LD-Carboxypeptidases (EC 3.4.17.13) are named for their ability to cleave amide bonds between L- and D-amino acids, which occur naturally in bacterial peptidoglycan. They are specific for the link between meso-diaminopimelic acid and d-alanine and therefore degrade GlcNAc-MurNAc tetrapeptides to the corresponding tripeptides. As only the tripeptides can be reused as peptidoglycan building blocks, LD-carboxypeptidases are thought to play a role in peptidoglycan recycling. Despite the pharmaceutical interest in peptidoglycan biosynthesis, the fold and catalytic type of LD-carboxypeptidases are unknown. Here, we show that a previously uncharacterized open reading frame in Pseudomonas aeruginosa has LD-carboxypeptidase activity and present the crystal structure of this enzyme. The structure shows that the enzyme consists of an N-terminal β-sheet and a C-terminal β-barrel domain. At the interface of the two domains, Ser115 adopts a highly strained conformation in the context of a strand-turn-helix motif that is similar to the “nucleophilic elbow” in αβ-hydrolases. Ser115 is hydrogen-bonded to a histidine residue, which is oriented by a glutamate residue. All three residues, which occur in the order Ser-Glu-His in the amino acid sequence, are strictly conserved in naturally occurring LD-carboxypeptidases. Therefore, the physiological role of LD-carboxypeptidases may be in peptidoglycan recycling (1), although other physiological roles have also been discussed (1–4).

LD-Carboxypeptidase activity can be detected in many Gram-negative and Gram-positive bacteria, but is absent in Caulobacter crescentus (5). It is still not clear whether all LD-carboxypeptidase activity is due to a single enzyme. Metz et al. (6, 7) reported that they could distinguish two LD-carboxypeptidase activities in Escherichia coli (termed I and II) by their sensitivity to D-amino acids and to the β-lactam antibiotic thienamycin. Activity I was purified and attributed to a 12-kDa norcardicin A-sensitive enzyme (7). There are no reports on purification of activity II. In later independent work, Ursinus et al. (2) reported the purification of a norcardicin A-sensitive and thienamycin-insensitive activity, which was originally ascribed to a 43-kDa dimer-forming protein in the periplasm. When this activity was cloned, the gene was found to code for a 33.6-kDa protein without leader sequence, and a reassessment of its localization suggested that the enzyme is cytosolic (1).

Deletion of the cloned LD-carboxypeptidase gene has little effect on the phenotype during logarithmic growth, but makes the strain prone to lysis in the stationary phase, unless a compensating mutation in AmpD, another peptidoglycan amidase, is present. In contrast to the results of Metz et al. (6, 7), Templin et al. (1) found a complete loss of all soluble LD-carboxypeptidase activity in the deletion strain. In this study, we therefore use the term LD-carboxypeptidase for the cloned enzyme, even though unrelated LD-carboxypeptidases may remain to be discovered.

The substrate specificity of the E. coli LD-carboxypeptidase has been well characterized (8). The enzyme is specific for tetrapeptides and discriminates against pentapeptides, but is permissive with regard to the attached sugar moieties: free peptides and peptides linked to MurNAc, GlcNAc-MurNAc, and UDP-MurNAc are substrates. High molecular mass murein sacculi or cross-linked muropeptides are not cleaved (see Fig. 1) (1, 8).

The inhibitor sensitivity of LD-carboxypeptidases is less clear, in part because many studies have been done with poorly characterized enzyme preparations that may not have been homogeneous (6, 7, 9–11). LD-Carboxypeptidase is sensitive to norcardicin A, but biochemical results suggest that the interaction is noncovalent and does not involve an opening of the lactam ring of the antibiotic (2). The sensitivity of LD-carboxypeptidase to other lactam antibiotics varies widely between antibiotics of the same class, but correlates with the chirality of the amino acid substituent of the antibiotic, again suggesting that lactam ring opening is not essential (2, 6). EDTA, which was used for standard periplasm extractions when the enzyme was thought to reside in the periplasm, does not inactivate the enzyme (1, 12). EDTA, which was used for standard periplasm extractions when the enzyme was thought to reside in the periplasm, does not inactivate the enzyme (1, 12). EDTA, which was used for standard periplasm extractions when the enzyme was thought to reside in the periplasm, does not inactivate the enzyme (1, 12). EDTA, which was used for standard periplasm extractions when the enzyme was thought to reside in the periplasm, does not inactivate the enzyme (1, 12). EDTA, which was used for standard periplasm extractions when the enzyme was thought to reside in the periplasm, does not inactivate the enzyme (1, 12).
is probably not an aspartic protease (2). As peptidases of unknown fold and catalytic class, LD-carboxypeptidases are currently classified as family U61 (U for "unknown") in the MEROPS Database (13). Here, we (a) show that the U61 enzyme from Pseudomonas aeruginosa has LD-carboxypeptidase activity, (b) present the crystal structure of the enzyme at 1.5-Å resolution, (c) demonstrate that the enzyme is a serine peptidase with a Ser-His-Glu catalytic triad, and (d) highlight an unexpected similarity of the region around the active-site serine residue to the "nucleophilic elbow" in αβ-hydrolases.

