AmpH, a Bifunctional dd-Endopeptidase and dd-Carboxypeptidase of Escherichia coli

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Received 8 July 2011/Accepted 6 October 2011

In Escherichia coli, low-molecular-mass penicillin-binding proteins (LMM PBPs) are important for correct cell morphogenesis. These enzymes display dd-carboxypeptidase and/or dd-endopeptidase activities associated with maturation and remodeling of peptidoglycan (PG). AmpH has been classified as an AmpH-type class C LMM PBP, a group closely related to AmpC β-lactamases. AmpH has been associated with PG recycling, although its enzymatic activity remained uncharacterized until now. Construction and purification of His-tagged AmpH from E. coli permitted a detailed study of its enzymatic properties. The N-terminal export signal of AmpH is processed, but the protein remains membrane associated. The PBP nature of AmpH was demonstrated by its ability to bind the β-lactams Bocillin FL (a fluorescent penicillin) and cefmetazole. In vitro assays with AmpH and specific muropeptides demonstrated that AmpH is a bifunctional dd-endopeptidase and dd-carboxypeptidase. Indeed, the enzyme cleaved the cross-linked dimers tetratetrapeptide (D45) and tetra-acetemperateptide (D44) with efficiencies (kcat/km) of 1,200 M⁻¹ s⁻¹ and 670 M⁻¹ s⁻¹, respectively, and removed the terminal N-alanine from muropeptides with a C-terminal d-Ala-d-Ala dipeptide. Both dd-peptidase activities were inhibited by 40 μM cefmetazole. AmpH also displayed a weak β-lactamase activity for nitrocefin of 1.4 × 10⁻³ nmol/μg protein/min, 1/1,000 the rate obtained for AmpC under the same conditions. AmpH was also active on purified sacculi, exhibiting the bifunctional character that was seen with pure muropeptides. The wide substrate spectrum of the dd-peptidase activities associated with AmpH supports a role for this protein in PG remodeling or recycling.

Bacterial peptidoglycan (PG) is an essential and specific structural component of the cell wall which is critical for preserving cell integrity and providing a defined cell shape (29). Escherichia coli PG assembly (murein synthesis) requires the polymerization of glycan strands composed of alternating N-acetylmuramic acid (NAMur) residues and subsequent cross-linking by short peptides (28). Penicillin-binding proteins (PBPs) are a family of enzymes of common evolutionary origin responsible for the polymerization and cross-linking of PG. PBPs share the ability to bind to β-lactam antibiotics that are substrate analogues of PG constituents, the natural substrates of PBPs in vivo (6). The PBPs have been organized into three classes based on sequence similarities (9). Class A high-molecular-mass PBPs (HMM PBPs) synthesize nascent glycan chains and cross-link them (transglycosylation and transpeptidation), but class B HMM PBPs catalyze cross-linking reactions only between stem peptides (transpeptidation). Peptidoglycan remodeling, and possibly some aspects of synthesis, is mediated by class C LMM PBPs. Class C PBPs display two predominant catalytic activities in vivo: dd-carboxypeptidase activity, which removes the terminal d-alanine from muropeptides with C-terminal d-Ala-d-Ala dipeptides, and dd-endopeptidase activity, which hydrolyzes peptide bridges linking adjacent glycan strands (23).

LMM PBPs are monofunctional or bifunctional dd-peptidases (12, 17, 16), but up to now it has not been clear what their roles are and which of these activities are predominant in vivo.

The ampH gene of E. coli codes for AmpH, a class C LMM PBPs of the AmpH type (23), and is included in the cluster of orthologous genes COG1680 (26), a family of genes whose products include AmpC-type β-lactamases and dd-carboxypeptidases. Although closely related to AmpC and other class C β-lactamases, AmpH did not show β-lactamase activity in a previous study (11). The phenotypes of certain (multiple) mutants suggest that although it is dispensable under laboratory conditions, AmpH might be relevant for PG metabolism and morphogenesis (11).

In this study, we wanted to define the enzymatic activities of E. coli AmpH on a broad range of purified muropeptides as well as on intact, purified sacculi. According to our results, AmpH is a bifunctional dd-endopeptidase-dd-carboxypeptidase which accepts a wide variety of muropeptides as substrates for both activities. Additionally, we have shown that AmpH appears to be processed when exported to the periplasm but remains membrane associated. The possible significance of these findings is discussed.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and enzymes. Escherichia coli DH5α (F⁻ lacZAM15 recA1) and E. coli BL21 (DE3) (F⁻ ompT thiS16 (F− mcrB−mut-1) gal dcm) were used as cloning hosts, and DV900 (CS-109 Δ[nosB ducA ducB dacC dacD pheG ampH ampic(pG)] (27) was used for peptidoglycan and muropeptide purification. DV900(Δ[DE3]) was constructed by using a ΔDE3 lysogenization kit (Novagen, Merck KGaA, Darmstadt, Germany), following the manufacturer’s recommendations, and was used to overexpress and purify the AmpH-End2 protein. Bacterial cultures were grown in one of the following media (1): Luria-

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† Published ahead of print on 14 October 2011.
Bertani (LB) medium, Super Optimal broth with catabolite repression (SOC) medium, or minimal M9 medium supplemented with 1 mM MgSO4 and 0.2% (wt/vol) Casamino Acids. Ampicillin (Amp) (100 µg/ml) and kanamycin (Kn) (30 µg/ml) were added as required.

