was carried out with a peptide synthesized according to the putative structure of this endorphin as inferred from the cDNA structure of *Xenopus* POMC (11).

Methods

Animals

Adult *Xenopus laevis* with a weight of approximately 25 g were obtained from our laboratory stock. Toads were kept under constant illumination on either a black or a white background for at least 3 weeks (22°C). For the experiments, animals were decapitated and pituitary neurointermediate lobes were rapidly dissected out. Lobes were homogenized in 1 ml 0.1 N ice-cold HCl and the extracts were freeze-dried under vacuum (Speed-Vac Concentrator Savant, New Brunswick Scientific, Watford, UK) or immediately submitted to reversed-phase HPLC.

Reversed-phase HPLC

A BioRad RSil C18 column (Richmond, CA) was used with 0.5 M formic acid/0.14 M pyridine as the primary solvent and n-propanol as the secondary solvent: Flow rate was 2 ml/min. One ml fractions were collected and dried under vacuum. Dried samples were resuspended in 0.02 M veronal acetate buffer containing 0.2 g/l NaN_3, 3 g/l bovine serum albumin (Sigma, St.Louis, MO, USA) and 100 KIU/ml trypsin inhibitor (Serva, Heidelberg, Germany). As markers, standard human acetylated endorphins [1-16], [1-27] and [1-31] (Sigma) were submitted to reversed-phase HPLC.

Radioimmunoassay

The N-terminal acetylated endorphin antiserum was raised against salmon acetylated β-endorphin, and is specific for the acetylated N-terminal of salmon β-endorphin (12). The serum has full cross-reactivity with the acetylated forms of mammalian β-endorphin (9). Cross-reactivity with nonacetylated forms of endorphin is less than 0.1% (9). For radioimmunoassay the antiserum was used in a dilution of 1:250,000. Bound and free antibodies were separated by precipitation with 15% polyethylene glycol and 2.4% ovalbumin as described previously (13).

Peptide purification

Reversed-phase HPLC fractions containing the major immunoreactive product of lobes of animals adapted to a white background were dried and resubmitted to chromatography

using the Smart system (Pharmacia LKB Biotechnology, Uppsala, Sweden). A C_2/C_{18} precision column (SC2.1/10) was used with 0.0169% TFA in H_2O and acetonitrile as elution buffer. On-line detection was performed with a UV-monitor. Flow rate was 200 µl/min. Fractions of 30 sec were collected, dried and redissolved in buffer for radioimmunoassay, or in 10% acetic acid in methanol for FAB mass spectrometry.

Fast-atom bombardment tandem mass spectrometry
Fast-atom bombardment tandem mass spectrometry (FAB-MS/MS) was performed on a JEOL JMS SX/SX102A four sector instrument using 6kV fast xenon atoms. Approximately 50 ng of peptide was placed on the probe tip in a glycerol matrix. Collisionally induced MS/MS spectra of the $[M+H]^+$ ion were obtained by scanning the second mass spectrometer at a 50% main beam reduction using helium as a collision gas.

Superfusion
Neurointermediate lobes from black background adapted *Xenopus laevis* were dissected and superfused using the microsuperfusion system described previously (13). Four 10 minute fractions were pooled and peptides were separated on reversed-phase HPLC. HPLC fractions were submitted to radioimmunoassay.

Peptide synthesis
N-terminal acetylated endorphin [1-8], with a purity of over 95%, was custom synthesized by The American Peptide Company (Santa Clara, CA, USA).

Results and Discussion

The purpose of this study was to identify the terminal product(s) of processing of the endorphins in *Xenopus* melanotropes. HPLC-analysis coupled to a radioimmunoassay specific for acetylated endorphins revealed that neurointermediate lobe extracts of *Xenopus* possess at least six immunoreactive products (Fig. 1). The elution profile was similar to that found in mammals (14-16) except for two peaks designated peak I and peak II (Fig. 1). An oxidation experiment with H_2O_2 showed that peak I represents the sulfoxide form of peak II. On the basis of elution positions of the standard acetylated β-endorphins, peaks III, IV and V very likely represent the N-terminal acetylated endorphins [1-16], [1-31] and [1-27], respectively. Likewise, based on the elution profiles found in rat neurointermediate lobes, peak VI probably is acetylated endorphin [1-26] (16). The HPLC profiles of acetylated endorphins of neurointermediate lobes of white- and black-background adapted *Xenopus laevis* displayed noteworthy differences in the relative amounts of the various peptides. In the lobes of white-background adapted animals the majority of immunoreactive material was associated with peak I/II (77.2 ± 2.0%, n=3), while this same product represented only 44.5 ± 2.6% (n=3) in lobes of black-background adapted animals. Apparently, under conditions of adaptation to a white background, the large endorphins (products III to VI) are processed to a small peptide represented in peak I/II. The observed accumulation of this peptide is in agreement with previous findings that melanotrope cells in white animals store peptides in the absence of secretion (6). Altogether our results

