Kinetic Investigation of the Specificity of Porcine Brain Thyrotropin-releasing Hormone-degrading Ectoenzyme for Thyrotropin-releasing Hormone-like Peptides

Evidence indicates that neuronally released thyrotropin-releasing hormone (TRH) is selectively inactivated by TRH-degrading ectoenzyme (TRH-DE) (EC 3.4.19.6). TRH-DE inhibitors may be used to enhance the therapeutic actions of TRH and to investigate the functions of TRH and TRH-DE in the central nervous system. Although TRH-DE appears to exhibit a high degree of specificity toward TRH, systematic specificity studies, which would facilitate inhibitor design, have not been previously conducted for this enzyme. In this paper we present the first description of TRH-DE specificity across a directed peptide library in which the histidyl (P₁) residue of TRH was replaced by a series of amino acids. Peptides were synthesized using standard solid phase chemistry. Kinetic parameters were measured either by continuous or discontinuous fluorometric assays or by quantitative high pressure liquid chromatography. The P₁ residue was found to influence significantly both the ability of the peptides to bind to TRH-DE, as measured by their Kᵣ values, and the ability of TRH-DE to catalyze their hydrolysis. Moderately bulky, uncharged P₁ residues were found to bind preferentially to TRH-DE. Results from this screen provide valuable information for the development of TRH-DE inhibitors and have led to the identification of two potent, reversible TRH-DE inhibitors, L-pyroglutamyl-L-asparaginyl-L-prolineamide (Kᵣ = 17.5 μM) and Glp-Asn-Pro-7-amido-4-methyl coumarin (Kᵣ = 0.97 μM).

Thyrotropin-releasing hormone-degrading ectoenzyme (TRH-DE) (EC 3.4.19.6) is a type II cell surface peptidase located on synaptosomal membranes in the central nervous system (1–4). TRH-DE catalyzes the hydrolysis of the Glp-His bond in thyrotropin-releasing hormone (TRH), a tripeptide with the amino acid sequence L-pyroglutamyl-L-histidyl-L-prolineamide (Glp-His-ProNH₂) (5–10). This enzyme is strategically placed to play a significant role in extracellular inactivation of TRH, and current evidence strongly indicates that TRH-DE is the principal enzyme responsible for terminating the actions of neuronally released TRH (11–14).

Although first recognized as a hypothalamic regulatory hormone, TRH is now believed to function as a neurotransmitter and/or neuromodulator within the central nervous system (15, 16) where it displays a broad spectrum of stimulatory actions independent of its neuroendocrine functions (15–17). Based on its central nervous system effects, TRH has been found to have potential use in the treatment of brain and spinal injury (18, 19) and several central nervous system disorders, including spinocerebellar degeneration, cognitive deficits, and spinal cord pain transmission (16, 17). The mechanisms by which TRH improves these conditions are not fully elucidated but appear to involve the potentiation by TRH of other neurotransmitter systems. Despite its promise, the use of TRH as a therapeutic agent is critically undermined by its susceptibility to proteolytic degradation (20).

Compounds that potently and selectively inhibit TRH-DE may be used to enhance the therapeutic actions of TRH in the central nervous system by either potentiating endogenous TRH and/or protecting exogenously administered TRH or TRH analogs from degradation. TRH-DE inhibitors may also be powerful tools for investigating the respective biological roles of TRH-DE and TRH within the central nervous system (13, 20, 21, 22). TRH-DE appears to be an exceptional example of a neuropeptide-specific peptidase (23) because no other ectopeptidase has been shown to be capable of degrading TRH, and TRH-DE appears to exhibit remarkable specificity for TRH (24). This peptidase does not catalyze the hydrolysis of other, larger neuropeptides that also contain an NH₂-terminal Glp residue, such as luteinizing hormone releasing hormone, neurotensin, bombesin, and gastrin (5, 8–10). With this unusual dual selectivity, TRH-DE is more similar to acetylcholinesterase than to angiotensin-converting enzyme. Inhibition of TRH-DE as a means of enhancing TRH signaling is attractive because it should affect TRH signals exclusively, and consequently it may offer significant investigative and therapeutic advantages. On the other hand, the design of TRH-DE inhibitors is made difficult by the narrow specificity of this enzyme (13).

