Title: AmpH, a bifunctional DD-endopeptidase and DD-carboxypeptidase of *Escherichia coli*

Running title: AmpH a new DD-endopeptidase and DD-carboxypeptidase.

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Key words: LMM PBPs, DD-peptidases, AmpH, *Escherichia coli*

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ABSTRACT

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 remodelling of peptidoglycan (PG). AmpH has been classified as an AmpH-type Class C-LMM-PBP, a group closely related with 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 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 tetra-pentapeptide (D45) and tetra-tetrapeptide (D44) with efficiencies (Kcat/Km) of 1200 M⁻¹ s⁻¹ and 670 M⁻¹ s⁻¹ respectively, and removed the terminal D-alanine from muropeptides with a C-terminal D-ala-D-ala dipeptide. Both DD-peptidase activities were inhibited by 40 µM Cefmetazol. AmpH also displayed a weak β-lactamase activity toward nitrocefin of 1, 4 × 10⁻³ nmol/µg prot/min, one thousandth the rate obtained for AmpC under the same conditions. AmpH was also active on purified sacculi, exhibiting the same bifunctional character shown with pure muropeptides. The wide substrate spectrum of the DD-peptidase activities associated to AmpH support a role of this protein in PG remodelling or recycling.
INTRODUCTION

Bacterial peptidoglycan (PG) is an essential and specific structural component of the cell wall critical to preserve cell integrity and provide a defined cell shape (29). *Escherichia coli* PG assembly (murein synthesis) requires the polymerization of glycan strands composed of alternating N-acetyl glucosamine (NAcGlc) and N-acetylmuramic acid (NAcMur) 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 crosslinking 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 classified into three classes based on sequence similarities (9). Class A high molecular mass PBPs (HMM-PBPs-A) synthesize nascent glycan chains and crosslink them (transglycosylation and transpeptidation) but class B high molecular mass PBPs (HMM-PBPs-B) only catalyse crosslinking reactions between stem peptides (transpeptidation).

Peptidoglycan remodelling, and possibly some aspects of synthesis, are mediated by class C low molecular mass PBPs (LMM-PBPs-C). Class C PBPs display two predominant catalytic activities *in vivo*, DD-carboxypeptidase activity that removes the terminal D-alanine from muropeptides with C-terminal D-alanyl-D-alanine dipeptides, and DD-endopeptidase activity that hydrolyse peptide bridges linking together adjacent glycan strands (23). LMM-PBPs are monofunctional or bifunctional DD-peptidases (12, 17, 16), but up to now their roles and which of these activities are predominant *in vivo* remain unclear.

The *ampH* gene of *E. coli* codes for AmpH, a class C, LMM-PBPs of type-AmpH (23), and is included in the cluster of orthologous genes COG1680 (26), a family of proteins containing AmpC-type β-lactamases and DD-carboxypeptidases. Although closely related with AmpC and other class C β-lactamases, AmpH did not show β-lactamase activity in a previous report.
(11). The phenotypes of certain (multiple) mutants suggest that although 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−, lacZΔM15, recA1) and *E. coli* BL21 (DE3) (F−ompT hsdSB [rB−, mB−]) gal dcm [DE3]) were the bacterial strains used as cloning hosts, and DV900 (CS-109, Δ[ponB, dacA, dacB, dacC, dacD, pbpG, ampH, ampC, pbp4b]) (27) was used for peptidoglycan and muropeptides purification. DV900 (DE3) was constructed by using the λDE3 Lysogenization Kit (Novagen, Merck KGaA, Darmstadt, Germany), following the manufacturer recommendations and used to overexpress and purify the AmpH-End2 protein.

Bacterial cultures were grown in one of the following media (1) Luria-Bertani (LB) medium, Super Optimal broth with catabolite repression (SOC) medium and minimal M9 medium supplemented with 1mM MgSO₄ and 0.2% (w/v) casamino acids. Antibiotics ampicillin (Amp) 100µg/ml and kanamycin (Kn) 30µg/ml were added as required.

Plasmid pGEM®-T Easy Vector (Promega, Madison, WI, USA) carrying *E. coli* ampH (pGEM-H) was from our laboratory collection, and pET-28b (+) Kn, Novagen® was
purchased from Merck Chemicals Ltd. (Nottingham, UK). Restriction enzymes were from Fermentas, Life Sciences (Madrid, Spain) and T4 DNA ligase and Pfu DNA polymerase were from Biotools B&M Labs, S.A (Madrid, Spain). All DNA manipulations were performed using standard methods and DNA samples were purified using PromegaWizard® Plus SV minipreps (Promega, Madison, WI, USA) DNA purification kit. PCR DNA products were cleaned using PromegaWizard® SV Gel and PCR Clean-up System (Promega, Madison, WI, USA).

