Identification of β -endorphins in the pituitary gland and blood plasma of the common carp (*Cyprinus carpio*)

E H van den Burg, J R Metz, R J Arends, B Devreese¹,
I Vandenberghe¹, J Van Beeumen¹, S E Wendelaar Bonga
and G Flik

Department of Animal Physiology, Faculty of Science, University of Nijmegen, Toernooiveld 1, 6525 ED Nijmegen, The Netherlands

¹Laboratory of Protein Biochemistry and Protein Engineering, University of Gent, KL Ledeganckstraat 35, B9000 Gent, Belgium

(Requests for offprints should be addressed to G Flik; Email: gertflik@sci.kun.nl)

Abstract

Carp β -endorphin is posttranslationally modified by N-terminal acetylation and C-terminal cleavage. These processes determine the biological activity of the β -endorphins. Forms of β -endorphin were identified in the pars intermedia and the pars distalis of the pituitary gland of the common carp (*Cyprinus carpio*), as well as the forms released *in vitro* and into the blood. After separation and quantitation by high performance liquid chromatography (HPLC) coupled with radioimmunoassay, the β -endorphin immunoreactive products were identified by electrospray ionisation mass spectrometry and peptide sequencing. The release of β -endorphins by the pituitary gland was studied after stimulation with corticotrophin-releasing factor (CRF) *in vitro*. In the pars intermedia, eight N-acetylated truncated forms were identified. Full length N-acetyl β -endorphin(1–33) coeluted with

N-acetyl β -endorphin(1–29) and these forms together amounted to over 50% of total immunoreactivity. These products were partially processed to N-acetyl β -endorphin(1–15) (30.8% of total immunoreactivity) and N-acetyl β -endorphin(1–10) (3.1%) via two different cleavage pathways. The acetylated carp homologues of mammalian α - and γ -endorphin were also found. N-acetyl β -endorphin(1–15) and (1–29) and/or (1–33) were the major products to be released *in vitro*, and were the only acetylated β -endorphins found in blood plasma, although never together. CRF stimulated the release of opioid β -endorphin from the pars distalis. This non-acetylated β -endorphin represents the full length peptide and is the most abundant form in plasma.

Journal of Endocrinology (2001) **169**, 271–280

Introduction

Beta-endorphin is a pro-opiomelanocortin (POMC)-derived peptide and is predominantly produced in the pituitary gland and the brain. Its bioactivity depends on C-terminal truncation and N-terminal acetylation. For instance, β -endorphin(1–31) is a very potent opiate, whereas its acetylated congener is essentially devoid of opioid activity (Akil *et al.* 1981). On the other hand, C-terminal cleavage produces the very effective opioid antagonist β -endorphin(1–27) (Nicolas & Li 1985).

In addition to its opioid function, β -endorphin was found to be involved in a variety of physiological processes (reviewed by Dalayeun *et al.* 1993), including regulation of the immune (Heijnen *et al.* 1987, Shahabi *et al.* 1991, 1996) and reproductive (e.g. Faletti *et al.* 1999) systems. Furthermore, β -endorphin plays a role in the vertebrate stress response (e.g. Akil *et al.* 1985, Kjær *et al.* 1995, Wendelaar Bonga 1997, Mosconi *et al.* 1998).

Since forms of β -endorphin exert different biological effects, research so far has focused on the occurrence of β -endorphins that may each have different actions. Post-translationally modified β -endorphins have been described in a variety of vertebrates, including teleost fish (Takahashi *et al.* 1984) and mammals (Weber *et al.* 1981, Smyth 1984, Millington *et al.* 1992). However, data on the release and functions of β -endorphins are very scarce. In human blood plasma, the major form is β -endorphin(1–31) (Silberring *et al.* 1998). In fish, N-acetyl β -endorphin immunoreactivity has been reported in plasma (Rodrigues & Sumpster 1984, Sumpster *et al.* 1985, Mosconi *et al.* 1998), although the exact nature of the forms responsible for this immunoreactivity is unknown.

Recent cloning of carp POMC (Arends *et al.* 1998) showed that full length β -endorphin in this species consists of 33 amino acid residues, rather than 31 amino acids as in mammals and other vertebrates, and contains one potential dibasic and five potential monobasic cleavage sites. Therefore, identification of β -endorphins in the pituitary gland

predicts how processing of carp β -endorphin takes place and how this relates to processing in mammals. In addition to this evolutionary perspective, it is also of great interest to study processing of β -endorphin isoforms from a physiological point of view, as N-acetyl β -endorphin may have corticotrophic activity in teleosts (Balm *et al.* 1995).

