Volume 238, number 2, 338–342 FEB 06396 October 1988

Identification of zymogen and mature forms of human carboxypeptidase H

A processing enzyme for the synthesis of peptide hormones

Vivian Y.H. Hook and Hans-Urs Affolter[+]

Department of Biochemistry, Uniformed Services University of the Health Sciences, Bethesda, MD 28014, USA and *[+]Institute for Brain Research, University of Zurich, Switzerland*

Received 23 August 1988

Carboxypeptidase H (CPH) is one of several processing enzymes required for the conversion of peptide hormone precursors into their smaller active forms. In this study, high levels of CPH activity was found in a liver metastasis of a human ileal carcinoid which expresses β-preprotachykinin mRNA and the tachykinin neuropeptides, substance P and substance K. This human CPH showed properties of a zinc-metallopeptidase that is structurally similar to bovine and rat CPH. Immunoblots of the human ileal carcinoma with anti-bovine CPH showed that CPH activity is represented by two proteins of apparent molecular masses 57 and 55 kDa. Cell-free translation of poly(A)$^+$ RNA followed by immunoprecipitation with anti-bovine CPH showed that human CPH mRNA encodes a precursor protein of apparent molecular mass 75 kDa. These data demonstrate that human CPH is synthesized as a zymogen, prepro-CPH, which must be cleaved to form catalytically active CPH.

Prohormone processing enzyme; Limited proteolysis; Substance P; (Human ileal carcinoid)

1. INTRODUCTION

Peptide hormones and peptide neurotransmitters such as adrenocorticotropic hormone (ACTH) [1], enkephalins [2,3], substance P [4], etc. are synthesized as large protein precursors of approx. 10–30 kDa which must be specifically cleaved to form the smaller active peptides. Carboxypeptidase H (H = hormone [5]; previously referred to as 'carboxypeptidase B-like' [6–8], or 'enkephalin convertase' [9–11]), is one of several proteases required for peptide precursor processing. Following the initial cleavage of the precursor at pairs of basic residues (Lys-Arg, and less commonly Arg-Arg, Lys-Lys, or Arg-Lys sequences) by trypsin-like endopeptidase(s) [12], carboxypeptidase H (CPH) then removes the COOH-terminal Lys and/or Arg extensions on the peptide. Although CPH resembles pancreatic carboxypeptidase B (CPB) in its specificity for basic amino acid residues, differences in the molecular properties of CPH compared to CPB [6–11], and lack of cross-reactivity of anti-CPH antiserum with CPB [8] have shown that CPH is a unique carboxypeptidase that is involved in the production of a variety of peptide hormones and neurotransmitters.

The peptide precursors lack biological activity and it is only the smaller mature peptides which possess the ability to function as the physiologic mediators of cell-cell communication. Because it is at the step of precursor processing that inactive forms are converted to potent active peptides, the processing enzymes should be important for the control of peptide hormone production. Defective processing enzymes could lead to abnormal hormone production in endocrine or neurological

Correspondence address: V. Hook, Dept of Biochemistry, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Rd, Bethesda, MD 20814, USA

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50 © 1988 Federation of European Biochemical Societies

diseases. However, determination of the role of these processing enzymes in human physiology requires identification of the human isozymes that convert the peptide precursors to the small hormones.

A liver metastasis of a human ileal carcinoid has been found to contain large amounts of the neuropeptide substance P [13]. It contains corresponding high levels of β-preprotachykinin mRNA [14] which encodes a 15 kDa precursor that must be cleaved to form substance P and substance K (neurokinin A) which are peptides of 11 and 10 amino acid residues, respectively. The pairs of dibasic amino acid sequences within the human β-preprotachykinin precursor [15] predict that CPH is required for processing and, thus, should be present in this tumor. In this study, we show that the human ileal carcinoid tumor contains high levels of CPH activity that possesses properties similar to bovine and rat CPH. Furthermore, analysis of the molecular forms of CPH in this tumor shows that CPH itself is synthesized as a zymogen that must be converted to the smaller active enzyme.

