Carboxypeptidase H in the Hypothalamo–Neurohypophyseal System: Evidence for Processing and Activation of a Prohormone-Processing Enzyme During Axonal Transport

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Investigations of peptide precursor processing in nerve cells, including studies on prooxytocin and provasopressin processing in the rat hypothalamo–neurohypophyseal system, show that prohormone processing occurs during axonal transport of maturing secretory vesicles. Recent studies (Fricker et al., 1989; Rodriguez et al., 1989) show that carboxypeptidase H (CPH), one of several proteases required for prohormone processing, is synthesized as a proenzyme that presumably requires activation. To determine if pro-CPH, like prohormone precursors, is processed and activated during axonal transport, we have analyzed the molecular forms of CPH present at several levels in the rat hypothalamo–neurohypophyseal system. These biochemical and immunochemical studies showed that the supraoptic nucleus (SON), a region enriched in neuronal cell bodies, possesses primarily an inactive 65-kDa species of CPH. The median eminence and pituitary stalk regions that are enriched in axons possess both the inactive 65-kDa and the active 55-kDa forms of CPH, and nerve terminals of the posterior pituitary contain primarily the active 55-kDa CPH. These results support the hypothesis that pro-CPH is processed and activated during axonal transport from neuronal perikarya of SON to nerve terminals of the posterior pituitary. Furthermore, analysis of immunoreactive CPH in the rat and bovine pituitary showed that each tissue possessed different relative amounts of zymogen compared to mature forms of CPH, suggesting that tissue-specific processing of pro-CPH occurs. Thus, the biosynthesis of active peptide hormones requires the simultaneous processing of proenzyme and prohormone.

It is well known that numerous peptide hormones and neurotransmitters are synthesized as prohormone precursors that must undergo limited proteolysis to yield the smaller, physiologically active peptides. Carboxypeptidase H (CPH) (Webb, 1986) is one of the later processing enzymes in the proteolytic cascade

required for conversion of prohormones such as provasopressin, prooxytocin, proenkephalin, and others into the smaller, active peptides. Subsequent to the action of trypsin-like endopeptidase(s) that cleave at pairs of dibasic amino acid residues (lysine, arginine) within the precursor (Docherty and Steiner, 1982; Gainer et al., 1985), CPH [previously referred to as “carboxypeptidase B-like” (Hook and Loh, 1984; Hook et al., 1985b), “enkephalin convertase” (Fricker and Snyder, 1982, 1983), and “carboxypeptidase E” (Fricker et al., 1989)] removes the COOH-terminal lysine and/or arginine extensions on the peptide. Biochemical studies (Fricker and Snyder, 1982, 1983; Hook et al., 1982; Hook and Loh, 1984; Supattapone et al., 1984; Davidson and Hutton, 1987) show that CPH can be distinguished from carboxypeptidase B and carboxypeptidase N, which also possess specificities for basic amino acid residues, by its acidic pH optimum, thiol dependence, potency of guanidinoethylmercaptosuccinic acid (GEMSA) inhibition, and molecular weight. Immunochemical studies (Hook et al., 1985b) showed that a specific anti-CPH antibody does not crossreact with carboxypeptidases B, N, A, Y, or P, suggesting that CPH is a structurally distinct carboxypeptidase. Analysis of the amino acid sequence deduced from the CPH cDNA (Fricker et al., 1989; Rodriguez et al., 1989) confirms that CPH is a unique carboxypeptidase that is involved in the production of a variety of peptide hormones and neurotransmitters.

Earlier investigations (Hook et al., 1985a) on the regulation of CPH showed that catalytically inactive and active forms exist. CPH activity was increased without changes in cellular levels of CPH immunoreactivity during elevated rates of enkephalin peptide biosynthesis in primary cultures of adrenal medullary chromaffin cells. Therefore, while the total number of carboxypeptidase enzyme molecules remained constant, there may be a conversion of existing enzyme molecules to a more active form. Other studies (Hook and Eiden, 1985) show that CPH is more active in mature chromaffin granules containing fully processed enkephalin peptides than in a mixed population of immature and mature granules containing partially and fully processed proenkephalin. These findings indicate that CPH may undergo a process of activation during secretory vesicle maturation.

