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ARTICLE

Chemical shift perturbations induced by the acylation of *Enterococcus faecium* L,D-transpeptidase catalytic cysteine with ertapenem

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Abstract Penicillin-binding proteins were long considered as the only peptidoglycan cross-linking enzymes and one of the main targets of β-lactam antibiotics. A new class of transpeptidases, the L,D-transpeptidases, has emerged in the last decade. In most Gram-negative and Gram-positive bacteria, these enzymes generally have nonessential roles in peptidoglycan synthesis. In some clostridiae and mycobacteria, such as *Mycobacterium tuberculosis*, they are nevertheless responsible for the major peptidoglycan cross-linking pathway. L,D-Transpeptidases are thus considered as appealing new targets for the development of innovative therapeutic approaches. Carbapenems are currently investigated in this perspective as they are active on extensively drug-resistant *M. tuberculosis* and represent the only β-lactam class inhibiting L,D-transpeptidases. The molecular basis of the enzyme selectivity for carbapenems nevertheless remains an open question. Here we present the backbone and side-chain ^{1}H, ^{13}C, ^{15}N NMR assignments of the catalytic domain of *Enterococcus faecium* L,D-transpeptidase before and after acylation with the carbapenem ertapenem, as a prerequisite for further structural and functional studies.

Keywords L,D-Transpeptidase · Carbapenem · β-Lactam · Peptidoglycan · NMR resonance assignment

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Biological context

Peptidoglycan is a major component of the bacterial cell wall and is essential to maintain cellular integrity and cell shape. It is assembled outside the cytoplasm from lipid II precursors by high molecular-weight penicillin-binding proteins (PBPs) in two steps, transglycosylation and D,D-transpeptidation, the latter step consisting in cross-linking of the formerly polymerized disaccharide entities through their peptide stems (Sauvage et al. 2008).

Penicillin-binding proteins have long been considered as the sole essential transpeptidase enzymes. An L,D-transpeptidase activity was however characterized in 2000 in *Enterococcus faecium* strain D344S (Mainardi et al. 2000). The enzyme responsible for this activity was identified by reverse genetics in 2005 (Mainardi et al. 2005) and since then L,D-transpeptidases (Ldts) were isolated in other Gram-negative and Gram-positive bacteria. In many organisms they only play a minor role in peptidoglycan synthesis, but in *Clostridrium difficile*, in *Mycobacterium tuberculosis*, and in *Mycobacterium abscessus* (Lavollay et al. 2011) they predominantly carry out peptidoglycan cross-linking.

Penicillin-binding proteins (Sauvage et al. 2008) and Ldts (Bielnicki et al. 2005; Biarrotte-Sorin et al. 2006; Erdemli et al. 2012) are structurally unrelated, with drastically different folds and catalytic sites (active nucleophiles are a serine in PBPs and a cysteine in Ldts). The two enzyme families also show different susceptibility towards β-lactam antibiotics. While all β-lactams, including penams, cephems and carbapenems, potentially inactivate PBPs in vivo, Ldts are only inhibited by carbapenems (Mainardi et al. 2007).

In order to decipher the origins of the Ldt specificity towards carbapenems, which appears as a promising strategy against drug-resistant *M. tuberculosis*, different approaches were developed at the molecular level. Kinetic studies on the *E. faecium* Ldt showed that the binding of imipenem to the enzyme was limiting with a kinetic constant of 0.061 μM min^{-1} and that acylenzyme hydrolysis was negligible. Further analyses with three commercially available and one synthetic carbapenems showed that the bulky carbapenem side-chain has both positive and negative effects in preventing hydrolysis of the acylenzyme and impairing drug binding (Dubée et al. 2012). Structural studies of the acylenzyme at atomic resolution were also pursued with NMR in the absence of good diffracting crystals. The first study on the *Bacillus subtilis* enzyme revealed that the acylenzyme with imipenem showed increased dynamics at the cysteine active site following acylation by β-lactam antibiotics (Lecoq et al. 2012), preventing high-resolution description of the binding site in the drug-enzyme covalent complex.

Here we report the essentially complete ^{1}H,^{13}C,^{15}N-backbone and side-chain resonance assignments of the catalytic domain (residues 341–466) of the *E. faecium* L,D-transpeptidase (Ldt_{fm}) in the absence and in the presence of ertapenem. We show that the secondary structure elements of the domain in the absence of carbapenem in solution are consistent with the structural elements identified in the crystal structure of a larger construct (Biarrotte-Sorin et al. 2006; PDB code 1ZAT). Furthermore, the acylation induces only modest perturbations in the catalytic domain. On the contrary to the *B. subtilis* enzyme, acylation does not seem to induce marked increased dynamics in the *E. faecium* enzyme.

