Mutational Analysis of Peptidoglycan Amidase MepA†

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ABSTRACT: Murein endopeptidase A (MepA) from *Escherichia coli* is a periplasmic peptidoglycan amidase that cleaves D,D amide bonds between D-alanine and *meso*-2,6-diaminopimelic acid in *E. coli* peptidoglycan. MepA and its homologues in other proteobacteria share overall structural similarity with D-Ala-D-Ala metallopeptidases and local similarity around the active site with lysostaphin-type enzymes, which has prompted the classification of these enzymes as LAS enzymes. LAS enzymes contain a single divalent cation in the active site, which is tetracoordinated in the crystal structures. Three of the metal ligands are identical in all structures, but the identity of the fourth ligand varies. Two residues in proximity to the metal might act as a general acid/base, but their role is not clear. Here, we report a new MepA expression system, which allows the separation of MepA variants from the endogenous wild-type enzyme, and an HPLC assay with a defined peptidoglycan fragment, which allows assessment of MepA activity without a refolding step. We find that the conserved metal ligands are required for folding (D120) or catalysis (H113, H211). Separate mutations of the candidate catalytic residues H206 or H209 and of the "fourth" metal ligand H110 are tolerated for folding but drastically reduce activity. Mutation of residue W203 to aspartate impairs substrate binding.

Murein endopeptidase A (MepA) from *Escherichia coli* is a nonessential, periplasmic enzyme that acts as an autolysin and cleaves the cell walls of the producer organism, probably during cell wall remodeling, growth, or recycling (*1*). The enzyme is specific for amide bonds that cross-link the linear peptides that are attached to *N*-acetylmuramic acid. In *E. coli*, these amide bonds connect the carboxylate group of D-alanine residue to the amino group of *meso*-2,6-diaminopimelic acid (m-DAP) that is linked to the D-configured stereo center (Figure 1) (*2*, *3*). Therefore, MepA from *E. coli* belongs to the group of D,D-peptidases that also includes numerous transpeptidases and amidases with specificity for the D-Ala-D-Ala link in peptidoglycan precursors (*4*). Unlike the transpeptidases with a catalytic serine residue, but like the cation-dependent D-Ala-D-Ala peptidases, MepA is insensitive to β-lactam antibiotics but can be inhibited by metal-chelating agents such as EDTA[1] or 1,10-phenanthroline (*3*).

MepA homologues have been found in some, but not all, proteobacteria for which genomic sequences are available. No homologues have been detected in Gram-positive bacteria or archaebacteria. At the level of amino acid sequence, MepA-like enzymes (MEROPS family M74 peptidases) (*5*) share only two short motifs with some other groups of peptidoglycan hydrolases, but clear similarities emerge at the level of tertiary structure. The overall structural similarity is greatest between MepA-like enzymes and metal-dependent D-Ala-D-Ala amino- and carboxypeptidases, which are all specific for amide bonds between D-configured amino acids and are now grouped in a single peptidase clan in the MEROPS database. Locally, in the region of the active site, MepA-like enzymes are also strikingly similar to lysostaphin-like enzymes, but the overall structural similarity between the two groups of enzymes is low (*3*). Therefore, they are classified as separate peptide clans but are collectively referred to as LAS (*l*ysostaphin, D-*A*la-D-Ala peptidase, *s*onic hedgehog) enzymes to highlight their active site similarities (*6*).

LAS enzymes contain a single divalent cation in the active site. From electron paramagnetic resonance (EPR) experiments, it has been concluded that the metal ion is pentacoordinated in the active conformation of VanX and, by implication, probably also in other LAS enzymes (*7*). In contrast, the metal ion in the crystallographic structures of LAS enzymes is consistently tetrahedrally coordinated except in the complexes of VanX with phosphinate and phosphonate transition state analogues (*3*, *8*−*11*). Three metal ligands are conserved and occur in the order histidine (H113 in MepA), aspartate (D120 in MepA), histidine (H211 in MepA) in the amino acid 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 30−100 amino acids (*6*).

The identity of the fourth metal ligand varies. It is a water molecule in the structures of D-Ala-D-Ala carboxypeptidase (PDB accession code 1LBU) (P. Wery, Ph.D. Thesis) and

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; DTT, dithiothreitol; TCEP, tris(2-carboxyethyl)phosphine.

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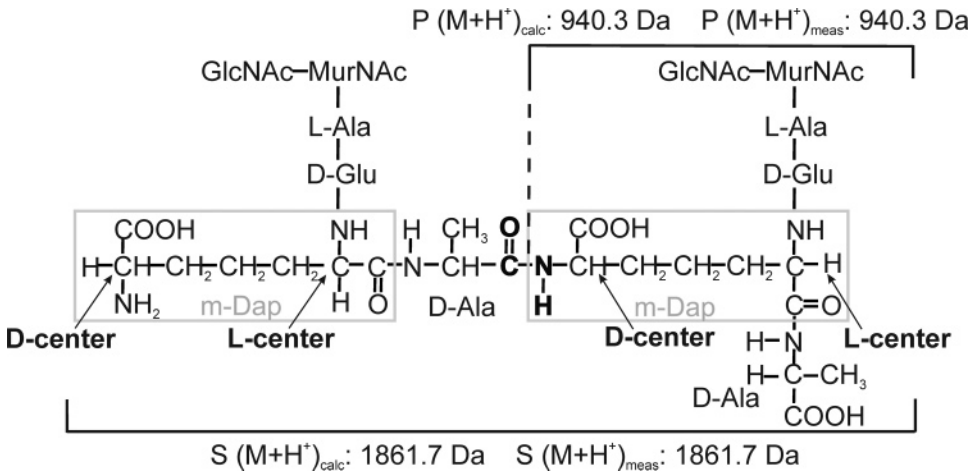

FIGURE 1: Schematic diagram of the GlcNAc-MurNAc tetrapeptide dimer and monomer that results from lysozyme digestion of *P. putida* peptidoglycan. MepA cleaves the peptide bond (bold) between D-alanine and *meso*-2,6-diaminopimelic acid. The calculated and experimentally measured monoisotopic masses of the substrate (labeled S) and product (labeled P) are indicated. The stereochemical information is taken from the literature. The break in the carbohydrate chain is based on the specificity of lysozyme, which is known to cleave the glycosidic bond between C-1 of MurNAc and C-4 of GlcNAc but not between C-1 of GlcNAc and C-4 of MurNAc (*13*).

