Mutational Analysis of the Thyrotropin-Releasing Hormone-Degrading Ectoenzyme. Similarities and Differences with Other Members of the M1 Family of Aminopeptidases and Thermolysin†

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ABSTRACT: Thyrotropin-releasing hormone-degrading ectoenzyme (TRH-DE) is a TRH-specific peptidase which catalyzes the inactivation of the peptidergic signal substance TRH. As indicated by sequence alignment, TRH-DE and the other members of the M1 family of aminopeptidases have a distinct set of conserved amino acid residues in common. By replacing amino acid residues that are putatively involved in catalysis, we could demonstrate that the enzymatic activities of the mutants E408D, E442D, E464Q, E464D, Y528F, H507R, and H507F are dramatically decreased, essentially due to the changes of V_{max}. The mutant enzymes E408Q and E442Q are inactive, whereas the specific enzymatic activity of the mutants R488Q, R488A, and Y554F are similar to that of the wild-type enzyme. These data strongly suggest that E408, E442, Y528, and H507 are involved in the catalytic process of TRH-DE while E464 presumably represents the third zinc-coordinating residue and may be equivalent to E166 in thermolysin. In contrast, amino acid residues R488 and Y554 seem not to be involved in the catalytic mechanism of TRH-DE.

Thyrotropin-releasing hormone (TRH;[1] pyroGlu-His-ProNH$_2$) was the first hypothalamic releasing factor to be purified and characterized (reviewed in refs *1* and *2*). In addition to its endocrine function TRH also acts as a neuromodulator or neurotransmitter in the CNS where it elicits a wide range of pharmacological and behavioral effects in extrahypothalamic brain areas (reviewed in refs *3*−*5*). These functions are mediated by high-affinity TRH receptors (*6*−*8*) and imply the existence of an efficient inactivation system. Current evidence strongly indicates that TRH-degrading ectoenzyme (TRH-DE) (EC 3.4.19.6) is responsible for the specific inactivation of neuronally released TRH (*9*−*19*).

After enzyme purification (*20*) and cloning the cDNA of rat (*21*) and human (*22*) TRH-DE this enzyme could be identified as glycosylated type II metalloproteinase (*20*, *21*, *23*). The extracellular domain contains the HEXXH + E motif that is characteristic of Zn-dependent aminopeptidases (reviewed in ref *24*). We recently identified by site-directed mutagenesis studies rat TRH-DE as a new member of mammalian cell-surface peptidases that exist as disulfide-linked dimers (*25*). In the study described herein we have used site-directed mutagenesis to investigate the functional role of defined amino acids at the active site of this unusual peptidase. The amino acids to be mutated were selected on the basis of structural data that are available for some metallopeptidases as well as on the results of functional studies on the catalytic mechanism of metallopeptidases belonging to the same family or closely related families of metallopeptidases. A comparison of the complete amino acid sequence of rat TRH-DE and those in the translated GenBank revealed significant homology with rat aminopeptidase N (*21*, *26*) (34%) and mouse aminopeptidase A (32%) (*27*). Comparison of rat and human TRH-DE sequences with homologous aminopeptidases of the M1 family (*26*−*34*) and other members of the gluzincin family [thermolysin (*35*) and human neprilysin (*36*)] shows the greatest degree of sequence conservation in the region surrounding the zinc-binding motif (Figure 1). Chemical modification (*37*, *38*) and site-directed mutagenesis experiments (*39*−*50*) have suggested that the

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[1] Abbreviations: TRH, thyrotropin-releasing hormone; TRH-DE, TRH-degrading ectoenzyme; CNS, central nervous system; pyroGlu and Glp, pyroglutamic acid; PAP II, pyroglutamyl aminopeptidase II; PAP I, pyroglutamyl aminopeptidase I; APN, aminopeptidase N; APA, aminopeptidase A; APB, aminopeptidase B; PSA, puromycin-sensitive aminopeptidase; AP II, aminopeptidase II; LTA4H, leukotriene A4 hydrolase; PILS-AP, puromycin-insensitive leucyl-specific aminopeptidase; NEP, neprilysin; BHK, baby hamster kidney; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylendiaminetetraacetic acid; EBSS, Eagle's balanced salts; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Glp-SH, (*S*)-5-mercaptomethyl-2-pyrrolidinone; DEPC, diethyl pyrocarbonate; AMC, 7-amino-4-methylcoumarin; DPP IV, dipeptidyl peptidase IV; DTT, dithiothreitol; blLAP, bovine lens leucine aminopeptidase. Enzymes: thyrotropin-releasing hormone-degrading ectoenzyme (EC 3.4.19.6); pyroglutamyl aminopeptidase I (EC 3.4.19.3); aminopeptidase A (glutamyl aminopeptidase; EC 3.4.11.7); aminopeptidase N (EC 3.4.11.2); meprin A (EC 3.4.24.18); membrane dipeptidase (EC 3.4.13.19); endothelin-converting enzyme-1 (EC 3.4.24.71); aminopeptidase B (EC 3.4.11.6); puromycin-sensitive aminopeptidase (EC 3.4.11.14); leukotriene A4 hydrolase (EC 3.3.2.6); puromycin-insensitive leucyl-specific aminopeptidase (EC 3.4.11.-); neprilysin (EC 3.4.24.11); dipeptidyl peptidase IV (EC 3.4.14.5); thermolysin (EC 3.4.24.27); leucine aminopeptidase (EC 3.4.11.1).

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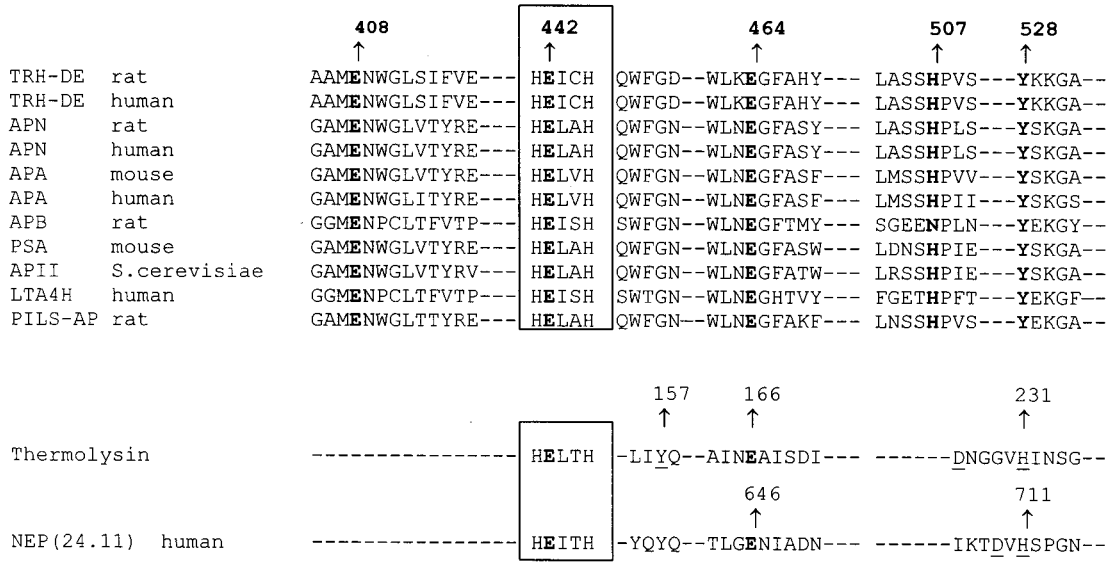

