ACTIVITY-STRUCTURE RELATIONSHIPS OF THE THYROTROPIN RELEASING HORMONE

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SUMMARY. Analogs of the thyrotropin releasing hormone (TRH) have been studied for activity. Although the structure of TRH is highly specific for activity, some analogs released TSH at levels of about 20 to 10,000 times that of TRH. A similarity in the activity of the analogs to that of TRH is indicated by the inhibition in vivo by triiodothyronine and their activity in vitro. TRH, but not the active analogs, was inactivated in human serum showing a specificity of the inactivation. The in vivo activity of an analog at microgram levels is that of a relatively potent substance although these levels are up to 10,000 times higher than those of TRH; TRH has extraordinary potency. The potency of some of these analogs may be due to no inactivation by serum.

The initial data of Folkers et al.(1) showing that synthetic pyroglutamyl-histidyl-proline amide, I, exhibited the hormonal activities of the porcine thyrotropin releasing hormone (TRH) were directly followed by additional data showing the identity of porcine TRH and synthetic pGlu-His-Pro-NH_2**, I, according to the comparison of their chemical and hormonal properties (Bøler et al.(2)).

Structural and synthetic studies (Burges et al.(3)), showed that ovine TRH also has structure I, and continuing studies added to the structural evidence

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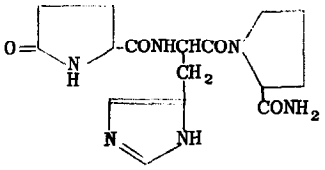
*Hypothalamic Hormones XIII.

**We have previously used the symbol (pyro)Glu- in the expression (pyro)Glu-His-Pro(NH_2) for TRH, because (pyro)Glu- was previously used in peptide nomenclature (16). Others (8) working on TRH have used the symbol "PCA" in place of (pyro)Glu. On principles of nomenclature, it has been pointed out that "PCA" is not self-evident in amino acid chemistry. Although pyroglutamic acid and 2-pyrrolidone-5-carboxylic acid are chemically equivalent, only the traditional name pyroglutamic acid is self-evident in respect to glutamic acid. The symbol "Glp-" is self-evident in respect to Glu and Gln and could be used (Edelmann and Kabat, J. Biochem., in press). Since (pyro)Glu has been used for years, it seems appropriate for peptide nomenclature to contract this symbol to pGlu, and which also shows a terminal amino acid. We greatly appreciate the advice of Dr. Waldo E. Cohn, Director, Office of Biochemical Nomenclature, National Research Council, Washington, D. C.

for porcine TRH which confirmed structure I (Nair et al. (4)).



I

Now that structure I is established for porcine and ovine TRH and indicated for the bovine and human hormones (Bowers et al.(5)), it is evident that TRH of several mammalian species may have the same chemical structure and species specificity may be minimal or absent. Present investigations include a study of the hormonal activity-peptide structure relationships of TRH. Preliminary studies (Bowers et al.(6,7) and Burgus et al.(8)) have already indicated that the chemical nature of the tripeptide is, in general, highly specific for activity. However, the number of structural modifications of pGlu-His-Pro-NH_2 which can be synthesized and bioassayed for hormonal activity are large. Systematic studies of activity-structure relationships may help reveal the biochemical mechanism of the hormonal activity as well as add to knowledge of activity-structure relationships for peptide hormones; such studies may also lead to synthetic inhibitors of TRH which may be useful. Studies have been reported describing some of the mechanisms involved in the interactions between triiodothyronine and TRH which regulate release of TSH. Although the site of action of each of these regulators is in the anterior pituitary gland, evidence indicates they do not directly interact (9, 10).

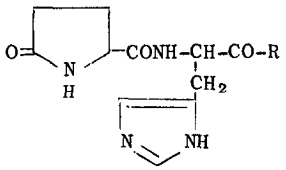
The synthesis of Gln-His-Pro-NH_2 has been described (Folkers et al.(11)). This tripeptide is of unique interest, because of its facile chemical conversion to TRH. Gln-His-Pro-NH_2 is an analog of low or no activity, but which might exist in the hypothalamus and have a direct biological relationship to TRH as a precursor.