Only a few inhibitors have been synthesized so far which exhibit a significant effect on TRH-DE activity in vitro, none of which have been tested for activity in vivo.
which has been found to be sufficiently effective for pharmacological studies in vivo (13, 21, 22). With a $K_1$ of 8 $\mu M$, N-[1-carboxy-2-phenylethyl]N-imidazole benzyl histidyl-$\beta$-naphthylamide (CPhNA) appears to be the most potent of these (21). CPhNA has been shown to increase the recovery of TRH released from rat brain slices, indicating that TRH levels, and thus, TRH neurotransmission, may be increased through TRH-DE inhibition (21).

Protease inhibitors often incorporate structural features that enable the inhibitor to interact with the enzyme's active site (20, 25, 26). On the basis that TRH-DE has been shown to be a zinc metalloprotease (27) and that it is weakly inhibited by Glp and His-ProNH$_2$ (35), Bauer et al. (13) synthesized derivatives of Glp and His-ProNH$_2$ incorporating various functionalities, such as hydroxamate, thiol, and phosphamidate groups known to inhibit other metalloproteases (26). All proved to be poor inhibitors of TRH-DE (13).

The design of TRH-DE inhibitors is hampered by the fact that a three-dimensional structure has not been ascertained for TRH-DE or the aminopeptidases A and N, with which, according to cDNA sequence analysis, TRH-DE shares approximately 30% homology (1). In the absence of a target structure, however, structure-activity studies to identify structural requirements for interaction with the enzyme's active site can be used as a basis for the rational design of enzyme inhibitors (25, 28, 29). No systematic structure-activity studies of TRH-DE have been previously undertaken, and there is no active site model for TRH-DE. Thus, to date, there has been insufficient information to identify the structural features that need to be incorporated into TRH-DE inhibitors.

Several investigators previously examined the ability of TRH derivatives, with modifications to TRH's COOH- and/or NH$_2$-terminal residues, to act as substrates or inhibitors of TRH-DE (6, 8, 10, 30). None was found to inhibit TRH-DE activity to any significant extent. Until recently, a histidine residue in the P$_1$' position was thought to be essential for TRH-DE activity (5, 8, 22), yet it has now been shown that the naturally occurring TRH-like peptide Glp-Phe-ProNH$_2$ is a substrate for bovine brain TRH-DE (10, 31).

To investigate further the influence of P$_1$' residues on ligand binding and catalytic activity of TRH-DE and to facilitate the design of TRH-DE inhibitors, we have conducted kinetic studies on a directed peptide library in which the central histidyl residue of TRH was replaced by a series of amino acids. New and improved TRH-DE assays (32) were employed to investigate the kinetic properties of the peptide library rigorously. The data presented here provide the first illustration of TRH-DE specificity across a library of TRH-like peptides. Furthermore, two potent, reversible TRH-DE inhibitors were identified from the screen of this library.

**EXPERIMENTAL PROCEDURES**

Pyroglutamyl-histidyl-prolylamido-4-methyl coumarin (TRHAMC) and 7-amino-4-methyl coumarin (AMC) were purchased from Bachem U. K. Ltd. Glp-Asn-ProAMC was custom synthesized by the American Peptide Company (Sunnyvale, CA). All other chemicals, except those specified below, were of analytical grade and obtained from either Sigma-Aldrich (Ireland) or Merck (Germany).

**Peptide Library**—Glp-His-ProNH$_2$, Glp-Glu-ProNH$_2$, and Glp-His-ProOH were obtained from Sigma-Aldrich. Glp-Gln-ProNH$_2$ and Glp-Asn-ProNH$_2$ were purchased from Peninsula Laboratories Inc. (U. K.). Glp-Asn-ProNH$_2$, Glp-Tyr-ProNH$_2$, and Glp-o-Asn-ProNH$_2$ were obtained from the American Peptide Company in addition to being synthesized by the method described below. All other TRH-like peptides in the library were synthesized either manually using a bubbler system (for details, see Ref. 33) or using a Synergy Peptide Synthesizer (Applied Biosystems, U. K.). In both cases, standard solid-phase Fmoc chemistry was employed (33). Pyroglutamic acid and Fmoc amino acid derivatives were purchased from Calbiochem-Novabiochem U. K. Ltd. Trifunctional amino acids were obtained with side chain-protecting groups as follows: Fmoc-Sert(Bu)-OH, Fmoc-Thr(Bu)-OH, Fmoc-n-Asn(Trt)-OH, Fmoc-Arg(Pmc)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Asp(0Bu)-OH, Fmoc-His(Trt)-OH, and Fmoc-Cys(Trt)-OH.

