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# Forces and Structural Limitations of Binding of Thyrotrophin-Releasing Factor to the Thyrotrophin-Releasing Receptor: the Pyroglutamic Acid Moiety

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SUMMARY

GOREN, H. JOSEPH, BAUCE, LORENZO G. & VALE, WYLIE (1977) Forces and structural limitations of binding of thyrotrophin-releasing factor to the thyrotrophin-releasing receptor: the pyroglutamic acid moiety. *Mol. Pharmacol.*, 13, 606–614.

Thyrotrophin-releasing factor (TRF) and analogues of its pyroglutamyl ring were tested for their potencies in eliciting thyrotrophin release *in vivo*. The ratio of potency of an analogue to the potency of TRF was used to predict changes in binding energy of association between the TRF molecule and the TRF-thyrotroph receptor. Assumptions made in this correlation were that the analogue has efficacy and stability to degradation *in vivo* equal to TRF, and a similar temperature dependence of binding. Types of noncovalent bonds were assigned according to the changes in binding energy. Using the potencies of 23 analogues of TRF in which only the pyroglutamyl residue was modified (some taken from the literature), the following inferences have been drawn about the TRF molecule–TRF-thyrotroph receptor interaction. (a) The ring amide proton is a hydrogen bond acceptor. The strength of this hydrogen bond is approximately 1.5 kcal/mol. (b) The δ-ketone oxygen is a hydrogen bond donor. The strength of this hydrogen bond is approximately 3.5 kcal/mole. (c) The γ-methylene group is involved in formation of a hydrophobic bond whose strength is approximately 0.8 kcal/mole. (d) The receptor molecule in the region of the ring amide proton cannot accommodate a group larger than a hydrogen atom. (e) There is a tight fit between the receptor and the β- and γ-carbons of the pyroglutamyl ring. (f) The 5-membered ring structure and its puckering are important in maintaining the right spatial location of the ring amide and the δ-ketone. (g) There is room for substitution on the α-carbon of pyroglutamic acid for at least one methyl group. (h) The pyroglutamyl residue may contribute up to 50% of the total binding energy of TRF.

## INTRODUCTION

Thyrotrophin-releasing factor, when injected into humans or animals, exhibits a variety of biological effects (1). Physiologically, it appears to stimulate the secretion

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of thyrotrophin and perhaps prolactin (1-4) from the anterior pituitary. Several reports on the structural requirements of TRF[2] for its biological activity have appeared (e.g., refs. 1, 3, 5), but rarely have these been interpreted in terms of noncovalent bonds formed between the hormone and its receptor. The present studies were directed toward an understanding of the types of binding forces available in and the spatial limitations on the TRF-TSH receptor.

TRF is L-pyroglutamyl-L-histidyl-L-prolylamide (5). The currently accepted solution conformation for TRF has the molecule in an extended form (6). In nonpolar solvents (and possibly at the receptor binding site) a single hydrogen bond between the proline amide proton and the carbonyl oxygen of histidine may exist (7, 8). Assuming that the TRF-thyrotroph receptor recognizes one of the solution stable conformations of TRF (8), changes in the TSH-releasing potency of TRF analogues modified at position 1 can be attributed to alterations in the hormone-receptor interaction, since pyroglutamic acid is not considered to be involved in stabilization of any of the solution conformations of TRF.

The potencies of most TRF analogues reported to date may be related to their ability to bind with the TRF-thyrotroph receptor (5, 9). The two antagonists that have been synthesized are believed to act at a site different from the TRF-TSH receptor (10). Thus TRF and its analogues display affinity constants, determined from dose-response curves, and dissociation constants, determined by dissociation of [$^3$H]TRF from pituitary extracts (11), pituitary membranes (12, 13), or from pituitary $GH_3$ tumor cells (14), in fair agreement with each other. In contrast, [formyl-Pro$^1$]-TRF has been found to have a higher potency relative to TRF when determined by bioassay (8%) than when determined by radioreceptor assay (1%).[3] This discrepancy has been related to the observation that [formyl-Pro$^1$]-TRF is resistant to serum inactivation (15). In this report the TSH-releasing potencies of a series of synthetic position 1 TRF analogues are used to elucidate some features of the pyroglutamyl binding site of the TRF-TSH receptor.

