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Metabolism of Thyrotropin Releasing Hormone in Brain Extracts

ISOLATION AND CHARACTERIZATION OF AN IMIDOPEPTIDASE FOR HISTIDYLPROLINEAMIDE*

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An extract of porcine brain acetone powder incubated with thyrotropin-releasing hormone (TRH; pGlu-His-ProNH$_2$) produces acid TRH (pGlu-His-Pro), histidine, and prolineamide. Fractionation of the brain extract by DEAE-cellulose chromatography produces three protein fractions which metabolize TRH. The activity of these fractions was characterized using TRH with a ^{3}H-label on the histidine or proline as well as [*His*-^{3}H]His-ProNH$_2$. Fraction I contains pyroglutamate aminopeptidase and Fraction II contains TRH deamidase. Fraction III was found to contain a previously unrecognized enzyme which cleaves His-ProNH$_2$ to histidine and proline. The histidylprolineamide imidopeptidase has been characterized. A competition study using a variety of compounds containing histidine or proline suggests that the best substrates for the imidopeptidase contain a free α-amino group on histidine and a blocked carboxyl group on proline, as is found in His-ProNH$_2$. A survey of a variety of polypeptide hormones indicates that many of them inhibit the imidopeptidase activity. A kinetic study of the inhibition of the enzyme by adrenocorticotropic hormone (1-24) shows that the inhibition by polypeptide hormones is noncompetitive. We hypothesize that pituitary hormones may stimulate the production of (cyclo)-His-Pro by inhibiting alternate routes of TRH metabolism.

Thyrotropin-releasing hormone (TRH, pyroglutamylhistidylprolineamide) is a hypothalamic releasing hormone which plays a role in regulating the release of the pituitary hormone thyrotropin. Under some circumstances, TRH[1] can also stimulate the release of growth hormone and prolactin (1). In addition, it exerts some effects at the level of the central nervous system, impinging on both behavioral and thermoregulatory centers (1).

The metabolism of TRH has been the subject of considerable investigation. The various reactions that may be involved in the degradation of TRH are summarized in Fig. 1. It has been established that TRH is metabolized by a TRH deamidase in serum to pGlu-His-Pro (acid TRH) (2, 3). Knigge and Schock (4) have suggested that plasma enzymes further metabolize pGlu-His-Pro to pGlu-His and proline, since they found proline to be a major product of TRH metabolism in plasma. Incubation of TRH with hypothalamic extracts produces a different pattern of products. Studies in this laboratory (5) have indicated that, in addition to TRH deamidase activity, there is also a pyroglutamate aminopeptidase activity which converts TRH to His-ProNH$_2$. The His-ProNH$_2$ produced may then undergo cyclization to (cyclo)-His-Pro[2] (6). While some investigators using fresh brain extracts (7, 8) reported that acid TRH is a major product of TRH metabolism, other investigators (9, 10) showed that extracts from freeze-dried brain tissue produced both acid TRH and prolineamide. Fig. 1 outlines two possible pathways for the origin of prolineamide. The studies presented here provide evidence that prolineamide may be produced from TRH primarily by the indirect pathway (see Fig. 1, Reaction B) involving the cleavage of His-ProNH$_2$ produced from TRH by pyroglutamate aminopeptidase. An imidopeptidase catalyzing the cleavage of His-ProNH$_2$ to histidine and prolineamide has been purified from brain extracts and characterized. The studies herein have revealed that the enzyme can be inhibited by a variety of polypeptide hormones.

EXPERIMENTAL PROCEDURES

Materials—DEAE-cellulose (DE23) was from Whatman. SP-Sephadex was a Pharmacia product. [*Pro*-^{3}H]TRH (21.8 Ci/mmol) was from New England Nuclear. [*His*-^{3}H]TRH and [*His*-^{3}H]His-ProNH$_2$ were prepared as described under "Methods." Porcine brain acetone powder, trypsin inhibitor from soya bean and lima bean, *N*-iodosuccinimide, triiodothyronine, and thyrotropin were from Sigma. Oxytocin, vasopressin, luteinizing hormone-releasing factor, and TRH were obtained from Calbiochem. ACTH (1-24, Synacthen, Ciba) was a gift from Drs. Steven Sabol and Jan Wolff, National Institutes of Health. ACTH (1-10) and ACTH (1-13) were generously provided by Drs. Harold Gainer and Peng Loh, National Institutes of Health. ACTH (5-10) and ACTH (11-24) were gifts from Dr. George Sayers, Case Western Reserve University School of Medicine. Angiotension II (Ciba) and cholera toxin were generously provided by Dr. James Harwood, National Institutes of Health. The isolated α and β chains of thyrotropin and luteinizing hormone were generous gifts from Dr. Leonard Kohn, National Institutes of Health. Human chorionic gonadotrophin was a product of Ayerst Labs. Insulin was obtained from Mann. Insulin (chain A) was from Sigma; insulin (chain B, oxidized) was from Boehringer. Methionine enkephalin was a product of Peninsula Laboratories. (Cyclo)-His-Pro was prepared in this laboratory as previously described (6). Bachem supplied (cyclo)-Gly-Pro and (cyclo)-His-Gly. Chemical Dynamics was the source for Gly-Pro, Asp-Pro, His-Pro, Glu-Pro, and pGlu-His-OCH$_3$. pGlu-His was prepared by incubation of a sample of pGlu-His-OCH$_3$ (generously provided by Dr. Arthur Felix of Hoffmann-La Roche) at room temperature for 1 h at pH 9.

Methods—Pyroglutamate aminopeptidase was prepared from porcine brain acetone powder by the following procedure. Acetone powder (5 g) was suspended in 75 ml of Tris-HCl buffer (0.02 M, pH 7.5) containing 50 mM NaCl (Buffer I). The suspension was sonicated for 10 min in a Raytheon 10 kc sonic oscillator at 1.0 A output, then centrifuged at 27,000 × *g* for 30 min. The supernatant solution was further centrifuged for 45 min at 190,000 × *g*. The supernatant

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[1] The abbreviations used are: TRH, thyrotropin-releasing hormone; ACTH, adrenocorticotropic hormone; TSH, thyroid-stimulating hormone.