Plasmid pGEM-T Easy vector (Promega, Madison, WI) carrying E. coli ampH (pGEM-H) was from our laboratory collection, and pET-28b (+) (Novagen) was purchased from Merck Chemicals Ltd. (Nottingham, United Kingdom). Restriction enzymes were from Fermentas, Life Sciences (Madrid, Spain), and T4 DNA ligase and Pfu DNA polymerase were from Biotools B&K Labs, S.A. (Madrid, Spain). All DNA manipulations were performed using standard methods, and DNA samples were purified using a Promega Wizard Plus SV miniprep (Promega, Madison, WI). DNA purification kit. PCR DNA products were cleaned using Promega Wizard SV gel and a PCR cleanup system (Promega, Madison, WI).

Chemical reagents. All chemicals were of analytical grade (Merck, Darmstadt, Germany). Imidazole, sodium dodecyl sulfate (SDS), and N-lauroylsarcosine sodium salt, ≥94% (Sarkosyl), were from Sigma-Aldrich (Saint Louis, MO), high-pressure liquid chromatography (HPLC)-grade methanol was obtained from Scharlau S.L. (Santematan, Spain), and ultrapure water (for the preparation of HPLC eluants) was generated on a Millipore Super-Q water purification system. Nitrocellulose was from Osoid (Cambridge, United Kingdom). Protein assays were performed using a standard procedure described by Bradford (1). E. coli His6-tagged AmpH, His6/ampH, and His6/ampH(NcI-H) were purified, digested with NdeI and EcoRI, and cloned into plasmid pET28b following the manufacturer's instructions. Vectors carrying ampH were used to transform E. coli DH5α, and after verification by DNA sequencing, were transformed into E. coli BL21(DE3), in which the induction assay was done. After transformation, competent E. coli cells were recovered in SOC medium with vigorous shaking for 1 h at 37°C; cells were then plated on LB agar plates for each sample. The change in absorbance at 482 nm over time was measured for each sample. The OD600 was determined with a D-C protein assay kit (Bio-Rad, Hercules, CA) and adjusted to 1.0 with water.

Cloning, overexpression, and purification of E. coli AmpH. The E. coli ampH gene (10), previously inserted forward (opposite to p-lac) into pGEM-T Easy (pGEM-H), was amplified by PCR, using the primers (Sigma-Aldrich, Saint Louis, MO) NcI-H (5′-CCATGGGCTTGAAACGTAGTCTGCT-3′) and RI-H (5′-TCCGATACCCGGCGGATAACCA-3′). The resulting fragments from PCR (a 1.28-kbp NdeI-EcoRI fragment with primers NdeI-H and RI-H and a 1.221-kbp Ncol-EcoRI fragment with primers Ncl-H and RI-H) were purified, digested with Ndel and EcoRI and with Ncol and EcoRI, and cloned into plasmid pET28b, following the manufacturer's instructions. Vectors carrying ampH were used to transform E. coli DH5α and, after verification by DNA sequencing, were transformed into E. coli BL21(DE3), in which the induction assay was done. After transformation, competent E. coli cells were recovered in SOC medium with vigorous shaking for 1 h at 37°C; cells were then plated on LB agar plates supplemented with kanamycin (Kn) (30 µg/ml) and incubated at 37°C overnight. The recombinant proteins His6 tags either at both termini (AmpH-ENd2) or at the C terminus (AmpH-Enc1). For overexpression, E. coli BL21(DE3) p28H-Enc1 (producing AmpH-Enc1) and BL21(DE3) p28H-ENd2 (producing AmpH-ENd2) were grown in a 30-liter fermentor (UD-30 B; Braun, Germany) in minimal M9 medium supplemented with 30 µg/ml Kn at 37°C for 1 to 2 h with vigorous agitation (220 rpm) until an optical density at 600 nm of 0.3 was reached. Induction of protein expression was achieved by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and incubation for a further 2 h at 37°C. Cells were then harvested and frozen at −70°C. To purify the expressed proteins, a portion of the cell paste (5 g) was thawed and suspended in 30 ml of saline phosphate buffer (PBS; 150 mM NaCl, 5 mM KCl, 25 mM NaH2PO4, 1.45 mM NaH2PO4, 70°C) and kept on ice for 10 to 20 min. EDTA (250 mM final concentration) was added to cell suspensions at 50°C. Supernatant (cytoplasmic fraction) was recovered and stored at 4°C, and pellets (membrane fraction) were resuspended in 50 µl of 30 mM Tris-HCl (pH 8.0) and 1 mM EDTA.

