Peptidoglycan amidase MepA is a LAS metallopeptidase

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**Abstract:**

LAS enzymes are a group of metallopeptidases that share an active-site architecture and a core folding motif and have been named according to the group members lysostaphin, D-Ala-D-Ala carboxypeptidase and sonic hedgehog. *Escherichia coli* MepA is a periplasmic, penicillin insensitive murein endopeptidase that cleaves the D-alanyl-meso-2,6-diaminopimelyl amide bond in *E. coli* peptidoglycan. The enzyme lacks sequence similarity with other peptidases, and is currently classified as a peptidase of unknown fold and catalytic class in all major databases. Here, we build on our observation that two motifs, characteristic of the newly described LAS group of metallopeptidases, are conserved in MepA-type sequences. We demonstrate that recombinant *E. coli* MepA is sensitive to metal chelators and that mutations in the predicted Zn$^{2+}$ ligands H113, D120 and H211 inactivate the enzyme. Moreover, we present the crystal structure of MepA. The active site of the enzyme is most similar to the active sites of lysostaphin and D-Ala-D-Ala carboxypeptidase, and the fold is most closely related to the N-domain of sonic hedgehog. We conclude that MepA-type peptidases are LAS enzymes.

Key words: peptidoglycan, MepA, LAS, metallopeptidase
**Introduction:**

Peptidoglycan amidases are important for bacterial cell wall remodelling during cell growth and division (1). It has become clear in recent years that peptidoglycan hydrolases exist for nearly every amide linkage that occurs in bacterial cell walls (2). In many species of bacteria, the number of peptidolglycan hydrolases is even higher than the number of different amide linkages, pointing to a functional redundancy in peptidoglycan hydrolases or to a role of specific peptidoglycan hydrolases only at certain stages of the bacterial life cycle (2). Although the different peptidoglycan hydrolases have either no or no significant similarity at the sequence level, work in several laboratories has established that most of these peptidoglycan hydrolases are structurally related (3-6).

We have recently proposed to classify lysostaphin-type enzymes, metallopeptidases with specificity for D-Ala-D-Ala and sonic hedgehog as LAS enzymes (6). LAS enzymes contain a single, tetrahedrally coordinated Zn\(^{2+}\) in their active sites. Three Zn\(^{2+}\) ligands are conserved and occur in the order histidine, aspartate, histidine in the sequence. They are part of two motifs, H-x(3,6)-D and H-x-H (E-x-x-H in VanX-type enzymes), that are separated by 30 to 100 amino acids in the sequence. In the crystal structures, the histidine of the first motif coordinates the Zn\(^{2+}\) via the N\(_e\) atom and is oriented by a hydrogen bond from its N\(_d\) atom to a main chain carbonyl oxygen atom. The aspartate acts as a monodentate ligand, with one of its O\(_d\) atoms in contact with Zn\(^{2+}\) and with the other exposed to solvent in the active enzymes. The glutamate of the E-x-x-H motif of VanX-type enzymes and the first histidine of the H-x-H motif in all other LAS enzymes are close to the Zn\(^{2+}\) in the crystal structures, but never in direct contact with it, suggesting that these residues may act as the catalytic base. The histidine of the E-x(2)-H motif and the second histidine of the H-x-H motif contact the Zn\(^{2+}\) directly via their N\(_d\) atoms, and, except in VanX, are oriented by hydrogen bonds from their N\(_e\) atoms to varying hydrogen bond acceptors. The identity of the forth Zn\(^{2+}\) ligand differs...
between structures. It is a water molecule in the structures of D-Ala-D-Ala carboxypeptidase (PDB-accession code 1LBU) (P. Wery, Ph.D thesis), VanX (PDB-accession code 1R44) (7) and sonic hedgehog (PDB-accession code 1VHH) (8). In the case of the lysostaphin-type peptidase LytM (PDB-accession code 1QWY), a latent form of the enzyme was crystallized. In this structure, a poorly conserved asparagine residue from the N-terminal part of the enzyme contacts the metal via its Oδ. Biochemical data are consistent with a role of this residue as an “asparagine switch” (9).

Spatial superposition of LAS enzymes shows that the conservation of active-site architecture includes the presence of two additional residues that can be either glutamate or histidine in conserved locations. As these residues are not Zn²⁺ ligands, it has been speculated that they play a role as general base/general acid in the catalytic mechanism. Beyond the catalytic machinery, LAS enzymes share a core folding motif of four antiparallel β-strands with conserved topology and strand order 1 2 4 3 that is typically part of a larger central β-sheet with poorly conserved periphery. Outside the core folding motif, LAS enzymes are very diverse, making the similarity between different LAS families undetectable by PSI-BLAST similarity searches (6).

*E. coli* MepA is a periplasmic murein endopeptidase that is believed to play a role in the removal of murein from the sacculus and could also play a role in the integration of nascent murein strands into the sacculus (10). Mechanistically, the enzyme is an amidase that cleaves the alanyl-meso-2,6-diamino-pimelyl peptide bond that connects peptidoglycan strands in gram-negative cell walls (11). Partially purified preparations of *E. coli* MepA were reported to be sensitive to metal chelating agents, deoxyribonucleic acid and lipoteichoic acid, but insensitive to penicillin (12). Due to the low abundance of MepA in the *E. coli* periplasm, the previously reported purification procedure required large amounts of cells (12).