Synthesis—The seven amino acids used in the synthesis of the library were monitored by high performance liquid chromatography (HPLC). The purity of the peptide was 95% or greater. Peptide coupling was achieved using Fmoc chemistry. The resin was washed with acetic acid and then dissolved in formamide and deprotected with 20% piperidine in N,N-dimethylformamide. Coupling was performed twice for each amino acid using the Synergy Peptide Synthesizer and once when the bubbler system was employed. Three equivalents (i.e. 3-fold excess over the resin loading capacity) of each amino acid were coupled onto the resin with HBTU/ HOBT/DIPEA (1:1:2 equivalents) at each step. No deprotection step was necessary after the coupling of pyroglutamic acid. On completion of peptide assembly, the resin was washed thoroughly with dichloromethane followed by methanol and allowed to dry overnight. In the absence of labile amino acids and side protection groups, cleavage of the peptide from the resin was achieved by placing the dry resin in a round bottom flask and adding 95% trifluoroacetic acid in water (10 ml/g of dry resin). This reaction mixture was stirred at room temperature for approximately 1 h before filtering the suspension through a sintered glass funnel. Sequences containing Asn were deprotected and cleaved using a trifluoroacetic acid solution containing 95% trifluoroacetic acid, 2.5% water, and 2.5% trisopropylsilane (v/v/v). For sequences containing Ser, Trp, or Arg, cleavage/deprotection was achieved using reagent K (82.5% trifluoroacetic acid, 5% water, 5% thioanisole, 5% phenol, 2.5% 1,2-ethanediol, pH 2) in place of trifluoroacetic acid (33, 34).

These small peptides proved difficult to precipitate directly from the filtrate using diethyl ether, so the trifluoroacetic acid and scavengers were first removed by rotary evaporation under vacuum. The residue was washed with petroleum ether. Diethyl ether was then added to the semisolid residue to crystallize the peptide. Because of the hygroscopic nature of these peptides during isolation, after decantation of the bulk of the solvent, a steady stream of nitrogen was used to evaporate the diethyl ether and to dry the peptide pellet simultaneously. The peptide pellets were dried thoroughly under nitrogen before transferring the dried material to preweighed glass containers for storage in a desiccator. After weighing the dried material, the peptides were stored at 20°C. Stock solutions were prepared from this material.

Peptides were analyzed and judged to be homogeneous by HPLC. HPLC analysis was conducted using a Thermo Separation Products Inc. Spectrum System HPLC. Standard 1 mM solutions of each peptide in 20 mM potassium phosphate buffer, pH 7.5, were analyzed on a C-18 reverse phase column (Hypersil U. K.) using a linear gradient of 0–70% acetonitrile in 0.08% trifluoroacetic acid as described previously (31).

**Enzyme Purification—TRH-DE**—TRH-DE was purified almost 20,000-fold from porcine brain as described previously (32, 35). A Coomassie-stained gel of the purified enzyme after SDS-polyacrylamide gel electrophoresis showed one major band, the molecular mass of which was consistent with that of 116 kDa reported previously for TRH-DE (35). This highly purified TRH-DE preparation did not contain any of the various peptidase activities tested, including aminopeptidases, carboxypeptidases, dipeptidyl aminopeptidases, and dipeptidyl aminopeptidases, and it was completely devoid of other TRH-degrading enzymes, including pyroglutamin aminopeptidase I (EC 3.4.21.8) and 3-prolyl oligopeptidase (EC 3.4.19.3) (32). The preparation was found to have (i) a protein concentration of 0.8 mg ml$^{-1}$ using a modification of the Lowry method (36) with bovine serum albumin as a standard and (ii) a specific activity of 0.17 unit mg$^{-1}$ with TRHAMC as substrate under standard conditions of a continuous assay described previously (32) and outlined below. Dipetidyl peptidase IV (DPP-IV) (EC 3.4.14.5) was purified 370-fold from bovine kidney (37) and was found to have a protein concentration of 7.7 mg ml$^{-1}$ using the method of Markwell et al. (38). This preparation did not contain any measurable amount of either dipeptidase or oligopeptidase activity. The specific activity was 17.5 units mg$^{-1}$ with Gly-ProAMC as substrate (32).