Recent molecular cloning of rat CPH (Fricker et al., 1989; Rodriguez et al., 1989) showed that the enzyme is initially synthesized as a precursor, prepro-CPH, which requires processing and activation. In neurons, pro-CPH is presumably transported with prohormones in secretory vesicles from the neuronal peri-

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Figure 1. Rat HNS. Hypothalamic cell bodies located in the SON and PVN send axons through the ME and pituitary stalk, which terminate in the posterior pituitary (neural lobe) of the rat pituitary.

karya via axons to nerve terminals; this hypothesis, however, has not been tested. Studies of prohormone processing in the rat hypothalamo-neurohypophyseal system (HNS) (Fig. 1) have shown that secretory vesicle maturation and prohormone processing take place as vesicles are axonally transported from hypothalamic cell bodies of the supraoptic nucleus (SON) and paraventricular nucleus (PVN) to nerve terminals in the posterior pituitary (Gainer et al., 1977; Brownstein et al., 1980; Liston et al., 1984). The HNS should be an excellent system to determine if an inactive form of CPH may be activated during vesicle maturation and axonal transport. Therefore, in this study, we have analyzed the molecular forms of CPH present at several levels in the rat HNS using a specific, well-characterized anti-bovine CPH antibody (Hook et al., 1985b) in conjunction with enzyme activity assays.

The CPH antiserum has been used in immunocytochemical studies (Hook et al., 1985b) to visualize the discrete localization of CPH in neurons of the rat SON and PVN, where it is involved in the processing of provasopressin (Hook and Loh, 1984), prooxytocin (Kanmura and Chaiken, 1985), and proenkephalin (Hook and Loh, 1984). Further resolution by electron microscopic immunohistochemistry confirmed that CPH in these neurons is localized to secretory vesicles in cell body, dendrite, axon, and nerve terminals (Hook et al., 1985b). This antibody recognizes CPH from the rat, monkey, and cat (Hook et al., 1985b; Chesselet and Hook, 1988) indicating that CPH from several species share some structural similarities.

In this report, analysis of zymogen and active forms of CPH in the rat HNS suggest that a precursor form of CPH in the SON appears to be converted during axonal transport to catalytically active CPH, the major form of CPH present in nerve terminals of the posterior pituitary. These studies suggest that conversion of zymogen to the mature form of CPH may play a role in the control of peptide hormone biosynthesis. Furthermore, the maturation of secretory vesicles during axonal transport may include mechanisms for the activation of CPH and other prohormone-processing enzymes.

Materials and Methods

Tissue dissection. From freshly decapitated male Sprague-Dawley rats (250 gm, Zivic Miller), the median eminence (ME) and pituitary stalk were dissected together and taken as the axon region (ME-stalk). The SON was microdissected separately. The posterior pituitaries were separated from the anterior and intermediate lobes while tissues were kept moist. All tissues were dissected under an operating microscope and then immediately frozen on dry ice.

Stalk transected, sham-operated, and control (no surgery) male rats

were obtained from Zivic Miller. Thirteen days after surgery, the posterior pituitaries from 6 rats in each group were removed and frozen on dry ice. Degeneration of the pituitary stalk was confirmed by microscopic examination. Posterior pituitary tissue weight after stalk transection has been shown to be reduced to 50% of sham controls (Laszlo and DeWied, 1966), and protein content in this experiment was reduced to 25% of controls.

Preparation of homogenates, and soluble and membrane fractions. After tissue dissection, ME-stalks and posterior pituitaries from 10 rats were pooled and sonicated on ice in 100 μ l 0.1 M Na-acetate (pH, 5.7) for preparation of tissue homogenates. Soluble and membrane-associated fractions were prepared by centrifuging the homogenate in a Beckman airfuge for 10 min at 4°C. The supernatant was taken as the soluble fraction, and the pellet, which was washed 2 times in homogenate buffer (100 mM Na-acetate; pH, 5.7), was taken as the membrane-associated fraction. The membrane-associated fraction represents proteins "associated" with the membrane and integral membrane proteins. Protein content was determined by the method of Lowry (Lowry et al., 1951) with bovine serum albumin as standard.

Homogenates from bovine pituitary were prepared by dissecting anterior, intermediate, and neural lobes from fresh pituitaries (Biological Research and Development, Gaithersburg, MD), and homogenization was in 5 vol 0.1 M Na-acetate (pH, 5.7). Aliquots (8–10 μ g protein) were subjected to CPH immunoblot analysis.

Carboxypeptidase H activity. CPH activity was measured by following the conversion of 3 H-benzoyl-Phe-Ala-Arg (New England Nuclear) to 3 H-benzoyl-Phe-Ala, and CPH activity was calculated as the difference between CoCl₂-stimulated (3 mM) and guanidinopropylsuccinic acid (GPSA)- (1.2 μ M) inhibited activities, as previously described (Stack et al., 1984; Hook and LaGamma, 1987). Enzyme activities were quantitated in the linear range of the assay.