Thus, the present study aimed to identify post-translationally modified β -endorphin isoforms in the pars intermedia and the pars distalis of the pituitary gland of the common carp (*Cyprinus carpio*). Furthermore, we analysed which truncated forms are released *in vitro* and which are present in the blood plasma. Finally, the effects of corticotrophin-releasing factor (CRF) on β -endorphin release by the pituitary gland were assessed by means of *in vitro* superfusion. CRF stimulates the release of adrenocorticotrophic hormones such as adrenocorticotrophic hormone (ACTH) and α -melanocyte-stimulating hormone (α -MSH) during stress (Wendelaar Bonga 1997). The analysis of CRF-driven β -endorphin release may indicate whether any β -endorphins are secreted during stress.

Materials and Methods

Animals

Male carp (70–120 g) were obtained from laboratory stock. Fish were held in 100 litre tanks within a recirculating system supplied with UV-filtered and biologically filtered tap water. The water temperature was maintained at 22 °C and the daily photoperiod was 16 h. Fish were fed commercial fish food (Trouvit, Trouw, Putten, The Netherlands) at 1.5% of body weight per day.

Tissue preparation

Fish were anaesthetised in 0.1% 2-phenoxyethanol. One millilitre blood was collected from the caudal blood vessels using a 21-gauge syringe with Na₂EDTA as anti-agglutinant. One trypsin inhibitor unit (TIU) aprotinin (Sigma, St Louis, MO, USA) per ml plasma was added to prevent proteolytic breakdown. After centrifugation (500 g, 10 min, 4 °C), plasma was collected and used immediately. To obtain the pituitary gland, the fish were placed on ice directly after the blood had been collected. The pituitary gland was then removed and the pars intermedia and pars distalis were separated with the aid of a dissection microscope. The tissue was homogenised mechanically on ice in 100 μ l 10 mM HCl and 0.2 TIU aprotinin. After centrifugation (10 000 g, 10 min, 4 °C) to remove membranes and cellular debris, the supernatant was submitted to reversed-phase HPLC immediately or after storage at –20 °C.

Prepurification of plasma

A Pharmacia LKB Superdex-75 HR 10/30 column (Pharmacia LKB Biotechnology, Uppsala, Sweden) was

used for initial purification of plasma. The eluent consisted of an 0.36 M acetate buffer (0.088 M ammonium acetate, 0.272 M acetic acid, 19% v/v ethanol, pH 4.4), as described by Sælsen *et al.* (1994). The flow was 0.5 ml/min, and 300 μ l fractions were collected. One hundred microlitres of the fractions were vacuum-dried and redissolved in veronal acetate buffer: VAT-buffer, pH 8.6, 0.02 M sodium barbital, 0.2 g/l NaN₃, supplemented with 0.3% bovine serum albumin (Sigma), and 100 KIU/ml trypsin inhibitor (Trasylol; Bayer, Leverkusen, Germany), and analysed by radioimmunoassay.

Radioimmunoassay

N-acetyl β -endorphin was quantitated in duplicate by radioimmunoassay with an antiserum against salmon N-acetyl β -endorphin (developed and characterised by Takahashi *et al.* 1984). This antibody has full cross-reactivity with acetylated forms of mammalian (Dores *et al.* 1991) and *Xenopus* (Van Strien *et al.* 1993) β -endorphin. Cross-reactivity of this antibody with non-acetylated forms is less than 0.1% (Dores *et al.* 1991). The final dilution of the antiserum was 1:250 000. *Xenopus* N-acetyl β -endorphin(1–8) used as tracer was iodinated using the iodogen method (Salacinsky *et al.* 1981) and purified through solid phase extraction (octadecyl Bakerbond column). Immunocomplexes were separated from free tracer by precipitation of the complexes with 15% polyethylene glycol and 2.4% ovalbumin.

Reversed-phase HPLC

Immunoreactive Superdex-75 fractions obtained from blood plasma samples were vacuum dried and resuspended in 100 μ l 10 mM HCl. These samples, as well as the pars intermedia and pars distalis homogenates, were submitted to reversed-phase HPLC using a μ RPC C2/C18 SC2-1/10 column (Amersham Pharmacia Biotech AB, Uppsala, Sweden). Beta-endorphins were separated with an acetonitrile gradient (22–36%) in water containing 0.1% trifluoroacetic acid (Merck, Darmstadt, Germany) at 150 μ l/min. Fractions (300 μ l) were dried under vacuum and redissolved in VAT-buffer for radioimmunoassay. Standards were human α -endorphin, human γ -endorphin, N-acetyl β -endorphin(1–27) (Sigma) and *Xenopus* N-acetyl β -endorphin(1–8) (American Peptide Company, Santa Clara, CA, USA).

Affinity chromatography

To assess the presence of non-acetylated β -endorphin, homogenates of pars intermedia and pars distalis, as well as Superdex-75-fractions of plasma, were submitted to affinity chromatography to deplete the samples of N-acetyl β -endorphins. An affinity column was prepared according

to the manufacturer's instructions (Pharmacia) using the anti-N-acetyl β -endorphin antibody. The samples were eluted with 2 ml 0.1 M Na_2CO_3 , pH 8.0, and subsequently vacuum-dried. The pellets were redissolved in 10 mM HCl and submitted to reversed-phase HPLC. To measure non-acetylated β -endorphins, samples were chemically acetylated as described by Van Strien *et al.* (1993). Briefly, 100 μl of sample was dried under vacuum and resuspended in 100 μl 50 mM phosphate buffer, pH 8.0. Acetylation was started by adding 2 μl acetic anhydride (Sigma), followed by vigorous vortexing. Samples were dried and resuspended in VAT-buffer for radioimmunoassay for acetylated endorphins.