VanX (PDB accession code 1R44) (*8*), a sulfate or phosphate molecule from the crystallization buffer in the structures of the *in vitro* activated forms of LytM (PDB accession codes 2BOP and 2B44) (*9*), and an asparagine oxygen atom in the structure of the full-length, latent form of LytM (PDB accession code 1QWY) (*11*). In the crystals of *E. coli* MepA, the fourth metal ligand is a H110, which completes the coordination sphere of the metal. As this leaves no coordination site for the carbonyl oxygen atom of the scissile amide bond of a substrate, the crystallized conformation cannot be catalytically relevant, and therefore the role of H110 remains unclear (*3*).

In all LAS enzymes, two additional residues that might act as general acid/base during catalysis are present in spatially conserved positions. These two residues are normally histidines, but in the D-Ala-D-Ala peptidase VanX, the place of one histidine residue is taken by a glutamate (*8*). In MepA, the two histidines are H206 and H209. The imidazole ring of H206 superimposes very well with its counterparts in other LAS enzymes, including the unusual glutamate in VanX. In contrast, H209 is present in a rotamer conformation that precludes the perfect overlap with the equivalent histidines in the other LAS enzymes. As the crystal structure captures an inactive state, we expect that the "unusual" rotamer conformation is a crystallization artifact that will not be present in the active form (*3*).

In prior work, we have established a zymography assay for MepA activity and used it to test the catalytic importance of the conserved histidines and aspartate of the H-x(3,6)-D and H-x-H motifs. We found that mutation of the signature residues to alanines led to a reduction of activity, but quantitative conclusions could not be drawn, because the mutants were contaminated with endogenous wild-type protein and also because the refolding efficiency of the mutant enzymes was not known (*3*).

Here, we report a new MepA expression system, which makes it possible to separate MepA variants from the endogenous wild-type enzyme, and an HPLC assay with a defined peptidoglycan fragment for the quantitative determination of enzyme activities. Our kinetic results are in agreement with earlier qualitative results and show that the candidate catalytic residues H206 and H209 and the "fourth" ligand residue H110 are all required for activity.

EXPERIMENTAL PROCEDURES

Protein Cloning, Expression, and Purification. The wild-type *mepA* gene was amplified by PCR with primers to introduce *Eco*RI and *Xho*I restriction sites and cloned via these sites into pETMM82 (a kind gift of G. Stier and D. Drechsel) to give the pETMM82-*mepAwt* plasmid. This vector is based on the pET24d backbone but additionally contains the coding sequences for DsbC, a hexahistidine tag, and a tobacco etch virus (TEV) protease cleavage site immediately downstream of the ribosome binding site and upstream of the polylinker. For the overexpression of the fusion protein, the construct was transformed into BL21-(DE3) *E. coli* cells. Cells were grown in LB medium supplemented with 25 μg/mL kanamycin at 37 °C until they reached an OD_{600} of 0.7−0.9, transferred to 20 °C for 15 min for cooling, induced with 0.5 mM isopropyl β-D-thiogalactopyranoside, and further grown at 28 °C for 4 h. Protein was purified by Ni^{2+}-affinity chromatography on nitrilotriacetic acid−agarose (Qiagen). Fractions containing 90% pure DsbC-MepA fusion protein (~57.4 kDa) were concentrated to 1 mg/mL protein and dialyzed against buffer T (50 mM Tris-HCl, pH 8.0, 0.5 mM EDTA). TEV protease cleavage was performed overnight at room temperature in buffer T with the addition of 0.5 mM DTT and 10 μg of TEV protease per 1 mg of the fusion protein. The progress of the TEV-mediated cleavage was monitored by SDS−PAGE. When the reaction was complete, the proteins were dialyzed overnight against 10 mM Tris-HCl, pH 7.5, 200 mM NaCl buffer and subjected to gel filtration chromatography on a Superose 12 HR 10/30 column in 10 mM Tris-HCl, pH 7.5, 200 mM NaCl buffer. MepA eluted partly as a monomer and partly as higher molecular weight species. Fractions with monomeric MepA were dialyzed against 10 mM Tris-HCl, pH 7.5, concentrated to 5 mg/mL, and used for activity assays.

All mutants were obtained by PCR-based site-directed mutagenesis according to the Stratagene QuickChange protocol with Pfu Turbo DNA polymerase (Stratagene), using

pETMM82-*mepAwt* plasmid as a PCR template. The mutants were overexpressed and purified like the wild-type protein as described above. In order to confirm that only the intended mutations were introduced, all of the DNA constructs of the mutants were sequenced.

Circular Dichroism Spectroscopy. All of the enzymes were dialyzed to 10 mM Tris-HCl, pH 7.5, buffer prior to CD analysis. Spectra were recorded with a Jasco J-710 spectropolarimeter using a thermostated cuvette of 0.2 mm path length. Data were collected in the wavelength range from 195 to 260 nm with a response time of 1.0 s and a 1.0 nm data pitch. The final spectra are averages of three independent runs. Spectra were normalized to 40 μM protein concentration, corrected for buffer, and transformed to molar ellipticities.

Determination of Zn^{2+} Content in MepA Proteins by X-ray Fluorescence. The Zn^{2+} content of MepA and its mutants was assessed by an X-ray fluorescence experiment with Synchrotron radiation at beamline BW6 of the Deutsche Elektronensynchrotron (DESY, Hamburg), which is equipped with a Si-PIN diode detector connected to a multichannel analyzer (Amptek) for energy-dispersive X-ray photon detection. Samples were mounted in a thin quartz cuvette and excited with $\lambda = 1.2820$ Å (9.646 keV) photons. This incident energy is optimal for efficient K-hole generation in Zn^{2+} but leads to overlap of (undesired) inelastic scattering from the buffer with the characteristic fluorescence spectrum [literature values for Zn: $\lambda_{2KLII} = 1.4390$ Å, $E_{2KLII} = 8.6158$ keV and $\lambda_{1KLIII} = 1.4352$ Å, $E_{1KLIII} = 8.6387$ keV (*12*), not resolved] from Zn^{2+}. Therefore, a reference spectrum with buffer alone was collected for each sample spectrum and subtracted to isolate the contribution due to X-ray fluorescence. Although all measurements were recorded with a fixed slit width and exposure time and although care was taken to preserve the geometry of the setup between measurements, all spectra were normalized to the integral of the emission spectrum prior to the subtraction step. All measurements were carried out in duplicate and averaged. For calibration, we used 50, 125, 250, 500, and 1000 μM $ZnCl_2$ solutions in buffer. Protein solutions were concentrated to 200 μM, except for the H110AH211A double mutant, which tended to precipitate and was only concentrated to 160 μM. As all proteins had been extensively washed with Zn^{2+}-free solutions prior to the measurements, we assumed that all Zn^{2+} in the samples was specifically bound at the active sites.