A aliquots of periplasmic (15 µl), cytoplasmic (5 µl), and membrane (1 µl) fractions were analyzed by SDS-PAGE in 10% acrylamide gels, and AmpH-ENd2 and AmpH-Enc1 were identified by Western blot immunodetection. Aliquots of the membrane fraction were washed with KCl, NaCl, and LiCl at final concentrations of 0.5 and 1 M to attempt dissociation of His-tagged AmpH forms from cell membranes.

β-Lactamase assay. β-Lactamase activity was assayed using the chromogenic substrate nitrocefin as described by O’Callaghan et al. (19). Nitrocefin stock solution (500 µg/ml) was made in PBS, pH 8.0. Samples with 125 µl (25 µg, 4 µM final concentration) of the purified AmpH-Enc1 or AmpH-ENd2 fraction or 20 µl of a pure stock solution of AmpC (0.1 µg, 1.7 × 10−3 µM final concentration) were transferred to 250 µl of nitrocefin stock solution (100 µM final concentration) in a final volume of 150 µl of PBS, pH 8.0, and incubated at 37°C for 5 min, then 3 µl of 0.25 M sodium HPO4, 1 M NaCl, 50 mM EDTA, 200 units penicillin G/mL, and 0.1 units β-Lactamase/mL were added to samples, and the mixture was kept on ice for 30 min. Insoluble samples without enzyme were used as blanks for each sample. The change in absorbance at 482 nm over time was measured on a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific Inc., Wal-ltham, MA).

β-Lactam binding assays. The in vitro assays for PBP activity were based on modifications of the procedures described by Sprent and Pardie (24) and Koyasu et al. (14). Membranes of E. coli BL21/p28H-ENd2 and E. coli CS109 were prepared as described before. Membranes from E. coli CS109 were used as a standard for the molecular weights of E. coli PBPs. Protein concentration was determined with a D-C protein assay kit (Bio-Rad, Hercules, CA) and adjusted to 5 mg/ml in PBS, pH 8.0. Purified AmpH-Enc1 (9.75 µg/15 µl) was preincubated with 5 µl of cefmetazole (20-µg/ml final concentration) at 37°C for 5 min, and nitrocefin stock solution (100 µM final concentration) was added to the mixture, and the mixtures were incubated for 30 min at 37°C. SDS sample buffer (10 µl) was added, and samples were boiled for 5 min. Insoluble samples were separated by SDS-PAGE in 8% acrylamide gels and detected directly on the gels on a ThermoFisher Scientific H10000 variable-mode imaging system (Gel Documentation System) at 37°C with aeration. The
cells from a 1-liter culture were harvested by centrifugation for 15 min at 4,300 × g at room temperature, resuspended in 20 ml of culture medium, and slowly mixed with an equal volume of 8% (wt/vol) boiling SDS with vigorous stirring. The suspension was boiled for 4 h and left overnight with moderate stirring at room temperature. Saccules were concentrated by centrifugation for 15 min at 265,000 × g. The pellet was washed with water until no SDS was detected by the method of Hayashi (10). The last pellet of the washing procedure was suspended in 10 ml of 10 mM Tris-HCl (pH 7.2) and digested first with 100 μg/ml α-amylase (EC 3.2.1.1; Sigma-Aldrich, Saint Louis, MO) for 1 h at 37°C and then with 100 μg/ml preactivated pronase E (EC 3.4.24.4; Merck, Darmstadt, Germany) at 60°C for 90 min. The enzymes were inactivated by boiling for 20 min in 1% (final concentration) SDS. The cell walls were collected by centrifugation as described above and washed three times with water. The peptidoglycan was stored in water at 4°C.

Preparation and separation of muropeptides. Peptidoglycan was digested in 50 mM phosphate buffer (pH 4.9) with Cellosolves (Hoechst AG, Frankfurt, Germany) 100 μg/ml final concentration at 37°C overnight. The enzyme reaction was stopped by boiling the sample for 2 min in a water bath and centrifuged (Eppendorf centrifuge at maximum speed for 10 min) to remove insoluble debris. The supernatant was mixed with 1/3 volume of 0.5 M sodium borate buffer (pH 9.0) and reduced with excess sodium borohydride (NaBH4) at 30 min at room temperature. The pH was tested with pH indicator strips (Acilit, Merck) and adjusted to 3 with phosphoric acid. All samples were filtered (Millex-GV filters; 0.22-μm pore size, 2.5-mm diameter; Millipore, Cork, Ireland) and stored at −20°C.

Separation of the reduced muropeptides by HPLC (325 system; Kontron Instruments) was performed essentially by the method of Glauner et al. (7, 8). The eluted muropeptides were monitored by measuring absorbance at 204 nm (Jasco UV-1570 spectrophotometer). When required, the individual peaks were collected, vacuum dried, and stored at −20°C.

Quantification of muropeptides. Individual muropeptides were quantified from their integrated areas using samples of known concentration as standards. Concentration of the standard muropeptides was determined as described by Work (30).

