The primary structure of human β-lipotropin

Further peptide sequencing resolves the controversy and suggests the existence of only one human β-lipotropin

N.G. Seidah, K.L. Hsi+, M. Chrétien†, E. Barat*, A. Patthy* and L. Graf*

Clinical Research Institute of Montreal, 110 Pine Avenue West, Montreal H2W 1R7, Canada and * *Institute for Drug Research, PO Box 82, 1325 Budapest, Hungary*

Received 27 August 1982

Human β-lipotropin isolated in Hungary from frozen pituitary glands was purified by high-performance liquid chromatography in Canada. The amino acid sequence of the first 30 residues was determined. Trypsin, trypsin/papain, and trypsin/thermolysin fragments were obtained for the disputed region containing residues 9–25 of β-lipotropin. Their amino acid composition and sequence established beyond doubt that only one human β-lipotropin sequence is present. These results suggest the presence of only one gene coding for human pro-opiomelanocortin, the precursor of adrenocorticotropin and β-endorphin and resolve the controversy over the sequence of human β-lipotropin.

Human β-lipotropin *Sequence* *HPLC purification*

1. INTRODUCTION

It is now well-established that β-lipotropin (β-LPH) and adrenocorticotropin (ACTH) are part of a common precursor in various species [1–4], called pro-opiomelanocortin [4]. The complete amino acid sequence of the various human segments isolated has also been reported. These include the amino-terminal fragment 1–76 [5,6], the joining peptide [7], ACTH [8], β-LPH [9,10], γ-LPH [11] and β-endorphin [12,13]. The human pro-opiomelanocortin DNA sequence has been revised [14] and so has been the protein amino acid sequence of human β-LPH [15]. These two revised sequences are identical as the primary structure of β-LPH is concerned. However, both reports [14,15] disagree with the sequences in [10,11] and proposed by the Hungarian group (see footnote in [11]) for residues 9–25 of the β-LPH structure. It was originally thought that polymorphism might be the cause of such a controversy [14,15]. This was likely since evidence for the presence of more than one gene coding for both rat [16,17] and porcine [18] pro-opiomelanocortin was presented. The purpose of this joint study was to reexamine the possibility that human β-LPHs prepared by the Hungarian [9] and Canadian workers [15] originate from two different pro-opiomelanocortin genes. Crude human β-LPH and all the purified β-LPH fragments were prepared in Hungary and the sequence work was done in the Canadian laboratory. Our results confirm the sequence in [15], thus suggesting that the differences between the published sequences are more likely due to difficulties in polypeptide sequencing than to polymorphism of the human pro-opiomelanocortin gene.

+ Visiting scholar from: Shanghai Institute of Materia Medica, Academia Sinicia, China

† To whom reprint requests should be addressed

2. MATERIALS AND METHODS

2.1. *Isolation of human β-LPH*

β-LPH was prepared as in [19]. This preparation was then further purified by reverse-phase, high-performance liquid chromatography (HPLC) as shown in fig.1. The column used was a μ-Bondapak C_{18} (Waters Co. 0.39 × 30 cm). The HPLC

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$2.75

apparatus consisted of two Beckman 110A pumps, a model 450 controler, a Hitachi variable wavelength spectrophotometer and a Waters model 730 data module. Elution was made using a linear gradient of 2-propanol and 0.1% trifluoroacetic acid (TFA) from 20–60% in 60 min at 1 ml/min. Absorbance was read at 235 nm (see fig.1).

2.2. *Purification of trypsin/papain and trypsin/thermolysin fragments of human β-LPH*

The large tryptic fragment (residues 9–37) was purified by chromatography on a Sephadex G-25 column of the tryptic digest of human β-LPH (see [10]) and then by high-voltage paper electrophoresis at pH 5.0 (see method in [20]). Digestion of this fragment with papain (Sigma, type III) was performed in 0.1 M pyridine acetate at pH 5.5 containing 5 mM EDTA and 5 mM dithiothreitol, at 1:20 (w/w) of enzyme/peptide at 37°C for 3 h. Digestion with thermolysin (Serva, 3× crystallized) was carried out with 1:100 enzyme/substrate (w/w), in 0.1 M NH_4HCO_3 (pH 8.0) at 37°C for 3 h. The fragments were separated by two-dimensional paper electrophoresis at pH 5.0 and pH 2.0 as in [9,20].

2.3. *Amino acid analysis*

Amino acid analysis of purified trypsin/papain and trypsin/thermolysin fragments of human β-LPH was carried out on an updated Beckman 120C amino acid analyser following 24 h hydrolysis using 5.7 mM HCl at 108°C. Quantitation of sample amount of the papaic and thermolysin fragments was done by amino acid analysis in order to calculate the sequence yields of these fragments (see table 1).