Chemical reagents

All chemicals were of analytical grade (Merck, Darmstadt, Germany). Imidazole, sodium dodecyl sulphate (SDS), and N-lauroylsarcosine sodium salt, ≥94% (Sarkosyl) were from Sigma-Aldrich (Saint Louis, MO, USA), HPLC grade methanol was obtained from Scharlau S.L (Sentmenat, Spain), ultrapure water, for the preparation of HPLC eluents, was generated on a Millipore super Q water purification system. Nitrocefin was from Oxoid (Cambridge, UK). Protein assays were performed with the RC DC protein assay (Bio-Rad, Hercules, CA, USA).

Cloning, overexpression and purification of E.coli AmpH

The E.coli ampH (10) gene, previously inserted forward (opposite to p-lac) into pGEM®-T Easy plasmid (pGEM-H), was amplified by PCR, using the primers (Sigma-Aldrich, Saint Louis, MO, USA): 5’-CCATGGGCTTGAAACGTAGTCTGCT-3’ Ncl-H, 5’-GCCATATGGCTTTGAAACGTAGTCTGCT-3’ Nde-H, and 5’-TCGAATTCGAGGACGCGGGGATAACCA-3’ Ndi-H. The resulting fragments from PCR (1,28 Kbp NdeI-EcoRI, with primers Ndi-H and RI-H; and 1,221 Kbp NcoI-EcoRI, with
primers NcI-H and RI-H) were purified, digested with NdeI and EcoRI and with NcoI 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 made. Initial recovery of E. coli competent cells after transformation was performed in SOC medium with vigorous shaking for 1 h at 37°C, cells were then plated onto LB agar plates supplemented with Kanamycin (Kn) 30μg/ml, and incubated at 37°C overnight. The recombinant proteins carried His₆ tags either at both terminals (AmpH-ENd2), or at the C-terminal (AmpH-ENC1).

For overexpression, strains 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 hs with vigorous agitation (220 rpm) until an OD600 of 0.3 was reached. Induction of protein expression was achieved by addition of 1mM 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 (5g) was thawed and suspended in 30 ml of saline phosphate buffer (PBS: 150 mM NaCl, 2.5 mM KCl, 8 mM NaH₂PO₄•12H₂O and 1.45 mM NaH₂PO₄) pH 8.0. Cells were broken by two passes through a French press (American Instrument co, Urbana, III) at 20 000 psi and the lysate was centrifuged (257000 × g, 15 min, 4°C). The resulting pellet was suspended to a final volume of 10 ml in PBS pH 8.0 with 2% Sarkosyl and stirred in a rotating wheel for 4 hs at room temperature. Insoluble material was removed by ultracentrifugation (345000 × g, 15 min, 20 °C). The supernatant (10 ml) was dialyzed three times, for four hours each, against 1-liter of
PBS-0.2% Triton X-100, pH 8.0 (solubilization buffer) at room temperature. The dialyzed supernatant was mixed with 2 M imidazole (20 mM final concentration) and with 1 ml (2mg of protein/ml of resin) of high density Nickel 6BCL-QHNi-25 resin (ABT, Tampa, FL, USA) that had been equilibrated with PBS-0.2% Triton X-100-20 mM imidazole, pH 8.0 (rinse buffer). The protein solution and resin were mixed in a rotary wheel for 4 hs at room temperature. Unbound proteins were removed from the resin washing with 3 ml of rinse buffer three times. Proteins bound to the nickel-resin were eluted stepwise with 3 ml of each 125 mM, 250 mM and 500 mM imidazole in solubilization buffer. Each 3 ml-elution fraction was dialyzed three fold against 1-liter of solubilization buffer at room temperature for hours and then fractions were aliquoted and stored at -20ºC.

Fractions containing AmpH-END2 or AmpH-ENc1 were analyzed by NuPAGE, Novex 10% Bis-Tris Gel in MOPS SDS running buffer and SeeBlue® Plus2 pre-stained standard molecular marker (Invitrogen, Carlsbad, CA, USA). The gels were stained with Coomassie blue G-250, and the protein was electroblotted onto Immobilon-P transfer membrane (Millipore Corporation, Bedford, MA, USA) by means of a Criterion™ blotter (Bio-Rad) by a modification of the method described (22). The membrane was immersed in blocking buffer, Tris-buffered saline-0.05% Tween 20 (TBS-T) containing 3% (w/v) non-fat dry milk for 1 h. Immobilized proteins were probed with anti-His α-rabbit (SC-803, SantaCruz Biotechnology, Heidelberg, Germany), 3000 fold diluted in TBS-T solution as primary antibody. The blot was washed three times for 5 min in TBS-T and exposed to a second antibody goat anti-rabbit/horseradish peroxidase conjugate 3000 fold diluted in TBS-T (GAR-HRPO 170-651, Bio-Rad, Hercules, CA, USA) for 1 h. After wash in TBS-T, protein was identified by Luminol visualization by adding a premixed solution of 5 µl Luminol [200 ×
stock solution: 88.6 mg Luminol (A8511, Sigma-Aldrich, Saint Louis, MO, USA) in 1 ml DMSO] and 4.3 µl Luciferin [218 × stock solution: 10 mg D-Luciferin (411400, Roche Diagnostics, Mannheim, Germany) in 2.1 ml Tris 100 mM, pH 7.8-8.0] in 1ml Tris 100 mM, pH 7.8-8.0, to 1ml of H₂O₂ (Merck, Darmstadt, Germany) 15% solution in Tris 100 mM, pH 7.8-8.0. The blot was placed between two squares of plastic wrap in contact with 1 ml of Luminol solution and layed over an X-ray film for a few minutes. The peptide sequence was characterized by MALDI-TOF analysis and the N-terminal amino acid sequence was determined with standard Edman chemistry.

