MpaA is a murein-tripeptide-specific zinc carboxypeptidase that functions as part of a catabolic pathway for peptidoglycan-derived peptides in \( \gamma \)-proteobacteria

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The murein peptide amidase MpaA is a cytoplasmic enzyme that processes peptides derived from the turnover of murein. We have purified the enzyme from *Escherichia coli* and demonstrated that it efficiently hydrolyses the \( \gamma \)-d-glutamyl-diaminopimelic acid bond in the murein tripeptide (L-Ala-\( \gamma \)-D-Glu-meso-Dap), with \( K_m \) and \( K_{cat} \) values of 0.41±0.05 mM and 38.3±10 s\(^{-1}\). However, it is unable to act on the mureine tetrapeptide (L-Ala-\( \gamma \)-D-Glu-meso-Dap-D-Ala). *E. coli* MpaA is a homodimer containing one bound zinc ion per chain, as judged by mass spectrometric analysis and size-exclusion chromatography. To investigate the structure of MpaA we solved the crystal structure of the orthologous protein from *Vibrio harveyi* to 2.17 \( \AA \) (1\( \AA \)=1.0 nm). \( V_h \) MpaA, which has identical enzymatic and biophysical properties to the *E. coli* enzyme, has high structural similarity to eukaryotic zinc carboxypeptidases. The structure confirms that MpaA is a dimeric zinc metalloprotein. Comparison of the structure of MpaA with those of other carboxypeptidases reveals additional structure that partially occludes the substrate-binding groove, perhaps explaining the narrower substrate specificity of the enzyme compared with other zinc carboxypeptidases. In \( \gamma \)-proteobacteria *mpaA* is often located adjacent to *mppA* which encodes a periplasmic transporter protein previously shown to bind murein tripeptide. We demonstrate that MppA can also bind murein tetrapeptide with high affinity. The genetic coupling of these genes and their related biochemical functions suggest that MpaA amidase and MppA transporter form part of a catabolic pathway for utilization of murein-derived peptides that operates in \( \gamma \)-proteobacteria in addition to the established murein recycling pathways.

Key words: *Escherichia coli*, murein tripeptide, protein structure, transporter, *Vibrio harveyi*, zinc carboxypeptidase.

INTRODUCTION

Peptidoglycan (murein) is the major structural component of bacterial cell walls that forms a mesh-like layer outside the cytoplasmic membrane and is essential for maintaining cell growth with high turgor pressure and for conserving cell shape [1,2]. It consists of glycan strands composed of alternating units of GlcNAc (N-acetylmuramic acid) and MurNAc (N-acetylmuramuramic acid), linked together by \( \beta \)-1,4 glycosidic bonds [3]. In *Escherichia coli* the glycans are decorated with pentapeptides [4]. They are linked by bonds between the amino group of m-Dap (meso-diaminopimelic acid) at position 3 of one peptide and the carboxy group of MurNAc in nascent peptidoglycan. The strength of the wall is a result of approximately half of these peptides forming covalent bonds with peptides on adjacent glycan strands [4]. They are linked by bonds between the amino group of m-Dap (meso-diaminopimelic acid) at position 3 of one peptide and the carboxy group of the D-Ala residue at position 4 of adjacent peptide [2]. In mature peptidoglycan there is subsequent cleavage of one or both of the D-Ala residues, leaving the tetrapeptide, or the tripeptide as the common form present in the mature polymer, with only traces of unprocessed pentapeptide [4,5].

The biosynthesis of peptidoglycan is a complex process that takes place in three subcellular compartments: the cytoplasm, the cytoplasmic membrane and the periplasm (Figure 1). The pentapeptide is assembled on a UDP-MurNAc molecule through the action of a series of Mur enzymes to create the UDP-MurNAc-pentapeptide [6]. The phospho-MurNAc-pentapeptide motif of the latter precursor is then transferred on to the undecaprenyl-phosphate carrier lipid by the MraY membrane translocase, generating lipid I. Subsequent addition of a GlcNAc motif by the MurG transferase yields the lipid II intermediate which is flipped to the periplasmic face of the inner membrane where disaccharide-pentapeptide units are finally inserted into peptidoglycan by murein synthases [5].

The cell wall of bacteria is a dynamic structure which is continually remodelled during cell growth and division. In *E. coli* 40–50% of the peptidoglycan is broken down and reused each generation for the synthesis of new murein [7]. In this process, murein is degraded by the action of murein-hydrolysing enzymes which include lytic transglycosylases, amidases and endopeptidases. The resulting anhydro-muropeptides (GlcNAc-anhMurNAc-L-Ala-\( \gamma \)-D-Glu-meso-Dap-D-Ala) are imported into the cytoplasm by a specific permease (AmpG), where they are efficiently reused in a well-studied murein recycling pathway [7,8] (Figure 1). In the cytoplasm, the muropeptides are broken down by an amidase AmpD to yield GlcNAc-anhMurNAc and the tetrapeptide (L-Ala-\( \gamma \)-D-Glu-meso-Dap-D-Ala) [9]. The tetrapeptide is subsequently hyrolysed by LD-carboxypeptidase LdcA [10] to produce D-Ala and Mtp (murein tripeptide, L-Ala-\( \gamma \)-D-Glu-meso-Dap-D-Ala). Under normal growth conditions these tripeptides are ligated to UDP-MurNAc by Mpl (murein peptide amidase)}
Murein hydrolases break down peptidoglycan (PG) into muropeptide (GlcNAc-anhMurNAc-L-Ala-γ-D-Glu-m-Dap-D-Ala), Mtp and murein tetrapeptide. Muropeptides are taken up by AmpG permease and processed in the cytoplasm by AmpD and LdcA to liberate free Mtp which is then ligated to UDP-MurNAc by Mpl to produce UDP-MurNAc-Mtp and re-enters the pathway for de novo synthesis of peptidoglycan. Mtp and murein tetrapeptide are taken up via MppA/OppBCDF. Murein tetrapeptide is hydrolysed by LdcA to Mtp in the cytoplasm. Mtp either enters into the recycling pathway by the action of murein peptide ligase enzyme (Mpl) or is hydrolysed into its component amino acids by the concerted actions of an amidase (MpaA), an epimerase (YcjG) and a peptidase (PepD). CM, cytoplasmic membrane; OM, outer membrane.

A second pathway of peptidoglycan turnover uses the outer membrane protein AmiD, which is homologous with the cytoplasmic AmpD protein [12]. AmiD activity releases tri- and tetra-peptides in the periplasm from both the intact murein, but also from released muropeptides, and indeed there is strong evidence that E. coli actually secrete both peptides into the growth medium [13]. The released tri- and tetra-peptides are poor substrates for AmpG [8] and the tripeptide is recycled via the MppA/OppBCDF ABC transporter (ATP-binding cassette transporter) [14,15]. A route for uptake of the tetrapeptide has yet to be identified.

