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Adrenocorticotropin. Synthesis of [6-Phenylalanine]-α^{1-19}-adrenocorticotropic Hormone and Its Steroidogenic, Melanocyte-Stimulating, and Lipolytic Activity†

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ABSTRACT: [6-Phenylalanine]-α^{1-19}-adrenocorticotropic hormone has been synthesized by the solid-phase method. A comparison of its steroidogenic, melanocyte-stimulating, and lipolytic activity to that of α^{1-19}-adrenocorticotropic hormone indicates that the replacement of histidine by phenylalanine results in a marked lowering of the biological activity.

A continuing effort in the study of structure and function of adrenocorticotropin has been directed toward determining the relative importance of each of the amino acid residues in the ACTH structure (Figure 1) for high steroidogenic activity. The results thus far indicate: (1) the carboxyl-half of the molecule plays no vital role in steroidogenic activity, and 50–100% activity can be obtained from the amino-terminal nonadecapeptide amide (Ramachandran *et al.*, 1965); (2) the basic core at positions 15–18 is important for full steroidogenic response, and a partial deletion of the basic residues (Ramachandran *et al.*, 1965) or their substitution with neutral residues (Brugger *et al.*, 1970) lowers activity; (3) an intact Lys-Pro-Val sequence is important for high activity (Blake and Li, 1967). Of the nine amino-terminal residues only arginine (Chung and Li, 1967; Tesser and Rittel, 1969) and tryptophan (Hofmann *et al.*, 1970) seem to be specifically required for high steroidogenic potency. Replacement of any of the first seven amino acid residues by amino acids with similar side chains gives peptides of comparable biological activity. Of particular interest to us was the report that the replacement of histidine with β-(pyrazolyl-3)-alanine in the amino-terminal eicosapeptide of ACTH[1] gave a peptide possessing 40–70% of the steroidogenic activity of the histidine peptide (Hofmann *et al.*, 1967, 1970). To gain further insight into the contribution of the histidine residue toward the steroidogenic and other activities of ACTH, the synthesis and biological assay of [6-phenylalanine]-α^{1-19}-ACTH were carried out; results of these studies are herein reported.

[6-Phenylalanine]-α^{1-19}-ACTH (I) was synthesized by the solid-phase method (Merrifield, 1964) as described for the synthesis (Blake *et al.*, 1972) of α^{1-19}-ACTH (II). Amino acids were coupled as their N^{α}-Boc derivatives throughout the synthesis; side-chain protecting groups were as reported previously. Treatment of the peptide-resin with liquid HF (Sakakibara *et al.*, 1967; Lenard and Robinson, 1967), followed by chromatography of the crude peptide on Sephadex G-25 and carboxymethylcellulose (Peterson and Sober, 1956), gave peptide I (Figure 2). The analytical data of peptide I were as expected for [6-phenylalanine]-α^{1-19}-ACTH (see Experimental Section).

The biological properties of peptides I and II are listed in Table I. The data indicate that replacement of histidine by phenylalanine has a profound effect on the biological activity. The steroidogenic and melanocyte-stimulating activities of peptide I are reduced to approximately 1 and 3%, respectively, of the activities of peptide II. It is of interest that the lipolytic activity of the phenylalanine analog in isolated fat cells of rats and rabbits is very much lower than that expected from its steroidogenic potency. Thus, the histidine residue plays an important role for the function of ACTH. The chemical differences between these amino acids are that the aromatic ring in histidine is smaller than the aromatic ring in phenylalanine and that histidine contains the imidazole ring which may form a hydrogen bond with the receptor in the membrane of the adrenal gland. Whether such an interaction exists remains to be determined.

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[1] Abbreviations used are: ACTH, adrenocorticotropic hormone; Boc, *tert*-butyloxycarbonyl. All the asymmetric amino acids occurring in the peptides mentioned in this paper are of the L configuration.

TABLE I: Biological Activity[a] of [Phe^6]-α^{1-19}-ACTH and α^{1-19}-ACTH.

	In Vivo Steroidogenesis (Units/mg)	*In Vitro* Melanocyte-Stimulating Act. (Units/g)	*In Vitro* Lipolysis[b]	
			Rabbit	Rat
α_s-ACTH	100	1×10^8		
α^{1-19}-ACTH	92 (76–122) [3]	1.0×10^8 ($(0.3–1.3) \times 10^8$) [3]	1.7×10^{-8} [3]	4.3×10^{-10} [2]
[Phe^6]-α^{1-19}-ACTH	1.3 (0.6–2.3) [3]	3.0×10^6 ($(0.9–6.2) \times 10^6$) [3]	$>1.4 \times 10^{-5}$ [3]	1.5×10^{-5} [2]

[a] Numbers in parentheses represent the 95% confidence limits; numbers in brackets represent the number of assays. [b] Concentration, moles/liter, at one-half maximal response.

