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Report

Kinetics and Pattern of Degradation of Thyrotropin-Releasing Hormone (TRH) in Human Plasma

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The kinetics and mechanism of degradation of the tripeptide TRH (pGlu-His-Pro-NH_2) and its various primary and secondary degradation products (TRH-OH, His-Pro-NH_2, and His-Pro) have been determined in human plasma at 37°C. The rates of degradation of both TRH and TRH-OH (pGlu-His-Pro) showed mixed zero-order and first-order kinetics. At low substrate concentrations first-order kinetics occurred, with TRH and TRH-OH degradation half-lives of 9.4 and 27 min, respectively. The initial step in the plasma-catalyzed degradation of TRH is due to hydrolysis of the pGlu-His bond by the TRH-specific pyroglutamyl aminopeptidase serum enzyme, resulting in the exclusive formation of histidyl-proline amide (His-Pro-NH_2). Using specific HPLC methods the major degradation route (67%) of this dipeptide in human plasma was hydrolysis of the peptide bond to yield His and Pro-NH_2, whereas deamidation to yield His-Pro accounted for 29% of the total degradation. A minor pathway (≤4%) was spontaneous cyclization to yield cyclo(His-Pro). Both His-Pro-NH_2 and His-Pro degraded by first-order kinetics and faster than TRH, with half-lives of 5.3 and 2.2 min, respectively, in 80% plasma.

KEY WORDS: thyrotropin-releasing hormone (TRH); histidyl-proline amide; enzymatic degradation; plasma-catalyzed hydrolysis.

INTRODUCTION

Thyrotropin-releasing hormone (TRH; pGlu-L-His-L-Pro-NH_2) is the hypothalamic peptide that regulates the synthesis and secretion of thyrotropin from the anterior pituitary gland. Since its discovery in 1969, TRH has been shown to have not only a variety of endocrine and central nervous system-related biological activity, but also potential as a drug in the management of various neurologic and neuropsychiatric disorders including depression, shock and schizophrenia (for reviews see Refs. 1–7). However, the clinical utility of TRH has been hampered by its rapid metabolism and clearance and its poor access to the brain (1,7,8). Intravenous studies in rats and humans have shown that TRH has a plasma half-life of only 6–8 min (9–12), because of rapid degradation of the tripeptide by enzymes endogenous to all body fluids and tissues.

The initial enzymatic degradation of TRH in the blood and various tissues occurs either by deamidation of the terminal proline amide residue to yield TRH-OH (pGlu-His-Pro) or by removal of the N-terminal pyroglutamyl residue to yield His-Pro-NH_2 (Scheme I) (12–16). The deamidation is effected by prolyl endopeptidase (16,17), whereas the hydrolysis of TRH at the pGlu-His bond is catalyzed by the less specific pyroglutamyl aminopeptidase (type I), the TRH-specific pyroglutamyl aminopeptidase (type II), and a "TRH-degrading serum enzyme" (13,14,18–21). The latter two pyroglutamyl aminopeptidases exhibit similar physical and chemical characteristics and are probably identical or derived from the same gene (13). In serum or plasma the deamidation appears to play no significant role (16,19,22) and the degradation of TRH is due solely to hydrolysis of the pGlu-His bond by the TRH-specific pyroglutamyl aminopeptidase serum enzyme (13,14). The histidyl-proline amide (His-Pro-NH_2) formed upon this hydrolysis is subsequently enzymatically hydrolyzed to its constituent amino acids or converted nonenzymatically to the stable histidyl-proline diketopiperazine [cyclo(His-Pro)] (14,23,24). On the other hand, in organs such as the liver, kidney, and gut and in various brain tissues, the deamidation is an important degradation pathway of TRH (15,16,25).

The biological half-life of TRH may be enhanced by derivatization of the peptide to produce prodrugs that are resistant to the TRH-degrading enzymes but can regenerate the parent TRH cleavable by other mechanisms (26). A clear understanding of the rate and mechanism of the enzymatic inactivation of the peptide is crucial to this approach (27). Information on the kinetics and pattern of degradation of the primary degradation product (His-Pro-NH_2) in plasma is also important because of the possible biological activities of its degradation products His-Pro (28,29) and cyclo(His-Pro) (23,30).