It was proposed that tri- and tetra-peptides were recycled via another enzyme, MpaA, which was identified on the basis of its homology with a sporulation-related enzyme ENP1 from Bacillus sphaericus that cleaves the γ-D-Glu-meso-Dap amide bond from both tri- and tetra-peptide [16,17] and which is a member of the peptidase family M14 [CPA (carboxypeptidase A) family] [18]. Although in vitro biochemical characterization of this enzyme has not been reported, there is genetic evidence which suggests a function for this enzyme in E. coli [17]. In the normal recycling pathway described by Park and Uehara [7], cytoplasmically released Mtp is ligated to UDP-MurNAc by Mpl. Strains lacking mpl accumulate Mtp in the cytoplasm and a strain lacking both mpl and mpaA accumulated significantly higher levels of Mtp, suggesting that its normal function is to breakdown Mtp in the cell [17]. This enzyme clearly competes with Mpl for substrate and so possibly has a catabolic function that is regulated to ensure that it is active only when peptidoglycan biosynthesis is not a limiting factor for growth. The product of the MpaA-catalysed reaction on the Mtp substrate, L-Ala-D-Glu, is then converted into L-Ala-L-Glu and subsequently into L-Ala and L-Glu by YcjG epimerase and PepD peptidase respectively [20]. The amino acids produced can be used as carbon and nitrogen sources by E. coli.
γ-Vibrio harveyi solved the structure of the characterized protein from LGC standards. V. harveyi Invitrogen. MurNAc-L-Ala-[11]. GlcNAc-MurNAc (anhydro)-Mtp and GlcNAc-MurNAc Dap-D-Ala, L-Ala-

To determine the precise function of MpaA in E. coli, we have characterized the protein in vitro to study its kinetic parameters and substrate specificity, determined its oligomeric state and solved the structure of the Vibrio harveyi MpaA (Vh_MpaA) protein by X-ray crystallography, revealing the structural basis of the action of a zinc carboxypeptidase with a very narrow substrate specificity.

MATERIALS AND METHODS

Chemicals

Restriction endonucleases were purchased from New England Biolabs, oligonucleotides were produced by MWG-Biotech, and DNA purification kits were obtained from Macherey-Nagel. DNA sequencing was performed at the facility in the University of York. Tripeptides L-Ala-γ-D-Glu-L-Lys, L-Pro-L-Phe-L-Lys and L-Lys-L-Glu-L-Lys were purchased from Alta Bioscience. L-Ala-γ-D-Glu-

Bacterial strains, plasmids and growth conditions

E. coli strain DH5α (Invitrogen) was used for cloning and maintenance of plasmid constructs, whereas E. coli strain BL21 (DE3) (Novagen) was used for expression of recombinant protein. Both strains were grown routinely in LB (Lennox broth) with antibiotic (kanamycin at a concentration of 30 μg/ml) and growth was monitored at A600, using a Jenway 6305 UV–Vis spectrophotometer. Selenomethionine-labelled Vh_MpaA was produced in a methionine auxotroph strain, B834 (DE3), of E. coli. Strains, plasmids and primers used in the present study are listed in Table 1.

Cloning, expression and purification of MpaA and MppA

The LIC (ligation-independent cloning) method was used for cloning of mpaA into pETYSBLIC3C vector. This vector is based on the pET-28a vector (kanamycin-resistant, 30 μg/ml) and allows expression of recombinant proteins with a His6 tag on N-terminus of the protein [21]. The mpaA gene was amplified from genomic DNA of E. coli K-12 MG1665 using PCR primers Ec_MpaAR and Ec_MpaAF and from genomic DNA of V. harveyi ATCC BAA-1116 (gene VIBHAR_07057) using the PCR primers Vh_MpaAR and Vh_MpaAF (Table 1). Primers were designed such that a LIC tail was added to the PCR product, which was designed to be complementary to the LIC cassette in pETYSBLIC3C. PCR-amplified fragments were cloned into pETYSBLIC3C. The resultant expression vectors were named as pAM6092 and pAM6093. The nucleotide sequence was verified by DNA sequencing.

For synthesis of recombinant proteins, each construct (pAM6092 and pAM6093) was introduced into BL21 (DE3) cells. For enzymology, the transformed cells were grown aerobically in 5 ml of LB for 8 h, and used to inoculate 50 ml of LB for overnight aerobic growth at 37 °C. This was then used to inoculate 625 ml of LB at 37 °C. Cells were allowed to grow aerobically to an A600 of 0.4–0.6 before production of recombinant protein was induced with 0.4 mM IPTG (isopropyl β-D-thiogalactopyranoside), followed by further overnight aerobic incubation at 37 °C. Cells were harvested by centrifugation at 4430 g for 15 min at 4 °C and resuspended in buffer A [50 mM Tris/HCl (pH 8), 500 mM NaCl and 10 mM imidazole] supplemented with a cocktail of protease inhibitors (Complete™ EDTA-free tablets, Roche). The cells were lysed by sonication and cell debris was removed by centrifugation at 38 000 g for 30 min at 4 °C and the supernatant containing overexpressed protein was collected. Ni2+-affinity chromatography was used for the purification of the His6-tagged protein using a 5 ml His-trap column (GE Healthcare), connected to an ÄKTA purifier P-900. The soluble fraction containing the protein of interest was passed over the column pre-equilibrated with buffer A and the recombinant protein was eluted by applying a linear gradient of imidazole (0–500 mM). To remove the His6 tag, purified protein was incubated with HRV3C protease in a ratio of 1:100 at 4 °C overnight and then separated from the protease by a second Ni2+-affinity chromatography step [a 5 ml His-trap column (GE Healthcare)] where the protease is retained on the column through an N-terminal His6 tag. Four residues from the affinity tag (Gly-Pro-Ala-Met) remain attached to protein following HRV3C cleavage. Fractions containing cleaved protein were pooled, dialysed and concentrated using Vivaspin 5 kDa MWCO (molecular-mass cut-off) concentrators. Protein concentrations were determined from the absorbance at 280 nm by the method of Bradford.
at 280 nm ($A_{280}$) using calculated molar absorption coefficients of 45790 cm$^{-1}$·M$^{-1}$ and 28360 cm$^{-1}$·M$^{-1}$ for Ec_MpaA and Vh_MpaA respectively. *E. coli* MppA was purified as described previously [15].

For crystallization, pAM6903 was transformed into a methionine auxotroph strain, B834 (DE3) of *E. coli*. The transformed cells were grown aerobically in 5 ml of LB for 8 h, and used to inoculate 50 ml of LB for overnight aerobic growth at 37°C. This was then used to inoculate 625 ml of M9 minimal medium supplemented with 2.5 g of glucose, 2 mM MgSO$_4$, 25 μg/ml FeSO$_4$, 7H$_2$O, 25 mg of each amino acid except methionine, 25 mg of L-selenomethionine, a cocktail of vitamins (riboflavin, niacinamide, pyridoxine monohydrochloride and thiamine, each at 1 μg/ml) and the antibiotic kanamycin at 30 μg/ml. Cells were allowed to grow aerobically to an $A_{600}$ of 0.4–0.6 before synthesis of recombinant protein was induced with 0.4 mM IPTG, followed by a further 4 h aerobic incubation at 37°C. A purification protocol similar to that for the native enzyme/protein was used for purification of selenomethionine-labelled protein. Crystallization experiments used protein with a His$_6$ tag.