[2] The abbreviations used are: TRF, thyrotrophin-releasing factor; TSH, thyrotrophin (thyroid-stimulating hormone).

[3] W. Vale, G. Grant, and M. Monahan, unpublished results.

## METHODS

TRF and its analogues were synthesized in solution.[4] All compounds were pure according to amino acid analyses, elemental analyses, mass spectra,[5] and thin-layer chromatography in several solvent systems.

*Bioassays*. The bioassay *in vivo* is based on the ability of TRF or active analogues to release TSH from the anterior pituitary, thereby increasing the blood level of $^{125}$I-labeled thyroid hormones in appropriately prepared mice (16). Analogues and standards were usually given at three dose levels each. Following covariance adjustment of the raw data, potencies were calculated according to a three-, four-, or six-point bioassay design (17).

*Calculations*. At equilibrium the change in free energy ($\Delta F$) of a reaction is

$$\Delta F = \Delta F^\circ + RT \ln K \qquad (1)$$

where $K$ is the equilibrium constant at temperature $T$ (absolute), $R$ is the gas constant, and $\Delta F^\circ$ is the change in free energy of the reaction under standard temperature (25°) and pressure (1 atm) conditions. In principle this equation may be applied to the binding of drugs to their specific receptors (18), where $K = K_a$ (association constant). Thus each analogue of TRF has a free energy of binding ($\Delta F$) associated with it:

$$\Delta F = \Delta F^\circ + RT \ln K_a \qquad (2)$$

Assuming that for TRF and its analogues $|\Delta F| << |\Delta F^\circ|$, then, for all temperatures,[6]

[4] L. G. Bauce and H. J. Goren, manuscript in preparation.

[5] Courtesy of Dr. N. Ling, Salk Institute, La Jolla, Ca.

[6] For this assumption to be valid, $K_a$ must be temperature-dependent in such a way that, as the temperature rises, $K_a$ decreases. This would indicate an exothermic binding reaction. Grant and Vale (19) have shown that whole $GH_3$ cells bind [$^3$H]TRF much better at 0° than at 37°, in keeping with the assumption.

$$\Delta F^\circ = -RT \ln K_a \quad (3)$$

The difference between the abilities of two analogues of TRF to bind to the TRF-TSH receptor may in such instances be expressed as a difference in standard free energies of binding ($\Delta\Delta F^\circ$) of the analogues to the receptor:

$$\Delta\Delta F^\circ(\text{binding}) = RT \ln \frac{K_d(2)}{K_d(1)} \quad (4)$$

where $K_d(1)$ and $K_d(2)$ are the dissociation constants ($K_d = 1/K_a$) of analogues 1 and 2, respectively. Since affinity constants and dissociation constants of TRF analogues have been shown to be inversely proportional to their potency in stimulating TSH release (9), an alternative to Eq. 4 is

$$\Delta\Delta F^\circ(\text{activity}) = -RT \ln \frac{\text{potency}(1)}{\text{potency}(2)} \quad (5)$$

In the interaction of TRF with the TRF-TSH receptor the bonds formed may be assumed to be noncovalent. Noncovalent bonds have certain energies (bond strengths) associated with them (20). The differences in binding energy, $\Delta\Delta F^\circ$(binding) or $\Delta\Delta F^\circ$(activity), may be used to predict the types of bonds lost (positive $\Delta\Delta F^\circ$) or gained (negative $\Delta\Delta F^\circ$) when the structure of TRF is changed.

An analogue with a positive $\Delta\Delta F^\circ$ (relative to TRF) may in some instances bind with strength equal to TRF, but some of the binding energy may be required to alter the conformation of the hormone analogue or the receptor binding site to obtain the appropriate bond, as in the relationship between structure and strength of binding of chemical agents to the cholinergic receptor (21, 22). Since it is the pyroglutamyl residue of TRF that is modified and because the changes to that part of the hormone are minimal in terms of bulk, loss of binding energy because of contribution to conformational transitions has not been considered in the following discussion.