FIGURE 1: Alignment of amino acid sequences in the active site of several members of the M1 family of aminopeptidases, thermolysin and neprilysin. Alignment of sequences of rat TRH-DE (*21*), human TRH-DE (*22*), rat APN (*26*), human APN (*28*), mouse APA (*27*), human APA (*29*), rat APB (*30*), mouse PSA (*31*), *S. cerevisiae* AP II (*32*), human LTA4H (*33*), PILS-AP (*34*), *B. thermoproteolyticus* thermolysin (*35*), and human neprilysin (*36*). The mutated residues of rat TRH-DE and their positions are indicated in bold type. Arrows indicate the amino acid residue, which corresponds to the number. The HEXXH consensus sequence is boxed. The residues of thermolysin and neprilysin that are proposed to be involved directly or indirectly in the stabilization of the transition state are underlined.

zinc coordination and the general organization of the active site of these enzymes resembles that of thermolysin, with the involvement of E/D, Y, H, and R residues in the catalytic mechanism.

A tyrosine residue 65−68 amino acids along from the respective third zinc-coordinating glutamic acid residues seems to be conserved in most members of the M1 family (Figure 1). In aminopeptidase A, Y471 has been shown by site-directed mutagenesis to be involved in the stabilization of the transition state (*49*). We were therefore interested in studying the functional significance of Y528 of rat TRH-DE, the presumable counterpart of Y471 in APA, especially with respect to its direct involvement in the catalytic mechanism. In addition, we hypothesized that E464 located in the WLKEG motif of TRH-DE contributes to the third zinc ligand and that E442 located in the zinc-binding motif HEXXH (HEICH in TRH-DE) plays a crucial role in catalysis as observed for other gluzincins. Moreover, we were interested in exploring the potential role of E408 of TRH-DE, located in the AMEN conserved motif (Figure 1) as site-directed mutagenesis studies previously suggested that the counterparts of this residue in APN (E350) (*51*) and APA (E352) (*52*) interact via a hydrogen bond with the free α-amino group of substrates or inhibitors to stabilize the transition state, which is believed to be formed in a manner similar to that of thermolysin (*53*). In leukotriene A_4 hydrolase (LTA4H) (*54*), the free amino group of bestatin makes a similar interaction with E271, the corresponding residue of E350 in APN and E352 in APA. Since TRH (pyroGlu-His-ProNH_2) does not have a free α-amino group at the N-terminus, the question arises as to the role of this highly conserved residue in the reaction mechanism of TRH-DE.

Recently, a site-directed mutagenesis study on APA suggested that H450, located in the S_1 subsite of this enzyme, seems to be involved in catalysis and substrate binding via hydrogen bonding between the imidazole group of the histidine residue and the negatively charged side chain carboxylate of the N-terminal acidic amino acid of substrates (*55*). This residue (H507 in TRH-DE) is conserved in the most members of the M1 family of aminopeptidases as shown in Figure 1, except in APB, in which the histidine residue is replaced by an asparagine residue. In APA, H450 and Ca^{2+} seem to control together the specificity of this enzyme for substrates with N-terminal acidic amino acid residues. Since the activity of TRH-DE is not Ca^{2+} dependent and the terminal amino acid of TRH is pyroGlu, we were further interested in studying whether H507 is implicated in the catalytic mechanism of this enzyme. In addition, we hypothesized that R488 of TRH-DE plays a crucial role in substrate binding. The results of a previously reported study on the chemical modification of aminopeptidase N (*37*) show that in this enzyme a single arginine is essential for substrate binding. This arginine might correspond to R488 of rat TRH-DE which has been previously suggested to align with R203 of thermolysin (*38*). In the latter enzyme, R203 has been shown to form a hydrogen bond with the carbonyl of the $P_1'-P_2'$ amide bond of the substrate, allowing the binding of an extended peptide in agreement with the endopeptidase nature of this peptidase (*56*).

Thus, site-directed mutagenesis has been used in the study described here to assess the role of E408, E442, E464, R488, H507, Y528, and Y554 of rat TRH-DE. Each amino acid residue was modified in a cloned cDNA, the mutant cDNAs were expressed in BHK cells, and the properties of the recombinant mutant enzymes were compared to the wild-type enzyme.

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis and Construction of Expression Plasmid. Site-directed mutagenesis was performed according to the one-step overlap extension PCR method of Urban et al. (*57*). For mutagenesis, the target DNA fragments were cloned into pBluescript II KS and SK (Stratagene). These

fragments served as templates in a single PCR reaction in the presence of the universal primer T7 (5′-GTAATACGACTCACTATAGGGC-3′) and two mutagenic primers which contained an overlapping region of 19 nucleotides. The PCR products were loaded onto a 1% (w/v) agarose gel and purified using the JetSorb purification kit (Genomed). The presence of the mutation and the absence of nonspecific mutations was confirmed by DNA sequencing with an Applied Biosystems 371 automated sequencer using fluorescent ddNTPs (ABI). Sequence analysis was performed using the program MACMOLLY TETRA (SoftGene GmbH). Each mutant was then subcloned back to the wild-type plasmid pBs II KS+/rat TRH-DE. *Blg*II and *Bsm*I were used for subcloning the mutated PCR fragments, and the region around the mutation was sequenced. The full-length rat TRH-DE cDNA for wild type and the mutants was then restricted by *Not*I and *Xba*I and subcloned into the expression vector pcDNA3.1/His A (Invitrogen).