[2] (Cyclo)-His-Pro is the diketopiperazine of histidine and proline. It is also designated in some figures as His-Pro.

solution was applied to a column (5 × 25 cm) of DEAE-cellulose equilibrated with Buffer I. Elution was continued with Buffer I. A single peak of absorbance at 280 nm was obtained in Fractions 15 to 22 (10-ml fractions). The fractions were combined and concentrated to 10 ml by pressure filtration through an Amicon PM30 membrane filter.

[*His*-^{3}H]TRH was prepared from TRH by the following procedure. TRH (10 mg, approximately 30 μmol) was dissolved in 10 ml of sodium acetate buffer (0.2 M, pH 6.5). *N*-Iodosuccinimide (300 μmol in 5 ml of acetonitrile) was added. After 2 min at room temperature, the reaction was terminated by the addition of solid sodium thiosulfate until the brown reaction mixture became colorless. The reaction mixture was concentrated *in vacuo* to 1 ml. A fractionation procedure using SP-Sephadex previously described by Ling *et al.* (11) for the separation of TRH, monoiodo-TRH, and diiodo-TRH was followed. The sample was applied to a column (2.5 × 9 cm) of SP-Sephadex previously equilibrated with 0.01 M ammonium acetate, pH 3.6 (11). Elution of the column was with the same buffer. Fractions (5 ml) were screened for Pauly-positive material (12). Fractions 5 and 6 contained (2,4-diiodohistidyl)-TRH. The diiodo-TRH was repurified by passage over another SP-Sephadex column (2 × 19 cm). The purified diiodo-TRH was concentrated to dryness. Diiodo-TRH (13 mg, 21 μmol) was exposed to tritium gas in the presence of a palladium catalyst (procedure performed by New England Nuclear). Analysis of the labeled product indicated that approximately 60% of the radioactivity was localized in TRH. Purified [*His*-^{3}H]TRH was obtained by chromatography on an SP-Sephadex column (2 × 19 cm). Elution was with 0.01 M ammonium acetate, pH 3.6 (250 ml), followed by 0.01 M ammonium acetate, pH 5.2 (250 ml), then with 0.01 M ammonium acetate, pH 7.5 (250 ml). TRH was recovered in the last fraction. The specific activity of this product was assumed to be theoretical (58 Ci/mmol).

[*His*-^{3}H]His-ProNH$_2$ was prepared from [*His*-^{3}H]TRH by the following procedure. [*His*-^{3}H]TRH (200 μl, 1 mCi/ml) was mixed with unlabeled TRH (100 μl, 4 mg/ml), then added to a solution of porcine brain pyroglutamate aminopeptidase (10 ml, 100 mg). After incubation at 37°C for 90 min, the mixture was centrifuged and the supernatant solution was filtered through Amicon CF25 membrane cones by centrifugation for 2 h at 1500 × *g*. Before use, [*His*-^{3}H]His-ProNH$_2$ was purified by passage through a DEAE-cellulose column (0.8 × 5 cm). This accomplished the removal of any histidine or His-Pro generated during storage. The radioactive material which passed through the column was concentrated by lyophilization.

Examination of the purity of the radioactive His-ProNH$_2$ preparation by high voltage electrophoresis showed that it was contaminated with two other materials. One contaminant (approximately 20%) had the same mobility as TRH, but did not disappear on further incubation with pyroglutamate aminopeptidase. This is therefore a biologically inactive metabolite of TRH. Bauer and Lipmann (10) have suggested that this material is a stereochemical impurity. The other contaminant is (cyclo)-His-Pro which is formed by nonenzymatic cyclization of the His-ProNH$_2$ (see Fig. 4*B*).

The assay for imidopeptidase activity was based on the cleavage of His-ProNH$_2$ to histidine followed by resolution of the two compounds by DEAE-cellulose chromatography. Reaction mixtures (250 μl) contained: Tris-HCl buffer (pH 7.0, 0.05 M), [*His*-^{3}H]His-ProNH$_2$ (5×10^{-6} M, 6×10^5 cpm/nmol). Incubation at 37°C was initiated by the addition of enzyme. At 0, 10, 20, and 30 min, aliquots (50 μl) were deposited on DEAE-cellulose columns (0.8 × 5 cm). The columns were prepared from DEAE-cellulose (free base form) suspended in H_2O and then equilibrated with H_2O. Alternatively, 50-μl aliquots were transferred to test tubes which were held in a boiling H_2O bath for 5 min. H_2O (0.5 ml) was then added and duplicate samples of 200 μl were deposited on DEAE-cellulose columns. The columns were washed with 5 ml of H_2O, followed by 1 ml of 0.1 N HCl. Elution of histidine was accomplished by the addition of 3 ml of 0.5 N HCl. This fraction was collected directly into scintillation vials for counting. Calibration of this column with radioactive His-ProNH$_2$ and histidine indicated that the 0.5 N HCl fraction contained approximately 13% of added His-ProNH$_2$ and 89% of added histidine. Velocities were calculated by plotting counts per min *versus* time for the four time points and drawing the best line through these points (see *e.g.* Fig. 7). When the substrate was incubated with buffer in the absence of enzyme, the blank value did not increase over a period of 30 min.

Several pieces of evidence indicate that the product found in the 0.5 N HCl wash following incubation of [*His*-^{3}H]His-ProNH$_2$ with Fraction III is actually histidine. TRH, (cyclo)-His-Pro, and His-ProNH$_2$ are not adsorbed to DEAE-cellulose and appear in the preliminary H_2O wash. Possible contaminants that are bound to DEAE-cellulose are histidine, His-Pro, and acid TRH. As indicated in the description of the preparation of [*His*-^{3}H]His-ProNH$_2$, the radioactive substrate was freed of contaminating histidine or His-Pro by passage through a DEAE-cellulose column. Further, the other contaminant in the His-ProNH$_2$ with a mobility similar to TRH is biologically inert and cannot be converted to acid TRH by the deamidase present in Fraction III. A definitive demonstration that the product of the reaction of His-ProNH$_2$ with Fraction III was histidine came from an examination of the reaction products by paper chromatography (see "Methods"). Two peaks of radioactivity were observed. The major component had an R_F of 0.14 corresponding to histidine, while there was a minor component at R_F 0.70 corresponding to (cyclo)-His-Pro. The diketopiperazine forms spontaneously from residual His-ProNH$_2$ during the process of paper chromatography. There was no evidence by paper chromatography for the presence of pGlu-His, His-Pro, or acid TRH. Taken together, we conclude that the assay is specific for the formation of histidine.