Isolation of MepA Muropeptide Substrate from Gram-Negative Bacterial Cell Walls. The GlcNAc-MurNAc tetrapeptide dimer, which is a MepA substrate, was isolated from purified cell walls of *Pseudomonas putida*. First, purified peptidoglycans were obtained as described previously (*3*) and incubated with lysozyme (0.2 mg/mL), which cleaves the β-1,4 glycosidic links between C-1 of MurNAc and C-4 of GlcNAc but not between C-1 of GlcNAc and C-4 of MurNAc (*13*). Incubation was at 37 °C for 48 h with gentle shaking. Insoluble contaminations and uncleaved, polymeric peptidoglycans were removed by high-speed centrifugation. Then lysozyme was removed by ultrafiltration (10 kDa cutoff; Viva Science). The flow-through fraction was Speed-Vac-concentrated and subjected to gel filtration chromatography on a Superdex Peptide 10/300 GL column connected with an Ettan LC fast protein liquid chromatography system (Amersham Biosciences) in water. The peak containing muropeptide with a molecular mass of 1861.7 Da (identified by mass spectrometry) corresponds to the disaccharide tetrapeptide dimer. Its fractions were collected, Speed-Vac-concentrated, and stored at −20 °C.

Determination of GlcNAc-MurNAc Tetrapeptide Monomer and Dimer Concentrations. In order to determine MepA substrate concentration, it was derivatized with Sanger's reagent [DNFB (dinitrofluorobenzene)]. As an indicator of the reaction efficiency, tetraglycine of known concentration was also derivatized under identical conditions. The reaction was performed by mixing a muropeptide/tetraglycine water solution with the derivatization solution (2 mM DNFB, 0.5% TEA in acetonitrile) and incubating for 30 min at 60 °C. After the reaction, samples were cooled down to ambient temperature, acidified to pH 6.0, and separated by gel filtration chromatography on a Superdex Peptide 10/300 GL column. The amount of the derivatized reaction product was determined by the absorption measurement at 360 nm ($\epsilon_{360} = 17300$ M^{-1} cm^{-1}). Finally, the concentration of the disaccharide tetrapeptide dimer was calculated, taking into account the reaction yield, which was estimated to be ∼50% based on the yield of tetraglycine derivatization.

HPLC-Based Activity Assay. The enzyme reactions were carried out in 125 or 250 μL in 10 mM Bis-Tris, pH 6.0, 0.1% Triton X-100, and 5 mM $MgCl_2$ at 37 °C. Before the reaction, the substrate was preincubated in the reaction buffer for 10 min at 37 °C, and then the reaction was initiated by the addition of enzyme. Substrate concentrations were chosen in the range of approximately 0.35−4 times the K_M. Initial reaction velocities were measured in duplicate or triplicate per substrate concentration. The concentrations of the wild-type enzyme and the mutants were 1 and 10−400 nM, respectively. Reactions were stopped by acidification to pH 3.0 with traces of 10% TFA. To separate the substrate and the product of the enzyme reaction, HPLC was performed on a NUCLEOSIL 100-5 C18 column (3 mm i.d., 150 mm) (Marcherey Nagel) equilibrated with eluent A (30 mM phosphate, pH 4.3). For the elution, a linear gradient was generated from 0% to 100% of eluent B (50 mM phosphate, pH 4.95, 15% methanol) in 30 min. The HPLC separation was performed at room temperature at a flow rate of 0.4 mL/min. The absorbance of both the substrate and the product was monitored at 205 nm. The amount of the product was measured by peak integration and calculated by comparison with the standards of known concentration. K_M and k_{cat} values and their errors were calculated by hyperbolic regression analysis with the program Hyper, version 1.1s, assuming standard Michaelis−Menten kinetics. The standard assumptions for Michaelis−Menten kinetics were met in all cases, even though large quantities of some MepA variants had to be used in the assays. The concentration of the substrate was at least 12.5-fold larger than the concentration of enzyme (so that enzyme binding does not deplete the substrate pool significantly), and initial velocities were measured throughout (so that substrate depletion and/or product inhibition does not interfere with the measurements).

Pull-Down Assay for Peptidoglycan Binding. Bacillus megaterium peptidoglycans were isolated and purified as described previously (*14*). The pull-down assay was performed at 37 °C by incubating 2 μg of MepA wild-type or mutant proteins with 0.5 mg of purified *B. megaterium* peptidoglycans in 30 μL of the binding buffer (10 mM Bis-

Tris, pH 6.0, 5 mM $MgCl_2$) for 30 min. The peptidoglycan fraction was collected by centrifugation at 15000*g* for 15 min. The supernatant containing the unbound fraction was directly analyzed by SDS−PAGE. Peptidoglycans with bound protein were further washed with 1.0 mL of the binding buffer supplemented with 250 mM NaCl and centrifuged once again. To extract bound proteins from peptidoglycan, the pellet was resuspended in Laemmli loading buffer, boiled for 5 min, centrifuged to remove insoluble material, and then analyzed by SDS−PAGE.

RESULTS

A New Overexpression System for MepA. MepA has three disulfide bonds and occurs naturally in the oxidizing environment of the *E. coli* periplasm, where its folding is assisted by the disulfide bond isomerase DsbC (*15*). To prepare MepA without contamination from the wild-type enzyme, we cloned the open reading frame of the *mepA* gene without its periplasmic leader sequence into the pETMM82 expression vector. The resulting construct directs the cytoplasmic expression of a fusion protein that consists of an N-terminal DsbC domain, followed by a hexahistidine tag, a TEV protease cleavage site, and the MepA amino acid sequence starting from residue 20. The fusion protein was purified by Ni^{2+}-affinity chromatography and then cleaved off from its tags by the cysteine protease TEV. The dithiothreitol (DTT) concentration in the cleavage buffer was limited to 0.5 mM to minimize its effect on the disulfide bridges of MepA. Although the completeness of cleavage was confirmed by SDS−PAGE, the majority of MepA migrated as a multimeric complex together with DsbC in gel filtration experiments. Nevertheless, a fraction of MepA migrated as a monomer and was used for all further experiments. In the case of the wild-type enzyme, protein that was purified in this way had similar specific activity as the protein that was obtained by the purification according to the previous protocol (data not shown). Despite the cytosolic expression and tag cleavage in the presence of DTT, mass spectrometry indicated that all three disulfide bridges were intact in the monomeric MepA fraction (see below).

