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Thimet oligopeptidase: site-directed mutagenesis disproves previous assumptions about the nature of the catalytic site

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Abstract Zinc metallopeptidases that contain the His-Glu-Xaa-Xaa-His (HEXXH) motif generally have a third ligand of the metal ion that may be either a Glu residue (in clan MA) or a His residue (in clan MB) (Rawlings and Barrett (1995) Methods Enzymol. 248, 183–228). Thimet oligopeptidase has not yet been assigned to either clan, and both Glu and His residues have been proposed as the third ligand. We mutated candidate ligand residues in the recombinant enzyme and identified Glu, His and Asp residues that are important for catalytic activity and/or stability of the protein. However, neither of the Glu and His residues close to the HEXXH motif that have previously been suggested to be ligands is required for the binding of zinc. We conclude that thimet oligopeptidase is not a member of clan MA or clan MB and it is likely that the enzyme possesses a catalytic site and protein fold different from those identified in any metallopeptidase to date. The definitive identification of the third zinc ligand may well require the determination of the crystallographic structure of thimet oligopeptidase or one of its homologues.

Key words: Thimet oligopeptidase; Metallopeptidase family M3; Site-directed mutagenesis; Zinc ligand

1. Introduction

Thimet oligopeptidase (EC 3.4.24.15) is a cytosolic zinc peptidase that is widely distributed in cells of eukaryotic organisms (reviewed in [1]). The enzyme hydrolyzes peptides in the size range 5–17 amino acid residues [2,3]. Many reports have suggested that natural substrates of the enzyme are biologically active peptides including enkephalin precursors, bradykinin, luliberin and neurotensin, but it seems equally possible that the major substrates are oligopeptides produced inside the cell in the course of protein turnover. These would include peptides generated by the proteasome, some of which become antigenic when presented in the context of an MHC I complex [4]. The restriction of the activity of thimet oligopeptidase to substrates containing not more than 17 amino acid residues may be an adaptation that prevents it from causing proteolytic damage in the cytosol where it seems to exist in a fully active state with no natural inhibitor.

The thimet oligopeptidase family of metallopeptidases (M3) also contains neurolysin (EC 3.4.24.16), saccharolysin (EC 3.4.24.57), mitochondrial intermediate peptidase (EC 3.4.24.59) and bacterial peptidases such as oligopeptidase A (EC 3.4.24.70) and peptidyl-dipeptidase Dcp (EC 3.4.15.5) [5,6]. These enzymes perform important functions in both prokaryotic and eukaryotic organisms, and it is desirable that their enzymology is well understood. However, nothing is known directly of the structure of the catalytic site as no crystallographic structure is yet available for any member of the family. Conserved throughout the sequences is the motif His-Glu-Xaa-Xaa-His (HEXXH) (Fig. 1). The HEXXH motif is found in many other families of zinc metallopeptidases also, and most of these can be placed in one of two clans. A clan is a group of families of peptidases that are distantly related and thus share similar protein folds, and the clans into which the HEXXH metallopeptidases are placed are termed MA and MB [7]. In the peptidases of both clans the His residues of the HEXXH motif provide two of the amino acid side chains that coordinate the catalytic zinc atom, but a third ligand is required and the nature of this distinguishes clans MA and MB. In clan MA, example peptidases from which are thermolysin (EC 3.4.24.27) and neprilysin (EC 3.4.24.11), the third ligand is a Glu residue, and it is located 18 to 72 residues C-terminal to the HEXXH motif. In contrast, in clan MB containing astacin (EC 3.4.24.21) and stromelysin (EC 3.4.24.17), the third ligand is a His (or very occasionally Asp), and with few exceptions it is much closer to the HEXXH motif, being the third His in the motif 'HEXXHXXGXXH' [7,8].