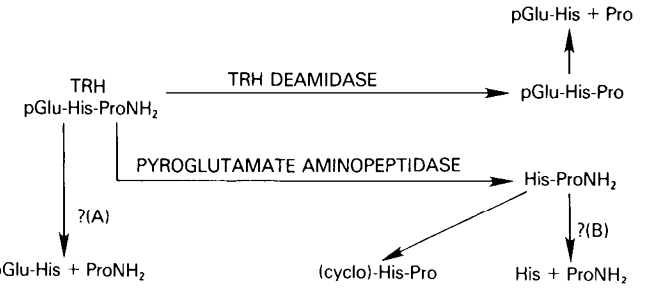

FIG. 1. Pathway of TRH metabolism. Conversion of TRH to pGlu-His-Pro catalyzed by TRH deamidase takes place in both serum and brain. pGlu-His-Pro is further metabolized by serum enzymes to pGlu-His and proline. Pyroglutamate aminopeptidase activity which converts TRH to His-ProNH$_2$ is demonstrable in brain tissue, but has not been found in serum. His-ProNH$_2$ spontaneously cyclizes to (cyclo)-His-Pro. Preliminary evidence suggests that brain tissue contains an enzyme that catalyzes the cyclization. *Pathways A* and *B* outline two possible routes for the formation of prolineamide which has been observed in brain extracts. It is shown in this study that an enzyme in brain catalyzes the reaction (*Pathway B*) leading to the cleavage of His-ProNH$_2$ to histidine and prolineamide.

Paper electrophoresis was run on Whatman No. 3MM paper at 3 kV for 80 min at pH 3.6 (pyridine:acetic acid:H_2O, 12:100:3000). Co-electrophoresed standards were localized by spraying with either the Pauly (12) reagent which detects acid TRH, TRH, (cyclo)-His-Pro, and histidine or the ninhydrin reagent which detects proline and prolineamide. Dried electrophoretograms were cut into 0.5-inch segments for counting. Methanol (90%, 1 ml) was added to the counting vials to elute the radioactive compounds from the strips. Then scintillation fluor (10 ml, toluene:Triton X-100:Liquifluor, 12:6:1) was added to the vials for determination of the distribution of radioactivity. The recovery of applied radioactivity was generally 25%.

Paper chromatography was performed as described in Table I, where the R_F values for a variety of compounds related to TRH are listed. Detection of radioactive samples on chromatograms was performed as outlined above for paper electrophoresis.

RESULTS

The object of these studies was to delineate the enzymatic reactions involved in the metabolism of TRH in brain extracts. As shown in Fig. 2, incubation of labeled TRH with crude brain extract leads to the formation of acid TRH (pyroglutamylhistidylproline), histidine, and prolineamide. The peak of radioactivity with a mobility of 10 inches is clearly prolineamide rather than His-ProNH$_2$, since it contains radioactivity derived from [*Pro*-^{3}H]TRH but not from [*His*-^{3}H]TRH. As outlined in Fig. 1, the prolineamide derived from TRH could arise by Pathway A involving a direct cleavage of TRH to form pGlu-His and prolineamide or by Pathway B which involves an initial degradation of TRH by pyroglutamate aminopeptidase to His-ProNH$_2$ followed by a cleavage of His-ProNH$_2$ to prolineamide. The following studies were designed

TABLE I

R_F values of TRH and some of its metabolites

Ascending paper chromatography was carried out on Whatman No. 3MM paper using CH_3Cl_3:CH_3OH:H_2O, 5:5:1. Histidine-containing compounds were detected by spraying with the Pauly reagent (10). Proline and prolineamide were detected with the ninhydrin reagent.

Compound	R_F
Histidine	0.14
pGlu-His	0.17
His-Pro	0.29
Acid TRH	0.40
Proline	0.45
Prolineamide	0.50
TRH	0.60
(cyclo)-His-Pro	0.70

to distinguish which of these pathways account for the formation of prolineamide.

