Confirmation of the 1–20 Amino Acid Sequence of Human Adrenocorticotrophin

By H. P. J. BENNETT, P. J. LOWRY and C. McMARTIN
Research Division, CIBA Laboratories, Horsham, Sussex RH12 4AB, U.K.

(Received 29 November 1972)

The tryptic fragments of natural human adrenocorticotrophin, were separated by countercurrent distribution and a correction in positions 25, 26, 27 and 30 was made by Riniker *et al.* (1972) in a study of the fragment containing residues 22–39. We have purified the remaining tryptic fragments, namely residues 1–8, 9–15, 16–21 and 17–21, by using ion-exchange chromatography on CM-cellulose and have carried out sequence determination by using the subtractive Edman degradation procedure and digestion with aminopeptidase M and carboxypeptidase B. These results have confirmed the proposed sequence for human adrenocorticotrophin in regions 6–7, 10–14 and 17–20, which had previously been arrived at only by analogy with the invariant sequence found in the three other mammalian adrenocorticotrophin species that had been investigated.

Investigations by Riniker *et al.* (1972) have led to revision of the widely accepted 22–39 sequences of human and porcine corticotrophins. The structure of human ACTH* proposed by Lee *et al.* (1961) also included the following sequence for the region that is responsible for its steroidogenic properties:

Ser-Tyr-Ser-Met-Glu(His,Phe)Arg-Trp(Gly,Lys,Pro,-Val,Gly)Lys-Lys(Arg,Arg,Pro,Val)Lys-Val-Tyr-Pro

The sequences shown in parentheses were not established by chemical characterization but were proposed on the basis of homology with known corticotrophins. In view of the clinical importance of human ACTH it was decided to complete sequence studies in these regions, and we here present evidence confirming the proposed structure.

Materials and Methods

Materials

Partially purified tryptic fragments from natural human ACTH were kindly supplied by Dr. B. Riniker and Dr. W. Rittel (CIBA-Geigy Ltd., Basle, Switzerland). These fragments had been isolated by countercurrent distribution [composition: 1-butanol–acetic acid–water (4:1:5, by vol.)] as described by Sieber *et al.* (1972).

CM-cellulose (CMC 32) was obtained from Whatman Biochemicals Ltd., (Maidstone, Kent, U.K.).

Carboxypeptidase B (di-isopropyl phosphorofluoridate-treated) was purchased from Sigma (London) Chemical Co. Ltd. (London S.W.6, U.K.), and Rohm G.m.b.H. (Darmstadt, Germany) supplied the aminopeptidase M. This was treated before use with di-isopropyl phosphorofluoridate by the method of Hill & Smith (1957).

Trimethylamine was obtained from BDH

* Abbreviation: ACTH, adrenocorticotrophin.

Chemicals Ltd., (Poole, Dorset, U.K.) and *N*-ethylmorpholine was from Koch-Light Laboratories Ltd. (Colnbrook, Bucks., U.K.).

Purification of reagents

Pyridine, phenyl isothiocyanate, butyl acetate and trifluoroacetic acid were purified by the method of Lowry & Chadwick (1970). Chlorobutane was shaken overnight with aq. 5% (w/v) KMnO₄ and then washed with water. It was then dried with anhydrous Na₂SO₄ and redistilled. The fraction that boiled at 78°C was collected and stored at room temperature.

Trimethylamine was distilled at 4°C from anhydrous trimethylamine containing 10% (v/v) acetic anhydride and stored at 4°C as an aqueous solution (approx. 3M).

Purification of peptides

Fractionated microgranular CM-cellulose (McMartin & Vinter, 1969) was packed as a thick slurry in 0.5M-trimethylamine acetate buffer, pH 5, in a constant-bore glass-tubing column (0.6cm internal diam. × 15cm length). This was equilibrated with 50mM-trimethylamine acetate buffer, pH 5, before use.

All flow rates were controlled by Technicon proportioning pumps (Technicon Instruments Co., Chertsey, Surrey, U.K.).

A concave gradient was used to develop the CM-cellulose column. Typically this ran from 50mM- to 0.8M-trimethylamine acetate buffer (both pH 5). The mixing chamber was a 150ml beaker and the final buffer was contained in a 60ml conical flask, the two being connected by a syphon. The column flow rate was approximately 0.05ml/min and fractions were collected every 20min.