Homologues of *E. coli* MepA occur in many gram-negative bacteria, consistent with the universal presence of amide bonds between D-alanine and meso-2,6-diamino-pimelate in
these species (13). MepA-type enzymes have not been detected in gram-positive organisms (14), even though some of them, such as *B. subtilis*, contain alanyl-meso-2,6-diamino-pimelyl peptide bonds in their peptidoglycan (13). Consistent with the penicillin insensitivity of the *E. coli* enzyme, MepA-type peptidases are not related in sequence to the largest class of murein endopeptidases, the penicillin-binding proteins. MepA-type enzymes are poorly understood at the mechanistic level. Their fold and catalytic class have not been determined. In the MEROPS peptidase database, they are currently classified as family U6 (“unknown”) (14).

In this communication, we show that MepA-type peptidases are LAS enzymes. We demonstrate that the recombinant *E.coli* enzyme is inhibited by Zn$^{2+}$ chelators, and that mutations in any of the signature sequence residues strongly reduce or abolish activity. Moreover, we present the crystal structure of *E.coli* MepA to prove that the fold and active site architecture of the enzyme are consistent with the classification as a LAS enzyme.
Experimental procedures:

Cloning, expression, and protein purification: Standard PCR techniques were used to amplify \textit{mepA} from genomic DNA of \textit{E.coli} XL-1Blue. The amplified DNA fragment was cloned into pET15b with restriction enzymes \textit{NcoI} and \textit{XhoI} and the construct was checked by double strand sequencing. For protein expression, the plasmid was transformed into \textit{E.coli} BL21(DE3) cells. Bacteria were initially grown in 37° C to an \textit{OD}_{600} of 0.5 - 0.8, induced with 0.2 mM IPTG and then grown for up to three hours in 25° C. For selenomethionine containing protein, the plasmid was transformed into the methionine auxotroph \textit{E. coli} strain B834(DE3). Cells were cultured in 1 liter medium in 37° C to an \textit{OD}_{600} of 1.0 and additionally up to 6 hours after induction with 0.5 mM IPTG, according to the published protocol (15).

Cells were harvested and resuspended in buffer A (50 mM Tris, pH 7.5). After sonication and high-speed centrifugation at 40,000 g, the supernatant was fractionated with ammonium sulfate. The material precipitating between 35 \% and 60 \% saturation was dissolved in buffer A and dialyzed overnight against 100 volumes of buffer A with one buffer change. The dialyzed supernatant was applied to a DEAE Sepharose FF column (Amersham Pharmacia) equilibrated with buffer A and the flow-through fractions containing MepA were collected and subsequently applied on SP Sepharose column (Sigma). After extensive washing of the column with 100 mM NaCl/buffer A, the protein was eluted with 200 mM NaCl/buffer A. The pooled fractions with MepA were concentrated to 1 ml (YM-10 Centricons) and subjected to a Sephacryl S-300 (Amersham Biosciences) gel filtration column in 10 mM Tris-HCl (pH 7.5)/100 mM NaCl. The pure MepA was dialyzed overnight against 100 volumes of 5 mM Tris-HCl (pH 7.5) and concentrated to 10 – 15 mg/ml (Amicon 10 kDa cut-off regenerated cellulose fibers).
**Crystallization:** All crystals were grown at room temperature (21° C) by sitting drop vapor diffusion with 0.5 ml of reservoir buffer. Orthorhombic, monoclinic and triclinic crystals could be grown.

Orthorhombic crystals appeared after four weeks by mixing equal amounts of reservoir buffer containing 0.2 M ammonium acetate, 0.1 M tri-sodium citrate dihydrate pH 5.69, 30% w/v Polyethylene Glycol 4000 and 10 mg/ml protein solution in 5 mM Tris-HCl (pH 7.5). One asymmetric unit consisted of two molecules. For cryoprotection crystals were soaked in the mixture containing 85% reservoir buffer and 15% PEG 400 (Tab. I).

Monoclinic crystals were grown against a reservoir containing 30% w/v Polyethylene Glycol Monomethyl Ether 5000, 200 mM ammonium sulfate, 100 mM MES pH 6.5, by mixing 2 µl of the reservoir buffer with 2 µl protein solution (15 mg/ml ) and 0.4 µl 1,4 – butanediol. Crystals with two monomers of MepA in the asymmetric unit appeared within 3 – 4 days. They could be flash cryocooled directly from the mother liquor (Tab. II).

Triclinic crystals were grown under the same conditions as monoclinic ones if the additive 1,4-butanediol was omitted from the drop, by mixing 2 µl of the reservoir buffer with 2 µl protein solution (10 mg/ml ). Crystals contained 6 molecules per asymmetric unit, and could be reproduced also with the selenomethionine version of the protein (Tab. II).

**Structure determination:** All datasets were collected at beamline BW6 at DESY synchrotron in Hamburg (Germany). Fluorescence scanning showed that the orthorhombic and triclinic forms contained Zn$^{2+}$, whereas Zn$^{2+}$ was unexpectedly absent from the monoclinic form.