2. MATERIALS AND METHODS

2.1. *Tissue homogenates*

Human ileal carcinoid tumor, derived from a liver metastasis, was obtained from Dr F. Sundler (University of Lund, Sweden). The tissue had been stored postmortem at -80°C for several years. The carcinoid tumor was homogenized in 0.1 M sodium acetate (pH 5.7). Fresh bovine adrenal medulla (Biological Research and Delivery Development Services, Mt. Airy, MD) homogenate was prepared similarly. Samples were assayed for CPH activity and subjected to immunoblot analysis. Protein content in homogenates was determined according to Lowry et al. [16] with bovine serum albumin as standard.

2.2. *CPH assay*

CPH activity in tissue homogenates was assayed by following the conversion of [^{3}H]benzoyl-Phe-Ala-Arg to [^{3}H]benzoyl-Phe-Ala using a modification [17] of the method of Stack et al. [18]. This procedure measures CPH activity by taking advantage of its characteristic simulation by $CoCl_2$ and inhibition by guanidinopropylsuccinic acid (GPSA), a zinc-metalloprotease inhibitor [19]. Activity was measured in the presence of [Co^{2+}] or [Co^{2+} + GPSA], CPH activity being taken as the difference. The reaction mixture contained, in a final volume of 60 μl, 0.1 M sodium acetate (pH 5.7), 100000 cpm [^{3}H]benzoyl-Phe-Ala-Arg (30 Ci/mmol, Dupont-New England Nuclear, Boston, MA), 3 mM Co^{2+} and/or 1.2 μM GPSA (a gift from Dr Thomas Plummer, State of New York Dept of Health, Albany, NY). After vortex-mixing and being left to stand on ice for 30 min, samples were incubated at 37°C for 10 min, the reaction subsequently being arrested by the addition of 50 μl of 1 N HCl in ice. The product [^{3}H]benzoyl-Phe-Ala was extracted with 1.0 ml $CHCl_3$ and aliquots counted in a scintillation counter.

2.3. *Immunoblotting*

Immunoblot analysis of tissue homogenate was performed as in [8]. Homogenate samples were heated to 100°C with SDS-PAGE sample buffer (final concentrations: 5 mM Tris-glycine, 3.3% β-mercaptoethanol, 10% glycerol, 2% SDS). Samples were electrophoresed on 10% SDS-PAGE gels, transferred electrophoretically to nitrocellulose (0.45 μm, Schleicher and Schuell, Keene, NH), and probed with rabbit anti-bovine CPH (lot no. A(2); final dilution 1:500). Rabbit antibody-CPH complexes were visualized with peroxidase-linked goat anti-rabbit immunoglobulins (Boehringer Mannheim, Indianapolis, IN).

2.4. *Isolation of poly(A)$^+$ RNA and cell-free translation*

Frozen tumor tissue was pulverized in liquid N_2 and RNA isolated by homogenization in 5.5 M guanidine thiocyanate, 2.5 mM EDTA, 0.5% sarcosyl, 0.22 M β-mercaptoethanol, and 20 mM Tris-HCl (pH 7.4) [20]. The slurry was passed three times through a 23-gauge needle and layered on a 1.5 ml CsCl-trifluoroacetic acid (Pharmacia, Piscataway, NJ) cushion (density: 1.51 with 250 mM EDTA). After centrifugation in a Beckman SW 50.1 rotor at 36000 rpm for 16–18 h, the pelleted RNA was dissolved in 0.3 ml of 0.2% SDS, heated to 58°C for 5 min and precipitated with 3 vols ethanol in the presence of 0.4 M sodium acetate (pH 5.5). Poly(A)$^+$-enriched RNA was selected using Hybond-mAP (Amersham) according to the supplier's protocol. Aliquots of poly(A)$^+$ RNA (1–5 μg) were translated with [^{35}S]methionine in a rabbit reticulocyte cell-free translation system (New England Nuclear).