Carboxypeptidase H immunoreactivity. CPH immunoreactivity (IR) in homogenate samples was measured by radioimmunoassay using a specific CPH antiserum (Hook et al., 1985b). Homogenates were diluted 1:2 with a triton-containing buffer [final concentrations: 100 mM Na-phosphate (pH, 7.4), 0.5 trypsin inhibitor units/ml of Trasylol (Sigma), 0.1% bovine serum albumin, 0.01% merthiolate, 0.1% Triton X-100, and 10 mM EDTA] to solubilize the membrane-bound enzyme. After remaining on ice for 2 hr, the solubilized samples were centrifuged for 5 min in a Beckman microfuge at 4°C, and the supernatants were assayed for CPH IR as described previously (Hook et al., 1985b).

Immunoblots. Immunoblots were performed essentially as described previously (Hook et al., 1985b). Briefly, samples were subjected to 10% SDS-PAGE and electrophoretically transferred to nitrocellulose membranes (Schleicher and Schuell). Membranes were blocked with 10% fetal calf serum in PBS (pH, 7.4) for 2 hr. They were then incubated with anti-CPH antibody at a final dilution of 1:1000 in PBS (pH, 7.4), washed with 0.05% NP-40/PBS 3 times, incubated with goat anti-rabbit peroxidase-conjugated secondary antibody [10 μ l in 10 ml 10% fetal calf serum/PBS (pH, 7.4), Boehringer Mannheim], and washed 3 times with 0.05% NP-40/PBS. The peroxidase was visualized with 4-chloro-1-naphthol (Aldrich) as substrate. Selected lanes on the immunoblots were scanned with a Shimadzu laser densitometer.

p-Aminobenzoylarginine-Sepharose affinity chromatography. SON, ME-stalk, and neural-lobe tissues (20 rats for each region) were homogenized in 200 μ l buffer A, composed of 50 mM Na-acetate (pH, 6.0), 0.5 M NaCl, 1 mM PMSF, and 1% Triton X-100. After 1 freeze-thaw, and sitting on ice for 1 hr, 200 μ l buffer B [50 mM Na-acetate (pH, 6.0), 0.5 M NaCl, 1 mM PMSF, and 0.1% Triton X-100] was added to bring the total volume of the homogenate to 400 μ l. This sample was centrifuged at 4°C for 5 min in a Beckman microfuge to remove the pellet of insoluble material, which was resuspended in 200 μ l buffer B. To the resultant supernatant was added 100 μ l of a slurry of the affinity resin p-aminobenzoylarginine-Sepharose (a gift from Dr. T. H. Plummer, New York State Department of Health, Albany, NY) in buffer B. After rocking at 4°C for 45 min, the p-aminobenzoylarginine-Sepharose was pelleted by centrifugation for 5 min in the microfuge. The resultant supernatant was taken as the unbound fraction. The p-aminobenzoylarginine-Sepharose was washed 3 times with 400 μ l buffer B, and proteins were eluted with 200 μ l of 0.5 M arginine in buffer B. Bound proteins were collected by pelleting the p-aminobenzoylarginine-Sepharose and collecting the supernatant. Bound and unbound fractions were subjected to immunoblot analysis, and total CPH activity was determined in each fraction. The bound fraction was dialyzed against 20 mM Na-acetate (pH, 6.0) prior to measurement of CPH activity.

CNBr cleavage. Peptide maps, generated by cyanogen bromide (CNBr)

cleavage (Gross, 1967), of the 55- and 65-kDa forms of CPH were compared. The SON and posterior pituitary dissected from 40 rats were each homogenized in 500 μ l 25 mM Tris-HCl (pH, 7.5). The 55- and 65-kDa forms of CPH from each tissue homogenate was isolated by SDS-PAGE in tube gels (5 mm diameter, 10 cm length). These CPH species were eluted from tube gel slices with an Isco electroeluter. Aliquots of isolated 55- and 65-kDa CPH were subjected to CNBr cleavage (Gross, 1967) by incubation at room temperature in 70% formic acid and 100 μ g/ml CNBr for 20 hr. These conditions have been commonly used for CNBr digestion of numerous proteins (Gross, 1967). Samples were lyophilized, and cleavage products were analyzed by CPH immunoblots on 14% SDS-PAGE gels.

