Identification of *N*- and *C*-terminal corticotropin peptides in the M_r 80 000 form of neurophysin

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The ^{125}I-labeled M_r 80 000 form of neurophysin has been purified from bovine neurohypophysi. Tryptic digests of this species were analyzed, prior to or after treatment with carboxypeptidase B, by high-pressure liquid chromatography followed by isoelectric focusing and the fragments compared with those generated by a similar treatment of reference bovine 1–39 adrenocorticotropin. The ACTH peptides 22–39 and 1–8, as well as the 1–7 derivative of the latter were identified by those two independent criteria. This provides chemical evidence supporting the hypothesis [8] that high M_r neurophysin may contain the sequence of ACTH.

Corticotropin *Neurophysin* *Neuropeptide precursor*
High-performance liquid chromatography *Tryptic peptide mapping* *Hypothalamus*

1. INTRODUCTION

A body of evidence now indicates that both neurophysin and Arg^8-vasopressin are synthesized in hypothalamic neurons via an M_r 17 000–20 000 common precursor [1–5]. Moreover, the sequence of a cloned cDNA has been shown to code for a gene product of M_r 17 300 containing both neurophysin and vasopressin [5]. However, analysis of extracts of bovine hypothalamus or neurohypophysis [3,6] as well as of lysates made from neurosecretory granules [7] from the same organ, indicate that they contain in addition to the M_r 17 000–20 000 molecules, species of M_r 80 000 bearing both neurophysin and vasopressin antigenic determinants and resistant both to denaturation and to disulfide-bridge reduction [6]. Tryptic peptide analysis of a fragment derived from this molecule established sequence homologies with neurophysin [6]. This large species indeed cross-reacts both with anti-corticotropin and anti-β-endorphin antibodies and generates biologically active corticotropin by proteolysis [8]. Therefore it might include in its structure the sequences of these neuropeptides; in [8] it was proposed as a new example of pluripotent pro-hormone.

Here we show that this type of high M_r neurophysin called coenophorin [8] (from the Greek κοινος = common and φερω = I bear) indeed possesses sequences stretches homologous to the N- and C-terminal sequences of ACTH.

Abbreviations: SDS, sodium dodecyl sulfate; CPB, carboxypeptidase B; DFP, diisopropylfluorophosphate; ACTH, adrenocorticotropin hormone; HPLC, high-performance liquid chromatography; IEF, isoelectric focusing; pI, isoelectric point

2. METHODS

2.1. *Purification of ^{125}I-labeled coenophorin*

The labeled M_r 80 000 form of neurophysin was purified to homogeneity as in [6]. Briefly, partially purified high M_r immunoreactive species from bovine neurohypophysis were labeled with ^{125}I and immunoprecipitated with antibodies directed against bovine neurophysin II. Labeled antibody-bound material was solubilized by boiling in 62.5 mM Tris–HCl (pH 6.8) containing 10% glycerol, 5% mercaptoethanol and 2% SDS for 5 min and then submitted to SDS–polyacrylamide gel electrophoresis [6]. The M_r 80 000 species was

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detected by autoradiography, cut out and digested with trypsin as in [6].

2.2. *Identification of ACTH peptides*

Tryptic peptides derived from ACTH were identified in the digest by comparing both their retention time, in reverse-phase, high-pressure liquid chromatography (HPLC), and their isoelectric point (pI) with those of reference peptides derived from $_{125}$I-labeled standard bovine ACTH 1–39.

Cleavage with carboxypeptidase B: Tryptic peptides recovered from the M_r 80 000 species or from 1–39 ACTH were further treated with 0.1 μg porcine carboxypeptidase B (DFP-treated, Sigma, St Louis MO) in 200 μl 0.1 M ammonium bicarbonate for 1 h at 37°C and the reaction stopped by 10 min boiling.