**EXPERIMENTAL PROCEDURES**

**Cloning**—The gene for LD-carboxypeptidase from *P. aeruginosa* strain PA01 was amplified by standard PCR methods from genomic DNA, adding EcoRI and BamHI sites for cloning. The fragment was digested with EcoRI and BamHI, and the digestion product was ligated with T4 ligase into a derivative of pET15b (Novagen) that lacks the original EcoRI site of the vector and carries six histidine residues and an original EcoRI site downstream of the original Ncol site. Thus, the N-terminal sequence of our construct was MGHHHHHHHFMTS . . . , where MTS comprises the first residues of the native LD-carboxypeptidase sequence. Restriction analysis and automated DNA sequencing confirmed that the expression construct harbored the full LD-carboxypeptidase gene. The conceptually translated sequence differed from the Swiss-Prot sequence (accession number Q9HTZ1) at two positions. Asp^{109} and Gln^{114} were both mutated to glutamate. As the recombinant protein is active (see below), it is likely that the point mutations are due to strain differences, but this was not rigorously checked. Site-directed mutations were introduced into the expression construct by the QuickChange method (Stratagene) essentially according to the manufacturer’s protocol, but with Pfu polymerase (EURx Ltd.) instead of Pfu Turbo polymerase.

**Expression**—For recombinant protein expression, plasmids were transformed into BL21(DE3) cells. Bacteria were grown in 37 °C to *A*_{600} = 0.5, cooled to 22 °C, induced with 0.5 mM isopropyl β-D-thiogalactopyranoside at *A*_{600} = 0.7, and grown in LB medium for 4 h prior to cell harvesting. To produce selenomethionine-containing protein, the expression construct was transformed into the methionine-auxotrophic *E. coli* strain B834(DE3). Cells were grown in 37 °C to *A*_{600} = 0.8, shifted to 28 °C, induced at *A*_{600} = 1.0 with 0.5 mM isopropyl β-D-thiogalactopyranoside, and cultured for an additional 8 h in minimal medium containing 0.3 mM L-selenomethionine (14).

**Protein Purification**—Cells were harvested; resuspended in buffer A (50 mM Tris-HCl (pH 7.5) and 200 mM NaCl), and treated with lysozyme, DNase I, and phenylmethylsulfonyl fluoride. After sonication and centrifugation at 145,000 × *g*, the supernatant was applied to a column (CC250/3 Nucleosil 100-5-C18, Macherey Nagel). The column was equilibrated with buffer B (50 mM sodium dihydrogen phosphate/sodium dihydrogen phosphate, 0.1 sodium acetate disodium hydrogen phosphate/sodium hydrogen phosphate (pH 4.95) and 15% methanol) for 40 min. The flow rate was 0.5 ml/min throughout. Retention times are relative to the appearance of the injection peak. Crystallization—The wild-type protein could be concentrated only to 6 mg/ml. Needle-shaped crystals were grown at room temperature (21 °C) by vapor diffusion of 4 μl of sitting drops with 0.5 ml of reservoir buffer from a 6 mg/ml protein solution mixed in 1:1 ratio with reservoir buffer containing 0.02 mM calcium chloride dihydrate, 0.1 sodium acetate, and centrifugation at 145,000 × *g* for 1 h at room temperature and washed several times to remove traces of SDS. The polymer was resuspended in 1.2 ml of 10 mM Tris (pH 7.0) and treated first with 120 μg of o-amylase (Fluka) for 2 h at 37 °C to degrade high molecular mass glyco- gen and then incubated with 240 μg of Pronase (Fluka) for 1.5 h at 60 °C to release covalently bound lipoprotein. After another wash with boiling 8% SDS, the pellet was resuspended in 1.2 ml of 10 mM Tris (pH 7) and treated overnight with 200–300 μg of lysozyme at 37 °C. Material that remained insoluble after the lysozyme treatment was pelleted and discarded. The supernatant was filtered through a 5-kDa cutoff filter (Vivascience) and subjected to time-of-flight mass spectrometric analysis. For the most prominent peak, a molecular mass of 940.4 ± 0.1 Da was found, consistent with the molecular mass of the GlcNAc-MurNAc disaccharide with a tetrapeptide side chain (see Fig. 1). A priori, the glycosidic linkage in the disaccharide could connect either C-1 of GlcNAc and C-4 of MurNAc or, alternatively, C-1 of MurNAc and C-4 of GlcNAc. The assignment in Fig. 1 is based on the known specificity of lysozyme (16) and was not checked experimentally. To estimate the amount of GlcNAc-MurNAc tetrapeptide, several methods were tried. Derivatization of the free amino group in *meso*-diaminopimelic acid with Sanger reagent (fluorodinitrobenzene) failed; thus, reduction of the muramic acid to muramitol was used to titrate the amount of di- saccharide. The calculations assumed that 4 mol of disaccharide can be reduced per mol of NaBH₄ with 100% reaction efficiency and are therefore likely to overestimate the true amount of GlcNAc-MurNAc tetrapeptide.

**Activity Assay**—2 μg of GlcNAc-MurNAc tetrapeptide (~2 nmol) was incubated with 20 ng of LD-carboxypeptidase (0.57 pmol of protomer) or control for 20 min at 37 °C in either 10 mM Tris (pH 7.0) or crystallization buffer (50 mM citric acid/citrate (pH 4.5)). The wild-type enzyme was active in both buffers. The activity of mutants with altered triad residues was undetectable under these conditions. To establish the limits of their activity, 2 μg of mutant enzyme (57 pmol of protomer) was incubated with 2 μg of substrate (~2 nmol) for 20 min or overnight at 37 °C. The reaction was monitored by HPLC² (Waters) using a C18 column (CC250/3 Nucleosil 100-5-C18, Macherey Nagel). The column was equilibrated with buffer B (50 mM phosphoric acid/sodium dihydrogen phosphate (pH 4.3)), washed for 5 min with buffer B after injection, and ramped to buffer C (75 mM sodium dihydrogen phosphate/sodium hydrogen phosphate (pH 4.95) and 15% methanol) for 40 min. The flow rate was 0.5 ml/min throughout. Retention times are relative to the appearance of the injection peak.