Results

Carboxypeptidase H in SON, ME-stalk, and posterior pituitary

The relative specific activities of CPH were assessed in rat ME-stalk (axon-enriched region) and posterior pituitary (nerve terminals) by determining the CPH activity:IR ratio in tissue homogenates (Fig. 2*a*). CPH activity was measured by following the conversion of 3 H-benzoyl-Phe-Ala-Arg to 3 H-benzoyl-Phe-Ala (Stack et al., 1984), and CPH IR was measured by radioimmunoassay (Hook et al., 1985b). The CPH activity:IR ratio serves as an index of the level of catalytic activity per unit number of enzyme molecules and allows differentiation between highly active and inactive populations of CPH. These ratios were 0.06 ± 0.004 and 0.30 ± 0.01 pmol ($\pm$ SEM) 3 H-benzoyl-Phe-Ala/min·ng IR CPH in the ME-stalk and posterior pituitary, respectively, showing that CPH molecules in the posterior pituitary were 5 times more active than those in ME-stalk. In addition, CPH activities (pmol 3 H-benzoyl-Phe-Ala/min· μ g protein) per total homogenate protein in the ME-stalk and posterior pituitary were 5- and 33-fold greater, respectively, than that in the SON (Fig. 2*b*).

To confirm that CPH in nerve terminals of the posterior pituitary is axonally transported from neurons of the SON and PVN, CPH activity was determined in the posterior pituitary after stalk transection (Fig. 2*c*). At 13 days after stalk transection, when axons and nerve terminals are maximally degenerated (Laszlo and DeWied, 1966), CPH activity in the posterior pituitary was reduced to 8% of that in the sham-operated controls. Disappearance of CPH activity from the nerve terminals after transecting their axonal route indicates that the source of CPH in the posterior pituitary is from the magnocellular hypothalamic neurons.

These results (Fig. 2, *a-c*) suggest that CPH enzyme molecules may be activated during axonal transport from the SON to nerve terminals of the posterior pituitary.

Multiple forms of CPH

The activation of CPH that we observed implies that inactive or partially active forms of the enzyme exist. To investigate these possibilities, the molecular forms of CPH present in tissue homogenates of the SON, ME-stalk, and posterior pituitary were analyzed by immunoblots with anti-CPH serum (Fig. 3*a*). Two CPH immunoreactive proteins of 65 and 55 kDa (apparent molecular weight) were present in different amounts in each of these regions. The primary CPH species in the posterior pituitary was the 55-kDa form, which corresponds to active CPH because it possesses the identical molecular weight as CPH purified from rat insulinoma (Davidson and Hutton, 1987). The ME-stalk and SON regions contained much higher levels of the 65-kDa species. In the SON, a doublet of 67 and 65 kDa was consistently observed.

Figure 2. CPH in rat HNS. *a*, Ratio of CPH activity to immunoreactivity in the ME-stalk (*Stalk*) and neural lobe (*NL*). $n = 3$; $p < 0.005$ (statistically significant) by Student's *t*-test. *b*, CPH activity in the SON, ME-stalk, and neural lobe is expressed as pmol benzoyl-Phe-Ala/min· μ g protein for each region. *c*, Total CPH activity in the neural lobe after stalk transection. $n = 3$ for control, and $n = 2$ for sham and transected groups; $p < 0.01$ (statistically significant compared to control or sham-operated controls) by Student's *t*-test. Vertical lines represent SEM.

The percentage of total CPH IR represented by the 55-kDa species was 81%, 23%, and 1%, in the posterior pituitary, ME-stalk, and SON, respectively, as determined from the peak areas of densitometric scans of the immunoblots (Fig. 3*b*). The percentage of total immunoreactive 65-kDa species in each region was 17%, 72%, and 98% for the posterior pituitary, ME-stalk, and SON, respectively. Therefore, it appears that the SON contains almost exclusively the 65-kDa form, while the ME-stalk contains a 3:1 ratio of 65- and 55-kDa species, and the posterior

Figure 3. Immunoblot of CPH in rat HNS. *a*, Immunoblot of CPH in the SON, ME-stalk (ST), and neural lobe (NL) tissue homogenates. For tissue homogenates (H), relative amounts of tissue applied to each lane were as follows: lane 1, neural lobe from 1 rat; lane 2, ME-stalk from 2 rats; lane 3, SON from 1 rat. For soluble (S) and membrane-associated (M) fractions of homogenates, lanes 4, 6, 9, and 10 represent tissue from 1 rat and lanes 5, 7, and 8 represent tissue from 2 rats. *b*, Relative levels of 65- and 55-kDa CPH in homogenates of SON, ME-stalk (Stalk), and neural lobe (NL). Laser densitometric scans were taken of lanes 1, 2, and 3 of *a*. *c*, Immunoblot of CPH in the rat anterior (lane 1), intermediate (lane 2), and neural lobe (lane 3) pituitary homogenates.

pituitary exhibits primarily the 55-kDa species. CPH immunoblots of PVN (cell bodies) of the rat HNS were similar to the SON (data not shown).