Generation and Overexpression of MepA Variants. We used the pETMM82-*mepAwt* plasmid as a template for PCR-based site-directed mutagenesis and constructed three groups of mutants: first, mutations in Zn^{2+}-ligating residues, H110A, H113A, D120A, H211A, and H110A/H211A; second, mutations to identify the general acid/base residue, H206A, H206D, H206E, H209A, and H209D; and third, a mutation in a putative substrate-binding residue, W203D. The Zn^{2+}-ligating residues and the putative general base histidines were selected on the basis of the Zn^{2+}-containing MepA crystal structure (PDB accession code 1U10) (*3*). W203 was found to interact hydrophobically with diaminopimelic acid, a substrate component located in the vicinity to the scissile bond, in the structure of MepA crystallized in the presence of 100 mM diaminopimelic acid (unpublished data). The mutation of this residue to the hydrophilic aspartate should substantiate its role. Expression and purification of the mutant proteins were carried out as described for the wild-type protein. The DsbCMepAD120A mutant protein was less soluble, unstable, and could not be prepared in sufficient amounts for quantitative assays. All other mutant proteins behaved like the wild-type protein. The homogeneity of the wild-type and mutant proteins at the final stage of the purification was confirmed by SDS−PAGE (Figure 2A). All mutants with the exception of the D120A had CD spectra that were indistinguishable from the CD spectrum of the wild-type protein, indicating that the secondary structure of mutant proteins was not perturbed by the mutations (Figure 2B). Moreover, samples were subjected to ESI mass spectrometry analysis prior to and after reduction with tris(2-carboxyethyl)phosphine (TCEP; 50 mM TCEP, 50 °C, 60 min). In all cases, the TCEP treatment increased the mass by 6 Da, which is consistent with reductive cleavage of all three disulfide bonds in MepA. The result indicates that the disulfide bonds were intact despite the cytosolic expression and the presence of the mild reduction agent DTT in some purification steps and were destroyed only by the strong reducing agent TCEP at elevated temperature.

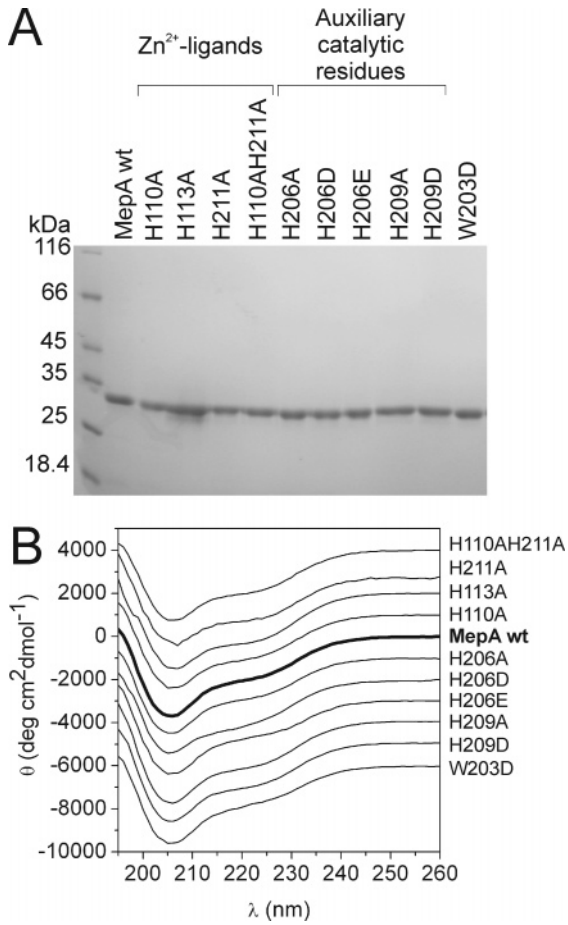

FIGURE 2: Homogeneity (A) and structural integrity (B) of the wild-type MepA and the mutant proteins. (A) 12% polyacrylamide gel loaded with 2 μg of each protein at the final step of the purification after SDS−PAGE separation, stained with Coomassie Blue R-250. (B) CD spectra expressed in molar ellipticities. The absolute values of the ordinate apply to the wild-type spectrum only. To avoid overlap, the spectra of mutants have been shifted by integer multiples of 1000 deg cm^2 $dmol^{-1}$.

Determination of Zn^{2+} Content in MepA Proteins. The expression and purification procedure of MepA involves folding in the reducing environment of the cytosol, Ni^{2+}-affinity chromatography, and tag cleavage in the presence of 0.5 mM EDTA and DTT. Nevertheless, the activity of wild-type MepA was comparable to the activity of MepA after expression in the periplasm and purification according to a different protocol (*3*) (data not shown). Although wild-

