

# Identification by site-directed mutagenesis of three essential histidine residues in membrane dipeptidase, a novel mammalian zinc peptidase

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Membrane dipeptidase (EC 3.4.13.19) is a plasma membrane zinc peptidase that is involved in the renal metabolism of glutathione and its conjugates, such as leukotriene D<sub>4</sub>. The enzyme lacks the classical signatures of other zinc-dependent hydrolases and shows no homology with any other mammalian protein. We have used site-directed mutagenesis to explore the roles of five histidine residues in pig membrane dipeptidase that are conserved among mammalian species. When expressed in COS-1 cells, the mutants H49K and H128L exhibited a specific activity and  $K_m$  for the substrate Gly-D-Phe comparable with those of the wild-type enzyme. However, the mutants H20L, H152L and H198K were inactive, but were expressed at the cell surface at equivalent levels to the wild-type, as assessed by immunoblotting and immunofluorescence. These three mutants

were compared with regard to their ability to bind to the competitive inhibitor cilastatin, which binds with equal efficacy to native and EDTA-treated pig kidney membrane dipeptidase. Expressed wild-type enzyme and mutants H20L and H198K were efficiently bound by cilastatin-Sepharose, but H152L failed to bind. Thus His-152 appears to be involved in the binding of substrate or inhibitor, whereas His-20 and His-198 appear to be involved in catalysis. Membrane dipeptidase shares some similarity with a dipeptidase recently cloned from *Acinetobacter calcoaceticus*. In particular, His-20 and His-198 of membrane dipeptidase are conserved in the bacterial enzyme, as are Glu-125 and His-219, previously shown to be required for catalytic activity.

## INTRODUCTION

Membrane dipeptidase (MDP; renal or microsomal dipeptidase; dehydropeptidase-I; EC 3.4.13.19) is a plasma membrane zinc peptidase that is abundant in the renal brush border, from where it was first purified [1,2]. MDP hydrolyses a wide range of dipeptides, including those with a D-amino acid at the C-terminus, as well as certain dehydropeptides (e.g. glycyl-dehydrophenylalanine) (see [3] for a review). The physiological role of MDP involves the metabolism of glutathione and its conjugates, especially leukotriene D<sub>4</sub> [4]. MDP is unique among mammalian peptidases in also possessing  $\beta$ -lactamase activity towards the carbapenem class of antibiotics [5–7]. The recognition of this activity for MDP led to the development of a specific competitive inhibitor, cilastatin [8], which has proved useful for affinity purification of the enzyme [2,6].

Relatively limited structural information is available for MDP. In particular, its catalytic mechanism that allows both peptidase and  $\beta$ -lactamase activity is not understood. The enzyme is anchored to the plasma membrane by a glycosylphosphatidylinositol (GPI) moiety, and was the first peptidase to be so identified [9]. The complete structure of the GPI anchor on pig MDP and the glycan core structure of the GPI anchor on the human enzyme have recently been reported [10]. MDP has now been cloned from six mammalian species (pig, human, rabbit, rat, mouse and sheep) [11–16], which has revealed that it is highly conserved among species (> 80% identity at the amino acid level), but it displays no significant sequence similarity with any other mammalian proteins. MDP exists as a disulphide-linked homodimer of subunit molecular mass 47 kDa in the pig [17]. The sole cysteine residue involved in its dimerization has recently been identified as Cys-361 by using site-directed mutagenesis, but other cysteine residues do not appear to be involved in enzyme

action [18]. Although MDP is a metalloenzyme with one atom of zinc bound per subunit [19], its amino acid sequence contains none of the recognized zinc peptidase motifs [20,21], and it therefore constitutes a novel zinc peptidase.

Site-directed mutagenesis has established that His-219 [22] and Glu-125 [23] are critical residues that are essential for catalytic activity. We have now employed site-directed mutagenesis of all the remaining conserved histidine residues in order to identify further residues important for enzyme activity. Mutation of His-20, His-152 and His-198 in pig MDP resulted in the complete loss of enzyme activity, although the mutant protein was still expressed at the cell surface at normal levels. Two of these histidines (positions 20 and 198) are found to be conserved in a recently cloned bacterial metallo-dipeptidase (*Acinetobacter calcoaceticus* dipeptidase; ACDP) [24], which shows 46.9% overall similarity with pig MDP.