2.4. *Sequence determination*

Automated Edman degradations were performed on a Beckman 890C sequenator equipped with a Sequemat P-6 autoconverter using a 0.3 M Quadrol program (Beckman no. 121178) and 3 mg Polybrene (Aldrich) [6]. Following 4 dummy cycles, 0.3 mg HPLC purified human β-LPH (see fig.1C, peak d_1) were applied to the sequenator cup and double coupling performed for the first cycle only. The sequencing was extended to 30 cycles. For papain and thermolysin fragments the amounts deposited on the sequenator cup, based on quantitative amino acid analysis are shown in table 1. Phenylthiohydantoin (PTH) amino acids were identified and quantitated by HPLC as in [6].

3. RESULTS

The HPLC purification of human β-LPH eluted from the CM-Sephadex column [19] is shown in fig.1. Based on previous work [15], the elution position of human β-LPH coincided with peak d (fig.1A). Upon repurification of material under peak d twice, using the same column and gradient elution (fig.1B,C), a homogeneous preparation of human β-LPH was obtained (peak d, fig.1C) and used for sequencing studies. The sequenator PTH yields of each amino acid obtained from the material under peak d_1 (fig.1C) are shown in fig.2. The deduced sequence of the first 30 residues is shown

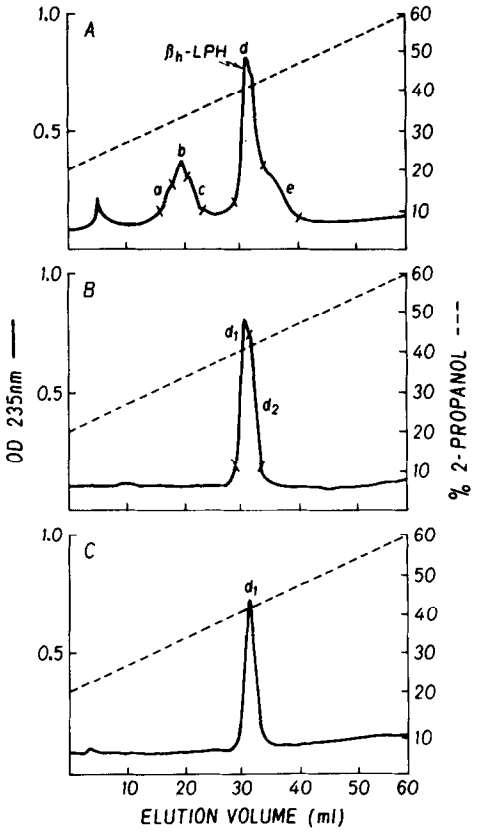

Fig.1. Purification of human β-LPH by reverse-phase, high-performance liquid chromatography: (A) preliminary purification of human β-LPH; (B,C) the repurification on HPLC under same conditions as in (A); (- - -) linear gradient of 2-propanol used.