Tissue Culture and Transfection of BHK Cells. BHK cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) supplemented with 10% fetal bovine serum in a 37 °C incubator, gassed with 10% CO_2. For transient expression, BHK cells were plated onto six-well plates at 2×10^5 cells per well in 2 mL of DMEM supplemented with serum or on 150 cm^2 petri dishes at 2×10^6 cells per dish in 20 mL of DMEM supplemented with serum. After 18 h of growth, the cells (80% confluent) were washed twice with serum-free DMEM (2 mL per well or 20 mL per dish), and then 400 μL/well or 5 mL/dish of serum-free medium was added to the cells. LipofectAmine reagent (Life Technologies) and DNA were diluted separately with serum-free medium (2 μg of DNA and 12 μg of LipofectAmine in 100 μL of medium for transfection of the six-well plates or 10 μg of DNA and 60 μg of LipofectAmine in 250 μL of medium for transfection of the 150 cm^2 petri dishes). PLUS reagent (Life Technologies) was added to the DNA solution (7 or 20 μL, respectively), and the solution was incubated at room temperature for 15 min. The DNA-PLUS reagent was mixed with diluted LipofectAmine reagent and then incubated for 15 min at room temperature. The DNA-PLUS reagent−LipofectAmine reagent complex was added to the cells kept in serum-free DMEM. After incubation at 37 °C and 10% CO_2 for 4 h, 2 mL/well or 20 mL/dish of DMEM containing 10% fetal bovine serum was added. The cells were collected 48 h after transfection, washed with phosphate-buffered saline, and homogenized in ice-cold 20 mM potassium phosphate buffer, pH 7.4. After centrifugation (20000*g* for 30 min at 4 °C) the cell pellet was washed in the same buffer and then resuspended in ice-cold 20 mM potassium phosphate and 500 mM NaCl, pH 7.4. After 30 min on ice the membranes were collected by centrifugation (20000*g* for 30 min at 4 °C). This step was repeated once. The final membrane pellet was homogenized in 20 mM potassium phosphate, pH 7.4, and stored at −80 °C until used for Western blot analysis and enzyme assays. The protein was determined by a modification of the Lowry method as described by Peterson (*58*). For stable expression, BHK cells were plated onto six-well plates at 2×10^5 cells per well and transfected by using LipofectAmine-PLUS as described above. Twenty-four hours after transfection, the cells were treated with 1 mL of trypsin−EDTA (1×) in EBSS W/O (Life Technologies) for 5 min and transferred onto 150 cm^2 petri dishes containing 20 mL of DMEM supplemented with 10% fetal bovine serum and 1 mg/mL G418 (Life Technologies). Cells were grown in a 37 °C incubator aerated with 10% CO_2 and were selected for resistance to G418. Individual resistant colonies producing large amounts of TRH-DE (indicated by enzyme assays and Western blot analysis) were cloned by limiting dilution techniques.

SDS−PAGE and Western Blot Analysis. Polyacrylamide gel electrophoresis was carried out according to Laemmli (*59*) using a 4% stacking and a 7.5% separating gel. After being blotted (*60*) onto nitrocellulose membranes (BAS83, Schleicher and Schuell) by use of a tank blot (Bio-Rad trans blot cell), the proteins were detected by the chemical luminescence technique (SuperSignal HisProbe Western blotting kit, Pierce). The protein expression levels were estimated by densitometric scanning (Duoscan T1200, Agfa) and calculated using the program NIHIMAGE (NIMH).

Enzyme Assays. (A) Radiochemical Assay. The activity of the TRH-DE was measured by the radiochemical assay in the presence of the inhibitors of the soluble TRH-degrading enzymes as described in ref *16* using [pyroGlu-^{3}H]TRH as substrate. The kinetic parameters (K_m and V_{max}) were determined from Lineweaver−Burk plots using concentrations of TRH ranging from 2 to 200 μM. As reference, the specific activity of the wild-type recombinant enzyme was established by Western blot analysis and enzyme assays, whereby purified rat TRH-DE (*20*) was used as standard. To determine the specific activities of the mutants at 2 μM [pyroGlu-^{3}H]TRH concentration, the substrate hydrolysis rates were reported for an equivalent level of expression of each mutant. The sensitivity of wild-type and mutated TRH-DEs to several inhibitors and chemical modifying agents of the enzyme was determined by establishing dose-dependent inhibition curves. IC_{50} values were considered to be approximately equal to K_i values, as the interactions were shown to be competitive [$IC_{50} = K_i(1 + [S]/K_m)$] and the concentration of the substrate used (2 μM) was less than its K_m for the enzyme ($K_m \cong 35$ μM). The inactivation of TRH-DE with (*S*)-5-mercaptomethyl-2-pyrrolidinone (Glp-SH) was performed in the presence of 1 mM dithiothreitol. For the inactivation by diethyl pyrocarbonate (DEPC), the compound was dissolved in ethanol at adequate concentrations and 2% (v/v) of the stock solutions was added to the incubation mixture to adjust given concentrations of DEPC. The reaction was allowed to proceed at 25 °C for 30 min and was then stoped with NaN_3 (final concentration 20 mM). After addition of [pyroGlu-^{3}H]TRH the enzyme activity was determined as described above.

(B) Fluorometric-Coupled Enzyme Assay for TRH-DE. A modification of a recently developed coupled fluorometric assay (*61*) was used to investigate the ability of the membrane-bound, wild-type recombinant enzyme and the E408D mutant to hydrolyze Glp-His-ProAMC (Bachem), Glp-Phe-ProAMC, and Glp-Orn-ProAMC (PolyPeptide Laboratories). In this assay, dipeptidyl aminopeptidase IV (Sigma) is the coupling enzyme. In the case of Glp-His-ProAMC as substrate, the reaction follows the scheme:

$$\text{Glp-His-ProAMC} \xrightarrow{\text{TRH-DE}} \text{His-ProAMC} \xrightarrow{\text{DPP IV}} \text{His-Pro} + \text{AMC}$$

The liberation of the final degradation product AMC was measured fluorometrically. To determine the activity of the DPP IV solution under the standard conditions employed for the TRH-DE coupled assay, 1 μL of DPP IV (0.063 μg) was incubated with 100 μM Gly-ProAMC (Bachem) in 20 mM potassium phosphate buffer in a total volume of 600 μL. The DPP IV activity was monitored continuously by measuring the increase in AMC fluorescence using a Perkin-Elmer LS-3B fluorescence spectrometer fitted with a thermostated cell holder. Wavelengths for excitation and emission were set at 370 and 440 nm, respectively. The maximum velocity for DPP IV was found to be 8.7 units mg^{-1}, whereby one unit of enzyme activity was defined as the amount of enzyme catalyzing the formation of 1 μmol of AMC in 1 min under the conditions used. At 30 °C, 30 μL of this DPP IV solution (1.89 μg) in a total volume of 600 μL of 20 mM potassium phosphate buffer, pH 7.5, was sufficient for the complete degradation of 1 μM Gly-ProAMC within 2 min. Under the incubation conditions used, DPP IV was stable for long periods of time. Even after incubation at 30 °C for 9 h, no decrease in the enzymatic activity could be observed.

