Orientation Restrictions of the Peptide Hormone, Thyrotropin-Releasing Factor, Due to Intramolecular Hydrogen Bonding†

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ABSTRACT: As part of the study of its structure–activity relationships and the analysis of biological and receptor binding data, the hypothalamic peptide hormone, thyrotropin-releasing factor (TRF) (<Glu-His-Pro-NH_2), and a variety of closely related analogs were examined by potentiometric titration; the pK_a of the imidazole proton was determined. In TRF, the imidazole proton has a pK_a of 6.25, well below the value for free imidazole (pK_a = 7.08). The titration data obtained for the TRF analogs suggest that there is a hydrogen-bonding interaction between the N^{π}-imidazole nitrogen atom and the pyroglutamylhistidyl, peptide-bond amide hydrogen. Titration of appropriate analogs demonstrates that while modification of the <Glu moiety has only minor effects upon this interaction, a rigid peptide bond at the carboxyl group of histidine is necessary to presumably restrict the freedom of the histidine α- and β-carbons and hence the imidazole ring. The C-terminal amide moiety does not affect the hydrogen bonding (except when alkylated with a sufficiently large group which might interfere sterically, *e.g.*, piperidine, or when the C-terminal possesses a free carboxyl group and thereby electrostatically interferes). Methylation of the N^{τ}-nitrogen atom of the imidazole ring in TRF stabilizes the hydrogen bonding as indicated by a lower pK_a value, yielding a biologically hyperactive analog of TRF.

Thyrotropin-releasing factor (TRF) is a small peptide, pyroglutamylhistidylprolylamide, <Glu-His-Pro-NH_2, isolated from ovine and porcine hypothalami, that stimulates the release of thyroid-stimulating hormone (TSH) from the pituitary glands of every mammalian species tested (Burgus *et al.*, 1969; Nair *et al.*, 1970). The bioassay of synthesized TRF analogs has demonstrated that the parent molecule is extremely conservative and full biological activity obligatorily requires the entire tripeptide (Guillemin *et al.*, 1971). Recently it has also been shown that the analogs of TRF are recognized by pituitary thyrotroph membrane receptors at an affinity proportional to their relative biological potencies (Grant *et al.*, 1972a,b). The ability of the receptor to recognize TRF analogs is affected by modifying either any of the functional groups of TRF or any of the three five-membered ring structures in the molecule. The determination of the relative biological potencies and pituitary receptor binding affinity of each of the TRF analogs has allowed us to assess the relative involvement of each part of the peptide in its role in the release of TSH.

Interpretation of nuclear magnetic resonance (nmr) spectra suggests that TRF exists in a definable, restricted molecular orientation (Burgus *et al.*, 1969; J. Rivier, unpublished observations). Unfortunately, it has not yet been established whether the modified TRF analogs possessing lower biological potencies exist in the same or some alternate, thermodynamically preferred conformations than are possible for the parent TRF molecule. A preference for a particular conformation would be expected to affect an analog's ability to react at high affinity with the TRF receptor. The changed activity of an analog might be due, conversely, to variations in the ability of modified TRF molecules to be recognized by the cellular TRF receptors while still maintaining the same relative orientations as exist in the parent molecule.

In this publication, we have attempted to define an intramolecular interaction of TRF. Specifically, we have measured the pK_a value of the imidazole ring in selective TRF-derived analogs. The observed internal hydrogen bonding of the molecule affects predictably the pK_a value of the imidazole group. On the basis of the observed data and deductions about the thermodynamic preference of such an interaction, taken in concert with the determined, preferred orientation of histidine and histamine (Kier, 1968; Sachs *et al.*, 1971), a plausible, imidazole group orientation in the TRF molecule is proposed.

Materials and Methods

Potentiometric titrations were carried out using a radiometer (Copenhagen) Type TTT 1C autotitrating apparatus. Peptide samples were titrated at 5×10^{-4} to 5×10^{-2} M. Samples (2 ml) were adjusted to pH 3.5 with HCl and then titrated with NaOH (0.001–0.01 M). Peptides used were synthesized either in our laboratory or provided by Dr. Nicolaides of Parke-Davis Co. (Gillessen *et al.*, 1970).

Results

Figure 1 shows simple titration curves of TRF, histidine, imidazole, and <Glu-His-OMe. The TRF curves are unchanged over a 30-fold concentration range (180 μg to 6 mg/ml). That the pK_a value of the imidazole proton in TRF (pK_a = 6.25) is lower than that in free imidazole (pK_a = 7.08) and similar to the value for histidine (pK_a = 6.0) is due, presumably, to an intramolecular interaction. A study of this interaction permitted a definition of the orientation of the imidazole ring in TRF. It was hoped that by systematically altering the various functional groups on the TRF molecule it would be possible to establish the basis for the interaction.