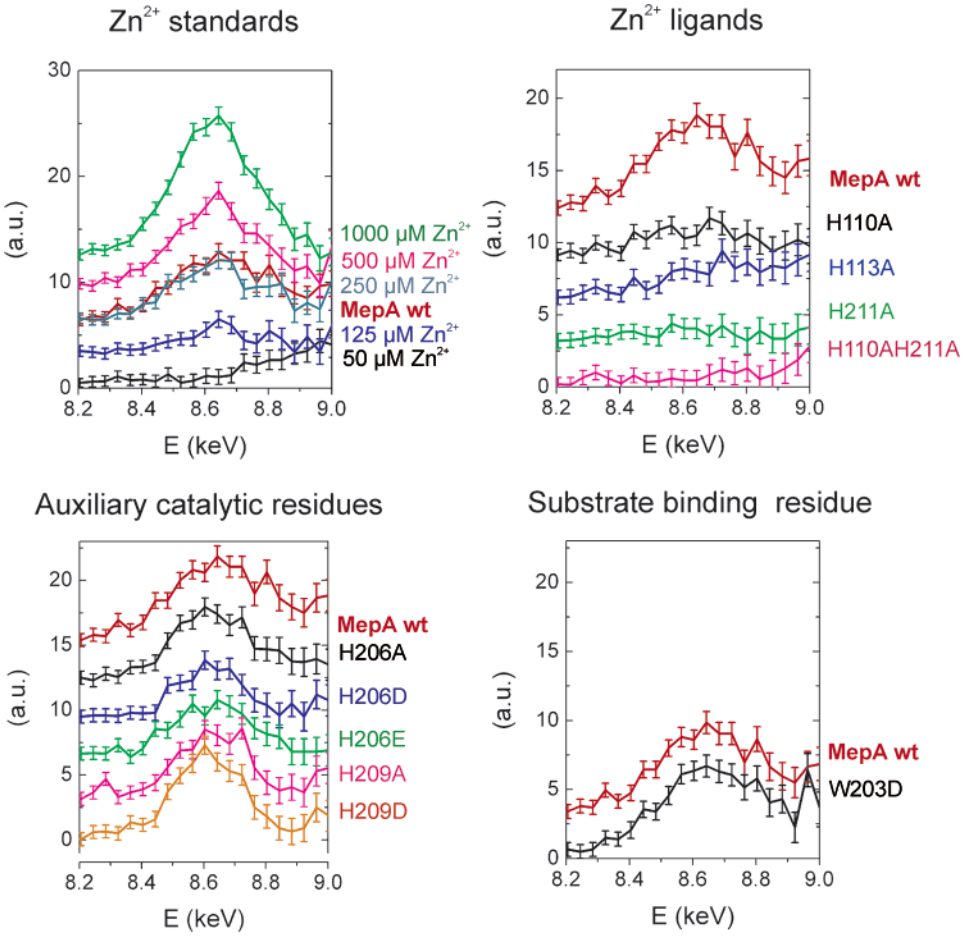

FIGURE 3: Zn^{2+} content in wild-type and mutant MepA proteins. MepA and its variants were extensively dialyzed against Zn^{2+} free buffer and concentrated to 200 μM (160 μM for the H110AH211A mutant). X-ray fluorescence spectra of the samples and of various Zn^{2+} standards were recorded as described in Experimental Procedures. Spectra were shifted by integer multiples of 3 (au) to avoid overlap. Error bars were calculated assuming Poisson statistics for the count rate.

type MepA retained Zn^{2+} in the presence of 0.5 mM EDTA, the effect of this treatment on the MepA mutants had to be assessed experimentally, which was done by X-ray fluorescence spectroscopy. Preliminary calibration experiments with standard $ZnCl_2$ concentrations in sample buffer indicated that Zn^{2+} above 100 μM could be reliably detected. The spectrum collected for 200 μM wild-type MepA was most similar to that of 250 μM $ZnCl_2$, which is consistent with the expected 1:1 ratio of protein and metal (Figure 3). However, the Zn^{2+} signal was substantially weaker for the H110A and H113A MepA mutants and below the 100 μM detection threshold for the H211A and H110/H211A double mutant, which lacks two Zn^{2+}-ligating residues (Figure 3). For all other MepA variants with mutations in putative catalytic or substrate binding residues we obtained Zn^{2+} signals that were indistinguishable from the signal of wild-type MepA within the error of the experiment (Figure 3).

Purification of the MepA Substrate. The GlcNAc-MurNAc tetrapeptide dimer (Figure 1), a substrate for the MepA activity assay, was obtained from the cell walls of Gram-negative *P. putida* bacteria. Briefly, peptidoglycan was isolated and purified using standard procedures, cleaved with lysozyme, and further fractionated by gel filtration on Superdex peptide resin to yield almost pure MepA substrate. Mass spectrometry confirmed that the muropeptide mass corresponds to the expected mass of the disaccharide tetrapeptide dimer generated by lysozyme cleavage of Gram-negative murein (1861.7 Da) (Figure 1). The concentration of the substrate was determined by A_{360} after derivatization with Sanger's reagent.

HPLC analysis of the GlcNAc-MurNAc tetrapeptide dimer on a reversed-phase C18 column in phosphate buffer according to Glauner's procedure (*16*) produced two peaks (Figure 4A, retention times $R_{ts1} = 17.9$, $R_{ts2} = 19.6$ min), which were attributed to two different conformations of the anomeric carbon atom in the substrate. In support of this interpretation, we found that (a) both peaks had the correct mass, (b) reinjection of the material from a single peak reproduced both peaks, (c) borohydrate reduction of the material prior to HPLC coalesced the two peaks into one, and (d) both peaks, or alternatively the single peak, after sodium borohydrate treatment were absent from material that was subjected to a prolonged incubation with MepA prior to the HPLC analysis. It remains unclear why only two peaks were observed for the GlcNAc-MurNAc tetrapeptide dimer, which has two anomeric carbon atoms and should have given rise to a peak triplet or quartet instead of the observed doublet (*16*, *17*). It is possible that the peaks were not resolved, or alternatively, that two forms were overrepresented in our sample and did not equilibrate with the other forms on the time scale of the experiment (about 1 h) (Figure 4A).

MepA Activity Assay. The cleavage of the GlcNAc-MurNAc tetrapeptide dimer was assessed by HPLC in an end-point assay with fixed incubation time. Two new peaks

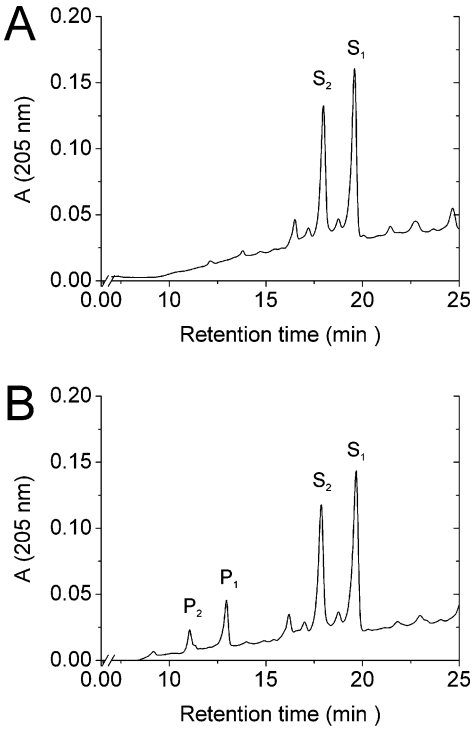

FIGURE 4: HPLC chromatogram showing the MepA activity. (A) HPLC trace of the purified 10 μM GlcNAc-MurNAc tetrapeptide dimer prior to incubation with the enzyme. (B) HPLC trace of the reaction mixture after 20 min incubation of 10 μM GlcNAc-MurNAc tetrapeptide dimer with 1 nM wild-type MepA. The assignment of peaks as GlcNAc-MurNAc tetrapeptide dimer (S_1 and S_2) and GlcNAc-MurNAc tetrapeptide monomer (P_1 and P_2) is based on the mass spectrometry analysis of each of the four peaks.

were observed only after incubation with the enzyme (Figure 4B, retention times $R_{tp1} = 11$ min, $R_{tp2} = 13$ min). Mass spectrometry analysis, sodium borohydride treatment, and reinjection experiments were all consistent with the interpretation that these two new peaks represented different conformations of the reaction product, GlcNAc-MurNAc tetrapeptide monomer. The presence of two peaks is consistent with earlier reports in the literature and with chemical expectation, because the GlcNAc-MurNAc tetrapeptide monomer has only a single anomeric carbon atom (*17*). Derivatization of the reaction mix after incubation with Sanger's reagent increased the sensitivity of peptidoglycan detection but also introduced additional variability into the assay and was therefore not used. Instead, peptidoglycan fragments were detected directly by their absorption at 205 nm.