1 unit of enzyme activity was defined as that amount catalyzing the formation of 1 $\mu M$ of product in 1 min under the standard conditions employed. All incubations in the assays described below were carried out in 20 mM potassium phosphate buffer, pH 7.5, at 37°C. Fluorescence measurements were made using a Perkin-Elmer LS 50B luminescence spectrometer fitted with a thermostated cell holder. Wavelengths for excitation and emission were set at 370 and 440 nm, respectively, with slit widths of 10 nm and 5 nm, respectively.

**Kinetic Analysis of the Peptide Library Using HPLC**—The ability of each peptide in the library to act as a TRH-DE substrate was assessed using HPLC. As an initial screen, 1 mM peptide was incubated with 0.8 $\mu g$ of TRH-DE in a total volume of 1 ml for 18 h at 37°C. Control
samples were included in which peptide (1 mM) or Glp (0.2–0.8 mM) was incubated under identical conditions in the absence of TRH-DE. TRH-DE activity was terminated by the addition of trifluoroacetic acid (0.15%, v/v), and samples were then analyzed using HPLC as described previously (31). Products resulting from TRH-DE hydrolysis were separated on a C-18 reverse phase column using an linear gradient of 0–70% acetonitrile in 0.08% (v/v) trifluoroacetic acid. The concentration of Glp formed by the action of TRH-DE on the peptide library was measured by UV absorbance at 206 nm with a quantitative detection limit of 0.1 μM for a 40-μl injection volume, employing a signal to noise ratio of 10. Following the incubation of each peptide with TRH-DE, evidence of Glp in the sample was taken to indicate that the peptide was a TRH-DE substrate.

The rates of hydrolysis for each of those peptides identified as substrates were then compared by measuring Glp production using microarrays with shorter incubation times (39). In these assays, peptide (1 mM) was incubated at 37 °C in a total volume of 100 μl, and incubation times and TRH-DE concentration were adjusted to produce a detectable amount of Glp while remaining within the initial rate period of the reaction. Typically, 0.8–3.2 μg of TRH-DE and incubations times of 5 min to 18 h were used. Measurements were made in triplicate. The rate of TRH-OH hydrolysis was also measured by this method.

**Determination of Inhibitor Constants for Selected TRH-DE Substrates**—The HPLC assay lacked sufficient sensitivity for determining kinetic constants. Therefore, those peptides undergoing significant hydrolysis (i.e., those that exhibited rates of hydrolysis ≥ 0.5 unit mg⁻¹) were examined as competitive substrates of TRH-DE using a recently published discontinuous fluorometric TRH-DE assay (32). This assay employs TRHAMC as the substrate and depends on the measurement of the fluorescence of AMC produced as shown in Reactions 1 and 2.

| TRH-DE       | Glp-His-Pro-AMC → His-Pro-AMC |
|--------------|--------------------------------|
| 5 h, 80 °C   | His-Pro-AMC → His-ProDKP + AMC |
| (cyclization) |                                 |

**REACTIONS 1 AND 2**

We have shown that the amount of AMC formed under these conditions is a quantitative measure of TRHAMC cleavage (32). Initial rates for the hydrolysis of TRHAMC by TRH-DE were determined in triplicate at five different substrate concentrations, both in the absence and presence of at least three concentrations of peptide. Ki values were obtained by nonlinear regression analysis of the data collected. When determined in this way (i.e. by treating the peptide substrates as inhibitors of TRHAMC hydrolysis), these Ki values correspond to the Michaelis constants for those library peptides hydrolyzed by TRH-DE (40).

**Kinetic Analysis of Peptides That Are Not Hydrolyzed by TRH-DE**—A recently developed continuous fluorometric assay (32) was used to investigate the ability of those library peptides that were not hydrolyzed by TRH-DE to inhibit TRHAMC degradation by TRH-DE. Glp-Asn-Pro-AMC was also included in this study. In this assay, TRHAMC is the substrate, and DPP-IV is the coupling enzyme. The sequence is shown in Reaction 3.

| TRH-DE       | Glp-His-Pro-AMC → His-Pro-AMC → His-Pro + AMC |

**REACTION 3**

The reaction was monitored continuously by measuring the increase in AMC fluorescence. A linear progress curve with no discernible lag period was observed when the reaction was monitored over a period of 10 min. Nevertheless, data sampling was not commenced until 100 s from the start of the reaction to ensure that measurements were taken after a steady state had been reached (see Ref. 32). This continuous assay permits accurate assessment of nonlinear progress curves that may arise in the presence of tight binding inhibitors.