The orthorhombic crystal form was solved by multiple anomalous diffraction (MAD) at the Zn$^{2+}$ edge. Three highly redundant datasets with both Friedel mates were collected to a resolution of about 2.6 Å (Tab. I), and the dataset at the remote wavelength was then extended in a final pass with long exposures to a resolution of 1.9 Å. AUTOSHARP (16) was used to identify the two Zn$^{2+}$ sites and to calculate initial phases with an overall figure of merit of
0.57 to 2.6 Å that were subsequently improved by SOLOMON (17). These phases combined with the high resolution dataset served as an input for ARP/WARP (18) that delivered an approximately 50% complete model (without sequence assignment) for subunit A, and almost no model for subunit B. No further building was attempted in this crystal form.

Two copies of the partial model for subunit A were subsequently located in the monoclinic crystal form using MOLREP (19) and used to calculate starting phases for an ARP/WARP-run at 1.4 Å resolution, that yielded near-complete models and a confident sequence assignment for both monomers in this crystal form.

The model from the monoclinic crystal form was then used to solve the triclinic crystal form, again by molecular replacement using MOLREP (19). The MOLREP search for six monomers worked with default settings, probably because of the high quality of the model from the monoclinic crystal form and because of the presence of pseudotranslation vectors relating several monomers in identical or near-identical orientations. As the success of the molecular replacement procedure was not expected at the stage of data collection, we had collected anomalous data at both the Zn$^{2+}$ and Se edges for a selenomethionine crystal of this form. With the help of the model phases, the Zn$^{2+}$ and Se scatterers could be identified with excellent signal in anomalous difference Fourier maps. This assignment confirmed Zn$^{2+}$ in the active sites, identified three additional Zn$^{2+}$ sites, and confirmed all selenium sites predicted by the model, strongly supporting the sequence assignment. Heavy atom sites were then used to calculate a model-independent map with MLPHARE (20), and this map was improved by combined solvent flattening and six-fold averaging with DM (20) using model-derived NCS operators and a model-derived averaging mask. The resultant averaged map was very clear and allowed to model differences between the triclinic and monoclinic forms with confidence.

Structure refinement: The orthorhombic crystal form proved unexpectedly difficult to refine even with structural information from the other crystal forms. We were unable to lower the free R factor significantly below 30% (for 1.9 Å data), suggesting that changes or more
likely disorder in the poorly defined subunit B in this crystal form are not adequately described by the model.

The monoclinic form has well-defined electron-density for both subunits, and could be satisfactorily refined with REFMAC (21) to an effective resolution of 1.4 Å with good stereochemistry (Tab. II). The final model comprises residues 20 to 244 and 260 to 274 for subunit A, residues 23 to 244 and 259 to 272 for subunit B, 560 water molecules and 9 sulfate anions from the crystallization buffer. The assignment of one molecule of 1,4-butanediol from the crystallization buffer is very tentative.

The triclinic form at 2.4 Å resolution was refined with CNS (22) and with REFMAC (21) without NCS restraints in the final stage. Models are similarly complete as for the P21 form, but all six copies of MepA contain Zn$^{2+}$ in their active sites. Three additional Zn$^{2+}$ ions are located at the interfaces between monomers that are related by local two-fold symmetry. Sulfates from the buffer are bound to sites near the active centers that also accommodate sulfates in the P21 form.

*Generation and purification of mutants:* MepA mutants H113A, D120A, H209A and H211A were obtained by PCR-based site-directed mutagenesis according to the Stratagene protocol with Pfu Turbo DNA polymerase (Stratagene). The engineered MepA variants were all soluble and expressed at least at the same level as wild type MepA. For their purification, the same protocol as for the wild type MepA could be used. Alternatively, a simplified procedure was applied, with periplasmic fraction isolation as a first step. This included growing cells at 30°C to an optical density (OD$_{600}$) of 0.6, induction with 0.1 mM IPTG and further incubation for two hours at the same temperature. Harvested cells were then incubated in Buffer B (20% sucrose, 30 mM Tris-HCl pH 7.5) for 10 min. at room temperature and centrifuged to collect cells. Osmotic shock was accomplished by the rapid addition of 5 mM MgSO$_4$ to washed cells and gentle mixing. The sample was then incubated on ice for an additional 5 minutes and centrifuged at 12,000 x g to remove the spheroplasts and intact cells.
from the supernatant, which was the periplasmic fraction. MepA mutants were the dominant band on SDS polyacrylamide gel in this solution. For final purification, the solution was subjected to ion-exchange chromatography on SP-Sepharose resin as described above for native MepA. After dialysis to 5 mM Tris-HCl buffer (pH 7.5), the protein could be concentrated and stored in –70° C.

**Peptidoglycan isolation:** Gram-negative peptidoglycans were isolated according to (23) with modifications. Briefly, *Pseudomonas putida* were grown in 30° C on LB medium and harvested at OD 600 of ~ 1.5. The cell pellet was resuspended in cold water and added slowly to 8% boiling SDS. Samples were boiled for another 30 min and then incubated 12 hours at room temperature. Polymeric peptidoglycan was recovered by high-speed centrifugation (100 000 x g) and washed several times to remove traces of SDS. Harvested, insoluble material was then treated with α-amylase in 37° C and subsequently with pronase in 60° C. After dilution with water, the sacculi were washed several times and finally resuspended in 10 mM Tris-HCl buffer (pH 7.5).