The influence of genome-encoded MepA on the measured activity of recombinant MepA preparations was tested in a control experiment: 20 mL *E. coli* BL21(DE3) cultures were transformed with either empty pETMM82 (mock) or pETMM82-*mepAwt* vector and grown as described in Experimental Procedures for a standard MepA preparation. Proteins with the affinity to the Ni^{2+}-NTA resin were batch purified. Fractions that eluted at 100 mM imidazole or higher were pooled, quantified by the Bradford assay, and assayed for MepA activity with 10 μM GlcNAc-MurNAc tetrapeptide dimer as the substrate. A clear product peak was observed with 20 ng of protein from the cells transformed with pETMM82-*mepAwt* but not with a 1000-fold excess (20 μg) of protein from the mock purification (Figure 5A). We conclude that the background of endogenous MepA is efficiently eliminated by the Ni^{2+}-NTA chromatography step and that the measured MepA activities can all be attributed to the overexpressed proteins.

Activity of Wild-Type MepA. In order to find optimal conditions for MepA activity, two major factors were taken into account: pH optimum and divalent metal ion influence. The pH activity profile turned out to be consistent with the pH dependence in the zymography assay (*3*). The presence of Mg^{2+} appeared to be activatory up to 5 mM concentration. The activating effect was not specific for Mg^{2+} but could be observed for Mn^{2+} and Ca^{2+} ions as well. Finally, the reaction was carried out in 10 mM Bis-Tris, pH 6.0, 0.1% Triton X-100, and 5 mM $MgCl_2$ at 37 °C.

Assuming standard Michaelis−Menten kinetics, we obtained a V_{max} of 6.4 ± 1.1 nM/s, which translates into a k_{cat} of 6.4 ± 1.1 s^{-1}, because the enzyme was used in 1 nM concentration. For the binding constant, we obtained a K_M of 13.3 ± 5.0 μM (Figure 5B,C). The quoted numerical errors include only the statistical uncertainty but not the systematic error from the substantial uncertainty in the GlcNAc-MurNAc tetrapeptide dimer concentration, because the latter introduces a constant correction factor to the K_M of wild-type and mutant proteins.

Activity of MepA Variants with Altered Metal Ligands. The divalent metal cation in the MepA crystal structures is tetracoordinated in direct contact with H110, H113, D120, and H211. With the exception of D120, each of the metal ligands could be individually mutated to alanine without major effects to the secondary structure of the protein. Mutant proteins were assayed in identical conditions as wild-type MepA, but more enzyme (between 20 and 400 nM) was required to generate detectable amounts of product (Supporting Information, Figure 1). For single residue mutants, we found only minor variations in the K_M value for the substrate, but the k_{cat} values were significantly reduced to approximately 2% of the wild-type value. In case of the double mutant H110A/H211A, K_M was slightly higher, but the k_{cat} value decreased 20-fold in comparison to any of the single mutants. Together with the control experiment described above (Figure 5A), these data suggest that the observed trace activity is rather due to genuine residual activity than to contamination (Table 1, Figure 6, and Supporting Information, Figure 1).

Activity of MepA Variants with Altered Candidate Active Site Residues. H206 and H209 are located in close proximity to the active site metal ion and (after a rotamer adjustment of H209) have their side chains in spatial positions that are invariably taken by histidine or glutamate residues in most other LAS enzymes. To test the role of these histidines, we tested the activity of the H206A, H206D, H206E, H209A, and H209D MepA mutants. In all cases, we found a significant drop of the activity, to k_{cat}/K_M values lower than 2% of the activity of the wild-type protein. However, aspartate and glutamate did not substitute for histidine (Table 1, Figure 6, and Supporting Information, Figure 1).

Activity of the W203D MepA Variant. There is no protein−substrate complex among previously reported MepA structures (*3*). However, when MepA was crystallized in the presence of 100 mM diaminopimelic acid, which is a component of MepA substrate, some additional density was observed close to the active site Zn^{2+} and in proximity to F177 and W203 (unpublished data). To test the significance