The K_m and V_{max} values for (a) Glp-His-ProAMC, (b) Glp-Phe-ProAMC, and (c) Glp-Orn-ProAMC were determined by the fluorometric coupled enzyme assay as described above by measuring initial rates at five different substrate concentrations in the range of 0−10 μM (a) or 0−50 μM (b, c). The substrates were incubated at 30 °C with 1.89 μg of DPP IV and 0.0168 μg of the membrane-bound, recombinant wild-type TRH-DE or 0.2 μg of the E408D mutant, in a total volume of 600 μL of 20 mM potassium phosphate buffer, pH 7.5, under continuous shaking (Eppendorf thermomixer 5436). Incubation times of 25 min to up to 8 h were used. TRH-DE and DPP IV activity was terminated by immersing the samples in an 80 °C water bath for 2 min. The samples were then cooled on ice for 2 min and centrifugated at 14000*g* for 1 min. The resulting supernatants were kept on ice prior to measurement of fluorescence intensity. Control samples were included, in which substrates and DPP IV were incubated under identical conditions in the absence of TRH-DE. The quantity of AMC produced (nanomoles) was determined by comparison to an AMC standard curve. The kinetic parameters (K_m and V_{max}) were calculated from Lineweaver−Burk plots.

RESULTS

Site-Directed Mutagenesis and Expression of Wild-Type and Mutated Enzymes in BHK Cells. To investigate the functional role of conserved amino acid residues in TRH-DE, glutamate residues E408, E442, and E464 were replaced by glutamine (Q) or aspartate (D), histidine residue H507 by arginine (R) or phenylalanine (F), tyrosine residues Y528 and Y554 by phenylalanine (F), and arginine residue R488 by glutamine (Q) or alanine (A). The wild-type and the mutant cDNAs were then subcloned into the expression vector pcDNA3.1/His A and expressed transiently or stably in BHK cells. Transfection under the same conditions with control plasmid pcDNA3.1/HisB/lacZ or pcDNA3.1/HisA was also carried out. Western blot analysis of the membrane fractions prepared from these cells showed that each mutant enzyme had the same apparent molecular mass (130 kDa) as the recombinant wild-type enzyme (Figure 2).

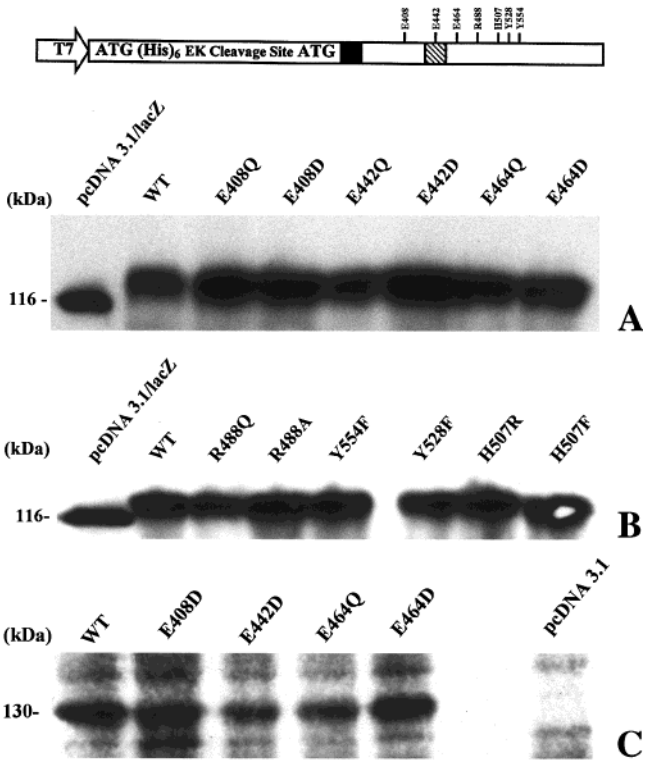

FIGURE 2: Western blot analysis of wild-type and mutated rat TRH-DEs expressed in BHK cells. The top diagram shows the schematic representation of the primary structure of rat TRH-DE in pcDNA3.1/HisA. The complete open reading frame was fused in-frame C-terminal to the polyhistidine tag. The mutated residues are indicated as vertical lines on the top of the diagram. The closed box indicates the transmembrane spanning domain, and the hatched box indicates the Zn-binding consensus motif. Following transient (A, B) or stable (C) transfection of the BHK cells with pcDNA3.1, pcDNA3.1/lacZ, wild type, and mutants, the membrane fractions containing the ectoenzyme were subjected to SDS−PAGE as described under Experimental Procedures. The His-tagged proteins were detected using the SuperSignal HisProbe Western blotting kit (Pierce). The protein expression levels were estimated by densitometric scanning (Duoscan T1200, Agfa) and calculated using the program NIHIMAGE (NIMH). The positions of the molecular mass markers (in kDa) are indicated.

Enzymatic Activity of Wild-Type and Mutated TRH-DEs and Inhibitory Potencies of Various Classes of Inhibitors or Chemical Agents Modifying Amino Acid Residues. The specific activity of each mutant enzyme was determined in the membrane fractions by the radiochemical assay as described in ref *16*. The specific activity of the wild-type recombinant enzyme was established by Western blot analysis and enzyme assays, in which purified rat TRH-DE (*20*) was used as standard. The kinetic parameters for the hydrolysis of [pyroGlu-^{3}H]TRH by wild-type and mutated enzymes are reported in Table 1. None of the K_m values that could be determined seem significantly different from those of the wild type.