In vitro superfusion

The pars distalis and pars intermedia were separated and were placed on a cheese-cloth filter in a superfusion chamber and superfused with a Hepes-buffered (15 mM; pH 7.38) Ringer's solution containing NaCl (128 mM), KCl (2 mM), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (2 mM), with 0.25% (w/v) glucose and 0.03% (w/v) bovine serum albumin. Medium was pumped through the chambers at 30 $\mu\text{l}/\text{min}$ by a Watson-Marlow 503U multichannel peristaltic pump (Smith and Nephew Watson-Marlow, Falmouth, Cornwall, UK). After 180 min superfusion, when the release of endorphins had reached an apparent steady state, ovine CRF (Sigma) was added to the medium to a final concentration of 1 nM to test its effect on the release of β -endorphin isoforms. A 20-min fraction before and a 20-min fraction during the pulse were collected in 50 mM HCl and analysed for the presence of β -endorphin isoforms as described above.

Molecular weight determination by electrospray ionisation mass spectrometry (ESMS)

Partially purified β -endorphin immunoreactive peptides were analysed on a Micromass Q-TOF mass spectrometer (Wythenshawe, Manchester, UK), fitted with a nano-electrospray ion source. Lyophilised peptides were dissolved in 50% acetonitrile/1% formic acid in water; the final peptide concentration was approximately 1 μM (based on the theoretical molecular weight of the peptide). Four microlitre aliquots of sample solutions were loaded into borosilicate Nanoflow tips (Protana, Odense, Denmark). The mass spectrometer (MS) was operated in positive ion mode with a source temperature of 30 °C. A potential of 0.8–1.5 kV applied to the Nanoflow tip combined with a nitrogen backpressure of 5–10 psi produced a sample flow of 10–30 nL/min into the analyser. All data, both MS and MS/MS, were acquired with the TOF analyser. In MS/MS mode the quadrupole was used in resolving mode to select the precursor ion for fragmentation in the hexapole collision cell. MS/MS was performed with argon gas in the collision cell at a pressure of 6×10^{-5} mbar

measured in the analyser. All data were processed using Masslynx software delivered with the instrument.

Peptide sequencing

Peptides were sequenced by automated Edman degradation in a model 476 pulsed-liquid sequenator (Applied Biosystems, Foster City, CA, USA).

Peptide synthesis

Carp N-acetyl β -endorphin(1–10) was synthesised by the Institute of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, The Netherlands. Carp β -endorphin(1–29) was synthesised by the Department of Immunohaematology and Bloodbank, Leiden, The Netherlands.

Immunohistochemistry

Brain–pituitary gland complexes were fixed in Bouin's fixative. Sagittal sections (7 μm) were immunostained with the peroxidase-anti-peroxidase method, according to Van der Heijden *et al.* (1999) with minor modifications, using the salmon N-acetyl β -endorphin antibody as described above or a carp β -endorphin(20–29) antiserum (1:2000). The latter antibody was produced in our laboratory by immunising rabbits with carp β -endorphin(20–29) coupled to keyhole limpet haemocyanin. The antiserum recognises acetylated as well as non-acetylated full length carp β -endorphin and carp β -endorphin(1–29). Serum specificity was assessed histochemically by omitting the antiserum and by replacing the antiserum with normal rabbit serum. Immunostaining was fully abolished in these tests. The sections were subsequently incubated with this antibody (overnight), goat-anti-rabbit IgG (1:150; 1 h; Nordic Immunology, Tilburg, The Netherlands), rabbit peroxidase-anti-peroxidase (1:150; 1 h; Nordic) and 3,3-diaminobenzidine tetrahydrochloride (0.25 mg/ml) in the presence of 0.0005% (v/v) H_2O_2 for 5 min.

Statistics

Six fish were sampled to quantitate and identify the forms of β -endorphin in blood plasma, pars intermedia and pars distalis. Basal and CRF-stimulated release of β -endorphins was studied in quadruplicate, whereas the immunohistochemical analysis of distribution of β -endorphins in the brain–pituitary gland complexes was performed on three fish. As little variation between replicates occurred, results are presented as representative examples, unless stated otherwise.