**Crystallization**—Much better, plate-like crystals were obtained by vapor diffusion at room temperature (21 °C) when a 6 mg/ml protein solution was mixed in 1:1 ratio with reservoir buffer and 25% (2 viewing) 2,3-butanediol. Crystals belonged to ~2.9 Å and showed clear signs of high anisotropic mosaicity and disorder.

Much better, plate-like crystals were obtained by vapor diffusion at room temperature (21 °C) when a 6 mg/ml protein solution was mixed in 1:1 ratio with reservoir buffer containing 0.02 mM calcium chloride dihydrate, 0.1 sodium acetate trihydrate (pH 4.6), and 30% (v/v) 2-methyl-2,4-pentanediol. Crystals could be flash-cryocooled from mother liquor and diffracted in-house to ~2.9 Å and showed clear signs of high anisotropic mosaicity and disorder.

²The abbreviations used are: HPLC, high performance liquid chromatography; MAD, multwavelength anomalous diffraction.
to space group P2₁, with cell constants \( a = 52.2, b = 78.3, \) and \( c = 71.3 \) Å and \( \beta = 104.3^\circ \). The presence of a strong signal for a local 2-fold axis in the self-rotation function and the solvent content indicated that the crystals contained two subunits of \( \text{LD-carboxypeptidase} \) in the asymmetric unit.

**Structure Determination**—A native data set to 1.5-Å resolution and a three-wavelength selenomethionine MAD data set to 2.4-Å resolution were collected at beamline BW6 (Deutsches Elektronen-Synchrotron, Hamburg, Germany). As each protomer could contain up to four selenomethionine residues (the initiator methionine of the N-terminal His tag, the native initiator methionine, and two methionine residues in the sequence), up to eight selenomethionine sites were expected, but only four sites were identified by SHELXD (17). Phasing and solvent flattening suggested a clear preference for one hand over its enantiomeric alternative, but the resulting map was clearly of insufficient quality for model building. Nevertheless, it was good enough for the program GETAX (18) to define the location of the dimer axis, which turned out to be consistent with the positions of four selenium atoms. To further improve phases, we screened heavy atom soaks for the possible generation of derivatives in-house. A tungstate soak showed a clear peak in the \( y \) coordinate (15). The behavior of the monoisotopic mass of the dominant fragment in lysozyme digests matched the expected mass of the disaccharide tripeptide. When material from a reverse phase column, four peaks instead of the expected two peaks were found (Fig. 2A). Such peak splitting was reported previously for lysozyme digests of \( E. \text{coli} \) peptidoglycan and was attributed to the equidistant \( \alpha \) and \( \beta \) positions (15). The behavior of \( P. \text{putida} \) peptidoglycan digests is consistent with this interpretation. When material from a

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**TABLE ONE**

| Data collection and phasing statistics for the selenomethionine crystals and the tungstate soak |

The DM program phase improvement step combined solvent flattening and 2-fold averaging. FOM, figure of merit.

| Derivative | Selenomethionine | Tungstate |
|------------|-----------------|-----------|
| Unit cell dimensions | | |
| \( a (\text{Å}) \) | 52.41 | 52.29 |
| \( b (\text{Å}) \) | 78.08 | 77.77 |
| \( c (\text{Å}) \) | 71.24 | 71.18 |
| \( \beta \) | 104.18° | 104.42° |
| Wavelength (Å) | 0.9500 | 0.9792 | 0.9797 |
| Total no. of reflections | 172,955 | 158,313 | 158,155 |
| Unique no. of reflections | 47,559 | 43,215 | 43,249 |
| Resolution (Å) | 2.3 | 2.4 | 2.4 |
| Completeness (%) | 99.0 (80.1) | 99.3 (88.4) | 99.1 (81.6) | 89.5 (77.1) |
| \( I/\sigma \) | 17.7 (4.1) | 19.7 (6.0) | 19.0 (5.4) | 21.1 |
| \( R_{\text{sym}} \) (last shell) (%) | 8.3 (28.7) | 7.4 (21.2) | 7.8 (22.6) | 8.3 (12.6) |
| No. of sites | 4 | 1 | |
| FOM | 0.43 (20 to 2.5 Å) | 0.48 (20 to 2.5 Å) | 0.32 (15 to 2.8 Å) |
| Combined FOM | | |
| FOM after DM | 0.74 (20 to 2.5 Å) | |

**RESULTS**

**Choice of Enzyme**—The best characterized \( \text{LD-carboxypeptidase} \) is the enzyme from \( E. \text{coli} \), which is very unstable (2). Therefore, we decided to concentrate on homologs from other bacterial species. The conserved hypothetical protein from \( P. \text{aeruginosa} \) (Swiss-Prot accession number Q9HTZ1) could be expressed in \( E. \text{coli} \), was stable enough for purification, and could be crystallized. As the open reading frame had never been characterized, a substrate was required to test its activity.