Much is known about the catalytic sites of peptidases in clans MA and MB, including many crystallographic structures, so it would be very helpful to be able to link the thimet oligopeptidase family to one of these clans. This has been attempted in the past, with links to both clans being postulated. Thus thimet oligopeptidase has been described as 'thermolysin-like' [9] because it contains a residue Glu-501 that is 25 amino acids C-terminal to the HEXXH motif (see Fig. 1), and in support of this, site-directed mutagenesis of the residue corresponding to Glu-501 in mitochondrial intermediate peptidase inactivated the enzyme [10]. An alternative proposal about the relationships of the family has been made by the curators of the SWISSPROT database (Release 36). The annotations to the sequence records for thimet oligopeptidase (e.g. P24155) and mitochondrial intermediate peptidase (e.g. P51980) indicate that His-479 is a catalytic residue, presumably on the basis of similarity to the motif 'HEXXHXXGXXH' that occurs in clan MB (see above).

The purpose of the present study was to re-examine the question of the third zinc ligand in thimet oligopeptidase by mutagenesis of candidate residues including the two that have been proposed, His-479 and Glu-501. All zinc ligands known from peptidases containing the HEXXH motif are Glu, His or Asp, so it was reasoned that the third ligand would probably be a residue of one of these amino acids that is conserved in

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Abbreviations: DTT, dithiothreitol; IPTG, isopropyl-β-D-thiogalactopyranoside; QF02, 7-methoxycoumarin-3-carboxylyl-Pro-Leu-Gly-Pro-Lys (2,4-dinitrophenyl)

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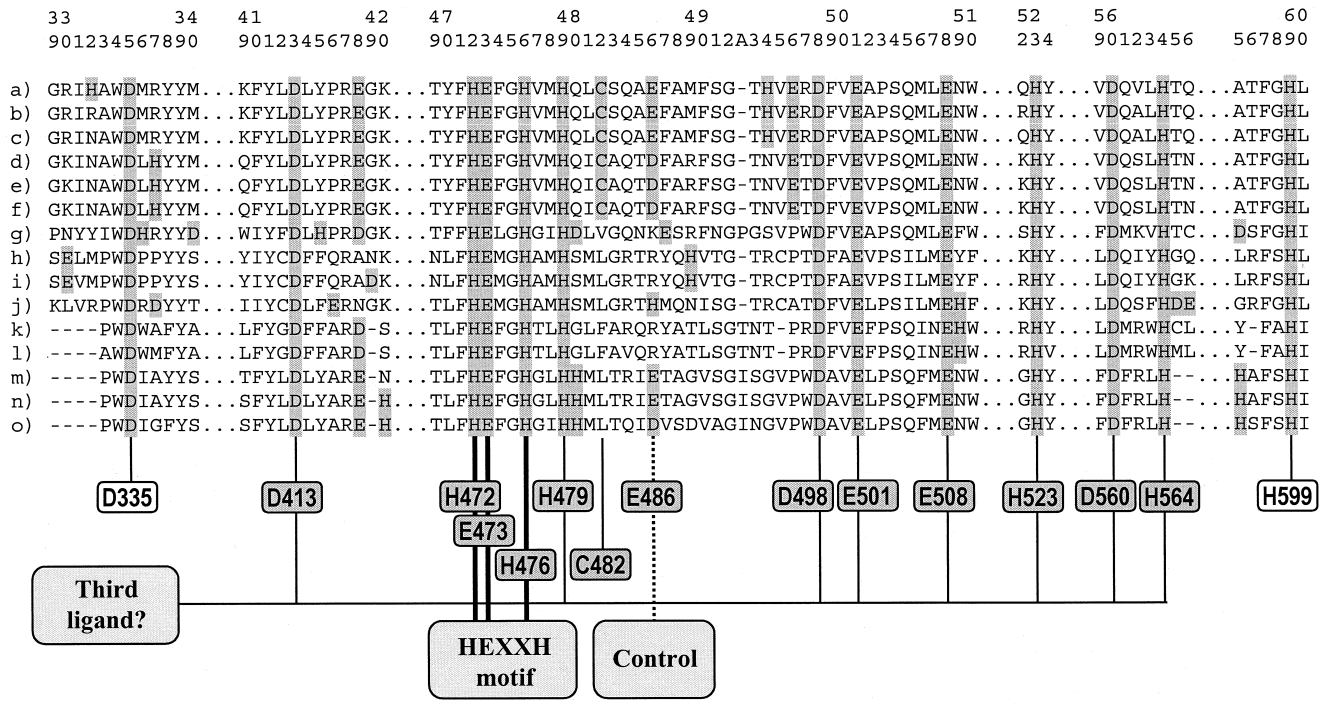