## EXPERIMENTAL

### Materials

Restriction enzymes and T4 polynucleotide kinase were obtained from New England Biolabs (Hitchin, U.K.). Sequenase kit (version 2.0) and the enhanced chemiluminescence (ECL) Western blotting kit were from Amersham International PLC (Amersham, Bucks., U.K.). [ $\alpha$ -<sup>35</sup>S]dATP (1000 Ci/mmol) was from Du Pont Ltd. (Stevenage, U.K.). Tissue culture media, serum, trypsin-EDTA, penicillin/streptomycin, L-glutamine, transfection reagent (Lipofectamine) and T4 DNA ligase were purchased from Gibco-BRL, Life Technologies Ltd. (Paisley, Scotland, U.K.). Gly-D-Phe, bicinchoninic acid and high-molecular-mass protein standards were obtained from Sigma (Poole, Dorset, U.K.). The mammalian expression vector pEF-

Abbreviations used: ACDP, *Acinetobacter calcoaceticus* dipeptidase; ECL, enhanced chemiluminescence; GPI, glycosylphosphatidylinositol; MDP, membrane dipeptidase; PI-PLC, phosphatidylinositol-specific phospholipase C.

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BOS was generously donated by Dr. Shigekazu Nagata (University of Osaka, Japan) [25]. Cilastatin (MK 0791) was a gift from Merck, Sharp & Dohme Research Laboratories (Rahway, NJ, U.S.A.). *Bacillus thuringiensis* phosphatidylinositol-specific phospholipase C (PI-PLC) was a gift from Dr. M. G. Low (Columbia University, New York, NY, U.S.A.). Cilastatin was attached to CNBr-activated Sepharose 4B as described in [2,6]. All other reagents were of analytical grade.

#### Enzyme expression in transfected COS-1 cells and membrane preparation

Transient expression was performed as described previously [22]. Plasmid DNA was transfected into COS-1 cells by using Lipofectamine reagent, and membranes were isolated from the cells 48 h post-transfection. Cells were washed three times with PBS, scraped and centrifuged (1000 *g*, 10 min). The pellet was resuspended in 50 mM Tris/HCl, pH 7.5, containing 20 mM CaCl<sub>2</sub> and the cells were disrupted by N<sub>2</sub> cavitation. After a brief centrifugation at 1000 *g*, the supernatant was centrifuged at 100000 *g* for 1 h and the resulting membrane pellet was resuspended in 50 mM Hepes buffer, pH 7.5. Membranes were solubilized by incubation with 60 mM *n*-octyl  $\beta$ -D-glucopyranoside for 1 h at 4 °C, followed by centrifugation at 12000 *g* for 10 min to remove insoluble material. The solubilized membranes were stored in aliquots at -20 °C.

#### Site-directed mutagenesis

Mutations were introduced into MDP in the pEF-BOS vector by the method of Kunkel et al. [26]. *Escherichia coli* CJ236 was transformed with the plasmid pEF-MDP. From an ampicillin-resistant colony, single-strand DNA (containing uracil) was isolated using helper phage R408 (with the standard protocol of Boehringer-Mannheim). All the mutagenic primers were designed to be antisense. For construction of the different mutants, the following primers were used: H20L, 5'-AAGGCAGTCAT-TGAGCCCGTCAATGAC-3'; H49K, 5'-TTGGGGATGTTG-GTCTTCGTGTGGGCCAGG-3'; H128L, 5'-TGTCGATGG-AGAGGCCACCCTCCA-3'; H152L, 5'-TGTTGCAGCTGA-GGGTGAGGGTCAT-3'; H198K, 5'-TGGCCACGGACAC-CTTGGCCAAGTCGAT-3'. Mutations were verified by DNA sequencing. Each mutant was then subcloned back to the wild-type plasmid pEF-MDP and the entire region was sequenced. Mutant H20L was restricted by *Sma*I and a fragment of 200 bp was purified. Mutants H152L and H198K were digested using *Sun*I/*Eag*I, resulting in 600 bp fragments. All the purified fragments were subcloned back to the wild-type using the same sites. Mutant H49K and H128L cDNAs were confirmed from three different clones, retransformed and the region around the mutation was again sequenced.

