The HELLGH Motif of Rat Liver Dipeptidyl Peptidase III Is Involved in Zinc Coordination and the Catalytic Activity of the Enzyme

Katsuhiko Fukasawa,*,‡ Kayoko M. Fukasawa,‡ Hiroyuki Iwamoto,§ Junzo Hirose,§ and Minoru Harada‡

Department of Oral Biochemistry, School of Dentistry, Matsumoto Dental University, Shiojiri, Nagano 399-0781, Japan, and Department of Applied Biological Science, Faculty of Engineering, Fukuyama University, Fukuyama, Hiroshima 729-0251, Japan

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ABSTRACT: The role of the HELLGH (residues 450−455) motif in the sequence of rat dipeptidyl peptidase III (EC 3.4.14.4) was investigated by replacing Glu^{451} with an alanine or an aspartic acid residue and by replacing His^{450} and His^{455} with a tyrosine residue by site-directed mutagenesis. Mutated cDNAs were expressed three or four times in *Escherichia coli*, and the resulting proteins were purified to apparent homogeneity. None of the expressed mutated proteins exhibited DPP III activity. The mutants of Glu^{451} contained 1 mol of zinc per mole of protein, but mutants His^{450} and His^{455} did not contain significant amounts of zinc as determined by atomic absorption spectrometry. The Leu^{453}-deleted enzyme (having the zinc aminopeptidase motif HExxH-18-E) had almost the same order of binding affinity (for Arg-Arg-2-naphthylamide) as the wild-type enzyme, but the specificity constant was about 10%. These results provide evidence that the suitable number of amino acids included between Glu^{451} and His^{455} is three residues for the enzyme activity and confirm that residues His^{450}, His^{455}, and Glu^{451} are involved in zinc coordination and catalytic activity.

Since the determination of the three-dimensional structure of thermolysin (*1*), many metalloproteases have been cloned and some of them crystallized in the past 10 years (*2*, *3*). Bode et al., who determined the crystal structure of astacin (*4*) and adamalysin (*5*), classified them into a superfamily, the "metzincins", as they possess a methionine-containing turn with a similar conformation (the Met turn). They also suggested that the larger superfamily of zinc metalloproteases possessing the HExxH motif be termed the "zincins" (*6*). Hooper proposed the name "gluzincins" for the zinc metalloproteases of the HExxH short zinc binding consensus sequence containing the first two residues as zinc ligands and a glutamic acid as the third zinc binding ligand, as found in thermolysin, endopeptidase 24.11, and the aminopeptidase family, to distinguish them from the metzincins (*7*). In any case, the majority of zinc proteases exhibit a characteristic HExxH sequence integrated into an "active-site helix".

Recently, we determined that dipeptidyl peptidase (DPP)[1] III (EC 3.4.14.4) should be classified as a zinc metalloexopeptidase, as it exhibited a zinc dissociation constant of 2.5×10^{-13} M at pH 7.4 in a zinc binding study. When the gene was first cloned and sequenced, we noted that there was a region in the predicted protein (HELLGH, residues 450−455) that was closely similar to the HExxH zinc binding motif (*8*). In the study presented here, we replaced Glu^{451}, His^{450}, and His^{455}, the residues corresponding to the catalytic amino acid and the zinc ligands in the zinc metalloprotease motif (HExxH), with an alanine or an aspartic acid and a tyrosine. We also employed site-directed mutagenesis for the conserved glutamic acid residues (as conserved in human placental cDNA and predicted yeast protein 01232) to identify other residues that are important for enzyme activity. To examine how deletion of Leu^{453} would alter the substrate specificity and enzyme kinetics of the enzyme, we changed the HELLGH-18-E motif of rat DPP III into the zinc aminopeptidase motif HELGH-18-E. A preliminary account has already been presented (*9*).

We have discussed the validity of why the HELLGH motif of rat DPP III is a metal binding and catalytic domain.

EXPERIMENTAL PROCEDURES

Materials. Arg-Arg-NA and bestatin were obtained from Sigma and antipain, chymostatin, pepstatin A, and E-64 from Peptide Institute, Inc. (Osaka, Japan). Restriction and modifying enzymes were from Toyobo Co. (Osaka, Japan) and Takara Co. (Tokyo, Japan). All other reagents were of analytical grade and were purchased from Nakarai Tesque (Kyoto, Japan) or Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Isolation and Sequencing of cDNA Encoding Human DPP III. A λ gt 11 cDNA placental library purchased from Clontech Laboratories was screened with rabbit anti-human DPP III antiserum prepared as described previously (*8*). After 10^6 plaques had been screened, four positive clones were isolated. The nucleotide sequence was determined by the dideoxynucleotide chain-termination reaction method (*10*).

