Structure of Porcine Thyrotropin Releasing Hormone*

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ABSTRACT: The structure of thyrotropin-releasing hormone of porcine origin has been systematically investigated by a series of degradation reactions. *N*-Bromosuccinimide cleavage followed by the dansyl reaction and Edman degradation revealed a C-terminal prolyl end group, preceded by the histidyl moiety. Mild alkaline hydrolysis and subsequent dansyl reaction proved the N-terminal residue to be (pyro)-glutamic acid. The fragmentation patterns in the mass spectra of free as well as permethylated thyrotropin-releasing hormone supported the Glu-His-Pro sequence for the constituent amino acids. Methanolysis and hydrazinolysis indicated that an acyl substituent is unlikely to be present at the N-terminus. The possibility of substituents at the N- or C-terminus was also investigated by partial acid hydrolysis, followed by separation of the products by thin-layer chromatography and their spectroscopic characterization.

The results obtained support L-(pyro)Glu-L-His-L-Pro-NH_2 as being the structure of the porcine thyrotropin-releasing hormone.

In previous reports from this laboratory (Schally *et al.*, 1966; Schally *et al.*, 1969), we have described the isolation from porcine hypothalami of a neurohormone, designated thyrotropin-releasing hormone (TRH),[1] which controls the secretion of thyrotropic hormone from the anterior pituitary gland (Schally *et al.*, 1968). We have also reported its amino acid sequence (Schally *et al.*, 1969) and described a synthetic tripeptide with biological activity equivalent to that of natural TRH (Folkers *et al.*, 1969; Bøler *et al.*, 1969). This synthetic work was based on the modification of the N- and C-terminus of the tripeptide Glu-His-Pro, since the free peptide has no TRH activity (Schally *et al.*, 1968). Burgus *et al.* (1969a,b) have also reported synthetic derivatives of Glu-His-Pro with TRH activity.

TRH occurs in the hypothalamus only in very minute quantities and systematic structural studies on the pure hormone, therefore, were restricted by the amount of material available (Schally *et al.*, 1966). However, our recent efforts in the isolation of TRH from nearly a quarter of a million porcine hypothalami (Schally *et al.*, 1969) have furnished the necessary quantity of the hormone for the elucidation of its structure. This communication reports the systematic degradation work on porcine TRH, which paralleled the successful formulation of its structure by synthesis.

Experimental Procedure

Materials. TRH, isolated from porcine hypothalami and which was found active in doses of 10 pg *in vitro* and 1 ng *in vivo* (Schally *et al.*, 1966, 1968, 1969; Schally and Redding, 1967; Bowers *et al.*, 1967), was used in the degradation studies. Homogeneity tests on this TRH, by thin-layer chromatography and thin-layer electrophoresis, revealed one spot which was negative to ninhydrin and positive to Pauly's reagent, chlorine–*o*-tolidine reaction, and iodine vapor (Schally *et al.*, 1969).[2]

1-Dimethylaminonaphthalene-5-sulfonyl chloride (DNS-Cl) was obtained from Calbiochem and *N*-bromosuccinimide (NBS) from Nutritional Biochemical Corp. Phenyl isothiocyanate (PITC) purchased from Matheson Coleman and Bell was redistilled (71°(1.5 mm)) before use. Silver oxide was prepared essentially by the method described by Thomas *et al.* (1968), and dried *in vacuo* over P_2O_5. Picryl chloride was prepared as suggested by Pollard *et al.* (1956), and stored in a desiccator. Anhydrous hydrazine, obtained from Aldrich Chemicals, was redistilled from KOH under reduced pressure and stored dry for use. *t*-Butyl hypochlorite and *o*-tolidine, used in the chlorine–*o*-tolidine reaction, were obtained from Mann Laboratories and Aldrich Chemicals, respectively. Anyhydrous trifluoroacetic acid was purchased from MCB.

MN 300 HR-cellulose and silica gel GF-254 for thin-layer chromatography were from E. Merck, Darmstadt, W. Germany. The cellulose powder was washed repeatedly with 1 N acetic acid and distilled water before use.

Mass spectra were recorded on a Hitachi–Perkin–Elmer Model RMU-4 instrument at different temperature settings and at a 0 to 900 mass range. Both Varian A-60 and 100 MHz nuclear magnetic resonance spectrometers were used for recording the nuclear magnetic resonance spectra.

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[1] This substance was formerly called thyrotropin-releasing factor (TRF). However a new nomenclature proposed by Schally *et al.* (1968) will now be used.