**Substrate Preparation**—If the \( P. \text{aeruginosa} \) hypothetical protein (Swiss-Prot accession number Q9HTZ1) were an \( \text{LD-carboxypeptidase} \), it should be able to cleave the GlcNAc-MurNAc tetrapeptide, an abundant fragment in lysozyme digests of many Gram-negative bacteria. As \( P. \text{aeruginosa} \) is a human pathogen and subject to handling restrictions, we purified and digested the peptidoglycan from the closely related \( P. \text{putida} \). Mass spectrometry showed that the monoisotopic mass of the dominant fragment in the lysozyme digests matched the expected mass of the disaccharide tripeptide fragment. When material from a reverse phase column, four peaks instead of the expected two peaks were found (Fig. 2A). Such peak splitting was reported previously for lysozyme digests of \( E. \text{coli} \) peptidoglycan and was attributed to the equilibrium between the anomic forms of muramic acid with the C-1 OH in the \( \alpha \) - and \( \beta \) positions (15). The behavior of \( P. \text{putida} \) peptidoglycan digests is consistent with this interpretation. When material from a
single chromatography peak was re-injected, a peak doublet was again observed (data not shown). When the mixture was pretreated with the reducing agent sodium borohydride, only two instead of the usual four peaks were observed. The altered positions of the new peaks indicated that reduction of muramic acid to muramitol had indeed taken place (Fig. 2D).

The P. aeruginosa Hypothetical Protein Has Ld-Carboxypeptidase Activity—The recombinant P. aeruginosa hypothetical protein (SwissProt Accession Number Q9HTZ1) converted the GlcNAc-MurNAc tetrapeptide efficiently to the tripeptide. As both substrate peaks disappeared, we concluded that both anomerically forms of the GlcNAc-MurNAc tetrapeptide are substrates. The GlcNAc-MurNAc tetrapeptide with muramitol instead of muramic acid, which resulted from the sodium borohydride treatment, was still a substrate (Fig. 2, D and E), suggesting that the region around the anomic carbon of the GlcNAc-MurNAc tetrapeptide is not important for substrate recognition. As the Ld-carboxypeptidase substrate preparations were contaminated with the product, the reaction was best monitored by subtracting the HPLC trace prior to the reaction from the trace after the reaction. In this mode of presentation, the destruction of substrates gives rise to negative ("upward") peaks, and the emergence of products manifests itself as positive peaks (Fig. 2, C and F).

Structure Determination—To identify candidate active-site residues of Ld-carboxypeptidase, the crystallographic approach was taken. P21 crystals of Ld-carboxypeptidase from P. aeruginosa with two protomers in the asymmetric unit could be grown and diffracted to a resolution of 1.5Å on beamline BW6 (Deutsches Elektronen Synchrotron). The selenomethionine variant of the protein was produced and crystallized, but because it contained only two internal well ordered selenomethionine residues/protomer, the MAD phases were not sufficient for model building and had to be combined with the single isomorphous replacement phases for a tungstate derivative. The combined phases were improved and extended by 2-fold averaging and solvent flattening as described under "Experimental Procedures." A partially built model served as the starting point for automatic density interpretation by the ARP/wARP procedure (21). The final refinement statistics appeared satisfactory (TABLE TWO). The C-α trace of the Ld-carboxypeptidase dimer is presented in Fig. 3.

A Dimer in the Crystal and Likely in Solution—The P21 crystals contain two protomers in the asymmetric unit, which are related by a pure 2-fold rotation without a screw component (Fig. 3). The interface buries a total of 2800Å² and extends over 1400Å² and is therefore sufficiently large to be potentially relevant in solution. We therefore scored this contact region with the DCOMPLEX server, which applies statistical potentials to distinguish biologically relevant from purely crystallographic protein-protein contacts (22). The server suggested a value of ~25 kcal/mol for the affinity between the protomers and classified the interface as a "true interface." To confirm this conclusion experimentally, we performed size chromatography runs in two different salt concentrations. P. aeruginosa Ld-carboxypeptidase migrated with apparent molecular masses of 56 and 51 kDa in low salt (10 mM Tris (pH 7.5) and 50 mM NaCl) and high salt (10 mM Tris (pH 7.5) and 200 mM NaCl) buffers, respectively. These values are in between the calculated protomer mass of 34.6 kDa and the calculated dimer mass of 69.2 kDa (Fig. 4, A and B) and favor the dimer, at least for the high protein concentrations that were used for size chromatography. As the E. coli Ld-carboxypeptidase is known to dimerize as well, we suspected that dimerization may be a general feature of Ld-carboxypeptidases. To assess the conservation of the dimerization interface, we used the ConSurf server to map amino acid conservation scores to the protein surface (Fig. 5) (23). Fig. 5 (compare A, B, and C) shows that the interface is only slightly more conserved than other regions of the protein surface. Therefore,
the oligomerization state of LD-carboxypeptidases from other species remains undetermined.