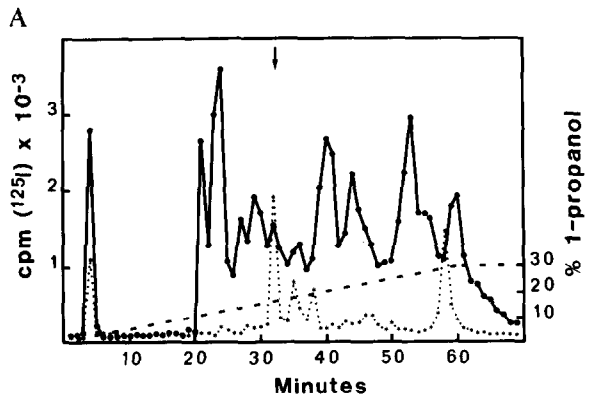
High-performance liquid chromatography: Peptides derived from the ^{125}I-labeled M_r 80 000 form of neurophysin or from ^{125}I-labeled bovine 1–39 ACTH were taken up in H_2O: MeOH, 50:50 (v/v) and injected on a C_{18} μ-Bondapack column (Waters Assoc.). The column was eluted with an increasing proportion of 1-propanol (0–30%) in aqueous trifluoroacetic acid (1/1000) at a flow rate of 1 ml/min. Each 1 min fraction was counted for ^{125}I-radioactivity.

Isoelectric focusing: Peptides recovered from HPLC fractionation were focused on 12%, ultrathin (0.5 mm), polyacrylamide gels. A pH gradient 9.5–3.5 or 5–3.5 was used (ampholines, LKB, Uppsala). The gels were dried and the radioactive peptides detected by autoradiography as in [6].

3. RESULTS

The sequence of bovine ACTH [9] predicts 2 tryptic peptides bearing a tyrosine residue which could be labeled with ^{125}I: the N-terminal peptide from residues 1–8; the C-terminal peptide from residues 22–39. Each one represents ≤ 1–2% of a M_r 80 000 polypeptide chain. Peptide 1–8 should be observed if it is either N-terminal or preceded by a basic amino acid in the sequence of high-M_r neurophysin. Peptide 22–39 ends with a phenylalanine; it is thus unlikely to be found in a tryptic digest of high-M_r neurophysin unless it is located at the C-terminal end. However, if it is followed, in the M_r 80 000 species, by a basic amino acid residue (as it is the case in another high-M_r form of ACTH, pro-opiomelanocortin) [10,11], it should be possible to generate the corresponding peptide by treating a tryptic digest with carboxypeptidase B (CPB) in order to remove the extra basic amino acid at its C-terminal end. Comparison (not shown) of the labeled fragments obtained by tryptic digestion of reference ACTH 1–39 and 1–24, respectively, indicated unambiguously that the C-terminal peptide corresponds to the species with a retention time of 58 min (seen in fig.1A). The tryptic fragments recovered in the 30–40 min region were found in both cases and are common to ACTH 1–24 and 1–39. Therefore, they correspond to the N-terminal region of ACTH (not shown). Microheterogeneity was observed, possibly due either to complex iodination reaction or incomplete proteolytic cleavage, or both. But a predominant peak was obtained eluting at 32 min (fig.1A) and probably corresponding to ^{125}I-ACTH 1–8. Analysis of the tryptic digest of purified M_r 80 000 neurophysin prior to treatment with CPB showed the presence of a peptide with the same HPLC-chromatographic properties and pI as ACTH 1–8 (fig.1A; fig.1B, lanes b,c).

If the tryptic peptides are treated with CPB any fragment ending with either a lysine or arginine should be modified by loss of the C-terminal residue. After treatment with CPB the ACTH 1–8 reference peptide was shifted toward longer retention time on HPLC and lower pI (fig.2A; fig.2B, lanes a,d); as expected, ACTH 22–39, ending with a phenylalanine residue, remained unaffected (fig.1A,2A). Treatment of the tryptic digest of high M_r neurophysin with CPB generated a peptide with the same HPLC-retention time and pI as the CPB-treated N-terminal sequence of ACTH (fig.2A; fig.2B, lanes c–e). Similarly, the CPB-treated tryptic digest of M_r 80 000 form of neurophysin contains a set of peptides identified in a similar way to ACTH 22–39 (fig.2A; fig.2C, lanes c–e).