In order to exclude any possible contamination of the purified protein with any putative activity form other PBPs or β-lactamases, the ENd2 protein was also overproduced and purified, by the same procedure describe above, from the strain DV900(DE3)-pH28-ENd2 strain, which is defective for all known LMW-PBPs.

Separation of soluble and envelope fractions from spheroplasts

Spheroplasts were prepared by a protocol based on the procedure of Birdsell and Cota-Robles (3) from 25 ml of cell culture at an OD550 nm of 1.0. Cultures were harvested and cell pellets resuspended in 1ml of 30 mM Tris-HCl pH 8.0-20% sucrose (T-S buffer) with 10 µl of EDTA 0.5 M pH 8.0, then lysozyme was added to cell suspensions at a final concentration of 250 µg/ml and kept on ice for 10-20 min. After addition of Cl₂Ca (20 mM final concentration) and NaCl (100 mM final concentration), samples were centrifuged at 40 000 × g for 15 min at 4°C. The supernatants constituted the periplasmic fraction and were stored. The pellets were resuspended in low osmotic strength buffer (0.5 ml of 30 mM Tris-HCl pH 8.0, MgCl₂ 5 mM) with DNasa (1µg/ml) to favour disruption of spheroplasts, and centrifuged at 40 000 × g for
30 min at 4°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 EDTA 1 mM. Aliquotes of periplasmic (15 µl), cytoplasmic (5 µl) and membrane fractions (1 µl) were analyzed by SDS-PAGE in 10% acrylamide gels and AmpH-End2 and AmpH-ENc1 were identified by western blot immunodetection. Aliquotes 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 membrane.

**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 purified AmpH-ENc1 or AmpH-ENd2 fraction or 20 µl of a pure stock solution of AmpC (0.1 µg, 1.7 × 10⁻² µM final concentration) were added to 25 µl of nitrocefin stock solution (160 µM final concentration) in a final volume of 150 µl of PBS, pH 8.0 and incubated at 37°C taking samples every five min during 1h. Parallel samples without enzyme were used as blanks for each sample. The change in absorbance overtime at 482 nm was measured on a NanoDrop 2000C Spectrophotometer (Thermo Fisher Scientific Inc, Wattham, USA).

**β-Lactam binding assays**

The *in vitro* assays for PBP activity were based on modifications of the procedures described by Spratt and Pardee (24) and Koyasu et al (14). Membranes of *E. coli* BL21/p28H-ENd2, and *E. coli* CS-109 were prepared as described before. Membranes from *E. coli* CS109 were used
as a standard for the molecular weight of *E. coli* PBPs. Protein concentration was determined with the D-C protein assay Kit (Bio-Rad, Hercules, CA, USA) and adjusted to 5 mg/ml in PBS pH 8.0. AmpH-ENc1 purified form (9.75 µg/15µl) was preincubated with 4 µl of cefmetazol (20 µg/ml final concentration) at 37°C for 5 min, then 3 µl of Bocillin™ FL (Invitrogen, Carlsbad, CA, USA) were added (10 µM final concentration) in a final volume of 40 µl and the mixtures were incubated for 30 min at 37°C. SDS sample buffer (10 µl) was added, and samples were boiled for 10 min. Insoluble materials were removed by centrifugation (Eppendorf centrifuge at maximum speed for 10 min at 20°C). Proteins in the sample were separated by SDS-PAGE in 8% acrylamide gels and detected directly onto the gels on a Thyphon 9410 Variable Mode Imager (General Electric) at 588 nm, with a 520BP40 emission filter.