#### Purification and assay of MDP

MDP was purified to apparent homogeneity from pig kidney cortex by solubilization with PI-PLC followed by affinity chromatography on cilastatin-Sepharose [2,27]. For the determination of the enzyme activity of expressed wild-type MDP and its mutants, solubilized COS-1 cell membranes (10  $\mu$ l; 3  $\mu$ g of protein) were incubated in 0.1 M Tris/HCl, pH 8.0, containing 3 mM Gly-D-Phe with or without the inhibitor cilastatin (0.25 mM) in a total volume of 100  $\mu$ l. After incubation for 3 h at 37 °C, samples were heated to 100 °C for 4 min and then centrifuged at 12000 *g* for 10 min at 4 °C. The product, D-Phe, was resolved from the substrate and quantified by HPLC on a

$\mu$ Bondapak C<sub>18</sub> column using a linear gradient of acetonitrile from 4.5 to 30% in 0.08% H<sub>3</sub>PO<sub>4</sub>, pH 2.5, with detection at 214 nm [9].

#### SDS/PAGE and immunoelectrophoretic blot analysis

PAGE was performed as described by Laemmli [28], using a 5% acrylamide stacking gel and a 10% acrylamide separating gel. Proteins were electrophoretically transferred to PVDF membranes (Immobilon P; Millipore) as described by Towbin et al. [29]. The PVDF membranes were then incubated with a polyclonal antibody raised against affinity-purified pig kidney MDP (1:1000 dilution) [2], and bound antibody was detected using the ECL kit. Levels of MDP protein expression were estimated by immunoblot analysis (densitometric scanning of bands; Scanmaster 3; Howtek) using purified pig kidney MDP as standard. The linearity of exposure with protein concentration in the range used was checked routinely.

#### Binding of MDP to cilastatin-Sepharose

Octyl glucoside-solubilized membranes from either the expressed wild-type MDP or the various mutants (7.5  $\mu$ g of protein) were incubated in 10 mM Tris/HCl, pH 7.5, containing 100 mM NaCl (40  $\mu$ l total volume) for 1 h at 37 °C in the presence of 0.1 unit of PI-PLC. After the incubation, samples were mixed with 100  $\mu$ l of cilastatin-Sepharose for 16 h at 4 °C. Purified pig MDP was incubated similarly with cilastatin-Sepharose. Subsequently the Sepharose was washed four times with 50 mM Tris/HCl, pH 7.5, containing 500 mM NaCl. SDS sample buffer was added, and the samples were heated to 100 °C for 5 min and then centrifuged at 3000 *g* for 1 min. The supernatants were then subjected to immunoelectrophoretic blot analysis. To assess whether the expressed mutants had been cleaved to a hydrophilic form by PI-PLC, the samples were incubated at 4 °C for 5 min after PI-PLC treatment in the presence of 1% (w/v) Triton X-114. The samples were then subjected to phase separation at 30 °C for 3 min [30]. The hydrophilic and amphipathic forms were resolved by centrifugation at 3000 *g* through a sucrose cushion, and the hydrophilic form was recovered and subjected to immunoelectrophoretic blot analysis.

#### Immunocytochemistry

COS-1 cells were plated in 24-well plates and transfected with wild-type or mutant MDP cDNAs. Cells were prepared for immunofluorescence 48 h post-transfection as described previously [22]. The cells were washed gently three times at 37 °C with warmed PBS and fixed at room temperature in 4% paraformaldehyde for 20 min. Subsequently the cells were washed three times with Tris-buffered saline and blocked for 30 min at room temperature in the same buffer containing 0.2% gelatin. After an overnight incubation at 4 °C with affinity-purified anti-(pig MDP) antibody (1:500 dilution), the cells were washed and incubated for 30 min with goat anti-rabbit IgG conjugated with fluorescein isothiocyanate (1:50 dilution). After washing three times with Tris-buffered saline, the coverslips were mounted on microscope slides in Vectashield (Vector Laboratories Inc., Peterborough, U.K.) and viewed using a Leitz confocal microscope.