**Protein analysis by MS**

A Micromass LCT Premier XE mass spectrometer using a nanospray source was used for ES (electrospray)-MS. For analysis of MpaA under denaturing conditions, purified MpaA (after cleavage of the His$_6$ tag) was dialysed into 10 mM Tris buffer (pH 8) and concentrated to 100 μM. Concentrated protein was diluted to 1 μM in 1:1 acetonitrile/water containing 0.1 % formic acid. The sample was passed into the ES-MS and the data were collected over 3 min within an $m/z$ range of 100–2000. For analysis under native conditions, purified MpaA was dialysed into 200 mM ammonium acetate and then concentrated to 100 μM. Data were collected over 30 min within an $m/z$ range of 100–8000. The raw $m/z$ data were deconvoluted to mass spectra using the MaxEnt1 routine in the MassLynx software provided by the manufacturer (Waters).

**SEC (size-exclusion chromatography)-MALLS (multi-angle laser light scattering)**

For SEC-MALLS, samples of MpaA (100 μl of 1 mg/ml in 20 mM Tris buffer, pH 8) were applied to a Superdex 200 10/300 GL SEC column (GE Healthcare) at 0.5 ml/min with an HPLC system (Shimadzu), linked to a Wyatt Dawn Heles MALLS detector and a Wyatt Optilab rEX unit, to determine the refractive index. The buffer was 150 mM NaCl and 20 mM Tris (pH 8). Data were analysed with the program Wyatt ASTRA version 5.3.4.14.

**Enzymatic assays for MpaA amidase activity**

The standard assay mixture (25 μl) contained 100 mM Tris/HCl (pH 8), 0.2 mM tripeptide-L-[14C]Ala-$\gamma$-D-Glu-meso-Dap (40 Bq) and purified MpaA [5 ng of protein in 5 μl of 50 mM Tris buffer (pH 8), containing 0.5 mg/ml BSA]. Mixtures were incubated at 37°C for 30 min, and the reactions were stopped by addition of 10 μl of acetic acid. Radiolabelled substrate [14C]-L-Ala-$\gamma$-D-Glu-meso-Dap and reaction product [14C]-L-Ala-D-Glu were separated by TLC on silica gel plates LK6D (Whatman), using ethanol/1 M ammonium acetate (pH 3.8) (5:2) as a mobile phase. The radioactive spots were located and quantified with a radioactivity scanner (Multi Tracemaster LB285; Berthold). When other compounds were tested as substrates, assay conditions were modified: the amount of MpaA enzyme was appropriately adjusted (from 5 ng to 10 μg), as well as the time of incubation (from 30 min to 16 h). Some enzymatic assays used unlabelled compounds as substrates; in that case, reaction mixtures were analysed by HPLC, using a C$_8$ ODS Hypersil 3 μm column (250 mm×4.6 mm; Grace). Elution was performed either using isocratic conditions with 0.05% TFA (trifluoroacetic acid) or by applying a gradient of acetonitrile (0–20% acetonitrile in 0.05% TFA) at a flow rate of 0.6 ml/min. Detection was at 214 nm. In some cases, the enzymatic digestion of tripeptide-derivative compounds was also analysed by measuring the release of the C-terminal amino acid residue (Dap or lysine) using a Hitachi L8800 amino acid analyser (ScienceTec).

For determination of the kinetic constants, the same assay was used with various concentrations of Mtp. In all cases, the substrate consumption was <20% and linearity was observed within the interval even at the lowest substrate concentration. The data were fitted to the equation $v = V_{max}S/(K_m + S)$, using the Simplex algorithm (MDFit software developed by M. Desmadr, IBBMC, Orsay, France).

**Crystallization of Vh_MpaA**

For crystallization, selenomethionine-labelled Vh_MpaA was concentrated to 20 mg/ml in 50 mM Tris/HCl (pH 8). Crystallization screening experiments were performed using several commercial kits (Index, Hampton I and II, Newcastle, PACT and Morpheus) by hanging-drop vapour diffusion procedures in 96-well plates set up with a Mosquito nanolitre pipetting robot (TTP Labtech). The plates were sealed to avoid evaporation and incubated at 20°C. The initial crystallization droplet contained 150 nl of a 20 mg/ml protein solution in 50 mM Tris/HCl (pH 8.0) mixed with 150 nl of well solution containing 0.1 M Mes/imidazole (pH 6.5), a 0.02 M mix of monosaccharides (Morpheus condition F2) and a mix of PEG [poly(ethylene glycol)] 8000 and ethylene glycol. Optimization experiments led to crystal growth over 7–10 days in 0.12 M Mes/imidazole (pH 6.5), 0.01 M d-glucose, 8 % PEG 8000 and 20 % ethylene glycol. Prior to flash-freezing in liquid nitrogen, crystals were soaked for 30 s in a cryoprotectant solution of 0.12 M Mes/imidazole (pH 6.5), 8 % PEG 8000 and 40 % ethylene glycol. SAD (single-wavelength anomalous dispersion) data were collected from a cryo-cooled crystal (100 K) on beamline I02 at the Diamond Light Source, UK, using a Mar CCD (charge-coupled device) detector. The SAD data were processed using the programme Xia2 [22]. The diffraction data statistics are summarized in Table 2.

**X-ray data collection, structure solution and refinement**

ShelxCD [23] was used to solve the structure of Vh_MpaA using SAD data collected from a selenomethionine crystal. ShelxD was used to locate the two selenium atoms present in the two molecules of the Vh_MpaA asymmetric unit. The location of the two selenium atoms allowed phasing of the Vh_MpaA structure and the space group was unambiguously determined to be $P6_1$22 following the density modification step performed by ShelxE. The resultant electron-density map was of sufficient quality to allow 244 of the 258 residues to be built using the programme Buccaneer [24]. The Vh_MpaA model produced by Buccaneer was refined iteratively by maximum likelihood methods in REFMAC5 [25] and model building using COOT [26] to give $R_{	ext{merge}}$ and $R_{	ext{free}}$ values of 19.6 % and 23.2 % respectively. The refinement statistics are given in Table 2. The structure was validated using COOT.
Table 2  Vh_MpaA X-ray data collection and refinement statistics

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Value</th>
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<tbody>
<tr>
<td>Wavelength (Å)</td>
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</tr>
<tr>
<td>Resolution range (Å)</td>
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</tr>
<tr>
<td>Space group</td>
<td>P61,22</td>
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<tr>
<td>Unit cell parameters (Å)</td>
<td>a = b = 73.6 Å, c = 208.7 Å</td>
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<tr>
<td>Number of unique reflections (overall/outer shell)</td>
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<tr>
<td>Completeness (%) (overall/outer shell)</td>
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<tr>
<td>Redundancy (overall/outer shell)</td>
<td>40.8/42.7</td>
</tr>
<tr>
<td>l/σ(l) (overall/outer shell)</td>
<td>33.5/7.4</td>
</tr>
<tr>
<td>Rmerge (%) (overall/outer shell)</td>
<td>10.5/78.5</td>
</tr>
</tbody>
</table>

Refinement and model statistics

- R-factor: 19.6 (23.2)
- Reflections: 17580/948
- Outer shell R-factor: 20.3 (26)
- Outer shell reflections: 1093/61
- Number of protein non-hydrogen atoms: 1933
- Number of zinc ions: 1
- Number of water molecules: 46
- Rmsd from target: 4
- Bond lengths (Å): 0.02
- Bond angles (°): 2.11
- Average B-factor (Å²): 33
- Ramachandran plot:
  - 100/0/0/0.0

The outer shell corresponds to 2.23–2.17 Å.