2.5. *Immunoprecipitation*

Immunoprecipitations were carried out with (0.5–2) $\times$ 10^6 cpm ^{35}S-labeled proteins as determined by trichloroacetic acid precipitation. Translation reaction mixtures (25–100 μl) were diluted to 300 μl to give final buffer concentrations of 0.1 M Tris-HCl (pH 7.4) with 2.6×10^{-4} M PMSF, 1×10^{-5} M leupeptin, and 5×10^{-3} M iodoacetamide as protease inhibitors. ^{35}S-proteins were immunoprecipitated twice with normal rabbit serum (nrs) and then with rabbit anti-CPH (lot A5) as follows.

5 μl nrs was added to each 300 μl sample. Following incubation at 4°C for 2 h with rocking, 30 μl protein A-Sepharose (Pharmacia) was added and samples were incubated for an additional hour at 4°C with rocking. Antigen-antibody-protein A-Sepharose complexes were pelleted by centrifugation in a microfuge for 5 min. The supernatant was again immunoprecipitated with 5 μl nrs, followed by 5 μl rabbit anti-bovine CPH. For CPH absorption controls, anti-CPH was preincubated with purified soluble form of bovine pituitary CPH, purified according to Fricker and Snyder [10] (the *p*-aminobenzoylarginine-Sepharose 4B affinity column was a gift from Dr T.H. Plummer, New York State Dept of Health) before addition of these components to ^{35}S-proteins for immunoprecipitation.

After immunoprecipitation, protein A-Sepharose pellets were washed with 500 μl NET buffer and 500 μl NET/NaCl (twice) [NET: 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM

EDTA, 0.5% NP-40; NET/NaCl: NET with 1.0 M NaCl]. ^{35}S-proteins were liberated from protein A-Sepharose by boiling in 25 μl sample buffer (final concentrations: 1.25 mM Tris-glycine, 1% SDS, 2.5% β-mercaptoethanol, 5% glycerol). The supernatant was collected by pelleting the protein A-Sepharose, and subsequent analysis by 7.5% SDS-PAGE. Autoradiographs were scanned with a Shimadzu laser densitometer.

3. RESULTS

3.1. *CPH activity*

The presence of high levels of β-preprotachykinin mRNA [14] and substance P peptide [13] in the human ileal carcinoid suggests that the presence of CPH activity is necessary for processing of β-preprotachykinin. The carcinoid displayed CPH activity which removed the COOH-terminal arginine from the substrate [^{3}H]benzoyl-Phe-Ala-Arg, yielding the product [^{3}H]benzoyl-Phe-Ala (fig.1A). The activity was stimulated 1.5-fold by 3 mM Co^{2+} under assay conditions of linear product formation which corresponded to 20–25% conversion of substrate to product. This is represented by assay of CPH present in less than 1 μg homogenate protein (fig.1). In addition, human CPH was inhibited by 1.2 μM GPSA, a zinc-metallopeptidase inhibitor. The difference between Co^{2+}-stimulated and GPSA-inhibited activity represents CPH (fig.1B).

CPH specific activity in the carcinoid was compared with that in several other neuroendocrine

Table 1

CPH specific activity in tissue homogenates

Tissue	CPH specific activity (pmol [^{3}H]benzoyl-Phe-Ala/min per μg protein)
Human carcinoid	0.13
Human rhabdomyosarcoma	0
Bovine adrenal medulla	0.026
Rat adrenal medulla	0.026
Rat posterior pituitary	0.71

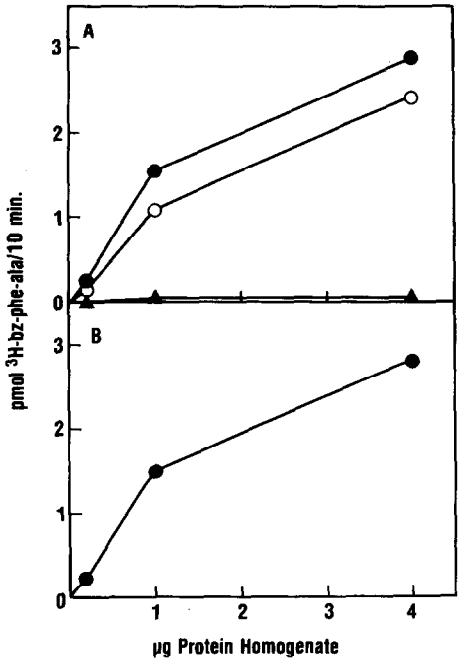

Fig.1. CPH activity in human ileal carcinoma. (A) CPH activity assayed in tissue homogenates: (○) no additions, (●) with 3 mM Co^{2+}, or (▲) with 3 mM Co^{2+} plus 1.2 μM GPSA. (B) Difference between Co^{2+}-stimulated and GPSA-inhibited (in the presence of Co^{2+}) CPH activities.