#### Determination of protein concentration

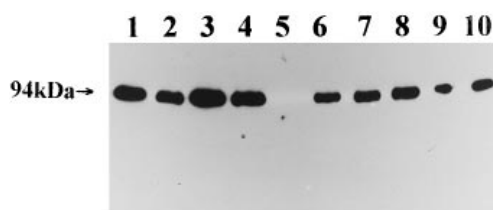
Protein concentrations were determined using the bicinchoninic acid method of Smith et al. [31] modified for use in 96-well microtitre plates [32], with BSA as standard.

## RESULTS

### Characterization of histidine mutants of MDP expressed in COS-1 cells

Site-directed mutagenesis was used to change each of five conserved histidines (residues 20, 49, 128, 152 and 198) in pig MDP to either Leu or Lys. After the mutations had been verified by sequencing, the mutated cDNAs were subcloned back into the pEF-MDP plasmid and expressed in COS-1 cells. An initial analysis of the activity of the various His mutants was conducted on whole cells in 24-well plates. Wild-type enzyme and mutants H49K and H128L were fully active, whereas mutants H20L, H152L and H198K revealed no detectable activity under these conditions (results not shown). All the mutants were then expressed in COS-1 cells, and a detergent-solubilized membrane fraction was prepared from each and subjected to immunoelectrophoretic blot analysis. Wild-type MDP protein and the various mutants exhibited identical mobilities compared with purified pig MDP (Figure 1), although there were small differences in the level of protein expression.

The specific activity of each mutant was determined and



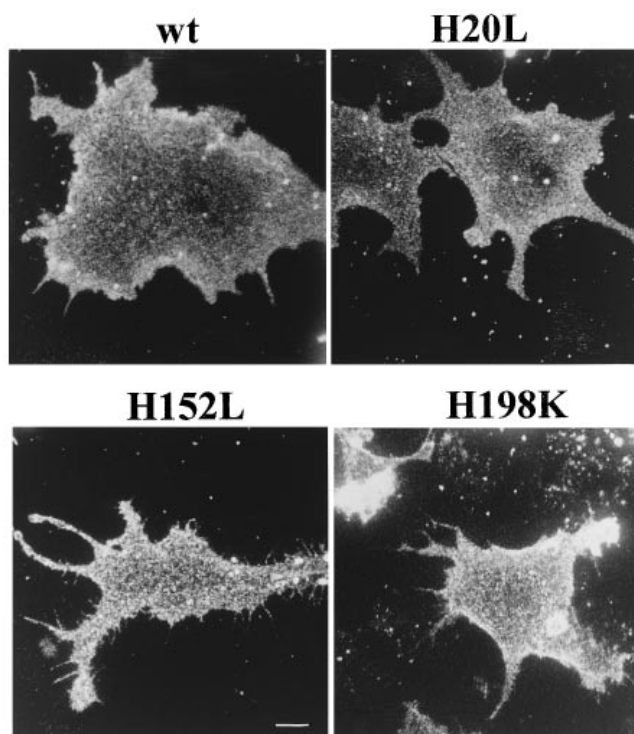
**Figure 1** Immunoelectrophoretic blot of wild-type and histidine mutants of MDP expressed in COS-1 cells

COS-1 cells were transfected with either the wild-type MDP cDNA or the indicated mutant cDNAs (see below). Membranes were prepared from the cells and, after detergent solubilization, 3  $\mu$ g portions of protein were subjected to electrophoresis under non-reducing conditions on an SDS/polyacrylamide gel, as described in the Experimental section. The proteins were then transferred to PVDF membranes and incubated for 16 h at 4 °C with a 1:1000 dilution of the anti-(pig MDP) antibody. The amount of MDP present in the different membrane preparations was quantified by densitometry (Scanmaster 3). Lane 1, 75 ng of purified pig kidney MDP; lane 2, wild-type MDP; lane 3, H49K; lane 4, H128L; lane 5, untransfected COS-1 cells; lane 6, H20L; lane 7, H152L; lane 8, H198K; lane 9, 25 ng of purified pig kidney MDP; lane 10, 50 ng of purified pig kidney MDP.