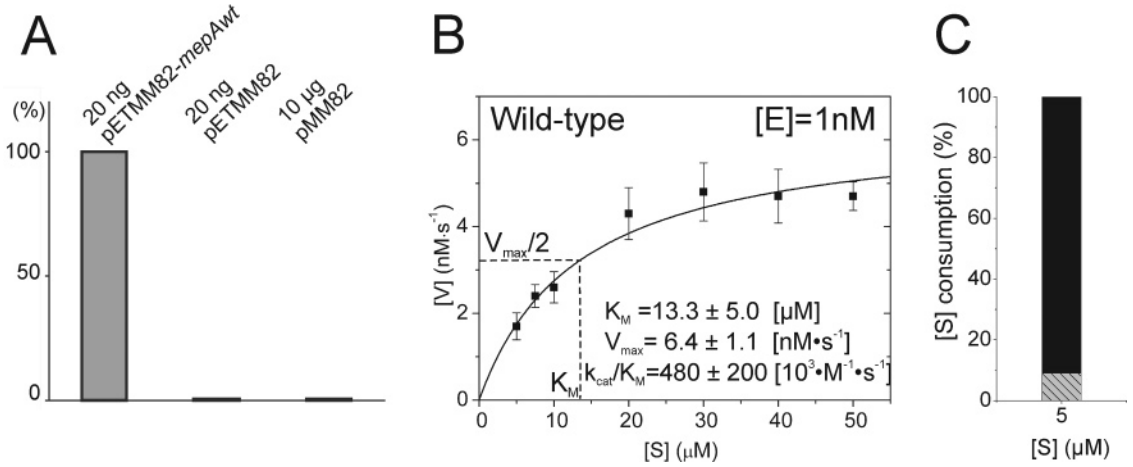

FIGURE 5: Wild-type MepA kinetics. (A) The cleavage of 10 μM GlcNAc-MurNAc tetrapeptide dimer by the protein pool with an affinity to Ni^{2+}-NTA resin obtained from *E. coli* BL21(DE3) cells transformed with a control, empty vector pETMM82 and with the same vector containing the *mepA* gene, pETMM82-*mepAwt*. (B) Michaelis−Menten plot of the initial velocity (V) versus the substrate concentration [S]. Assays were performed with 1 nM enzyme concentration and 5−50 μM substrate. The initial velocity, which is the mean of three independent measurements, was determined from the product accumulation after 10 min of the reaction. The error bars illustrate the standard deviation from the mean value. The observed initial velocities were fitted to the equation $V = (V_{max}[S])/(K_M + [S])$ by hyperbolic regression analysis. (C) Bar representation showing the substrate consumption at the end of the reaction for 5 μM substrate concentration (consumed substrate, hatched gray; remaining substrate, black).

Table 1: K_M and k_{cat} of Wild-Type and Mutant MepA Proteins

protein	K_M (μM)	k_{cat} (s^{-1})
wild type	13.3 ± 5.0	6.4 ± 1.1
H110A	7.5 ± 2.6	0.090 ± 0.013
H113A	9.7 ± 5.7	0.090 ± 0.026
H211A	14.8 ± 2.5	0.160 ± 0.015
H110A/H211A	18.4 ± 5.9	0.005 ± 0.001
H206A	8.1 ± 4.1	0.050 ± 0.012
H206D	9.4 ± 3.7	0.090 ± 0.016
H206E	10.9 ± 4.1	0.030 ± 0.005
H209A	10.5 ± 2.4	0.070 ± 0.008
H209D	6.2 ± 3.6	0.040 ± 0.007
W203D	50.0 ± 11.0	2.60 ± 0.38

of this finding, W203 was mutated to aspartate, which has very different properties. We found a substantial increase in K_M with respect to the wild type ($K_{M\ W203D} = 50$ μM versus the $K_{M\ wild-type} = 13.3$ μM) and a mild, approximately 2-fold decrease in k_{cat}, which is consistent with a role of residue W203 in substrate binding (Table 1, Figure 6, and Supporting Information, Figure 1).

MepA-wt and Mutants Binding to Peptidoglycan Polymer. A simple pull-down assay was performed to check the effect of the point mutants on MepA binding to polymeric peptidoglycan from *B. megaterium*, which is easy to isolate in quantity and similar to *E. coli* peptidoglycan, also with respect to the presence of the D-alanyl-*meso*-2,6-diaminopimelic acid amide bond cleaved by MepA (*14*). Bovine serum albumin was included as a control for unspecific binding but did not show affinity for peptidoglycan in the assay. In contrast, wild-type MepA was efficiently depleted from the supernatant and quantitatively pulled down to the pellet. The same was true for the other MepA variants, except for the H110A/H211A and W203D mutants. The histidine double mutant was depleted from the supernatant but not efficiently recovered in the pellet, suggesting that it binds weakly to peptidoglycan and is lost from the pellet during the washing steps. In contrast, the W203D mutant was quantitatively recovered in the supernatant and not in the pellet, which agrees with the kinetic analysis that this variant is a poor peptidoglycan binder (Figure 7).

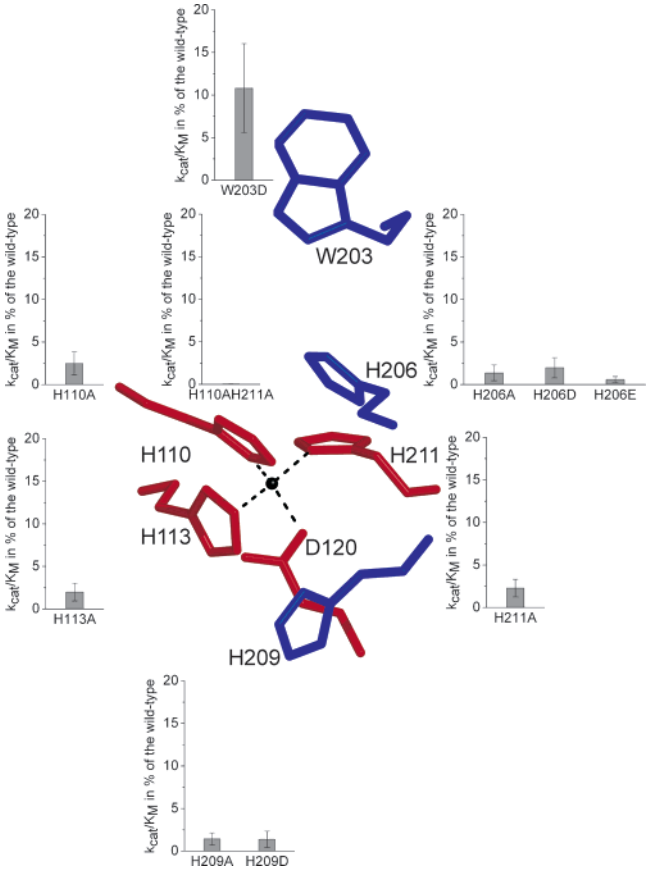

FIGURE 6: Activity of mutant enzymes. The mutated active site residues and the Zn^{2+} ion are shown in ball and stick representation. Zn^{2+}-ligating residues, H110, H113, D120, and H211, are colored red, and the other active site residues are drawn in blue. The k_{cat}/K_M activity of mutant proteins is expressed in % of the wild type.