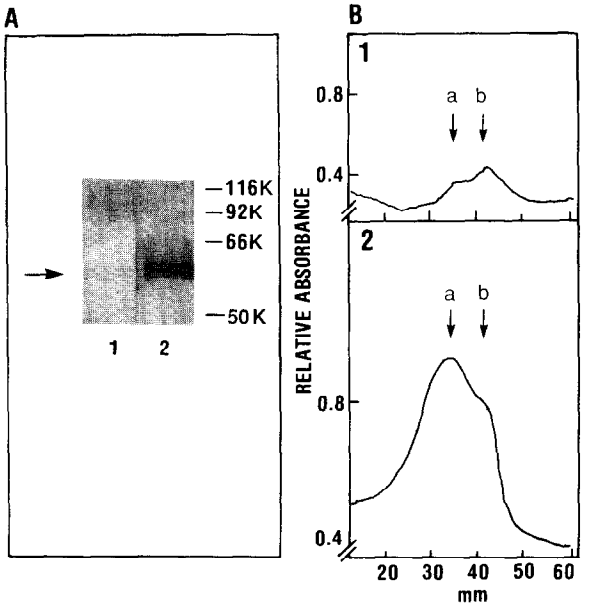

Fig.2. Immunoblot of CPH in human ileal carcinoid and bovine adrenal medulla. (A) Immunoblots of human ileal carcinoid (lane 1) and bovine adrenal medulla (lane 2) utilized 16 and 20 μg homogenate protein, respectively. The arrow denotes the position of the doublet detected by CPH antisera. (B) Densitometric scanning of (1) human ileal carcinoid and (2) bovine adrenal medulla immunoblots from (A). Peaks a and b (indicated by arrows) correspond to proteins of 57 and 55 kDa apparent molecular mass.

tissues that synthesize enkephalins (adrenal medulla), and vasopressin and oxytocin (posterior pituitary) (table 1). The human carcinoid showed 5-fold greater CPH specific activity than that in adrenal medulla of bovine or rat. CPH activity in the human carcinoid, however, was not as large as that in rat posterior pituitary. In contrast, human rhabdomyosarcoma (from pelvic muscle), a tissue which contains no peptide hormone, showed no CPH activity.

3.2. *Immunoblot analysis*

Characterization of CPH immunoreactive protein in human ileal carcinoma was accomplished by immunoblot analysis of tissue homogenate using a specific rabbit anti-bovine CPH serum that has been previously characterized [8]. The human ileal carcinoid, like bovine adrenal medulla, possessed two immunoreactive protein bands of apparent molecular masses 57 and 55 kDa (fig.2). When similar amounts of the two tissues (16–20 μg protein) were analyzed, the human ileal carcinoid tissue showed less CPH immunoreactivity than bovine adrenal medulla, as demonstrated by the lower intensity of CPH immunostaining.