Enzymatic assay for determination of d-carboxypeptidase activity in vitro. Purified His-tagged AmpH forms were assayed using the tripeptide N-acetyl-Lys-D-Ala-D-Ala (8.3 mM final concentration), 3 μl of 10% buffer (Tris-HCl buffer, 300 mM, pH 7.5), and 17 μl each of purified His-tagged AmpH forms (1 μg, 0.8 μM final concentration) were incubated at 37°C for 60 min. At this time, 5 μl of O-dianisidine (10 mg/ml; Sigma-Aldrich, Saint Louis, MO) (in methanol) and 70 μl of enzyme/coenzyme mix (flavin adenine dinucleotide [FAD], peroxidase, and d-amino acid oxidase) were added to each sample. After a further 5 min at 37°C, 400 μl of 50% (vol/vol) methanol-water was added, and samples were incubated for another 2 min. The absorbance of each sample at 460 nm was measured immediately. Control reaction mixtures containing only enzyme and controls for natural degradation of the tripeptide without enzyme were made for each sample. Standard samples with known amounts of d-alanine and unknown samples were tested in triplicate.

HPLC assay of E. coli AmpH d-peptidase activities. All enzymatic reactions were analyzed in triplicate. The d-carboxypeptidase activity was assayed by monitoring the appearance of the monomeric reaction product disaccharide tetrapeptide, NAcGlcNAcMur-L-Ala-D-Glu-DAP-D-Ala (M4) (where DAP is diaminopimelic acid), in mixtures containing PBS buffer (pH 7.3), enzyme (17 μg; final concentration, 2.05 μM), and various concentrations (6.3 × 10−5 to 9.5 × 10−2 mM) of monomeric disaccharide pentapeptide, NAcGlcNAcMur-L-Ala-D-Glu-DAP-D-Ala-D-Ala (M5), as a substrate in a final volume of 2.5 ml. The absorbance of each sample at 460 nm was measured immediately. Control reaction mixtures containing only enzyme and controls for natural degradation of the tripeptide without enzyme were made for each sample. Standard samples with known amounts of d-alanine and unknown samples were tested in triplicate.

Analysis of kinetic data. The dependence of the reaction rate on concentration of substrates for the d-peptidase activities considered here was examined under the conditions described above for concentrations in the range of 10−5 to 10−3 M. Apparent Km and Vmax values were obtained from double-reciprocal Lineweaver-Burk plots of the data. kcat was determined as Vmax/[E0], where [E0] = nmol of protein/ml (His-tagged AmpH) (18). Graphical and statistical analyses were performed using Microsoft Excel (Microsoft Inc., Redmond, WA).

RESULTS

Cloning, overexpression, and identification of AmpH. Upon induction with 1 mM IPTG, the E. coli BL21 derivatives containing the plasmids p28H-ENd2 (producing AmpH-ENd2, containing His, tags at both termini) and p28H-ENC1 (producing AmpH-ENC1, containing a His tag at the C terminus) overexpressed a single protein that migrated to a position around 45 kDa as determined by SDS-PAGE (Fig. 1, lanes 2 and 4) in accordance with the molecular masses of 46.39 kDa and 44.22 kDa expected from the known DNA sequences of the fusion proteins, respectively. Furthermore, the DNA sequences of the 1.28-kb EcoRI-NdeI AmpH-ENd2 and 1.22-kb EcoRI-NcoI AmpH-ENC1 fragments were identical to the sequence of E. coli ampH deposited in GenBank (accession number AAC73479). MALDI-TOF analyses of purified His-tagged AmpH-ENd2 and AmpH-ENC1 peptide sequence determined that both clones contain a major fragment of a 41.86-kDa protein with a pI value of 9.33 calculated by the Mascot program. This molecular mass is compatible with the mobility of the protein band in SDS-PAGE (Fig. 2) but is smaller than the 46.393-kDa and 44.215-kDa theoretical molecular masses calculated from the nucleotide sequences of AmpH-ENd2 and AmpH-ENC1, respectively. However, that mass correlated precisely with the band observed by Western blotting of...
the purified proteins extracted from the membrane of the overproducing strain (Fig. 2), suggesting that both proteins, having a cleavable sequence, are actually cleaved at that site. Liquid chromatography-tandem mass spectrometry confirmed the mass loss of a 23-amino-acid peptide (MGLKRSLLFSAV LCAASLTSVHA) at the N terminus of AmpH-ENc1. Although the final products for both fusion proteins were identical, we recovered mainly the mature form (His labeled at the C terminus) for ENd2 protein but most often got two bands for ENc1 protein but most often got two bands for ENd2, a major component corresponding to the mature form (labeled at the C terminus) for ENc1 protein but most often got two bands for ENd2, a major component corresponding to the mature form (labeled at the C terminus) and a second band with the expected size of the precursor (His labeled at both termini). We speculate that presence of the His tag at the N terminus might slow maturation of the protein. Mapping of the C terminus showed that it remained intact in both proteins. Although both His-tagged forms of AmpH seem to be cleaved at the amino terminus, the mature protein remains membrane associated (see below). However, inspection of the amino acid sequence did not reveal potential hydrophobic membrane-anchoring sequences other than the N-terminal signal peptide.