Preparation of peptidoglycan

Peptidoglycan was prepared from *E. coli* DV900 (a deletion mutant in 8 PBPs and AmpC and depleted of DD-CPase activity) cultures grown in LB medium at 37°C with aeration. The cells from a 1 liter culture were harvested by centrifugation for 15 min at 4300 × g at room temperature, resuspended in 20 ml of culture medium, slowly mixed with an equal volume of 8% (w/v) boiling SDS with vigorous stirring. The suspension was boiled for 4 h and left overnight with moderate stirring at RT. Sacculi 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 into 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, USA); for 1h at 37°C, and then with 100 µg/ml)
preactivated pronase-E at 60ºC for 90 min. (EC 3.4.24.4; Merck, Darmstadt, Germany). 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 4ºC.

Preparation and separation of muropeptides

Peptidoglycan was digested in 50 mM phosphate buffer (pH 4.9) with Cellosyl (Hoechst AG, Frankfurt, Germany) 100 µg/ml final concentration at 37ºC 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) for 30 min at room temperature. The pH was tested with pH indicator strips (Acilit, Merck) and adjusted to 3 with orthophosphoric acid. All samples were filtered through (Millex-GV, 0.22 µm, Ø2.5mm, Millipore, Cork, Ireland) and stored at -20ºC.

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

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 DD-carboxypeptidase activity *in vitro.*

Purified His-tagged AmpH forms were assayed using the tripeptide $\text{N}_{\alpha}\text{N}-\text{diacetyl-Lys-D-Ala-D-ala}$ (Sigma-Aldrich, Saint Louis, MO, USA) as substrate using a modification of method of Frère et al (5) as described by Vega et al (27).

Reaction mixtures containing 10 µl of $\text{N}_{\alpha}\text{N}-\text{diacetyl-Lys-D-Ala-D-ala}$ (8.3 mM final concentration), 3 µl of buffer 10X (Tris-HCl buffer 300 mM pH 7.5) and 17 µl of purified each His-tagged AmpH form (1 µg, 0.8 µM final concentration) were incubated at 37ºC for 60 min. At this time 5 µl of O-Dianisidine (Sigma-Aldrich, Saint Louis, MO, USA) 10 mg/ml (in methanol) and 70 µl of enzymes/coenzymes mix (flavin adenine dinucleotide, FAD, peroxidase and D-aminoacid oxidase were added to each sample. After a further 5 min at 37ºC, 400 µl methanol-water 50% (v/v) were added and samples were incubated for another 2 min. The absorbance of each sample was measured immediately at 460 nm. Control reactions containing only enzyme and controls of natural degradation of the tripeptide without enzyme were made for each sample. Standard samples with known amounts of D-alanine and unknown samples were carried out by triplicate.

HPLC assay of *E. coli* AmpH DD-peptidase activities

All enzymatic reactions were analyzed by triplicate. The DD-carboxypeptidase activity was assayed by following the appearance of monomeric reaction product dissacharide tetrapeptide: $\text{NAcGlc-NAcMur-L-ala-D-glu-dap-D-ala}$ (M4) in mixtures containing PBS buffer pH 7.3, enzyme (17 µg, final concentration 2.05 µM) and various amounts ($6.3 \times 10^{-3}$ to $9.5 \times 10^{-2}$ mM) of monomer dissacharide pentapeptide: $\text{NAcGlc-NAcMur-L-ala-D-glu-dap-D-ala-D-ala}$ (M5) as a substrate, in a final volume of 200 µl. Reactions were incubated at...
37°C for 240 min. The DD-endopeptidase activity was determined with dimeric compounds bis-disaccharide tetra-pentapeptide (D45) or disaccharide tetra-tetrapeptide (D44), as well as their (1→6) anhydro muramic acid containing derivatives D45N and D44N respectively as substrates. Sample mixtures consisted of 200 µl PBS pH 7.3, 0.4 µM of enzyme (3.4 µg) at various concentrations of substrates: for D45 and D44 (4 × 10^{-3} to 8x10^{-2} mM) and for D45N and D44N (1 × 10^{-3} to 2x10^{-2} mM). Reactions were incubated at 37°C during 15 min (for D45) or 30 min (for D44, D44N and D45N).

To study the activity of His-tagged AmpH on macromolecular PG reaction mixtures containing 160 µg of PG and 24.5 µg (2.17 µM) of AmpH-ENc1 in 270 µl PBS pH 7.3 were incubated overnight at 37 ºC. All enzymatic reactions were terminated by boiling the samples for 2 min. Once cooled down samples were digested with muramidase (100 µg/ml, final concentration) overnight and further processed for HPLC analysis as described above. Enzyme activities were estimated from the variation in the abundance of presumed substrate and product muropeptides relative to a control sample in the HPLC analyses of digested sacculi.