Results

Seven immunoreactive fractions were observed in homogenates of the pars intermedia (Fig. 1) containing

Figure 1 Reversed phase analysis of carp N-acetyl β -endorphin in the pars intermedia. The dashed line represents the acetonitril gradient in water, ranging from 22% in fraction 3 to 36% in fraction 40. Seven immunoreactive fractions were obtained containing N-acetyl β -endorphin(1–10) (I), N-acetyl β -endorphin(1–15) (II), N-acetyl β -endorphin(1–16) (III), N-acetyl β -endorphin(1–18) (IV), N-acetyl β -endorphin(1–17) (V), N-acetyl β -endorphin(1–19) (VI) and N-acetyl β -endorphin(1–29) and (1–33) (VII). The following standards were chromatographed separately: A, *Xenopus* N-acetyl β -endorphin(1–8); B, human N-acetyl β -endorphin(1–27).

8 N-acetyl β -endorphins. Peak VII contained 50.5% of the immunoreactivity and represented N-acetyl β -endorphin(1–29) plus (1–33), as determined by ESMS and peptide sequencing. The other predominant β -endorphin eluted in peak II (30.8%), and was found to be N-acetyl β -endorphin(1–15). N-acetyl β -endorphin(1–16) (peak III, 3.8%), N-acetyl β -endorphin(1–17) (peak V, 1.8%), N-acetyl β -endorphin(1–18) (peak IV, 9.4%), and N-acetyl β -endorphin(1–19) (peak VI, 0.6%) were the other constituents. The identity of the N-acetyl β -endorphin in peak I (3.1%) could not be assessed by ESMS. However, we observed the presence of β -endorphin(12–29) in fraction 14, indicating that N-acetyl β -endorphin(1–29) is cleaved at the single basic residue Arg¹¹ to yield N-acetyl β -endorphin(1–10). Synthetic carp N-acetyl β -endorphin(1–10) was found to elute in fraction 6, indicating that peak I contains this peptide.

One peak reflecting non-acetylated β -endorphin was obtained (peak VIII, 1.7%, Fig. 2). We were unable to identify this peptide with ESMS. The immunoreactive product was found to coelute with synthetic carp β -endorphin(1–29) (data not shown), and, in parallel to the acetylated forms, probably also with β -endorphin(1–33). Beta-endorphin(1–29) and (1–33) are the most

hydrophobic forms (as predicted from their amino acid composition) and thus the apparent immunoreactivity eluting in later fractions cannot represent β -endorphin. Immunoreactivity in these fractions (e.g. fractions 18 and 19 in Fig. 2), when present, is very low and therefore considered not specific. Fraction 5 always shows some immunoreactivity, but we were unable to identify its nature.

The pars intermedia released four forms *in vitro* (Fig. 3), of which N-acetyl β -endorphin(1–29) and/or (1–33), and N-acetyl β -endorphin(1–15) were predominant. The other secreted products were N-acetyl β -endorphin(1–10) and (1–18). The two former peptides were also found in plasma, but never simultaneously in the same fish (Fig. 4). Blood plasma from three fish contained N-acetyl β -endorphin(1–15), whereas plasma from the other three fish contained N-acetyl β -endorphin(1–29). The N-acetyl β -endorphin concentration in blood plasma was 3.7 ± 0.6 nmol/l (mean $\pm$ S.E.M., $n=6$). The concentration of the non-acetylated β -endorphin in plasma (Fig. 5, peak VIII) varied considerably among individual fish and ranged from 7 to 84 nmol/l.

In vitro stimulation of the pars intermedia with 1 nM CRF did not stimulate release of any β -endorphin (data not shown). On the other hand, the secretion of

Figure 2 Reversed phase analysis of carp β -endorphin in the pars intermedia. (Dashed line: acetonitril gradient in water; conditions as in Fig. 1.) Aliquots of immunoreactive fractions were further analysed by electrospray mass spectrometry, which did not reveal the identity of β -endorphins. Synthetic carp β -endorphin(1–29) coeluted in fraction 8 with peak VIII. The following human standards were chromatographed separately: C, α -endorphin; D, γ -endorphin.

non-acetylated β -endorphin was promoted in the pars distalis: basal release was almost undetectable but when stimulated with CRF the release increased to 57 fmol/min per pars distalis (Fig. 6).

Non-acetylated β -endorphin was localised in the nucleus preopticus (NPO) and in the hypothalamo-pituitary tract (Fig. 7). The β -endorphin-containing neurons spread throughout the pars intermedia (PI).

Figure 3 Basal *in vitro* release of carp N-acetyl β -endorphin(1–10) (peak I), (1–15) (peak II), (1–18) (peak IV), and (1–29)/(1–33) (peak VII). (Conditions as in Fig. 1.)

Figure 4 Reversed phase analysis of carp N-acetyl β -endorphin in blood plasma. Samples from six fish were analysed, three of which contained N-acetyl β -endorphin(1–15) (peak II, Ac-15), whereas plasma from the other fish contained N-acetyl β -endorphin(1–29) or (1–33) (peak VII, Ac-29/33). (Conditions as in Fig. 1.)

Non-acetylated β -endorphin was not detectable by immunohistochemistry in the pars distalis (PD). The immunostaining of the melanotroph cells in the PI reflects the presence of N-acetyl β -endorphin(1–33) and (1–29). Immunostaining with the antibody against acetylated β -endorphins only stained the melanotroph cells in the pars intermedia.