In the present study, we have investigated the kinetics of degradation of TRH, TRH-OH, His-Pro-NH_2, and His-

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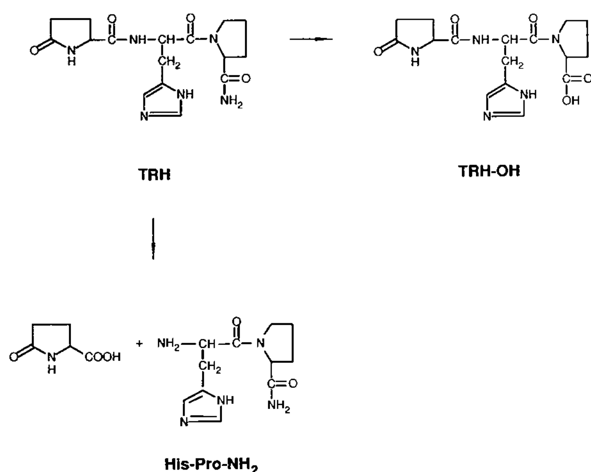


Scheme I

Pro in human plasma and elucidated the pattern of degradation of these peptides using specific HPLC methods.

MATERIALS AND METHODS

Apparatus

High-performance liquid chromatography (HPLC) was performed with a system consisting of a Kontron 420 LC pump, a Kontron 432 LC detector operated at 215 nm, and a Rheodyne 7125 injection valve with a 20-μl loop. A reversed-phase Nova-Pak CN HP Radial Pak column (100 × 8 mm) equipped with a Resolve CN Guard Pak precolumn (both from Waters) was used in all measurements except for the determination of histidine, in which case an Ultrapac TSK ODS-120T (5-μm) column (250 × 4.6 mm) (LKB Products, Sweden) was used. Readings of pH were carried out on a Radiometer PHM 83 Autocal instrument.

Chemicals

TRH, TRH-OH (pGlu-His-Pro), and cyclo(His-Pro) were obtained from Sigma Chemical Company, St. Louis, MO., whereas L-histidine, L-proline amide, and L-His-L-Pro were purchased from Bachem AG, Bubendorf, Switzerland. L-His-L-Pro-NH_2 (as the dihydrochloride salt) was kindly supplied by Carlbiotech A/S, Copenhagen.

Kinetic Measurements

All studies were performed at 37.0 ± 0.2°C. The reactions were initiated by adding 100 μl of a stock solution of the compounds in water to 5 ml of preheated plasma solutions. The solutions were kept in a water bath at 37°C and at appropriate intervals samples were taken, deproteinized as described below, and centrifuged for 3 min at 13,000 rpm^{-1}. A sample (20 μl) of the clear supernatant obtained was analyzed by HPLC. The compounds were quantified by measuring the peak heights in relation to those of standards chromatographed under the same conditions.

Degradation of TRH and TRH-OH. These compounds were incubated in undiluted human plasma at an initial concentration of 5×10^{-4} *M*. Plasma samples of 200 μl were deproteinized by mixing with 400 μl of a 2% (w/v) solution of zinc sulfate in methanol–water (1:1, v/v). The mobile phase used consisted of methanol–0.02 *M* acetate buffer, pH 5.0 (1:19, v/v), and the flow rate was 1.5 ml min^{-1}. Under these conditions TRH and TRH-OH showed a retention time of 5.1 and 4.4 min, respectively, whereas the constituent amino acids eluted with the solvent front.

Degradation of His-Pro-NH_2 and His-Pro. These compounds were incubated in 80% human plasma containing 0.01 *M* phosphate buffer of pH 7.4 at initial concentrations of 10^{-3} *M* (His-Pro-NH_2) or 3×10^{-4} *M* (His-Pro). Plasma samples of 250 μl were deproteinized by mixing with 250 μl of a 2% (w/v) solution of zinc sulfate in methanol–water (1:1, v/v). The compounds were chromatographed using the same mobile phase as for TRH and TRH-OH, the flow rate being 2 ml min^{-1}. Under these conditions His-Pro-NH_2 showed a retention time of 7.4 min, whereas His-Pro eluted after 4.4 min. Cyclo(His-Pro) appeared after 3.8 min.