In a preliminary experiment, the peptides were screened for their ability to inhibit TRH-DE activity. The initial rate of TRHAMC hydrolysis by TRH-DE was measured by incubating 5 μM TRHAMC with 1.23 μM of DPP-IV and 0.32 μg of TRH-DE, both in the absence and presence of each library peptide (1 mM final concentration) under standard assay conditions (32).

Peptides showing < 20% inhibition (Ki > 1 mM) were not examined further. The Ki values for the remainder of the peptides were determined by measuring their effects on TRHAMC degradation by TRH-DE using the continuous assay. Data were collected in duplicate at five different substrate concentrations and at least three different concentrations of each peptide.

All peptides that were observed to inhibit AMC production in the continuous fluorometric assay were assessed for their ability to inhibit the coupling enzyme, DPP-IV, using a direct continuous assay for DPP-IV which employed Gly-Pro-AMC as the substrate (32). Peptide concentrations similar to those used to determine the Ki values above were employed in the DPP-IV assay. None of these peptides was found to inhibit DPP-IV hydrolysis of Gly-ProAMC. Thus, the effects produced by these peptides can be attributed solely to their inhibition of TRH-DE.

Reversibility and time dependence of the inhibition produced by Glp-Gln-ProNH2, Glp-Asn-ProNH2, and Glp-Asn-ProAMC were examined by initially preincubating TRH-DE with each peptide at 37 °C for various periods up to 75 min. To investigate time dependence, the enzyme-peptide solution was subsequently added to a reaction mixture containing TRHAMC, DPP-IV, buffer, and peptide, and TRH-DE activity was measured using the continuous assay. The final concentration of Glp-Gln-ProNH2, Glp-Asn-ProNH2, and Glp-Asn-ProAMC used was 400, 160, and 1 μM, respectively. To test for reversibility, the enzyme-peptide solution was added to a reaction mixture that did not contain peptide. TRH-DE activity was not affected by preincubation at 37 °C.

**Analyses of Results**—All kinetic parameters were determined by nonlinear regression analysis using the computer program Prism (Graph Pad Software Inc.). Linear regression analysis employing proportional weighting was used to fit data to linear plots for display purposes only. Unless otherwise stated, all values are shown as the mean ± S.D.

**RESULTS**

**HPLC Analysis of the Peptide Library**—HPLC analysis revealed that TRH-DE catalyzed the removal of the NH2-terminal Glp residue from 15 out of the 25 members of the peptide library, including TRH. It can be seen from the representative HPLC traces shown in Fig. 1 that 1 mM TRH was fully degraded after overnight incubation with TRH-DE. No detectable Glp was released from those peptides where the P1 position was occupied by D-Asn, Gly, or the L-amino acids Asn, Gln, Trp, L-α-phenylglycine, homoproline, Glu, Asp, and Pro. The HPLC-based assay also showed that there was no detectable cleavage of Glp-Cys-ProNH2 by TRH-DE. This peptide was observed, however, to undergo oxidation with disulfide bond formation during incubation and was not examined further. Table I shows the rates of hydrolysis of those peptides that were found to be substrates for TRH-DE.

**Inhibitor Constants for Selected TRH-DE Substrates**—Because of the difficulty in obtaining reliable Ki values for library peptides acting as substrates, we measured instead their Ks values as competitive substrates. Ks values for those library peptides that were significantly hydrolyzed by TRH-DE are shown in Table II. All of these peptides were found to act as simple competitive inhibitors of the degradation of TRHAMC by TRH-DE (example shown in Fig. 2). Nonlinear regression analysis of the data collected in this study gave a Ks value for TRHAMC of 3.1 ± 0.5 μM (n = 8). This compared with the value of 3.4 ± 0.7 μM (n = 5) which we published recently for the discontinuous fluorometric assay (32). The observed hydrolysis and Ks value obtained for Glp-Phe-ProNH2 are consistent with previous reports for TRH-DE purified from bovine brain (10, 31). Vmax values for these peptides correspond to Michaelis constants (40). It can be seen from Table II that TRH is the most favorable substrate of those tested.