**Analysis of kinetic data**

The dependence of the reaction rate on substrate concentration of the DD-peptidase activities considered here was examined under the conditions described above for concentrations in the range of 10^{-3} to 10^{-1} mM. Apparent $K_m$ and $V_{max}$ values were obtained from double-reciprocal Lineweaver-Burk plots of the data. $K_{cat}$ constant was determined as $K_{cat}=\frac{V_{max}}{[E]}$, where $[E]$ = nmol of protein (His-tagged AmpH) (18). Graphical and statistical analyses were performed using Microsoft Excel (Microsoft Inc., Redmond, WA, USA).
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<sub>6</sub> tags at both terminals) and p28H-ENc1 (producing AmpH-ENc1, containing His<sub>6</sub> tag at the C-terminal) overexpressed a single protein that migrated to a position around 45 kD as determined by SDS-PAGE (Figure 1, lanes 2 and 4) in accordance with the molecular weights 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 sequences determined that both clones contain a fragment of 41.86 kD protein with a pI value of 9.33 calculated by Mascot program. This molecular mass is compatible with the mobility of the protein band in SDS-PAGE (Figure 2), but is smaller than the 46.393 kDa and 44.215 kDa theoretical mass calculated from the nucleotide sequences of AmpH-ENd2 and AmpH-ENc1 respectively. However, that mass correlated precisely with the band observed by western blot with the purified proteins extracted from the membrane of overproducing strain (Figure 2), suggesting that both proteins, having a cleavable sequence, are actually cleaved at that site. LC-MS/MS confirmed the mass loss of a 23 amino acids peptide (MGLKRSLLFSAVLCAASLTSVHA) at the N-terminal of AmpH-ENc1. Although the final products for both fusion proteins were identical, we recovered mainly the mature form (His labelled at the C-terminal) for ENc1 protein, but most often got two bands for ENd2; a major component corresponding to the mature form...
(labelled at the C-terminal), and a second band with the expected size of the precursor (His labelled at both terminals). We speculate that presence of His, tag at the N-terminal might slow down maturation of the protein. Mapping of the C-terminal 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 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 clones overexpressing AmpH-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 figure 4 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 sarkosylate) could AmpH be removed from the membranes (Figure 3). 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 1mM IPTG and its corresponding purified AmpH-ENc1 form were used in a binding assay with the fluorescent antibiotic. The four major PBPs of E. coli (PBP1a/b, PBP2, PBP3 and PBP5) were easily
detected on the SDS-PAGE, as well as a new, intense band matching the molecular weight calculated for the AmpH derivatives (Figure 5). Cefmetazole is a β-lactam with high affinity for the LMW-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 form containing samples with 20 µg/ml of cefmetazol abolished binding to Bocillin-FL (Figure 5). The two bands corresponding to AmpH-Nde2, and overlapping to PBP5 on line 1, are most probably due to partial cleavage of the signal peptide, producing both precursor and mature form.

**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 using the chromogenic β-lactam nitrocefin as substrate (1.4 x 10^{-3} nmol/µg prot/min), about one thousandth the rate for AmpC (8.7 x 10^{-4} nmol/µg prot/min) under identical conditions.

The potential DD-peptidase activities of His-tagged AmpH derivatives were studied following the effect of the protein on a series of purified muropeptides. Initial assays indicated that AmpH derivatives exhibited both, DD-carboxypeptidase activity as it was able to convert M5 and M5N into M4 and M4N respectively, and DD-endopeptidase activity because it cleaved the DD-peptide bridge in cross-linked muropeptides releasing the monomeric subunits (Figure 6). Once the activities were confirmed, a more detailed analysis was performed with AmpH-ENc1 form 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 $\alpha$-$\alpha$-diacetyl-Lys-D-Ala-D-ala, often used as an alternative DD-carboxypeptidase substrate (27, 13). AmpH-DD-carboxypeptidase had low turnover number and a low specificity constant for M5 (Table 1). Activity of Histag-AmpH on the synthetic tripeptide was $6 \pm 0.8 \times 10^{-3}$ nmol of D-ala/µg prot/min, which was comparable to the rates obtained using the complete muropeptide M5 as substrate ($4.98 \pm 0.48 \times 10^{-3}$ nmol/µg prot/min) as shown in Table 1. To gain further insight on the properties of His-tagged AmpH DD-endopeptidase activity, the cross-linked dimers D44, D45, and their anhydrous derivatives D44N and D45N were assayed as substrates to define the basic kinetic parameters (Table 1). The kinetics of *E. coli* AmpH-END1 DD-endopeptidase activity showed a higher efficiency for dimers D45 and D44 compared to D45N and D44N respectively. Interestingly, the presence or absence of D-ala at position 5 in the acceptor stem peptide had little or no influence on endopeptidase activity, but presence of anhydrous muramic acid strongly affected the reaction. Cross-linked trimers and tetramers were also substrates for both *E. coli* AmpH-END1 and AmpH-END2 forms (data not shown), but detailed data could not be gathered because of the difficulty to obtain appropriate amounts of these muropeptides. Both DD-peptidase activities were inhibited by cefmetazol 40 µM (20µg/ml) with isolated muropeptides as substrates (Figure 6) in accordance with the results of pencilling binding experiments reported above. In order to exclude any contamination from other LMW-PBPs, we cloned, expressed and purified the End2 protein using a strain lacking all LMW-PBPs and β-lactamases.
(DV900(DE3)). Analysis of the three enzymatic activities (DD-endopeptidase, DD-carboxypeptidase, and β-lactamase) on this preparation produced the same or equivalent results (2.94±0.40 x 10⁻³ nmol/μg prot/min on M5, 134±40 x 10⁻³ nmol/μg prot/min on D45 and 2.6 x 10⁻³ nmol/μg prot/min with nitrocefin) as first found, therefore supporting the multi-functional character of AmpH.

