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Site of Inactivation of Thyrotropin-Releasing Hormone by Human Plasma*

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ABSTRACT: Thyrotropin-releasing hormone (TRH) (synthetic, L-(pyro)Glu-L-His-L-Pro-NH_2) and its ^{14}C analog (L-(pyro)-Glu-L-[^{14}C]His-L-Pro-NH_2) after incubation with human plasma at 37° for 1 hr showed a 72% decrease in activity. After the recovery and purification, the peptide obtained exhibited different chromatographic and electrophoretic mobilities from TRH, but the amino acid composition was identical. The recovery of the hormone after incubation in terms of either amino acid analysis or radioactivity was 40–50%. An analogous experiment at 0° did not inactivate TRH and the recovery was 60%. The recovery of 40–50% of radioactivity from the purified Pauly-positive, inactive peptide showed that histidine is not cleaved away during incubation. Inactivated TRH did not show a free N terminus by Edman-dansyl procedure suggesting that the (pyro)glutamyl ring is also intact. On treatment with [^{14}C]diazomethane, the inactivated TRH was quantitatively converted into a [^{14}C]methyl ester, indicating a free C terminus. Inactivated TRH was compared chromatographically and electrophoretically to synthetic (pyro)Glu-His-Pro-OH and found to have the same mobilities. These suggest that during incubation with plasma, TRH undergoes cleavage of the amide group at the prolyl end which is probably the site of inactivation.

Previous reports from this laboratory (Nair *et al.*, 1970; Schally *et al.*, 1970) described the elucidation of the structure of thyrotropin-releasing hormone (TRH),[1] which controls the secretion of the thyrotropic hormone (TSH) from the anterior pituitary gland (Schally *et al.*, 1968). Determination of the structure of TRH as L-(pyro)Glu-L-His-L-Pro-NH_2 was paralleled by its successful synthesis (Bøler *et al.*, 1969; Flouret, 1970). The comparison of the chemical, spectroscopic (Nair *et al.*, 1970; R. M. G. Nair and A. V. Schally, in preparation[2]), and biological (Schally and Bowers, 1971; Bowers *et al.*, 1970a,b) properties of the synthetic L-(pyro)Glu-L-His-L-Pro-NH_2 and the natural porcine TRH proved that they are identical. The structure of ovine TRH was simultaneously shown by Burgus *et al.* (1969, 1970) to be also (pyro)Glu-His-Pro-NH_2. In the course of our previous investigations on the biological activity of TRH, we have undertaken a series of studies on inactivation of TRH by animal and human plasma (Redding and Schally, 1969). After the correct molecular structure of this neurohumor became known to us, it was thought that the rapid inactivation of TRH observed during its incubation with plasma needed further investigation as to how and at what particular site the inactivation occurred. An insight into the "site of inactivation" could shed more light on the metabolism and mechanism of action of this hormone which is now being extensively used in clinical studies (Bowers *et al.*, 1970a,b; Fleischer *et al.*, 1970; Herschman and Pittman, 1970).

Materials

Synthetic L-(pyro)Glu-L-His-L-Pro-NH_2, (pyro)Glu-His-Pro-OH, His-Pro-NH_2, His-Pro-NH_2-HCl, (pyro)Glu-His, and L-(pyro)Glu-L-[^{14}C]His-L-Pro-NH_2 (specific activity 0.7 mCi/mg) were obtained from Abbott Laboratories. The syn-

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[1] Abbreviations used are: TRH, thyrotropin-releasing hormone; TSH, thyrotropin-secreting hormone; DNS-Cl, dimethylaminonaphthalenesulfonyl chloride; PITC, phenyl isothiocyanate.

[2] A paper on the nmr and mass spectroscopic comparison of the free and derivatized porcine TRH and L-(pyro)Glu-L-His-L-Pro-NH_2 is sent to *Biochemistry*.

TABLE I: Results of TRH Bioassay on Rats.