**Zymography:** The activity of MepA was detected on 12% polyacrylamide-sodium dodecyl sulfate gels containing 0.05% *P.putida* PCM 2124 murein sacculi, according to the described protocol (24) with modifications. Before the gels were cast, peptidoglycans were homogenized by sonication. Samples containing whole cell supernatants (cytoplasmic and periplasmic fraction) were prepared from cells grown as described above. The supernatants were loaded on the gel in amounts corresponding to 5 µg of whole protein content. In case of purified proteins, about 0.2 µg of protein was loaded per lane. Electrophoresis was done in the cold room. Gels were incubated in renaturation buffer (0.5% Triton X-100, 5 mM MgCl₂, 20 mM Bis-Tris buffer, pH 6.0) for 16 hours, with one buffer exchange. After incubation, the zymograms were rinsed with Milli-Q water, stained with 0.1% methylene blue in 0.01 % KOH and destained in deionized water. Lytic zones indicating the enzyme hydrolytic activity appeared as clear zones on the blue background. For testing the dependence of MepA activity
on pH of the buffer, zymography gels were incubated in renaturation buffers of different pH, using 20 mM sodium citrate buffer for pH 4 and 5, and 20 mM Tris-HCl buffer for pH 7, 8 and 9.
Results:

LAS motifs in MepA enzymes: Our recent definition of LAS enzymes (6) prompted us to search for additional protein families that could belong to this group. Therefore, we searched alignments of poorly characterized protease families for the H-x(3,6)-D and H-x-H motifs that are characteristic for LAS enzymes and found that both are present in MepA peptidases. We further inferred from consensus secondary structure predictions that the characteristic histidines and the aspartate occurred in secondary structure contexts that would be expected for LAS enzymes. These observations together with the peptidoglycan amidase activity of MepA suggested that MepA enzymes could be an additional family in the LAS group of peptidoglycan amidases. Based on the sequence data alone, this conclusion remained very tentative. First, the similarity between MepA enzymes and other LAS enzymes could not be substantiated by iterative PSI-BLAST searches. Second, many other residues, including serines, cysteines and histidines outside the H-x(3,6)-D and H-x-H motifs are conserved among MepA proteins, leaving room for many other hypotheses about potential catalytic dyads or triads. Therefore, we decided to test our prediction about the similarity of MepA amidases and LAS enzymes experimentally.

MepA purification: The *E. coli* mepA gene was amplified by PCR and various *E. coli* expression constructs were prepared. A number of attempts to express the protein in the cytoplasm, both alone and as a fusion protein, yielded large amounts of insoluble protein. However, when overexpressed MepA was targeted to the periplasm by its own native leader sequence, soluble protein in moderate yield could be recovered from whole cell lysates. As recombinant overexpression of MepA rendered cells prone to lysis, direct isolation of MepA from the periplasm was not possible. Nevertheless, mass spectrometry analysis of MepA purified from whole cell lysates showed that the overexpressed protein had a mass of 28295 Da, corresponding to the protein after cleavage of the predicted 19 residue periplasmic leader.
sequence. The protein was purified as described in Experimental Procedures, and obtained in an overall yield of approximately 2 milligrams per litre of culture.

Zymography assay for MepA activity: With sufficient amounts of enzyme in hand, we next attempted to develop a simple, non-radioactive assay for MepA activity. Previous experience with peptidoglycan peptidases suggested that the activity could be conveniently assayed by zymography with purified peptidoglycans polymerized as a substrate into denaturing SDS-PAGE gels. As E. coli peptidoglycan is difficult to isolate in quantity, we settled for peptidoglycan from Pseudomonas putida that is similar or identical to E. coli peptidoglycan and gave clear signals for MepA activity (Fig. 1A).

Inhibitor sensitivity of MepA: Our hypothesis about the similarity of MepA to LAS enzymes suggested that the enzyme should be sensitive to the metal chelating agents EDTA and 1,10-phenanthroline, but not to serine and cysteine peptidase inhibitors such as PMSF and E-64. This turned out to be the case. Zn$^{2+}$ at 10 mM concentration was also inhibitory to the enzyme, consistent with previous reports about the inhibition of zinc-dependent metallopeptidases by unphysiologically high concentrations of Zn$^{2+}$ (9,25) (Fig. 1B).

Role of conserved residues in the H-x-(3,6)-D and H-x-H motifs: The hypothesis further implied that the conserved histidines and the aspartate in the H-x(3,6)-D and H-x-H motifs should be catalytically important (9,26). To test this assumption for MepA, each of the four conserved residues was separately mutated to alanine. E. coli cells overexpressing any of the four mutants were significantly less prone to lysis than cells overexpressing the wild-type protein. As described in Experimental Procedures, this allowed the isolation of periplasmic fractions and thus considerably simplified the purification procedure. More importantly, it suggested a difference in activity between the wild-type and mutant proteins. This difference was also clearly apparent from the zymography results. As the mutants were expressed in mepA+ background, the preparations of mutant protein were contaminated with traces of
wild-type protein, which could be responsible for the residual activity that was observed in these lanes (Fig. 1C).