**LD-Carboxypeptidase Architecture**—The LD-carboxypeptidase protomers are continuously traceable except for the very N-terminal residues and residues 143–151 and 143–149 in the two protomers. Superposition showed that the main chains of the independently traced and refined protomers are essentially identical except for the loop regions from residues 177 to 182, which are involved in extensive, but non-

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### TABLE TWO

**Data collection and refinement statistics for wild-type and mutant LD-carboxypeptidases**

We note that each LD-carboxypeptidase protomer contains two residues (Ser115 and Arg57) at position i+1 of a type II’ β-turn, which is normally reserved for glycines. Arg57 is far away from the active site and therefore not discussed. r.m.s.d., root mean square deviation.

| LD-Carboxypeptidase | Wild-type | S115A | H285A |
|---------------------|-----------|-------|-------|
| **Data collection statistics** | | | |
| Space group | P2₁ | P2₁ | P2₁ |
| a (Å) | 52.2 | 52.5 | 52.4 |
| b (Å) | 78.3 | 77.8 | 78.0 |
| c (Å) | 71.3 | 71.1 | 71.3 |
| β (°) | 104.3 | 104.5 | 104.2 |
| Wavelength | 1.05 | 1.54 | 1.54 |
| Total no. of reflections | 187,515 | 60,648 | 66,616 |
| Unique no. of reflections | 86,224 | 21,396 | 21,640 |
| Resolution range (Å) | 20.0 to 1.5 | 15.0 to 2.4 | 15.0 to 2.4 |
| Completeness (last shell) (%) | 97.1 (95.1) | 98.6 (96.4) | 99.3 (99.2) |
| I/σ (last shell) | 8.2 (3.6) | 7.8 (3.2) |
| R_sym (last shell) (%) | 3.7 (25.2) | 10.3 (22.6) | 13.7 (36.3) |
| B_min from Wilson (Å²) | 16.0 | 22.2 | 23.3 |
| **Refinement statistics** | | | |
| Resolution range (Å) | 20.0 to 1.5 | 15.0 to 2.4 | 15.0 to 2.4 |
| No. of reflections (work/test) | 81,899/4325 | 20,308/1082 | 20,550/1078 |
| No. of protein atoms (excluding H) | 4505 | 4503 | 4495 |
| No. of water molecules | 313 | 313 | 317 |
| R-factor (%) | 16.5 | 24.2 | 21.3 |
| R_free (%) | 17.8 | 24.7 | 24.5 |
| r.m.s.d. bond lengths (Å) | 0.01 | 0.01 | 0.01 |
| r.m.s.d. angles (°) | 1.4 | 1.4 | 1.4 |
| r.m.s.d. non-crystallographic symmetry (Å) | 0.5 | 0.5 | 0.5 |
| Ramachandran core region (%) | 91.8 | 91.9 | 91.9 |
| Ramachandran allowed region (%) | 7.0 | 6.9 | 6.9 |
| Ramachandran additionally allowed region (%) | 1.2 | 1.2 | 1.2 |
| Ramachandran disallowed region (%) | 0.0 | 0.0 | 0.0 |

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**FIGURE 3. Stereo Cα trace of the LD-carboxypeptidase dimer viewed along the 2-fold axis.** Every 10th residue is marked by a small circle, and residues 50, 100, 150, 200, and 250 are indicated by large circles. N-terminal domains are in black, and C-terminal domains are in gray. The dotted lines represent the disordered linkers between the N- and C-terminal domains. The side chains of the active-site residues Ser115, Glu217, and His285 are shown in atom representation. This figure was made with the MolScript program (54).
equivalent crystal contacts (data not shown). As this region is far away from the active site, the protomers were treated as equivalent in the following experiments. Each protomer can be divided into an N-terminal "sheet domain" (Fig. 3, black) and a C-terminal "barrel domain" (gray).

**N-terminal Domain**—The N-terminal sheet domain is organized around a five-stranded parallel β-sheet with a strand order of 2-1-3-4-5. The strands are connected by four helices. Three helices occur in β-α-β motifs (β1-α1-β2, β3-α-β4, and β4-α-β5) (Figs. 3 and 6 and supplemental Fig. 1, A and B), which are all right-handed as usual (24).

**C-terminal Domain**—The C-terminal domain is built around a seven-stranded parallel β-sheet with a strand order of 1-7-6-2-3-4-5 and sheet number 10, which is intermediate between the minimal sheet 8 and the maximal sheet 12, which have been found for seven-stranded β-barrels (25, 26). The barrel is made from a three-stranded parallel sheet and a four-stranded antiparallel sheet that have been joined together on the edges. The preference for right-handed β-α-β motifs in the antiparallel part of the barrel places all connecting helices on the outside of the barrel (Figs. 3 and 6 and supplemental Fig. 1, C and D).

**Active Site**—The insensitivity of *E. coli* LD-carboxypeptidase to EDTA (1, 12) and our failure to locate zinc ions in a crystal of LD-carboxypeptidase that had been soaked with 1 mM Zn²⁺ prior to the collection of a two-wavelength MAD data set suggested that the enzyme is probably not a metallopeptidase. We therefore inspected all serine and cysteine residues of LD-carboxypeptidase for environments that could render them more nucleophilic. The only strong candidate for an enzyme nucleophile from this search was Ser¹¹⁵. As shown in Fig. 7, the O-γ atom of this serine residue is located in the plane of the imidazole ring of His²⁸⁵ and only 3.0 Å away from the N-ε atom. We presume that His²⁸⁵ is unchanged and remains in the tautomeric state with the proton on the N-δ atom. In this scenario, the serine O-γ atom could donate a hydrogen bond to the N-ε atom of the imidazole ring either directly or via a bridging water molecule, and the N-δ atom of the imidazole ring could donate a hydrogen bond to an oxygen atom of the terminal carboxylate of Glu²¹⁷. We point out that such a hydrogen-bonding arrangement would not only orient the plane of the imidazole ring, but would also be expected to make His²⁸⁵ N-ε more basic and thus better suited as a general base in catalysis.