Fig. 1. Selection of targets for mutagenesis in recombinant rat thimet oligopeptidase. The partial alignment of sequences of a selection of peptidases from family M3 showing all of the residues of Asp, Glu and His that are conserved throughout all of these sequences. The alignment is numbered according to rat thimet oligopeptidase, and the same numbering scheme is applied to amino acids in the other members of the family throughout the present paper. All residues of Asp, Glu and His are highlighted (gray background) as is Cys-482. The conserved residues are labeled in the boxes below, as are also Glu-486 that was used as a control and Cys-482. The labels for the residues that were subjected to mutagenesis are filled in gray. The sequences are those of (a) rat thimet oligopeptidase, (b) human thimet oligopeptidase, (c) pig thimet oligopeptidase, (d) pig neurolysin, (e) rat neurolysin, (f) rabbit neurolysin, (g) saccharolysin, (h) rat mitochondrial intermediate peptidase, (i) human mitochondrial intermediate peptidase, (j) *Saccharomyces cerevisiae* mitochondrial intermediate peptidase, (k) *Escherichia coli* peptidyl-dipeptidase Dcp, (l) *Salmonella typhimurium* peptidyl-dipeptidase Dcp, (m) *Escherichia coli* oligopeptidase A, (n) *Salmonella typhimurium* oligopeptidase A and (o) *Haemophilus influenzae* oligopeptidase A.

all active peptidases of family M3. The potential zinc ligands were selected as shown in Fig. 1.

2. Materials and methods

2.1. Materials

cDNA c44 for thimet oligopeptidases described in [11] had been given earlier by Dr. J.L. Roberts, and the expression construct pEXTOP was produced in our laboratory [12]. The expression vector pT7-7 for the prokaryotic system [13] was a gift of Dr. P. Caffrey (Department of Biochemistry, University of Cambridge). *Escherichia coli* strains were DH5α (Life Technologies) and BL21(DE3)pLysS (Stratagene). Culture media 2TY and Terrific Broth (TB) were prepared as described [14].

Affinity-purified polyclonal antibody to rat thimet oligopeptidase was as previously [15]. The substrate 7-methoxycoumarin-3-carboxyl-yl-Pro-Leu-Gly-Pro-Lys (2,4-dinitrophenyl) (QF02) was purchased from Dr. C.G. Knight (Department of Biochemistry, University of Cambridge) or Calbiochem/Novabiochem. The isotope $^{65}ZnCl_2$ (specific activity 37 MBq/mg zinc) was purchased from Amersham Life Science Products.

2.2. Mutagenesis and vector construction of thimet oligopeptidase mutants

Mutagenesis was performed by PCR site-directed mutagenesis generally as described by Picard et al. [16] but with modifications. Mutagenic primers shown in Table 1 were based on the targets of interest in the thimet oligopeptidase sequence. Polymerase *Pfu* was used for all PCR reactions with a typical run of 95°C×1 min, 48°C×1 min, 72°C×2 min for 20 cycles. PCR was first performed using a mutagenic primer, the downstream primer of thimet oligopeptidase (5′-GCGTCCTAGGCACCAGCGAGGTGGCCAAAAGTGGCTGGC-3′), and the cDNA c44 as the template to generate a megaprimer. The megaprimer and the upstream primer (5′-CCGCGAATTCCA-GAACCTGCTGAAGGAGTACTTCCC-3′) were used for the second run of PCR, with c44 as the template, to generate an 870 bp product containing the mutated nucleotide(s). This product was purified from the agarose gel by use of the Gene Clean kit (BIO 101) and double digested with *Mlu*I and *Kpn*I. The corresponding sequence was removed from wild-type pEXTOP by treatment with the same enzymes to generate pEXTOP-(*Mlu*I/*Kpn*I). The digested, mutated