Discussion

We identified eight acetylated β -endorphins in the pars intermedia. N-acetyl β -endorphin(1–33) is the full length peptide (Arends *et al.* 1998), and its cleavage follows two pathways to yield either N-acetyl β -endorphin(1–10) or (1–15). The first pathway involves cleavage of N-acetyl

Figure 5 Reversed phase analysis of carp β -endorphin in plasma. Peak VIII coelutes with carp β -endorphin(1–29). (Conditions as in Fig. 1.)

Figure 6 Reversed phase analysis of *in vitro* CRF-driven β -endorphin release from the pars distalis. The control consists of non-acetylated β -endorphin release during the 20 min preceding the 20-min pulse with CRF on the same tissue. Peak VIII coelutes with carp β -endorphin(1–29). (Conditions as in Fig. 1.)

β -endorphin(1–33) to N-acetyl β -endorphin(1–29) at the dibasic cleavage site Lys³⁰–Lys³¹ by prohormone convertase 2 (PC2; Zhou *et al.* 1993). Next, this peptide is cleaved at Arg¹¹ to N-acetyl β -endorphin(1–11) and β -endorphin(12–29). The former is further processed to N-acetyl β -endorphin(1–10) by a carboxypeptidase H-like enzyme. The second pathway includes cleavage of either N-acetyl β -endorphin(1–33) or (1–29) at Lys²¹ to N-acetyl β -endorphin(1–21). Processing at monobasic cleavage sites has been reported in the toad, *Bufo marinus* (Dores *et al.* 1994) and in rat prodynorphin processing (Day *et al.* 1998). N-acetyl β -endorphin is further truncated to N-acetyl β -endorphin(1–15) by carboxypeptidase activity. All of the predicted N-acetyl β -endorphin truncated forms were present in pars intermedia homogenates of the carp, except for N-acetyl β -endorphin(1–11), (1–20), and (1–21). Therefore, we predict the conversion of the latter isoforms to be immediate and complete. In fact, it is well known that carboxypeptidases effectively cleave off C-terminal basic amino acid residues. Takahashi *et al.* (1984) demonstrated the presence of N-acetyl β -endorphin(1–20) in chum salmon (*Oncorhynchus keta*) pituitary gland. However, the C-terminal amino acid residue in this species is Leu rather than Arg or Lys, which is less effectively removed by such enzymatic activity. The absence of carp N-acetyl β -endorphin(1–20) and (1–21) can also be explained when the full length peptide is immediately cleaved at the Leu¹⁹–Phe²⁰ bond, as has been described in rats (Burbach *et al.* 1981).

The terminal products of the two processing routes are released by the carp pars intermedia *in vitro*, together with N-acetyl β -endorphin(1–29) and/or (1–33) and trace amounts of N-acetyl β -endorphin(1–18). In plasma, we found either N-acetyl β -endorphin(1–15) or N-acetyl β -endorphin(1–29)/(1–33). As the occurrence of these forms appeared to be individual-specific, we speculate that their release is under different hypothalamic control. The presence of N-acetyl β -endorphin(1–10) and (1–18) could not be demonstrated in plasma and their concentration may be under the detection limit of the assay (50 pM) or, alternatively, their secretion is under inhibitory hypothalamic control *in vivo*.

The processing pathway of N-acetyl β -endorphin appears to be similar to the mechanism in other vertebrates, since N-acetyl β -endorphin(1–29) (the homologue of mammalian N-acetyl β -endorphin(1–27)), as well as N-acetyl β -endorphin(1–18), (the homologue of N-acetyl α -endorphin), and N-acetyl β -endorphin(1–19) (the homologue of N-acetyl γ -endorphin) were found. On the other hand, we could not identify the carp homologue of β -endorphin(1–26), which may be due to the presence of Gln²⁹ that is less susceptible to carboxypeptidase activity (Smyth *et al.* 1989) than is His²⁷ in mammals. Cleavage to N-acetyl β -endorphin(1–10) resembles processing as observed in the amphibian *Xenopus laevis*, where the homologue of this carp β -endorphin, N-acetyl β -endorphin(1–8), is the terminal product of endorphin processing (Van Strien *et al.* 1993). Although a minor product in the carp pituitary gland (3.1%), N-acetyl

Figure 7 Sagittal section of carp brain showing the hypothalamo-pituitary complex. (a) β -endorphin staining is observed in the nucleus preopticus (NPO), the hypothalamo-hypophyseal tract (arrow), and in the pars intermedia (PI). The antibody recognises β -endorphin(1–29) and (1–33) as well as their acetylated counterparts. (b) The antibody against acetylated β -endorphins only stains the melanotroph cells in the PI, demonstrating that acetylation does not occur in the NPO and the hypothalamo-hypophyseal tract. The sections shown are 35 μ m apart, and both contain cells from the NPO as demonstrated by staining for CRF (not shown). ON, optic nerve; PD, pars distalis. Scale bar is 1 mm.