**Determination of the MepA structure:** The definition of LAS enzymes requires the presence of a conserved catalytic metal center in the context of a core folding motif of four antiparallel β-strands with characteristic topology. To investigate the fold and detailed active-site architecture of MepA enzymes, we decided to solve the crystal structure of *E. coli* MepA. To increase the chances that the structure of the enzyme in the crystal would be physiologically relevant, we first tested the dependence of the enzyme activity on the pH of the buffer. Although the zymography requires proper refolding and activity, we can be sure from the results in Fig. 2 that the enzyme is active at least in the range from pH 5 to pH 8. Gratifyingly, the enzyme could be crystallized in three different crystal forms in this pH range (Experimental Procedures). Scans of the X-ray fluorescence showed that the triclinic and orthorhombic crystal forms contained Zn$^{2+}$. Surprisingly, fluorescence scans of the best-diffracting monoclinic form demonstrated that Zn$^{2+}$ was entirely absent from these crystals. They were grown from a different batch of protein, and we suspect that the Zn$^{2+}$ was already lost at the stage of protein purification, because no exogenous Zn$^{2+}$ was added to the chromatography buffers.

The crystal structures were solved as described in Experimental Procedures, exploiting information from all three crystal forms at different stages of the structure solution process. In all forms, MepA was present as a dimer, and one dimer each was present in the P21 and P21212 asymmetric units, whereas the P1 form contained three dimers that were essentially translationally related. Two crystal forms were fully refined: the P1 form with Zn$^{2+}$ and the P21 form without Zn$^{2+}$ in the active center. The effects of the presence or absence of Zn$^{2+}$ are confined to the region around the active site and to the dimer-dimer interface. Although remote from both the active sites and the dimer interface, there is also substantial variation in the order and location of the C-terminal helix downstream of the disordered region of MepA.
If this region is excluded from the superposition, monomers superimpose with an rmsd of ~0.5 Å (Tab. I and II).

*MepA active center:* The crystal structure of the Zn\(^{2+}\) containing form of MepA shows an active site that fits the LAS consensus perfectly well. The Zn\(^{2+}\) is tetrahedrally coordinated by histidine 113 and aspartate 120 of the H-x-(3,6)-D motif and histidine 211, the second histidine of the H-x-H motif. H113 contacts the Zn\(^{2+}\) with its N\(\varepsilon\), and thus has to be protonated on the N\(\delta\) that donates a hydrogen bond to the main chain carbonyl oxygen of methionine 98. For histidine 211, it is the N\(\delta\) that contacts the metal, thus the N\(\varepsilon\) is protonated and indeed the structure shows the N\(\varepsilon\) within hydrogen bonding distance and properly oriented to donate a hydrogen bond to aspartate 118 O\(\delta\), a strictly conserved residue in the MepA family (Fig. 3).

As MepA is active at the crystallization pH, we would have expected a solvent molecule as the forth Zn\(^{2+}\) ligand. This is clearly not the case in our crystal structure, where histidine 110 N\(\delta\) plays this role. Although the Zn\(^{2+}\) site resembles a structural Zn\(^{2+}\) site, the analogy with other LAS enzymes suggests that histidine 110 would be displaced by a substrate. Several features of the MepA crystal structure support this idea. First, histidine 110 appears more mobile than the other histidine zinc ligands, because its imidazole side chain is not fixed in space by a hydrogen bond to an ancillary residue. Second, histidine 110 is defined only moderately well in electron density, and anchored on a loop that is among the most flexible regions in the structure. Third, this residue and the loop that anchors it are among the residues in the MepA structure that differ most between the Zn\(^{2+}\) bound and Zn\(^{2+}\) free forms.

Histidines 206 and 209 are in close proximity to the active site, but they do not coordinate the Zn\(^{2+}\) directly. Superposition shows that these two residues are spatially equivalent to two histidine residues that are present both in the structures of LytM (PDB-accession code 1QWY) and D-Ala-D-Ala-carboxypeptidase (PDB-accession code 1LBU) and have been considered as possible general base residues. In proximity to these two histidines, a
sulfate molecule from the crystallization buffer is bound to all copies of MepA, both in the Zn$^{2+}$ bound and Zn$^{2+}$ free crystal forms, where it apparently balances some of the charge of the histidines (Fig. 3).

**MepA fold:** The MepA fold is arranged around a central, six-stranded, mixed β-sheet. The four central strands of this sheet are antiparallel and connected as expected for LAS enzymes, with strand order 1,2,4,3 in LAS nomenclature and +1, +2x, -1 connectivities in Richardson nomenclature (27,28). Active-site residues are anchored on this core motif as expected for LAS enzymes. Quantitative DALI (29) structure comparisons of MepA with all proteins in the Protein Data Bank confirm the similarity to LAS enzymes. The highest DALI scores are obtained for the superposition of MepA with the LAS proteins sonic hedgehog (DALI-score 8.3) and D-Ala-D-Ala-carboxypeptidase (DALI-score 5.2). The similarity between MepA and these two LAS enzymes goes beyond the previously defined consensus, and includes a helix upstream of strand 3 and another helix downstream of strand 4. The central β-sheet and these two helices are the most rigid parts of the MepA structure. High B-factors are found for residues on the periphery, especially residues 123 to 158 and for the last ~60 residues. Within this stretch of 60 residues at the C-terminus, around 20 residues between two helices are disordered in all crystal forms (Fig. 4A).