**Structural Features in Support of the Proposed Triad**—Many features of the crystal structure support the interpretation that LD-carboxypeptidase is a serine protease and that Ser¹¹⁵, His²⁸⁵, and Glu²¹⁷ form a functional catalytic triad. (a) It is known that active sites are often located in regions with rare backbone conformations (27, 28). Ser¹¹⁵ is located in such a region. For both subunits, our model places this residue at position 2 (also called “+1”) of a perfect type II’ turn, which is normally reserved for glycines (29). The serine residue at this position in the LD-carboxypeptidase structure has (φ, ψ) = (−60°, −130°) and falls in either the generously allowed or disallowed region of the widely used versions of the Ramachandran plot for non-glycine residues (30–33). (b) Ser¹¹⁵ is strategically located at the N terminus of a helix, where the helix dipole moment would be expected to favor deprotonation and thus catalysis. For cysteines in this location, it is known that deprotonation can occur prior to catalysis (34); for serine residues, which are more basic, deprotonation is more likely to happen during catalysis. (c) The proposed active site is strategically located at the interface between the sheet and barrel domains. The proposed nucleophile (Ser¹¹⁵) is anchored on the N-terminal sheet domain, whereas the other two triad residues (His²⁸⁵ and Glu²¹⁷) are placed on the C-terminal barrel domain, allowing a putative substrate to bind to the clef at the interface between the two domains. Moreover, such a location would place a substrate essentially at the “top” of the seven-stranded barrel in the C-terminal domain, thought to be the preferred binding place for substrates in both β- and αβ-barrels. (d) A large number of ordered water molecules are found in the vicinity of the active site, some of which may be bound to otherwise unfilled substrate-binding subsites. (e) All three proposed triad residues are strictly conserved among LD-carboxypeptidases and their homologs. Moreover, ConSurf mapping of alignment scores to the protein surface showed that the active-site region is among the most conserved regions of the protein (Fig. 5A).

**Mutagenesis Data Support the Proposed Triad**—The role of the proposed triad residues was checked experimentally by mutating them individually to alanine. In a direct comparison of the activities of the wild-type enzyme and the S115A, H285A, and E217A mutant proteins,
all mutant proteins behaved indistinguishably from the control without enzyme (data not shown). To make the comparison more quantitative, we incubated 2 nmol of substrate in a 24-μl sample volume with either 20 ng of wild-type enzyme or a 100-fold excess of the mutant proteins. Total substrate turnover after 20 min and after an overnight incubation was quantified by HPLC (Fig. 8). We concluded that any residual activity...
of the mutants was within experimental error and at least 500-fold lower than the activity of the wild-type enzyme.

Crystallography and Structure Determination of Active-site Mutants—The mutagenesis results showed that S115A, H285A, and E217A are required for enzyme activity, but did not necessarily prove that these residues are directly involved in catalysis because the defects of the mutant enzymes could also be due to impaired substrate binding or an overall folding problem. To test these possibilities, we assayed the ability of the mutants to protect substrates from the activity of the wild-type enzyme. The results were inconclusive and poorly reproducible, suggesting that the mutants may not be very stable (data not shown). The CD spectra of the wild-type and mutant enzymes suggested that all proteins were folded, but it remained unclear whether significant differences in the spectra could be attributed to different protein contaminations alone (data not shown). Therefore, we decided to verify the structures of the mutant proteins crystallographically. The S115A and H285A mutants could be crystallized under the same conditions as the wild-type enzyme with very similar cell constants. The S115A mutant was essentially indistinguishable from the wild-type enzyme, except that there was no density in the place of the Ser115 O-atom, which confirmed the mutation. The H285A mutant enzyme was also indistinguishable outside the active-site region, but Glu217, anchored by hydrogen bonds from His285 and Asn192 in the wild-type enzyme, flips into a new position in the H285A mutant enzyme (Fig. 7B). Altogether, the crystallographic analysis of the mutants showed that, for the S115A and H285A mutant LD-carboxypeptidases, the lack of activity cannot be attributed to a folding defect.

FIGURE 7. LD-Carboxypeptidase active site in ball-and-stick representation. Only selected residues and the electron density covering these residues are shown for clarity. The densities are annealed omit maps, calculated by omitting all residues and water molecules shown. Selected hydrogen-bonding or salt-bridging interactions are highlighted by green lines. A, wild-type enzyme (stereo view); B, H285A mutant protein. Note the flip and change in hydrogen bonding of Glu217. The density for Ser115 in the mutant is poor, probably reflecting disorder in this region as a result of the mutation. The figure was made with the MolScript program (34).

FIGURE 8. Comparison of the activities of wild-type and mutant LD-carboxypeptidase proteins. 24-μl reactions with 2 nmol of substrate were set up with either 20 ng of wild-type (WT) enzyme or 2 μg of mutant proteins to make the comparison more stringent. Total substrate turnover was quantified after the indicated times by HPLC and was not normalized for the amount of protein in the reaction.