Table 1
Mutagenic primers

Mutants	Primers
D413A	GTTCTACCTCG**C**CCTGTACC
H472R	CCTACTTCC**G**CGAGTTCGGGC
E473D	ACTTCCAC**GA**CTTCGGGCACG
H476R	GAGTTCGGGC**G**CGTCATGCACC
H479D	CACGTCATG**GA**CCAGCTCTGC
H479R	CGTCATGC**G**CCA**A**CTCTGCTCACAGG
C482A	GCACCAGCTC**GC**CTCACAGGC
C482S	CACCAGCTC**A**GCTCACAGGC
E486D	GCTCACAGGCAGA**C**TTTGCTATG
D498A	GTGGAGCGGG**C**ATTTGTGGAG
E501A	GGGACTTTGTGG**C**GGCACCGTC
E501Q	GGGACTTTGTG**CA**GGCACCG
E508Q	CACAGATGCTG**CA**GAACTGG
H523Q	GTCCCAGC**AG**TACCGCACAGG
D560A	CCAAGGTAG**C**TCAGGTCCTG
H564A	GGTCCTG**GC**CACACCGACAG
H564R	CAGGTCCTGC**G**CACACAGACAG

Codons for the mutated residues are underlined, and the bolded characters represent the base changes introduced. Nucleotides CAA (double underlined) in H479R encode a silent mutation.

PCR product was ligated into pEXTOP-(*Mlu*I/*Kpn*I) to produce the pEXTOP mutants. Plasmid DNA of the pEXTOP mutants was propagated in *E. coli* strain DH5α. The sequences of all mutated constructs were confirmed by DNA sequencing [17].

2.3. Expression of recombinant wild-type and mutant forms of rat thimet oligopeptidase

The recombinant wild-type and mutant thimet oligopeptidases from the pT7-7 vector were expressed in *E. coli* strain BL21(DE3)pLysS [13]. Briefly, a single colony of each transformant was picked from 2TY/agar plates containing ampicillin (100 μg/ml) and chloramphenicol (33 μg/ml) and grown in 2 ml of 2TY medium with ampicillin and chloramphenicol. After 6 h the culture became turbid and PCR using thimet oligopeptidase-specific primers was performed to confirm that the cells were bearing plasmid pEXTOP (wild-type or mutants). The cells were centrifuged, resuspended in 2 ml of fresh Terrific Broth (TB) containing appropriate antibiotics, and stored at 4°C overnight. The cell suspension (0.5 ml) was used to inoculate 50 ml of TB and grown until the OD_{600} reached 0.6. Protein expression was induced by addition of IPTG to 0.4 mM, and cells were harvested 4 h later. Cells were suspended in 50 mM Tris-HCl, pH 7.8, containing 0.1 mm $ZnCl_2$, and 0.05% BRIJ 35 and lysed by three cycles of freeze/thaw. Nucleic acids were removed by precipitation with protamine sulfate (2 mg/ml final concentration) and centrifugation. Expression of the recombinant thimet oligopeptidase protein in the cell lysate was analyzed by immunoblotting developed with polyclonal anti-rat thimet oligopeptidase antibody.

Recombinant wild-type and E508Q mutant forms of thimet oligopeptidase were expressed on a large scale and purified to homogeneity as described previously [12].

2.4. Detection of catalytic and zinc-binding activities

The enzymatic activities of wild-type and mutant thimet oligopeptidase preparations were measured fluorometrically by use of the quenched fluorescence substrate QF02 [1].