Replacement of E408 yielded enzymes that were either completely inactive (E408Q) or had greatly diminished (E408D) catalytic activity. The effect of the mutation on activity was generally due to a decrease in the value of V_{max}. The K_m value of TRH for the E408D mutant was not significantly altered, and the ratio V_{max}/K_m was about 30-fold lower than that of the wild-type enzyme. To characterize the role of E408 further, the inhibitory potencies of several TRH derivatives and Glp-SH were determined. As shown

Table 1: Kinetic Parameters for the Hydrolysis of [pyroGlu-^{3}H]TRH by Wild Type and Various Rat TRH-DE Mutants[a]

enzyme	specific activity (nmol·min^{-1}·mg^{-1})	K_m (μM)	V_{max} (nmol·min^{-1}·mg^{-1})	V_{max}/K_m (mL·min^{-1}·mg^{-1})
wild type	250 ± 44	32.5 ± 1	3727 ± 104	114.67
E408Q	−	−	−	−
E408D	6.8 ± 1.2	36.97 ± 0.9	126.25 ± 1.8	3.4
E442Q	−	−	−	−
E442D	3.63 ± 0.5	44.64 ± 2.6	74.27 ± 1.96	1.6
E464Q	1.66 ± 0.02	−	−	−
E464D	21 ± 0.7	40 ± 4.5	520.24 ± 0.06	13
H507R	39.1 ± 1.3	38.24 ± 3.1	878.55 ± 32.7	22.97
H507F	16.15 ± 0.5	36.67 ± 1.9	285.89 ± 6.	7.8
Y528F	12.26 ± 0.4	22.3 ± 8	90.58 ± 5.7	4
Y554F	237.5 ± 17.6	−	−	−
R488Q	208.68 ± 30.1	25.4 ± 3.2	2178.85 ± 92.1	85.8
R488A	190 ± 49.5	−	−	−

[a] Specific activities were determined at 2 μM substrate concentration. Reactions were carried out as described under Experimental Procedures. Values are the mean ± SD from three independent transfections. −, not detected.

Table 2: K_i Values (μM) of TRH Derivatives and Glp-SH for Wild-Type and E408D Mutant Enzyme[a]

inhibitor	wild type	E408D
Glp-His(3Me)-ProNH$_2$	54	75
Glp-Asn-ProNH$_2$	5.33	44
Glp-Asn-ProAMC	0.25	3.5
Glp-Gln-ProNH$_2$	70	200
Glp-Glu-ProNH$_2$	2000	6000
Glp-SH	40	400

[a] Enzyme activity was determined radiochemically as described under Experimental Procedures at different concentrations of the peptides and Glp-SH. K_i values were estimated from the dose-dependent inhibition curves as described under Experimental Procedures.

in Table 2, the K_i values of Glp-Asn-ProNH$_2$ and Glp-Asn-ProAMC for the E408D mutant were increased by 1 order of magnitude as compared to the wild-type enzyme. No significant alteration in the binding of Glp-His(3Me)-ProNH$_2$ was found by replacing E408 by D, whereas this mutation induced a 3-fold decrease in the inhibitory potency of Glp-Gln-ProNH$_2$. The peptide Glp-Glu-ProNH$_2$, with the negatively charged residue Glu at the P_1' site, displays a poor affinity for both the wild type (2 mM) and the E408D mutant (6 mM). The inhibitory potency of the thiol inhibitor Glp-SH for the mutant enzyme was decreased by 1 order of magnitude (Table 2 and Figure 4). The influence of the metal-complexing agents 1,10-phenanthroline and EDTA on the enzymatic activity of the wild-type and mutant enzymes is summarized in Table 3. No differences in the inhibition of the residual activity of mutant E408D by EDTA and moderate differences in its inhibition by 1,10-phenanthrolin were observed as compared to the wild-type enzyme.

The kinetic parameters of the membrane-bound recombinant wild-type enzyme and the E408D mutant were compared directly by carrying out kinetic analysis of the synthetic peptides Glp-His-ProAMC, Glp-Phe-ProAMC, and Glp-Orn-ProAMC (Table 4). All were found to act as substrates of TRH-DE. The K_m value of the wild-type enzyme for Glp-His-ProAMC (4.1 μM) compared well with the value of 3.8 ± 0.5 μM, which was published recently for the continuous

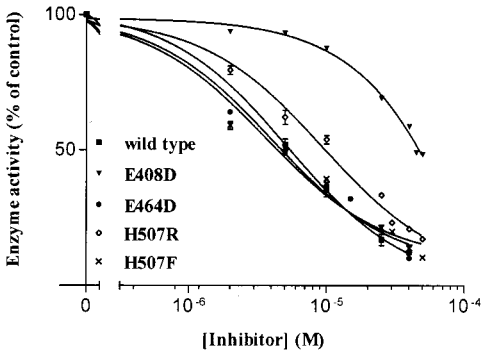

FIGURE 3: Effect of Glp-Asn-ProNH$_2$ on the enzymatic activity of wild-type and mutated rat TRH-DEs. Enzyme activity was determined at various concentrations of Glp-Asn-ProNH$_2$. The vertical error bars indicate the SD of the values where it exceeds the size of the symbol. K_i values were calculated to be 5.33 μM for wild type, 44 μM for E408D, 5 μM for E464D, 12 μM for H507R, and 6 μM for H507F.

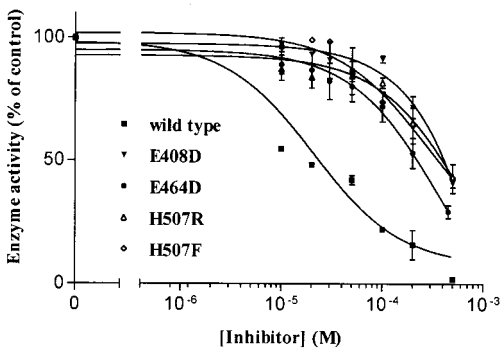

FIGURE 4: Effect of Glp-SH on the enzymatic activity of wild type and various rat TRH-DE mutants. Enzyme activity was determined at different concentrations of Glp-SH. The inactivation with Glp-SH was performed in the presence of 1 mM dithiothreitol. Control reactions, without inhibitor but in the presence of 1 mM DTT, were incubated in parallel for the 100% values. The vertical error bars indicate the SD of the values where it exceeds the size of the symbol. K_i values of Glp-SH were calculated to be 40 μM for wild type, 400 μM for E408D, 240 μM for E464D, 380 μM for H507R, and 410 μM for H507F.