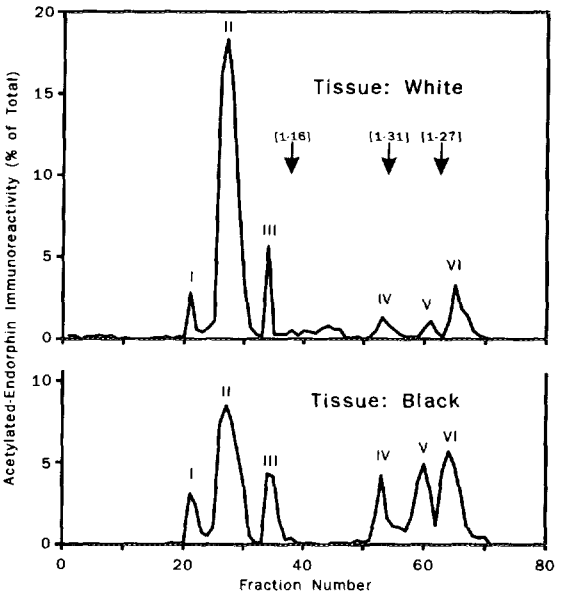

Fig. 1.
Reverse-phase HPLC analysis of N-acetylated endorphin immunoreactive material in extracts from neurointermediate lobes of white- and black-background adapted *Xenopus laevis*. Immunoreactive products are numbered from I to VI according to their retention time. Elution positions of synthetic human standard peptides (N-terminal acetylated β-endorphin [1-16], [1-27] and [1-31]) are indicated by arrows.

permit the conclusion that the product present in peak I/II represents the terminal product of processing of the acetylated endorphins.

The immunoreactive endorphin associated with peak II was further purified on the Smart system and studied with FAB tandem mass spectrometry. The FAB mass spectrum showed $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$ ions at *m/z* 943, 965 and 981 respectively. The collisionally induced dissociation MS/MS spectrum of the $[M+H]^+$ ion (Fig. 2a) revealed sufficient fragment ions to determine the amino acid sequence of the peptide unambiguously. This amino acid sequence is in exact agreement with that of the amino terminal eight amino acid residues of β-endorphin [1-31], including the acetylated amino group at the N-terminus. In this calculation the amino acids were deduced from the nucleotide sequence of *X. laevis* POMC cDNA (11). On the basis of this sequence, we had the acetylated β-endorphin [1-8] of *X. laevis* synthesized. This synthetic peptide had identical chromatographic characteristics as product II on reversed-phase HPLC (not shown) and gave an identical FAB tandem mass spectrum (Fig. 2b). Therefore, we conclude that this product is α,N-acetyl β-endorphin [1-8]. This confirms the proposal of Maruthainar *et al.* (10) and it seems likely that our product is identical to the terminal product of processing detected by Dores *et al.* (9). Analysis of the medium from