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TABLE I

Type of Mol Modification	TRF Analog Primary Structure	Potentiometric pK_a Value (±0.05)	Biol Potency (U/mg)
Prototype	<Glu-His-Pro-amide	6.25	50,000
Amide substitutions	-methyl ester	6.10	10,000
	-(free acid)	6.75	10
Alkylated C-terminal amide	<Glu-His-Pro-dimethylamide	6.25	250
	-diethylamide	6.45	25
	-piperidine	6.45	100
	-monoethylamide	6.25	7,000
Carboxyl deletion	<Glu-His-pyrrolidide	6.2	100
Proline deletions	<Glu-His-amide	6.7	2–10
	-methyl ester	7.0	<1
<Glu substitutions	Pro-His-Pro-amide	6.05	5
	Cyclobutyl-His-Pro-amide	6.35	8
	(*N*-Me)<Glu-His-Pro-amide	6.25	600

In analogy to histidine and its derivatives (Sachs *et al.*, 1971), an intramolecular hydrogen-bond structure between an amino or amide hydrogen of TRF and a nitrogen of the imidazole ring would lead to a decreased affinity of the imidazole for a proton, an increased acidity of the imidazolium group, and thus a lower pK_a. On the other hand, the hydrogen involved in a hydrogen bond, *i.e.*, to a carbonyl group, would be expected to be less available, and its pK_a value would be higher as has been demonstrated by hydrogen-exchange experiments (Printz *et al.*, 1972). In TRF the imidazole proton has a pK_a lower than that of the free imidazole proton. Only three amide hydrogens exist in TRF: (a) the one on the nitrogen of the lactam ring, (b) the <Glu-His peptide bond amide hydrogen, and (c) the C-terminal amide group. The feasible hydrogen-bonding structures of the C-terminal and lactam amides can be ascertained directly by titrating various derivatives; the <Glu-His amide hydrogen cannot, owing to the unavailability of proper analogs.

The pK_a of the imidazole proton in various TRF analogs is distinctly changed (Table I). Removal of the C-terminal amide group, yielding the free acid, raises the pK_a value of the imidazole proton from 6.25 to 6.75, close to that obtained for free imidazole (7.05); esterification of the carboxylic acid group with a methyl residue reestablishes an interaction which lowers the imidazole pK_a value to 6.10. The free carboxylic acid analog of the TRF molecule has very low biological activity. Deletion of the entire proline-terminal amino acid residue raises the pK_a value similar to C-terminal deamidation even when the dipeptide, <Glu-His, has a blocked C terminus.

Derivatization of the C-terminal amide group only appears to interfere slightly with the titratability of the imidazole proton. The blocking of the C-terminal amide function with mono- or dialkyl groups does not appear to affect the availability of the imidazole proton. Dimethylation or monoethylation has no detected significant effect (pK_a = 6.25) while the addition of a larger alkyl group, such as a cyclic amide, *e.g.*, piperidine, has a slightly more pronounced influence (pK_a = 6.45). Total removal of the proline carboxyl group to yield the peptide <Glu-His-pyrrolidide demonstrates that the increased availability of the imidazole proton is not dependent upon an interaction of the imidazole ring directly or indirectly with C-terminal amide group (pK_a = 6.2). The influence of the larger modifications in this proline amide group can be interpreted as being the consequence of a direct steric effect.

It is evident that the intramolecular hydrogen bonding, as revealed by a reduction in the pK_a of the imidazole proton, has an apparent requirement for any amino acid amide in the C-terminal position of the molecule (Table I and IIa). In the absence of a peptide bond at the carboxyl group of the histidyl residue no hydrogen bonding is detected.

Alteration of the pyroglutamyl (<Glu) residue either by removal of the keto group to yield proline or by N-methylation has no effect upon the pK_a value of the peptide. Similarly, substitution of a four-carbon ring (cyclobutyl) for the pyrrolidone has only a slight effect. Although we have but a few analogs with N-terminal <Glu modifications, the finding that