Table 3: Inhibition of Wild Type and Mutants E464D and E408D by the Metal-Complexing Agents 1,10-Phenanthroline and EDTA[a]

		inhibition of activity (%)		
treatment	concn	WT	E464D	E408D
1,10-phenanthroline	0.1 mM	12	84	21.3
	0.3 mM	57.2	98	86.8
	0.5 mM	82.5	98	99
	1 mM	94	100	100
	2 mM	99	100	
EDTA	30 μM	17	89.3	18
	100 μM	21	94	25
	300 μM	24	100	

[a] Enzyme activity was determined by the radiochemical assay as described under Experimental Procedures in the presence of 1,10-phenanthroline or EDTA at the concentrations indicated.

fluorometric coupled assay with TRH-DE purified from porcine brain (*61*). The K_m values of the mutant enzyme for Glp-His-ProAMC, Glp-Phe-ProAMC, and Glp-Orn-ProAMC were higher (3.5, 2.2, and 2.6 times, respectively) than those found for the wild-type enzyme. The V_{max} value of the mutant for Glp-Orn-ProAMC was reduced by a factor of 4, resulting in a 10-fold decrease of the ratio V_{max}/K_m of E408D as

Table 4: Comparison of Kinetic Parameters for the Hydrolysis of Glp-His-ProAMC, Glp-Phe-ProAMC, and Glp-Orn-ProAMC by Wild Type and the E408D Mutant[a]

	V_{max} (units mg^{-1})		K_m (μM)		V_{max}/K_m (units mg^{-1} μM^{-1})	
peptide	wild type	E408D	wild type	E408D	wild type	E408D
Glp-His-ProAMC	0.57	0.0058	4.1	14.3	0.14	0.0004
Glp-Phe-ProAMC	0.1	0.001	4.6	10	0.021	0.0001
Glp-Orn-ProAMC	0.04	0.01	12.5	33	0.0032	0.0003

[a] The kinetic parameters (V_{max} and K_m) were calculated from Lineweaver−Burk plots of data obtained from the fluorometric coupled enzyme assay for TRH-DE as described under Experimental Procedures.

Table 5: Inhibition of Wild Type and Mutants H507R and H507F by Diethyl Pyrocarbonate[a]

	concn	inhibition of activity (%)		
treatment	(mM)	wild type	H507R	H507F
DEPC	0.1	24	20	20
	0.5	41	42	38.8
	1	87.6	75.9	90
	2	89.5	96.1	100
	5	100	100	100

[a] Membrane fractions of cells expressing recombinant wild-type or mutant enzymes were incubated at 25 °C with DEPC at the concentrations indicated. After 30 min, the reaction was stopped by addition of NaN_3 (final concentration 20 mM), and the enzyme activity was determined radiochemically as described under Experimental Procedures.

compared to the wild-type enzyme. In contrast, the V_{max} values of E408D for Glp-His-ProAMC and Glp-Phe-ProAMC were about 100-fold lower, leading to a V_{max}/K_m decrease of 350- and 210-fold, respectively. Interestingly, the V_{max} of the mutant for Glp-Orn-ProAMC was higher than those for the two other substrates, whereas Glp-Orn-ProAMC displayed the poorest hydrolysis rate by the wild-type enzyme, as compared to Glp-His-ProAMC and Glp-Phe-ProAMC.

Intrigued by the possibility that the product Orn-ProAMC may act as an inhibitor of the wild-type TRH-DE, we used pyroglutamyl aminopeptidase I (PAP I) to degrade Glp-Orn-ProAMC into Glp and Orn-ProAMC and tested the products for their ability to inhibit the [pyroGlu-^{3}H]TRH hydrolysis by the wild-type enzyme and the E408D mutant. None of these products was found to inhibit wild-type or mutant enzyme at concentrations up to 30 μM (data not shown). Thus, the effects produced by the substrate Glp-Orn-ProAMC cannot be attributed to a product inhibition of TRH-DE.

In the case of residue H507, the K_m values of the mutants H507R and H507F for the substrate TRH, were almost similar to those of the wild-type enzyme, indicating that mutating H507 does not provoke any major changes in ground-state binding. For the mutant enzymes H507R and H507F, however, V_{max} values were lower, leading to decreases in V_{max}/K_m of 5- and 15-fold, respectively (Table 1). Mutating H507 to either R or F did not significantly modify the K_i values of the peptide Glp-Asn-ProNH_2 (Figure 3). The largest differences in K_i values were found with the thiol inhibitor Glp-SH. For the mutant enzymes, the inhibitory potency of this inhibitor was decreased by 1 order of magnitude (Figure 4).

It has been shown that TRH-DE is sensitive to modification by diethyl pyrocarbonate, and thus it has been postulated that a histidine residue in the active site of the enzyme might be responsible for the inactivation by DEPC (*38*). To ascertain whether H507 was the target of DEPC, we exposed the wild-type enzyme and the H507R and the H507F mutants to this reagent. Surprisingly, the residual activity of the mutants was still sensitive to the action of DEPC (Table 5), suggesting that another functional histidine or tyrosine residue in or near the active site of the enzyme is modified by this reagent.

Replacement of E442, the glutamate residue in the consensus sequence HEICH, also yielded enzymes that were either completely inactive (E442Q) or had greatly diminished (E442D) catalytic activity. Changing the glutamate for an aspartate led to an enzyme with a specific activity representing only 1.4% of that of the wild-type enzyme. To characterize further the properties of the mutant E442D, we determined the kinetic parameters K_m and V_{max} for the hydrolysis of [pyroGlu-^{3}H]-TRH. E442D had a K_m of 44.6 $\pm$ 2.6, close to that of wild-type TRH-DE (32.5 $\pm$ 1), whereas the V_{max} value was considerably decreased (Table 1), resulting in a 70-fold decrease in the V_{max}/K_m ratio of the mutant as compared to that of the wild-type enzyme. These results indicate that the reduction of the specific activity of the mutant enzyme is probably due to a much lower efficiency of hydrolysis.

When residue E464 was replaced by glutamine, we obtained a mutant enzyme in which the rate of hydrolysis was 150-fold reduced, preventing measurement of kinetic parameters. In contrast, when the negative charge at position 464 was restored by substitution with aspartate (E464D), a low but significant activity could be detected, representing 8.4% of the wild-type TRH-DE specific activity. As shown in Table 1 the E464D mutant exhibits a K_m value very similar to that of the nonmutated enzyme, suggesting that the structure of the active site of the enzyme has not been grossly altered by the mutation. In contrast, the V_{max} value was significantly reduced. The V_{max}/K_m ratio was decreased to 11.3% of that of the wild-type enzyme. To characterize further the role of E464, the inhibitory potencies of several TRH derivatives and Glp-SH were determined. As shown in Figure 3, Glp-Asn-ProNH_2 had the same inhibitory potency for both the wild type and the E464D mutant, indicating that the S_1' subsite of the enzyme was not altered by the mutation. In contrast, the inhibitory potency of Glp-SH was about six times lower for the mutant compared with the wild-type enzyme (Figure 4). As shown in Table 3 the mutant E464D exhibited a lower affinity for zinc than the wild type, as suggested by its higher sensitivity to chelating agents.