We now report data from the biological testing of some new synthetic tripeptides which are analogs of TRH with changes in the moieties of proline and histidine. These new analogs of TRH were tested for hormonal activity in mice by the T_3-TRH method (Bowers et al. (6,12,13). In this quantitative method, ^{125}I blood levels are measured before and two hours after the intravenous injection of TRH or other compounds. Differences in these radioiodine levels, recorded as the ^{125}I Δcpm, are proportional to the amount of TSH released from the pituitary gland. Each value recorded represents the mean obtained from five mice.

A category of TRH analogs, summarized in Table I, consists of modifica-

tions of the proline amide moiety. The dose levels of 1, 3, and 9 ng of synthetic TRH which release TSH in mice are useful levels for reference. Results of responses of these amounts of synthetic TRH are recorded in the footnote of

TABLE I. ACTIVITY OF MODIFICATIONS OF THE PROLINE MOIETY OF PGLU-HIS-PRO-NH_2* (TRH)



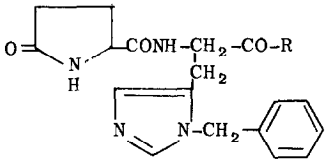
	Compound**	I^{125} Δcpm with dosage increase		
		0.1 μg	1 μg	10 μg
II	R = -N(CONHCH$_3$) pGlu-His-Pro-NHMe	3503	3675	6955
III	R = -N(COOCH$_3$) pGlu-His-Pro-OMe	3588	4200	5150
IV	R = -NH-CH(CONH$_2$)-CH(CH$_3$)$_2$ pGlu-His-Val-NH_2	351	1564	5090
V	R = -NH-CH(CONH$_2$)-CH$_2$-CH(CH$_3$)$_2$ pGlu-His-Leu-NH_2	137	250	3000
VI	R = -NH-CH(CONH$_2$)-CH$_3$ pGlu-His-Ala-NH_2	88	850	4360
VII	R = -NH-CH$_2$-CONH$_2$ pGlu-His-Gly-NH_2	130	202	114
VIII	R = -NH-CH(CONH$_2$)-CH$_2$C$_6$H$_5$ pGlu-His-Phe-NH_2	140	306	420

*The activity of pGlu-His-Pro-NH_2 (TRH) was, 1 ng-638, 3 ng-2382 and 9 ng-3446 I^{125} Δcpm. Saline showed 94 and 127 I^{125} Δcpm.

**The symbols of the amino acids and the peptides in the tables are following IUPAC-IUB Commission on Biochemical Nomenclature. Abbreviated Designation of Amino Acid Derivatives and Peptides. Tentative Rules. Biochem. 5, 2485 (1966).

Table I. Another category of TRH analogs, summarized in Table II, consists of modifications in which the N^{im}-benzyl group is attached to the histidine moiety

TABLE II. ACTIVITY OF MODIFICATIONS OF THE HISTIDINE AND PROLINE MOIETIES OF PGLU-HIS-PRO-NH_2* (TRH)



Compound**		I^{125} Δcpm with dosage increase		
		0.1 μg	1 μg	10 μg
IX	R = -N(CONHCH3)-Bzl; pGlu-His-Pro-NHMe	100	168	210
X	R = -N(COOCH3)-Bzl; pGlu-His-Pro-OMe	42	103	43
XI	R = -NH-CH(CONH2)-CH(CH3)2 (Bzl); pGlu-His-Val-NH_2	378	175	376
XII	R = -NH-CH(CONH2)-CH2-CH(CH3)2 (Bzl); pGlu-His-Leu-NH_2	220	290	166
XIII	R = -N(CONH2)-Bzl; pGlu-His-Pro-NH_2	310	1272	3510
XIV	R = -NH-CH(CONH2)-CH3 (Bzl); pGlu-His-Ala-NH_2	80	102	60
XV	R = -NH-CH2-CONH2 (Bzl); pGlu-His-Gly-NH_2	110	93	75
XVI	R=-NH-CH(CONH2)-CH2-C6H5 (Bzl); pGlu-His-Phe-NH_2	177	225	102

*For TRH activity see Table I.

**See Table I.

as well as changes in the proline amide moiety. If the proton affiliated with the two nitrogen atoms of the imidazole ring is essential for activity by hydrogen bonding, then the presence of the benzyl group could prevent this structural involvement. It is also possible that an altered response of the benzyl analogs could result from steric hindrance.