Fractionation of a crude brain extract by DEAE-cellulose chromatography (Fig. 3) indicated that the protein could be resolved into three major fractions. Each of these fractions contained enzymes that would degrade TRH. An examination of the enzyme activity in Fraction I is shown in Fig. 4. It can be seen that this fraction converts [*His*-^{3}H]TRH to His-ProNH$_2$ (Panel A). Since the peak which forms at a mobility of about 10 inches contains label derived from [*His*-^{3}H]TRH, the product is His-ProNH$_2$ rather than prolineamide. Therefore, the activity in Fraction I is identifiable as pyroglutamate aminopeptidase. Fig. 4*B* shows that this enzyme fraction does not act on [*His*-^{3}H]His-ProNH$_2$. As indicated under "Methods," the radioactive substrate which was prepared from TRH is contaminated with some residual radioactivity in the TRH region (mobility approximately 5 inches) which is not biologically active TRH, as well as some (cyclo)-His-Pro (mobility approximately 7 inches) which is formed by spontaneous cyclization of His-ProNH$_2$. An analysis of the products formed by the action of Fraction II on [*His*-^{3}H]TRH is shown in Fig. 5*A*. A single peak of radioactivity (mobility approximately 2 inches) co-migrating with acid TRH and pGlu-His is formed. A similar peak of radioactivity is formed when the enzyme is incubated with [*Pro*-^{3}H]TRH (data not shown), suggesting that the product is acid TRH. However, since pGlu-His and proline have mobilities similar to that of acid TRH (see Figs. 2 and 5), the possibility was considered that the products were a mixture of pGlu-His and proline. This possibility was eliminated by examination by paper chromatography of the product formed by incubation of Fraction II with [*His*-^{3}H]TRH (data not shown). In addition to residual TRH (R_F 0.60, see Table I), there is a peak of radioactivity at R_F = 0.40 corresponding to acid TRH, but none at R_F = 0.17 corresponding to pGlu-His. This indicates that Fraction II contains TRH deamidase which converts TRH to acid TRH and does not catalyze the formation of pGlu-His from TRH. Panel B of Fig. 5 indicates that this enzyme fraction has essentially no activity toward His-ProNH$_2$. Fig. 6, Panel A, indicates that incubation of Fraction III with either [*Pro*-^{3}H]TRH or [*His*-^{3}H]TRH leads to the formation of a peak of radioactivity (mobility approximately 2 inches) that co-migrates with acid TRH. An examination of the product formed from [*His*-^{3}H]TRH by paper chromatography (data not shown; see above for similar analysis with Fraction II) established that it was acid TRH. This fraction, therefore, contains TRH deamidase which is probably a contamination from Fraction II (see Fig. 3). An examination of the data in Fig. 6, Panel B, indicates the presence of a hitherto unrecognized enzymatic activity active on His-ProNH$_2$. Incubation of the enzyme with [*His*-^{3}H]His-ProNH$_2$ leads to the formation of a peak of radioactivity (mobility approximately 7 inches) that co-migrates in the same region as histidine and (cyclo)-His-Pro. Further analysis of the product by paper chromatography (see Table I) (data not shown) indicated that the radioactive peak chromatographs in the region characteristic of histidine (R_F = 0.1) rather than (cyclo)-His-Pro (R_F = 0.7). Therefore, the enzyme splits His-ProNH$_2$ to histidine and prolineamide and provides an explanation for the formation of prolineamide from TRH in brain extracts. It is clear from these experiments that the mechanism by which prolineamide arises from TRH involves Pathway B of Fig. 1 whereby TRH is first converted to His-ProNH$_2$ followed by an imidopeptidase-dependent cleavage of His-ProNH$_2$ to prolineamide.

In order to assist in the characterization of the imidopeptidase from Fraction III, a rapid assay for cleavage of His-ProNH$_2$ by this enzyme was developed (see "Methods"). This assay is based on the inability of His-ProNH$_2$ to bind to DEAE-cellulose while the labeled cleavage product of [*His*-^{3}H]His-ProNH$_2$ (^{3}H-His) does bind to DEAE-cellulose. The radioactive histidine eluted from the DEAE-cellulose column provides a measure of the enzymatic activity. Using this assay, it was possible to show (data not shown) that the imidopeptidase activity could be assayed under conditions that demonstrate both linearity to time (0 to 30 min) and enzyme concentration (60 to 240 μg of protein).

An examination was made of the pH dependency for imidopeptidase activity (data not shown). When assays were performed in phosphate buffer, the activity gradually increased between pH 6 to 7.5. When assays were carried out in Tris buffer, there was a steeper drop in activity between pH 7 to 8.5. Maximum activity was observed between pH 7 to 7.5.

A test of some possible inhibitors of the imidopeptidase was

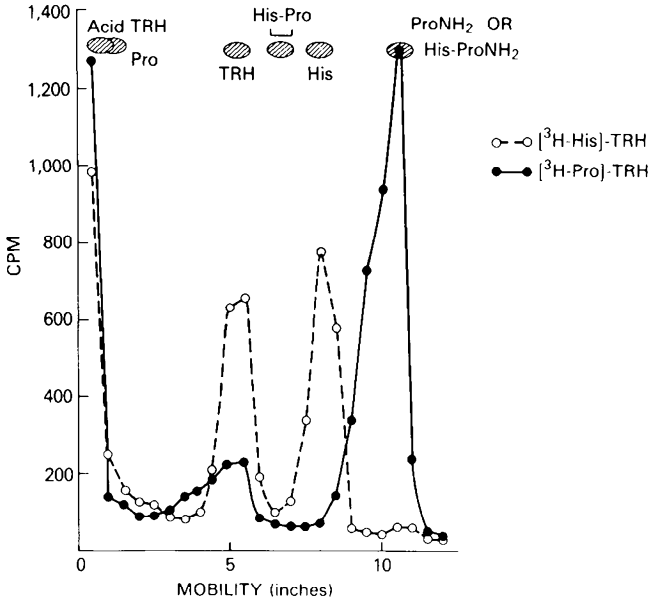

FIG. 2. Formation of histidine and prolineamide from TRH by brain extract. One gram of porcine brain acetone powder was suspended in 15 ml of Tris-HCl (0.02 M, pH 7.5) containing NaCl (50 mM) and subjected to sonication for 10 min in a Raytheon sonic oscillator (10 Kc) at 1.0-A output. The suspension was then centrifuged for 30 min at 27,000 × *g*. Aliquots (100 μl) of the supernatant solution were incubated with 8.6 pmol of either [*Pro*-^{3}H]TRH or [*His*-^{3}H]TRH in a total volume of 105 μl at 37°C. After 30 min, aliquots (10 μl) were spotted on Whatman No. 3MM paper in 1-inch strips. Electrophoresis and counting was carried out as described under "Methods." The position at 0 inches corresponds to the region where the sample was applied. Migration of the designated compounds is in the direction of the cathode.