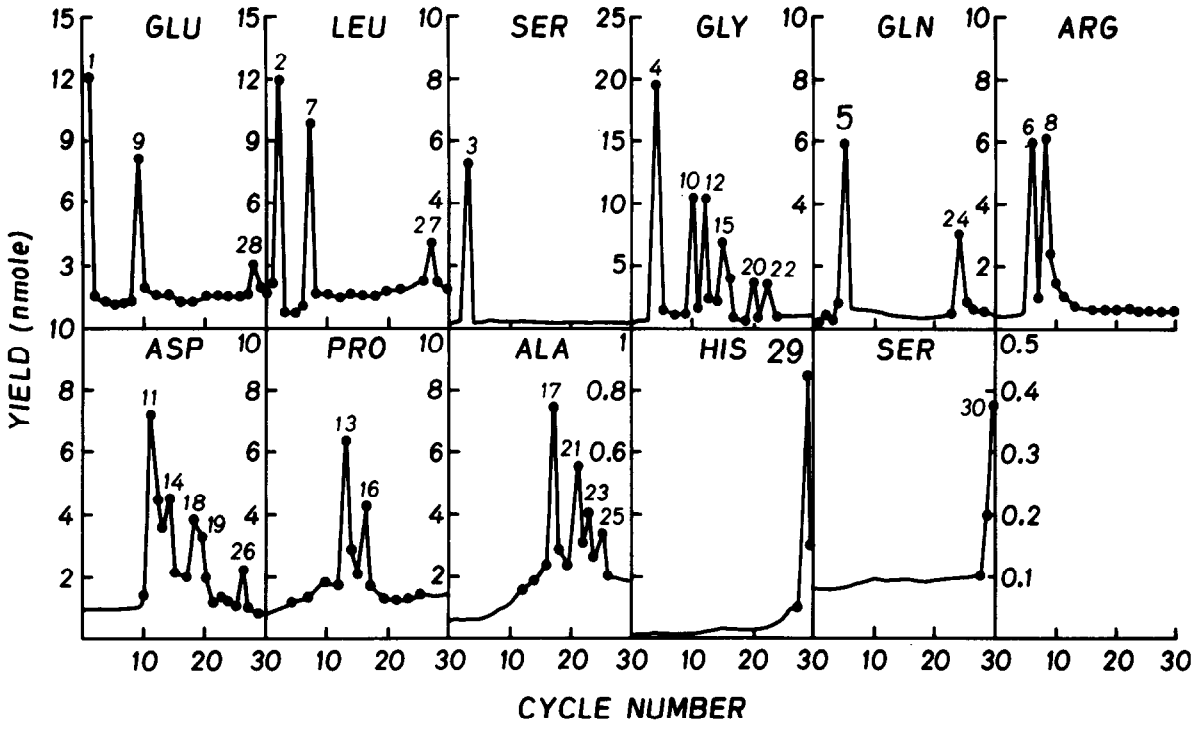

Fig.2. Yield of each PTH-amino acid obtained during the sequence of human β-LPH, as a function of sequencer cycle number. The numbers above each peak denote the assigned sequence position of that particular residue.

Table 1

Amino acid composition and deduced sequences of papaic and thermolysin fragments of human β-LPH

Amino acid	Papaic fragments				Thermolysin fragments	
	(8/1S9)	(2S71)	(2S8)	(2S9)	(82a4)	(82a5)
Asp	4.1 (4)	4.1 (4)	4.0 (4)	4.3 (4)	5.0 (5)	5.2 (5)
Ser	–	–	–	–	1.0 (1)	–
Glu	1.0 (1)	1.4 (1)	1.9 (2)	1.1 (1)	3.2 (3)	2.2 (2)
Pro	1.8 (2)	1.5 (2)	1.6 (2)	1.6 (2)	1.9 (2)	2.1 (2)
Gly	5.2 (5)	5.3 (5)	5.4 (5)	5.2 (5)	4.7 (5)	5.0 (5)
Ala	2.1 (2)	2.5 (3)	2.8 (3)	2.3 (2)	3.7 (4)	3.9 (4)
Leu	–	–	–	–	1.2 (1)	–
His	–	–	–	–	0.4 (1)	–
Corresponding human β-LPH peptide	9–22	9–23	9–24	9–22	9–30	9–26
Fragments identification in fig.3	e	f	i	h	d	g
Residues confirmed by sequence	9–22	9–18	9–11	9–13	9–23	9–17
Sequenced sample amount (nmol)	4	2	2	2	8	15
Sequenator yield	11%	18%	10%	10%	31%	23%

in fig.3 (line c). It is seen that the sequence of all 30 residues is identical to that reported either for a human β-LPH preparation [15], or deduced from the revised DNA sequence of human pro-opiomelanocortin [14].

To reconfirm beyond doubt that the sequence obtained for this preparation is identical with that in [15], trypsin/papain and trypsin/thermolysin fragments of human β-LPH originating from the disputed sequence region were also analyzed. The amino acid composition of these trypsin/papain and trypsin/thermolysin fragments is shown in table 1. It can be seen that the amino acid composition of all the fragments obtained fit the reported human β-LPH sequence [15] and that obtained in this work (fig.3, line c) for residues 9–30. Moreover, each papain and thermolysin fragment was sequenced. The low amounts of some fragments available (fragments h, i, table 1) allowed the identification of only few amino-terminal residues. For those which were available in large quantities (fragments d–g, table 1), sequencing could be extended for 10–15 residues and in the case of fragment e up to the COOH-terminal residue. The yields obtained although low (10–31%), could be explained by the possible extraction losses of these peptides during sequencing, due to their small size. Indeed as the peptide size and amount available are larger, the sequenator yield is higher (cf. yields of fragments d and h in table 1). Nevertheless, in all cases the partial sequences obtained are identical to that reported earlier for human β-LPH [14,15].