A Novel Peptidase—Dali quantitative structure comparisons (35) between LD-carboxypeptidase and the proteins in the Protein Data Bank indicated that LD-carboxypeptidase is structurally more similar to uroporphyrinogen III synthase (code 1wd7) (36), the receiver domain of a plant ethylene receptor (code 1dcf) (37), and a signaling protein (code 1tij) (38). The Dali Z-scores, which measure the similarity in S.D. above average, were 7.5, 6.2, and 6.0, respectively. The high values probably result from the canonical architecture of LD-carboxypeptidase, which is built around a β-sheet and a β-barrel, and provide no insight about function or mechanism. Altogether, the Dali search algorithm identified >60 proteins with Dali Z-scores >4, with no clear cutoff between “true” and “false” positives. We therefore searched this list for hydrolases. S-Adenosylhomocysteinase (Protein Data Bank code 1b3r), a thioether hydrolase that uses reversible redox chemistry to facilitate the hydrolysis reaction (39), scored highest and is likely a purely structural match. Interestingly, the second highest scoring hydrolase, with a Dali Z-score of 4.7, was dienelactone hydrolase (Protein Data Bank code 1din), an αβ-hydrolase with a Cys-His-Asp catalytic triad (40). The good structural match between this enzyme and LD-carboxypeptidase is the result of the nearly identical β-sheet topologies of the two enzymes. The optimal structural superposition brings the nucleophiles of the active sites in roughly the same region, but places them in nonequivalent loops of the two proteins (data not shown).

A Strained Active-site Serine Residue—The most remarkable feature of the suggested active site in LD-carboxypeptidase is the very unusual main chain conformation of the active-site serine residue, which has Ramachandran angles in the lower right quadrant of the Ramachandran plot. We believe that the unusual serine conformation is a feature of the model and not a crystallographic error: the structure was solved at 1.5-Å resolution, which is sufficient to locate carbonyl oxygen atoms with confidence. Weighted 2Fo – Fc and omit maps show robust density for this region, and B-factor values for all non-hydrogen atoms are below 15 Å² (except for the Ser115 O-γ atom, which has B-factors slightly below 20 Å²) in the two subunits. The modeled conformation implies hydrogen bonds from Phe114 O to Ile117 NH and from Ser115 O to Ser118 NH.
and O-γH, which together could compensate for the repulsion between Ser\textsuperscript{115} C-β and Phe\textsuperscript{114} O. In our model (which was refined with standard van der Waals terms), the distance between these two atoms is 2.9 Å and is thus significantly shorter than the 3.3-Å van der Waals equilibrium distance (41), but still longer than the 2.8-Å "normally allowed" distance that was used in the original work of Ramachandran et al. (30). Thus, the clash between Ser\textsuperscript{115} C-β and Phe\textsuperscript{114} O cannot be very severe.

We note that the conformation of the active-site serine residue in LD-carboxypeptidase is not unprecedented and that similar conformations have been described in two recent surveys of Ramachandran outlier residues. Gunasekaran et al. (42) found such conformations in the second position (i+1) of perfect type II′ β-turns. In a follow-up on this work, Pal and Chakrabarti (43) reported that such residues (in their nomenclature, "region II" residues) are "common in the first position of 3\textsubscript{10}-helices, which in the majority of cases lead into α-helices." As type II′ β-turns are essentially short 3\textsubscript{10}-helices, the results of Gunasekaran et al. and Pal and Chakrabarti are perfectly consistent. The active-site serine residue in LD-carboxypeptidase fits their description: it is the i+1 residue of a type II′ β-turn, which itself is part of a 3\textsubscript{10}-helix, which leads into a regular α-helix.

A Nucleophilic Elbow in LD-Carboxypeptidase and αβ-Hydrolases—The location of a serine residue at a sharp kink between a β-strand and an α-helix reminded us of the nucleophilic elbow in αβ-hydrolases. αβ-Hydrolases form a structurally defined, very large group of hydrolases that use serine or cysteine as the nucleophile, histidine as the general base, and aspartate or glutamate as the third triad residue (44, 45). In the lipase from Geotrichum candidum, which was analyzed at 1.8-Å resolution, the triad is Ser-His-Glu, as in LD-carboxypeptidase (46, 47). Therefore, we chose this enzyme as a representative of the αβ-hydrolases for a detailed comparison (Fig. 9).
In both structures, the joint in the nucleophilic elbow is a type II' β-turn with the serine in the i+1 position, even though the turn in αβ-hydrolases was originally and inaccurately described as "γ-like" (44). In both structures, the serine residue has the same unusual main chain conformation, which in αβ-hydrolases is thought to make the serine easily approachable by substrate and the hydrolytic water molecule (45). αβ-Hydrolases and LD-carboxypeptidase superimpose perfectly well in the immediate vicinity of the active-site serine residue, but they differ downstream of the type II' β-turn: in LD-carboxypeptidase, it is part of a 310-helix, whereas in the lipase from G. candidum, it is immediately followed by a standard α-helix. This difference results in a significantly different orientation of the helix relative to the serine residue. Remarkably, there is an almost equivalent difference in the orientation of the β-strand upstream of the active-site residue, so the overall shape of the nucleophilic elbow is very similar in both proteins. For αβ-hydrolases, it has been noted that the sharp turn at the nucleophilic elbow, which packs the helix very tightly against the preceding strand, constrains the sequence around the active-site serine residue to GXSXG (44, 45). In LD-carboxypeptidases, which have a slightly different backbone, the motif is GXSDX, without the requirement for the second glycine residue. The conservation of the aspartate residue immediately downstream of the active-site serine residue is understandable: its side chain carboxylate oxygen atoms accept hydrogen bonds from main chain amides and thus help to fix the sharp turn in the nucleophilic elbow (Fig. 9).