For determination of binding of zinc, recombinant thimet oligopeptidase was partially purified from bacterial lysates by ion exchange chromatography with the Pharmacia FLPC system. The Mono Q column (HR5/5) was equilibrated with 20 mM Tris-HCl, pH 7.8, 0.05% BRIJ 35, 0.1 mM $ZnCl_2$. The column was washed with the starting buffer and bound proteins were eluted with a gradient of 0–300 mM NaCl in the buffer over 25 min. Fractions enriched in thimet oligopeptidase were identified either by fluorometric assay or by Western immunoblotting and were subjected to zinc-binding analysis as described below.

Zinc binding was determined as described [18]. Samples were partially purified preparations as described above together with purified recombinant thimet oligopeptidase expressed from the wild-type and E508Q constructs and thermolysin, a known zinc metallopeptidase, as a positive control. The samples were run in SDS/PAGE and the proteins were transblotted to nitrocellulose [19]. The nitrocellulose was incubated in 10 mM Tris-HCl pH 7.5 for 1 h, followed by a second 1 h incubation in 40 ml of 10 mM Tris-HCl, pH 7.5, containing 0.1 M KCl and 50 μCi $^{65}ZnCl_2$. The blot was then washed in 10 mm Tris-HCl, pH 7.5, 0.1 M KCl (200 ml) five times, 15 min each. The blot was dried and exposed to Fuji RX film at −70°C for 48–72 h and the autoradiograph was processed for detection of bound ^{65}Zn. Two identical blots were prepared for each sample, one being used for the zinc-binding assay and the other for immunodetection of thimet oligopeptidase to correlate the protein with the zinc-binding activity.

3. Results and discussion

3.1. Targets for site-directed mutagenesis of thimet oligopeptidase

When the sequence of thimet oligopeptidase was aligned with those of several other members of peptidase family M3, a conserved HEXXH motif was evident (residues 472–476) together with a number of conserved Glu, Asp and His residues (Fig. 1). Accordingly Asp-413, His-472, Glu-473, His-476, His-479, Asp-498, Glu-501, Glu-508, His-523, Asp-560 and His-564 were selected as targets for mutagenesis. Asp-335 and His-599 were not examined because they are much further from the HEXXH motif than any third ligand so far known. Glu-486 is not conserved throughout the family but it was selected as an additional control target. Cys-482 was also mutated in the light of the proposal that this residue might be responsible for the thiol dependence of the enzyme [11]. Replacement amino acids were selected in the light of the secondary structure of each mutant predicted by the PEPTIDESTRUCTURE program of the GCG package [20]. Only for E508Q and H479D was any significant change in structure from the wild-type predicted.

3.2. Protein expression and enzymatic activity of thimet oligopeptidase mutants

Constructs for mutants D413A, H472R, E473D, H476R, H479D, H479R, C482A, C482S, E486D, D498A, E501A, E501Q, E508Q, H523Q, D560A, H564A and H564R were generated and expressed as described in Section 2. Fig. 2A shows the Western immunoblot of the lysates from bacteria expressing the wild-type and mutant enzymes. The wild-type and all mutant recombinant proteins appeared as bands with the predicted mass of 78 kDa together with some degraded fragments also recognized by the antibodies. The controls for the bacterial strain and the vector produced no immunoreactive material. The mutant proteins E473D, H479D, H479R and H564R were less stable than the others during storage at 4°C.