Fluorescence spectroscopy

Protein fluorescence experiments used a FluoroMax 4 fluorescence spectrometer (Horiba Jobin-Yvon) with connecting water bath at 25 °C. Ligand free protein (MpaA+) was purified as described previously [15] and was used at a concentration of 0.05 μM in 50 mM potassium phosphate buffer (pH 7.8). Protein was excited at 297 nm with slit-widths of 3.5 nm and emission was monitored at 330 nm with slit-widths of 3.5 nm. To determine the K₅₄ for tetrapeptide binding, the protein was titrated with increasing concentrations of the tetrapeptide and the corresponding fluorescence change was monitored in temperature intervals with a stirring speed of 307 rev./min. Raw titration data were integrated and fit to a single-site model of binding using MicroCal Origin version 7.0.

RESULTS

Overexpression and purification of Ec_MpaA

To investigate the biochemical properties of Ec_MpaA, a recombinant mpaA gene was expressed in E. coli to produce an N-terminally His₆-tagged protein. SDS/PAGE analysis of cell extracts showed that induced cells accumulated a recombinant protein with a molecular mass of approximately 28 kDa with 30–40% of the overproduced protein present in the soluble fraction (Supplementary Figure S1 at http://www.BiochemJ.org/bj/448/bj4480329add.htm). Soluble protein was purified using Ni²⁺-affinity chromatography and the His₆-tag was removed using HRV3C protease to produce native MpaA with four additional amino acid residues (Gly-Pro-Ala-Met) at the N-terminus. After purification, the protein was ~99 % pure as judged by Coomassie Blue staining of SDS/PAGE gels (Figure 2A, inset), with a final yield of 12 mg/l. ES-MS under denaturing conditions was used to determine the molecular mass of the recombinant MpaA protein to be 26783±1 Da (Figure 2A), which is consistent with the predicted mass of the protein after removal of the His₆-tag (26782 Da).

MpaA is a homodimer in solution containing two bound zinc ions

The purified Ec_MpaA was further examined using ES-MS, this time under native conditions that often preserves non-covalent interactions between proteins and their ligands [29,30]. We observed a major species with mass of 26846 Da and a minor species with a mass of 53692 Da (Figure 2B). The difference in mass of the species observed under denaturing conditions (26783±1 Da) and that observed under native conditions (26846±1) is 63 Da. These data indicate that Ec_MpaA contains a zinc ion (65.3 Da) consistent with its assignment based on structure to the family of Zn²⁺-dependent amidases. Also, the second peak of 53692±2 Da is exactly twice that of the native zinc-bound form, suggesting that MpaA forms a homodimer which bind two molecules of zinc. To investigate the native oligomeric state of Ec_MpaA further we analysed the protein using SEC-MALLS (Figure 2C), which revealed a single symmetric peak for MpaA with a calculated mass of 54±1kDa. This is a close match to that observed by native ES-MS for a dimer species and so confirms that Ec_MpaA is a dimer in solution.

The enzymatic activity of MpaA is highly specific to L-Ala-γ-D-Glu-meso-Dap (Mtp)

The MpaA enzyme has been demonstrated to cleave L-Ala-γ-D-Glu-meso-Dap (Mtp) to L-Ala-γ-D-Glu and Dap in a qualitative assay [17] and we used the purified enzyme to characterize this.

and MOLPROBITY [27]. The co-ordinates and experimental structure factors have been deposited in the RCSB PDB under the accession code 4AXV. The dimer interface was analysed using PISA [28].
reaction further in vitro. MpaA was able to hydrolyse Mtp at a rate of 200 nmol·s\(^{-1}\)·mg\(^{-1}\) (Table 3). The kinetic parameters of MpaA towards Mtp were determined using substrate concentrations ranging from 0.07 to 0.7 mM. The calculated \(K_m\) and \(k_{cat}\) values were 0.41±0.05 mM and 38.3±10 s\(^{-1}\) respectively (Table 3). The pH-dependence of the MpaA-catalysed reaction was investigated revealing a rather flat curve between pH 7.2 and pH 9, with an optimum value at approximately 8.0 (Supplementary Figure S2 at http://www.BiochemJ.org/bj/448/bj4480329add.htm). The activity of MpaA was totally inhibited by incubation with 10 mM EDTA (results not shown) suggesting that the enzyme requires the presence of metal ions.

It has been suggested that Mtp is also a substrate for MpaA [17], but surprisingly we saw no activity with this substrate. Although the enzyme cannot tolerate a C-terminal extension in the peptide substrate, we investigated the ability of the related tripeptide L-Ala-γ-D-Glu-L-Lys to be used as a substrate and this did give a low rate of cleavage, approximately 1.5 % of that seen with Mtp. The enzyme also had extremely low, but detectable, activities with the MurNAc tripeptide and the dipeptide γ-D-Glu-meso-Dap, and did not act on a number of other peptidoglycan precursors and their analogues (Table 3).

These data suggest that Mtp is the physiological substrate of MpaA and that the enzyme cannot tolerate many changes in this structure, including the length of the peptide and the presence of a γ-D-Glu-meso-Dap bond. The lack of activity on the tetrapeptide demonstrates that MpaA does not exhibit any endopeptidase activity.

The structure of Vh_MpaA resembles eukaryotic carboxypeptidases

In order to probe further into the mechanism of MpaA action we sought to determine the structure of E. coli MpaA, but were unable to grow crystals. However, we were able to grow crystals and determine the crystal structure to 2.17 Å (1 Å = 0.1 nm) resolution of MpaA from V. harveyi (Vh_MpaA, VIBHAR_07057) (Table 2). This orthologue was chosen because V. harveyi is a γ-proteobacterium that contains a divergently transcribed mpaA/mppA gene pair described later in the present paper. We achieved a high level of production of Vh_MpaA and confirmed its mass using ES-MS (Supplementary Figure S3A at http://www.BiochemJ.org/bj/448/bj4480329add.htm). Incorporation of L-selenomethionine to Vh_MpaA was confirmed using ES-MS (Supplementary Figure S4 at http://www.BiochemJ.org/bj/448/bj4480329add.htm). Vh_MpaA was also found to be a dimer in solution by SEC-MALLS and ES-MS suggested that, as for Ec_MpaA, the V. harveyi MpaA dimer also binds two zinc ions (Supplementary Figures S3B and S3C). Vh_MpaA is able to hydrolyse Mtp (specific activity of 190 nmol·s\(^{-1}\)·mg\(^{-1}\)) (Supplementary Table S1 at http://www.BiochemJ.org/bj/448/bj4480329add.htm), but not murein tetrapeptide, confirming that it is a functional orthologue (the two proteins are 56 % identical and 67 % similar over 230 amino acids) (Supplementary Figure S5 at http://www.BiochemJ.org/bj/448/bj4480329add.htm) of the E. coli MpaA protein. We crystallized a 258 residue polypeptide consisting of Vh_MpaA fused at its N-terminus to a 22 residue sequence containing a His\(_6\) tag. The first 15 residues of