For the determination of histidine, formed upon degradation of the peptides, a method utilizing ion-pair chromatography was used. In this method an Ultrapac TSK ODS-120T column was eluted with 0.02 *M* acetic acid containing 0.15% (w/v) of sodium heptane sulfonate monohydrate at pH 3.2. The flow rate was 1 ml min^{-1} and the column effluant was monitored at 215 nm. Under these conditions histidine showed a retention time of 4.0 min. The plasma samples (250 μl) to be analyzed were deproteinized by mixing with 250 μl of a 5% (w/v) perchloric acid solution. A blank plasma sample showed a peak with a retention time identical to that for histidine. This peak was assumed to be histidine, which is known to be present in human plasma at a concentration of about 6×10^{-5} *M* (31). The height of the peak from the plasma used in this study corresponded to a concentration of histidine of 7×10^{-5} *M*.

RESULTS AND DISCUSSION

Kinetics of Degradation of TRH and TRH-OH

The rates of degradation of TRH and TRH-OH were determined in undiluted human plasma at 37°C. Only one batch of plasma was studied. The experiments were carried out in triplicate; the rate parameters obtained therefrom varied within ±5%. At an initial concentration of 5×10^{-4} *M*, the progress of hydrolysis of both TRH and TRH-OH followed mixed zero- and first-order kinetics. As seen from Figs. 1 and 2, the rate of degradation initially followed zero-order kinetics, and as the substrate depleted, it changed to follow first-order kinetics. This behavior is typical for enzyme-catalyzed reactions following Michaelis–Menten kinetics in which the initial substrate concentration is higher than the Michaelis constant K_m (32).

The differential form of the Michaelis–Menten equation is

$$-\frac{dS}{dt} = \frac{V_{max}S}{K_m + S} \quad (1)$$

where V_{max} is the maximum rate of substrate consumption, K_m is the Michaelis constant, and S is the substrate concentration. Integration of Eq. (1) gives (33)

$$V_{max}t = S_0 - S + K_m \ln (S_0/S) \quad (2)$$

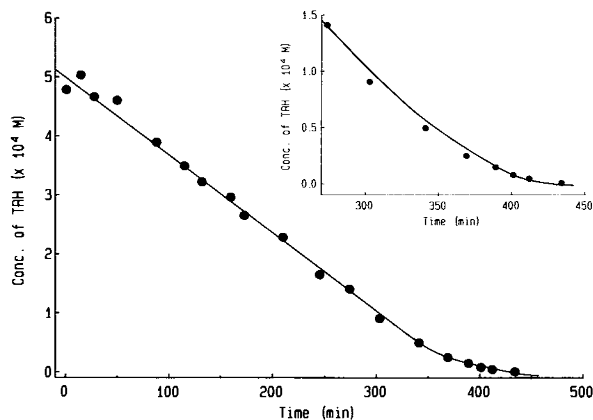


Fig. 1. Plot showing the rate of degradation of TRH in human plasma at 37°C. The inset is an enlarged plot of the rate data at low TRH concentrations. The curves are calculated from Eq. (2) and the rate parameters K_m and V_{max} given in Table I.

where S_0 is the initial substrate concentration and S is the substrate concentration at time t.