[2] This porcine TRH preparation was estimated to contain 33% of the amino acid material (Schally *et al.*, 1969). However, since no correction was made for recovery of amino acid after hydrolysis on a microscale, the actual amino acid content was about 50–60% (C. Y. Bowers *et al.*, unpublished). The remainder of the weight was accounted for by inert impurities derived from Nalgon tubing (Schally *et al.*, 1969), moisture, bound acetate and filter paper fibres, which were at first erroneously assumed to contribute little to the original weight (A. V. Schally and C. Y. Bowers unpublished).

TABLE I

Amino Acid Fragments	*m/e*	Others	*m/e*
Free TRH at 245°			
Prolyl less one =CO	70	CH_3COO	59
Prolyl	97	CH_3CO	43
(Pyro)Glu	112		
Pyrrolidone ring	84		
Imidazole ring with a CH_2 side chain	81, 82		
His	137		
Diketopiperazine of histidine and proline	234		
Permethylated TRH at 175°			
Parent molecular ion of the pentamethyl derivative (M^+)	432		
Prolyl	97		
Methyl-His	150		
Dimethyl-His	165		
(Pyro)Glu	111		
$N(CH_3)_2$	44		
CH_3	15		
TRH after Methanolysis and Permethylation			
Parent molecular ion (M^+)	419		
Prolyl ester	128		
Methyl-His	150		
Dimethyl-His	165		
(Pyro)Glu	111		
CH_3	15		

Methods

Cleavage with NBS was by the method of Shaltiel and Patchornik (1963) and the subsequent dansyl reaction and Edman degradation were carried out essentially by the method described by Gray (1967), with slight modification. Thus 80 μg of TRH was mixed with 90 μl of a solution of NBS (0.003 M) in pyridine acetate [pyridine–acetic acid–water (1:10:19)], sealed under nitrogen, and kept at 23° for 30 min at first and subsequently at 100° for 1 hr. The solvents were evaporated *in vacuo* over NaOH and P_2O_5 and redissolved in 200 μl of 50% aqueous pyridine. One-half of this solution was transferred to another tube, dried *in vacuo*, and subjected to the dansylation procedure. The other half was used for the Edman degradation, first by reaction with PITC (5%, in pyridine) and then reaction with trifluoroacetic acid, followed by reaction with DNS-Cl. The dansyl amino acids were identified by thin-layer chromatography on silica gel GF, using the solvent system chloroform–ethanol–acetic acid (38:4:3) as developing agent.

TRH (15 μg) was kept in contact with 1 M NaOH (50 μl) at 23° for 65 hr, dried *in vacuo*, neutralized with 100 μl of 0.5 M acetic acid, and dried again. This was then redissolved in 50 μl of 0.2 M $NaHCO_3$ and mixed with 50 μl of DNS-Cl solution (2.5 mg/ml of acetone). The reaction mixture was kept in the dark for 3 hr and dried *in vacuo*, and 50 μl of 6 N HCl was added. The tube was sealed and the hydrolysis conducted at 100° for 12 hr. The excess HCl was removed *in vacuo* over NaOH and the hydrolysate chromatographed on silica gel GF (250 μ layer), using the solvent system chloroform–ethanol–acetic acid (38:4:3) (Dekker *et al.*, 1949; Battersby and Robinson, 1961; Blomback and Doolittle, 1963; Ikenaka and Schmid, 1965).

Permethylation of TRH and mass spectrometry were carried out by the methods previously described by Agarwal *et al.* (1968, 1969) and Das *et al.* (1967), using a 30-μg sample for each experiment.

Hydrazinolysis of TRH was performed essentially by the method previously described by Blomback *et al.* (1966). The dry hydrazides were dissolved in ethanol and chromatographed on silica gel GF, with a solvent system 1-butanol–pyridine–acetic acid–water (30:20:6:24, v/v). The spots were revealed by spraying with either 0.5% ethanolic picryl chloride (Schmer and Kreil, 1969) and exposure to ammonia or 1-butanol saturated with ammoniacal silver nitrate (Dawson *et al.*, 1959). Hydrazinolysis was repeated after methanolysis of TRH with 2 N HCl in absolute methanol at 23° for 12 hr.

TRH was subjected to methanolysis with 2 N HCl in absolute methanol in a sealed tube at 100° for 4 hr (Blomback *et al.*, 1966). After removal of excess HCl and methanol *in vacuo* over NaOH, the products were chromatographed on cellulose (250 μ, layer) with 1-butanol–ethyl acetate–acetic acid–water (1:1:1:1, v/v) as solvent. The spots were located by ninhydrin.