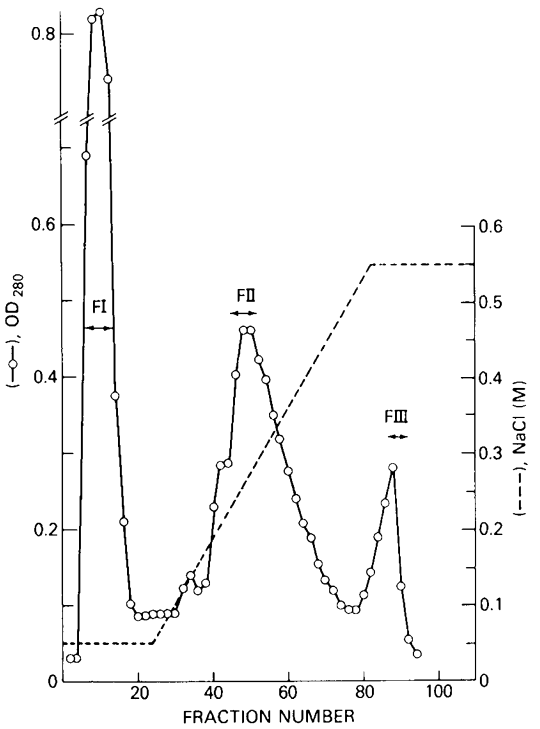

FIG. 3. DEAE-cellulose chromatography of brain extracts. One gram of porcine acetone powder was suspended in 15 ml of Tris-HCl buffer (0.02 M, pH 7.5) containing NaCl (50 mM), EDTA (1 mM), dithiothreitol (1 mM), and glycerol (10%) (Buffer A). The suspension was homogenized with a motor-driven Teflon homogenizer for 2 min, then centrifuged at 27,000 × *g* for 30 min. The supernatant solution was applied to a column (1.5 × 25 cm) of Whatman DE23 cellulose, previously equilibrated with Buffer A. After washing the column with 150 ml of Buffer A (Fraction 24), a linear gradient of Buffer A containing NaCl from 50 to 550 mM was initiated (total volume = 300 ml). At Fraction 80, elution with Buffer A containing 500 mM NaCl was initiated (150 ml). Fractions (5 ml) were assayed for absorbance at 280 nm. The designated fractions were pooled and concentrated by pressure filtration through an Amicon UM10 membrane to final volumes of 1 ml to produce Fractions I (*FI*), II (*FII*), and III (*FIII*). The protein concentrations were approximately 41, 5, and 3 mg/ml, respectively.

carried out (data not shown). The enzyme appears to have a sensitive sulfhydryl group. When *N*-ethylmaleimide at a concentration (0.2 mM) in excess of the dithiothreitol concentration (0.1 mM) was included in assays, the imidopeptidase activity was completely inhibited. The enzyme activity was not inhibited by benzamidine (5 mM), or soya bean or lima bean trypsin inhibitor (100 μg/ml). A variety of metals (magnesium, manganese, cobalt, calcium, or zinc) added at 1 mM had no effect on the enzyme activity. The inclusion of EDTA (10 mM) in standard reaction mixtures led to a 3-fold increase in velocity compared to controls. These data suggest that the imidopeptidase has no requirement for metal for catalytic activity. The presence of tightly bound essential metal cannot be excluded by these studies.

As indicated above, the Fraction III enzyme is contaminated with TRH-deamidase (see Fig. 6*A*). There may be other contaminating peptidase in this fraction as well. In order to test the specificity of the imidopeptidase, a competition approach was therefore adopted. This analysis was based on the idea that imidopeptidase-dependent formation of histidine from His-ProNH$_2$ would be decreased in the presence of an alternate specific substrate or an inhibitor of imidopeptidase activity. Fig. 7 shows this type of competition analysis with respect to TRH. Incubation mixtures containing 5.5×10^{-6} M tritium-labeled His-ProNH$_2$ were challenged with various concentrations of unlabeled TRH. It can be seen that a concentration of TRH in 100-fold excess over that of His-ProNH$_2$ was required in order to achieve a 50% inhibition of the degradation of His-ProNH$_2$. In this study, it is not clear whether TRH is serving as a substrate or an inhibitor for the imidopeptidase activity. In either case, it is evident that His-ProNH$_2$ is by far a better substrate for the imidopeptidase activity than is TRH. These data provide further support for the notion that the origin of prolineamide from TRH is via imidopeptidase activity on His-ProNH$_2$ rather than directly on TRH (see Fig. 1).

As shown in Table II a variety of other compounds containing histidine or proline were tested as competitors of radioactive His-ProNH$_2$ for imidopeptidase activity. As expected, unlabeled His-ProNH$_2$ gives an apparent competition of the activity that can be explained on the basis of an isotope dilution effect. All the other compounds tested produced substantially less competition for the cleavage of radioactive His-ProNH$_2$ than did unlabeled His-ProNH$_2$. These data provide further support for the notion that the enzyme is specific for cleavage of His-ProNH$_2$. However, a comparison of the relative competition efficiencies of the various compounds shown in Table II suggests that the best substrate for the imidopeptidase contains a free amino group on the histidine and a blocked carboxyl group on the proline moiety.

The requirement for a free amino group on histidine is demonstrated by a comparison of the competition by His-ProNH$_2$ and (cyclo)-His-Pro. A 10-fold excess of (cyclo)-His-

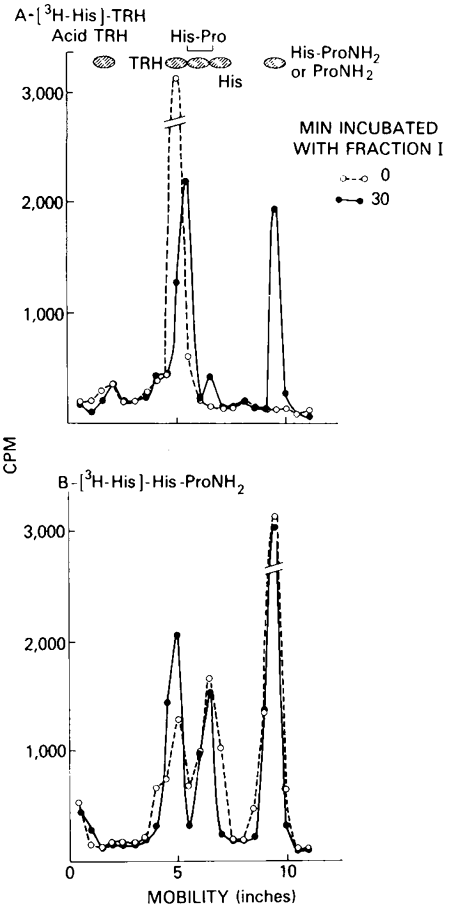

FIG. 4. Action of Fraction I on TRH or His-ProNH$_2$. Fraction I (see Fig. 3) (100 μl) was incubated with either [*His*-^{3}H]TRH (*Panel A*) or [*His*-^{3}H]His-ProNH$_2$ (*Panel B*) (5 μl, 5 × 10^5 cpm, 8.6 pmol) at 37°C. At 0 and 30 min, aliquots of the reaction mixtures (10 μl) were examined by electrophoresis as described in Fig. 1.