Cell fractionation experiments (Fig. 3*a*) showed that the 65-kDa form was primarily a soluble protein in the SON, while in the ME-stalk and posterior pituitary regions, it was associated with the membrane fraction. The 55-kDa form in the posterior pituitary was present in soluble and membrane-associated fractions.

The relative distribution of the 65- and 55-kDa species in the rat anterior and intermediate pituitary was also examined on immunoblots (Fig. 3*c*). The 55-kDa CPH found in the intermediate lobe appeared to be identical to that seen in neural lobe (posterior pituitary). However, the anterior lobe showed a doublet of 57- and 55-kDa apparent molecular weight. Almost no 65-kDa species were found in the 3 lobes of the rat pituitary.

Analysis of the molecular forms of CPH in the bovine pituitary (Fig. 4) showed that substantial amounts of a 64–65-kDa CPH exist in the anterior, intermediate, and neural lobes. The 64–65-kDa species was present in greater amounts than the

smaller, 55- and 52-kDa species. The anterior pituitary possessed both 55- and 52-kDa forms that presumably correspond to the previously purified membrane (Supattapone et al., 1984) and soluble (Fricker and Snyder, 1983) forms of CPH, respectively. Intermediate and neural lobes contained the 52-kDa CPH, but the 55-kDa form was not detected.

Analysis of active and inactive forms

To determine whether the 65- and/or 55-kDa species of CPH possess catalytic activity, the 2 forms from homogenates of rat SON, ME-stalk, and posterior pituitary were separated from one another by p-aminobenzoylarginine-Sepharose affinity chromatography (Fig. 5*a*), and their relative activities were measured. Immunoblots of the fractions show that the 65-kDa form does not bind to the p-aminobenzoylarginine-Sepharose and that the 55-kDa form binds and is eluted with arginine.

The unbound fraction, containing the 65-kDa species, showed virtually no enzyme activity, whereas the bound fraction, containing the 55-kDa species, had almost all of the CPH activity present in each region (Fig. 5*b*). All enzyme activity in the

Figure 4. Immunoblot of CPH in bovine pituitary. Homogenates of anterior (10 μ g protein, lane 1), intermediate (8 μ g protein, lane 2), and neural lobes (8 μ g protein, lane 3) of the bovine pituitary was subjected to CPH immunoblot as described in Materials and Methods.

unbound fraction of the posterior pituitary was bound after a second passage through p-aminobenzoylarginine-Sepharose. The binding of the 55-kDa CPH to p-aminobenzoylarginine-Sepharose is consistent with purification of 55-kDa rat CPH by this same affinity column (Davidson and Hutton, 1987). Purification of a single catalytically active, 55-kDa rat CPH (Davidson and Hutton, 1987) supports the proposal that the 65-kDa immunoreactive CPH is an inactive form of CPH.

Kinetic properties of the 55-kDa CPH from the SON, ME-stalk, and posterior pituitary were compared using enzyme isolated by affinity chromatography and, therefore, free from the 65-kDa form. The apparent K_m values (determined on Lineweaver-Burk plots) for the 55-kDa CPH in the SON, ME-stalk, and posterior pituitary were 1.2, 1.0, and 1.2 μ M benzoyl-Phe-Ala-Arg, respectively. In the presence of 1 mM CoCl_2 , CPH

activities in the SON, ME-stalk, and posterior pituitary were stimulated 2-fold. The similar K_m values and similar degree of elevation of activity by CoCl_2 for the enzyme in the SON, ME-stalk, and posterior pituitary demonstrates that the 55-kDa form of CPH is the same in each of the 3 regions.

The levels of total enzyme activity were compared with the amounts of the 65- or 55-kDa forms in the 3 regions (Fig. 6). Total CPH activity and levels of the 55-kDa CPH had a correlation coefficient of 0.997. However, levels of enzyme activity and levels of the 65-kDa form of CPH had a correlation coefficient of -0.964 . As vesicles are transported from the SON to nerve terminals of the posterior pituitary, the decrease in levels of inactive, 65-kDa CPH and the concurrent increase in relative levels of activity and active, 55-kDa CPH support the hypothesis that the 65-kDa form is converted to active, 55-kDa CPH during axonal transport in the rat HNS.