Membrane localization of AmpH. Exponentially growing cultures of E. coli BL21/p28H-ENd2 and AmpH-ENc1 were subjected to cell fractionation to separate periplasmic, cytoplasmic, and membrane fractions and determine His-tagged AmpH location. As shown in Fig. 3, Western blot analysis of the fractions indicated that His-tagged AmpH-ENd2 derivatives were mostly associated with the membrane fractions but were essentially absent in both the periplasmic and the cytoplasmic fractions. Furthermore, AmpH derivatives could not be extracted when membrane fractions were washed with NaCl, KCl, or LiCl at high concentrations (0.5 M to 1 M) (data not shown), and only in the presence of strong detergents (1% SDS or 2% sodium sarcosylate) could AmpH be removed from the membranes (Fig. 4). The same results were obtained for AmpH-ENc1 (data no shown).

β-Lactam-binding capacity of purified AmpH. Membrane extracts from induced E. coli BL21/p28H-ENc1 cells with 1 mM IPTG and its corresponding purified AmpH-ENc1 form were used in a binding assay with fluorescent antibiotic. The four major PBPs of E. coli (PBP1a/b, PBP2, PBP3, and PBP5) were easily detected by SDS-PAGE, as well as a new, intense band matching the molecular weight calculated for the AmpH derivatives (Fig. 5). Cefmetazole is a β-lactam with high affinity for the LMM PBPs of E. coli. To confirm the β-lactam binding ability of AmpH-ENc1, cefmetazole was used as a competitor for Bocillin FL in competition assays. Indeed, preincubation of His-tagged AmpH-containing samples with 20 μg/ml of cefmetazole abolished binding to Bocillin FL (Fig. 5). The two bands corresponding to AmpH-ENd2, and overlapping PBPs in lane 1 of Fig. 5, are most probably due to partial cleavage of the signal peptide, producing both precursor and mature forms.
Enzymatic activity of AmpH. Because AmpH is closely related to the class C β-lactamases, we tested purified His-tagged AmpH forms for β-lactamase activity, with purified AmpC as a reference. AmpH displayed a clearly positive, although reduced, level of β-lactamase activity in assays using the chromogenic β-lactam nitrocefin as the substrate \( \left( 1.4 \times 10^{-3} \text{ nmol/µg protein/min} \right) \), about 1/1,000 the rate for AmpC \( \left( 8.7 \times 10^{-3} \text{ nmol/µg protein/min} \right) \) under identical conditions.

The potential DD-peptidase activities of His-tagged AmpH derivatives were studied by monitoring the effect of the protein on a series of purified muropeptides. Initial assays indicated that AmpH derivatives exhibited both DD-carboxypeptidase activity, as they were able to convert M5 and M5N into M4 and M4N, respectively, and DD-endopeptidase activity, because they cleaved the DD-peptide bridge in cross-linked muropeptides, releasing the monomeric subunits (Fig. 6). Once the activities were confirmed, a more detailed analysis was performed with AmpH-ENc1 and a number of substrates to define both the specificity of each reaction and the basic kinetic parameters. In all instances, the enzyme activity followed saturation kinetics and could be fitted to double-reciprocal Lineweaver-Burk plots to determine the apparent \( K_m \), \( V_{max} \), and \( k_{cat} \) by nonlinear regression.

dD-Carboxypeptidase activity was measured with the natural substrate M5 and the synthetic tripeptide \( \text{NacGlc-NAcMur-L-Ala-D-Glu-DAP-D-Ala} \) and M5 \( \left( \text{NacGlc-NAcMur-L-Ala-ε-DiaC-Lys-DAP-D-Ala} \right) \) in mixtures containing dimer D45 as the substrate. The DD-carboxypeptidase activity of AmpH-ENc1 \( \left( 1.38 \mu M \right) \) was assayed by monitoring the appearance of M4 in mixtures containing M5 as the substrate. Reactions were performed in the presence of cefmetazole (CF) \( 40 \mu M \) (a and d) or without CF (b and c) as described in Materials and Methods. Control samples with muropeptides without enzyme (c and f) were incubated simultaneously under the same conditions.

In order to exclude any contamination from other LMM PBPs, we cloned, expressed, and purified the ENd2 protein using a strain lacking all LMM PBPs and β-lactamases [DY900(DE3)]. Analysis of the three enzymatic activities (DD-endopeptidase, DD-carboxypeptidase, and β-lactamase) on this preparation produced the same or equivalent results \( \left( 2.94 \pm 0.40 \times 10^{-3} \text{ nmol/µg protein/min} \right) \) on M5, \( \left[ 134 \pm 40 \times 10^{-3} \text{ nmol/µg protein/min} \right] \) on D45, and \( 2.6 \times 10^{-3} \text{ nmol/µg protein/min with nitrocefin} \) as first found for the protein purified from
the BL21(DE3) strain, supporting the multifunctional character of AmpH.