For the determination of the activity of the mutated forms of thimet oligopeptidase at least two clones were used for each

Table 2
Activities of mutant thimet oligopeptidases

Clone	Relative activity (%)
Controls	
BL21(DE3)pLysS	0
PT7-7 vector	0
Wild-type	100
'*HEXXH*'	
H472R	0
E473D[a]	0
H476R	0
Histidine	
H479D[a]	2
H479R[a]	2
H523Q	60
H564R[a]	0
H564A	40
Glutamate	
E486D	100
E501A	0
E501Q	1
E508Q	250
Aspartate	
D413A	0
D498A	0
D560A	0
Cysteine	
C482A	100
C482S	50

Cells of *E. coli* strain BL21(DE3)pLysS carrying the wild-type (pEXTOP) and mutant constructs were induced for protein expression, and lysates of the cells were assayed for enzymatic activity. The activity from the cell lysate of each mutant was compared to that of the wild-type enzyme, which was assigned an arbitrary value of 100. At least two clones were used for each mutant and activities were assayed on at least three separate occasions. The figures shown are means of the results.

[a]Unstable during storage.

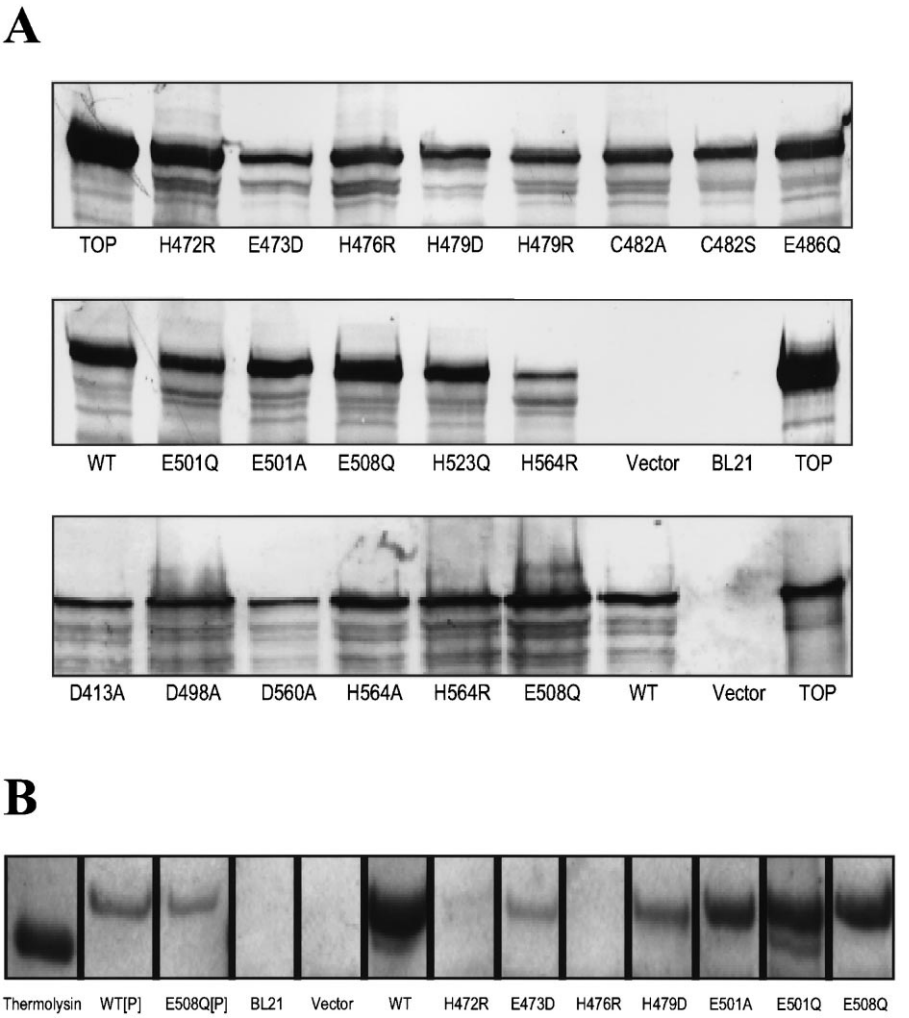

Fig. 2. Expression of mutant forms of thimet oligopeptidase and their binding of zinc. A: The recombinant forms of the enzyme were expressed as described in Section 2, and cell lysates (~60 μg of total protein) were subjected to SDS/PAGE and Western immunoblot analysis. Control samples included the purified recombinant thimet oligopeptidase (TOP), bacterial strain (BL21) and bacteria transfected with the vector alone (Vector). All mutants expressed the intact protein of 78 kDa identical to that of the wild-type (WT). The expressed protein from mutant H564R was unstable and became partially degraded during storage. B: Analysis of ^{65}Zn binding to wild-type and mutant thimet oligopeptidase. Samples were run in SDS/PAGE and transblotted to nitrocellulose paper. The blot was incubated in $^{65}ZnCl_2$ solution and processed for autoradiography as described in Section 2. Control samples were thermolysin (10 μg), purified wild-type and E508Q mutant thimet oligopeptidase (WT[P], E508Q[P], each 5 μg) and lysates from the bacterial strain (BL21) and bacteria transfected with vector alone (Vector). The experimental samples (40 μg protein) were partially purified preparations from lysates of bacteria expressing the wild-type enzyme and the mutated forms as labeled.

mutant and activities were assayed on at least three separate occasions. The mean relative activities are shown in Table 2. The mutants H472R, E473D, and H476R were catalytically inactive, consistent with the expectation that His-472 and His-476 in the HEXXH motif are zinc ligands and Glu-473 is catalytic [21].