Table 3 Substrate specificity and kinetic parameters of Ec_MpaA

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific activity (nmol·s(^{-1})·mg(^{-1}))</th>
<th>(K_m) (mM)</th>
<th>(k_{cat}) (s(^{-1}))</th>
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</thead>
<tbody>
<tr>
<td>Mtp</td>
<td>200</td>
<td>0.41±0.05</td>
<td>38.3±10</td>
</tr>
<tr>
<td>L-Ala-γ-γ-Glu-MurNAc(tripeptide)</td>
<td>2.8</td>
<td>0.005</td>
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<tr>
<td>γ-γ-Glu-Meso-Dap</td>
<td>0.007</td>
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</tbody>
</table>
this sequence are not defined in the electron-density maps and they are assumed to be disordered. The remaining seven residues of the tag are observed as they make crystal packing interactions (results not shown). In the refined model of Vh_MpaA, residue numbering starts at the first methionine residue of the native protein (1–235). There is one Zn$^{2+}$ ion (at the active site) and 46 water molecules associated with each molecule of Vh_MpaA.

The overall fold is one seen in zinc metallocarboxypeptidases [31] of both prokaryotes and eukaryotes. It comprises a central eight-stranded mixed $\beta$-sheet with surrounding $\alpha$-helices (Figure 3A). A DALI search of the PDB revealed that Vh_MpaA has the highest structural similarity to a number of eukaryotic general carboxypeptidases giving Z-scores of 20±0.5 for ~200 equivalent residues with an rmsd (root mean square deviation) of 2.7±0.1 Å. The three best matches were CPD (carboxypeptidase D) from duck (PDB code 1HSL) [32], the catalytic domain of human CPN (carboxypeptidase N) (PDB code 2NSM) [31] and the Drosophila melanogaster CPD (PDB code 3MN8) [33], with the closest bacterial match being the carboxypeptidase T from Thermococcus litoralis [34] (Supplementary Figure S6 at http://www.BiochemJ.org/bj/448/bj448o329add.htm). A sequence alignment of Vh_MpaA with duck CPD, human CPN and D. melanogaster CPD is shown in Supplementary Figure S7 (at http://www.BiochemJ.org/bj/448/bj448o329add.htm), which reveals that all three eukaryotic carboxypeptidases have an additional C-terminal TT (transthyretin) domain, characteristic of the CPN/E subfamily of carboxypeptidases, that is missing in Vh_MpaA [31,32], but that the catalytic domains of all four proteins align well at the sequence and structural level (Supplementary Figures S6 and S7).

Active site of Vh_MpaA and a possible structural basis for substrate specificity

Comparison of the active site of MpaA with that of the well-studied human CPA reveals that there is conservation of both the zinc-co-ordinating residues His88, Glu31 and His186 and other residues integral to catalysis in this enzyme, Glu209, Arg98, Arg88 and Asn97 (Figure 3B). Tyr238 in human CPA, whose contribution to catalysis has long been controversial [35], corresponds to Tyr248 in Vh_MpaA. This residue is in an ‘up’ conformation in Vh_MpaA away from the active site. This conservation of catalytic residues, together with the close structural superimposition, suggests a common catalytic mechanism for CPA and Vh_MpaA [31]. According to this mechanism, upon binding of substrate, the $\alpha$-carboxylate forms a bidentate salt bridge to the side chain of Arg145 (corresponding to Arg88 in MpaA) and a polar interaction with the side-chain amide of Asn83 (Asn97 in Vh_MpaA). Glu270 (Glu209 in Vh_MpaA) acts as a base to activate a nucleophilic water molecule which attacks the carbonyl carbon (the carbonyl bond being polarized by the zinc) of the scissile peptide bond. This generates a tetrahedral anionic intermediate which is stabilized by interactions with the side chain of Arg127 (Arg88 in Vh_MpaA) and the zinc in a quasi-oxygen hole. Following collapse of this intermediate and peptide bond cleavage, Arg127 (Arg88 in Vh_MpaA) and Arg145 (Arg88 in Vh_MpaA) stabilize the carboxylates of the peptide and amino acid products respectively, with the newly formed amino group interacting with Glu270 (Glu209 in Vh_MpaA) [36].

Unlike the carboxypeptidases, MpaA exhibits narrow substrate specificity. In the absence of structural data on the MpaA–Mtp complex, we displayed the amino acid and pentapeptide ligands from the human CPA-cleaved hexapeptide product complex [36] in the active site of Vh_MpaA following least squares superimposition of the secondary structure elements in the two proteins. As can be seen in Figure 3C, the Arg113–Ser122 loop serves as a flap which closes around the peptide-binding groove and clashes with the N-terminus of the N-terminal pentapeptide cleavage product. This argues that this loop restricts the length of substrates in Vh_MpaA.

Next we attempted to introduce the Mtp ligand from the MpaA–Mtp crystal structure into Vh_MpaA. This is not straightforward as the D-stereochemistry at Glu1 of the Mtp and its $\gamma$-peptide linkage differs from the structures of conventional peptides [15]. A pose derived by fitting Mtp into the electron density for the pentapeptide ligand in the carboxypeptidase-cleaved hexapeptide complex, placed the L-Ala–D-Glu residues over the P3–P4 residues of the pentapeptide with the oxygen of the scissile peptide bond in co-ordination distance of the zinc ion (Figure 3C) and with the $\alpha$-carboxylate of Dap in salt-bridging distance of Arg98. As shown in Figure 3C, the rest of the Dap molecule extends into the protein beyond the volume occupied by the P3 amino acid product in the human carboxypeptidase structure. This highly speculative modelling shows that Mtp can be accommodated in the pocket, but it is not clear how the protein interacts with the various functional groups to determine specificity for Mtp. We note, however, that the S1 pocket in Vh_MpaA, where Dap would sit, is more accessible from the solvent than in the ‘classical’ human carboxypeptidase which has an enclosed ‘dead-end’ pocket [36], suggesting that there may not be multiple contacts between Dap and MpaA.

Analysis of the dimer interface in Vh_MpaA

Analysis of the intermolecular interactions in the crystal using the programme PISA [28] reveals a prominent protein–protein interface in which ~2050 Å$^2$ or 17% of the surface area on each MpaA subunit is buried, indicating that Vh_MpaA forms a homodimer (Figure 4). This observation is consistent with both the MS and SEC-MALLS data for the protein (Supplementary Figure S3). The residues involved in the dimer interface span the full length of the protein and the dimer interface is stabilized by a network of intermolecular hydrogen bonds (Supplementary Figure S7). At the heart of the interface, residues at the C-terminus of helix $\alpha 2$ are enclosed in a surface cavity in the partner subunit. Core residues in this interface are Ala10, Ala19, Ile55, Ser77, Asn83, Glu84, Gly86 and Val123 with Arg6, Thr7, Phe12, Pro27, Leu29, Thr52, Glu93, Trp114, Ser214, Asp216 and Glu220 prominent contributors (>50 Å$^2$ of surface area buried in the interface) to the rim [37]. As far as we are aware, this is the first observation of a dimeric bacterial zinc carboxypeptidase.