4. DISCUSSION

The data in fig.3 and table 1 clearly show that the sequence of human β-LPH obtained from the Hungarian laboratory [9,19,20] is identical with that reported by the Canadian group [15]. Further-

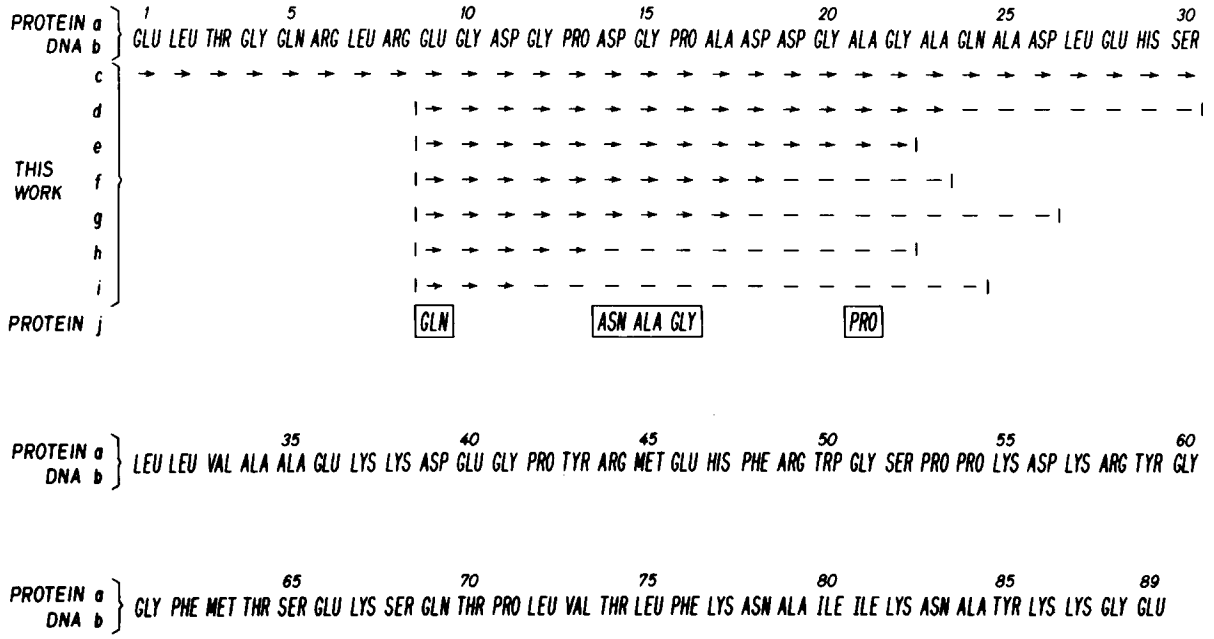

Fig.3. Confirmed human β-LPH amino acid sequence: (a) protein sequence determined in [15]; (b) DNA sequence determined in [14]; (c) protein sequence of purified human β-LPH in this work (see fig.1,2); (d) trypsin/thermolysin fragment of human β-LPH, 82a4; (e) trypsin/papain fragment of human β-LPH, 8/1 S8; (f) trypsin/papain fragment of human β-LPH, 2S71; (g) trypsin/thermolysin fragment of human β-LPH, 82a5; (h) trypsin/papain fragment of human β-LPH, 2S9; (i) trypsin/papain fragment of human β-LPH 2S8; (j) protein sequence deduced in [10,11]; amino acid residues differing from a, b are boxed in. In all other positions, the sequence reported in [10,11] is identical to that in [14,15]. The amino acid composition of the fragments is shown in table 1. (→) represents residues identified by sequence; (-) not sequenced residues due to fall off in yield during sequencing.

more, this human β-LPH sequence is the same as that deduced from the revised DNA sequence of the gene coding for human β-LPH [14].

Of course, it is still possible that the amino acid sequence, between residues 9–22, of human β-LPH isolated in [10] is different from that of human β-LPHs obtained by our laboratories, independently. This possibility is, however, rather unlikely. In this regard it is interesting to note that the amino acid composition of a thermolysin fragment, Thl (containing residues 9–24) isolated in [10] is identical with that of a papaic fragment, 2S8 (i) included in table 1 of this paper. Furthermore, it might well be that one of the key-fragments, Pl, originally assigned to positions 9–16 in [10], is actually a fragment of double size, with a composition indistinguishable from that of 2S71 or 2S9 in table 1 here. To further illustrate this kind of difficulty in locating the enzymic fragments purely on the basis of amino acid composition and partial sequence data, it is worth mentioning that Graf and coworkers had originally thought that all the papaic and thermolysin fragments (except 8/1S8) in table 1 contain only 1 proline instead of 2. A preliminary sequence proposal by the latter group was based on this erroneous assumption (see footnote in [11]).

