EVIDENCE FOR THE OCCURRENCE OF TWO FORMS OF β-LIPOTROPIN IN RAT PITUITARY

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1. Introduction

β-Lipotropin (β-LPH) was originally isolated from ovine pituitary extracts in 1965 [1]. β-LPH aroused much interest as it was shown to serve as a precursor for pituitary peptides with opiate activity [2]. The primary structure of rat β-LPH has been deduced from the nucleotide sequence of a rat pituitary pro-opiomelanocortin gene [3]. Studies with purified rat pituitary β-LPH however, have given contradictory results concerning the amino acid composition and size of the polypeptide [4–6].

When trying to develop efficient methods for the purification of β-endorphin/ACTH related peptides from the pituitary we noticed that immunoreactive β-LPH from rat pituitary separated into two immunologically indistinguishable components by cation-exchange chromatography. These components are stable in concentrated urea solution, have M_r 8000–10 500 as assessed by polyacrylamide gel electrophoresis and gel filtration, and most likely do not represent artefactually generated deamidation forms of a single β-LPH molecule.

There are at least 2 pro-opiomelanocortin genes in the rat [3]. Here, we provide evidence which indicates that a difference between these genes resides in the part coding for β-LPH.

2. Materials and methods

Sixty-eight Sprague-Dawley rats aged 5–15 days were sacrificed by decapitation. Pituitary glands were removed, weighed and immediately extracted with 10 vol. 0.2 M HCl by sonication [7].

The freeze-dried extract was dissolved in 7.5 ml 10% HOAc and applied into a 2.6 × 90 cm column of Sephadex G-75 (Pharmacia) eluted with 10% HOAc at 40 ml/h. Fractions of 5.4 ml were collected and 10 μl from each fraction was lyophilized for β-LPH radioimmunoassay [7]. The peak of immunoreactivity corresponding to the elution of human β-LPH marker was divided into 2 pools, a and b (see fig.1). Both pools were lyophilized, dissolved in 10 ml 1 mM HCl and loaded into columns (0.7 × 18 cm) packed with SP-Sephadex C-25 (Pharmacia). Stepwise salt gradient at pH 6.7 with or without 6.5 M urea was run as follows: wash with 0.05 M NH_4OAc until 18 fractions of 1.8 ml were eluted. The eluant was changed to 0.15 M NH_4OAc and 27 fractions were collected, followed by 0.40 M NH_4OAc for 20 fractions and finally 1.0 M NH_4OAc for 18 fractions. β-LPH radioimmunoassay was performed with eluate diluted 300 times. Fractions corresponding to the 2 major peaks of immunoreactivity (designed as pools 1 and 2, see fig.2) were pooled, divided into aliquots, lyophilized and stored at −20°C.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS–PAGE) was performed in 12.5% gels according to [8]. The gels were sliced and prepared for radioimmunoassay as in [9].

Gel filtration on Sephadex G-75 (0.7 × 49 cm columns) in the presence of urea was performed as in [7].

For β-LPH radioimmunoassays [7] 4 different antisera were used each directed to the C-terminal part of β-LPH and thus crossreacting equimolarly with β-endorphin and pro-opiomelanocortin [7,10,11].

Dansylation of standard amino acids (Merck) was performed according to [12]. The dansylated amino acids were separated by reverse-phase, high-performance liquid chromatography (HPLC, Spherisorb 5 μm, 4.5 × 250 mm column from Phase Separations)

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using a Varian model 5000 liquid chromatograph. The column was eluted with a linear gradient from 12–16% 2-propanol in 10 mM NH_4OAc (pH 4.00) (adjusted with HOAc) during 10 min with a flow rate of 1 ml/min at 38°C. Fluorescence detection was used (Varian Fluorichrom, excitation at 340–380 nm, emission at 460 nm).

All chemicals used were purchased from Merck or Serva and were of analytical grade.

3. Results and discussion

Acid extract of 68 rat pituitaries yielded 16 nmol β-LPH immunoreactivity against porcine β-endorphin standard. Gel filtration on Sephadex G-75 separated the immunoreactivity into 3 components, of which the middle one eluted at the same position as human β-LPH (fig.1). This peak (fractions 37–46 in fig.1, 3 nmol) was divided into 2 pools, a and b, and they were analyzed separately on SP C-25 columns. The elution profiles thus obtained are depicted in fig.2. Two major peaks of immunoreactivity were detected both for pools a and b, the sizes of which varied so that the early eluting (more acidic) material (designated as pool 1) was detected in greater amounts in pool a from G-75 chromatography. The relative sizes of components 1 and 2 remained unchanged when the SP C-25 chromatography was run in the presence of 6.5 M urea. Moreover, it was found that component 1 could be eluted with 0.10 M NH_4OAc while component 2 eluted with 0.40 M NH_4OAc as in the earlier runs (not shown). The fact that component a, which consisted of material from the ascending limb of the β-LPH peak in gel filtration (fig.1), contained more immunoreactive material eluting with 0.15 M NH_4OAc in SP C-25 chromatography suggested that there could be a difference in the M_r-values of the 2 components of β-LPH. However, both components migrated identically in SDS–PAGE in the 9000–10 500 M_r region (fig.3), and no significant difference in the M_r-values was detected by gel filtration on G-75 in acid urea buffer (fig.4). Taking into account the sensitivity and accuracy of the methods used for M_r determination we conclude that both components have M_r 8000–10 500, and that the M_r-