By analyzing the progress curves in Figs. 1 and 2 according to Eq. (2); using iterative nonlinear regression analysis (33), a reasonable fit of this equation to the curves was obtained, indicating that the plasma-catalyzed degradation of TRH and TRH-OH followed Michaelis–Menten kinetics. Values of the rate parameters K_m and V_{max} for the degradation of the peptides are listed in Table I along with the half-lives of degradation. At a low substrate concentration (i.e., $S << K_m$), the enzymatic reaction is first-order with the rate equal to

$$-\frac{dS}{dt} = \frac{V_{max}}{K_m} S \qquad (3)$$

The half-lives given in Table I refer to this rate, that is,

$$t_{1/2} = 0.693/(V_{max}/K_m) \qquad (4)$$

Saturation kinetics for the degradation of TRH in human plasma or serum has previously been observed by a number of investigators (22,34,35). At low TRH concentrations, i.e., conditions where the rate of degradation follows first-order kinetics (concentrations lower than about 10 μM), half-lives of degradation of TRH in human plasma or serum of 8 min (34), 11 min (35), 18 min (36), 39 min (37), 15 min (38), 33 min (39), 8 min (40), and 17 min (12) have been reported. As observed by Iversen (12) there is considerable variation of the serum-catalyzed degradation of TRH between different individuals. With serum from 14 persons Iversen observed degradation half-lives *in vitro* at 37°C ranging from 4 to 38 min, the mean value being 16.8 ± 9.4 min. No significant difference between human whole blood, serum, or plasma in the rate of TRH degradation has been observed (8).

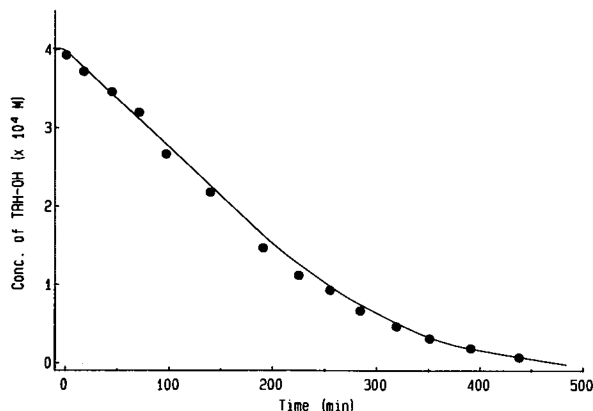


Fig. 2. Plot showing the rate of degradation of TRH-OH in human plasma at 37°C. The curve is calculated from Eq. (2) and the rate parameters K_m and V_{max} given in Table I.

Table I. Kinetic Data for the Degradation of TRH and TRH-OH in Human Plasma at 37°C

Compound	K_m (M)	V_{max} (M min^{-1})	$t_{1/2}$ (min)[a]
TRH	1.9×10^{-5}	1.4×10^{-6}	9.4
TRH-OH	1.0×10^{-4}	1.8×10^{-6}	38

[a] The values of the half-lives are calculated from Eq. (4).

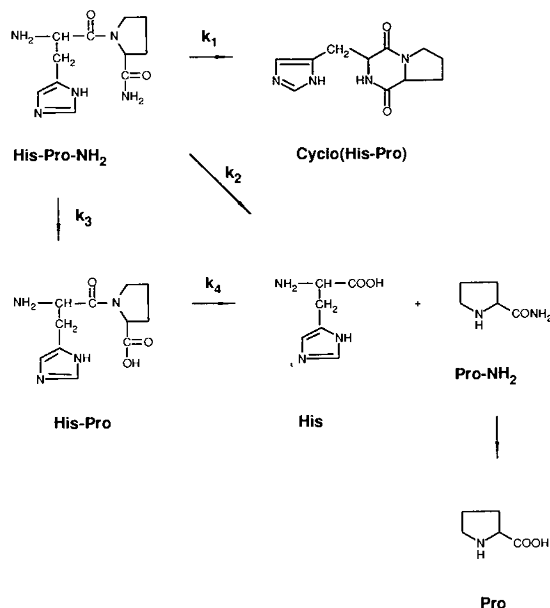
A K_m value for the degradation of TRH in human plasma or serum has not been reported before. In the case of rat plasma a K_m value of 4.1×10^{-6} M has been reported (41), and the partially purified TRH-degrading serum enzyme from rats had a K_m value of 5×10^{-5} M (21).

Whereas TRH and TRH-OH showed the same V_{max} values, the acid had a fivefold higher K_m value, resulting in a slower first-order rate of degradation in human plasma (Table I). This is in accordance with previous investigations reporting on a similarly slower rate of degradation of TRH-OH relative to TRH in human serum (22,34). The same applies for the *in vivo* metabolism in the rat following intravenous administration (42).