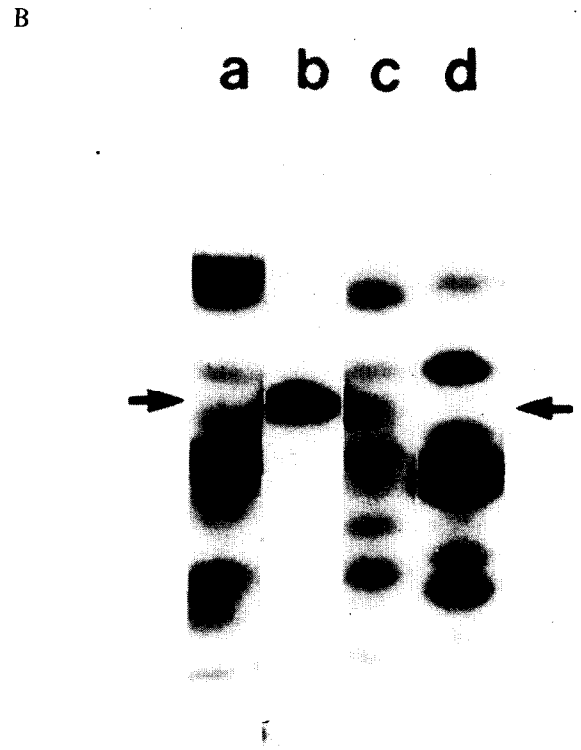


Fig.1. Analysis of ^{125}I tryptic peptides from the M_r 80 000 form of neurophysin, by reverse-phase HPLC followed by IEF on polyacrylamide gels. High M_r immunoreactive species were analysed on a SDS–polyacrylamide gel under reducing conditions, after ^{125}I-labeling and immunoprecipitation by bovine anti-neurophysin antibodies, as in section 2. The M_r 80 000 band was located on a dried gel by autoradiography, cut out and treated with trypsin [6].

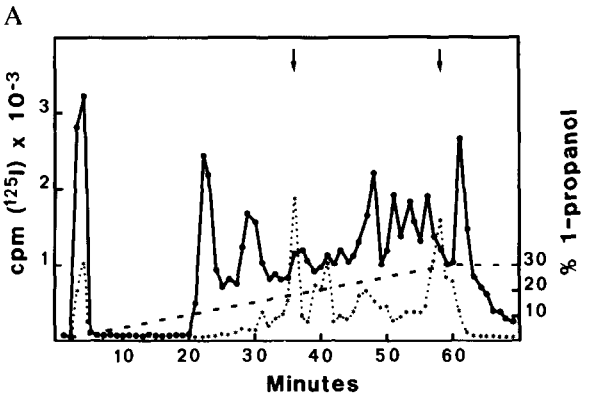
(A) Reverse phase HPLC:
The tryptic peptides recovered after digestion of the M_r 80 000 species were first analyzed on a C_{18} μ-Bondapack column (Waters Assoc.) (—•—). Tryptic peptides derived from ^{125}I-labeled bovine 1–39 ACTH were analyzed under the same conditions (•*•). The arrow indicates the elution peak corresponding to the N-terminal portion 1–8 of reference ^{125}I-ACTH.

(B) Isoelectric focusing on polyacrylamide gels:
Each fraction of the M_r 80 000 digests eluted in the relevant regions of the HPLC separation was analyzed by isoelectric focusing on polyacrylamide gels, together with reference ACTH peptides. A pH gradient from 9.5–3.5 (top to bottom) was used in this case. Lane: (b) fraction 32 min of the HPLC separation of trypsinized ^{125}I-ACTH; (a,c,d) fractions 31, 32 and 33 min, respectively of the HPLC separation of trypsinized M_r 80 000 ^{125}I-neurophysin. The arrows point to the radioactive peptide, with the same pI as the reference tryptic fragment 1–8 of ^{125}I-ACTH, which appears predominantly in fraction 32 min and was not detected in the fractions immediately preceeding and following this region of the chromatogram.

4. DISCUSSION

Thus, we have identified by two independent criteria the ACTH peptides 22–39 and 1–8 as well as the 1–7 derivative of the latter. This provides chemical evidence supporting the hypothesis that high M_r neurophysin may contain the sequence of ACTH [8]. Since, on the other hand, vasopressin- and β-endorphin-like immunoreactivities also copurify with this species [8], it appears that this very large form of neurophysin is, indeed, a composite molecule.