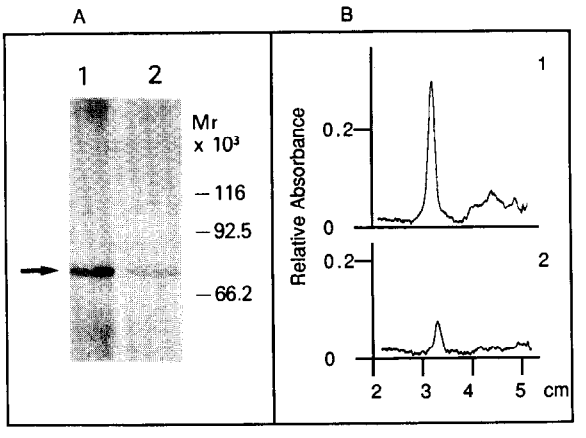

Fig.3. Cell-free translation of poly(A)$^+$ RNA and immunoprecipitation with anti-CPH. (A) Human ileal carcinoma mRNA was translated in a rabbit reticulocyte cell-free translation system and ^{35}S-labeled proteins were immunoprecipitated with anti-CPH serum in the absence (lane 1) and presence (lane 2) of partially purified bovine pituitary CPH. Immunoprecipitates were analyzed on 7.5% SDS-PAGE and autoradiographs are shown. The arrow indicates a protein of 75 kDa apparent molecular mass. (B) Densitometric scanning of lanes 1,2 from (A). These scans show that detection of the 75 kDa protein by the CPH antiserum is specific, since antiserum binding to this protein is reduced (scan of lane 2) when unlabeled CPH is included in the incubation mixture.

3.3. *Identification of zymogen form of CPH*

To compare the primary translation product of CPH encoded by its mRNA with the mature forms of CPH (apparent molecular mass: 55 and 57 kDa), cell-free translation experiments were performed. Poly(A)$^+$ RNA isolated from the human ileal carcinoid was translated with [^{35}S]methionine in a rabbit reticulocyte cell-free translation system, radiolabeled proteins being immunoprecipitated with anti-bovine CPH serum. Analysis of the immunoprecipitates by SDS-PAGE and autoradiography revealed that CPH is initially synthesized as a precursor protein of apparent molecular mass 75 kDa.

4. DISCUSSION

Previous investigations have characterized CPH in bovine and rat species. CPH has not been identified in human tissues. Here, we have demonstrated that a human carcinoid contains high levels of CPH activity, one of several processing enzymes required for conversion of the β-preprotachykinin precursor to the smaller active neuropeptides, substance P and substance K, that are present in this tissue. Furthermore, analysis of the molecular forms of CPH shows that it is first synthesized as a zymogen that must be cleaved to form mature active CPH.

The carcinoid contains levels of CPH activity which are comparable to those of other neuropeptide-synthesizing tissues. It contains 5-fold greater CPH specific activity than rat or bovine adrenal medulla which possess considerable amounts of proenkephalin peptides. On the other hand, it contains less CPH activity than rat posterior pituitary where high levels of CPH activity are present for the production of vasopressin, oxytocin, and enkephalin peptides [12].

Human CPH was stimulated by Co^{2+}, and the Co^{2+}-stimulated activity was completely inhibited by GPSA, a zinc-metallopeptidase inhibitor [19]. These data show that human CPH, like the bovine and rat forms, is a zinc-metallopeptidase.

The presence of CPH immunoreactivity in human tissue, as detected with an anti-bovine CPH antibody, suggests that human CPH shares struc-

tural similarities and some homologous amino acid sequences with bovine CPH. Using anti-bovine CPH antibody, CPH immunoreactivity has also been reported in rat pituitary [8] and in striatum of cats and monkeys [22]. Thus, CPH in several species appears to be a structurally similar enzyme protein.

Comparison of CPH catalytic activity and immunoreactivity in equivalent amounts of tissue homogenates shows that the carcinoid possesses levels of CPH specific activity and immunoreactivity that are 5- and approx. 0.25-fold of the corresponding values observed in bovine adrenal medulla. These data suggest that CPH in the carcinoid possesses greater catalytic activity per unit number of enzyme molecules than that in bovine adrenal medulla. This conclusion would be valid if the CPH antiserum has identical affinity for human and bovine isozymes. Alternatively, if human and bovine CPH possess some structural differences, the antiserum may have different affinities for the two forms. In this case, conclusions concerning the levels of CPH protein in the two species must await the determination of human and bovine CPH structures.