## RESULTS AND DISCUSSION

*Use of Eq. 5.* Table 1 lists the changes in standard free energy of binding for TRF and 12 of its analogues. These values were calculated from the dissociation constants and potencies reported by Grant *et al.* (9). The data of Table 1 are plotted in Fig. 1. If $\Delta\Delta F^\circ$ (activity) is exactly equal to $\Delta\Delta F^\circ$-

TABLE 1

*Differences in standard free energy of binding between TRF and some of its analogues*

| Compound[a] | $\ln[K_d(2)/K_d(1)]$[b] | $\Delta\Delta F^\circ$ (binding)[c] | ln [potency(1)/potency(2)][b] | $\Delta\Delta F^\circ$ (activity)[c] |
|---|---|---|---|---|
| | | *kcal/mole* | | *kcal/mole* |
| 1. TRF | 0 | 0 | 0 | 0 |
| 2. [$N_\tau$-Me-His$^2$]-TRF | 1.90 | −1.025 | 2.08 | −1.28 |
| 3. [Pro-Gly-$NH_2^3$]-TRF | −1.10 | +0.59 | −1.05 | +0.64 |
| 4. [Pro-NH-EtOH$^3$]-TRF | −2.30 | +1.24 | −1.83 | +1.12 |
| 5. [Pro-NH-Et$^3$]-TRF | −3.40 | +1.84 | −1.97 | +1.21 |
| 6. [Pro-OMe$^3$]-TRF | −3.69 | +1.99 | −2.30 | +1.41 |
| 7. [*N*-MepGlu$^1$]-TRF | −5.01 | +2.71 | −4.07 | +2.50 |
| 8. [Prolinol$^3$]-TRF | −5.08 | +2.74 | −4.42 | +2.71 |
| 9. [Met$^2$]-TRF | −5.52 | +2.98 | −4.61 | +2.83 |
| 10. [Pyrrolidine$^3$]-TRF | −4.09 | +2.21 | −4.83 | +2.96 |
| 11. [Pro-OEt$^3$]-TRF | −3.91 | +2.16 | −5.52 | +3.39 |
| 12. [Hexamethyleneimine$^3$]-TRF | −7.60 | +4.16 | −6.91 | +4.24 |
| 13. [Morpholine$^3$]-TRF | −6.40 | +3.46 | −5.81 | +3.57 |

[a] Abbreviations employed are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature (23).

[b] TRF and the TRF analogue in question are indicated by (2) and (1), respectively. Dissociation constants and potencies were taken from Grant *et al.* (9).

[c] $\Delta\Delta F^\circ$ (binding) $= -RT \ln [K_d(2)/K_d(1)]$, and $\Delta\Delta F^\circ$ (activity) $= -RT \ln$ [potency(1)/potency(2)], where $R$ = 1.98 cal deg$^{-1}$ mol$^{-1}$ and $T$ = 273° K [for $\Delta\Delta F^\circ$ (binding)] and 310° K [for $\Delta\Delta F^\circ$ (activity)].

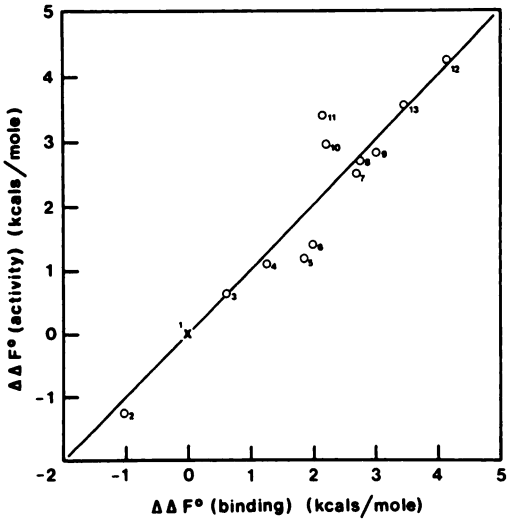



FIG. 1. *Relationship between two methods of calculating* $\Delta\Delta F°$.

$\Delta\Delta F°$(binding) and $\Delta\Delta F°$(activity) were calculated using Eqs. 4 and 5, respectively. Potencies and dissociation constants of TRF and its analogues were those reported by Grant *et al.* (9). Numbers correspond to the compounds in Table 1. The solid line is the theoretical curve if the two equations are exactly equal.