The additional mutants of histidine all showed low to moderate activity. When His-479 was replaced by Asp or Arg activity became very low, but was nevertheless detectable. Both proteins were unstable. Mutant H523Q was active at 60% of the control level. The mutant H564R was totally inactive and unstable, but H564A retained 40% of the activity of the wild-type. It appears that His-479 and His-564 are important for the proper folding of thimet oligopeptidase, but none of the conserved residues His-479, His-523 and His-564 is essential for catalytic activity. His-479 is probably too close to the HEXXH motif to serve as the third ligand (since this would require an excessively sharp turn in the polypeptide chain between His-476 and His-479), and indeed the H479R mutant of yeast mitochondrial intermediate peptidase was previously found to be partially active [10].

The two conserved Glu residues at positions 501 and 508 were mutated, as was the non-conserved Glu-486. Not surprisingly, the E486D mutant was fully active, and served simply as a control. E501A was totally inactive, but values for E501Q were consistently above zero. Taken together with the previous report that a mutant of mitochondrial intermediate peptidase equivalent to E501D was inactive [10], these data indicated that Glu-501 might contribute to the catalytic activity of thimet oligopeptidase. In striking contrast, mutant E508Q was superactivated to 250%. The E508D mutant of yeast mitochondrial intermediate peptidase had been found to be fully active [10].

Replacement of Asp-413, Asp-498 and Asp-560 with Ala

resulted in a total loss of activity in each case, even though the mutant enzymes were expressed in intact form. The D498E mutant of mitochondrial intermediate peptidase has been reported to be active however.

Both the C482A and C482S mutants were enzymatically active. Moreover, both showed normal thiol dependence in the form of activation by dithiothreitol and inhibition by *N*-phenylmaleimide (data not shown). This supports the conclusion of Shrimpton et al. [9] that Cys-482 is not responsible for the thiol dependence of thimet oligopeptidase despite its proximity to the catalytic site.

3.3. Zinc-binding activity of the mutants

Partially purified protein of mutants H472R, E473D, H476R, H479D, E501A, E501Q, and E508Q was evaluated for zinc-binding activity with ^{65}Zn, in comparison to the wild-type enzyme and thermolysin. The autoradiograph (Fig. 2B) shows the expected binding of zinc by wild-type thimet oligopeptidase and the active E508Q mutant. Also seen is loss of zinc binding in mutants H472R and H476R, consistent with the expectation that these two residues are the zinc ligands in the HEXXH motif. The inactive mutant E473D bound zinc as expected since it is known for other HEXXH metallopeptidases that this residue is involved in catalysis but not zinc binding [21]. However, mutants H479D, E501A and E501Q all retained the ability to bind zinc, indicating that although important for the catalytic activity of the enzyme, neither His-479 nor Glu-501 is the third ligand of the metal ion as has been suggested previously.

