Chemical Differences between Thyrotropin Isohormones

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Amide determinations have been carried out, with a newly developed g.l.c. method, on thyrotropin hormones prepared by isoelectric focusing. The observed differences in amide content fully account for the differences in the electrochemical properties of the isohormones.

Fawcett *et al.* (1969) reported the isolation of four thyrotropin isohormones from a purified bovine anterior-pituitary extract by isoelectric focusing at pH7–10 in a sucrose density gradient. These were at that time designated α -, β -, γ - and δ -thyrotropins, in order of increasing isoelectric point, but, in view of the subsequent widespread use of the α and β notation to denote the subunits of the glycoprotein hormones (including thyrotropins), we now propose the nomenclature thyrotropins A, B, C and D for these substances. The biological potencies of these preparations were: thyrotropin A, 30 i.u./mg; thyrotropin B, 20 i.u./mg; and thyrotropin C, 60 i.u./mg. The differences between the A and B isohormones may not be significant, being within the confidence limits of the bioassay, but thyrotropin C was clearly more active (confidence limits in one bioassay 43–90 i.u./mg). Thyrotropin D later proved to be contaminated with a fifth component, thyrotropin E, from which we were unable to separate it. The low biological potencies (< 10 i.u./mg) of the D and E isohormones suggested that they were degradation products, and they are not considered further here. On the other hand the A, B and C isohormones prepared by the method of Fawcett *et al.* (1969) appeared to be substantially pure, as

demonstrated by repeated isoelectric focusing at consistent pI values, and by the appearance of single zones on polyacrylamide-gel electrophoresis at pH6 (Fawcett *et al.*, 1969).

Results and Discussion

Chemical differences between the isohormones as prepared by isoelectric focusing were difficult to detect, mainly because of the very small amounts available. Differences in carbohydrate composition suggested by the initial analyses have not been confirmed by later results obtained with larger amounts of material (Table 1). The differences found now, with the possible exception of the low fucose content of thyrotropin A, are within the experimental error. The possibility of limited proteolytic digestion before or during isolation was also considered. Dansylation, followed by hydrolysis for 4 h at 110°C with 6M-HCl, and identification of the dansyl*-amino acids by two-dimensional chromatography on polyamide thin-layer sheets (Gray, 1967), revealed only phenylalanine as the sole *N*-terminal amino acid residue, in agree-

* Abbreviation: dansyl, 5-dimethylaminonaphthalene-1-sulphonyl.

Table 1. *Carbohydrate composition of thyrotropin isohormones*

Hexosamines were determined by the distillation methods of Cessi & Piliego (1960) and Cessi & Serafini-Cessi (1963). Fucose was determined by the method of Dische & Shettles (1948) and mannose (the only hexose identified by t.l.c.) by a scaled-down version of the orcinol/H₂SO₄ method of Winzler (1955). The results are of duplicate analyses. For other experimental details, see the text.

Thyrotropin isohormone	Carbohydrate composition [% (w/w) of dry protein]			
	Glucosamine	Galactosamine	Fucose	Mannose
A	3.66	2.69	0.76	4.44
	3.70	2.65	0.77	4.45
B	3.85	2.77	0.88	5.10
	3.75	2.87	0.94	5.06
C	3.67	2.75	0.98	4.92
	3.73	2.69	0.97	5.00

Table 2. *Amide contents and isoelectric points of thyrotropin isohormones*

Results are means $\pm$ 1 s.d. for the numbers of determinations given in parentheses. For experimental details, see Fawcett *et al.* (1969), Davy & Morris (1977) and the text.

Thyrotropin isohormone	Amide content (mol of NH ₃ /mol of protein)	pI
A	14.07 $\pm$ 0.63 (9)	8.39 $\pm$ 0.058 (9)
B	16.74 $\pm$ 0.46 (12)	8.61 $\pm$ 0.033 (12)
C	17.34 $\pm$ 0.57 (10)	8.75 $\pm$ 0.012 (9)

ment with the known structures of the α - and β -subunits (Liao & Pierce, 1971). Similarly, carboxypeptidase A digestion of the isohormones, followed by g.l.c. determination of the *N*-trifluoroacetyl methyl esters of the liberated amino acids (Davy & Morris, 1976), revealed the *C*-terminal sequence -Tyr-(His)-Lys-Ser in all cases, in agreement with the known structure of the α -subunit. (Histidine is the only amino acid that cannot be positively identified by the method used; its presence was inferred from the absence of any other residue.) The *C*-terminal methionine residue of the β -subunit was only partially released, in accordance with our previous findings (Davy & Morris, 1976), but approx. 0.2 mol of methionine residue per mol of protein was released from each isohormone preparation. These findings, together with the fact that no consistent differences could be found in the amino acid compositions of the different isohormones, make it unlikely that significant proteolytic digestion had occurred either during the storage of the pituitary glands or during the preparation of the isohormones.

The differences in pI between the isohormone species as prepared by the method of Fawcett *et al.* (1969) had earlier suggested that differences in amide content might account for the multiplicity of thyrotropins observed. However, existing methods (including ammonia determinations during amino acid analysis after prolonged acid hydrolysis) were insufficiently reliable and sensitive to establish significant differences in amide content on the small amounts of material available. The development in this laboratory (Davy & Morris, 1977) of a new method for protein amide determinations applicable to nanomolar amounts suggested a solution for this problem. This method involves hydrolysis of the protein with 2M-HCl for 3 h at 100°C, followed by isothermal g.l.c. determination of the ammonia liberated by on-column neutralization of a portion of the hydrolysate. Application of this method to the thyrotropin isohormones gave results consistent with

their isoelectric points as determined by isoelectric focusing (Table 2). These results suggest that thyrotropin C differs from thyrotropin B by only one amide group in a total of 17–18 amide residues, and that the latter differs from thyrotropin A by two amide residues, consistent with the greater difference in pI and therefore better separation of these species (Fig. 1 in Fawcett *et al.*, 1969). This charge difference is also confirmed by the mobility differences at pH 6 (Fawcett *et al.*, 1969).

It may be relevant that thyrotropin C, which occurs in the largest amount in the original concentrate and has the highest amide content, is also significantly the most potent biologically, and it may represent the native hormone synthesized by the pituitary gland. Uncovering of carboxyl groups by hydrolysis of amides may alter the charge distribution, possibly hindering the assembly of the α - and β -subunits and diminishing the biological activity.

This example shows that nanomolar amide analyses may be very valuable in characterizing multiple protein species detected by isoelectric focusing.

References

- Cessi, C. & Piliego, F. (1960) *Biochem. J.* **77**, 508–510
- Cessi, C. & Serafini-Cessi, F. (1963) *Biochem. J.* **88**, 132–136
- Davy, K. W. M. & Morris, C. J. O. R. (1976) *J. Chromatogr.* **116**, 305–314
- Davy, K. W. M. & Morris, C. J. O. R. (1977) *J. Chromatogr.* **136**, 361–369
- Dische, Z. & Shettles, L. B. (1948) *J. Biol. Chem.* **175**, 595–603
- Fawcett, J. S., Dedman, M. L. & Morris, C. J. O. R. (1969) *FEBS Lett.* **3**, 250–252
- Gray, W. R. (1967) *Methods Enzymol.* **11**, 139–159
- Liao, T.-H. & Pierce, J. G. (1971) *J. Biol. Chem.* **246**, 850–865
- Winzler, R. J. (1955) *Methods Biochem. Anal.* **2**, 279–311