* To whom correspondence and reprint requests should be addressed: Department of Oral Biochemistry, School of Dentistry, Matsumoto Dental University, Shiojiri, Nagano 399-0781, Japan. Telephone: +81-263-52-3100, ext. 3337. Fax: +81-263-53-3456. E-mail: kmf@po.mdu.ac.jp.

‡ Matsumoto Dental University.

§ Fukuyama University.

[1] Abbreviations: DPP, dipeptidyl peptidase; NA, naphthylamide; E-64, (L-3-*trans*-carboxyoxiran-2-carbonyl)-L-Leu-agmatin.

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Table 1: Primers Used for Mutagenesis of Rat DPP III cDNA

	mutation nucleotide sequence (5′ → 3′)	numbering
selection primer		
*Spe*I site change	CCGCTCTAGAACATGTGGATCCCC	
mutagenic primer		
Glu^{451} → Ala	GGGGTTACATGCGCTGTTGGGC	1341−1362
Glu^{451} → Asp	GGGGTTACATGACCTGTTGGGCC	1341−1363
Glu^{474} → Ala	TGACCAGGCGACAGTG	1411−1426
Glu^{474} → Asp	GACCAGGACCACAGTGATC	1412−1429
Glu^{493} → Ala	CCGGAGTGGAGCGACATGGGATAGC	1465−1489
Glu^{493} → Asp	CGGAGTGGAGACATGGGATAG	1466−1488
Glu^{508} → Ala	CCAGCTACGAAGCGTGCCGGGCTGAG	1511−1536
Glu^{508} → Asp	GCTACGAAGACTGCCGGGCTG	1514−1534
Glu^{555} → Ala	GTTGGCTTTGGCGTTCTACACCCC	1653−1676
Glu^{555} → Asp	GTTGGCTTTGGACTTCTACACCCC	1653−1676
His^{450} → Tyr	GCAGGTGGGGTTATGAGCTGTTGGG	1335−1361
His^{455} → Tyr	GAGCTGTTGGGCTATGGCAGCGGC	1351−1374
deleted Leu^{453}	GGGTTACATGAGCTG- - -GGCCATGGCAGCGGC	1342−1374

Restriction endonuclease fragments of the cDNA were subcloned into M13 mp18 or mp19, and all regions of these single-stranded DNA species were sequenced using a universal primer and specific primers. Specific oligomers were purchased from Sawaday Co. (Tokyo, Japan).

Site-Directed Mutagenesis. Mutagenesis was performed on the cDNA encoding rat DPP III with a Chameleon double-stranded site-directed mutagenesis kit from Stratagene, which was used according to the manufacturer's recommended protocol. The pBluescript phagemid containing a rat DPP III cDNA was heat denatured, and the *Spe*I selection primer and the appropriate mutagenic primer (Table 1) were annealed to one of the strands. Both of these primers were extended around the plasmid and ligated to create the new mutant DNA strand. After a restriction digestion with *Spe*I for linearization, the remaining circular (mutated) plasmid was used to transform repair-deficient mut S *Escherichia coli* (XLmut S competent cells), and the transformed cells were selected by growth on ampicillin. Then the cells were grown in liquid culture, and plasmid DNA was isolated. After a second restriction digestion with *Spe*I, *E. coli* SOLR competent cells were transformed with the resultant DNA. Single colonies were grown in liquid culture for identifying the expressed protein by SDS−PAGE. Single-stranded DNA was isolated by infecting the bacteria with VCSM 13 helper phages for sequencing. Sequence analysis of the entire cDNA inserts confirmed that no alteration of the protein primary structures, other than the desired mutation, had occurred. The recombinant single-stranded plasmids were sequenced by use-appropriate synthetic primers.