Activity of AmpH on macromolecular peptidoglycan.

Peptidoglycan hydrolases may or may not accept sacculi as substrate. Those that do not are generally associated with peptidoglycan turn-over or recycling rather than biosynthesis. Therefore we assayed the ability of AmpH-ENc1 to accept sacculi as substrate 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-alan-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, Figure 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 abundances of essentially all muropeptides cross-linked by DD-peptide bridges also fell upon AmpH digestion in 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 exhibit both DD-endopeptidase and DD-carboxypeptidase activities on sacculi.
A general understanding of PG structure and metabolism, and specifically on the bacteriolytic effect of β-lactam antibiotics, depends on detailed knowledge of 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 in six of them are monofunctional or bifunctional DD-carboxypeptidases or DD-endopeptidases (17, 16, 27, 2, 21). AmpH has been associated with PG recycling although up to date; 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 looks consistent with other well-characterized LMM-PBPs, whose amino termini are cleaved as the protein mature, but remain membrane associated (6).

Analysis *in silico* of the amino acid sequence of AmpH predicts a periplasmic protein associated with the bacterial membrane by a signal-like peptide segment that working as membrane anchor. However, our results show that the signal peptide is cleaved in the mature form of both, AmpH-ENd2 (both N- and C-terminal His-tagged protein) and AmpH-ENC1 (only C-terminal His-tagged protein). So, it seems that the N-terminal signal peptide function 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 as closely related to AmpC but no \( \beta \)-lactamase activity was detected (11). Interestingly, PG recycling has been related with the induction of particular class C \( \beta \)-lactamases that hydrolyse \( \beta \)-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, remodelling or recycling of PG (11). Here, we analyzed \( \beta \)-lactamase activity of both pure His-tagged AmpH derivatives and found a low but significant activity toward nitrocefin, about one thousandth the rates measured for AmpC under the same conditions, therefore these data suggests 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 dissacharide pentapeptide (M5) and from the synthetic tripeptide \( \text{N}_\alpha,\text{N} \)-diacetyl-Lys-D-Ala-D-ala exhibiting therefore a typical DD-carboxypeptidase activity. Activity on the synthetic tripeptide indicated that His-tagged AmpH derivatives displayed a low DD-carboxypeptidase activity with similar rates that those obtained using the complete muropeptide as substrate, but it was higher than maximum enzymatic activity (0.75 \( \times \) 10\(^{-3} \) nmoles min\(^{-1} \) µg \(^{-1} \)) on synthetic tripeptide reported for LMM PBPD2 (Lmo2812) of \textit{Listeria monocytogenes} under the same assay conditions (13). AmpH-ENc1 DD-carboxypeptidase activity has an apparent \( Km \) (\( Km=225 \) µM) for M5 that is lower than the \( Km \) of PBP5, the predominant DD-carboxypeptidase in \textit{E. coli}, for diacetyl-L-lysyl-D-ala-D-ala (\( Km>1 \) mM) (25). However, the DD-CPase activity associated to \textit{E coli} PBP4, the archetypal class C LMM-PBP, on N-acetylmuramyl-pentapeptide, a substrate structurally closer to M5, had a \( Km \) approximately one tenth (\( Km=20.4 \)µM) the value for AmpH (20).

Incubation of AmpH with DD-cross-linked muropeptides, clearly demonstrated an efficient DD-endopeptidase activity associated to this protein and the kinetic analysis indicates that this
is the predominant activity of the protein at least in vitro. In fact $K_{cat}$ was about ten to
hundred times higher for the DD-endopeptidase than for the DD-carboxypeptidase on natural
substrates. Interestingly, the DD-endopeptidase activity was essentially 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 substrate the amount of D44 detected was minimal and
the final amount of M5 (17.8%) was close to equimolar with M4 (18.6%) discards the
possibility of AmpH first converting D45 into D44 and then acting on the later. However, the
presence of the (1→6) anhydro form of muramic acid seems to have a significant influence on
the reaction. Indeed, presence of the anhydro form reduced $K_{cat}$ to one tenth of the value for
the normal muropeptides. It is important to notice that the muropeptides used here had been
subjected to NaBH$_4$ reduction and therefore contain 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 substrate 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 DD-carboxypeptidase
was also clearly manifested when pentapeptide enriched sacculi were used as substrate.