**MepA disulfide topology:** In the crystal structures, and presumably also in the *E. coli* periplasm, MepA contains three disulfide bonds per protomer. All three cysteine disulfide bridges are strategically placed to hold the otherwise very loosely packed C-terminal residues in place. This is particularly true for the isolated helix at the C-terminus, which lies downstream of the disordered region in the structure (Fig. 4B).

**MepA dimers in all crystal forms:** In all crystal forms, MepA packs as a dimer and buries 1300 Å$^2$ of mostly hydrophilic surface. The extent of the contact area, 650 Å$^2$, places the interaction between MepA monomers in the twilight zone of biologically significant homodimer interactions (30). The dimerization in the crystals brings two arginine aspartate
pairs into close proximity. This favorable dipole-dipole interaction appears to compensate for the penalty of stacking the two arginine guanidinium groups. In the Zn$^{2+}$ containing triclinic form, a Zn$^{2+}$ that is distinct from the catalytic Zn$^{2+}$ is bound at the dimer interface. This Zn$^{2+}$ is tetrahedrally coordinated by aspartates 147 and histidines 150 from both monomers. In the Zn$^{2+}$ free form, a water molecule takes the place of the Zn$^{2+}$ and hydrogen-bonds to both aspartates. The two histidines move out and capture two additional water molecules.

**MepA from E. coli versus MepA from other species:** Sequence alignments reveal that residues aspartate 147 and histidine 150 that form the Zn$^{2+}$ binding site in the *E. coli* MepA crystals are not conserved in other species. Mapping of MepA alignment scores on the molecular surface with Consurf (31) reveals an interesting pattern: the most conserved surface patches are the walls of the active-site cleft, and two patches on the surface that are connected to the active-site cleft and run essentially perpendicular to it (Fig. 5). The branched structure of the conserved surface patches is reminiscent of the branched structure of the MepA substrate, and thus it appears likely that the conservation pattern maps out the active site. This interpretation is also consistent with the location of a trapped sulfate in the crystal that is likely to exploit a binding site for a negatively charged carboxylate of the substrate.
Discussion:

*MepA latency:* The active site in MepA with its tetrahedrally coordinated Zn$^{2+}$ fits the LAS consensus, with one important exception: there is no direct equivalent of histidine 110 in any of the LAS structures. The place of the Nδ of this residue is taken by a water molecule in the structures of D-Ala-D-Ala carboxypeptidase and sonic hedgehog, and by the Oδ of an asparagine residue in the structure of latent LytM. Based on the analogy with the “cysteine switches” that keep matrix metallopeptidases (MMPs) proteolytically inactive (32), we have previously proposed that the asparagine in the LytM structure plays a similar role as an “asparagine switch”. By analogy with the MMPs, we have further shown that proteolytic removal of a LytM “proregion” activates the enzyme, at least *in vitro* (9). Removal of a proregion as the activation mechanism *in vivo* remains to be proven for LytM, and appears highly unlikely in MepA case for a number of reasons. First, the proteolytic cut in MepA would have to be very precise, since the occluding histidine 110 occurs only three residues upstream of the first Zn$^{2+}$ ligating histidine in the sequence. Second, the cut would not separate the N- and C-terminal cleavage products that would remain anchored to each other via a disulfide linkage. Third, even if the N-terminal fragment would dissociate, it would take part of the LAS core folding motif with it, and most probably destroy the integrity of the enzyme. Thus, it appears that despite the high conservation of the active site in LAS enzymes, there is substantial variation in the identity and role of the forth Zn$^{2+}$ ligand. Examples have so far been found for the absence of a forth amino acid ligand to the Zn$^{2+}$, for the presence of an amino acid ligand that is likely to be displaced by a substrate, and for amino acid ligands that require the proteolytic removal of the anchoring profragments for their activation.

*Convergent or divergent evolution of LAS enzymes?* The high similarity between LAS enzymes at the structural level (Fig. 6) contrasts sharply with the lack of sequence similarity between different families of LAS enzymes even in iterative PSI-BLAST searches and raises the question whether the similar active sites in different families of LAS enzymes have arisen
by convergent or divergent evolution. Several arguments support the existence of a common ancestor of LAS enzymes. First, the active sites occur in the context of similar folds. The MepA structure shows that the similarity between some LAS enzymes goes beyond the previously described consensus and includes two helices that are spatially separated from the active site by the central $\beta$-sheet and are thus unlikely to be required for function (Fig. 6). Second, the peptidoglycan amidase activity of MepA is consistent with the role of other LAS enzymes in bacterial cell wall hydrolysis, again reinforcing with idea of a common ancestor. So far, the N-domain of sonic hedgehog is the only LAS protein without role in peptidoglycan hydrolysis, and indeed without any enzymatic activity, since the signaling function of sonic hedgehog is not mediated by proteolysis (33).