β -endorphin(1–10) may be the most abundant β -endorphin in tilapia (*Oreochromis mossambicus*; Lee *et al.* 1999), indicating that utilisation of this pathway – which is not found in mammals – differs among fish species. Thus, in species where N-acetyl β -endorphin is preferentially truncated to N-acetyl β -endorphin(1–10), the β -endorphin may have a specific function not encountered in other species, or forms other than N-acetyl β -endorphin(1–10) exert similar effects.

The reason why mammalian α -endorphin is not further processed to β -endorphin(1–13), similar to carp β -endorphin(1–15), may relate to species-specific processing. Indeed, Takahashi *et al.* (1984) found no cleavage of the salmon homologue of N-acetyl α -endorphin. The C-terminal amino acid of γ -endorphin is Trp in all species examined, but the preceding amino acid varies. For instance, this amino acid is Leu in the carp (Arends *et al.* 1998), Ile in the chum salmon (Takahashi *et al.* 1984) and Val in humans (Takahashi *et al.* 1981). It appears

that proteolytic activity depends not only on the nature of the C-terminal amino acid residue, but also on the neighbouring amino acids.

We were unable to identify non-acetylated β -endorphins by ESMS, although the presence of one such form was indicated by radioimmunoassay. It coeluted with synthetic carp β -endorphin(1–29) (not shown), which is likely to have the same hydrophobicity as carp β -endorphin(1–33). As measuring of these forms is possible only after chemical acetylation, it could be argued that an artefact induced by the acetylation procedure accounts for the occurrence of this immunoreactivity in the assay. However, this method has been shown to acetylate non-acetylated β -endorphins effectively (Lorenz *et al.* 1986, Van Strien *et al.* 1993). A more likely explanation is that the amount of the non-acetylated forms was too small for detection by ESMS, especially since the β -endorphins were only partially purified. In mammals, the non-acetylated β -endorphin present in the anterior pituitary corticotroph cells is the full length peptide, which is not further processed, because PC2 is hardly expressed in these cells (Benjannet *et al.* 1991). Using 32 g chum salmon pituitary glands, Takahashi *et al.* (1984) were able to isolate and identify full length non-acetylated β -endorphin, which must have been produced by the corticotroph cells in the pars distalis. We assume that the same applies to carp, and therefore suggest the presence of β -endorphin(1–33) in the pars distalis homogenates. The release of this peptide would be under stimulatory control of CRF, as indicated by the strong induction of its release by this neuropeptide *in vitro*. The trace amounts of non-acetylated β -endorphin in the pars intermedia may originate from the hypothalamic-hypophyseal tract that we showed to contain non-acetylated β -endorphin(1–33) in carp. This peptide was not released by the melanotroph cells in the pars intermedia *in vitro*, not even in the presence of CRF (not shown). Thus, the non-acetylated β -endorphin in plasma must originate from the pars distalis and probably represents the full length peptide.

In human plasma, the most abundant β -endorphin isoform is the opioid β -endorphin(1–31) (Silberring *et al.* 1998). The existence of opiate active β -endorphins in teleosts is indicated by the localisation of μ -opiate receptors in the coho salmon (*Oncorhynchus kisutch*) brain (Ebbesson *et al.* 1996).

In addition to their opioid activity, β -endorphins may be involved in the stress response. We showed that CRF, which has a pivotal role in stress in all vertebrates and is a potent stimulator of ACTH and MSH secretion in fish (Van Enkevort *et al.* 2000), induces β -endorphin release from the pars distalis *in vitro*. This indicates co-release with ACTH, being a well-known corticotrophic hormone in fish (Wendelaar Bonga 1997). In contrast, CRF failed to increase the release of acetylated β -endorphins from the pituitary gland in our study, and this does not support a role for these peptides in the stress response of carp,

Figure 8 Summary of β -endorphin processing in the common carp. Immediately after cleavage out of POMC, β -endorphin is N-terminally acetylated in the pars intermedia to N-acetyl β -endorphin(1–33) (Ac-33). This peptide is further processed to N-acetyl β -endorphin(1–29) (Ac-29), which is partially processed to N-acetyl β -endorphin(1–10) (Ac-10) and N-acetyl β -endorphin(1–18) (Ac-18). The bulk of the latter is cleaved to N-acetyl β -endorphin(1–15) (Ac-15). N-acetyl β -endorphin(1–33) and/or (1–29), (1–18), (1–15) and (1–10) are released by the pituitary gland *in vitro*. In the pars distalis, β -endorphin is produced and released only when stimulated with CRF. No further processing of β -endorphin(1–33) (33) takes place. Percentages indicate the relative amount of the β -endorphins in the pars intermedia. For clarity, intermediate cleavage products are omitted from the scheme.

although increased plasma levels during stress have been reported for other fish species (Sumpter *et al.* 1985, Mosconi *et al.* 1998) as well as mammals (Akil *et al.* 1985). A factor other than CRF may be involved in the control of N-acetyl β -endorphin release in carp.