We therefore conclude that AmpH is a bifunctional LMW-PBP with DD-carboxypeptidase-
DD-endopeptidase activities on solubilized muropeptides and on whole sacculi, and with a
marginal $\beta$-lactamase activity. These traits suggest that AmpH may play roles in the course of
PG remodelling or recycling. PBP7, the other PBP DD-endopeptidase, accepts only high
molecular mass murein sacculi as substrates in vitro (21) and PBP4 preferentially cleaved
monomer or dimer muropeptides (4) which implies a possible difference in the function of
these two enzymes. AmpH enzyme activity appears to be comparable with 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, and useful to release the shorter glycan chains from purified sacculi.

ACKNOWLEDGMENTS

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

REFERENCES


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**Legends for figures.**

**Figure 1.** SDS-PAGE analysis of overexpressed His-tagged AmpH from *E. coli* BL21/pET28H.

Cells were grown in minimal M9 medium and induced with 1mM isopropyl-β-D-thiogalactopyranosid as described in material and methods. Line 1, protein molecular weights...
standard pre-stained SeeBlue®Plus2; lines 2, 3, 4 and 5, Coomassie blue staining of cell extracts from 50 µl of culture from BL21/pET28H-ENd2 non induced (line 2) and induced (line 3) and BL21/pET28H-ENC1 non induced (lines 4) and induced (line 5); lines 6, 7, 8 and 9, Western blot analysis using anti-His tag antibodies analysis of corresponding non induced (lines 6 and 8) and induced (line 7 and 9) samples were performed simultaneously.

**Figure 2.** Purified His-tagged AmpH from *E. coli* BL21/pET28H-ENd2. Cells overproducing AmpH-ENd2 were analyzed by Coomassie stained SDS-PAGE (A) and Western-blot (B) as described in materials and methods. Line 1, protein molecular weights pre-stained standard (SeeBlue® Plus2); lines 2 and 8, total membrane fraction treated with 2% Sarkosyl; lines 3 and 9, soluble fraction; Lines 4 and 10, flowthrough from the nickel affinity column; lines 5 and 11, first eluted fraction with 125 mM Imidazole; lines 6 and 12, second eluted fraction with 250 mM Imidazole; lines 7 and 13, third eluted fraction with 500 mM Imidazole. Protein molecular weights are represented on the right.

**Figure 3.** Western blot analysis of Histagged-AmpH in isolated and detergent-extracted membranes from *E. coli* BL21/pET28H-ENd2. Cells overexpressing AmpH-ENd2 were broken by French press. The resulting cell membrane pellet fraction was treated with 1% SDS and 2% Sarkosyl followed by ultracentrifugation to recover membrane pellet and soluble fraction. Line 1, whole cells overexpressing AmpH-ENd2; lines 2, total cells broken by French press; line 3, resulting membrane cell pellet; line 4, supernatant fraction; lines 5 and 6, membrane pellet and soluble fraction after 1% SDS treatment; lines 7 and 8, membrane pellet and soluble fraction after 2% Sarkosyl treatment. Positions of the protein standard are shown on the left.
Figure 4. Western blot analysis of Histagged-AmpH in cellular fractions from overexpressed E. coli spheroplasts.

Membrane, cytoplasm and periplasm fractions from E. coli BL21/pET28H-ENd2 strain induced by isopropyl-β-D-thiogalactopyranoside were prepared as described in material and methods. Line 1, purified AmpH-End2 extract (line 1); lines 2 and 3, total spheroplast non-induced (line 2) and induced (line 3); lines 4 and 5, membrane fraction non-induced (line 4) and induced (line 5); lines 6 and 7, periplasmic fractions non-induced (line 6) and induced (line 7); lines 8 and 9 citoplasmic fractions non-induced (line 8) and induced (line 9).

Figure 5. SDS-PAGE analysis of Bocillin-FL binding of membrane-bound and purified Histagged-AmpH.

Bocillin-FL binding assays were done with 35µg of membrane extracts (lines 1 and 2) and with 0.65 µg of purified AmpH-ENd2 (lines 3 and 4) obtained from induced E. coli BL21/pET28-H-ENd2 cultures. Membranes from E. coli CS109 (lines 5 and 6) were used as a pattern of molecular weight PBPs. On lines 2, 4 and 6 reactions were pre-incubated with cefmetazol 20 µg/ml. The major and minor bands of PBPs profile are indicated on the left of the panel.

Figure 6. HPLC analysis of Histagged-AmpH DD-peptidase activities on muropeptides.