With the classification of MepA as a LAS enzyme, five protein families, namely lysostaphin-type enzymes, D-Ala-D-Ala amino- and carboxypeptidases, MepA like enzymes and the N-domains of sonic hedgehog proteins are now known to belong to the LAS superfamily. It remains to be seen whether more families of LAS enzymes can be found, and, if so, whether these families would also have a role in peptidoglycan hydrolysis.
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References:

1. Holtje, J. V., and Heidrich, C. (2001) Biochimie 83, 103-108
2. Smith, T. J., Blackman, S. A., and Foster, S. J. (2000) Microbiology 146 (Pt 2), 249-262
3. Anantharaman, V., and Aravind, L. (2003) Genome Biol 4, R11
4. Bateman, A., and Rawlings, N. D. (2003) Trends Biochem Sci 28, 234-237
5. Rigden, D. J., Jedrzejas, M. J., and Galperin, M. Y. (2003) Trends Biochem Sci 28, 230-234
6. Bochtler, M., Odintsov, S., Marcyjaniak, M., and Sabala, I. (2004) Protein Sci 13(4):854-61.
7. Bussiere, D. E., Pratt, S. D., Katz, L., Severin, J. M., Holzman, T., & Park, C. H. (1998) Mol Cell 2, 75-84
8. Hall, T. M., Porter, J. A., Beachy, P. A., and Leahy, D. J. (1995) Nature 378, 212-216
9. Odintsov, S., Sabala, I., Marcyjaniak, M., and Bochtler, M. (2004) J Mol Biol 335, 775-785
10. Goodell, E. W., and Schwarz, U. (1983) J Bacteriol 156, 136-140
11. Kitano, K., Tuomanen, E., and Tomasz, A. (1986) J Bacteriol 167, 759-765
12. Tomioka, S., Matshuhashi, M. (1978) Biochem. Biophys. Res. Commun. 84, 978-984
13. Schleifer, K. H., and Kandler, O. (1972) Bacteriol Rev. 36, 407-477
14. Rawlings, N. D., O’Brien, E., and Barrett, A. J. (2002) Nucleic Acids Res 30, 343-346
15. Budisa, N., Steipe, B., Demange, P., Eckerskorn, C., Kellermann, J., & Huber, R. (1995) Eur. J. Biochem. 230, 788-796
16. Bricogne, G., Vonrhein, W., Paciorek, C., Flensburg, M., Schiltz, E., Blanc, P., Rovesi, R., and Evans, M. G. (2002) Acta Cryst. A58(Supplement), C239
17. Abrahams, J. P., and Leslie, A. W. G. (1996) Acta Cryst. D52, 30-42
18. Morris, R. J., Perrakis, A., and Lamzin, V. S. (2003) Methods Enzymol 374, 229-244
19. Vagin, A., and Teplyakov, A. (2000) Acta Crystallogr D Biol Crystallogr 56 Pt 12, 1622-1624
20. Collaborative Computational Project Number 4. (1994) Acta Crystallogr D Biol Crystallogr 50, 760-763
21. Murshudov, G. N., Vagin, A. A., and Dodson, E. J. (1997) D53, 240-255
22. Brunger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) Acta Crystallogr D Biol Crystallogr 54 (Pt 5), 905-921
23. Glauner, B. (1988) Anal. Biochem. 172, 451-464
24. Bernadsky, G., Beveridge, T., and Clarke, A. (1994) J Bacteriol 176, 5225-5232
25. Kessler, E., Safrin, M., Abrams, W. R., Rosenbloom, J., and Ohman, D. E. (1997) J Biol Chem 272, 9884-9889
26. Gustin, J. K., Kessler, E. & Ohman, D. E. (1996) J Bacteriol. 178, 6608-6617
27. Richardson, J. S., and Richardson, D. C. (2002) Proc Natl Acad Sci U S A 99, 2754-2759
28. Richardson, J. S. (1977) Nature 268, 495-500
29. Holm, L., and Sander, C. (1995) Trends Biochem Sci 20, 478-480
30. Jones, S., and Thornton, J. M. (1996) Proc Natl Acad Sci U S A 93, 13-20
31. Glaser F, P. T., Paz I, Bell RE, Bechor-Shental D, Martz E, Ben-Tal N. (2003) Bioinformatics 19, 163-164
32. Becker JW, M. A., Rokosz LL, Axel MG, Burbaum JJ, Fitzgerald PM, Cameron PM, Esser CK, Hagmann WK, Hermes JD, et al. (1995) Protein Sci. 4(10), 1966-1976
Abbreviations:

PMSF (phenyl methyl sulfonyl fluoride); E64 (trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane)
Figure legends:

Fig. 1:
Zymography assay of peptidoglycan hydrolysis activity. (A) Comparison of endogenous MepA activity (lane 2) with the activity of MepA overexpressing cells (lane 3) and of the purified MepA preparation (lane 4). 5 µg of total protein from whole cell extracts were loaded in lanes 2 and 3, and 0.2 µg of purified protein were loaded in lane 4. 0.1 µg chicken egg lysozyme in lane 1 were applied as a positive control for peptidoglycan hydrolysis. (B) Inhibitor sensitivity of MepA. Samples were renatured and stained in standard buffers without additions (lane 1), or with addition of the indicated protease inhibitors (lane 2-6). (C) Comparison of the activity of wild-type MepA (lane 1) with the activity of MepA mutants (lanes 2-5).

Fig. 2:
MepA activity in the zymography assay as a function of the pH of the renaturation buffer. The observed activity is a combination of refolding efficiency and enzyme activity at the different pH values.