Peptide maps of the 65- and 55-kDa forms of CPH

Peptide maps of the 65- and 55-kDa forms of CPH were compared to provide further evidence for a precursor-product relationship between these CPH proteins. Peptide fragments generated by CNBr cleavage were analyzed by CPH immunoblots (Fig. 7). Characterization of the CPH antibody demonstrated that this polyclonal antibody recognizes multiple epitopes, because proteolysis of purified bovine CPH by *S. aureus* V8 protease produced fragments of 46, 38, 30, 27, 20, and 15 kDa that were all recognized by the CPH antiserum in immunoblots (data not shown). CNBr treatment, which selectively attacks methionine residues within proteins to result in cleavage of methionyl peptide bonds (Gross, 1967), resulted in the formation of 4- and 3-peptide fragments from the 65- and 55-kDa CPH species,

Figure 5. p-Aminobenzoylarginine affinity chromatography of 65-kDa and 55-kDa CPH. *a*, Immunoblot of CPH in homogenate (*H*), triton-insoluble (*Ins.*) and p-aminobenzoylarginine-Sepharose unbound (*U*) and bound (*B*) fractions of SON, ME-stalk (*St*), and neural lobe (*NL*). The amounts of homogenate, insoluble, and unbound fractions applied to the gel lanes represent tissue from 1.5 rats; bound fractions represent 2 rats. *b*, Total CPH activity (from 20 rats) in unbound (*U*) and bound (*B*) fractions from the SON, ME-stalk (*Stalk*) and neural lobe (*NL*).

Figure 6. Correlation of activity with 55-kDa or 65-kDa forms of CPH in SON, ME-stalk, and neural lobe. Total CPH activity (dark bars), ng immunoreactive 65-kDa CPH (gray bars), and ng immunoreactive 55-kDa CPH (light bars) were measured and calculated for each rat. Total CPH IR represents both the 65- and 55-kDa forms. The amounts of immunoreactive 65-kDa and 55-kDa CPH were calculated from peak areas of densitometric scans of CPH immunoblots (shown in Fig. 3*a,b*) in combination with RIA measurement of total CPH immunoreactivity for each region. The correlation coefficient for CPH activity and levels of 55-kDa CPH was 0.997; for 65-kDa CPH, -0.964 .

respectively, as detected by CPH immunoblots. If the 65- and 55-kDa species are related, a greater number of fragments should be generated from the 65-kDa than from the 55-kDa form; this finding was observed. CNBr treatment resulted in 2 bands at 35.5 and 29.0 kDa that were common to peptide maps of both the 65- and 55-kDa CPH proteins. These similar peptide fragments support the hypothesis that the 65- and 55-kDa proteins appear to be structurally related.

Discussion

Peptide hormones are synthesized as large precursors that require the action of endopeptidases and CPH to generate the smaller, biologically active peptides. Little is known, however, about the regulation of these protease activities during prohormone processing. In this study, we have examined the molecular forms of CPH in the rat HNS, which allows biochemical analysis of anatomically distinct regions containing predominantly cell-body, axon, and nerve-terminal regions of a defined population of peptidergic neurons. We demonstrate that the SON, a cell-body-enriched region, possesses an inactive, 65-kDa CPH zymogen that is presumably processed during axonal transport to the active 55-kDa CPH, the major form present in nerve terminals of the posterior pituitary.

The predominant form of CPH in the SON was found to be a 65-kDa species of CPH. A minor 67-kDa form of CPH in the SON may represent prepro-CPH or an intermediate in the processing of prepro-CPH. The cDNA for rat prepro-CPH predicts a molecular weight of 53,315 Da for this precursor; therefore, the larger 67- and 65-kDa forms detected in the SON probably represent pro-CPH that has undergone posttranslational modifications, such as glycosylation. The major 65-kDa CPH intermediate in the SON represented 98% of the total CPH IR, whereas the active, 55-kDa form represented only 2% of the total CPH IR in this region. The 65-kDa CPH is inactive and does not bind to p-aminobenzoylarginine-Sepharose.