**Table 1** Properties of recombinant wild-type and histidine mutants of MDP expressed in COS-1 cells

COS-1 cells were transfected with pEF-vector containing either the wild-type MDP cDNA or the indicated mutant cDNAs. Solubilized membranes (3  $\mu$ g of protein) were assayed for 3 h, as described in the Experimental section. Specific activity is presented as cilastatin-sensitive hydrolysis of Gly-D-Phe, and is expressed relative to MDP protein estimated by immunoblot analysis (see the Experimental section). ND, activity not detectable. The results represent means  $\pm$  S.D. of three separate transfections.

Protein	Codon substitution	Specific activity ( $\mu$ mol of D-Phe/min per mg of MDP protein)
Untransfected cells	—	N.D
Wild-type	—	4.20 $\pm$ 0.16
H20L	CAC $\rightarrow$ CTC	ND
H49K	CAC $\rightarrow$ AAG	2.90 $\pm$ 0.20
H128L	CAC $\rightarrow$ CTC	14.70 $\pm$ 0.65
H152L	CAC $\rightarrow$ CTC	ND
H198K	CAC $\rightarrow$ AAG	ND



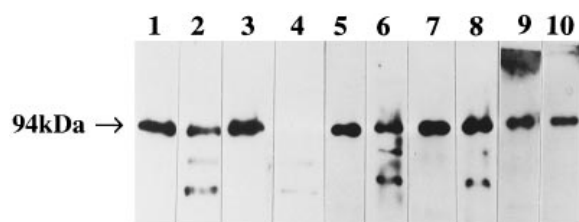
**Figure 2** Immunofluorescence of transfected COS-1 cells for wild-type and mutant MDPs using confocal microscopy

Cells were prepared and viewed as described in the Experimental section. All the micrographs represent a composite image taken at seven focal planes. wt, wild-type; bar = 10  $\mu$ m.

expressed relative to the amount of MDP protein quantified by immunoblot analysis in Figure 1, as described in the Experimental section; the data are summarized in Table 1. Mutants H49K and H128L were expressed at similar levels to the wild-type and were of comparable specific activity, suggesting that these histidine residues are not involved in catalysis. They also exhibited similar  $K_m$  and  $V_{max}$  values (H49K,  $K_m$  = 12.5 mM,  $V_{max}$  = 22.0  $\mu$ mol/min per mg; H128L,  $K_m$  = 6.25 mM,  $V_{max}$  = 68  $\mu$ mol/min per mg) to the wild-type ( $K_m$  = 7.1 mM,  $V_{max}$  = 14.3  $\mu$ mol/min per mg). In contrast, mutants H20L, H152L and H198K, although expressed in amounts comparable with the wild-type (Figure 1), displayed no detectable enzyme activity. These values should be regarded as approximate, since pronounced high-substrate inhibition seen above 10 mM substrate concentration with both the expressed wild-type protein and the active mutants precluded a more detailed kinetic analysis. The focus of the present work is therefore on the inactive mutants.

### Cell-surface expression of the inactive H20L, H152L and H198K mutants

In order to address whether the inactive mutants had failed to be targeted to the plasma membrane, immunofluorescence staining of COS-1 cells expressing either the wild-type enzyme or the non-active mutants was undertaken (Figure 2). All three of the non-active histidine mutants of MDP were seen to be expressed at the cell surface, with a punctate staining and fluorescent intensity similar to those observed for the wild-type.



**Figure 3** Determination of the ability of wild-type and mutant forms of MDP to bind to cilastatin-Sepharose

Aliquots (7.5  $\mu$ g) of membrane protein from COS-1 cells transfected with either the wild-type enzyme or the indicated mutants (see below) were first incubated with PI-PLC for 1 h at 37 °C. After incubation with PI-PLC, the samples were either subjected to phase separation in Triton X-114 or incubated with cilastatin-Sepharose. The aqueous phase after phase separation in Triton X-114 (odd-numbered lanes) and the material bound to the cilastatin-Sepharose (even-numbered lanes) were then electrophoresed under non-reducing conditions, transferred on to PVDF membranes and incubated with the anti-MDP antibody, as described in the Experimental section. The lanes represent: 1 and 2, wild-type MDP; 3 and 4, H152L; 5 and 6, H20L; 7 and 8, H198K; 9 and 10, H219K.