The DD-endopeptidase activity (Panel A) of AmpH-ENc1 (0.28µM) was assayed by following the appearance of monomeric compounds: M4 (NAcGlc-NAcMur-L-ala-D-glu-dap-D-ala) and M5 (NAcGlc-NAcMur-L-ala-D-glu-dap-D-ala-D-ala) in mixtures containing dimer D45 as substrate. The DD-carboxypeptidase activity (Panel B) of AmpH-ENc1 (1.38 µM) was assayed by following the appearance of M4 in mixtures containing M5 as substrate.
Reaction mixtures were performed in the presence of cefmetazol (CF) 40 µM (a, d) or without CF (b, e) as described in material and methods. Control samples with muropeptides without enzyme (c, f) were incubated simultaneously at the same conditions. Peaks corresponding to D45, M4, M5 and CF are indicated.

Figure 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) Peptidoglycan (PG) substrate from *E. coli* DV900 incubated with purified His-tagged AmpH enzyme as described in material and methods; B) PG substrate incubated at the same conditions without added enzyme. DD-peptidase activity is showed by decrease of dimeric and trimeric compounds: tetrapentapeptide (D45), tetratetrapeptide (D44), tetratetrapentapeptide (T445) and analogous (1→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 displays an increase of monomeric tetrapeptide (M4), analogous (1→6)-anhydro muramic acid containing derivatives M4N and M5N compounds.
Table 1. Kinetic of *E. coli* Histagged-AmpH DD-peptidase activities (*).  

<table>
<thead>
<tr>
<th>Enzymatic activity</th>
<th>Substrate</th>
<th>Km (µM)</th>
<th>Vmax [nmol. min⁻¹. µg prot⁻¹]</th>
<th>Kcat (s⁻¹)</th>
<th>Kcat/Km (M⁻¹. s⁻¹)</th>
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<tr>
<td>a) DD-Carboxypeptidase</td>
<td>M5</td>
<td>225±35</td>
<td>4.98±0.48 x10⁻³</td>
<td>3.4 x10⁻¹</td>
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<tr>
<td></td>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>b) DD-Endopeptidase</td>
<td>D45</td>
<td>102±5</td>
<td>174±15.6 x10⁻³</td>
<td>1.2 x10⁻¹</td>
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<tr>
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<tr>
<td></td>
<td>D44</td>
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<td>162±15.6 x10⁻³</td>
<td>9 x10⁻²</td>
<td>6.7 x10⁻¹</td>
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<tr>
<td></td>
<td>D44N</td>
<td>31.6±1.5</td>
<td>23.4±3 x10⁻³</td>
<td>1.6 x10⁻²</td>
<td>5 x10⁻¹</td>
</tr>
</tbody>
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a) DD-carboxypeptidase activity *in vitro* on tripeptide (Nα, NƐ- diacetyl-Lys-D-Ala-D-ala) as substrate was measured as described in material and methods. All kinetic constants were calculated using data obtained with 2.05 µM of purified Histagged-AmpH extract with various amounts (6.3x10⁻³ to 9.5x10⁻² mM) of monomer diisacharide pentapeptide (M5). b) All kinetic constants of DD-endopeptidase activity were calculated using data obtained with 0.4 µM of purified Histagged-AmpH extract and various amounts (4x10⁻³ to 8x10⁻² mM) of dimers diisacharide tetra-pentapeptide (D45) or diisacharide tetra-tetrapeptide, (D44) and various amounts of analogous 1,6-anhydro compounds D45N or D44N (1x10⁻³ to 2x10⁻² mM) as described in Material and methods. The enzymatic reactions were analyzed by HPLC assay as described in Material and methods.

ND, Not determined.

(*)All kinetic constants must be consider apparent values because the impossibility to calculate initial enzyme velocities by HPLC method.
<table>
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<tr>
<th>Strains</th>
<th>Muropeptide composition (%)</th>
<th>CS109</th>
<th>CS109/ AmpH(^{(1)})</th>
<th>DV900</th>
<th>DV900/ AmpH(^{(1)})</th>
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</table>

\(^{(1)}\) AmpH DD-peptidases activities were measured in reaction mixtures with enzyme and peptidoglycan as described in material and methods.

\(^{(2)}\) Relative molar abundance of muropeptides was calculated from the areas of the corresponding peaks as described (8). Muropeptides are abbreviated according to the following notation: First letter, M monomer, D cross-linked dimer, T cross-linked trimer; numbers indicate the length of stem peptides, 3 stands for L-ala-D-glu-mesoDAP, 4 stands for L-ala-D-glu-mesoDAP-D-ala, and 5 for L-ala-D-glu-mesoDAP-D-ala-D-ala; Terminal letter indicates, D a muropeptide cross-linked through a (L-D)- DAP-DAP peptide bridge, L a Braun's lipoprotein anchoring muropeptide, and N a muropeptide with a residue of (1-6)anhydro muramic acid.