The enzymatic activity of the Y528F mutant was decreased to 5% of that of the wild-type enzyme (Table 1). The K_m values were equivalent for the wild type and the Y528F mutant, but the V_{max} value was greatly decreased, leading to a decrease in V_{max}/K_m to 1/30 for this mutant. The presence of a tyrosine residue in the active site of TRH-DE was further investigated by testing the sensitivity of the wild-type enzyme and the Y528F mutant to *N*-acetylimidazole, a chemical reagent that acetylates tyrosine, lysine, and cysteine residues (*37*).

As shown in Figure 5, the mutant Y528F was still sensitive to the action of *N*-acetylimidazole, albeit at a much reduced

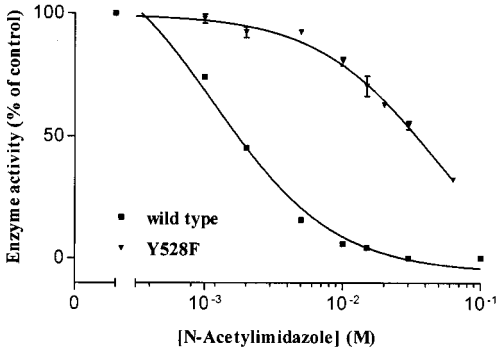

FIGURE 5: Inactivation of wild type and the Y528F mutant by *N*-acetylimidazole. Enzyme activity was determined at different concentrations of *N*-acetylimidazole. The vertical error bars indicate the SD of the values where it exceeds the size of the symbol. IC_{50} values of *N*-acetylimidazole were calculated to be 2 mM for wild type and 35 mM for Y528F.

level. The IC_{50} values determined for nonmutated TRH-DE and Phe528 mutant were 2 and 35 mM, respectively. In contrast to Y528, the Y554 residue, which resides 25 residues carboxy-terminal to Y528, could be replaced by phenylalanine without a significant change in the enzymatic activity (Table 1).

The results of a previously reported study on the chemical modification of aminopeptidase N (*37*) show that a single arginine is essential for enzymatic activity and more precisely for substrate binding by this enzyme. This arginine could well correspond to R488 of rat TRH-DE which has been previously suggested to align with R203 of thermolysin (*38*). Therefore, we studied the functionality of arginine 488 by substituting this arginine residue by a glutamine or alanine. As shown in Table 1, the specific activities of both mutant enzymes were similar to those of the wild-type enzyme.

DISCUSSION

The aim of this study was to investigate the organization of the active site of TRH-DE and to improve the characterization of its mechanism of action by replacing several conserved residues, known to be involved in the catalytic mechanism of other metallopeptidases.

E408, the glutamate residue located in the AMEN conserved motif, is the counterpart of E271 in LTA4H, E350 in APN, and E352 in APA. Previous studies on these three enzymes (*51*, *52*, *54*) have indicated that an interaction occurs between the negative charge of the side chain carboxylate of the corresponding glutamate residue and the N-terminal amino group of inhibitors or substrates. It has been proposed that in APN E350 forms a hydrogen bond with the free α-amino group of ligands to stabilize the transition state, which is believed to be formed in a manner similar to that of thermolysin (*51*). Likewise, it has been suggested that E352 in APA interacts with the free N-terminus of substrates, although it has also been recognized that E352 may act directly as a nucleophilic catalyst in the catalytic process of APA (*52*). To investigate the functional role of E408 in TRH-DE, radical and conservative replacement of this residue was carried out.

The mutation E408Q in TRH-DE led to a completely inactive enzyme. To maintain some measurable activity, E408 was replaced by D, which conserves, but displaces, the negative charge by one methylene group. Kinetic studies showed that for TRH the K_m value of the E408D mutant was similar to that determined for the wild-type enzyme, whereas the V_{max} value was decreased, leading to a 30-fold reduction in cleavage efficiency of the mutant. These results suggest that E408 is important in the hydrolytic mechanism of TRH-DE and are consistent with the results observed previously following site-directed mutagenesis of the counterparts of E408 in APN and APA.

In APN and APA the interacting partner of the glutamate residue is believed to be the α-amino group at the N-terminus of substrates or inhibitors. TRH cannot interact with E408 in precisely the same way since it does not possess a free N-terminal α-amino group. In TRH, however, the possibility exists that E408 of TRH-DE could form a hydrogen bond with either the NH group of Glp or the imidazole group of histidine. The results from a previous study (*62*) suggest that the imidazole group in TRH may not be protonated during the binding of TRH to TRH-DE, facilitating the possibility of hydrogen bonding between this residue and E408. Nevertheless, data from this previous study (*62*) and the study presented herein (Table 4) show that TRH analogues in which the P_1' residue cannot form a hydrogen bond with E408 are TRH-DE substrates. Thus, it appears extremely unlikely that the P_1' residue of TRH or TRH-like peptides forms a hydrogen bond with E408 and in a manner analogous to that described for APA and APN. Although the possibility remains that the NH group of Glp may interact with the carboxylate of E408, comparison of affinities of Glp-SH and Glp-containing peptides for wild-type and E408D enzymes (Table 2) seems to indicate that interactions between TRH or TRH analogues and E408 would be relatively weak. This interpretation is consistent with the observation that the counterpart of E408 in APN (E350) plays a more important role in stabilizing the transition state of the substrate, and consequently its rate of degradation, than in ensuring the energetically favorable specific binding of the free amino group of substrates or inhibitors (*51*). Nevertheless, the fact that the E408D mutation results in a more than 30-fold reduction in the cleavage efficiency for TRH and in a 350- and 210-fold reduction in the cleavage efficiencies for Glp-His-ProAMC and Glp-Phe-ProAMC, respectively, clearly indicates that E408 plays an important function in the catalytic mechanism of TRH-DE. To define the exact role of this residue, however, we have to await the availability of compounds acting as transition-state inhibitors.