Activity of AmpH on macromolecular peptidoglycan. Peptidoglycan hydrolases may or may not accept sacculi as substrates. Those that do not are generally associated with peptidoglycan turnover or recycling rather than biosynthesis. Therefore, we assayed the ability of AmpH-ENc1 to accept sacculi as substrates for both activities. As sacculi of wild-type *E. coli* are essentially free of pentapeptides, sacculi from DV900, a multiple DD-carboxypeptidase mutant which accumulates high proportions of D-Ala-D-Ala-containing muropeptides, were used to check AmpH DD-carboxypeptidase activity. HPLC analyses showed that AmpH-ENc1 displayed both DD-peptidase activities on macromolecular PG (Table 2; Fig. 7). The proportion of monomeric pentapeptide (peak M5), dimeric compounds (peaks D45, D44, and D45N), and trimeric compounds (peaks T445 and T445N) fell drastically when PG from *E. coli* DV900 was incubated in the presence of His-tagged AmpH. In addition, the relative abundance of essentially all muropeptides cross-linked by DD-peptide bridges also fell upon AmpH digestion of sacculi from both strains, indicating a broad substrate specificity of the DD-endopeptidase activity and a high efficiency on macromolecular peptidoglycan. These results indicate that His-tagged AmpH exhibits both DD-endopeptidase and DD-carboxypeptidase activities on sacculi.

TABLE 1. Kinetic of *E. coli* His-tagged AmpH DD-peptidase activitiesa

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<tr>
<th>Enzymatic activity</th>
<th>Substrate</th>
<th><em>K</em>&lt;sub&gt;m&lt;/sub&gt; (μM)</th>
<th><em>V</em>&lt;sub&gt;max&lt;/sub&gt; [mmol min&lt;sup&gt;−1&lt;/sup&gt; μg protein&lt;sup&gt;−1&lt;/sup&gt;]</th>
<th><em>k</em>&lt;sub&gt;cat&lt;/sub&gt; (s&lt;sup&gt;−1&lt;/sup&gt;)</th>
<th><em>k</em>&lt;sub&gt;cat&lt;/sub&gt;<em>K</em>&lt;sub&gt;m&lt;/sub&gt; (M&lt;sup&gt;−1&lt;/sup&gt; s&lt;sup&gt;−1&lt;/sup&gt;)</th>
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<tr>
<td>DD-Carboxypeptidase</td>
<td>M5</td>
<td>225 ± 35</td>
<td>(4.98 ± 0.48) × 10&lt;sup&gt;−3&lt;/sup&gt;</td>
<td>3.4 × 10&lt;sup&gt;−3&lt;/sup&gt;</td>
<td>15</td>
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<tr>
<td></td>
<td>Tripeptide</td>
<td>ND</td>
<td>(6 ± 0.8) × 10&lt;sup&gt;−3&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>DD-Endopeptidase</td>
<td>D45</td>
<td>102 ± 5</td>
<td>(174 ± 15.6) × 10&lt;sup&gt;−3&lt;/sup&gt;</td>
<td>1.2 × 10&lt;sup&gt;−1&lt;/sup&gt;</td>
<td>1.2 × 10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>D45N</td>
<td>28.7 ± 7.5</td>
<td>(27.6 ± 9) × 10&lt;sup&gt;−3&lt;/sup&gt;</td>
<td>1.9 × 10&lt;sup&gt;−2&lt;/sup&gt;</td>
<td>6.6 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>D44</td>
<td>134 ± 25</td>
<td>(162 ± 15.6) × 10&lt;sup&gt;−3&lt;/sup&gt;</td>
<td>9 × 10&lt;sup&gt;−2&lt;/sup&gt;</td>
<td>6.7 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>D44N</td>
<td>31.6 ± 1.5</td>
<td>(23.4 ± 3) × 10&lt;sup&gt;−3&lt;/sup&gt;</td>
<td>1.6 × 10&lt;sup&gt;−2&lt;/sup&gt;</td>
<td>5 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
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</table>

*<sup>a</sup> DD-Carboxypeptidase activity in vitro on tripeptide (Nα,Nα-diacetyl-Lys-D-Ala-D-Ala) as the substrate was measured as described in Materials and Methods. All kinetic constants were calculated using data obtained with 2.05 μM purified His-tagged AmpH extract with various amounts (6.3 × 10<sup>−3</sup> to 9.5 × 10<sup>−2</sup>mM) of monomeric disaccharide pentapeptide (M5). All kinetic constants of DD-endopeptidase activity were calculated using data obtained with 0.4 μM purified His-tagged AmpH extract and various amounts (4 × 10<sup>−3</sup> to 8 × 10<sup>−2</sup>mM) of dimers disaccharide tetratetrapeptide (D45) or disaccharide tetratetrapeptide (D44) and various amounts of the analogues [(→α)-anhydro compounds D45N and D44N (1 × 10<sup>−3</sup> to 2 × 10<sup>−5</sup>mM) as described in Materials and Methods. The enzymatic reactions were analyzed by HPLC assay as described in Materials and Methods. All kinetic constants must be considered apparent values because of the impossibility of calculating initial enzyme velocities by HPLC. Values are means ± standard deviations. ND, not determined.