Experimental Section

Seryltyrosylserylmethionylglutamylphenylalanylphenylalanylarginyltryptophylglycyllysylprolylvalylglycyllysyllysylarginylarginylproline (*I*). Peptide I was synthesized as described for α^{1-19}-ACTH (Blake *et al.*, 1972). The starting Boc-prolyl-resin (derived from Bio-beads S-X-I, 200–400 mesh, purchased from Bio-Rad Laboratories, Richmond, Calif.) contained 0.19 mmole of proline/g of resin. Amino acids were coupled exclusively as their N^{α}-Boc derivatives. Side chains were protected as follows: serine, *O*-benzyl; tyrosine, *O*-benzyl; glutamic acid, γ-benzyl ester; arginine, N^{G}-tosyl; lysine, N^{ϵ}-benzyloxycarbonyl.

A portion of the dried nonadecapeptide-resin (0.75 g, 0.094 mmole) was treated with 12 ml of liquid HF containing 2.1 ml of anisole for 1 hr at 0°. After evaporation of the HF at 0°, the resin was dried and stirred with 7 ml of trifluoroacetic acid for 5 min. Filtration of the mixture and washing of the resin with trifluoroacetic acid gave a filtrate that was evaporated to dryness. The residue was distributed between 30 ml of 0.2 N acetic acid and 15 ml of ether. The aqueous layer was further washed with two 10-ml portions of ether and evaporated to a volume of 5–10 ml. Chromatography on Sephadex G-25 (2.5 × 133 cm, 0.5 N acetic acid) and isolation of the material corresponding to the major peak (elution volume, 396 ml) gave 97 mg of crude peptide I. Chromatography on carboxymethylcellulose (Figure 2), followed by rechromatography of the major peak gave 28 mg of peptide I (peptide content 81% as determined by ultraviolet spectrum, 10% yield based on starting Boc-prolyl-resin).

Amino acid analysis (Spackman *et al.*, 1958) of an acid hydrolysate of peptide I gave $Lys_{3.1}Arg_{3.0}Ser_{1.7}Glu_{1.0}Pro_{1.9}Gly_{2.0}Val_{1.0}Met_{1.0}Tyr_{0.9}Phe_{2.0}$. Measurement of the ultraviolet spectrum in 0.1 N sodium hydroxide (Beaven and Holiday, 1952) gave Tyr:Trp ratio of 1.03. Paper electrophoresis in pyridine–acetate buffer (pH 3.7, 400 V, 3 hr) gave one ninhydrin-positive, Pauly-positive spot at R_F 0.76 (relative to lysine). Paper electrophoresis in collidine–acetate buffer (pH 6.9, 400 V, 3 hr) gave one ninhydrin-positive, Pauly-positive spot at R_F 0.62. Electrophoresis on polyacrylamide gel at pH 4.5 showed one band.

To 0.5 mg of peptide I in 0.3 ml of Tris buffer (pH 8.4, 0.01 M Mg^{2+}) was added 10 μg each of trypsin and chymotrypsin, and the mixture was incubated at 37° for 24 hr. The digest was then heated in boiling water for 15 min, cooled, and treated with 20 μg of leucine aminopeptidase for 46 hr at 37°. Amino acid analysis gave $Ser_{2.0}Tyr_{1.0}Met_{0.9}Glu_{1.0}Phe_{2.0}Trp_{1.0}Gly_{1.2}Arg_{2.6}Lys_{1.7}Pro_{0.6}Val_{0.9}$. The low values of the amino acids located in the carboxyl-half of the molecule are due to the well-known resistance to enzymatic digestion of peptide bonds involving proline.

H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-
1 5 10

Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val
15 20

Lys-Val----------Pro-Leu-Glu-Phe-OH
22 36 39

FIGURE 1: Structure of adrenocorticotropins.