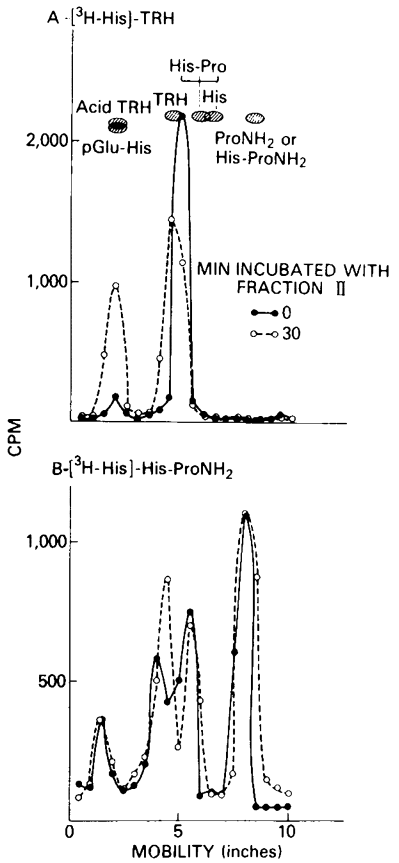

FIG. 5. Action of Fraction II on TRH or His-ProNH$_2$. Fraction II (see Fig. 3) (100 μl) was incubated with either [*His*-^{3}H]TRH (*Panel A*) or [*His*-^{3}H]His-ProNH$_2$ (*Panel B*) as in Fig. 4.

Pro reduces the activity to 83% of the control, while His-ProNH$_2$ reduces the activity to 20% of the control. The requirement for a blocked carboxyl group on proline is demonstrated by a comparison of the competition by His-ProNH$_2$ and His-Pro. Ten-fold excess of these compounds produce 20% and 80% of the control activity, respectively. There is an absolute requirement for proline, since (cyclo)-His-Pro gives a competition reaction, while (cyclo)-His-Gly is completely ineffective as a competitor. On the other hand, the requirement for histidine is not absolute. (Cyclo)-Gly-Pro, Gly-Pro, and Asp-Pro are ineffective competitors, but Glu-Pro produces a competition only slightly less efficiently than does His-Pro. On the basis of this survey, the enzyme can be evaluated as an imidopeptidase with a preference for histidine coupled to the imino acid.

Using the competition approach a survey of a variety of polypeptide hormones on the activity of the imidopeptidase was performed. It is apparent from the data in Fig. 8 that many polypeptide hormones compete with labeled His-ProNH$_2$ for the activity of the imidopeptidase. The most potent competition was achieved with the compounds labeled Group I (β chain of thyrotropin, human chorionic gonadotropin, insulin, and ACTH (1-24)). Less substantial inhibition of the activity was achieved with the compounds represented by Group II. They are: the α chain of luteinizing hormone, angiotension II, TSH, and the α chain of thyrotropin. By increasing the concentration of the polypeptide hormones in Group III, oxytocin and vasopressin, above that represented by the substrate His-ProNH$_2$ it was possible to achieve inhibition of imidopeptidase activity. A number of other compounds were tested for possible effects on the activity of the imidopeptidase. Hydrocortisone (10^{-4} M), triiodothyronine (10^{-4} M), luteinizing hormone-releasing factor (200 μg/ml), methionine enkephalin (7×10^{-5} M), cholera toxin (200 μg/ml), the β chain of luteinizing hormone (200 μg/ml), and bovine serum albumin (200 μg/ml) all gave no inhibition of

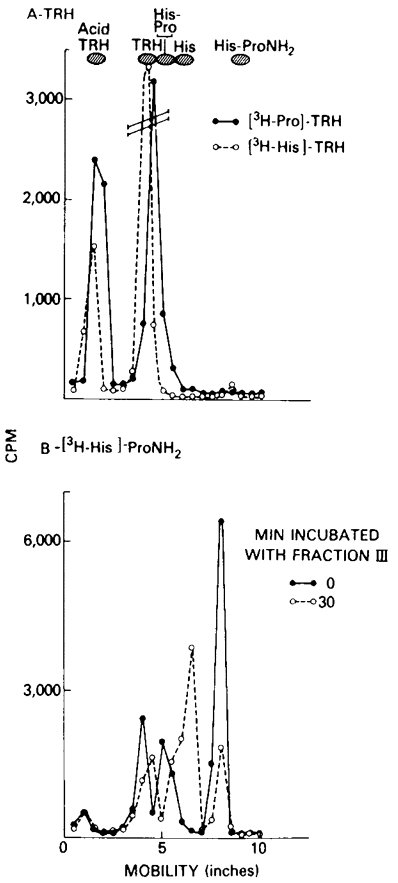

FIG. 6. Action of Fraction III on TRH or His-ProNH$_2$. *Panel A*, [*His*-^{3}H]TRH and [*Pro*-^{3}H]TRH were incubated with Fraction III for 30 min as described in Fig. 4, then examined by electrophoresis. *Panel B*, [*His*-^{3}H]His-ProNH$_2$ was incubated for either 0 or 30 min with Fraction III as described in Fig. 4, then assayed as in Fig. 4.

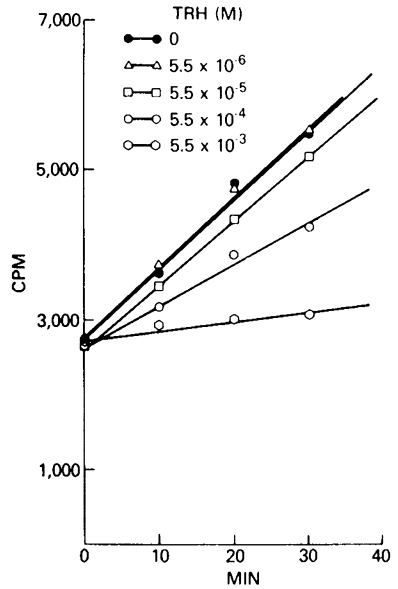

FIG. 7. Effect of TRH on imidopeptidase activity. Imidopeptidase activity was measured as described under "Methods" using Fraction III enzyme (see Fig. 3). The indicated concentrations of TRH were included in the assays.