Table 2. His-tagged AmpH activity on sacculi purified from *E. coli* strain<sup>b</sup>

<table>
<thead>
<tr>
<th>Muropeptide</th>
<th>CS109</th>
<th>CS109/AmpH&lt;sup&gt;b&lt;/sup&gt;</th>
<th>DV900</th>
<th>DV900/AmpH&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>M3</td>
<td>19</td>
<td>18.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M3G</td>
<td>1.3</td>
<td>2.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M4</td>
<td>29.2</td>
<td>43.5</td>
<td>6.34</td>
<td>46.62</td>
</tr>
<tr>
<td>M3L</td>
<td>4.2</td>
<td>4.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D34D</td>
<td>5.1</td>
<td>4.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D43</td>
<td>10.4</td>
<td>6.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M5</td>
<td>37.3</td>
<td>21.27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D44</td>
<td>19.5</td>
<td>10.7</td>
<td>6.53</td>
<td>3.08</td>
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<tr>
<td>D45</td>
<td>40</td>
<td>17.58</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M4N</td>
<td>0.51</td>
<td>6.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T444</td>
<td>2.1</td>
<td>1.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D43L</td>
<td>8.5</td>
<td>5.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T445</td>
<td>7.1</td>
<td>4.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M5N</td>
<td>0.98</td>
<td>2.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D44N</td>
<td>0.7</td>
<td>0.9</td>
<td>0.28</td>
<td>0.13</td>
</tr>
<tr>
<td>D45N</td>
<td>0.28</td>
<td>0.13</td>
<td></td>
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</tr>
<tr>
<td>T445N</td>
<td>0.49</td>
<td>0.16</td>
<td></td>
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</tr>
</tbody>
</table>

<sup>b</sup> Relative molar abundance of muropeptides was calculated from the areas of the corresponding peaks as described previously (8). Muropeptides are abbreviated according to the following notation: the first letter indicates monomer (M), cross-linked dimer (D), or cross-linked trimer (T); the numbers indicate the length of stem peptides, where 3 stands for L-Ala-L-Glu-meso-DAP, 4 stands for L-Ala-L-Glu-meso-DAP-D-Ala, and 5 stands for L-Ala-L-Glu-meso-DAP-D-Ala-D-Ala; the last letter indicates a muropeptide cross-linked through an (1→3)-DAP peptide bridge (D), a Braun’s lipoprotein anchoring muropeptide (L), or a muropeptide with a (1→α)-anhydromuramyl acid residue (N).

<sup>6</sup> Amh cp-peptidase activities were measured in reaction mixtures with enzymes and peptidoglycan as described in Materials and Methods.

### DISCUSSION

A general understanding of PG structure and metabolism, and specifically the bacteriolytic effect of β-lactam antibiotics, depends on detailed knowledge of the activity of enzymes involved in murein biochemistry. β-Lactam antibiotics exert their action through inhibition of HMM PBPs responsible for the polymerization of PG. The LMM PBPs are also inhibited by β-lactams, but since they are not essential for bacterial growth, their inhibition is not usually fatal to bacteria. The seven LMM PBPs of *E. coli* are involved in cell division, PG maturation, or recycling, and the major biochemical activities of six of them are monofunctional or bifunctional DD-carboxypeptidases or DD-endopeptidases (2, 16, 17, 21, 27). AmpH has been associated with PG recycling, although at the time of this study, it was the only LMM PBP that remained uncharacterized for enzymatic activity. Cell fractionation studies localized both forms of His-tagged AmpH protein exclusively in the membrane fraction. Furthermore, AmpH seems to be strongly anchored to the membrane, although no canonical hydrophobic anchoring sequences were found. This situation appears to be consistent with other well-characterized LMM PBPs, whose amino termini are cleaved as the proteins mature but remain membrane associated (6).

Analysis in *sito* of the amino acid sequence of AmpH predicts a periplasmic protein associated with the bacterial membrane by a signal-like peptide segment that functions as a...
membrane anchor. However, our results show that the signal peptide is cleaved in the mature forms of both AmpH-END2 (both N- and C-terminally His-tagged protein) and AmpH-ENC1 (only C-terminally His-tagged protein). So, it seems that the N-terminal signal peptide functions in translocation of the protein, but once AmpH is fully translocated to the periplasm, the signal sequence is removed and then the bulk of the protein binds to the outer surface of the inner membrane.

AmpH was previously reported to be closely related to AmpC, but no \(\beta\)-lactamase activity was detected (11). Interestingly, PG recycling has been related to the induction of particular class C \(\beta\)-lactamases that hydrolyze \(\beta\)-lactam compounds (20). The penicillin-binding characteristics and the phenotypes of \(\text{ampH}\) mutants suggested that AmpH (and AmpC) may play roles in the synthesis, remodeling, or recycling of PG (11). Here, we analyzed \(\beta\)-lactamase activity of both purified His-tagged AmpH derivatives and found a low but significant activity for nitrocefin, about 1/1,000 the rate measured for AmpC under the same conditions; therefore, these data suggest that the native form of AmpH does have \(\beta\)-lactamase activity.