(binding), the data of Table 1 will all fall on the solid line of Fig. 1. Generally this did occur, and on this basis $\Delta\Delta F°$(activity) was used to determine the differences in strength between TRF and its analogues in binding to the TRF-thyrotroph receptor (6).

Those compounds of Table 1 which deviate from the curve in Fig. 1 are either partial agonists (efficacy less than that of TRF), more susceptible than TRF to degradation *in vivo* (24–29), or more stable than TRF to proteolysis *in vivo*; or they display a proportionality constant between potency and dissociation constant containing a temperature function different from that of TRF.[7]

[7] Although $\Delta\Delta F°$(binding) and $\Delta\Delta F°$(activity) were determined at two different temperatures ($T_2$ and $T_1$, respectively), because of the relationship demonstrated in Fig. 1, it may be shown that

$$\Delta\Delta F°(\text{activity}) = \Delta\Delta F°(\text{binding}) = -\left(\frac{RT_1T_2}{\Delta T}\right)\ln\frac{k(2)}{k(1)}$$

where $\Delta T = T_1 - T_2$ and $k(2)$ and $k(1)$ are the proportionality constants between the potencies (measured at $T_1$) and dissociation constants (measured at $T_2$) of TRF and its analogue, respectively; i.e., $k(2)$ = potency (2) × $K_d(2)$ and $k(1)$ = potency(1) × $K_d(1)$. If $k(1)$ and $k(2)$ are functions of temperature, those compounds which fall on the curve in Fig. 1 probably contain the same function.

*Binding activity of pyroglutamic acid to TRF-TSH receptor.* Table 2 lists a series of analogues of TRF in which only the pyroglutamyl ring was substituted. The potencies of the TRF analogues reported were determined in mice as described in METHODS. These were then used to calculate $\Delta\Delta F°$(activity) with the aid of Eq. 5, where 2 was TRF and 1 the TRF analogue. It should be borne in mind in the following discussion that some of the conclusions drawn may be in error, as efficacy, susceptibility to degradation *in vivo*, and the temperature effect on binding are considered equivalent to those of TRF.

The types of binding forces by which the pyroglutamyl ring may participate with a receptor include (a) hydrogen bond donors—a pair of electrons from the carbonyl oxygens of both the α- and γ-carboxyl groups and the ring amide nitrogen; (b) hydrogen bond acceptors—the proton of the ring amide; (c) dipole-dipole or dipole-induced dipole interactions with the ketone function or the amide function participating; and (d) hydrophobic bonds, the source being the β- and γ-methylene groups.

The data indicate that some of these types of noncovalent bonds are formed in the hormone–TRF-TSH receptor interaction. The α-carboxyl of pyroglutamic acid is probably not involved in any binding, since *cycle*(L-histidyl-L-proline) (potency <0.1) is approximately 10 times more potent than [formyl[1]]-TRF (potency <0.01; compound 2, Table 2). Neither is the α-carbon involved in binding, since [acetyl[1]]-TRF (compound 3, Table 2) does not bind more strongly than [formyl[1]]-TRF. [Ureido[1]]-TRF (compound 4, Table 2), which has an amide nitrogen that could occupy the pyroglutamic acid ring amide position on the receptor, binds more strongly, by approximately 1.5 kcal/mole (6.3 kJ/mole),

TABLE 2

*Potencies and differences in standard free energies of binding between TRF and its analogue substituted at position 1*