Replacement of the Pro-NH_2 group with Pro-OMe, III, decreased the activity of the hormone. Burgus et al. (8), found the Pro-OMe analog about 50% as active as TRH while we have found that it has only about 2% of the activity of TRH in mice. Also, we have found that the release of TSH from 0.05 μg of the Pro-NHMe analog, II, corresponds to the release from 0.003 μg of TRH (Bowers et al.(14)). Thus, the Pro-NHMe analog is slightly more active than the Pro-OMe analog.

When Pro-NH_2 was replaced by Val-NH_2, IV, Leu-NH_2, V, or Ala-NH_2, VI, and administered to T_3-TRH assay mice, TSH was released with dose levels approximately 100-1000 times the active dose (1 ng) of TRH. The potency of the Val-NH_2 and Ala-NH_2 analogs were about the same. They had from 0.1-0.2% of the activity of TRH and both were slightly more active than the Leu-NH_2 analog. When doses of the active analogs were increased the response increased. Analogs IV, V, and VI had only 1/25 to 1/50 the activity of analogs II and III. Neither the analogs of Gly-NH_2, VII, nor Phe-NH_2, VIII, were active at 10,000 times the active dose of TRH.

To characterize further the biological activity of these analogs, studies were performed to determine if the in vivo response of the analogs was inhibited by triiodothyronine, if incubation in serum caused inactivation or if they stimulated release of TSH in vitro from rat anterior pituitary glands incubated in Krebs-Ringers bicarbonate solution. Each of these activities have been elicited with synthetic and natural TRH (6) and, thus, are significant ways of characterizing the TRH activity of an analog. As recorded in Tables III, IV and V, the in vivo response of the active analogs, in each instance, was inhibited by triiodothyronine and all were active in vitro; however, incubation in human serum obtained from a normal adult male failed to cause their inactivation. These same analogs were also incubated in the serum of rats, which had been pretreated with triiodothyronine. This method is known to increase the TRH inactivation activity of serum. The results showed that the Pro-NHMe (II) and Pro-OMe (III) analogs were completely inactivated after incubation for 30 minutes while the Val-NH_2 (IV), Leu-NH_2 (V), and Ala-NH_2 (VI) analogs had no loss of activity. Although the Pro-NHMe and Pro-OMe analogs are inactivated by serum this inactivation, compared to TRH, occurs more slowly or is less effective. If these analogs are not metabolized or excreted more rapidly than TRH, it is likely that the lack of serum inactivation of the analog, in part, accounts for

TABLE III. INACTIVATION BY SERUM

	Compound	Dose* μg	I^{125} △cpm Incubation** 0	I^{125} △cpm Incubation** +	p-value 0 vs +
I	pGlu-His-Pro-NH_2	0.009	2840	153	<0.001
II	pGlu-His-Pro-NHMe	0.060	1460	2190	ns
III	pGlu-His-Pro-OCH_3	0.060	1043	724	ns
IV	pGlu-His-Val-NH_2	4.0	3042	3067	ns
V	pGlu-His-Leu-NH_2	10.0	2830	2837	ns
VI	pGlu-His-Ala-NH_2	4.0	1500	1403	ns

*Dose per 0.2 ml normal human serum.

**After incubation at 37° C for 30 minutes, serum was assayed in T_3-TRH assay mice. Each result is the mean △cpm obtained from five mice.

TABLE IV. INHIBITION BY TRIIODOTHYRONINE (T_3)

	Compound	Dose* μg	I^{125} △cpm Inhibition** 0	I^{125} △cpm Inhibition** +	p-value 0 vs +
I	pGlu-His-Pro-NH_2	0.009	4275	20	<0.001
II	pGlu-His-Pro-$NHCH_3$	0.060	3887	-50	<0.001
III	pGlu-His-Pro-OCH_3	0.060	1847	-20	<0.001
IV	pGlu-His-Val-NH_2	10.0	5090	1490	<0.001
V	pGlu-His-Leu-NH_2	10.0	2918	525	<0.001
VI	pGlu-His-Ala-NH_2	10.0	6218	430	<0.001

*Dose per mouse.