The 65-kDa CPH appears to be processed during axonal transport, as demonstrated by the presence of both 65- and 55-kDa forms of CPH in the ME-stalk, which contains primarily axons. Processing during axonal transport results in the presence of

Figure 7. Peptide maps of 65- and 55-kDa forms of CPH. The 65- and 55-kDa forms were isolated from the SON and posterior pituitary (described in Materials and Methods) and subjected to CNBr cleavage. The resultant peptide fragments were separated on 14% SDS-PAGE gels and transferred to nitrocellulose membranes for CPH immunoblots shown in *a*: lane A, 55-kDa CPH, CNBr-treated; B, 55-kDa CPH, untreated; C, 65-kDa CPH, CNBr-treated; and D, 65-kDa CPH, untreated. Peptide fragments 1 and 2 (35.5 and 29.0 kDa, respectively) were common to peptide maps of both the 55- and 65-kDa CPH species. *b* and *c* show densitometric scans of lanes A and C (from *a*), respectively; these scans confirm the identical electrophoretic mobilities of peptides 1 and 2.

active 55-kDa CPH as the major form (80% of total CPH IR) present in nerve terminals of the posterior pituitary. The 55-kDa form of CPH binds to p-aminobenzoylarginine-Sepharose, an affinity resin used in purifications of CPH. The correlation between CPH activity and levels of 55-kDa CPH, and the inverse correlation between CPH activity and levels of 65-kDa CPH in cell bodies, axons, and nerve-terminal regions of the

HNS support the hypothesis that the 65-kDa protein appears to be converted to mature 55-kDa CPH during axonal transport.

The presence of some peptidergic inputs terminating in the SON and ME-stalk should be considered in the interpretation of our data implicating processing of pro-CPH during axonal transport. In the rat SON, previous immunohistochemical studies (Hook et al., 1985b) show that CPH is localized to the cytoplasm and is absent in nuclei of the neuronal perikarya. If the observed CPH IR was from peptidergic inputs terminating on SON neurons, the nuclear area of the cells would show immunostaining, representing terminals on plasma membranes above the nuclei. However, the lack of nuclear and presence of cytoplasmic immunostaining indicates that the majority of 65-kDa CPH in the SON is probably localized to cell bodies. In nerve terminals of the posterior pituitary, the 55-kDa CPH was predominant compared to the 65-kDa form; this finding may be representative of many neurons, suggesting that peptidergic terminals in the SON may contain small amounts of 55-kDa CPH detected in this region. In addition, in the ME-stalk, the presence of some peptidergic nerve terminals in this region may contribute to the content of 55-kDa CPH; therefore, the 65-kDa:55-kDa CPH ratio within axons of the ME-stalk may be higher than observed in homogenates of this region. Further investigation of the subcellular localization will require electron microscopic immunohistochemical studies with proCPH- and CPH-specific antibodies.

Peptide maps of the 65- and 55-kDa forms of CPH provided further evidence for a precursor-product relationship between these 2 proteins. After cleavage by CNBr at methionyl peptide bonds, 2 species of 29.0 and 35.5 kDa were common to peptide maps of both the 65- and 55-kDa forms of CPH. The apparent M_r values of the peptide fragments generated from the 65- and 55-kDa proteins are consistent with predicted molecular weights of CNBr-cleavage products resulting from the partial digestion of precursor and mature forms of CPH, as described below.

Based on the amino acid sequence of rat CPH deduced from its cDNA (Fricker et al., 1989; Rodriguez et al., 1989), production of the 29.0-kDa CNBr fragment would be consistent with cleavage of the 55-kDa CPH (residue 1-434, numbered according to Rodriguez et al., 1989) at Met-250 to result in a fragment of 1-250 with a calculated M_r of 28,134 Da (unglycosylated). Cleavage of the 65-kDa at Met(-6) and Met-250 would generate a fragment with an M_r of 28,904 Da (calculated without glycosylation), which would not differ in apparent electrophoretic mobility (on SDS-PAGE) from the 28,134-Da fragment generated from 55-kDa CPH. Because it is known that 55-kDa CPH is glycosylated (Davidson and Hutton, 1987), glycosylation of these fragments at Asn-97 could increase their apparent M_r to 29.0 kDa.

Production of the 35.5-kDa fragment would be consistent with cleavage of 55-kDa CPH at Met-284 to generate fragment 1-284 of 31,717 Da and would also be consistent with cleavage at Met-112 and Met-422 to produce fragment 113-422 of 34,090 Da. Similarly, cleavage of 65-kDa CPH at Met(-6) and Met-284 or at Met-112 and Met-422 would generate fragments of 32,487 and 34,090 Da, respectively. These calculated M_r values are for unglycosylated fragments. Glycosylation at Asn-97 or Asn-348 could increase the sizes of these fragments to 35.5 kDa. While mature rat CPH of 55 kDa is deglycosylated to 50 kDa (Davidson and Hutton, 1987), the degree of glycosylation at each Asn-97 or Asn-348 site is unknown.