### Interaction of purified pig MDP with cilastatin-Sepharose

The ability of purified pig MDP to bind to cilastatin-Sepharose was compared before and after treatment of the enzyme with EDTA to remove zinc. For these and subsequent experiments with the expressed mutants, the PI-PLC-cleaved form of MDP was used, since the amphipathic (membrane) form binds poorly to the immobilized inhibitor [17]. MDP was incubated with 50 mM EDTA for 30 min and then subjected to gel filtration to remove the EDTA. The EDTA-treated MDP was completely inactive after gel filtration. Both active and EDTA-treated MDP were then incubated with cilastatin-Sepharose, eluted into SDS sample buffer and subjected to immunoelectrophoretic blot analysis as described in the Experimental section. The two forms of MDP were able to bind to a similar extent to the affinity resin (results not shown).

### Purification of MDP mutants by cilastatin-Sepharose affinity chromatography

The wild-type and inactive His mutants of MDP were examined for their ability to bind to cilastatin-Sepharose after solubilization from membranes with PI-PLC (Figure 3, even-numbered lanes). The wild-type enzyme and mutants H20L and H198K were efficiently bound by the cilastatin-Sepharose (Figure 3,

		<b>20</b>	
pMDP	1	DQFRDLAVRIMQDT <b>PVIDGHND</b> LPWQ	26
hMDP	1	DFFRDEAERIMRDS <b>PVIDGHND</b> LPWQ	26
ACDP	1	MKPSH <b>PVFDGHND</b> ALTR	18
		<b>198</b>	<b>219</b>
pMDP	191	GVMIDLAHVSVATMRAALKLSQAPV <b>IFSHSSA</b> YSLC	226
hMDP	191	GVLIDLAHVSVATMKATLQLSRAPV <b>IFSHSSA</b> YSLC	226
ACDP	198	KMVIDVSHMNEQAFWDTVDLLQ <b>QPIVATHSNA</b> HALC	233

**Figure 4** Partial alignment of the amino acid sequences of pig and human MDPs and of ACDP

Regions of the sequence of pig MDP (pMDP; amino acids 1–26 and 191–226; [11]) are aligned with those of human MDP (hMDP; [12]) and of ACDP [24]. Residues conserved between all sequences are in bold. The conserved histidine residues in MDP are indicated above the sequences by their position number. The alignment was generated by using the Wisconsin GCG program BESTFIT.

lanes 2, 6 and 8), whereas H152L failed to bind (lane 4). To exclude the possibility that H152L was unable to bind to cilastatin-Sepharose because it had not been cleaved from the membrane by PI-PLC, the membranes from COS-1 cells expressing the wild-type enzyme and each of the mutants were separately incubated with PI-PLC and then subjected to temperature-induced phase separation in Triton X-114. The resulting aqueous phases were then subjected to immunoelectrophoretic blot analysis (Figure 3, odd-numbered lanes). The wild-type enzyme and all the mutants, including H152L, were detected as hydrophilic forms, indicating that all had been efficiently solubilized by PI-PLC. A mutant MDP in which His-219 (previously shown to be a critical catalytic residue in pig MDP [22]) was replaced by Lys also bound efficiently to cilastatin-Sepharose (Figure 3, lanes 9 and 10).

### DISCUSSION

Although MDP is a zinc-containing peptidase, its cDNA-derived amino acid sequence contains none of the major zinc peptidase motifs, such as the His-Glu-Xaa-Xaa-His sequence found in about half of all metalloproteases identified (e.g. the thermolysin, neprilysin and astacin families) or the His-Xaa-Xaa-Glu-His inverzincin motif found in the pitrilysin family [20,21]. Thus MDP is a novel zinc metallopeptidase. When MDP was first cloned and sequenced [11], we noted that there was a region (Asp-His-Leu-Asp-His; residues 269–273) of close similarity with the Asp-His-Thr-His zinc-binding motif identified crystallographically in the D-alanyl-D-alanine-cleaving carboxypeptidase of *Streptomyces albus* [33]. However, although both MDP and this carboxypeptidase are capable of cleaving substrates containing D-amino acids and both bind certain  $\beta$ -lactam compounds, site-directed mutagenesis of the Asp-His-Leu-Asp-His motif in MDP and subsequent expression of the mutants clearly indicated that this sequence was not essential for catalytic activity [22]. However, His-219 was identified as a critical residue in MDP in that study. The only other residue essential for catalysis identified to date in MDP is Glu-125 [23].