In summary, β -endorphin processing in carp, of which the main steps are emphasised in Fig. 8, displays the same pattern as reported for other vertebrates. α -N-acetylation of β -endorphin(1–33) and subsequent processing to truncated forms take place only in the melanotroph cells in the pars intermedia, whereas β -endorphin(1–33) becomes detectable in the corticotroph cells of the pars distalis when stimulated with CRF. The presence of at least three β -endorphins in the circulation suggests that post-translational modifications of β -endorphin expand the possibilities for multiple physiological effects. These findings warrant the search for regulatory mechanisms in the release of the different β -endorphins.

Acknowledgements

This work was partially supported by SLW 805–33–101-P. The authors gratefully acknowledge Mr T Spanings for animal care. BD is a postdoctoral fellow of the Funds for Scientific Research – Flanders. JVB is indebted to the same institute for research grant G.0422.98.

References

- Akil H, Young E, Watson SJ & Coy DH 1981 Opiate binding properties of naturally occurring N- and C-terminal modified β -endorphins. *Peptides* **2** 289–292.
- Akil H, Shiomi H & Matthews J 1985 Induction of the intermediate pituitary by stress: synthesis and release of a nonopioid form of β -endorphin. *Science* **227** 424–426.
- Arends RJ, Vermeer H, Martens GJM, Leunissen JAM, Wendelaar Bonga SE & Flik G 1998 Cloning and expression of two pro-opiomelanocortin mRNAs in the common carp (*Cyprinus carpio* L.). *Molecular and Cellular Endocrinology* **143** 23–31.
- Balm PHM, Hovens MLM & Wendelaar Bonga SE 1995 Endorphin and MSH in concert form the corticotropic principle released by tilapia (*Oreochromis mossambicus*; Teleostei) melanotrope. *Peptides* **16** 463–469.
- Benjannet S, Rondeau N, Day R, Chrétien M & Seidah NG 1991 PC1 and PC2 are proprotein convertases capable of cleaving pro-opiomelanocortin at distinct pairs of basic residues. *PNAS* **88** 3564–3568.
- Burbach JPH, De Kloet ER, Schotman P & De Wied D 1981 Proteolytic conversion of β -endorphin by brain synaptic membranes. Characterization of generated β -endorphin fragments and proposed metabolic pathway. *Journal of Biological Chemistry* **256** 12463–12469.
- Delayeun JF, Norès JM & Bergal S 1993 Physiology of β -endorphins. A close-up view and a review of the literature. *Biomedicine and Pharmacotherapy* **47** 311–320.
- Day R, Lazure C, Basak A, Boudreault A, Limperis P, Dong W & Lindberg I 1998 Prodynorphin processing by proprotein convertase 2. Cleavage at single basic residues and enhanced processing in the presence of carboxypeptidase activity. *Journal of Biological Chemistry* **273** 829–836.