Expression and Purification of Recombinant Rat DPP III Mutant Proteins. Mutated rat cDNAs were expressed as previously described (*8*). After centrifugation, cells in a 1 L culture were resuspended in 50 mL of PBS and homogenized in a glass homogenizer for 1 min (cell suspension). Aliquots (0.2−20 μL) of the cell suspension were preincubated with 0.1 mM bestatin and 25 mM sodium phosphate buffer (pH 7.4) at 37 °C for 10 min. Then, 0.2 mM Arg-Arg-NA was added to the assay mixture. After incubation at 37 °C for 20 min, 500 μL of 1 M acetate buffer (pH 4.0) containing 10% Tween 20 and 200 μL of Fast Garnet GBC (0.2 mg/mL in water) were added to the reaction mixture. The absorbance of the resulting diazo dye was measured at 530 nm. One unit of enzyme activity was defined as the amount catalyzing the formation of 1 μmol of β-naphthylamine/min at 37 °C. The protein of the cell suspension was assessed by the method of Hartree (*11*), and the protein content during the following steps was determined according to Bradford (*12*) with BSA as the standard. The protein content of each cell suspension was 513 ± 31 mg/L of culture. Extraction and purification of each protein were performed as described previously (*8*) with minor modification. Buffers used throughout the purification contained each of the following at a concentration of 0.1 mM: antipain, pepstatin, chymostatin, and E-64. In the case of inactive mutants, the expressed proteins were detected by dot blotting immunoassay using rabbit anti-human DPP antiserum in each step. Finally, 0.4−0.8 mg of each purified protein was obtained per liter of cell culture.

Zinc Content of Mutated Proteins. Zinc determinations were performed by electrothermal atomic absorption spectrometry according to a previously reported method (*8*) using 250−300 μg of each purified protein.

RESULTS

Isolation and Sequencing of cDNA Encoding Human DPP III. To specify the conserved amino acid residues in the region around the HELLGH motif of rat DPP, we isolated the cDNA encoding human DPP III. A human placental cDNA library was screened immunologically, and then four positive clones were isolated. From the nucleotide sequencing of these cDNAs, all four were the same, and the molecular mass of the insert cDNA was 1880 bp. The deduced amino acid sequence of the partial cDNA was 95% homologous to that of rat DPP III, but the N-terminal 243 amino acids residues were missing. Figure 1 shows an alignment of the deduced amino acid sequence among three species containing the HELLGH motif. We focused on the conserved glutamic acids (451, 474, 493, 508, and 555) and histidine residues (450 and 455) and replaced those in rat DPP III with an alanine or an aspartic acid and a tyrosine by site-directed mutagenesis.

Specific Activity of the Mutated Enzymes in the Cell Suspension. DPP III activity of the cells with the mutated plasmids is shown in Figure 2. There were three patterns regarding the expressed enzyme activity between alanine and aspartic acid mutant enzymes. Both mutants of Glu (474,

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rat   351 EQLLKELPWPPAFEKDKFLTPDFTSLDVLTFAGSGIPAGINIPNYDDLRQ 400
human     ----------T---------------------------------------
yeast     -EFISL---SKDY--PI-NP------E----T---------------V-L

rat   401 TEGFKNVSLGNVL AVAYATKREKLTFMEEEDKDLYIRWKGPSFDVQVGL 449
human     ------------ ---T-Q ----  L--D------L------------
yeast     KI---------I-S-A-KSSSKHPPS-ISQ--RPIFEKYQSD--E----I

rat   450 ***  *GSGKLFVQDEKGAFNFDQETVINPETGEQIQSWYRSGETWDSKFS 500
human     --------------------------------------------------
yeast     -----------LTEFTD- ----K-NPPLGLD-KPVSTY-KV----G---G

rat   501 TIASSYEECRAESVGLYLCLNPQVLQIFGFEGTDAED VIYVNWLNMVRAG 550
human     --------------------H----E------A---- -------------
yeast     QL-GPF------VIAMF-LT-KKI-D----HDVESQ-K---AGY-Q-A---

rat   551 LLALEFYTPETANWRQAHMQARFVILRVLLEAGEGLVTVTPTTGSDGRPD 600
human     ----------AF-------------------------I-----------
yeast     -----YWN-K-GK-G-P------S-MKTFMKHSTDKNFLKLEMN- TND-

rat   601 ARVHLDRSKIRSVGKPALERFLRRLQVLKSTGDVVAGRALYEGYAAVTDA 650
human     ---R-----------------------------AG---------T----
yeast     FAIK--K-L-KTA-HECVKDY-KH-H-Y-CS---EQ-SKYFIDRST--
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FIGURE 1: Alignment of the deduced amino acid sequences of rat and human DPP III and hypothetical protein 01232 (*Saccharomyces cerevisiae*; NBRF-PIR accession number S66749). Amino acid residues in rat DPP III are numbered from the amino-terminal methionine. Residues and gaps in human DPP III and hypothetical protein 01232 that are identical to those of rat DPP III are denoted by dashes and spaces, respectively. Glutamic acids replaced with alanine or aspartic acid, histidines replaced with tyrosine, and the deleted leucine in rat DPP III are denoted by asterisks.