In an attempt to identify other essential residues in MDP, we have employed a site-directed mutagenesis approach in which the five remaining histidines (residues 20, 49, 128, 152 and 198) in pig MDP [11] that are conserved in human, rabbit, rat, mouse and sheep MDPs [12–16] were each changed either to Leu or to Lys; these mutant proteins were subsequently expressed in COS-1 cells. All five mutants were expressed at levels comparable with that of the wild-type, as shown by immunoelectrophoretic blot analysis, but only two of them (H49K and H128L) were enzymically active. The three inactive mutants were shown to be expressed at the cell surface by immunofluorescence microscopy at levels comparable with that of the wild-type. They were all released efficiently by PI-PLC and were normally glycosylated, as indicated by their molecular mass on SDS/PAGE, which was similar to that of the wild-type. Thus all three mutants were normally processed and targeted to the plasma membrane, consistent with no conformational abnormalities as a result of mutation.

In order to distinguish whether the histidine residues that had been replaced in the three inactive mutants are involved in substrate/inhibitor binding or catalysis, the ability of the expressed mutants to bind to the competitive inhibitor cilastatin was examined. In parallel, it was shown that purified pig MDP that had been depleted of its zinc by EDTA treatment, and which was inactive, could still bind fully to cilastatin-Sepharose. Both H20L and H198K bound efficiently to cilastatin-Sepharose, whereas H152L, even though it was released from the cell surface

by PI-PLC, failed to bind. Thus it would appear that His-152 is involved in substrate/inhibitor binding rather than catalysis. In contrast, His-20 and His-198, along with His-219 [22], appear to be involved in catalysis.

MDP exhibits unusual catalytic activity in that, in addition to hydrolysing dipeptides, it can also hydrolyse the carbapenem class of  $\beta$ -lactam antibiotics. It is the only known example of a mammalian  $\beta$ -lactamase, but it shares no sequence or other similarities with the bacterial metallo- $\beta$ -lactamases [7]. However, during the course of the present study, the gene encoding a dipeptidase from *Acinetobacter calcoaceticus* was cloned and expressed in *E. coli* [24]. ACDP shares a number of enzymic similarities with MDP. It is a metalloenzyme, being inhibited by EDTA and containing 1.2 mol of  $Zn^{2+}$  per mol of enzyme. Like MDP it can hydrolyse dipeptides with either an L- or a D-amino acid at the C-terminus, although D-amino acids are preferred in this position. However, ACDP differs from MDP in that it is unable to hydrolyse unsaturated dipeptides (e.g. glycyl-dehydrophenylalanine) and it is not inhibited by cilastatin [24].

The deduced amino acid sequence of this bacterial dipeptidase shows 48.8 and 46.9% similarity (25.2% identity) with human and pig MDP respectively (the human and the pig MDP sequences share 90.3% similarity and 83.0% identity). The amino acid residues found previously to be important for catalytic activity in the human and pig enzymes, i.e. Glu-125 and His-219, are conserved in ACDP [24]. In addition, it is seen that His-20 and His-198 of MDP are also conserved in the bacterial enzyme, and lie within regions of high similarity between the six mammalian MDP sequences and the ACDP sequence (Figure 4). His-152 of MDP, which appears to be involved in cilastatin binding, is not conserved in ACDP, consistent with that enzyme being insensitive to the inhibitor. His-128 of MDP, the mutation of which did not affect activity, is also not conserved in ACDP. The mutagenesis and enzymic data reported here confirm that MDP and ACDP may represent the first members of a new family of metallopeptidases. Further structural studies on these enzymes may help to elucidate their catalytic mechanism and explain the ability of MDP to function both as a peptidase and as a  $\beta$ -lactamase.

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