The presence of the M_r 80 000 form of neurophysin is now firmly established on chemical grounds. Observations by others of M_r 80 000 form of immunoactive AVP (A. Cupo and M. Delaage, unpublished) and $M_r \geq 70\,000$ of neurophysin and vasopressin in extracts of rat hypothalamo–neurohypophyseal system [12] support this contention. In this respect, it is of interest that biosynthetic studies of ectopic vasopressin production in a lung carcinoma showed incorporation of ^{35}S-label in an



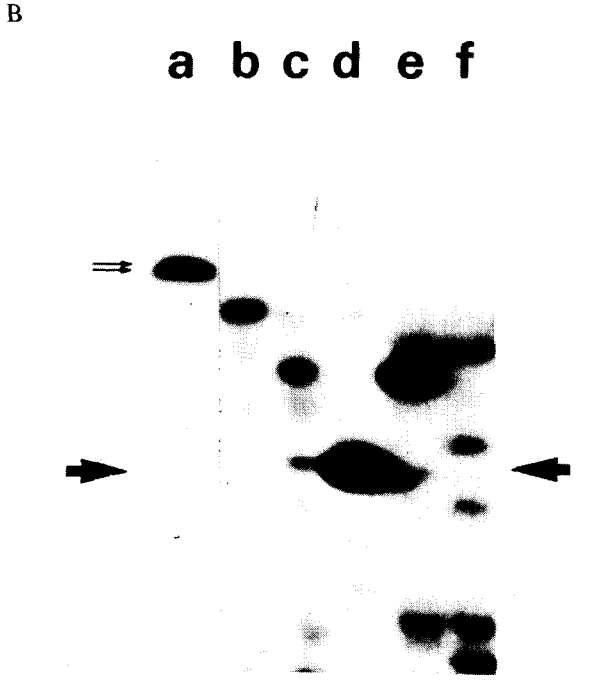


Fig.2. Analysis of the ^{125}I peptides generated by trypsinization of the M_r 80 000 form of neurophysin followed by treatment with carboxypeptidase B (CPB). Tryptic peptides derived from M_r 80 000 form of neurophysin or from reference 1–39 ACTH were first submitted to CPB and then analyzed as in fig.1.

(A) HPLC patterns of the M_r 80 000 digest (—•—) and of standard labeled ACTH processed under the same conditions (•*•). Arrows indicate fractions 36 and 58 min corresponding, respectively to fragments 1–7 and 22–39 of ^{125}I-ACTH.

(B) IEF analysis (pH 9.5–3.5; top-to-bottom) of material recovered under fractions 35–38 min (lanes b,c,e and f, respectively) of the HPLC separation of the M_r 80 000 digest. Lane d represents fractions 36 min of the HPLC separation of reference ^{125}I-ACTH treated by trypsin + CPB. Lane a (double arrow) is fragment 1–8 untreated by CPB (seen also in fig.1B, lane b). Arrows point to radioactive peptide with the same pI as the reference fragment 1–7 of ^{125}I-ACTH.

(C) IEF analysis (pH 5.0–3.5; top-to-bottom) of the material recovered under fractions 56–60 min (lanes a,b,d,e and f, respectively) of fig.2A (—•—). Lane c shows the material of fraction 58 min (fig.2A (•*•). The arrows point to a set of peptides corresponding to the C-terminal fragment 22–39 of ^{125}I-ACTH.

immunoreactive material larger than the predominant M_r 20 000 species [13]. It is not clear as yet whether these species are related to each other. However, none can be accounted for by the sequence of the M_r 17 300 neurophysin–vasopressin precursor predicted from the cloned cDNA characterized in [5]. Therefore, it is not unexpected that such large molecules might harbour the sequences of several, additional, biologically active peptides.

Relationships between the M_r 80 000 coenophorin, pro-opiocortin and pro-neurophysin–vasopressin remain to be established. As suggested in [3,6], these molecules may derive from distinct genes. Alternatively, they might arise either from differently arranged DNA sequences or from differently spliced mRNA. The biological function of coenophorin is still unknown. It is tempting to speculate that it may represent the common storehouse of a set of neuropeptides elaborated in specialized neurons of the hypothalamus. Anyway, it seems hardly compatible with the so-called 'one neuron–one hormone' theory [14]. The presence of several neuropeptide sequences on the same high-M_r form may provide a unitary explanation to a number of cytoimmunological observations which strongly argue in favor of the concomitant and ubiquitous presence of several immunoreactive peptides in the same neurons [15–17]: in particular to the detection of ACTH and β-endorphin immunoreactivities in neurons belonging to the magnocellular system of the rat hypothalamus [18,19].

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