Pattern of Degradation

The rapid inactivation of TRH in plasma or serum is due largely to hydrolysis of the pGlu-His bond, yielding pyroglutamic acid and His-Pro-NH_2 (13–15,18–21). This hydrolysis is effected by the "TRH-specific pyroglutamyl aminopeptidase" serum enzyme (13,14). Several investigators have reported that deamidation of TRH to give TRH-OH does not occur to any significant extent in human or rat plasma (16,19,22,34), although this degradation has previously been proposed to be a major pathway (43). In the present study, no TRH-OH formation was observed, thus confirming these more recent studies. As described above TRH-OH degrades at a rate similar to or slower than that of TRH (depending on the concentration), and therefore, any significant formation (>5%) of TRH-OH should have been detectable by the HPLC procedure. However, no TRH-OH peak in the chromatograms was found.

Little information exists on the degradation of His-Pro-NH_2 formed in the initial reaction. As depicted in Scheme II, His-Pro-NH_2 can be degraded by hydrolysis of the proline amide bond to yield His-Pro and subsequently to the free amino acids or by hydrolysis of the peptide bond, yielding histidine and proline amide. Both the former reaction involving a post-proline dipeptidyl aminopeptidase and the latter reaction mediated by an imidopeptidase have been



Scheme II

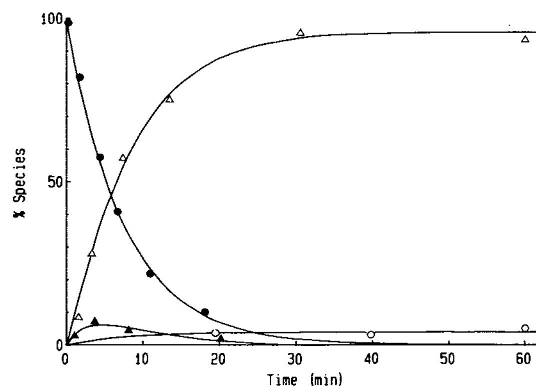


Fig. 4. Time courses for His-Pro-NH_2 (●), His-Pro (▲), histidine (△), and cyclo(His-Pro) (○) during incubation of His-Pro-NH_2 in 80% human plasma at 37°C.

demonstrated to occur upon incubation of His-Pro-NH_2 with various brain tissues (44–50). A third degradation pathway of His-Pro-NH_2 involves the spontaneous (nonenzymatic) cyclization to cyclo(His-Pro) (24,51,52). Using a TLC method Bauer *et al.* (18,19) have shown His-Pro to be a product of TRH degradation in rat serum, whereas Brewster *et al.* (37,39,53) found histidine to be the only significant product from degradation of TRH in human or rat plasma.

We studied the degradation of His-Pro-NH_2 and His-Pro in human plasma using an HPLC method. At initial concentrations of up to 10^{-3} *M* both peptides rapidly degraded according to first-order kinetics (Fig. 3). In 80% human plasma the half-life of disappearance of His-Pro-NH_2 was 5.3 min, whereas His-Pro degraded with a half-life of 2.2 min. The latter reaction is most likely mediated by a proline dipeptidase.

Analysis of the plasma solutions of His-Pro-NH_2 showed histidine to be the major product of degradation but small amounts of His-Pro as well as of cyclo(His-Pro) were also formed. The time courses observed for the various fragments are shown in Fig. 4. His-Pro formed from His-Pro-NH_2 is subsequently degraded, whereas cyclo(His-Pro), which is formed to an extent of 4%, is stable in plasma. The time course for His-Pro may be described by the following equation:

$$[\text{His-Pro}]_t = [\text{His-Pro-NH}_2]_0 \frac{k_3}{k_4 - K}(e^{-Kt} - e^{-k_4 t}) \quad (5)$$

where k_3 and k_4 are pseudo-first-order rate constants for the formation and degradation, respectively, of His-Pro, and K is the pseudo-first-order rate constant for the overall degradation of His-Pro-NH_2, i.e.,