In vitro assays with isolated muropeptides showed that His-tagged AmpH forms cleave the terminal d-Ala residue from disaccharide pentapeptide (M5) and from the synthetic tripeptide \(N_\alpha,N_\varepsilon\)-diacetyl-Lys-d-Ala-d-Ala, therefore exhibiting a typical \(\beta\)-carboxypeptidase activity. Activity on the synthetic tripeptide indicated that His-tagged AmpH derivatives displayed a low \(\beta\)-carboxypeptidase activity with rates similar to those obtained using the complete muropeptide as a substrate, but this activity was higher than the maximum enzymatic activity (0.75 \(\pm\) 0.10 \(\text{nmol min}^{-1} \text{g}^{-1}\)) on synthetic tripeptides reported for LMM PBPD2 (Lmo2812) of Listeria monocytogenes under the same assay conditions (13). AmpH-ENC1 \(\beta\)-carboxypeptidase activity has an apparent \(K_m\) for M5 that is lower than the \(K_m\) of PBP5, the predominant \(\beta\)-carboxypeptidase in \(E.\ coli\), for diacetyl-L-lysyl-d-Ala-d-Ala (\(K_m\) \(\approx\) 1 mM) (25). However, the \(\beta\)-carboxypeptidase activity associated with \(E.\ coli\) PBP4, the archetypal class C LMM PBP, on \(N\)-acetylmuramyl-pentapeptide, a substrate structurally closer to M5, had a \(K_m\) (20.4 \(\mu\)M) approximately 1/10 the value for AmpH (20).

Incubation of AmpH with \(\beta\)-cross-linked muropeptides clearly demonstrated that this protein has an efficient \(\beta\)-endopeptidase activity, and the kinetic analysis indicated that this is the predominant activity of the protein, at least in vitro. In fact, \(k_{cat}\) was about 10 to 100 times higher for the \(\beta\)-endopeptidase than for the \(\beta\)-carboxypeptidase on natural substrates. Interestingly, the \(\beta\)-endopeptidase activity was essen-

![FIG. 7. HPLC analysis of His-tagged AmpH DD-peptidase activities on macromolecular peptidoglycan. The changes in absorbance (Abs) at 204 nm of each muropeptide reaction product are displayed. (A) PG substrate from \(E.\ coli\) DV900 incubated with purified His-tagged AmpH enzyme as described in Materials and Methods; (B) PG substrate incubated under the same conditions without added enzyme. DD-Peptidase activity is showed by decrease of dimeric and trimeric compounds, i.e., tetrapentapeptide (D45), tetratetrapeptide (D44), tetratetrapentapeptide (T445), and analogous (1\(\rightarrow\)6)-anhydro-D45N and -T445N compounds. DD-Carboxypeptidase activity is displayed as a decrease of monomeric pentapeptide (M5). Both DD-endopeptidase and DD-carboxypeptidase activities display an increase of monomeric tetrapeptide (M4) and the analogous (1\(\rightarrow\)6)-anhydromuramic acid-containing derivatives M4N and M5N.](image-url)
tially unaffected by the presence of a d-Ala residue at the acceptor stem peptide of dimeric muropeptides. The facts that in the *in vitro* assays with D45 as the substrate, the amount of D44 detected was minimal and the final amount of M5 (17.8%) was close to equimolar with M4 (18.6%) eliminate the possibility that AmpH first converts D45 into D44 and then acts on the latter. However, the presence of the (1→6)anhydro form of muramic acid seems to have a significant influence on the reaction. Indeed, the presence of the anhydro form reduced *κ*~cat~ to 1/10 of the value for the normal muropeptides. It is important to note that the muropeptides used here had been subjected to NaBHi reduction and therefore contained muramicitol instead of the reducing sugar. The ability of AmpH to accept cross-linked trimers and tetramers as substrates reveals a relatively relaxed substrate specificity.

An important property of AmpH was its ability to accept intact sacculi as substrates for both DD-peptidase activities. Furthermore, our results are consistent with AmpH acting *in vivo* predominantly as a DD-endopeptidase, although its potential to work as a DD-carboxypeptidase was also clearly manifested when pentapeptide-enriched sacculi were used as substrates.

We therefore conclude that AmpH is a bifunctional LMM PBP with DD-carboxypeptidase and DD-endopeptidase activities on solubilized muropeptides and on whole sacculi and with a marginal β-lactamase activity. These traits suggest that AmpH may play roles in the course of PG remodeling or recycling. PBP7, the other PBP DD-endopeptidase, accepts muropeptide dimers and insoluble murein sacculi as substrates *in vitro* (21), and PBP4 preferentially cleaves monomer or dimer muropeptides (4), which implies a possible difference in the functions of these two enzymes. AmpH enzyme activity appears to be comparable to that of another DD-endopeptidase (MepA), a penicillin-insensitive enzyme that has been shown to cleave muropeptide dimers and insoluble murein sacculi in *vitro* (15). The functional overlap between PBP7, PBP4, MepA, and AmpH *in vivo* is unknown. From a methodological point of view, purified His-tagged AmpH proved to be a rather sturdy protein, useful for releasing the shorter glycan chains from purified sacculi.

**ACKNOWLEDGMENT**

This work was supported by grant BFU 2009-09200 from the Ministry of Science and Innovation (MICINN) of Spain.

**REFERENCES**