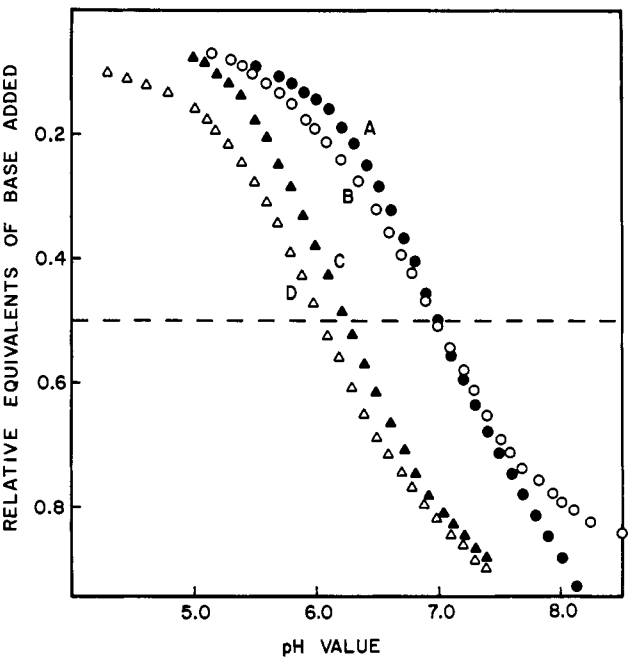

FIGURE 1: Titration curves of the imidazole proton of (A) imidazole, (B) <Glu-His-OMe, (C) TRF, and (D) histidine at ambient temperature (25°).

TABLE II

Peptide	Potentiometric pK_a value (±0.1)
a. <Glu-His-methyl ester	7.0
<Glu-His-amide	6.7
<Glu-His-prolinamide (TRF)	6.25
<Glu-His-glycinamide	6.1
<Glu-His-sarcosinamide	6.25
<Glu-His-leucinamide	6.3
b. <Glu-(N^{π}-Me)-His-Pro-amide	6.6
<Glu-(N^{τ}-Me)-His-Pro-amide	5.95

they show only slight effects upon the pK_a value of the imidazole proton in TRF indicates that <Glu does not directly contribute to the orientation of the imidazole group of TRF in solution. We therefore conclude that the functional groups of the <Glu moiety do not interact with the imidazole ring.

TRF peptide models built with scale-size LAB/QUIP push-fit and/or Corey–Pauling atomic models demonstrate that feasible interactions can occur between either the N^{π}- or N^{τ}-nitrogen atoms of the imidazole ring (IUPAC–IUB 1972 notations) and the amide hydrogens of the TRF molecule. The biological activity of the N^{π}- and N^{τ}-methylimidazole TRF derivatives [(N^{π}-Me)- and (N^{τ}-Me)-TRF] (Vale *et al.*, 1971) indicate that of the possible interactions the preferred hydrogen bond must form with the N^{π} atom of the imidazole ring. This is confirmed by the titration data in which the (N^{τ}-Me)-TRF has a pK_a value of 5.95, 0.65 unit lower than the (N^{π}-Me)-TRF analog (Table IIb).

Discussion

We had anticipated that a hydrogen bond involving the imidazole proton would decrease the availability of that proton, raising the pK_a, while an interaction of the electronegative imidazole nitrogen with an amide proton would decrease the affinity of imidazole for a proton, lowering the pK_a. The latter was observed in TRF. Using a number of synthesized TRF analogs we were able to rule out the interaction of the imidazole nitrogen with either the <Glu lactam or proline amide hydrogens. By elimination, lacking the appropriate analogs, we infer that the hydrogen-bonded interaction of the imidazole nitrogen is with amide hydrogen of the <Glu-His peptide bond. Titration data and biological potencies of methylated imidazole TRF analogs argue that the N^{π} position is the electronegative species in the imidazole ring interacting with the amide hydrogen. The amide hydrogen N^{π}-imidazole interaction is apparently strengthened by the formation of a peptide bond between the histidyl carboxyl group and the amino group of the C-terminal proline. The hydrogen bonded structure forms a six-membered ring structure and even though the N–H–N angle cannot be 180° (Figure 2), its effect distinctly influences the pK_a value of the imidazole proton. The angle of interaction is similar to that found for a preferred conformation of free histamine (Kier, 1968).

The intramolecular hydrogen bond does not appear to form (or at least is very much weaker) in the <Glu-His-amide dipeptide, but formation of a peptide bond on the histidyl carboxyl function using any of several amino acids similarly strengthens an imidazole–amide hydrogen-bonded interaction. This peptide-bond formation, producing rigidity in that part of the molecule, appears to be a requirement to establish the imidazole–amide hydrogen bond as any amino acid, even glycine with its minimal steric properties, in this C-terminal position produces a tripeptide with a low pK_a for the imidazole proton (Table IIa).