TRH (200 μg) was subjected to partial acid hydrolysis in a sealed tube using 2 N HCl at 100° for 4 hr. After drying over NaOH *in vacuo*, the hydrolysate was separated by thin-layer chromatography on acid-washed cellulose, using the system 1-butanol–ethyl acetate–acetic acid–water (1:1:1:1, v/v). The spots were located by iodine vapor and/or chlorine–*o*-tolidine reaction. Four components were subsequently eluted from a parallel channel on the chromatogram using methanol for the iodine positive spots and 0.1 M acetic acid for the chlorine–tolidine positive spots. After drying *in vacuo*, these fractions were used for nuclear magnetic resonance studies on a Varian A-60 spectrometer using a micro nuclear magnetic resonance tube. The solvents used were $CDCl_3$ for spots I and II which were eluted by methanol. For the acetic acid soluble spots (III and IV) a 1:1 mixture of D_2O and CD_3OD was used as the solvent. Tetramethylsilane was used as an internal standard. The nuclear magnetic resonance spectrum of TRH also was taken under similar conditions.

Results

NBS cleavage and the subsequent Dansyl–Edman procedure yielded DNS-Pro at the first step after hydrolysis. No dansyl amino acids were obtained at the subsequent degradation step.

Treatment of TRH with mild alkali under the conditions specified for the opening of the pyrrolidone ring (Blomback and Doolittle, 1963), followed by dansylation, hydrolysis, and identification by thin-layer chromatography, revealed dansyl-glutamic acid.

The fragmentation pattern of free TRH when subjected to mass spectrometry at 245° was as seen in Table I.

The fragmentation of free TRH did not proceed in an orderly fashion and it was not possible to obtain the parent

molecular ion, even after increasing the temperature to 300°, when pyrolysis occurred. However intense peaks were found at *m/e* 70, pyrrolidine ring (prolyl decomposition product); 97 (prolylcarbonyl); 67 (histidyl ring); 82 (histidyl ring with the CH_2 side chain); 137 (His); 84 (pyrrolidone ring); 170 (acetate of (pyro)glutamyl ring); and 59 (acetate ion).

A fast scan of the permethylated TRH gave a molecular ion of very weak intensity at *m/e* 432. TRH after methanolysis and permethylation showed a different molecular ion at 419 mass units. Peaks contributed by (pyro)glutamic acid (*m/e* 111), methyl-substituted histidine (*m/e* 150 and 165), and methyl groups were common for both derivatives of TRH. Permethylated TRH gave a peak at *m/e* 44 in the mass spectrum, showing the loss of $-N(CH_3)_2$. This was not observed in the other derivative which exhibited a peak due to the prolyl ester at 128 mass units. The infrared spectrum of TRH showed a broad, intense peak at 1675 cm^{-1} and 12 other peaks.

The hydrazinolysis experiments were carried out on TRH under different conditions to identify any substituent at either end of the peptide, but revealed only a single hydrazide equal in R_F value to that of an authentic sample of (pyro)-glutamyl hydrazide.

The hydrolysis of TRH with 2 N HCl followed by thin-layer chromatography gave 4 fragments: I and II were not Pauly or chlorine–tolidine positive, but were located by iodine vapor; III and IV could be revealed by iodine, but were also Pauly and chlorine–*o*-tolidine positive. A nuclear magnetic resonance spectrum of I in a micro tube with tetramethylsilane as internal standard and $CDCl_3$ solvent did not show any appreciable peaks. However the spectrum of II gave a weak methyl triplet at τ 8.9 ppm and an acid proton signal at a sweep offset of 245. The nuclear magnetic resonance spectrum of free TRH also gave a methyl triplet at τ 8.8 ppm and other signals at 6.8, 2.95, 2.2, and 3.85 ppm as broad multiplets. No acid protons were found in free TRH, at 0 to 300 sweep offset.

Discussion

The sequential degradation of porcine TRH confirmed that the N- and C-terminal residues are blocked in the molecule. NBS is known to effect specific cleavage of a peptide linkage which contains at its γ,δ position a double bond as in a histidyl peptide. After NBS-cleavage and dansylation of TRH, DNS-Pro was obtained in the first stage. This proves the rupture of the His-Pro link by NBS, and the liberation of the prolyl moiety. This degradation reaction establishes the His-Pro sequence in TRH. Mild alkaline hydrolysis of TRH opened the pyrrolidone carboxylyl ring, liberating the primary amino group of the N-terminal (pyro)-glutamic acid, which was then revealed by dansylation (Blomback and Doolittle, 1963). This evidence suggests that (pyro)glutamic acid is the N-terminal residue in TRH. The occurrence of (pyro)glutamyl residues has been reported in peptides like gastrin (Gregory *et al.*, 1964), fibrinopeptides (Blomback *et al.*, 1966), and eledoisin (Erspamer and Anastasi, 1962). The infrared spectrum of TRH shows an intense peak at 1675 cm^{-1}, proving the pyrrolidone carbonyl absorption (Barrass and Elmore, 1957).