DISCUSSION

MepA Expression. High overexpression of active MepA in the periplasm leads to cell lysis, presumably because the

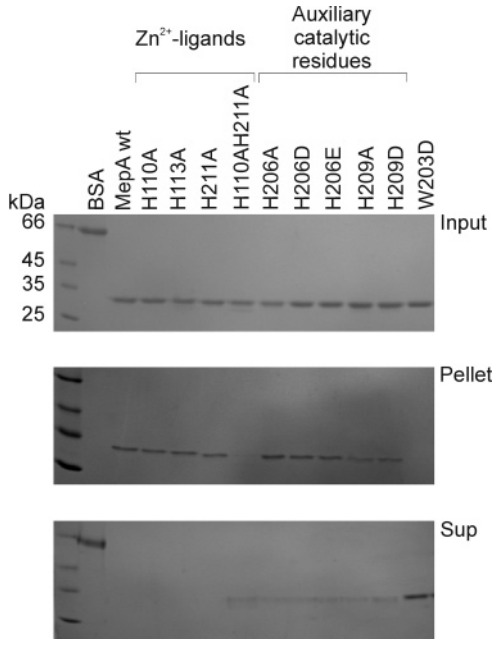

FIGURE 7: Affinity of MepA and mutant proteins to polymeric peptidoglycan. The binding was tested by a pull-down assay with purified peptidoglycan. 2 μg of BSA, wild-type MepA, and each mutant protein (Input) were incubated with 0.5 mg of purified *B. megaterium* peptidoglycan in 30 μL reaction volume. Insoluble peptidoglycan together with bound protein was collected by centrifugation. The peptidoglycan-bound fraction (Pellet) after extensive washing and the supernatant fraction (Sup) were analyzed by SDS−PAGE.

enzyme is in direct contact with its natural substrate, cell wall peptidoglycan. Expression of recombinant MepA in the *E. coli* cytoplasm solves this problem, because it separates the recombinant protein from its substrate, but is also problematic, because MepA has three disulfide bridges that are not expected to be stable in the reducing conditions of the *E. coli* cytoplasm. It is therefore no surprise that various attempts to express untagged MepA and various fusion constructs in the *E. coli* cytoplasm all yielded only protein in inclusion bodies. As MepA requires the assistance of disulfide bond isomerase DsbC for folding in the periplasm, we finally tested a DsbC-MepA fusion for expression in the cytosol and obtained soluble protein. As DsbC has chaperone and disulfide bond isomerase activities (*18*), it is not clear which of the two keeps MepA soluble in a reducing cytosolic environment, but once cells are opened and the environment becomes oxidizing, it is likely that the disulfide bond isomerase activity plays a role and keeps the formation of "false" disulfide bonds reversible and MepA soluble. Mass spectrometry demonstrates that the MepA proteins have the correct disulfide bridges at the end of the purification procedure and that these are stable against mild treatment with reducing agents but can be reduced upon 50 mM TCEP incubation at 50 °C for 60 min.

Choice of Substrate. We had difficulty obtaining purified peptidoglycan in quantity from *E. coli* and therefore focused on surrogate species. Gram-positive bacteria have thicker cell walls than Gram-negative ones and are therefore better suited for the isolation of peptidoglycan in quantity. Unfortunately, the peptidoglycan from most species cannot be used as a surrogate for *E. coli* peptidoglycan, because the amino acid composition and linkage are too different (*19*). The cell walls of the Gram-positive organism *B. megaterium* are an exception (*14*, *19*): because they resemble the cell walls of *E. coli*, and even contain the D-alanyl-*meso*-2,6-diaminopimelic acid amide bond that is cleaved by MepA, they were used for all pull-down experiments. Unfortunately, the digestion of *B. megaterium* peptidoglycan with lysozyme to generate GlcNAc-MurNAc peptide dimers is inefficient, so we had to settle for another species to obtain the substrate for the MepA HPLC assay. The Gram-negative *P. putida*, which has the same peptidoglycan composition as *E. coli* (*19*), proved to be a good source.

Are the Mutants Catalytically Impaired? Various indicators suggest that the low activity of the mutant proteins (except for the D120A variant) is not caused by a general folding defect. First, we found that all of the mutants yield a clear monomer peak in addition to the large molecular weight material, which suggests that a monodisperse species was isolated in the sizing chromatography procedure. In contrast, solubilized MepA from inclusion bodies, which was not properly folded, did not give rise to a monomer peak in gel filtration. Second, wild-type and mutant proteins appeared to be protected from unspecific degradation, in contrast to the D120A variant of MepA, which was partially degraded during the purification procedure. Third, the CD spectra of all MepA variants (except D120A) were indistinguishable from the spectrum of the wild-type protein, strongly indicating that the local amino acid substitutions did not induce overall structural perturbations. Fourth, mass spectrometry demonstrates that the disulfide bridges in wild-type MepA and all variants are intact. Finally, wild-type and mutant proteins differ mostly in k_{cat} and much less so in K_M, suggesting that the mutant proteins retain largely intact substrate binding sites. Finally, the quantitative results reported here are consistent with the qualitative results obtained with a different expression system and different activity assay (*3*).

Why Are the Mutants Inactive? H113 and H211 of MepA have direct equivalents in all other LAS enzymes and directly coordinate the active site Zn^{2+} ion in the crystal structures. Therefore, the experimental result that mutations of these residues impair both metal binding and activity was expected and is also consistent with our prior results from the zymography assay of MepA activity (*3*).

The effects of mutations to the metal ligand H110 in MepA were more difficult to predict. The LytM analogy would suggest that this "occluding" residue might act as an activity switch, similar to the "asparagine" switch that was found to operate in LytM to keep the full-length enzyme latent (*11*). If this analogy was correct, one might expect that H110A mutation might not interfere with metal binding and could instead activate the enzyme. However, the experiments show that the analogy does not hold. The H110A mutant has reduced metal content and reduced activity, which is still further reduced in the H110AH211A double mutant. The interpretation of this result is not clear, but the following model appears consistent with the data: H110 might protect against the loss of metal from MepA in the absence of substrate but might be displaced in its presence to allow direct contact of the Zn^{2+} ion to the carbonyl carbon atom of the scissile peptide bond. In this context, it might also be relevant that a recent electron paramagnetic resonance study has suggested that the active site of the MepA-related VanX has a pentacoordinated metal cation at the center, in contrast to

the X-ray structure of VanX (*7*), which shows a tetrahedrally coordinated metal ion in the active center of VanX (*8*), just as the MepA structures do (*3*). An EPR analysis of MepA may help to elucidate this point.

In the crystal structures of MepA, H206 and H209 are found in the vicinity of the catalytic metal ion but do not contact it directly. This is consistent with the X-ray fluorescence result that mutations to these residues do not significantly alter the metal content of MepA. As H206 and H209 have equivalents in all other LAS enzymes that could potentially change their protonation state during the reaction, we have previously conjectured that either H206 or His209 might act as a general acid/base residue (*6*). The present study shows that both residues are required for catalysis and that both charge preserving and nonpreserving mutations drastically reduce the activity. Our experimental findings are consistent with a role of H206 and H209 as a general acid/general base in catalysis, but they do not explain why both residues are required and they do not exclude alternative roles for either or both of these residues.

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SUPPORTING INFORMATION AVAILABLE

One figure showing the kinetic analysis of MepA mutant proteins. This material is available free of charge via the Internet at http://pubs.acs.org.

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