4. Conclusions

Both Glu-501 and His-479 have previously been suggested to be the third ligand of zinc in the thimet oligopeptidase family of metallopeptidase, which might have allowed the family to be assigned to clan MA or clan MB respectively. However we have found that mutants in both residues retain trace levels of catalytic activity, and more importantly, still bind ^{65}Zn. We therefore think it unlikely that either is in fact the expected ligand. Several of the other residues of histidine, glutamate and aspartate that were replaced led to loss of catalytic activity, but some of these effects may well have been due to perturbation of the structure of the molecule. We conclude that the metallopeptidase of family M3 apparently do not belong in clan MA or clan MB, and may well possess a quite distinctive catalytic site geometry and thus represent a unique clan of metallopeptidases. The elucidation of the details of this may well await the determination of a crystallographic structure.

References

[1] Barrett, A.J., Brown, M.A., Dando, P.M., Knight, C.G., McKie, N., Rawlings, N.D. and Serizawa, A. (1995) Methods Enzymol. 248, 529–556.
[2] Dando, P.M., Brown, M.A. and Barrett, A.J. (1993) Biochem. J. 294, 451–457.
[3] Knight, C.G., Dando, P.M. and Barrett, A.J. (1995) Biochem. J. 308, 145–150.
[4] Pamer, E. and Cresswell, P. (1998) Annu. Rev. Immunol. 16, 323–358.
[5] Rawlings, N.D. and Barrett, A.J. (1995) Methods Enzymol. 248, 183–228.
[6] Rawlings, N.D. (1998) in: A.J. Barrett, N.D. Rawlings and J.F. Woessner (Eds.), Handbook of Proteolytic Enzymes, Academic Press, London, pp. 1106–1107.
[7] Rawlings, N.D. (1998) in: A.J. Barrett, N.D. Rawlings and J.F. Woessner (Eds.), Handbook of Proteolytic Enzymes, Academic Press, London, pp. 989–991.
[8] Bode, W., Grams, F., Reinemer, P., Gomis-Rüth, F.X., Baumann, U., McKay, D.B. and Stöcker, W. (1996) Zool. Anal. Complex Syst. 99, 237–246.
[9] Shrimpton, C.N., Glucksman, M.J., Lew, R.A., Tullai, J.W., Margulies, E.H., Roberts, J.L. and Smith, A.I. (1997) J. Biol. Chem. 272, 17395–17399.
[10] Chew, A., Rollins, R.A., Sakati, W.R. and Isaya, G. (1996) Biochem. Biophys. Res. Commun. 226, 822–829.
[11] Pierotti, A., Dong, K.-W., Glucksman, M.J., Orlowski, M. and Roberts, J.L. (1990) Biochemistry 29, 10323–10329.
[12] McKie, N., Dando, P.M., Brown, M.A. and Barrett, A.J. (1995) Biochem. J. 309, 203–207.
[13] Studier, F.W., Rosenberg, A.H., Dunn, J.J. and Dubendorff, J.W. (1991) Methods Enzymol. 185, 60–89.
[14] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[15] Chen, J.-M., Changco, A., Brown, M.A. and Barrett, A.J. (1995) Exp. Cell Res. 216, 80–85.
[16] Picard, V., Ersdal-Badju, E., Lu, A. and Bock, S.C. (1994) Nucleic Acids Res. 22, 2587–2591.
[17] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[18] Mazen, A., Gradwohl, G. and de Murcia, G. (1988) Anal. Biochem. 172, 39–42.
[19] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[20] Genetics Computer Group (1994) Program Manual for the Wisconsin Package, Version 8, September 1994, University of Madison, Wisconsin.
[21] Cha, J. and Auld, D.S. (1997) Biochemistry 36, 16019–16024.