The most striking result obtained in this study is that mRNA isolated from the carcinoid encodes a protein of apparent molecular mass 75 kDa. Immunoblots showed that CPH activity in this tumor is represented by proteins of apparent molecular masses 57 and 55 kDa, which presumably correspond to the membrane-bound and soluble forms of CPH that have been identified in bovine pituitary and adrenal medulla [10,11]. These data lead to the conclusion that CPH is synthesized as a 75 kDa zymogen, prepro-CPH, that must undergo post-translational modification to form the mature active CPH.

The processing of prepro-CPH should require several steps. Secretory proteins such as CPH commonly contain an NH_2-terminal hydrophobic leader sequence of 15–30 amino acids which is removed by a signal peptidase at the rough endoplasmic reticulum [23]. The resultant pro-CPH must then be glycosylated and cleaved by (a) specific protease(s) to form the mature glycoprotein. Regulation of the steps involved in the post-translational modification of prepro-CPH can potentially play a role in the control of CPH activity and peptide hormone synthesis.

Acknowledgements: This work was supported by a grant from the National Institute of Drug Abuse (to V.H.). The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Department of Defense or the Uniformed Services University of the Health Sciences.

REFERENCES

[1] Nakanishi, S., Inoue, A., Kita, T., Nakamura, M., Chang, A.C.Y., Cohen, S.N. and Numa, S. (1979) Nature 278, 423–427.
[2] Gubler, U., Seeburg, P., Hoffman, B.J., Gage, L.P. and Udenfriend, S. (1982) Nature 295, 206–208.
[3] Noda, M., Furutani, Y., Takahashi, H., Toyosato, M., Hirose, T., Inayama, S. and Nakanishi, S. (1982) Nature 295, 202–206.
[4] Nawa, H., Kotani, H. and Nakanishi, S. (1984) Nature 312, 729–734.
[5] Webb, E.C. (1986) Eur. J. Biochem. 157, 16.
[6] Hook, V.Y.H., Eiden, L.E. and Brownstein, M.J. (1982) Nature 295, 341–342.
[7] Hook, V.Y.H. and Loh, P.Y. (1984) Proc. Natl. Acad. Sci. USA 81, 2776–2780.
[8] Hook, V.Y.H., Mezey, E., Fricker, L.D., Pruss, R.M., Siegel, R.E. and Brownstein, M.J. (1985) Proc. Natl. Acad. Sci. USA 82, 4745–4749.
[9] Fricker, L.D. and Snyder, S.H. (1982) Proc. Natl. Acad. Sci. USA 79, 3886–3890.
[10] Fricker, L.D. and Snyder, S.H. (1983) J. Biol. Chem. 258, 10950–10955.
[11] Supattapone, S., Fricker, L.D. and Snyder, S.H. (1984) J. Neurochem. 42, 1017–1023.
[12] Gainer, H., Russell, J.T. and Loh, Y.P. (1985) Prog. Neuroendocrinol. 40, 171–184.
[13] Alumets, J., Hakanson, R., Ingemansson, S. and Sundler, F. (1977) Histochemistry 52, 217–222.
[14] Affolter, H.U., unpublished.
[15] Harmar, A.J., Armstrong, A., Pascall, J.C., Chapman, K., Rossi, R., Curtis, A., Going, J., Edwards, C.R.W. and Fink, G. (1986) FEBS Lett. 208, 67–72.
[16] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[17] Hook, V.Y.H. and LaGamma, E.F. (1987) J. Biol. Chem. 262, 12583–12588.
[18] Stack, G., Fricker, L.D. and Snyder, S.H. (1984) Life Sci. 34, 113–121.
[19] McKay, T.J., Phelan, A.W. and Plummer, T.H. (1979) Arch. Biochem. Biophys. 197, 487–492.
[20] Chirgwin, J.M., Przybyla, A., MacDonald, R. and Rutter, W. (1979) Biochemistry 18, 5294–5299.
[21] Hook, V.Y.H., Eiden, L.E. and Pruss, R.M. (1985) J. Biol. Chem. 260, 5991–5997.
[22] Chesselet, M.-F. and Hook, V.Y.H. (1988) Regul. Peptides 20, 151–159.
[23] Evans, E.A., Gilmore, R. and Blobel, G. (1986) Proc. Natl. Acad. Sci. USA 83, 581–585.