- Dores RM, Steveson TC & Lopez K 1991 Differential mechanisms for the N-acetylation of α -melanocyte-stimulating hormone and β -endorphin in the intermediate pituitary of the frog, *Xenopus laevis*. *Neuroendocrinology* **53** 54–62.
- Dores RM, Gieseker K & Steveson TC 1994 The posttranslational modification of β -endorphin in the intermediate pituitary of the toad, *Bufo marinus*, includes processing at a monobasic cleavage site. *Peptides* **15** 1497–1504.
- Ebbesson LOE, Diviche P & Ebbesson SOE 1996 Distribution and changes in μ - and κ -opioid receptors during the midlife neurodevelopmental period of coho salmon, *Oncorhynchus kisutch*. *Journal of Comparative Neurology* **364** 448–464.
- Faletti AG, Mastronardi CA, Lomniczi A, Seilicovich A, Gimeno M, McCann SM & Rettori V 1999 β -endorphin blocks luteinizing hormone-releasing hormone release by inhibiting the nitricoxidergic pathway controlling its release. *PNAS* **96** 1722–1726.
- Heijnen CJ, Croiset G, Zijlstra J & Ballieux RE 1987 Modulation of lymphocyte function by endorphins. *Annals of the New York Academy of Sciences* **496** 161–165.
- Kjær A, Knigge U, Bach FW & Warberg J 1995 Stress-induced secretion of pro-opiomelanocortin-derived peptides in rats: relative importance of the anterior and intermediate pituitary lobes. *Neuroendocrinology* **61** 167–172.
- Lee J, Danielson P, Sollars C, Alrubaian J, Balm P & Dores RM 1999 Cloning of a neoteleost (*Oreochromis mossambicus*) POMC cDNA reveals a deletion of the γ -MSH region and most of the joining peptide region: implications for POMC processing. *Peptides* **20** 1391–1399.
- Lorenz RG, Tyler AN, Faull KF, Makk G, Barchas JD & Evans CJ 1986 Characterization of endorphins from the pituitary of the spiny dogfish, *Squalus acanthias*. *Peptides* **7** 119–126.
- Millington WR, Dybdal NO, Mueller GP & Chronwall BM 1992 N-acetylation and C-terminal proteolysis of β -endorphin in the anterior lobe of the horse pituitary. *General and Comparative Endocrinology* **85** 297–307.
- Mosconi G, Gallinelli A, Polzonetti-Magni AM & Facchinetti F 1998 Acetyl salmon endorphin-like and interrenal stress response in male gilthead sea bream, *Sparus aurata*. *Neuroendocrinology* **68** 129–134.
- Nicolas P & Li CH 1985 β -endorphin(1–27) is a naturally occurring antagonist to etorphine-induced analgesia. *PNAS* **82** 3178–3181.
- Rodrigues KT & Sumpter JP 1984 The radioimmunoassay of α -melanocyte-stimulating hormone and endorphin in trout (*Salmo gairdneri*) and the effect of blinding on the plasma levels of these peptides. *General and Comparative Endocrinology* **54** 69–75.
- Sælsén L, Andersen HB, Bratholm P & Christensen NJ 1994 Radioimmunoassay of plasma neuropeptide Y using HPLC for separation of related peptides and fragments. *Scandinavian Journal of Clinical and Laboratory Investigation* **54** 207–214.
- Salacinsky PRP, McLean C, Sykes JEC, Clement-Jones VV & Lowry P 1981 Iodination of proteins, glycoproteins, and peptides using a solid-phase oxidizing agent 1,3,4,6-tetrachloro-3 α ,6 α -diphenyl glycoluril (Iodogen). *Analytical Biochemistry* **117** 136–146.
- Shahabi NA, Burtress MZ & Sharp BM 1991 N-acetyl- β -endorphin_{1–31} antagonizes the suppressive effect of β -endorphin_{1–31} on murine splenocyte proliferation via a naloxone-resistant receptor. *Biochemical and Biophysical Research Communications* **175** 936–942.
- Shahabi NA, Heagy W & Sharp BM 1996 β -endorphin enhances concanavalin-A-stimulated calcium mobilization by murine splenic T cells. *Endocrinology* **137** 3386–3393.
- Silberring J, Li YM, Terenius L & Nylander I 1998 Characterization of immunoreactive dynorphin B and β -endorphin in human plasma. *Peptides* **19** 1329–1337.
- Smyth DG 1984 Chromatography of peptides related to β -endorphin. *Analytical Biochemistry* **136** 127–135.
- Smyth DG, Maruthainar K, Darby NJ & Fricker LD 1989 Catalysis of slow C-terminal processing reactions by carboxypeptidase H. *Journal of Neurochemistry* **53** 489–493.
- Sumpter JP, Pickering AD & Pottinger TG 1985 Stress-induced elevation of plasma α -MSH and endorphin in brown trout, *Salmo trutta* L. *General and Comparative Endocrinology* **59** 257–265.
- Takahashi H, Teranishi Y, Nakanishi S & Numa S 1981 Isolation and structural organization of the human corticotropin-beta-lipotropin precursor gene. *FEBS Letters* **135** 97–102.
- Takahashi A, Kawauchi H, Mouri T & Sakai A 1984 Chemical and immunological characterization of salmon endorphins. *General and Comparative Endocrinology* **53** 381–388.
- Van der Heijden AJH, Van der Meij JCA, Flik G & Wendelaar Bonga SE 1999 Ultrastructure and distribution dynamics of chloride cells in tilapia larvae in fresh water and sea water. *Cell and Tissue Research* **297** 119–130.
- Van Enckevort FHJ, Pepels PPLM, Leunissen JAM, Martens GJM, Wendelaar Bonga SE & Balm PHM 2000 *Oreochromis mossambicus* (tilapia) corticotropin-releasing hormone: cDNA sequence and bioactivity. *Journal of Neuroendocrinology* **12** 177–186.
- Van Strien FJC, Jenks BG, Heerma W, Versluis C, Kawauchi H & Roubos EW 1993 α ,N-acetyl- β -endorphin is the terminal product of processing of endorphins in the melanotrope cells of *Xenopus laevis*, as is demonstrated by FAB tandem mass spectrometry. *Biochemical and Biophysical Research Communications* **191** 262–268.
- Weber E, Evans CJ & Barchas JD 1981 Acetylated and non-acetylated forms of β -endorphin in rat brain and pituitary. *Biochemical and Biophysical Research Communications* **103** 982–989.
- Wendelaar Bonga SE 1997 The stress response in fish. *Physiological Reviews* **77** 591–625.
- Zhou A, Bloomquist BT & Mains RE 1993 The prohormone convertases PC1 and PC2 mediate distinct endoproteolytic cleavages in a strict temporal order during pro-opiomelanocortin biosynthetic processing. *Journal of Biological Chemistry* **268** 1763–1769.

Received 12 October 2000

Accepted 31 January 2001