As noted above, access to the active site is restricted by an extended hairpin element with flanking $\beta$-strands that resembles a flap (Figure 3C). In the dimer, the flap residues of each chain participate in three interchain ion pairs (His117–Glu209, Asp122–Arg98 and Lys124–Glu1) and a further three interchain hydrogen bonds. Furthermore, the side chain of Trp114 is packed into a cavity in the partner subunit formed by the N-terminal 310 helix and helices $\alpha 4$ and $\alpha 6$ (Supplementary Figure S7). The movement of the flap to allow entry of substrate into the active site would be expected to be accompanied by disruption of these predominantly polar interactions. Thus the opposing subunit in this unusual homodimeric carboxypeptidase has the potential to influence the structure and accessibility of the active site in the partner subunit through interactions that determine the conformation and dynamics of the flap. Whether, and to what extent, this determines the kinetics or the specificity of the MpaA-catalysed reaction is not yet established, but this question could be addressed through
Figure 3 Crystal structure of Vh_MpaA with its active centre and structural basis for substrate specificity

(A) Ribbon representation of the Vh_MpaA structure in stereo view with rainbow colouring from the N-terminus (blue) to the C-terminus (red). The black sphere represents a zinc ion which is labelled. (B) The active site of Vh_MpaA superimposed on that of human CPA (PDB code 2PCU). The side chains of active-centre residues are represented in ball and stick format (atoms are coloured by element with nitrogen in blue, oxygen in red and carbons in green (Vh_MpaA) or cyan (human CPA)). The closely superimposed zinc ions are shown as small spheres and coloured green in Vh_MpaA and cyan in human CPA. (C) Vh_MpaA in ribbon (light green) representation shown together with the hexapeptide cleavage products (cylinder format and atoms coloured by atom type with nitrogen in blue, oxygen in red and carbon in grey) taken from the complex with human CPA (PDB code 2PCU). The two protein chains were overlaid using the superpose proteins routine in the programme CCP4mg [45]. The flap region (magenta) of Vh_MpaA sterically clashes with the N-terminal pentapeptide product suggesting that access to the active site in Vh_MpaA is restricted to shorter peptides. Zinc in the active centre is shown as a black sphere. Mtp was modelled into electron density calculated for the pentapeptide ligand in the cleaved hexapeptide complex and is represented in cylinder format with atoms coloured as follows: nitrogen in blue, oxygen in red and carbon in green.
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Figure 4 Quaternary structure of Vh_MpaA

The quaternary structure of Vh_MpaA in the crystal is a homodimer. (A) Ribbon representation of the two chains of the MpaA dimer with subunits coloured in light green and blue and with the zinc ions shown as black spheres. The chain termini are labelled and distinguished by the presence or absence of an apostrophe ('). (B) Close-up view of the dimer interface in which the subunits are shown as transparent surfaces surrounding ribbon renderings of the backbones which are coloured by chain. Residues shown as cylinders contribute to intersubunit salt bridges which are denoted by broken lines. Residues from the lower blue subunit are labelled with an apostrophe (') to distinguish them from residues from the upper green subunit. The side chains of residues Trp114 together with those of Asn83 and Gln84 together with all atoms of Gly86 are shown as spheres to emphasize their projection into cavities in the surface of the opposing subunit. The zinc ions are shown as black spheres.

detailed comparative studies of MpaA mutants in which the dimer interface is disrupted. Site-directed mutagenesis of residues 83–86 may provide a route to monomeric MpaA derivatives.

Analysis of the genome context of mpaA in γ-proteobacteria reveals a potential scavenging pathway for peptidoglycan-derived peptides

In E. coli K-12 the mpaA amidase gene is likely to be monocistronic and is convergently transcribed from the ycjG gene which encodes an L-Ala-D-Glu epimerase that is probably needed for the cell to utilize the product of the MpaA reaction [20,38]. Examination of the genome context of mpaA across a range of γ-proteobacteria (Figure 5) reveals that the genetic association of mpaA and ycjG is conserved in Salmonella enterica, Klebsiella pneumoniae and Yersinia pestis. Furthermore, in these three genomes the divergently oriented mppA transporter gene is adjacent to the mpaA amidase gene, an arrangement that is not seen in E. coli due to the interposition of three additional coding sequences. The adjacent mppA/mpaA arrangement is also seen in other γ-proteobacteria such as Erwinia carotovora. In the species V. harveyi, V. parahaemolyticus and Vibrio sp. Ex25 we observed a similar arrangement of the mpaA amidase gene linked...
V. harveyi
V. parahaemolyticus

B. cereus

Figure 5 Genetic organization of mpaA and mppA in different bacteria and proposed peptide catabolic pathway

Putative mpaA and mppA genes are represented by dark and light boxes respectively. Genes with similar putative function are represented by boxes patterned appropriately. E. coli, Salmonella enterica serovar Typhimurium LT2; Kp, Klebsiella pneumoniae subsp. Pneumoniae MGH 78578; Yp, Yersinia pestis KIM; Eca, Erwinia carotovora subsp. Atroseptica SCTR1043; Vs, Vibrio sp. MEd 222; Vp, Vibrio parahaemolyticus AQ3810; Vh, Vibrio harveyi ATCC BAA-1116. mppA* indicates oppA orthologues that we predict are functionally homologous with mppA. tpX encodes a thiol-peroxidase-type protein. 01306 of K. pneumoniae encodes a putative transporter.

to a gene encoding an oligopeptide-binding protein, which we have called mppA* and which has the same amino acid sequence changes that we identified for the MppA transporter protein that distinguished it from its ancestor the general oligopeptide-binding protein OppA [15]. Examining the phylogeny of the OppA and MppA/MppA* proteins from these bacteria suggests that the Mtp-binding function has evolved twice from the oppA genes in these lineages (Supplementary Figure S8 at http://www.BiochemJ.org/bj/448/bj4480329add.htm).

The conserved gene organization of the mppA transporter genes with the mpaA amidase gene and ycjG seen in S. enterica, K. pneumoniae and Y. pestis, suggests that the three gene products have a functional and likely physiological connection. Given that MppA is the Mtp-binding protein component of the MppAOppBCDF transporter [14,15], the uptake of Mtp may be coupled to its catabolism via the MpaA amidase and YcjG epimerase (Figure 1). Although Mtp makes up approximately 7% of the total peptide content of the cell wall, the most abundant peptide is the murein tetrapeptide (L-Ala-γ-D-Glu-meso-Dap-L-Ala), making up approximately 36% [4]. To investigate whether the tetrapeptide could be taken up by E. coli we examined whether it would bind to E. coli MppA. Using tryptophan fluorescence spectroscopy we observed a ~5.5% quench in the fluorescence upon addition of excess ligand, which could be titrated to yield a $K_d$ of 0.140 ± 0.003 μM (Figure 6A). We also measured binding of tetrapeptide to MppA via ITC (Figure 6B) which yielded an equilibrium dissociation constant of 0.111 ± 0.003 μM, in close agreement with the data derived from fluorescence. Although binding tetrapeptide with high affinity, the protein is unable to bind murein pentapeptide (L-Ala-γ-D-Glu-meso-Dap-L-Ala-L-Ala) or muropeptides (results not shown).