The data show that modifications of the <Glu moiety do not affect the titration of the imidazole proton but modifications of the C-terminal amino acid to appear to have a pronounced influence. The lower pK_a value of the imidazole proton when the C-terminal carboxyl group is derivatized, ideally by an amino group, demonstrates that the electrostatic effect of a free carboxyl group reduces the availability of the proton on the imidazole ring.

As mentioned previously, deductions from model building and from biological data indicate that the preferred amide hydrogen–imidazole interaction is with the N^{π}-imidazole atom. In an attempt to ascertain more directly which position interacts with the hydrogen of the amide group we titrated the (N^{π}-Me)-TRF and (N^{τ}-Me)-TRF derivatives. The pK_a values of 6.6 and 5.95, respectively (Table IIb), reflect a situation consistent with our deductions and offer an explanation of their biological activities and the preferred orientation of the imidazole ring within the TRF molecule. The difference in pK_a of 0.65 unit produced by the hydrogen-bonding preference of N^{π} over N^{τ} is equivalent to a free-energy difference of 890 cal/mole favoring the N^{π}-bonding configuration of TRF. The stability produced by the methylation of the N^{τ} position, reflected in pK_a values, increases the biological activity as well as the stability of the TRF tripeptide (Vale *et al.*, 1971). These methylated imidazole TRF analogs help rule out any suggestion of intermolecular hydrogen bonding as, due to steric restrictions, only the N^{τ} position can take part in such an interaction.

The free energy of protonation of the imidazole ring of TRF, affected by the proposed hydrogen-bonded interaction of the imidazole nitrogen with the amide proton of the pyroglutamylhistidyl amide bond, is lower by 1140 cal/mole relative to the protonation of free imidazole (ΔG° imidazole = 9780 clal/mole, ΔG° imidazole of TRF = 8640 cal/mole). This energy difference is a monitor of our proposed preferred orientation of the histidine moiety in the TRF molecule. An internal hydrogen bond in proteins has been estimated to contribute 1–5 kcal/mole, this type of hydrogen-bonded interaction has been suggested to form the nucleus of folded protein structures during synthesis (Lewis *et al.*, 1971). Hydrogen-bonded interactions are cumulative in protein molecules, and form a basis of the three-dimensional structure of proteins (Kendrew, 1961). The postulated interaction requires peptide-bond formation (or the parallel steric interference of an alkylated amide group) restricting the rotational freedom of the terminal amino acid. The data indicate that this terminal amino acid is a major factor in the stability and allowed orientations of TRF.

Hydrogen bonding between the amide function and the N^{π}-imidazole atom is affected by the alkylation of the C-terminal amide. Certain orientations of the proline residue appear, in molecular models, to be unlikely due to overlapping electron orbitals. Such deductions suggest that the amide–imidazole interaction imposes restrictions upon the plausible histidylprolylamide dipeptide orientations but little restriction upon the orientation of the pyroglutamylhistidyl dipeptide relationships.

The data and its interpretations allow us to present a three-dimensional model of the TRF molecule (Figure 2). It stresses

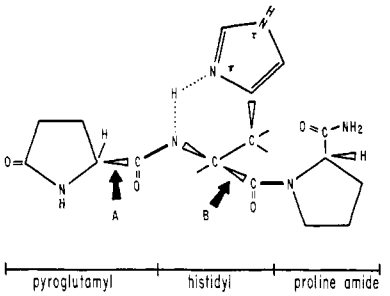

FIGURE 2: Diagrammed model of the proposed hydrogen-bonded interaction of the TRF imidazole N^{π} atom. Heavy lines indicate the planar peptide bonds. A and B are the bonds which rotate without restrictions imposed by the observations presented.

the hydrogen bonding between the N^{π} of the imidazole and the amide proton of the pyroglutamylhistidyl peptide bond. The preferred orientation between the single hydrogen on the histidyl α-carbon and the two hydrogens on the histidyl β-carbon is restricted by the hydrogen-bonded interaction. The orientation of the proline moiety and its amide function must be such that modifications of the amide can either sterically or, in the case of the free carboxylic acid, electrostatically interfere with the imidazole–amide hydrogen bonding. The proline ring can be arranged to give minimal steric interaction with the other moieties in TRF. The <Glu ring is not restricted by data presented; in the proposed model <Glu is allowed almost a full 360° rotational freedom on the carboxyl–α carbon bond of this amino acid residue; some positions however, demonstrate minor Van der Waals interactions. The information obtained from imidazole titration experiments does not place restrictions on the <Glu rotational freedom. The proposed structure will hopefully be confirmed by current investigations in our laboratory.

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