Indeed, technical problems of determining the amino acid sequence, between residues 9–22, of human β-LPH (see fig.3) by classical fragmentation and against Edman–dansyl methods appear to be quite serious. Apparently, there are no trypsin, chymotrypsin, papain, thermolysin and pepsin sensitive peptide bonds within this sequence region to obtain smaller overlapping fragments by these enzymes. Furthermore, unless quantitative sequencing procedures are employed, qualitative data by the Edman–dansyl procedure may be difficult to interpret, in view of the possible contamination of the fragments and also, in this particular case, of a partial resistance of the repetitive Asp–Gly sequences against Edman degradation [9].

In conclusion, our comparative studies on two independently prepared human β-LPH preparations, do not support the view of either the presence of polymorphic variations in the β-LPH sequence or that more than one gene code for human pro-opiomelanocortin, as suggested in [2,11,14,15]. Accordingly, the structures of all the segments isolated and sequenced of human pro-opiomelanocortin [5,8,12,13,15] agree perfectly with the structure deduced from the revised DNA sequence of this pluripotent precursor molecule [14]. In contrast, at least two genes coding for pro-opiomelanocortin have been reported in some other species as rat [16,17], pig [18] and salmon [21,22]. It has yet to be established that such multiplicity of genes also exists for human pro-opiomelanocortin.

ACKNOWLEDGEMENTS

Supported by the Medical Research Council of Canada (PG-2), and the National Institutes of Health (NS16315-02)

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] Chang, A.C.Y., Cochet, M. and Cohen, S.N. (1980) Proc. Natl. Acad. Sci. USA 77, 4890–4894.
[3] Drouin, J. and Goodman, H.M. (1980) Nature 288, 610–613.
[4] Chrétien, M., Benjannet, S., Gossard, F., Gianoulakis, C., Crine, P., Lis, M. and Seidah, N.G. (1979) Can. J. Biochem. 57, 1111–1121.
[5] Seidah, N.G. and Chrétien, M. (1981) Proc. Natl. Acad. Sci. USA 78, 4236–4240.
[6] Seidah, N.G., Rochemont, J., Hamelin, J., Lis, M. and Chrétien, M. (1981) J. Biol. Chem. 256, 7977–7984.
[7] Seidah, N.G., Rochemont, J., Hamelin, J., Benjannet, S. and Chrétien, M. (1981) Biochem. Biophys. Res. Commun. 102, 710–716.
[8] Lee, T.H., Lerner, A.B. and Buettner-Janusch, V. (1961) J. Biol. Chem. 236, 2970–2974.
[9] Cseh, G., Barat, E., Patthy, A. and Graf, L. (1972) FEBS Lett. 21, 344–346.
[10] Li, C.H. and Chung, D. (1981) Int. J. Pept. Prot. Res. 17, 131–142.
[11] Li, C.H., Chung, D. and Yamashiro, D. (1980) Proc. Natl. Acad. Sci. USA 77, 7214–7217.
[12] Dragon, N., Seidah, N.G., Lis, M., Routhier, R. and Chrétien, M. (1977) Can. J. Biochem. 55, 666–670.
[13] Li, C.H., Chung, D. and Doneen, B.A. (1976) Biochem. Biophys. Res. Commun. 72, 1542–1547.
[14] Takahashi, H., Teranishi, Y., Nakanishi, S. and Numa, S. (1981) FEBS Lett. 135, 97–102.

[15] Hsi, K.L., Seidah, N.G., Lu, C.L. and Chrétien, M. (1981) Biochem. Biophys. Res. Commun. 103, 1329–1335.
[16] Crine, P., Lemieux, E., Fortin, S., Seidah, N.G., Lis, M. and Chrétien, M. (1981) Biochemistry 20, 2475–2481.
[17] Vuolteenaho, O. and Lappalnoto, J. (1982) FEBS Lett. 138, 79–82.
[18] Seidah, N.G., Larivière, N., Boileau, G., Gossard, F. and Chrétien, M. (1981) in: Advances in Endogenous and Exogenous Opioids, pp. 113–116, Proc. Intl. Narcotic Res. Conf. Kyoto, July 26–30, 1981.
[19] Cseh, G., Graf, L. and Goth, E. (1968) FEBS Lett. 2, 42–44.
[20] Barat, E., Patthy, A. and Graf, L. (1979) Proc. Natl. Acad. Sci. USA 76, 6120–6123.
[21] Kawauchi, H., Tsubokawa, M., Kanezawa, A. and Kitagawa, H. (1980) Biochem. Biophys. Res. Commun. 92, 1278–1288.
[22] Kawauchi, H., Adachi, Y. and Ishizuka, B. (1980) Int. J. Pept. Prot. Res. 16, 79–82.