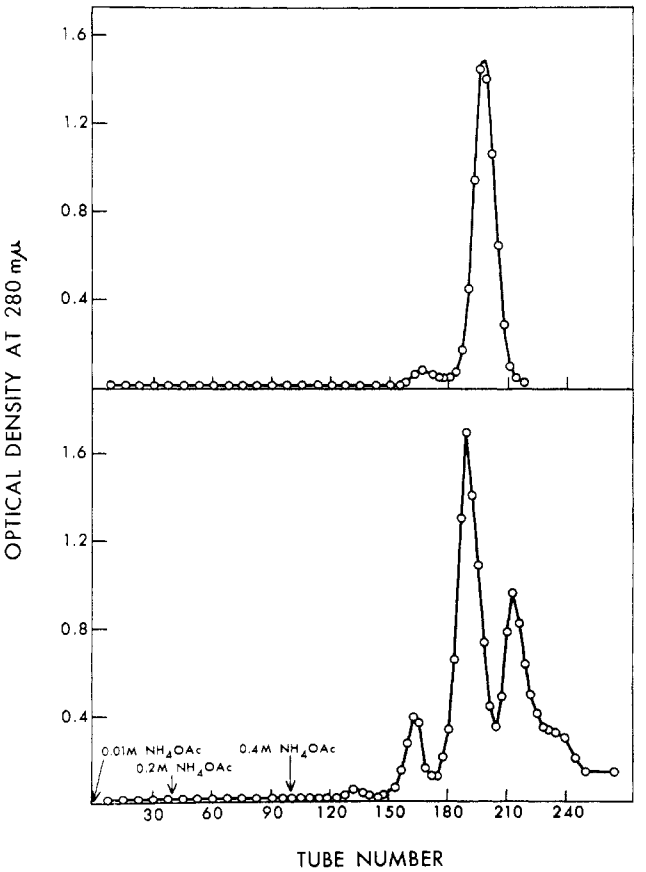

FIGURE 2: Lower: carboxymethylcellulose chromatography (column, 1.0 × 55 cm) of crude nonadecapeptide I. The initial buffer was 0.01 M ammonium acetate (pH 4.5). After 40 tubes (4 ml/tube), a gradient with respect to pH and salt concentration was started by introducing 0.2 M ammonium acetate buffer of pH 6.7 through a 500-ml mixing flask containing starting buffer. Later the gradient was increased by substituting 0.4 M ammonium acetate at the indicated position. Upper: rechromatography of the major peak under the same conditions.

Steroidogenic activity of peptide I was determined by the method of Vernikos-Danellis *et al.* (1966). The *in vitro* melanotropic activity was estimated by the procedure of Shizume *et al.* (1954). The isolated fat cells from rabbit and rat adipose tissues were used for lipolytic assay by the method of Ramachrandran and Lee (1970). Results are summarized in Table I. Highly purified sheep ACTH, obtained by the method previously described (Birk and Li, 1964), was used as the reference standard.

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Molecular Weight and Subunit Structure of Hagfish Transferrin†

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ABSTRACT: The molecular weight and subunit structure of hagfish transferrin was studied by sedimentation equilibrium, sedimentation velocity, determination of iron-binding capacity, sodium dodecyl sulfate–gel electrophoresis, and gel chromatography in a denaturing medium. Contrary to a previous report, the protein was found to consist of a single polypeptide chain of molecular weight in the range 75,000–80,000. Two similar or identical iron-binding sites, with optical and electron paramagnetic resonance spectra closely resembling those of human transferrin, are present in the native protein.

The transferrins comprise a class of proteins characterized by the ability to bind specifically, tightly, and reversibly a variety of transition metal ions of which the most important is Fe(III). Most studies of transferrins isolated from a variety of physiologic fluids and species have shown that they consist of single polypeptide chains of mol wt 75,000–80,000 (Green and Feeney, 1968; Querinjean *et al.*, 1971; Mann *et al.*, 1970), on which two identical binding sites are disposed (Aasa *et al.*, 1963; Aisen *et al.*, 1966). This has generated speculation about the possibility of gene duplication during the evolution of the proteins.

Recently Palmour and Sutton (1971) have reported that transferrin isolated from the serum of the California hagfish, a primitive vertebrate, has a molecular weight of only 45,000 and a single binding site for Fe(III). Because of the potential importance of this finding in the evolutionary biochemistry of proteins we have undertaken a further investigation of the molecular weight and subunit structure of hagfish transferrin.

Experimental Procedures

Freshly frozen hagfish serum was obtained in two separate batches from Pacific Biomarine Supply Co., the source used by Palmour and Sutton (1971). Transferrin was separately isolated from each batch by the method described by Palmour and Sutton (1971), using ^{59}Fe as a radioactive tracer during the isolation procedure. Over 98% of the counts recovered after the initial gel filtration step were present in the fraction used for the preparation of transferrin by ion-exchange chromatography. The final yield of purified protein in each preparation was about 1 mg/ml of serum. The optical

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