These data demonstrate that MppA transporter protein can recognize both the murein tri- and tetra-peptides and so we propose that both free peptides are taken up by the MppAOppBCDF transporter into the cell. The known cytoplasmic Ld-carboxypeptidase LdcA enzyme is able to cleave the tetrapeptide to tripeptide [10], which is then the substrate for the MpaA amidase and subsequently YcjG (Figure 1).

**DISCUSSION**

Cell-wall turnover is an enzymatic process that results in the loss of peptidoglycan components and has been reported in many bacteria [7]. The turnover products are generally re-utilized through a process known as cell-wall recycling [7]. In E. coli a major turn over product (muropeptide, GlcNAc-anhMurNAc-L-Ala-γ-D-Glu-meso-Dap-D-Ala) is known to be taken up by AmpG permease and further processed by lytic enzymes to release free cytoplasmic Mtp, which then re-enters the peptidoglycan biosynthetic pathway [7]. There is now clear evidence of an alternative route for the utilization of cell-wall-derived peptides by E. coli, which has been postulated for many years since E. coli was shown to secrete Mtp and murein tetrapeptide into the growth medium [13]. Release of these peptides follows their cleavage from the murein by AmiD, an outer membrane amidase [12]. The presence of a transporter MppAOppBCDF which recognises the murein tri- and tetra-peptides suggests that E. coli takes up these peptides for their re-utilization [14,15]. The genetic linkage of the mpaA amidase gene, the mppA transporter gene and the ycjG epimerase gene (Figure 5) suggests that E. coli has a route to facilitate uptake and catabolism of murein tri- and tetra-peptides. As the MpaA amidase does not recognize the tetrapeptide, the LdcA enzyme must be used to convert tetrapeptide transported by MppAOppBCDF into tripeptide that can be broken down by MpaA (Figure 1). LdcA is an important enzyme as, in its absence, free tetrapeptide is incorporated into new peptidoglycan via Mpl, which results in a less cross-linked and hence weaker
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This observation is also consistent with our data that the MpaA amidase does not recognize the tetrapeptide or it would have not accumulated in the ldcA background. In addition, in the present study we demonstrate that the transporter MppA recognizes both tri- and tetra-peptide with high affinity, yet it was notable that cytoplasmically expressed recombinant MppA purified with only tripeptide bound [15], suggesting that even though tetrapeptide is being taken up by these cells, the action of LdcA means that tripeptide is the only form that is present in significant concentrations in the cytoplasm.

The likely function of this catabolic pathway is to allow E. coli to utilize exogenous murein-derived peptides present in the growth medium from other Gram-negative bacteria as well as recycling its own murein peptides released in the periplasm. The relative affinity of the MpaA amidase for Mtp is approximately 4-fold lower than Mpl (K_m values of ~0.4 compared with ~0.1 mM) for Mtp and so, given a limiting amount (low micromolar) of substrate, it will be preferentially recycled into peptidoglycan rather than being catabolized. Only when higher concentrations are present is there likely to be significant catabolism via MpaA. It is interesting that the MpaA amidase can also recognize, albeit poorly, the murein tripeptide that would be derived from some Gram-positive bacteria where the Dap is replaced by lysine, although whether this is physiologically important needs to be determined. Little is known about the regulation either mpaA, mppA, ycjG or ldcA, but they do not appear to be within well-known catabolic regulons for carbon (CRP) or nitrogen (Ntr), suggesting that perhaps they are constitutively expressed.

The MpaA amidases from both E. coli and V. harveyi were easily overexpressed and purified in active forms. Previously efforts to overproduce E. coli MpaA in E. coli were unsuccessful [17], probably due to misidentification of the start codon, which resulted in the expression of a recombinant protein with an additional 20 amino acid residues at the N-terminus. We used the start codon as determined from the EcoGene database of E. coli [39] resulting in successful overproduction of MpaA. The structure of the Vh_MpaA protein revealed a classical zinc carboxypeptidase with complete conservation of key active-site residues. Clearly the MpaA enzyme is a special carboxypeptidase as it has high substrate specificity compared with more general carboxypeptidases such as the human CPA and CPB. The capping region that we propose constrains the size of the peptide substrate that can be recognized by MpaA (Figure 3C) is analogous to similar regions seen in the L-Ala-D-Glu epimerase enzyme that uses the products from the MpaA reaction and where a capping domain restricts the active site and plays a direct role in ligand recognition [40]. Similarly in the YkfC protein from Bacillus subtilis, which is functionally orthologous to MpaA, but is instead an NIpC/P60 cysteine peptidase, there is capping of the entry to the active site by an SH3 (Src homology 3) domain in the proteins which have been proposed to function in restricting the range of substrates that the enzymes can use [41,42]. The other unusual feature of MpaA as a member of the zinc carboxypeptidase family is that it forms a homodimer, and we have described how this dimerization might make a contribution to the ability of the enzyme to have a narrow substrate specificity for a zinc carboxypeptidase.

Using purified E. coli MpaA we were able to confirm much of the data of Uehara and Park [17], who demonstrated that MpaA specifically cleaves the γ-D-Glu-meso-Dap amide bond of Mtp and that replacement of the Dap by lysine resulted in a 98.5% drop in activity (Table 3). Uehara and Park [17] tested the activity of cell extracts prepared from E. coli mpaA-overproducing cells on several substrates and observed that MpaA was not active on UDP-MurNAc pentapeptide. We also confirmed that the purified MpaA

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**Figure 6** MppA will recognize murein tetrapeptide with high affinity

(A) Cumulative changes in tryptophan fluorescence of MppA upon titration with murein tetrapeptide. (B) Binding isotherms for the interaction of MppA with murein tetrapeptide. The top panel shows heat differences upon injection of ligand and the bottom panel shows the integrated heats of injection (H17039) and the best fit (solid line) to a single-site binding model using Microcal Origin.

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does not act on the tri- and penta-peptide-containing nucleotide precursor, confirming that MpaA does not interfere with the peptidoglycan biosynthetic pathway. Also, we show that MpaA is very poorly active with MurNAc-tripeptide (specific activity being 0.005 compared with 200 mmol·s⁻¹·mg⁻¹) and is totally inactive towards a disaccharide (anhydro)-tripeptide, demonstrating that it is a zinc carboxypeptidase with a rather narrow substrate range.