Peptides were detected in column effluents by

passing them through a refrigerated (4°C) Uvicord II u.v. absorptiometer (LKB Instruments Ltd.) set to record their extinction at 280nm. The Uvicord II was fitted with a 2.5cm path-length cell which was similar in construction to that described by McMARTIN *et al.* (1969) except that it had silica windows (H52/20mm; Jencons, Hemel Hempstead, Herts., U.K.) and these were sealed by means of Neoprene 'O' rings which fitted circular grooves cut in the ends of the Teflon body. To avoid the formation of air bubbles in

the cell the u.v. detector was kept at 4°C. The columns were run at room temperature.

Peptides which did not absorb at 280nm were detected with ninhydrin after t.l.c. of samples from the column effluent by a similar method to that of Sieber *et al.* (1972).

Sequence analysis

N-Terminal analysis of the tryptic fragments was carried out by the subtractive Edman-degradation

Table 1. *Amino acid compositions (molar ratios) of (a) corticotrophin-(1-8)-octapeptide, (b) corticotrophin-(9-15)-heptapeptide, (c) corticotrophin-(16-21)-hexapeptide, (d) corticotrophin-(17-21)-pentapeptide and of the residual peptides resulting from the stepwise subtractive Edman degradation of peptides a, b and c*

For experimental details see the text. N.D., Not determined because of breakdown during acid hydrolysis. At each step an *N*-terminal residue is removed and identification of the amino acid depleted between each successive step (indicated by *) therefore gives the sequence of the peptide.

(a) Residues 1-8

		Amino acid composition (mol/mol of peptide)						
		Ser	Glu	Met	Tyr	Phe	His	Arg
Whole peptide		1.5	1.1	0.9	1.0	1.1	1.0	1.0
		1.7	1.0	1.0	1.0	1.0	0.9	0.9
Subtractive Edman degradation:	Step 5	0.0	0.5*	0.2	0.1	1.0	0.8	1.1
	Step 6	0.0	0.2	0.1	0.1	0.8	0.4*	1.1

(b) Residues 9-15

		Amino acid composition (mol/mol of peptide)				
		Pro	Gly	Val	Lys	Trp
Whole peptide †		1.0	2.0	1.0	2.0	1.0
Subtractive Edman degradation:	Step 1	0.9	2.0	1.0	2.1	N.D.
	Step 2	0.9	1.1*	1.0	2.0	N.D.
	Step 3	0.8	1.0	1.1	1.1*	N.D.
	Step 4	0.0*	1.1	0.7	1.2	N.D.
	Step 5	0.0	1.1	0.3*	0.9	N.D.
	Step 6	0.0	0.0*	0.0	1.0	N.D.

(c) Residues 16-21

		Amino acid composition (mol/mol of peptide)			
		Pro	Val	Lys	Arg
Whole peptide		0.9	0.9	2.1	2.1
Subtractive Edman degradation:	Step 1	1.1	1.0	1.0*	1.8
	Step 2	1.2	1.0	0.9	1.0*
	Step 3	1.0	1.2	0.9	0.2*
	Step 4	0.0*	1.1	1.0	0.2
	Step 5	0.0	0.0*	1.0	0.1

(d) Residues 17-21

		Amino acid composition (mol/mol of peptide)			
		Pro	Val	Lys	Arg
Whole peptide		1.0	1.0	1.1	1.9

† Hydrolysis by Sepharose-bound enzymes (all others are acid hydrolysis).

method of Gray (1967) as modified by Lowry & Chadwick (1970). Where necessary butyl acetate was used instead of chlorobutane as a more polar organic extraction phase. Aminopeptidase M digestions were used for confirmation. These were performed in 0.03M-sodium phosphate buffer, pH 7.5, at 37°C. The peptide/enzyme ratio was either 40:1 or 200:1 (w/w).

C-Terminal analysis was carried out by using carboxypeptidase B with a peptide/enzyme ratio of 100:1 (w/w) in 0.2M-N-ethylmorpholine acetate buffer, pH 8.5, for various times at 37°C. Enzyme digestions were terminated by the addition of 0.1M-HCl and the solution analysed for amino acid content by using an AutoAnalyzer.

Peptide samples were either hydrolysed in 0.2ml of 6M-HCl in evacuated glass tubes for 16h at 110°C (with a crystal of phenol to prevent tyrosine loss, when necessary) or by digestion with Sepharose-bound peptidases as described by Bennett *et al.* (1972).