$$K = k_1 + k_2 + k_3 \quad (6)$$

where k_1–k_3 are pseudo-first-order rate constants for the reactions depicted in Scheme II. Fitting of the experimental data for His-Pro to Eq. (5) and knowing the values of K and k_4 from the slopes of the lines in Fig. 3, a value of 0.038 min^{-1} for k_3 was calculated, and the concentration–time curve for His-Pro was constructed from Eq. (5) (Fig. 4). The value of k_1 was calculated from K and the amount of cyclo-(His-Pro) formed and the value of k_2 from the identity $k_2 = K - k_1 - k_3$. In 80% human plasma the following values of the various rate constants were obtained:

$$k_1 = 0.0052\ \text{min}^{-1}$$
$$k_2 = 0.089\ \text{min}^{-1}$$
$$k_3 = 0.038\ \text{min}^{-1}$$
$$k_4 = 0.32\ \text{min}^{-1}$$
$$K = 0.13\ \text{min}^{-1}$$

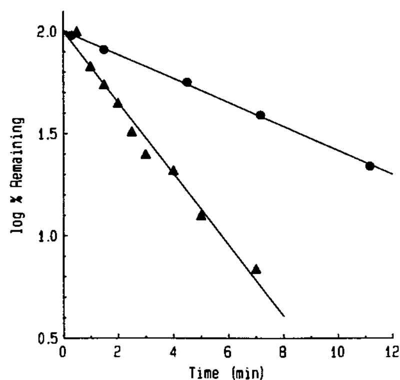


Fig. 3. Plots showing the first-order kinetics of degradation of His-Pro-NH_2 (●) and His-Pro (▲) in 80% human plasma at 37°C.

The major degradation pathway for His-Pro-NH_2 in human plasma is hydrolysis of the peptide bond by an imidopeptidase to yield histidine and proline amide. This pathway accounts for 67% of the overall degradation, whereas the pathway resulting in the formation of His-Pro makes a contribution of 29%. The remaining 4% degradation is due to spontaneous cyclization to cyclo(His-Pro). The 80% plasma solutions studied contained 0.01 *M* phosphate buffer of pH 7.4 and even this small buffer concentration is known to catalyze the cyclization of His-Pro-NH_2 (24). From the rate data given for this process (24) it can be calculated that in 80% plasma solutions without phosphate buffer the amount of cyclo(His-Pro) formed would be only about 2%. In a sep-

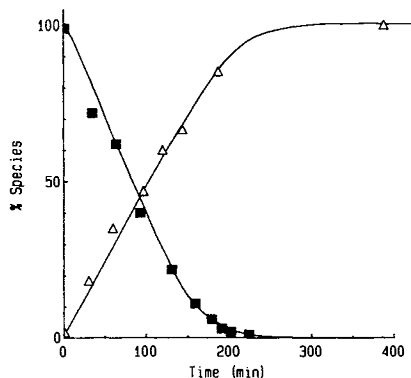


Fig. 5. Time courses for TRH (■) and histidine (△) during incubation of TRH at an initial concentration of 5×10^{-4} *M* in 80% human plasma at 37°C.

arate experiment with His-Pro-NH_2 in undiluted human plasma we confirmed that cyclo(His-Pro) was produced in only minute amounts (1–2%).

Following its formation His-Pro is rapidly hydrolyzed to the constituent amino acids. Therefore, the histidine arising from His-Pro-NH_2 through this route shows no significant lag period in agreement with the experimental data obtained (Fig. 4).

When the degradation of TRH was determined in human plasma at initial concentrations of 5×10^{-4} *M*, the only significant degradation product observed was histidine (Fig. 5). No His-Pro-NH_2 or His-Pro peaks were seen in the chromatograms of the solutions. This finding agrees with the degradation data described above for His-Pro-NH_2 and His-Pro since under the conditions used the rate of degradation of TRH proceeds according to zero-order kinetics and is much slower than the first-order rates of degradation of His-Pro-NH_2 and His-Pro. Therefore, no accumulation of these products occurs.

ACKNOWLEDGMENTS

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