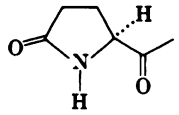
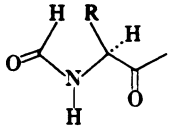
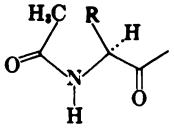
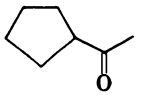
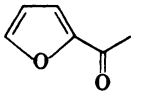
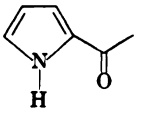
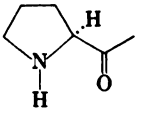
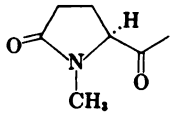
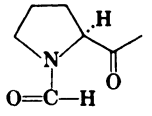
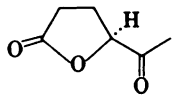
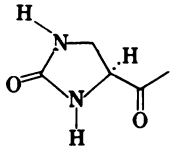
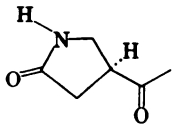
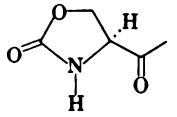
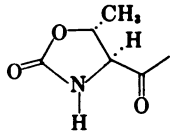
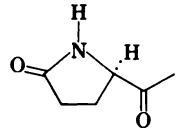
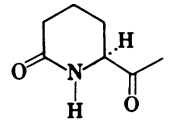
| Compound | Structure | Name[a] | Potency | | $\Delta\Delta F^{\circ}$ (activity)[b] |
|---|---|---|---|---|---|
| | | | This report | Literature | |
| | | | % | % | *kcal/mole* |
| 1 |  | pGlu (TRF) | 100 | 100 (5) | – |
| 2 |  | Formyl (R=H) | <0.01 | | >5.7 |
| 3 | | Acetyl ($R=CH_3$) | <0.01 | | >5.7 |
| 4 | | Ureido ($R=NH_2$) | <0.1 | | >4.3 |
| 5 |  | HCO-Gly (R=H) | 0.6 | | 3.2 |
| 6 | | HCO-Ala ($R=CH_3$) | 0.3 | | 3.6 |
| 7 | | HCO-Abu ($R=CH_2CH_3$) | 0.4 | | 3.4 |
| 8 | | HCO-Val [$R=CH(CH_3)_2$] | 0.2 | | 3.9 |
| 9 |  | Ac-Gly (R=H) | | 0.15 (30), 0.25 (31) | (4.0, 3.7) |
| 10 | | Ac-Ala ($R=CH_3$) | | <0.1 (31) | (>4.3) |
| 11 |  | Cyclopentanecarbonyl | 0.005 | <0.01 (31) | 6.1 (>5.7) |
| 12 |  | Furan-2-carbonyl | 0.004 | | 6.3 |
| 13 |  | Pyrrole-2-carbonyl | 0.04 | | 4.9 |
| 14 |  | Pro | | 0.01 (32) | (5.7) |
| 15 |  | MepGlu | | 1.7 (31) | (2.5) |
| 16 |  | HCO-Pro | 10 | 10 (31) | 1.4 |

TABLE 2—*Continued*

| Compound | Structure | Name[a] | Potency | | ΔΔF° (activity)[b] |
|---|---|---|---|---|---|
| | | | This report | Literature | |
| | | | % | % | *kcal/mole* |
| 17 |  | L-α-Hydroxyglutaryl-γ-lactone | 1.0 | 5.0 (31) | 2.9 (1.9) |
| 18 |  | L-Imidazolidone-4-carbonyl | 3.0 | | 2.2 |
| 19 |  | Iso-pGlu | | 0.3 (31) | (3.6) |
| 20 |  | L-Oxazolidone-4-carbonyl | 27 | 38[c] | 0.8 (0.6) |
| 21 |  | L-*trans*-5-Methyloxazolidone-4-carbonyl | 13 | | 1.3 |
| 22 |  | D-pGlu | 0.02 | 0.01 (33)<br>0.1 (34) | 5.3 (5.7)<br>(4.3) |
| 23 |  | L-Piperidone-6-carbonyl | 43 | | 0.5 |

[a] Abbreviations employed are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature (23). All amino acids except glycine are L-isomers. Other abbreviations are: HCO-, formyl-; pGlu, pyroglutamic acid; iso-pGlu, isopyroglutamic acid; MepGlu, *N*-methylpyroglutamic acid.

[b] Values in parentheses were calculated from the literature potency values.