From data obtained in this study it could be proposed that H507 in TRH-DE does not have a role in substrate binding, indicating some differencies in the evolution of the active sites and, consequently, in the mechanism of action between TRH-DE and APA. In the latter enzyme H450, the counterpart of H507 in TRH-DE, is involved in substrate binding via an interaction with the P_1 side chain carboxylate of the substrate, the extent of this interaction being increased by Ca^{2+} (*55*). In contrast, the results presented in our study would seem to be in favor of a minor role for H507 in catalysis since its replacement by either Arg or Phe led to the production of enzymes that still bound similarly to the wild-type enzyme and retained proteolytic activity, albeit reduced. The V_{max}/K_m value for the H507F mutant is 3-fold smaller as compared to that obtained for the H507R mutant. The presence of a phenylalanine at this position, preventing the formation of a hydrogen bond, probably leads to a less

efficient stabilization of the transition state by a local perturbation of the H-bond network. Similar K_i values of the inhibitor peptide Glp-Asn-ProNH_2 were obtained for the mutants (H507R and H507F) and the wild-type enzyme. In contrast, the H507 mutations resulted in a 10-fold reduction in affinities of the inhibitor Glp-SH. This increase in K_i values was perhaps unexpected although this could be due to more favorable interactions of electrons in the external d orbital of sulfur with the zinc cation in the case of the wild-type enzyme.

Our biochemical characterization of the expressed recombinant mutants demonstrates also that both glutamate residues E442 (in the HEICH consensus motif) and E464 (in the WLKEG conserved motif) are essential for TRH-DE enzymatic activity. Replacement of E442 yielded enzymes that were either completely inactive (E442Q) or had greatly diminished (E442D) catalytic activity (1.4% of wild-type activity). By replacing residue E464 by a glutamine, we obtained a mutant enzyme in which the rate of hydrolysis was 150-fold reduced. In contrast, when the negative charge at position 464 was restored by substitution with aspartate (E464D), a low but significant activity could be detected (8.4% of wild-type activity). This shows that, in addition to the charge, the length of the side chain of E464 and consequently the geometry of this amino acid are crucial for enzymatic activity. The similar affinity of E442D, E464D, and wild-type enzyme for TRH suggests that the loss of activity is due to an alteration of the ability of these mutants to cleave efficiently the peptide bond of the substrate rather than an alteration in substrate binding.

The mutant E464D exhibited a lower affinity for zinc than the wild type, as suggested by its higher sensitivity to chelating agents. Moreover, the differences observed between the inhibitory potencies of Glp-SH for the wild type and the E464D mutant indicate that the zinc ion is still present in the active site of E464D and allows the binding of the inhibitor, but its lower affinity suggests a decrease in the interaction with this inhibitor, probably due to a modification of the zinc ion position in the active site of the mutant.

Interestingly, the sequence surrounding the glutamate residue 464 in TRH-DE (WLXEG), is conserved among the different members of the M1 family of metallopeptidases, and the distance between glutamates of the HEXXH and WLXEG motifs (21 residues) is also conserved. Furthermore, in APA (*42*) and LTA_4 hydrolase (*39*, *54*) site-directed mutagenesis and crystal structure studies have shown that the glutamate in the WLXEG motif is the third zinc ligand, corresponding to E166 of thermolysin. Taken together, these results show the importance of E442 in the catalysis and E464 in zinc binding of TRH-DE and are consistent with the catalytic mechanism of TRH-DE being similar to that proposed for LTA_4 hydrolase and thermolysin.

TRH-DE mutant Y528F also showed a large decrease in specific activity (5% of wild-type activity) due to a large decrease in the V_{max} value, whereas no change in activity was detected when the nonconserved Y554 was replaced by F. Phenylalanine was chosen to alter the structure of the active site as little as possible. The kinetic measurements on Y528F are consistent with those observed for the mutation of the residues proposed to stabilize the transition state in thermolysin (H231), neprilysin (H711), and APA (Y471) (*47*−*49*). Furthermore, the 17-fold decrease in the inhibitory

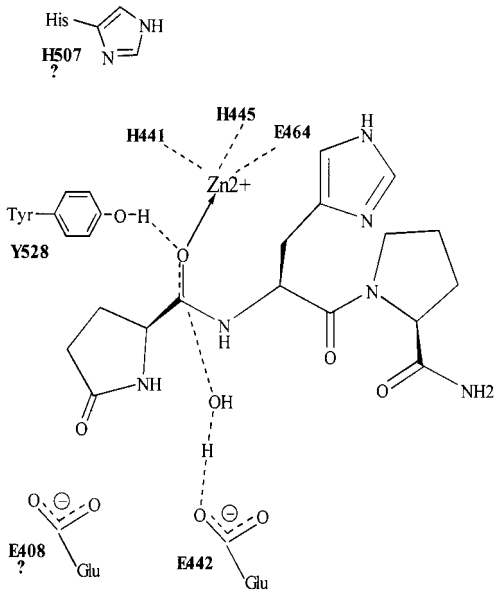

FIGURE 6: Hypothetical model for the hydrolysis of TRH by the TRH-degrading ectoenzyme. Amino acid residues of TRH-DE that play a putative role in catalysis or zinc coordination are indicated. Mutational analysis of the importance of H441 and H445 has not been reported. However, on the basis of the structure of other similar metallopeptidases of the M1 family, their interaction with zinc should be expected.

potency of *N*-acetylimidazole, a chemical modifying agent that acetylates tyrosyl, thiol, and lysyl groups, suggests that Y528 in TRH-DE is the counterpart of H231 in thermolysin, H711 in neprilysin, and Y471 in APA. However, in the thermolysin family, two residues (H231 and Y157) are involved directly in the stabilization of the transition state. In neprilysin, APA, TRH-DE, and [as discussed very recently (*54*)] LTA_4 hydrolase only one residue seems to have such a function, indicating that these enzymes are more related to the astacin family in which only one tyrosine residue is involved directly in the stabilization of the transition state (*63*, *64*).

On the basis of sequence analysis it has been hypothesized that arginine 488 of TRH-DE may be involved in substrate binding (*38*). R488 was thought to interact with the carbonyl of the $P_1'-P_2'$ amide bond of TRH and has been suggested to align with R203 of thermolysin (*56*). For APN, the involvement of a single arginine in substrate binding was also reported (*37*). Site-directed mutagenesis has now been used to evaluate the role of this residue, and no significant modification in enzyme activity and substrate binding was found in the R488Q and R488A mutants, suggesting that this residue is not involved in the activity of TRH-DE.

This is the first study to address the active site organization of TRH-DE and the functionality of seven residues postulated to play key roles in the mechanism of homologous zinc-dependent aminopeptidases. From the results of this investigation it has been possible to propose a hypothetical model for the hydrolysis of TRH by the TRH-DE by analogy with thermolysin and other metallopeptidases (Figure 6). In the absence of a crystal structure for TRH-DE, this model will be valuable for understanding the catalytic mechanism of TRH-DE, as well as the rational design of specific and potent TRH-DE inhibitors, which are essential tools for furthering

our knowledge of the physiological importance of this highly specific peptidase in health and disease.

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