**T_3, 0.2 μg, given SC to T_3-TRH assay mice 2 hours before injection of compound IV. Each result is the mean △cpm obtained from five mice.

their potency. Preliminary *in vitro* studies on plasma inactivation of TRH using both ^{14}C-His and ^{14}C-Pro labelled TRH indicate that the amide group of the proline moiety is changed first, then hydrolysis of the His-Pro peptide bond occurs and finally hydrolysis of the peptide bond of the dipeptide, pGlu-His (Bowers *et al.* unpublished). Both of the above results indicate the specificity of the serum inactivation mechanism which, as was postulated, may play an important physiological role (13). Furthermore, serum inactivation of the analogs must be considered in the interpretation of activity-structure relationships of compounds tested *in vivo*. It is possible that the relative

TABLE V. ACTIVITY IN VITRO

	Compound	Dose μg	I^{125} △cpm Activity* C	E	p-value C vs E
I	pGlu-His-Pro-NH_2	0.010	374	1506	<0.001
II	pGlu-His-Pro-$NHCH_3$	10.0	750	1826	<0.01
III	pGlu-His-Pro-OCH_3	10.0	700	3610	<0.001
IV	pGlu-His-Val-NH_2	10.0	496	2227	<0.001
V	pGlu-His-Leu-NH_2	10.0	274	1917	<0.001
VI	pGlu-His-Ala-NH_2	10.0	313	3383	<0.001
VII	pGlu-His-Gly-NH_2	10.0	343	510	ns
XII	pGlu-His(Bzl)-Leu-NH_2	10.0	690	340	ns
XIII	pGlu-His(Bzl)-Pro-NH_2	10.0	310	2080	<0.001
XIV	pGlu-His(Bzl)-Ala-NH_2	10.0	1670	1750	ns
XV	pGlu-His(Bzl)-Gly-NH_2	10.0	674	364	ns

*TRH or analogs added to medium of the experimental (E) but not the control (C) beaker. TSH measured in T_3-TSH assay mice. Values recorded represent amount of TSH present in medium and are the mean obtained from five mice.

lack of activity of TRH analogs with substitution for the histidine or pyroglutamic acid rather than the proline amide moiety may in part be due to their greater inactivation by serum. An additional understanding of the hormonal activities of the analogs in Table I may be aided by data in Table II.

The presence of the N^{im}-benzyl group in both the Pro-NHMe (VI) and Pro-OMe (VII) analogs eliminated any release of TSH, even at 10,000 times the effective dose level of TRH in the mouse. It was also found that the three analogs, X, XI, and XIII, were no longer active at 10,000 times the effective dose of TRH when a benzyl group was attached to the histidine moiety.

The addition of the N^{im}-benzyl group to the molecule of TRH, as in analog XIII, is particularly interesting and perhaps may help in understanding the responses of the other analogs. This "N^{im}-benzyl-TRH" analog released TSH. It had about 0.2% of the activity of TRH *in vivo*. Metabolic debenzylation of this analog to TRH does not seem probable, and the hormonal activity at the high dose level could be intrinsic to the analog. Although no one mechanism may be applicable for the activities of all these analogs, one may presently assume that one mechanism generally prevails for derivatives of this chemical nature especially since the analogs in Table IV were inhibited with triiodothyronine and were also active *in vitro* (Table V) but none were inactivated by serum (Table III). One may consider that the active analogs could be metabolically

transformed in the mouse to TRH, although such transformation would indeed have to be rapid in view of the nature of the assay. One of the benzyl analogs (XIII) is active and the others are not. Structurally, it seems very probable that the benzyl analogs IX through XVI would not be metabolically transformed into TRH.

A dose level of 10 μg for the mouse of an analog which fully releases TSH is a comparatively low amount; however, it is 10,000 times the effective dose of TRH. Such activity-structure relationships may be uncommon, but are observed in this study, now only because of the extraordinary potency of TRH at nanogram-dose levels but perhaps also because the active analogs are not inactivated by serum. One may consider that the molecular environment of the site of hormonal activity of TRH has some very low incidence of variation which permits certain analogs, perhaps some of those now described, to cause the release of TSH exactly like TRH at the normal sites which exist in overwhelming number. The plausability of this concept is supported by results of the in vitro studies which show a direct action of the active analogs on the pituitary gland and also results of the inhibition by triiodothyronine which would be expected of a compound which has a mechanism of action like TRH.

Studies of activity-structure relationships for the nonapeptide, bradykinin, led to the understanding that this hormone has three parts of the molecule which contribute significantly to its biological activity, namely, the center and two ends (Melrose et al.(15)). Although TRH is only a tripeptide, its center and two ends are also important for activity.

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