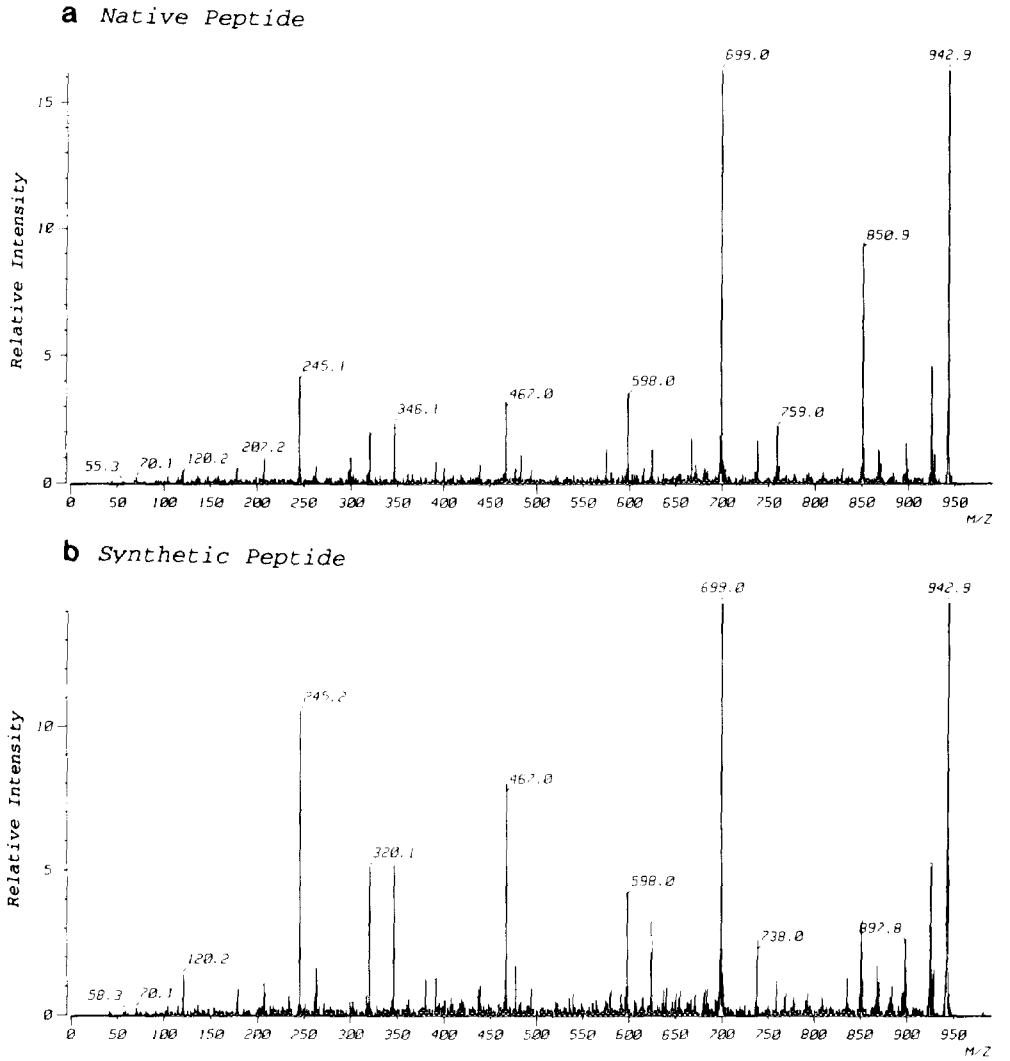

Fig. 2.
Collision-induced dissociation spectra of the $[M+H]^+$ ion from (a) product II and (b) 20 ng of synthetic *Xenopus* α,N-acetyl β-endorphin [1-8].

superfused neurointermediate lobe tissue shows that α,N-acetyl β-endorphin [1-8] is a major release product (Fig. 3). Therefore, the product constitutes an important component of the secretory signal from melanotrope cells.

Elucidation of the amino acid sequence of precursor molecules has clearly underlined the importance of pairs of basic amino acid residues (Lys-Arg, Arg-Arg, Lys-Lys) as potential recognition sites for specific proteases. However, such dibasics are not the only cleavage sites. From the structure of *Xenopus laevis* POMC it is clear that production of α,N-acetyl β-endorphin [1-8] must involve cleavage at a single arginine

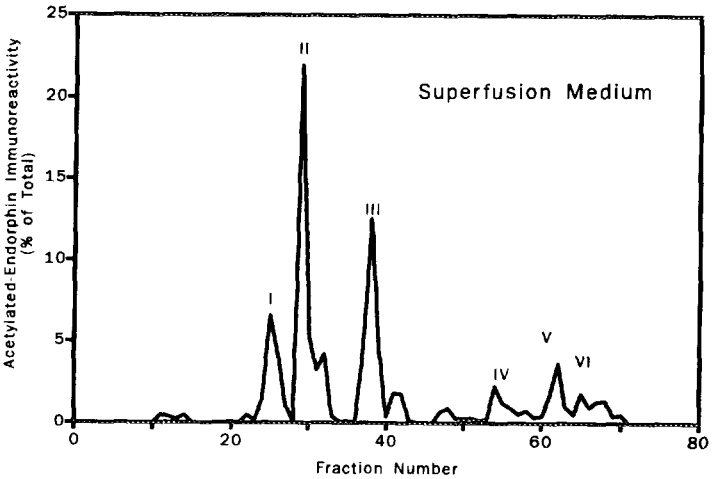

Fig. 3.
Reverse-phase HPLC analysis of N-terminal acetylated endorphin immunoreactive material released from superfused neurointermediate lobes of *X. laevis*.

residue at position 9 of β-endorphin in this species. This observation draws attention to the evolutionary significance of single arginine residues as sites for the processing of opioid peptides. In the atrial gland of the mollusk *Aplysia californica* prodynorphin is proteolytically cleaved to dynorphin at a single arginine residue (17). This suggests that philogenetically this type of opioid precursor cleavage is an old principle. Apparently, it is well conserved, because in mammals both dynorphin [1-8] and metorphamide are formed by cleavage at a single arginine residue (18,19). As to processing of β-endorphin, cleavage at a single arginine residue has been shown once before, viz. in the dogfish (20). It is to be expected that this mode of processing will be a general phenomenon for lower vertebrates because in all non-mammalian vertebrates studied, POMC shows a single arginine residue in the N-terminal region of β-endorphin (11,21-24).

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