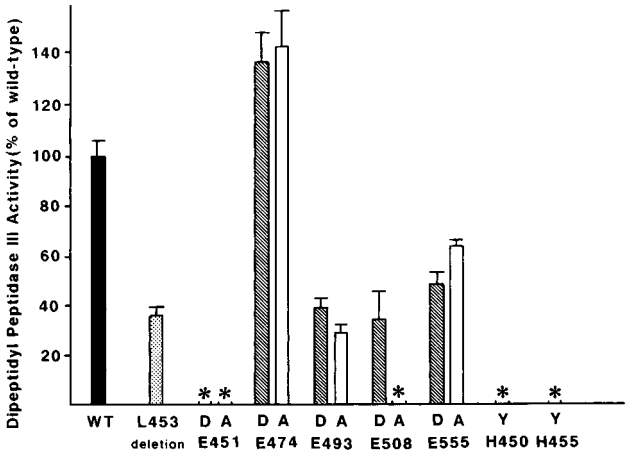

FIGURE 2: Enzyme activity of rat DPP III mutants in the cell suspensions. DPP III activity in suspensions of transfected *E. coli* cells is expressed as a percentage of the wild-type DPP III activity. Measurements are the mean of four or five independent cultures. The mean value for wild-type DPP III corresponds to 72.3 nmol of substrate hydrolyzed min^{-1} (mg of protein)$^{-1}$. The asterisks mean not detectable.

493, and 555) had significant enzyme activity, but neither of the Glu[451] mutants had any activity. In contrast to these mutants, in the case of Glu[508] mutants, only the aspartic acid mutant retained the enzyme activity. Both histidine mutants (H450Y and H455Y) did not have any DPP III activity, just like the Glu[451]-mutated proteins.

The Leu[453]-deleted mutant, which had the same motif as the M1 family (HExxH-18-E), also exhibited the enzyme activity. From these mutants, we purified nine mutated proteins, namely, both mutants of Glu (451, 474, and 508) and His (450 and 455) and the Leu[453]-deleted enzyme.

Expression and Purification of Rat DPP III Mutant Proteins. These mutated proteins were purified to apparent homogeneity (Figure 3), and each behaved like the wild-

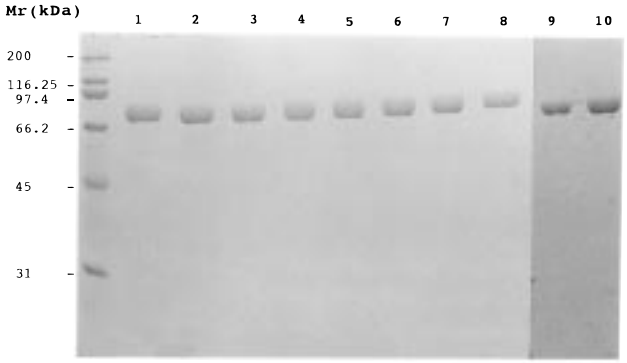

FIGURE 3: SDS−PAGE of wild-type and mutated rat DPP III proteins. Mutated proteins, L[453] deletion (lane 2), E451D (lane 3), E451A (lane 4), E474D (lane 5), E474A (lane 6), E508D (lane 7), E508A (lane 8), H450Y (lane 9), H455Y (lane 10), and wild-type DPP III (lane 1) (5 μg each), were electrophoresed on a standard 10% gel and stained with Coomassie brilliant blue. Molecular mass markers (kilodaltons) are shown (Mr).