A Ser-His-Glu Triad—A Ser-His-Glu triad is not only present in the lipase from G. candidum (47), but was also found in several other αβ-hydrolases such as acetylcholinesterase (48) and butyrylcholinesterase (49). Interestingly, all these enzymes cleave phosphate esters, not amide bonds. In peptidases with a serine nucleophile and histidine general base, aspartate and not glutamate is the norm for the third triad residue. The rule applies to trypsin-like (MEROPS clan PA), subtilisin-like (MEROPS clan SB), and prolyl oligopeptidase-like (MEROPS clan SC) peptidases, which are so different in their overall folds that they cannot have arisen from a single ancestor. The preference for aspartate over glutamate as the third triad residue in serine peptidases is not understood and may be fortuitous; nevertheless, to the best of our knowledge, aspartyl dipeptidase with its Ser-His-Glu triad is so far the only exception to this rule (50). Our present work on LD-carboxypeptidase adds another peptidase to the still very short list of hydrolases with a Ser-His-Glu triad that cleave peptide bonds and not phosphate esters.

Bacterial Versus Eukaryotic LD-Carboxypeptidases—Early biochemical data indicated that several different LD-carboxypeptidase activities may be present in bacteria (6), but only one such activity has been traced back to a cloned and characterized protein (1). Surprisingly, weak LD-carboxypeptidase activity has also been reported for an eukaryotic protein, the Drosophila peptidoglycan recognition protein-SA, which is involved in the sensing of bacterial infection and in the activation of the Toll pathway. Peptidoglycan recognition protein-SA shares the fold with T7 lysozyme, but has lost the metal-binding site and has a Ser-His dyad instead (51). Based on the crystal structure, a threonine residue might act as the third triad residue, but this hypothesis was not checked biochemically (51). Either way, it is clear that the LD-carboxypeptidases from Drosophila and P. aeruginosa are different.

LD-Carboxypeptidase Homologs with a Different Activity—Bacterial LD-carboxypeptidases are genome-encoded proteins. E. coli strains that produce the antibiotic microcin C7 additionally contain a plasmid-encoded LD-carboxypeptidase homolog, which mediates resistance against exogenously added microcin C7 and is therefore also known as microcin C7 self-immunity protein McCF (52). Sequence alignment of LD-carboxypeptidases and McCF showed that all three triad residues of LD-carboxypeptidase are conserved in McCF (Fig. 6), strongly suggesting that McCF shares not only the fold, but also the hydrolytic activity with LD-carboxypeptidases. Substrates of McCF are not known, but microcin C7 precursors are obvious candidates. However, microcin C7 maturation requires only one hydrolysis reaction, for which no enzyme has yet been found: the conversion of asparagine to aspartate (53). As this reaction could also occur spontaneously and because the link of this reaction to self-immunity is unclear, the question of the physiological activity of McCF remains unresolved.

More LD-Carboxypeptidases—At present, proteins with homology to LD-carboxypeptidase are variously annotated as conserved hypothetical proteins, (putative) muramoyl-tetrapeptidase carboxypeptidases, or (putative) microcin C7 self-immunity proteins in the sequence data bases. According to the annotations, there are about as many self-immunity proteins as LD-carboxypeptidases. This classification is suspicious because (a) most bacteria are not expected to produce microcin; (b) nearly all proteins are chromosomally encoded; (c) the presence of a microcin C7 self-immunity protein usually correlates with the lack of an LD-carboxypeptidase; and (d) the LD-carboxypeptidases and microcin C7 self-immunity proteins do not segregate into two separate branches in phylogenetic trees. Therefore, it is likely that many (but certainly not all) proteins that are currently annotated as (putative) microcin C7 self-immunity proteins are actually LD-carboxypeptidases.

Other LD-Carboxypeptidases—Our crystallographic and biochemical results have unexpected bearing on the still unanswered question of whether bacteria (and E. coli in particular) contain enzymes with LD-carboxypeptidase activity that are not homologous to the LD-carboxypeptidase described in this work. Previous work had traced some LD-carboxypeptidase activity to a 12-kDa protein that behaved as a monomer (7). When it was later found that the knockout of the only known LD-carboxypeptidase gene abolished all soluble LD-carboxypeptidase activity (1), it became likely that the 12-kDa protein was its proteolytically truncated version. Based on the results of this work, we can now rule out this possibility: Ser115 and His285 of the P. aeruginosa LD-carboxypeptidase triad align with Ser106 and His270 of E. coli LD-carboxypeptidase. As these residues are separated by 163 amino acids, any active degradation product with peptidase activity cannot be <18 kDa. To strengthen this conclusion, we looked for the minimal spacer between the active-site serine and histidine residues in LD-carboxypeptidase homologs and found that the spacer is always larger than the 12-kDa mass of the unknown LD-carboxypeptidase activity. Therefore, if 12 kDa is indeed the correct molecular mass of the unknown active enzyme, this enzyme cannot be a homolog of the LD-carboxypeptidase described in this work.

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Addendum—After deposition of the wild-type LD-carboxypeptidase coordinates in the Protein Data Bank and after submission of the original version of this manuscript, Osiępiuk et al. released their independently determined and still unpublished structure for “hypothetical protein Pa5198 from Pseudomonas aeruginosa” (I. Osiępiuk, E. Evdokimova, A. Savchenko, A. Edwards, and A. Joachimiak, Protein Data Bank code 1ZL0, deposition date May 4, 2005, release date June 21, 2005). Pa5198 is LD-carboxypeptidase. The LD-carboxypeptidase family U61 will be renamed family S66, clan SS in MEROPS (A. Barrett, personal communication). In Pfam, the entry Peptidase_U61 will be changed to Peptidase_S66 (A. Bateman, personal communication).