Group	Description of Sample	Dose/(ng)	Δcpm ^{125}I ± Std Dev	Decrease in Act. (%)
1	TRH after incubation with plasma at 37°, 1 hr	10.0	643 ± 171	72.1
2	TRH + saline at 37°, 1 hr	10.0	2293 ± 219	
3	[^{14}C]Histidyl TRH after incubation at 37° for 1 hr	10.0	649 ± 178	75.2
4	Above with saline at 37° for 1 hr	10.0	2501 ± 223	
5	Serum control	10.0	−33 ± 31	
6	Saline		0.6 ± 56	
7	TRH incubation at 0° with plasma for 1 hr	10.0	5106 ± 285	17.0
8	TRH + saline at 0°, 1 hr	10.0	6175 ± 435	
9	TRH synthetic	2.5	1351 ± 428	
	TRH synthetic	5.0	2505 ± 550	
10	TRH synthetic	10.0	6294 ± 1107	

thetic TRH and the [^{14}C]histidyl TRH were found active in doses of 1 ng *in vivo* (Redding and Schally, 1971) and were used for the incubation experiments. Human plasma was prepared from freshly collected blood. *N*-Methyl-[^{14}C]*N*-nitroso-*p*-toluenesulfonamide ([^{14}C]diazald, 5 mCi/mmole) was purchased from New England Nuclear Corp. Dimethylaminonaphthalenesulfonyl chloride (DNS-Cl) was obtained from Calbiochem and trichloroacetic acid phenyl isothiocyanate (PITC) from Matheson Coleman & Bell.

Microcrystalline MN-300 HR-cellulose for tlc was from E. Merck Darmstadt. Precoated "Polygram" Polyamide sheets were purchased from Brinkmann Instruments Inc.

The radioactivity was measured in a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co.). Thin-layer electrophoresis (tle) was conducted in a Brinkmann-Desaga tle apparatus.

Methods

Incubation Experiments. TRH (300 μg) was incubated in undiluted, fresh human serum (5 ml) at 37° for 1 hr. Aliquots of this were used for bioassay of TRH which was carried out essentially by the method of Redding and Schally (1969). A saline control with the same amount of TRH, but without the plasma, and plasma control without TRH were incubated simultaneously at 37°. Analogous experiments were also conducted at 0°. Incubation experiments under the same conditions were also performed, utilizing (pyro)Glu-[^{14}C]His-Pro-NH_2 ([^{14}C]histidyl TRH), and the radioactivity and biological activity were determined after recovery.

Recovery of TRH. After the incubation the plasma and saline samples were immediately treated with 2 ml of 5% aqueous trichloroacetic acid until precipitation was complete. The residue was separated after centrifugation. Clear supernatants were washed four times with 15-ml portions of redistilled diethyl ether to remove trichloroacetic acid and then lyophilized. The lyophilisate was then extracted three times with 5-ml portions of absolute methanol and filtered through Whatmann No. 541 filter paper. The filtrate was evaporated *in vacuo* and the materials thus obtained were further purified by tlc on prewashed cellulose (250-μ layer) using either 1-butanol–acetic acid–water (4:1:5, v/v) or pyridine–acetic acid–1-butanol–water (10:3:15:12, v/v) as the solvent systems (Nair *et al.*, 1971a). The peptide spots were revealed by Pauly's reagent (Bruce and Mitchell, 1952). Corresponding spots from parallel channels were cut out and eluted. The radioactivity was then counted and biological activity determined. The amino acid analyses on aliquots of this material were carried out in a Beckman Model 120C analyzer. The samples for analyses were hydrolyzed in 6 N HCl for 22 hr at 110° in sealed, evacuated tubes. Edman-dansyl sequential analyses were also carried out (Hartley, 1970).

The digestions with leucine aminopeptidase (Worthington, LAP 5926) and aminopeptidase M (Rohm and Haas, Darmstadt) were carried out in 0.1 M ammonium acetate buffer (pH 8.1). Subsequently the analyses were performed for free amino acids liberated during the digestion.

Tle was conducted on glass plates coated with cellulose (250 μ layer), in pyridine–acetate buffer (pH 4.5), at 310 V, 6 mA, 10° for 3–4 hr. The peptide spots were revealed with Pauly's reagent.

Labeling with [^{14}C]Diazomethane. Ethereal [^{14}C]diazomethane was prepared from [^{14}C]diazald in a special apparatus set up for distillation under controlled conditions.[3] This ^{14}C-labeled diazomethane was standardized by titration using peptides containing known quantities of free COOH groups. The standardized [^{14}C]diazomethane was utilized in estimating the free COOH group in the inactivated TRH. Similar determinations were also performed on TRH fractions recovered from the control experiments.

TRH bioassay was carried out by the procedure of Redding and Schally (1969), based on the ability of TRH injected *in vivo* to elicit a release of TSH from the anterior pituitary gland of mice pretreated with ^{125}I. The elevation of endogenous TSH increases the rate of release of labeled thyroid hormone from the thyroid gland. This is measured by an increase in blood radioactivity over that of control values.

[3] The method is described in a separate paper (Nair and Schally, 1971) to *Analytical Biochemistry* on "a quantitative method for the determination of free COOH groups by ^{14}C labeling in peptides."