Table 2: Kinetic Parameters and Zinc Content of Mutated Proteins

	K_m ($\times 10^{-4}$ M)	k_{cat} (s^{-1})	k_{cat}/K_m ($\times 10^4$)	zinc content (mol/mol of protein)
wild type[a]	3.71 ± 0.56	16.6	4.47	1.03 ± 0.04
L[453] deletion	2.36 ± 0.09	1.75	0.74	0.96 ± 0.04
Glu[451] → Asp	—[b]	—	—	1.03 ± 0.08
Glu[451] → Ala	—	—	—	1.10 ± 0.04
Glu[474] → Asp	2.83 ± 0.24	24.1	8.51	0.93 ± 0.03
Glu[474] → Ala	2.56 ± 0.19	27.4	10.70	0.97 ± 0.05
Glu[508] → Asp	5.85 ± 0.61	1.05	0.17	0.43 ± 0.10
Glu[508] → Ala	—	—	—	0.07 ± 0.05
His[450] → Tyr	—	—	—	0.10 ± 0.02
His[455] → Tyr	—	—	—	0.07 ± 0.03

[a] Quoted from ref *8*. [b] Not detectable.

type enzyme in all purification steps. Although neither the Glu[451], His[450], nor His[455] mutants nor the E508A mutant had any DPP III activity, both Glu[474] mutants, E508D, and the Leu[453]-deleted enzyme were significantly active proteins (Table 2). These data reflected the same pattern that was exhibited for the enzyme activity in the cell suspensions (Figure 2). The binding affinity of these enzymes was almost on the same order, but the specificity constant was different. The k_{cat} values of the Leu[453]-deleted mutant and E508D mutant decreased to 10 and 6% of that of the wild-type enzyme, respectively, but both mutants of Glu[474] had a specificity constant that was about 150% higher than that of the wild-type enzyme.

The Leu[453]-deleted enzyme retained a characteristic feature of dipeptidyl peptidase with regard to the substrate specificity and had the same pH optimum as the wild-type enzyme. Mono amino acid-, Gly-Phe-, Gly-Pro-, and Bz-Arg-NAs were not hydrolyzed at all, even if 100 times more of the mutant enzyme protein and reaction times longer than those used for the assay with Arg-Arg-NA as the substrate were employed. This mutant also hydrolyzed Lys-Ala-NA, and the activity with this substrate was about 20% of that observed with Arg-Arg-NA at physiological pH (7.4).

The zinc content of all mutants except Glu[508], His[450], and His[455] mutants was 1 mol of zinc per mole of protein. In the case of the Glu[508] mutant, the enzyme activity of the alanine mutant was completely abolished, and the zinc content of the protein was also at the background level. Both mutants (H450Y and H455Y) were the same as the E508A mutant

in terms of enzyme activity and zinc content. On the other hand, the zinc content of the aspartic acid mutant (E508D) was 0.43 ± 0.10 mol per mole of protein.

DISCUSSION

It is generally known that secondary structures for metalloproteases containing HExxH (zincins) show the motif in a helix. The region around the HELLGH motif of rat DPP III also was situated in a helix according to the protein secondary structure predicted by a Macintosh Vector analysis system (data not shown). It is evident that Glu[451] constitutes the active site of rat DPP III because mutants of Glu[451] had the same zinc contents as the wild-type enzyme, but the activity of them was completely abolished.

In the case of gluzincins, the third ligand is a remote glutamic acid, which is located toward the C-terminus from the second histidine residue of the HExxH motif. Glu[474] corresponds to the third ligand in the case of zinc aminopeptidases classified into the family M1 of the clan MA (*3*), whose members include aminopeptidase A (*13*), leukotriene A_4 hydrolase (*14*), and aminopeptidase B (*15*). However, both mutants of Glu[474] had the same zinc content as, and also the full activity of, the wild-type enzyme. So, Glu[474] does not take part in the enzyme activity or zinc binding. It is noteworthy that mutated protein E508D possessed obviously some activity but that E508A did not. Moreover, with regard to the zinc content of both mutated proteins, Glu[508] is probably one of the zinc ligands in rat DPP III.

It is not altogether inconceivable that the HELLGH motif of rat DPP III was produced in the evolutionary process by mutation. Therefore, we purified and characterized the Leu[453]-deleted mutant enzyme (Table 2). The result did not measure up to our expectation. This enzyme had the same pH optimum, substrate specificity, and inhibitory profiles (including the fact that *o*-phenanthroline inhibited it but not the 1,4- and 1,7-derivatives) as the wild-type enzyme (data not shown). However, the specificity constant of the deletion enzyme was about 10% of that of the wild-type enzyme, though its binding affinity was on the same order. Therefore, it is significant for the efficiency of the enzyme reaction that the number of amino acid residues between Glu[451] and His[455] is three rather than two.