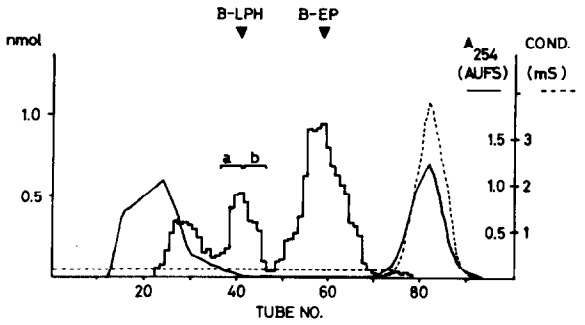

Fig.1. Gel filtration of an acid extract of 68 rat pituitaries on a 2.6 × 90 cm Sephadex G-75 column. Aliquots of each fraction (5.4 ml) were assayed for β-LPH immunoreactivity (columns). Arrows indicate the elution positions of purified human β-LPH [14] and synthetic porcine β-endorphin (B-EP) [15].

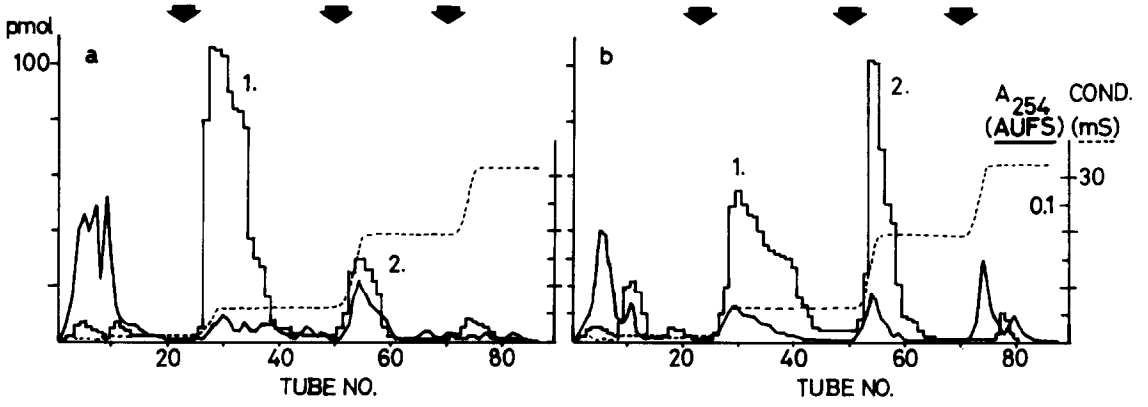

Fig.2. Cation-exchange chromatography on SP-Sephadex C-25 columns (0.7 × 18 cm) of pools a (left) and b (right) from G-75 chromatography. Aliquots of the fractions (1.8 ml) were assayed in β-LPH radioimmunoassay (columns). Arrows indicate the steps in the salt gradient. For further details see text.

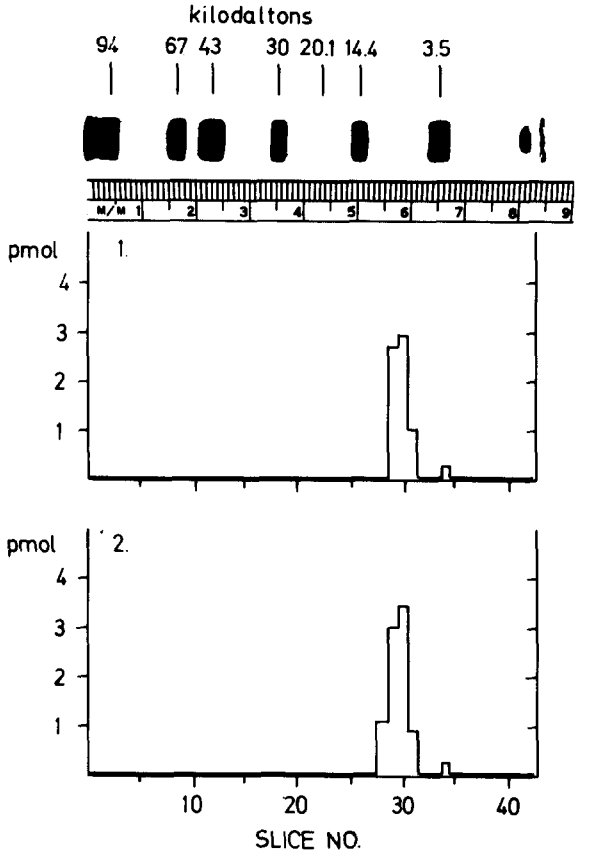

Fig.3. SDS–PAGE in 12.5% acrylamide gels of M_r marker peptides (top) and pools 1 (middle) and 2 (bottom) from SP C-25 chromatography.

values of the 2 forms may differ from each other up to 1500 M_r.