TABLE II: R_F Values and Amino Acid Composition of TRH Incubates (Solvent System: 1-Butanol–Acetic Acid–Water, 4:1:5, v/v).

Groups as in Table I	R_F Values (Major Pauly-Positive Spot)	Amino Acid Composition	Recov (%)	Recov of Radioactivity (%)
1	0.33 (inactivated)	Glu:1 His:1 Pro:1	51.2	
2	0.39 (active)	Glu:1 His:1 Pro:1	96.7	
3	0.33 (inactivated)	Glu:1 His:1 Pro:1	47.2	50.0
4	0.39 (active)	Not repeated		78.0
5	Traces of all amino acids			
6				
7	0.39 (active)	Glu:1 His:1 Pro:1	60.2	
8	0.39 (active)	Not repeated		
9 (Saline)				
10 (TRH, synthetic)				

Results

After incubation with human plasma at 37° for 1 hr, TRH showed a 72% decrease in biological activity (Table I), while a similar incubation at 0° did not significantly inactivate TRH. After the extraction and purification the inactivated TRH showed the amino acid composition Glu:His:Pro = 1:1:1 with 40–50% recovery (Table II). Similar experiments at 0° yielded 60% of the original TRH, whose amino acid composition was identical. Tlc of these materials in two different solvent systems showed that the inactivated peptide moved with a slower R_F 0.33 (Figure 1) than the active TRH recovered from 0° incubation experiment, or the synthetic hormone. Tle of these peptides revealed that the inactivated TRH moved about one-half times less fast toward the cathode than the synthetic TRH or TRH recovered from 0° incubation (Figure 2).

Attempts to use sequential degradation by Edman-dansyl procedure were unsuccessful on the inactivated TRH, as in the synthetic TRH. Digestion with aminopeptidase M and leucine aminopeptidase and subsequent amino acid analyses did not reveal any free amino acids.

Incubation of [^{14}C]histidyl TRH with plasma at 37° for 1 hr and subsequent bioassay also showed a 70% decrease in biological activity. The inactive peptide after recovery and tlc purification gave one major Pauly-positive spot with an R_F (0.33) equivalent to that of the inactive peptide obtained from the incubation of cold synthetic TRH at 37°. The recovery in terms of radioactivity also was 40–50%, which was in agreement with the recovery in terms of the amino acid analysis. Very little radioactivity was evinced by the plasma residue (15–20%). After incubation of [^{14}C]histidyl TRH at 0° there was a 60–65% recovery in terms of radioactivity and the bioassays showed no inactivation.

^{14}C-Esterification experiments (Nair *et al.*, 1971a,b; Nair and Schally, 1971)[2] on inactivated TRH and subsequent calculation of free COOH groups in terms of ^{14}C label showed one free carboxylic acid group. Synthetic TRH and TRH recovered after incubation at 0° did not show any ^{14}C labeling by this method. Comparison of inactivated TRH and (pyro)Glu-His-Proline (TRH-free acid) along with (pyro)Glu-His, His-Pro-NH_2 and synthetic TRH by tlc in two different solvent systems revealed that the inactivated TRH showed the same R_F value as that of TRH-free acid. Tle of these peptides exhibited identical electrophoretic mobilities for the TRH-free acid and the inactivated TRH.

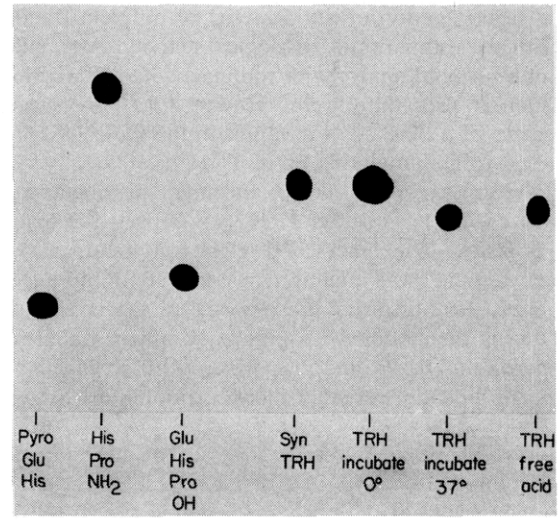

FIGURE 1: Thin-layer chromatogram (MN-300 HR-cellulose, 250 μ layer, solvent system: 1-butanol–acetic acid–water, 4:1:5, v/v) of TRH after incubation with human plasma at 37 and 0°, 1 hr, and recovery, along with TRH, (pyro)Glu-His-Pro-OH (TRH-free acid), Glu-His-Pro-OH, (pyro)Glu-His, His-Pro-NH_2, and His-Pro-HCl.