[c] Determined by Dr. R. Rippel of Abbott Laboratories and listed in ref. 3.

than [formyl[1]]-TRF. Whether the amide is involved in hydrogen bonding as a donor or as an acceptor can be resolved by comparing compounds 11, 12, and 13 (Table 2). Replacement of a methylene group by an oxygen (possible hydrogen bond donor) adjacent to the α-carbon of the carboxylic acid does not improve the binding of compound 11 over compound 12. Replacement with an amine (compound 13), however, does improve binding by about 1.3 kcal/mole (5.4 kJ/mole). Thus it appears that the proton of the pyroglutamic acid is a hydrogen bond acceptor. Saturation of the pyrrole ring of compound 13 produces [Pro[1]]-TRF (compound 14), with a resultant loss of binding energy of 0.8 kcal/mole (3.3 kJ/mole). Since the α-amine of [Pro[1]]-

TRF is protonated at physiological pH, the electron donor in the TRF-TSH receptor responsible for the hydrogen bond with the amide proton could form an ion dipole bond with this compound. The binding energies of such interactions are approximately equivalent to hydrogen bonds (20), and compounds 13 and 14 should be expected to have equivalent activity. The difference in energy of binding of the two compounds may be attributed to the larger amount of energy needed to expel the water of hydration of the ionized [Pro$^1$]-TRF analogue. This energy comes from the energy of binding of the analogue to the TRF-TSH receptor.

From the above considerations it would appear that the ring amide proton contributes 1–1.5 kcal/mole (4.2–6.3 kJ/mole) to the binding of TRF to the TRF-TSH receptor. Replacement of this proton with a methyl group, [MepGlu$^1$]-TRF (compound 15), produces a loss of 2.5 kcal/mole (10.5 kJ/mole) in binding strength relative to the native hormone. Thus the presence of a methyl group in this position results in a loss of 1–1.5 kcal/mole (4.2–6.3 kJ/mole) in binding strength in addition to the loss of the hydrogen bond. This further loss in binding strength is probably due to either steric hindrance or the placement of a hydrophobic group in a hydrophilic region. The former is the more likely explanation. [Formyl-Pro$^1$]-TRF (compound 16) binds more poorly than TRF by about 1.4 kcal/mole (5.9 kJ/mole) (Table 2). In view of the stability of compound 16 to serum proteases (15), a more realistic figure for binding strength of [formyl-Pro$^1$]-TRF may be obtained from its relative binding activity, 1% relative to TRF.[3] This means a loss of 2.9 kcal/mole (12.1 kJ/mole) in binding energy compared with TRF. Assuming that the formyl oxygen of compound 16 occupies the same region in the TRF-thyrotroph receptor as the δ-ketone oxygen of pyroglutamic acid, the loss in binding strength of both compounds 15 and 16 compared with TRF may be attributed to the loss of a proton donor (amide proton) of a hydrogen bond and to steric hindrance by the methyl and formyl groups, respectively.

Comparison of the loss of binding strength of [pyrrole-2-carbonyl$^1$]-TRF (compound 13) relative to TRF (compound 1) suggests that the C(5) carbonyl oxygen in pyroglutamic acid is an electron donor of a hydrogen bond and that its bond strength may be as high as 5 kcal/mole (20.9 kJ/mole). This figure is probably high, since [L-α-hydroxyglutaryl-γ-lactone$^1$]-TRF (compound 17) binds 3 to 4 kcal/mole (12.5 to 16.7 kJ/mole) better than [furan-2-carbonyl$^1$]-TRF (compound 12). The difference between these two compounds is the placement of a carbonyl oxygen at carbon 5 in the heterocyclic ring and the loss of aromaticity. Aromaticity changes both the electronic nature and the hydrophilic character of the nucleophilic atoms in position 1 of the ring. It also prevents ring puckering. Aromaticity in the pyroglutamyl ring may be estimated to produce a loss of about 1–1.5 kcal/mole (4.2–6.3 kJ/mole) in binding strength of TRF. On this basis the δ-ketone oxygen is an electron donor of a hydrogen bond and its bond strength is approximately 3.5 kcal/mole (14.6 kJ/mole).

The ring structure of residue 1 in TRF is essential. [Formyl-Abu$^1$]-TRF (compound 7) and [acetyl-Ala$^1$]-TRF (compound 10) have the same number of atoms as TRF. The alkyl groups in these analogues could theoretically assume the positions of the β- and γ-methylene groups of pyroglutamic acid. In order to assume these positions, 3.5–4 kcal/mole (14.6–16.7 kJ/mole) of binding energy must be expended. [Formyl-Gly$^1$]-TRF (compound 5) would have less steric hindrance, and positioning of the ketone oxygen and amide proton would require less energy. Thus the loss in binding energy of compound 5 is not as great as the loss in binding energy of any of compounds 6–10.