Immunological characteristics of the 2 forms of β-LPH were studied by subjecting serial dilutions of components 1 and 2 to β-LPH radioimmunoassay using 4 different antisera (RB 100, Bendo 2, K 2 and K 3; [7,10,11]). Both components were found to displace, in parallel, tracer from each of antisera compared to synthetic porcine β-endorphin used as standard (not shown). This indicates that both components 1 and 2 contain identical antigenic structures as porcine β-endorphin (i.e., β-LPH 61–91). Therefore, the differences in components 1 and 2 most probably reside in the N-terminal part of rat β-LPH. It was in the N-terminal portion that the controversy of the structure of rat β-LPH was located in the earlier studies [4–6]. In [4] a molecule of ~90 amino acids was isolated with 2 methionines in its structure, while in

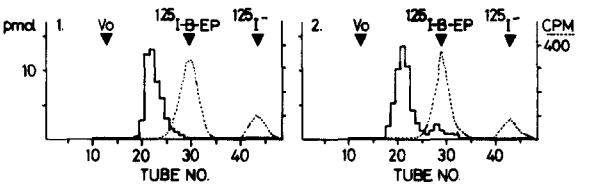

Fig.4. Gel filtration of pools 1 (left) and 2 (right) on 0.7 × 49 cm Sephadex G-75 columns equilibrated and eluted with 6.5 M urea/0.05 M HCl. Trace quantities of blue dextran, ^{125}I-labelled β-endorphin and $^{125}I^-$ were run as internal standards (arrows). The fractions (0.4 ml) were assayed in β-LPH radioimmunoassays (columns). As determined in separate runs, purified human β-LPH (M_r 11 700) eluted in fractions 19–20 and synthetic human ACTH (M_r 4500) in fractions 27–28.

[5,6] a molecule with only 1 methionine and ~70 amino acids total was found. Rat tissues contain at least 2 different genes for pro-opiomelanocortin (from which β-LPH is cleaved) thus giving rise to peptides differing in their amino acid sequences [3,13]. It was speculated [13] that one possible site of divergence in the different pro-opiomelanocortins might be the

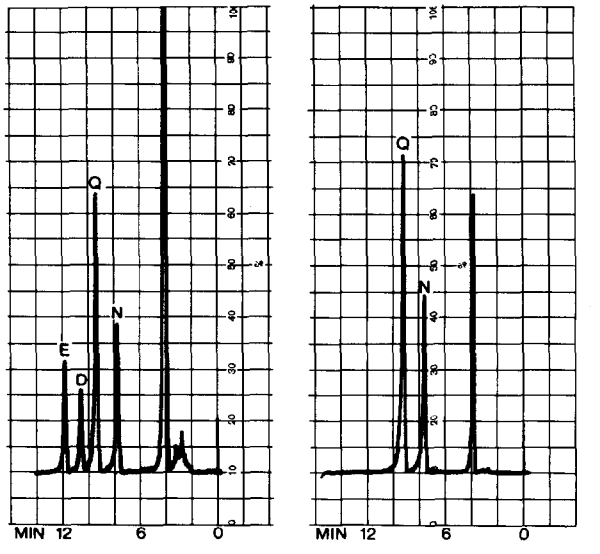

Fig.5. HPLC of dansyl derivatives of asparagine (N), glutamine (Q), aspartic acid (D) and glutamic acid (E). Profile to the left shows the elution of standard N, Q, D and E without pretreatment. Profile to the right shows the elution of N and Q after treatment with HCl, HOAc and NH_4OAc identical to that of pituitary β-LPH in section 2; ~500 pmol of each amino acid was injected to the column. Fluorescent material eluting during the first 5 min represents by-products of excess dansyl-Cl reagent.

N-terminal portion of β-LPH, submitted to considerable evolutionary drift. These findings provide evidence supporting this speculation. We have not observed heterogeneity in β-LPH prepared from human autopsy pituitaries by methods identical to those used here (unpublished).

Partial deamidation of glutaminyl or asparaginyl residues appeared to be the only reasonable possibility which could explain the clear difference in charge that was observed. Control experiments, however, in which we treated glutamine and asparagine in the same way as the immunoreactive material showed that no detectable conversion of glutamine or asparagine to glutamic acid or aspartic acid, respectively, takes place in the extraction and chromatography conditions employed (fig.5). This finding makes most unlikely the possibility that the observed charge heterogeneity of rat β-LPH is due to artefactual deamidation during the purification of the immunoreactive material.

These results indicate that rat pituitary contains 2 different forms of β-LPH differing from each other by charge and possibly by size. This paper also presents a simple method for separating these 2 forms, which will be useful in the isolation of the different β-LPHs.

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