Fig. 3:
Stereo superposition of the active sites of MepA (black lines), LytM (grey, continuous lines) and D-Ala-D-Ala carboxypeptidase (grey, dotted lines). The labeling of residues is consistent with the labeling in Fig. 2 of a previous publication on LAS enzymes (6). In *E. coli* MepA, residues H113 (“1”), D120 (“2”), H211 (“3”), M98 (“4”), D118 (“5”), H206 (“6”), H209 (“7”) and H110 (“8”) have been drawn. For MepA, the triclinic form with Zn$^{2+}$ in the active site has been used. In this crystal form that contains 6 monomers in the asymmetric unit, H209 is found in two conformations, and both are presented in this figure.
Fig. 4:
(A) PyMol ribbon representation of the MepA structure. Residues 20 and 270 at the N- and C-terminus of the structure and residues 244 and 261 upstream and downstream of the disordered part of MepA are marked. The four β-strands that form the core MepA folding motif are labeled according to LAS nomenclature. The catalytic Zn$^{2+}$ is shown as a pink sphere. Active-site residues and the six cysteines in MepA are presented in ball-and-stick representation. (B) Schematic diagram of the disulfide linkages in MepA.

Fig. 5: PyMol stereo representation of the MepA dimer in the crystals. The surface of the monomer at the bottom is colored green, and the surface of the monomer at the top is colored according to sequence conservation in the MepA family. Conservation scores were calculated with Consurf (31). Most conserved surface patches are shown in blue, and least conserved surface patches are in red.

Fig. 6: Comparison of (A) MepA, (B) D-Ala-D-Ala carboxypeptidase, (C) the truncated, active form of LytM, (D) the N-domain of sonic hedgehog. All structures are presented as Cα-traces in stereo. The core folding motifs and two helices common to MepA, sonic hedgehog and D-Ala-D-Ala carboxypeptidase are drawn as continuous, bold lines. The rest of the Cα-traces is presented as dotted lines. The catalytic Zn$^{2+}$ is shown as a sphere, and important catalytic residues (excluding the occluding Zn$^{2+}$ ligand) are shown in ball-and-stick representation. The numbering of β-strands is according to LAS nomenclature.
**Tables:**

**Tab. I:**

| Space group          | P21212                     |
|----------------------|----------------------------|
| a x b x c(Å)         | 66.53 x 165.29 x 45.63     |
| Wavelength (Å)       | 1.0500 1.2780 1.2820       |
| Total reflections    | 118 625 153 357 157 931    |
| Unique reflections   | 30 719 28 964 29 129       |
| Resolution (Å)       | 20.0 - 2.5 20.0 - 2.6 20.0 - 2.6 |
| Completeness (%)     | 91.5 (84.4) 97.3 (96.8) 97.6 (97.8) |
| I/σ                  | 24.7 (7.9) 28 (7.7) 28.0 (7.1) |
| Rsym (%)             | 6.9 (15.1) 7.7 (18.0) 7.5 (20.8) |
| Number of Zn sites   | 2                          |
| FOM (before solv)    | 0.57 (SHARP, 20.0 - 2.6 Å) |
| FOM (after solv)     | 0.88 (SOLOMON, 20.0 - 2.6 Å) |

**Tab. I:** Data collection and phasing statistics for the P21212 crystal form.
**Tab. II:** Data collection and refinement statistics for the P21 and P1 crystal forms.

| Space group | P21 | P1 |
|-------------|-----|----|
| a x b x c(Å) | 45.95 x 115.14 x 49.15 | 35.61 x 77.98 x 127.66 |
| α x β x γ (°) | 90 x 94.08 x 90 | 93.15 x 95.93 x 90.75 |
| Wavelength (Å) | 1.05 | 0.9793 |
| Total reflections | 314 504 | 103 423 |
| Unique reflections | 91 010 | 52 369 |
| Resolution range (Å) | 20.0 - 1.4 | 20.0 - 2.4 |
| Completeness (%) (last shell) | 91.3 (94.3) | 97.1 (93.5) |
| I/σ | 10 (5.5) | 14.1 (4.0) |
| Rsym (%) (last shell) | 3.6 (12.9) | 3.9 (15.1) |
| B(iso) from Wilson | 14.2 | 30.6 |

Refinement statistics

| Resolution (Å) | 20.0 - 1.4 | 20.0 - 2.4 |
| Protein atoms (excluding H) | 3728 | 11 217 |
| 1,4-butanediol | 1 | - |
| Zn²⁺ | - | 9 |
| Sulfate ions | 9 | 6 |
| Water molecules | 560 | 63 |
| R-factor (%) | 18.4 | 23.4 |
| R-free (%) | 20.6 | 26.6 |
| Rmsd bond distance (Å) | 0.013 | 0.011 |
| Rmsd angles (°) | 1.514 | 1.615 |
| Average B-factor (Å²) | 13.4 | 24.9 |
| Ramachandran core (%) | 89.4 | 89.1 |
| Ramachandran allowed (%) | 9.8 | 13.4 |
| Ramachandran add. allowed (%) | 0.8 | 0.6 |
| Ramachandran disallowed (%) | 0.0 | 0.0 |
Figures:

Fig. 1:

A

B

C

Fig. 2:
Peptidoglycan amidase MepA is a LAS metallopeptidase
Malgorzata Marcyjaniak, Sergey G. Odintsov, Izabela Sabala and Matthias Bochtler

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