The mass spectra of TRH were helpful in confirming the amino acid sequence of TRH. The fragmentation of free TRH indicated intense peaks characteristic of (pyro)glutamyl, histidyl, and prolyl residues and their degradation products (R. M. G. Nair and A. V. Schally, unpublished). A group of peaks at *m/e* 59 could not be accounted for by any one of the constituent amino acids, (pyro)Glu, His, or Pro. This may presumably be due to the existence of the TRH molecule as the acetate, derived by the salt-forming property of the electrophilic –NH group situated in the (pyro)glutamyl ring.

Mass spectra after permethylation revealed an orderly fission exhibiting the amino acid sequence with (pyro)glutamic at the N-terminus. There is no appreciable change in the splitting pattern of the (pyro)glutamyl end of both free TRH and its permethylation product. The permethylated TRH gave evidence of the $CON(CH_3)_2$ grouping at the carboxyl end of proline, during mass spectral fragmentation. But TRH after methanolysis and permethylation exhibited a peak due to the prolyl methyl ester in the mass spectrum. This proved the existence of a prolylamide C-terminus in the TRH molecule. The methanolysis of TRH hydrolyzed the prolylamide end with subsequent esterification. The prolyl ester was identified in the mass spectrum.

Hydrazinolysis did not reveal any acyl hydrazides in the chromatogram. This renders unlikely the possibility of any acyl substituent at either end of the blocked tripeptide. However (pyro)glutamyl hydrazide was identified on the chromatogram, exhibiting an R_F value equivalent to that of an authentic sample. This proves that the γ-carboxylic acid of the N-terminal glutamic acid is not free to react with the hydrazine, under the conditions of hydrazinolysis. The (pyro)glutamyl N-terminus remains intact under the influence of anhydrous hydrazine, while its α-carboxyl end involved in the peptide link preferentially opens with the formation of the α-hydrazide which is revealed as one of the reaction products.

Methanolysis at elevated temperature (100°) brings about an appreciable change in the TRH molecule. The product moves faster on a cellulose plate than TRH itself, and is ninhydrin positive, showing that the (pyro)glutamyl end as well as the prolyl amide group have been attacked by the acid and that presumably esterification has taken place. It is known that the ester and the primary amino group in a peptide increase the mobility on thin-layer chromatography in these solvent systems. Partial hydrolysis of TRH with 2 N acid also reveals minor iodine positive spots. One of these spots (II) exhibits a methyl triplet during nuclear magnetic resonance characterization.

The degradation studies on natural TRH, therefore, give evidence for a Glu-His-Pro sequence for the molecule, with a (pyro)glutamyl N-terminal end and prolylamide C-terminus. The presence of a substituent at the (pyro)glutamyl or at the prolylamide end cannot be fully excluded from the negative results in the hydrazinolysis and methanolysis experiments. Similarly the mass spectrometric fragmentation studies do not altogether exclude the possibility of a substituent. The methyl triplet obtained while scanning the nuclear magnetic resonance spectrum of free TRH and also the no. II fragment of its acid hydrolysate does not yet fit in with the other findings which corroborate the L-(pyro)Glu-L-His-L-Pro-NH_2 structural pattern for the TRH molecule. It is probable that the methyl signal in the nuclear magnetic resonance spectrum and the peak of 59 mass

units were due to impurities present in the TRH preparation or to bound acetate.

L-(Pyro)-Glu-L-His-L-Pro-NH_2 has been synthesized recently (Bøler *et al.*, 1969; Burgus *et al.*, 1969b; G. R. Flouret, unpublished results). The R_F values of natural TRH of porcine origin and synthetic L-(pyro)Glu-His-Pro-amide were identical in 17 chromatographic systems (Bøler *et al.*, 1969). The biological activity of the synthetic L-(pyro)Glu-L-His-L-Pro-NH_2 of Bøler *et al.* (1969) and G. R. Flouret (unpublished) was determined to be equivalent to that of natural porcine TRH (Schally *et al.*, 1970). However Burgus *et al.* (1969a,b) reported that their synthetic preparation had only one-fifth to one-third the activity of natural ovine TRH. This discrepancy has not been resolved, but the available synthetic evidence supports L-(pyro)Glu-L-His-L-Pro-NH_2 as corresponding to the structure of porcine TRH, or at least possessing the structural requirements for full biological activity.

Acknowledgment

We wish to thank Professor Karl Folkers, Director of the Biomedical Research Institute, University of Texas, Austin, Texas, for stimulating exchange of information and editorial suggestions and Dr. W. F. White who supplied L-(pyro)-Glu-L-His-L-Pro-NH_2, synthesized by G. R. Flouret at Abbott Laboratories.

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