The size of the 5-membered ring of residue 1 is important. [L-Piperidone-6-carbonyl$^1$]-TRF, in which the ring has been increased by one methylene group, exhibits a loss of 500 cal/mole (2.1 kJ/mole) in binding energy. This loss may be due to a change in ring puckering or to steric hindrance during binding. The latter is the more likely explanation, since [L-*trans*-5-methyloxazolidone-4-carbonyl$^1$]-

TRF (compound 21) displays a loss of about 500 cal/mole (2.1 kJ/mole) in binding strength compared with [L-oxazolidone-4-carbonyl[1]]-TRF (compound 20). This is equivalent to the loss in binding energy of [L-piperidine-6-carbonyl[1]]-TRF (compound 23) relative to TRF and of [formyl-Val[1]]-TRF (compound 7) relative to [formyl-Abu[1]]-TRF (compound 8).

The role of the β- and γ-methylene groups of pyroglutamic acid, aside from forcing the δ-carbonyl oxygen and the α-amide proton into the correct spatial configuration for recognition by the TRF-TSH receptor, may be to participate in hydrophobic bonding. From the presently available data it appears that the γ-methylene group contributes about 800 cal/mole (3.3 kJ/mole) to the binding energy of TRF. This may be surmised from the loss of binding when the γ-methylene group in TRF is replaced by an oxygen in [L-oxazolidone-4-carbonyl[1]]-TRF (compound 20). Replacement of the γ-methylene by an amino function, [L-imidazolidone-4-carbonyl[1]] (compound 18), produces a loss of about 2 kcal/mole (8.4 kJ/mole) in binding strength. The loss in binding energy over and above the loss of the hydrophobic group at the γ-position may be due to some binding of the γ-amide proton in the position of the α-amide proton. The latter binding would require energy to be expelled by the correct analogue–TRF-thyrotroph receptor interaction in order that the correct complex be formed. This would lower the total strength of binding of compound 18.

[D-pGlu[1]]-TRF (compound 22) has approximately 0.02% of the TSH-secreting ability of TRF. Thus substitution of a racemic pyroglutamyl residue for the L isomer would probably yield a TRF analogue with 50% potency. Since [DL-α-methyl-pGlu[1]]-TRF displays 50% of the potency of TRF in stimulation of secretion of TSH, [L-α-methyl-pGlu[1]]-TRF probably would have high potency. This result indicates that in the region of the α-proton of the pyroglutamyl ring of TRF in the TRF-thyrotroph receptor there is sufficient space to permit the placement of a methyl group without a consequence to the binding activity of the hormone. [MeHis[2]]-TRF, a position 2 analogue of TRF, exhibits 115% ± 8% of the potency of TRF (35). The methyl group of this analogue occupies essentially the same location in space as the methyl group of [α-methyl-pGlu[1]]-TRF, thus providing further evidence for the latter conclusion.

From the above discussion it follows that loss of the ring amide proton (1.5 kcal/mole; 6.3 kJ/mole), the δ-ketone oxygen (3.5 kcal/mole; 14.6 kJ/mole), and the γ-methylene group (0.8 kcal/mole; 3.3 kJ/mole) would reduce the strength of binding of TRF to the TRF-thyrotroph receptor by approximately 5.8 kcal/mole (24.3 kJ/mole). This loss in binding energy is equivalent to that of [formyl[1]]-TRF compared with the native hormone. Accordingly it may be predicted that the β-carbon of the pyroglutamyl ring will not participate in any form of binding in the interaction of TRF with the TRF-thyrotroph receptor.

The total contribution of the pyroglutamyl ring to the binding of TRF to the TRF-thyrotroph receptor is 5–6 kcal/mole (21–25 kJ/mole). This value may be almost half the total energy of binding of the TRF molecule to its receptor [$\Delta F^\circ \cong -12$ kcal/mole (50 kJ/mole), where $K_d = 2.5$ nM (5)], and therefore explains the strong dependence of the activity of TRF on an intact on pyroglutamyl residue.

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