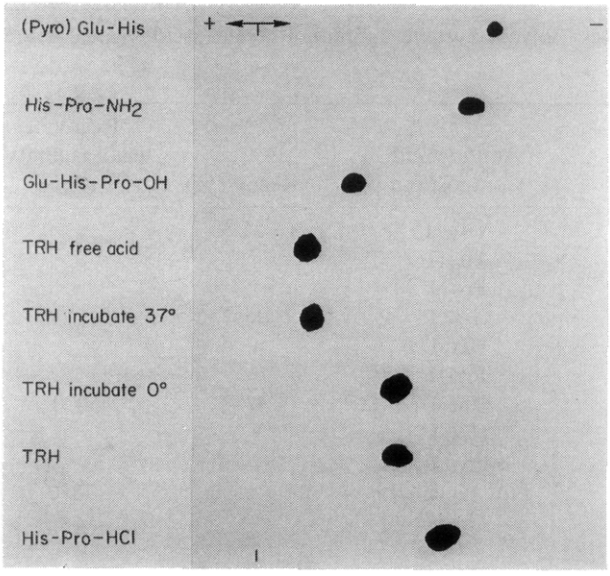

FIGURE 2: Thin-layer electrophoresis (MN-300 HR-cellulose, 250 μ layer, pyridine acetate buffer (pH 4.65), 310 V, 5 mA, 10°, 3 hr) of TRH after incubation with human plasma at 37 and 0°, 1 hr, and recovery, along with TRH, (pyro)Glu-His-Pro-OH (TRH-free acid), Glu-His-Pro-OH, (pyro)Glu-His, His-Pro-NH_2, and His-Pro-HCl.

Discussion

Synthetic TRH (L(pyro)Glu-L-His-L-Pro-NH_2) after incubation with fresh human plasma at 37° for 1 hr showed a 72% decrease in TRH activity. After recovery and purification the inactivated peptide obtained exhibited different chromatographic and electrophoretic mobilities from the parent molecule, even though the amino acid composition (Glu:His:Pro, = 1:1:1) was identical. The recovery in terms of amino acid analyses of the hormone incubated at 37° for 1 hr with human plasma was 40–50%. An analogous experiment at 0° did not inactivate the hormone and the recovery was 60%. These results were confirmed by incubating (pyro)Glu-[^{14}C]His-Pro-NH_2 at 0° and 37° with human plasma, followed by purification and determination of radioactivity of different fractions along with the estimation of biological activity. Attempts to carry out sequential analyses on the inactivated TRH by the Edman-dansyl procedure were unsuccessful. This suggests the existence of a blocked N terminus in the molecule, probably the (pyro)glutamyl ring as in TRH itself. Recovery of 40–50% radioactivity, while performing the inactivation experiments with [^{14}C]histidyl TRH, proved that the histidyl moiety is intact. The inactivated TRH, on treatment with [^{14}C]diazomethane, was quantitatively converted into a [^{14}C]-methyl ester, demonstrating the cleavage of the prolylamide group, during incubation with plasma. Comparison of synthetic (pyro)Glu-His-Proline (TRH-free acid) to inactivated TRH, by tle and tlc, revealed identical mobilities for both peptides.

These investigations, therefore, suggest that during incubation with human plasma 40–50% of TRH undergoes cleavage of the amide group at the prolyl end, giving rise to the free acid (pyro)Glu-His-Pro-OH. Since this reaction does not proceed at 0°, its mechanism is enzymatic, in agreement with earlier observations (Redding and Schally, 1969; Nair *et al.*, 1971b).

Recent evidence (Redding and Schally, 1971) indicates that when [^{14}C]TRH is injected into rats there is an accumulation of radioactivity in the anterior pituitary. Extraction of the whole pituitary, purification, and chromatographic and electrophoretic comparison of the major areas of radioactivity to the mobilities of TRH, its free acid, and the dipeptides (pyro)-Glu-His and His-Pro-NH_2 suggested that the radioactivity is located in the same area as that of TRH and the free acid. Dr. W. F. White (personal communication) has shown that when TRH is incubated with the nonparticulate portion of homogenates of anterior pituitaries of rats, the major metabolite obtained is the free acid of TRH. These findings indicate that there is no major breakdown of TRH into its constituent amino acids during the inactivation of the hormone either *in vitro* or *in vivo*, but only a process of deamidation takes place at the prolyl end, giving rise to a "pseudo" TRH with a free C terminus, possessing no hormonal activity.

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