**Kinetic Analysis of Peptides Not Hydrolyzed by TRH-DE**—Table III shows the percent inhibition of TRH-DE activity produced by those peptides not hydrolyzed by TRH-DE. Presented also are the Ki values obtained for those peptides exhibiting greater than 20% inhibition in the initial screening. The latter peptides were all found to act in a simple competitive manner as illustrated by a Lineweaver-Burk plot of data ob-
FIG. 1. Representative HPLC traces obtained after the incubation (18 h) of TRH with TRH-DE. The trace in the foreground represents a control sample for TRH. The trace in the background shows the products formed from TRH by the action of TRH-DE. TRH was degraded completely to give Glp and His-ProNH₂. Consistent with previously published results (31, 32, 39), His-ProNH₂ was found to undergo spontaneous intramolecular cyclization slowly to form His-prodike-topiperazine (His-ProDKP).

### Table I
Comparison of hydrolysis rates for library peptides (1 mM) found to be TRH-DE substrates

| Peptide          | Rate of hydrolysis (units mg⁻¹) |
|------------------|---------------------------------|
| Glp-His-ProOH    | 3.38 ± 0.18 (3)                 |
| Glp-His-ProNH₂   | 2.54 ± 0.25 (11)                |
| Glp-Thi-ProNH₂   | 2.12 ± 0.16 (6)                 |
| Glp-Phe-ProNH₂   | 1.56 ± 0.13 (5)                 |
| Glp-Tyr-ProNH₂   | 0.66 ± 0.04 (5)                 |
| Glp-Arg-ProNH₂   | 0.41 ± 0.02 (4)                 |
| Glp-Lys-ProNH₂   | 0.46 ± 0.02 (3)                 |
| Glp-Met-ProNH₂   | 0.21 ± 0.03 (4)                 |
| Glp-Leu-ProNH₂   | 0.14 ± 0.01 (5)                 |
| Glp-Thr-ProNH₂   | 0.14 ± 0.01 (5)                 |
| Glp-Ile-ProNH₂   | 0.06 ± 0.00 (4)                 |
| Glp-homoPhe-ProNH₂ | 0.05 ± 0.00 (3)              |
| Glp-Val-ProNH₂   | 0.05 ± 0.00 (3)                 |
| Glp-Ser-ProNH₂   | 0.05 ± 0.00 (3)                 |
| Glp-Nva-ProNH₂   | 0.04 ± 0.00 (6)                 |
| Glp-Ala-ProNH₂   | 0.02 ± 0.00 (3)                 |

*HomoPhe, 1-homophenylalanine; Nva, norvaline.

### Table II
Comparison of kinetic parameters for selected library peptides hydrolyzed by TRH-DE

| Peptide          | $V_{max}$ | $K_m$ | $V_{max}/K_m$ |
|------------------|-----------|-------|---------------|
| Glp-His-ProNH₂   | 2.63 ± 0.26 (11) | 35 ± 4 (3) | 0.08 ± 0.01 |
| Glp-Thi-ProNH₂   | 2.19 ± 0.16 (6)  | 34 ± 6 (3) | 0.06 ± 0.01 |
| Glp-Tyr-ProNH₂   | 0.67 ± 0.04 (5)  | 15 ± 3 (4) | 0.05 ± 0.01 |
| Glp-His-ProAMC   | 0.11 ± 0.01 (4)  | 3.1 ± 0.4 (9) | 0.04 ± 0.00 |
| Glp-Phe-ProNH₂   | 1.64 ± 0.14 (5)  | 55 ± 8 (3) | 0.03 ± 0.00 |
| Glp-His-ProOH    | 4.43 ± 0.24 (3)  | 311 ± 31 (5) | 0.01 ± 0.00 |

*From Ref. 32.

The Michaelis constant for Glp-His-ProAMC (TRHAMC) was measured directly, whereas the $K_m$ values for the library peptides were measured indirectly by treating them as competitive inhibitors of TRHAMC hydrolysis by TRH-DE. The $K_m$ values were all determined by nonlinear regression analysis of data obtained from the discontinuous fluorometric TRH-DE assay and represent the mean ± S.D. The number of determinations is shown in parentheses. Included for comparison are the corresponding $K_m$ values for TRH and TRH-0H, which we published recently (32). $V_{max}$ values were estimated from rates of hydrolysis measured by HPLC using the relationship $V_{max} = v_o(K_m + [S])/[S]$.