Amino acid analyses were performed on a Technicon AutoAnalyzer modified to give increased sensitivity. The modifications involved the use of a narrow-bore column (0.4cm internal diam. $\times$ 100cm) packed with Aminex A-6 resin (particle size $17.5 \pm 2 \mu\text{m}$; Bio-Rad Laboratories, Richmond, Calif., U.S.A.), and lower flow rates for the reagents.

Results and Discussion

Countercurrent-distribution fractions provisionally identified by amino acid analysis and t.l.c. as containing human ACTH tryptic fragments 1-8, 9-15, 16-21 and 17-21 were purified by ion-exchange chromatography on CM-cellulose as described in the Materials and Methods section.

The amino acid analyses of these purified fractions are shown in Table 1. The peptides containing labile amino acids were subjected to complete enzymic hydrolysis where indicated. Table 1 also shows the evidence for the sequence of these tryptic fragments obtained from subtractive Edman degradation.

Since the sequence of tryptic fragment 1-8 up to residue 5 is already known (Lee *et al.*, 1961), only subtractive Edman degradation steps 5 and 6 are shown. Although step 5 appears to be incomplete, the depletion of histidine on step 6 is reasonable evidence for its position at residue 6, in agreement with the assignment of phenylalanine to position 7 by carboxypeptidase B digestion.

Table 2 shows the results obtained from aminopeptidase M digestions of fragment 9-15 demonstrating the sequential release of tryptophan followed by glycine and lysine. This indicates that tryptophan is at the N-terminus and confirms the Edman-degradative results on the positions of glycine and lysine.

Table 2. Amounts of amino acids released after aminopeptidase M digestions of human corticotrophin-(9-15)-heptapeptide

Incubation conditions were: 0.03M-sodium phosphate buffer, pH 7.5; $\frac{1}{2}$ h; 37°C. Other details are given in the text.

Peptide/aminopeptidase M ratio (w/w)	Amino acids released (nmol)		
	Gly	Lys	Trp
200:1	1.5	0.3	2.8
40:1	4.4	1.1	4.7

Carboxypeptidase B digestion of three tryptic peptides gave the following release of amino acids: 1-8, after $\frac{1}{2}$ h digestion, Arg>Phe; 9-15, after 1h digestion, Lys; 16-21, after 1h digestion, Lys. This confirmed the C-terminal Phe-Arg sequence in the octapeptide and the presence of C-terminal lysine in the hexa- and hepta-peptides.

These results demonstrate that the sequences 6-7, 10-14 and 17-20 are as originally supposed and that the 1-24 region of ACTH is the same in all corticotrophins whose sequences have so far been determined.

In conjunction with the studies of Riniker *et al.* (1972) and Lee *et al.* (1961) the present investigations complete the chemical characterization of the primary sequence of human ACTH and provide convincing evidence for the following sequence:

Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro-Asn-Gly-Ala-Glu-Asp-Glu-Ser-Ala-Glu-Ala-Phe-Pro-Leu-Glu-Phe.

We thank Mr. E. J. Stevens for his assistance in the construction of the sensitive amino acid analyser, which was essential for these investigations.

References

- Bennett, H. P. J., Elliott, D. F., Evans, B. E., Lowry, P. J. & McMartin, C. (1972) *Biochem. J.* **129**, 695-701
- Gray, W. R. (1967) *Methods Enzymol.* **11**, 469-475
- Hill, R. L. & Smith, E. L. (1957) *J. Biol. Chem.* **228**, 577-600
- Lee, T. H., Lerner, A. B. & Buettner-Janusch, V. (1961) *J. Biol. Chem.* **236**, 2970-2974
- Lowry, P. J. & Chadwick, A. (1970) *Biochem. J.* **118**, 713-718
- McMartin, C. & Vinter, J. (1969) *J. Chromatogr.* **41**, 188-199
- McMartin, C., Simpson, P. & Thorpe, N. (1969) *J. Chromatogr.* **43**, 72-83
- Riniker, B., Sieber, P., Rittel, W. & Zuber, H. (1972) *Nature (London) New. Biol.* **232**, 114-115
- Sieber, P., Rittel, W. & Riniker, B. (1972) *Helv. Chim. Acta* **55**, 1243-1248