In the case of zincins, the first two ligands are two histidine residues located within the HExxH motif. In the case of rat DPP III, if Glu[451] acts as a catalytic base, both histidine residues (His[450] and His[455]) correspond to the zinc ligands. We also obtained the mutated cDNAs of His[450] and His[455] (H450Y and H455Y) and expressed them in *E. coli* independently. They did not have any DPP III activity, just like Glu[451]-mutated proteins. The zinc contents of these mutants as determined by the same purification methods were at the background level, just like that of E508A mutant protein.

This HELLGH motif exists in three kinds of monooxygenases (tyrosine, phenylalanine, and tryptophan hydroxylases) in almost all species, as determined by a search of the NBRF-PIR protein sequence database. These enzymes had in common the HExxGH motif, which was nearly the same as that in rat DPP III (HELLGH). In the case of the HELLGH motif in phenylalanine hydroxylase, two histidine residues and a remote glutamic acid (His[285], His[290], and Glu[330]) were identified as ligands binding to the active site iron by site-directed mutagenesis (*16*) and X-ray crystallography (*17*). Therefore, we analyzed the iron content of the rat DPP III by the same atomic absorption spectrometry, but it contained 0.04 ± 0.004 mol of iron per mole of enzyme.

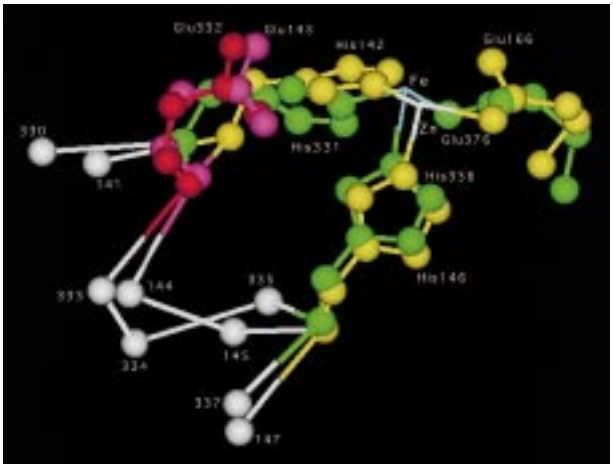

FIGURE 4: Superposition of the zinc ions in thermolysine onto the active site in tyrosine hydroxylase. In thermolysine, the yellow residues are His[142], His[146], and Glu[166] and the pink residue is Glu[143]. In tyrosine hydroxylase, the green residues are His[331], His[336], and Glu[376] and the red residue is Glu[332]. This figure was generated from PDB files 1HYT (*18*) and 1TOH (*19*).

In Table 2, the deletion of Leu[453] in the HELLGH motif of DPP III barely influenced the enzyme activity and the zinc content. To understand why the deletion of Leu[453] has little influence, the active site residues identified by crystallographic analysis in thermolysine, which has the HExxH motif, are superimposed onto those in tyrosine hydroxylase, which is the iron enzyme and has the HExxxH motif. To superimpose the zinc ions in thermolysine onto the active site in tyrosine hydroxylase, the minimization of the distances between Cα atoms of the residues (His[142], Glu[143], Thr[145], and His[146]) in thermolysine and those (His[331], Glu[332], Gly[335], and His[336]) in tyrosine hydroxylase was carried out using the least-squares methods in the Protein Advisor Program. The superposition of the residues is shown in Figure 4. The zinc binding residues in thermolysine were located in almost the same positions as the iron binding residues in tyrosine hydroxylase. Glu[143], which is involved in the enzyme activity of thermolysine, was also located in the same position of Glu[332] in tyrosine hydroxylase, and the distance between the zinc and the iron was 1.14 Å. The bond angles (88°) of the iron coordination geometry in tyrosine hydroxylase were slightly smaller than those (100°) of the zinc in theromolysine. However, it is known that in metalloderivatives (*20*) of bovine carboxypeptidase A, the bond angles in the metal binding sites are slightly scattered with the replacement of the metal ions. Despite the changing of the motif of the zinc binding site (HExxH) in thermolysine to that of the iron binding site (HExxxH), the arrangements of the residues in both active sites are very similar. Therefore, the deletion of Leu[453] in the HELLGH motif of rat DPP III may not influence the arrangement of the residues in the active site.

The data from this study have shown that Glu[451] is a crucial residue for the DPP III activity and His[450], His[455], and Glu[508] seem to be the zinc ligands. However, the answer should come following X-ray crystallographic analysis of this novel zinc metalloexopeptidase.

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