As the dipeptide meso-Dap-D-Ala has been detected in cell extracts of E. coli [13] this is presumably being produced in the periplasm and it was suggested that MpaA amidase would also be able to hydrolyse murein tetrapeptide [17], hence acting as an endopeptidase as well. This was unlikely as the MpaA protein lacks a signal peptide and is probably a cytoplasmic protein. Regardless, the results of the present study demonstrate that MpaA cannot hydrolyse murein tetrapeptide. This suggests that there must be an alternative route in E. coli to produce the meso-Dap-D-Ala product in the periplasm. The YkfC protein from B. subtilis is able to cleave tri-, tetra- and penta-peptides with L-lysine at the third position [20] and there are four members of this family in E. coli, two of which, YdhO and YafL, are predicted to be periplasmically localized [43], whereas two others, NlpC and Spr, are predicted to be outer membrane lipoproteins. One or more of these may encode the tetrapeptide endopeptidase activity responsible for the release of meso-Dap-D-Ala.

Our analysis of the genetic linkage between mpaA and mppA also suggests that the Mtp-binding function has evolved twice within the γ-proteobacteria from the ancestral oligopeptide binding protein (Supplementary Figure S8). The fact that the MppA transporter binds tripeptide and tetrapeptide, but not pentapeptide or muropeptides, reflects its ancestry as OppA has a preference for tri- and tetra-peptides [44]. We expressed the pentapeptide or muropeptides, reflects its ancestry as OppA has MppA transporter binds tripeptide and tetrapeptide, but not binding protein (Supplementary Figure S8). The fact that the Glu epimerase encoded by yakfB, is adjacent to yk/C and also to genes for uncharacterized oppA-like binding proteins (Figure 5), suggesting perhaps that a murein binding function has evolved independently for a third time in this bacterial lineage.

In conclusion, we have in the present study determined the biochemical and structural properties of the zinc metallooxazinocarboxypeptidase MpaA and confirmed its likely role in utilization of Mtp as a nitrogen and carbon source. Our discovery that MppA is able to bind murein tetrapeptide as well as tripeptide, and the genetic conservation of mppA, mpaA and the L-Ala-D-Glu epimerase encoded by ycf/G together suggest that these genes function in a catabolic manner to allow E. coli to utilize exogenous murein-derived peptides as nutrients.

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REFERENCES

19 Reference deleted

AUTHOR CONTRIBUTION

Abbas Maqbool and Gavin Thomas designed the research. Abbas Maqbool and Mireille Hervé performed the experiments. All authors analysed the results of the experiments. Abbas Maqbool, Anthony Wilkinson and Gavin Thomas wrote the paper.

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SUPPLEMENTARY ONLINE DATA

MpaA is a murein-tripeptide-specific zinc carboxypeptidase that functions as part of a catabolic pathway for peptidoglycan-derived peptides in γ-proteobacteria

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Figure S1 Overproduction and purification of Ec_MpaA protein

Overproduction and purification of Ec_MpaA, as determined by Coomassie-Blue-stained SDS/PAGE. Lane M, prestained protein ladder; lane 1, whole-cell lysate from IPTG-induced BL21 cells carrying pAM6092 plasmid; lane 2, soluble cell lysate; lanes 3–7, Ec_MpaA protein purified by Ni2+–chromatography. The molecular mass in kDa is indicated on the left-hand side.

Table S1 Substrate specificity of Vh_MpaA


<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific activity (nmol · s⁻¹ · mg⁻¹)</th>
</tr>
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<tbody>
<tr>
<td>Mtp</td>
<td>190</td>
</tr>
<tr>
<td>L-Ala-γ-D-Glu-L-Lys</td>
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</tr>
<tr>
<td>MurNAc-tripeptide</td>
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</tr>
<tr>
<td>γ-D-Glu-meso-Dap</td>
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Figure S2 L-Ala-γ-D-Glu-meso-Dap hydrolysing activity of Ec_MpaA as a function of pH

The optimum pH for MpaA activity was evaluated by incubating 26 ng of MpaA with 0.5 mM 14C-labelled tripeptide in Tris/HCl buffer at pH from 7.2 to 9.0, for 30 min at 37°C, in a final volume of 25 μl.

† Correspondence may be addressed to either of these authors (email ajw@ysbl.york.ac.uk or gavin.thomas@york.ac.uk). The co-ordinates and experimental structure factors have been deposited in the RCSB PDB under accession code 4AXV.
Figure S3 Biophysical evidence of zinc binding to Vh_MpaA and dimerization of Vh_MpaA

(A) ES-MS spectrum of Vh_MpaA under denaturing conditions. The molecular mass of the unbound protein (U) is indicated at 26313 atomic mass units (amu) and in agreement with the theoretical mass of Vh_MpaA after removal of the His6 tag (26314 Da). (B) ES-MS spectrum of Vh_MpaA under native conditions in water. Peak (L) gives the molecular mass of Vh_MpaA bound to zinc and peak (D) gives the molecular mass of Vh_MpaA dimer bound to two zinc atoms. (C) A SEC-MALLS trace of Vh_MpaA showing the relative absorbance at λ=280 trace normalized to 1 (thin line) and the molar mass of the species present in the sample (thick line). The molecular mass of Vh_MpaA determined by MALLS (53 KDa) is found to be consistent with the theoretical mass of a dimer of Vh_MpaA in solution (52.5 kDa).

Figure S4 Incorporation of L-selenomethionine to Vh_MpaA

ES-MS spectrum of selenomethionine-labelled Vh_MpaA under denaturing conditions. The molecular mass of purified selenomethionine–Vh_MpaA is indicated at 28422 atomic mass units (amu) and is in agreement with the theoretical mass of Vh_MpaA after incorporation of two L-selenomethionine residues (28422.18 Da).
MpaA is a murein-tripeptide-specific zinc carboxypeptidase

**Figure S5** Sequence alignment of Ec_MpaA with its orthologue Vh_MpaA

Amino acid sequences of Ec_MpaA and Vh_MpaA were aligned in ClustalW. Two proteins share 54% sequence identity. Identical amino acids and conserved amino acids are indicated by stars and colons respectively. Amino acid numbers for both proteins are indicated on the right-hand side. The putative zinc-binding triad His^{49}–Glu^{52}–His^{157} of Ec_MpaA are highlighted.

**Figure S6** Structural superimposition of Vh_MpaA with other carboxypeptidases

The proteins were superimposed in PyMOL and are shown in cartoon representation with Vh_MpaA in green, duck CPD in cyan and human CPN in yellow.
Figure S7  Sequence alignment and topological superimposition of Vh_MpaA with other carboxypeptidase domain-containing proteins

Sequence alignment of Vh_MpaA, duck CPD, human CPN and Drosophila CPD based on an alignment of the three-dimensional structures of the proteins generated using ESPript. The residues involved in the dimer interface are underlined. The residues involved in hydrogen-bond interactions for stabilizing two subunits in a dimer are marked by red triangles.
MpaA is a murein-tripeptide-specific zinc carboxypeptidase

Figure S8 Phylogenetic tree based on the maximum likelihood method using MppA and OppA protein sequences

The amino acid sequences were collected from NCBI and aligned in ClustaW. The phylogenetic tree was made using MEGAS software. Clearly OppAs of the Vibrio and Enterobacteriaceae family fall into separate clusters, suggesting that they evolve independently from a common ancestor. Similarly, MppAs of Vibrio and Enterobacteriaceae fall into separate clusters suggestive of their independent evolution. Node support is provided by bootstrap values.

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