Most of the inactive, 65-kDa CPH in the SON was present as a soluble protein, yet in the ME-stalk, it was primarily a membrane-associated protein. As the CPH precursor is routed through the rough endoplasmic reticulum and Golgi apparatus, it may be located within the soluble compartments of these organelles, and when it is packaged into secretory vesicles, it may be associated with the vesicle membrane. The 55-kDa form of CPH in the posterior pituitary appears to be soluble and membrane associated.

It is now apparent that previous immunocytochemical studies of CPH localization in the rat SON and PVN (Hook et al., 1985b) detected the presence of both the inactive 65-kDa and active 55-kDa species of CPH. Another study (Chesselet and Hook, 1988) of CPH localization by immunocytochemistry most likely represents detection of both 65- and 55-kDa species. It is not known, however, if the visualization of CPH in the SON and PVN by *in vitro* autoradiography with ^3H -GEMSA (Lynch et al., 1984, 1986), a potent, active site-directed inhibitor of CPH (McKay and Plummer, 1978), represents labeling of the 65- in addition to the 55-kDa form of CPH. Since *in vitro* autoradiography detects primarily membrane-bound components because of the washing procedure that results in removal of soluble proteins and since the 65-kDa CPH species in this study was present in a soluble fraction of the SON homogenate, it is likely that ^3H -GEMSA autoradiography of CPH in cell-body-enriched areas of the SON and PVN preferentially detected the 55-kDa CPH.

The results of this study suggest that the extent of zymogen conversion to active CPH could vary from tissue to tissue. Evidence for tissue differences in the relative amounts of the molecular forms of CPH was demonstrated in the rat and bovine pituitary. The 55-kDa form of CPH was the major form present in the rat anterior and intermediate pituitary. The rat anterior lobe also showed a 57-kDa form that was not present in intermediate or posterior lobes. The 65-kDa form of CPH was virtually absent in the 3 lobes of the rat pituitary, whereas it was the major species present in the SON.

In contrast to the rat, the 3 lobes of the bovine pituitary showed substantial amounts of a 64-65-kDa form of CPH that was present in higher quantities than the smaller, 55- and 52-kDa forms of CPH. The 55- and 52-kDa forms detected in this study appear to correspond to the previously described (Fricker and Snyder, 1983; Supattapone et al., 1984) membrane (apparent M_r , 52.5 kDa) and soluble (apparent M_r , 50.0 kDa) forms of bovine CPH, respectively. The small differences in apparent M_r of bovine CPH (55 and 52 kDa) detected in this study compared to reported purified CPH (52.5 and 50 kDa) can be due to our observation that the apparent molecular weights of proteins on SDS-PAGE can vary by 2-5 kDa from gel to gel. The anterior lobe contained both the 55- and 52-kDa forms of CPH. The intermediate and neural lobes possessed the 52-kDa form but not the 55-kDa form. These results suggest that the processing of pro-CPH is tissue specific.

Tissue-specific processing of pro-CPH implies that the relative levels of peptides extended at the COOH terminus with basic amino acid residues may vary from one cell to another. Evidence for differences in the extent of prohormone processing at the carboxypeptidase step has been shown (Altstein and Gainer, 1988) in developmental studies of prooxytocin and vasopressin processing in the rat HNS. After removal of the COOH-terminal basic residues by CPH, the products oxytocin-Gly and vasopressin-Gly undergo COOH-terminal amidation to form

the final physiologically active hormones. At one stage of embryogenesis, oxytocin-Gly¹⁰Lys¹¹Arg¹² was detected, but the completely processed (amidated) form was absent; however, fully processed vasopressin was present. Oxytocin and vasopressin precursors are synthesized in separate neurons. These studies show that there may be a block at the step of CPH processing in oxytocin cells but not in vasopressin cells. Whether or not this block may be due to incomplete processing of the CPH zymogen itself is unknown and requires further study.

What mechanism might control the conversion of the CPH zymogen to catalytically active CPH? Amino acid sequence analysis of the rat CPH cDNA shows that the COOH terminus of the prosegment within pro-CPH contains 5 arginine residues adjacent to the NH₂ terminus of CPH (Fricker et al., 1989; Rodriguez et al., 1989). These multiple basic residues are present at the COOH terminus of many prosegments within propeptides (Eipper et al., 1987; Furie and Furie, 1988) and are indicative of processing by endoproteolytic enzymes. Therefore, the hypothesis that a protease converts pro-CPH to the 55-kDa form suggests that zymogen activation could be an additional level of regulation in peptide hormone biosynthesis. It will be important to understand how the activities of multiple processing enzymes are coordinately regulated to result in complete pro-hormone processing.

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