TABLE II

Effect of amino acids and peptides on imidopeptidase activity

Imidopeptidase activity was measured as described under "Methods" using Fraction III enzyme (see Fig. 3) [*His*-^{3}H]His-ProNH$_2$ was used at a concentration of 5×10^{-6} M. The indicated amino acids or peptides were included in some reaction mixtures at the designated ratio of test compound (unlabeled compound added) to substrate (^{3}H-labeled His-ProNH$_2$). Activity is expressed as a percentage of the activity obtained in a control incubation with no added unlabeled compound.

Test compound	Ratio of test compound/substrate		
	10	100	1,000
	% control activity		
His-ProNH$_2$	20	2	0
(cyclo)-His-Pro	83	41	7
TRH(pGlu-His-ProNH$_2$)	100	60	15
His-Pro	80	61	39
Glu-Pro	100	71	50
Histidine	100	63	50
pGlu-His-OCH$_3$	100	88	50
Prolineamide	100	83	71
Ineffective: (cyclo)-His-Gly, (cyclo)-Gly-Pro, Gly-Pro, Asp-Pro			

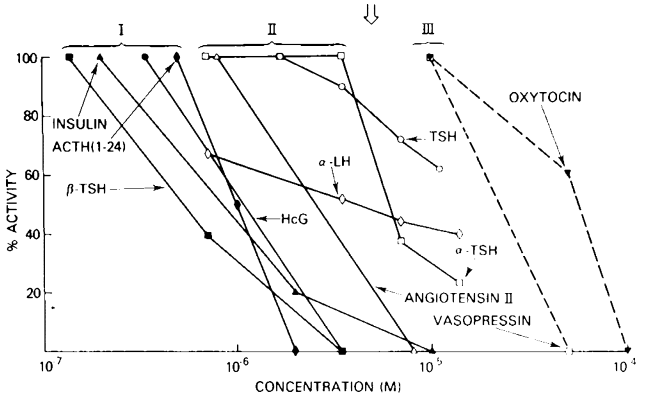

FIG. 8. Effect of polypeptide hormones on imidopeptidase activity. Imidopeptidase activity was measured as described under "Methods" using Fraction III enzyme (see Fig. 3). Polypeptide hormones were added where indicated at the designated concentrations. Activity is expressed as per cent activity observed in a control incubation with no added polypeptide hormone. The *vertical arrow* designates the concentration (5×10^{-6} M) of [*His*-^{3}H]His-ProNH$_2$ included in all incubation mixtures. *HcG,* human chorionic gonadotrophin; *LH,* luteinizing hormone.

the enzyme activity.

A further examination of the specificity of ACTH inhibition of imidopeptidase was carried out. The data in Table III show that the complete sequence of ACTH 1-24 is required for optimal inhibition of the imidopeptidase activity. Less substantial inhibition can be observed with fragments comprising approximately half the sequence (1-10, 1-13, or 11-24), but no inhibition is observed with the 5-10 sequence. These data suggest that the inhibition by ACTH is not a reflection of the content of a specific dipeptide sequence which serves as a competitive substrate for His-ProNH$_2$, but rather requires interaction of the complete polypeptide with the enzyme.

It is noteworthy that the polypeptide hormones which inhibit imidopeptidase activity are not uniformly characterized by the presence of a His-Pro sequence, further suggesting that the inhibition is not a result of their activity as substrates. It has been noted that different preparations of imidopeptidase show varying sensitivity to polypeptide hormones. For example, the preparation of enzyme used in Fig. 8 gave 50% inhibition of the activity by 10^{-6} M ACTH, while the preparation used for Fig. 9 required greater than 10^{-5} M ACTH to produce 50% inhibition of enzyme activity. In other experiments (data not shown), an enzyme preparation purified after sonication of the extract showed activity on His-ProNH$_2$ as a substrate but was completely insensitive to inhibition by ACTH. These data suggest that there is a regulatory site on the enzyme distinct from the substrate site.

A further insight into the nature of the inhibition of imidopeptidase activity by ACTH is presented in the data of Fig. 9. A kinetic analysis of the enzyme activity indicates that the inhibition by ACTH is noncompetitive (11). These data, therefore, provide strong evidence that the inhibition by ACTH of His-ProNH$_2$ cleavage is not explainable on the basis of ACTH being a competitive substrate, but rather that the imidopeptidase contains a regulatory site for inhibition by some polypeptide hormones.

DISCUSSION

Previous investigations concerned with the enzymology of TRH have demonstrated the presence in serum (2-4) and brain tissue (5, 7-10) of TRH deamidase (see Fig. 10). Since all efforts to demonstrate a biological activity associated with acid TRH have been negative (14), it appears likely that the deamidase pathway serves the purpose of inactivation of TRH. Recent studies from this laboratory (5, 6) have provided evidence for the presence in neural tissue of enzymes (a

TABLE III

Effect of ACTH fragments on imidopeptidase activity

Imidopeptidase activity was measured as described under "Methods" using Fraction III enzyme (see Fig. 3). [*His*-^{3}H]His-ProNH$_2$ was used at a concentration of 5×10^{-6} M and ACTH fragments at 10^{-5} M. Activity is expressed as a percentage of the activity obtained in a control incubation with no added ACTH fragment.