### DISCUSSION

The results from this study show that alteration of the $P_1$ residue in the tripeptide structure of TRH significantly affects both the ability of the resulting peptides to bind to TRH-DE, as measured by their $K_m$ values, and the ability of TRH-DE to catalyze their hydrolysis. The low turnover rates observed for library peptides containing Arg or Lys in the $P_1$ position compared with those containing thienylalanine and Phe suggest that although the $S_1$ site on the enzyme is capable of accepting a positively charged residue, a neutral, aromatic residue is preferred for catalysis (Fig. 4). These results imply that the imidazole group in TRH may not be protonated during the binding of TRH to TRH-DE. Further support for this is given by the observation that substitution of histidine by either of the non-basic isosteres, 1-Asn and 1-Gln, does not lead to significant loss of apparent binding affinity. On the whole, occupation of the $P_1$ site by residues with aromatic character appears to be particularly favorable for catalysis. Factors other than the nature of the residue, such as the size of the side chain, also seem to be influential because elongation of the side chain of Phe by one methylene group produced a significant loss in activity, and reduction in the length of the side chain by the same amount resulted in a peptide that exhibited a reduced affinity for TRH-DE and was not hydrolyzed by the enzyme. Moreover, the large indole ring replaced by Asp or Glu indicate that a negatively charged residue at the $P_1$ site is not favorable for binding or catalysis.

Kinetic data obtained for library peptides in which His was
of Trp appeared to be accommodated somewhat by the $S_1'$ subsite ($K_i = 232 \, \mu M$), but Glp-Trp-ProNH$_2$ was not hydrolyzed.

Although the presence of aromatic character in the $P_1'$ residue appears to be advantageous for substrates, it is clearly not essential for ligand binding because both Glp-Asn-ProNH$_2$ and Glp-Gln-ProNH$_2$ were able to bind to TRH-DE, with an apparent affinity similar to that of TRH, but they were not hydrolyzed. It is not obvious why Glp-Asn-ProNH$_2$ and Glp-Gln-ProNH$_2$ do not act as substrates, but it might be postulated that binding of these peptides to the enzyme is distorted, thus preventing catalysis. Lowe et al. (41) noted that it is possible to superimpose, two-dimensionally, the side chains of L-Asn and L-Gln onto that of L-His such that the amide nitrogen of L-Asn overlaps with the $\pi$N of L-His and that the amide nitrogen of L-Gln coincides with the $\pi$N of L-His. Because the TRH-like peptides that contain Asn, Gln, and His in the $P_1'$ position all bind relatively well to TRH-DE, it might be suggested that the nitrogen atoms in these side chains represent recognition moieties for binding to the enzyme's $S_1'$ subsite. The more favorable inhibitory properties of Glp-Asn-ProNH$_2$ compared with those of Glp-Gln-ProNH$_2$ may be related to the position of the nitrogen in the side chain and to the position of the amide carbonyl group relative to the $S_1'$ subsite on the enzyme. Because Glp-D-Asn-ProNH$_2$ displays poor affinity for TRH-DE,
the interaction of Asn with the $S_1'$ subsite on the enzyme appears to be stereospecific.

We were able to achieve significant improvement of the apparent binding affinity of Glp-Asn-ProNH$_2$ by substituting the amide group of the COOH terminus with AMC. The resulting compound, Glp-Asn-ProAMC, was found to have a $K_m$ of 0.97 ± 0.08 μM and is the most potent, competitive TRH-DE inhibitor described to date. Previously, the most potent TRH-DE inhibitor to be reported was CPHNA (21). This compound, though described to date. Previously, the most potent TRH-DE inhibitor to be reported was CPHNA (21). This compound, though described to date. Previously, the most potent TRH-DE inhibitor to be reported was CPHNA (21). This compound, though described to date. Previously, the most potent TRH-DE inhibitor to be reported was CPHNA (21). This compound, though described to date. Previously, the most potent TRH-DE inhibitor to be reported was CPHNA (21). This compound, though described to date. Previously, the most potent TRH-DE inhibitor to be reported was CPHNA (21). 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Julie A. Kelly, Gillian R. Slator, Keith F. Tipton, Carvell H. Williams and Karl Bauer

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