ACTH fragment	% control activity
5-10	100
1-10	64
1-13	59
11-24	50
1-24	18

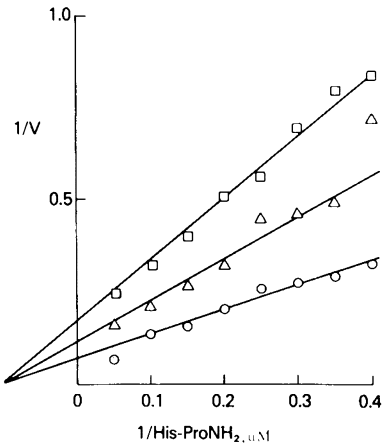

FIG. 9. Reciprocal plot of imidopeptidase activity at various ACTH concentrations. Imidopeptidase activity was measured as described under "Methods" using Fraction III enzyme (see Fig. 3). Assays were carried out using the indicated concentrations of [*His*-^{3}H]His-ProNH$_2$ and ACTH (1-24). The eight concentrations (M $\times$ 10^{-6}) of His-ProNH$_2$ used were: 20, 10, 7.5, 5, 4, 3.3, 2.85, and 2.5. The specific activities (cpm/nmol $\times$ 10^5) at these concentrations were 2, 2, 4.67, 4, 4.8, 8.33, 9.22, and 10, respectively. A value of 1.0 on the $1/V$ scale corresponds to an activity of 2.5 pmol of His-ProNH$_2$ degraded/30 min. ○——○, no ACTH; △——△, 10^{-5} M ACTH; □——□, 10^{-4} M ACTH.

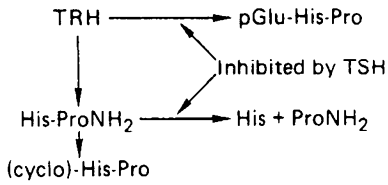

FIG. 10. Effect of TSH on enzymes involved with TRH metabolism. TSH inhibits both TRH deamidase and His-$ProNH_2$ imidopeptidase. The net result is that TRH is preferentially converted to (cyclo)-His-Pro.

pyroglutamyl aminopeptidase and probably a dipeptide amide cyclase) that result in the conversion of TRH to the diketopiperazine (cyclo)-His-Pro (see Fig. 10). Evidence has been presented that (cyclo)-His-Pro is a peptide with intrinsic biological activity (6); it has therefore been suggested that some of the hormonal activities attributed to TRH may involve its conversion to (cyclo)-His-Pro.

Incubation of brain tissue with TRH produces $ProNH_2$ as a major product (10). The isolation and characterization described in these studies of the brain imidopeptidase which splits His-$ProNH_2$ to histidine provides an explanation for the production of prolineamide from TRH and makes it unnecessary to invoke the action of an imidopeptidase acting directly on TRH (see Fig. 1). The scheme outlined in Fig. 10 indicates that the imidopeptidase may play a role in converting His-$ProNH_2$ to inactive products before it can be cyclized to the biologically active peptide (cyclo)-His-Pro.

Fraction III from the DEAE-cellulose chromatography (Fig. 3) is enriched for imidopeptidase activity, but is clearly not a pure enzyme. It contains TRH deamidase and is probably contaminated with other brain peptidases. Nevertheless, the use of a radioactive substrate provides a basis for examining the properties of the enzyme. All tests of the substrate specificity of the enzyme depended on the capability of various compounds to inhibit the enzyme-dependent cleavage of [*His*-^{3}H]His-$ProNH_2$ to ^{3}H-His. Using this approach, it was possible to establish that a suitable substrate for the enzyme is His-$ProNH_2$. The histidine residue cannot be replaced by a glycine or aspartic acid residue, but a Glu-Pro sequence may be acceptable (Table I). The α-amino group on the histidine residue must be free for good substrate activity, since TRH is neither a substrate (data not shown) nor an effective competitor of His-$ProNH_2$ cleavage (Table I). The carboxyl group of the proline residue must be blocked in order for the peptide to be effectively split; His-Pro does not compete for binding to the enzyme as effectively as does His-$ProNH_2$. (Cyclo)-His-Pro, of all the peptides tested, is the most effective competitor of His-$ProNH_2$ cleavage; however, a test of the action of the enzyme on [*His*-^{3}H](cyclo)-His-Pro indicated that it is not split by the imidopeptidase (data not shown).

Hui and Lajtha (15) recently studied prolidase activity in rat brain extracts. Their preparation split X-Pro peptides, where X could be alanine, glycine, leucine, or phenylalanine, but not histidine or proline. Blockade of the NH_2-terminal amino group or COOH-terminal carboxyl group substantially decreased the prolidase activity. The imidopeptidase activity described in this study is clearly different from the activity of the enzyme of Hui and Lajtha, since blockade of the COOH-terminal carboxyl group stimulates the His-$ProNH_2$ imidopeptidase and a His-Pro sequence is the preferred substrate.

The His-$ProNH_2$ imidopeptidase is not a trypsin-like enzyme. It is not inhibited by benzamidine or trypsin inhibitor. The enzyme does, however, appear to have an essential sulfhydryl group, since it is inhibited by *N*-ethylmaleimide.

Aminopeptidase P, purified from *Escherichia coli*, cleaves peptide bonds between the carboxyl group of an NH_2-terminal amino acid and the secondary amine of a proline residue (16). This enzyme is not specific for the NH_2-terminal amino acid and requires metal for activity. The imidopeptidase activity described here is different from that of the *E. coli* enzyme. It is not a general aminopeptidase (Table II) since it does not split substrates such as Gly-Pro and has no apparent requirement for metal.

An interesting property of imidopeptidase revealed by these studies involves the sensitivity of the enzyme to a variety of polypeptide hormones (see Fig. 8). The data of Fig. 9 suggest that polypeptide hormones inhibit the enzyme noncompetitively; it therefore appears that there is a regulatory site on the enzyme which responds to hormones that is distinct from the substrate binding site. It is noteworthy that TSH inhibits the imidopeptidase and also the TRH deamidase (5). The biological significance of the inhibition by TSH of multiple enzymes involved in the metabolism of TRH is not clear. However, as outlined in Fig. 10, it is possible that in the presence of TSH the metabolism of TRH is preferentially shunted toward the formation of (cyclo)-His-Pro. Since (cyclo)-His-Pro exerts some biological activities, the effect of TSH may provide a mechanism for eliciting a prolonged or amplified response to a TRH stimulus (17). A further elucidation of the endocrinology of TRH and its metabolites as well as the possible localization of pituitary hormones in TRH-degrading areas of brain will undoubtedly provide a more secure basis for evaluating these ideas.

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