Methods and experiments

Expression and purification of Ldt_{fm}

Recombinant ^{13}C,^{15}N-labeled [MGSSHHHHHHSSGENLYFQGHM-Ldt_{fm}(Glu341-Phe466)] (His-tagged Ldt_{fm}) containing an N-terminal oligo-histidine tag was produced from *Escherichia coli* BL21(DE3) harbouring a pETTEVΩ*ldt*$_{fm}$ derivative. Cells were grown overnight in an incubator at 37 °C and 180 rpm in 100 mL of M9 minimal medium containing 3 $g.L^{-1}$ [U-$^{13}C_6$]-D-glucose, 1 $g.L^{-1}$ $^{15}NH_4Cl$ and 50 $\mu g.L^{-1}$ kanamycin. This pre-culture was used to inoculate 4 L of fresh growth medium and cells were grown at 37 °C and 180 rpm to an OD_{600} of 0.6–1.0. IPTG (0.5 mM) was added and the cells were incubated further for 16 h at 16 °C and 180 rpm for His-tagged Ldt_{fm} overproduction. Bacteria were harvested by centrifugation (4,000×*g*, 20 min, 4 °C), resuspended in 100 mM sodium phosphate buffer (pH 6.4) containing 300 mM NaCl (buffer A), and disrupted by sonication. The enzymes were purified from clarified lysates by metal-affinity chromatography on NiNTA® beads (Qiagen) and by size-exclusion chromatography (Superdex® 75 HiLoad 26/60, GE Healthcare) in buffer A. Pure His-tagged Ldt_{fm} fractions were combined and concentrated. The resulting protein solution was then incubated with His-tagged TEV protease in a 10:1 protein:TEV mass ratio at 30 °C and 50 rpm for 1 h, leading to the cleavage of the MGSSHHHHHHSSGENLYFQ fragment from the Ldt construct. The His-tagged TEV and N-terminal His-tag were then separated from [GHM-Ldt_{fm}(Glu341-Phe466)] (Ldt_{fm}) through a metal affinity chromatography on NiNTA® beads (Qiagen). The eluted fractions containing pure Ldt_{fm} were then pooled and concentrated on an Amicon unit with a 10 kDa molecular weight cutoff. Sample quality was controlled by SDS-PAGE and mass spectrometry on a Qstar Pulsar I Applied Biosystem ESI-TOF instrument according to a previously described procedure (Mainardi et al. 2007). The experimental molecular weight of 15,363.87 Da is in complete agreement with the 15,362.92 Da value calculated for Ldt_{fm} with a 99 % ^{13}C and ^{15}N isotopic enrichment.

NMR spectroscopy

A 0.9 mM ^{13}C,^{15}N-labeled Ldt_{fm} sample was prepared in 100 mM sodium phosphate buffer (pH 6.4) containing 300 mM NaCl and 10 % $^{2}H_2O$ to study the apoenzyme and remained stable for over a year at 277 K. Acylenzyme samples were freshly prepared for the collection of each 3D NMR spectrum by addition of 2.0 molar equivalents of ertapenem (INVANZ) to prevent significant acylenzyme hydrolysis at 298 K during data collection. NMR experiments were performed on Agilent DirectDrive spectrometers equipped with cryogenic triple ^{1}H, ^{13}C, ^{15}N resonance probes operating at 600 and 800 MHz ^{1}H NMR frequencies, with the exception of the aliphatic ^{13}C-NOESY-HSQC collected on the acylenzyme on a 950 MHz Bruker US^2 spectrometer. Backbone $^{1}H_N$, ^{15}N, $^{13}C'$ and $^{13}C\alpha$ and side-chain $^{13}C\beta$ resonance assignments were achieved by using conventional [^{1}H,^{15}N]-HSQC, 3D HNCACB and 3D HNCO experiments. Side-chains were assigned using aliphatic and aromatic [^{1}H,^{13}C]-CT-HSQCs, a 3D H(C)CH-TOCSY, as well as a 3D ^{15}N-NOESY-HSQC, a 3D

aliphatic ^{13}C-NOESY-HSQC and a 3D aromatic ^{13}C-NOESY-HSQC with 150, 130 and 130 ms mixing time, respectively. Histidine side-chains were assigned using a [^{1}H,^{15}N]-SOFAST-HMQC to detect the $^{2}J_{H-N}$ and $^{3}J_{H-N}$ correlations. ^{1}H resonances assignment of the unlabeled ertapenem molecule covalently linked to the catalytic cysteine of Ldt$_{fm}$ was performed using a ^{13}C,^{15}N-filtered 2D NOESY experiment with 180 ms mixing time on a 1 mM acylenzyme sample in 100 % deuterated buffer. ^{1}H chemical shifts were externally referenced to DSS and ^{13}C and ^{15}N chemical shifts were indirectly referenced to ^{1}H as recommended by IUPAC. NMR data were processed with NMRPipe (http://spin.niddk.nih.gov/NMRPipe/) and analyzed using the CcpNmr Analysis 2.2 software (http://www.ccpn.ac.uk/software/analysis).

Extent of assignments and data deposition

Figure 1 shows the superposition of the 2D [^{1}H,^{15}N]-HSQC spectra recorded at 298 K on the Ldt$_{fm}$ sample before and after addition of two molar equivalents of ertapenem. Chemical shift changes induced by the addition of the antibiotic are moderate, suggesting a global similar fold for the protein in the two conditions.

Essentially complete resonance assignment was achieved for apo-Ldt$_{fm}$ (in the absence of ertapenem). Overall 92.5 % of all ^{1}H, ^{13}C, and ^{15}N resonances were assigned, including all backbone nuclei with the exception of the first G338 residue and the amide group of the second H339 residue reminiscent from the histidine-tag, the proline nitrogens, and the carbonyl carbon of residues sequentially followed by a

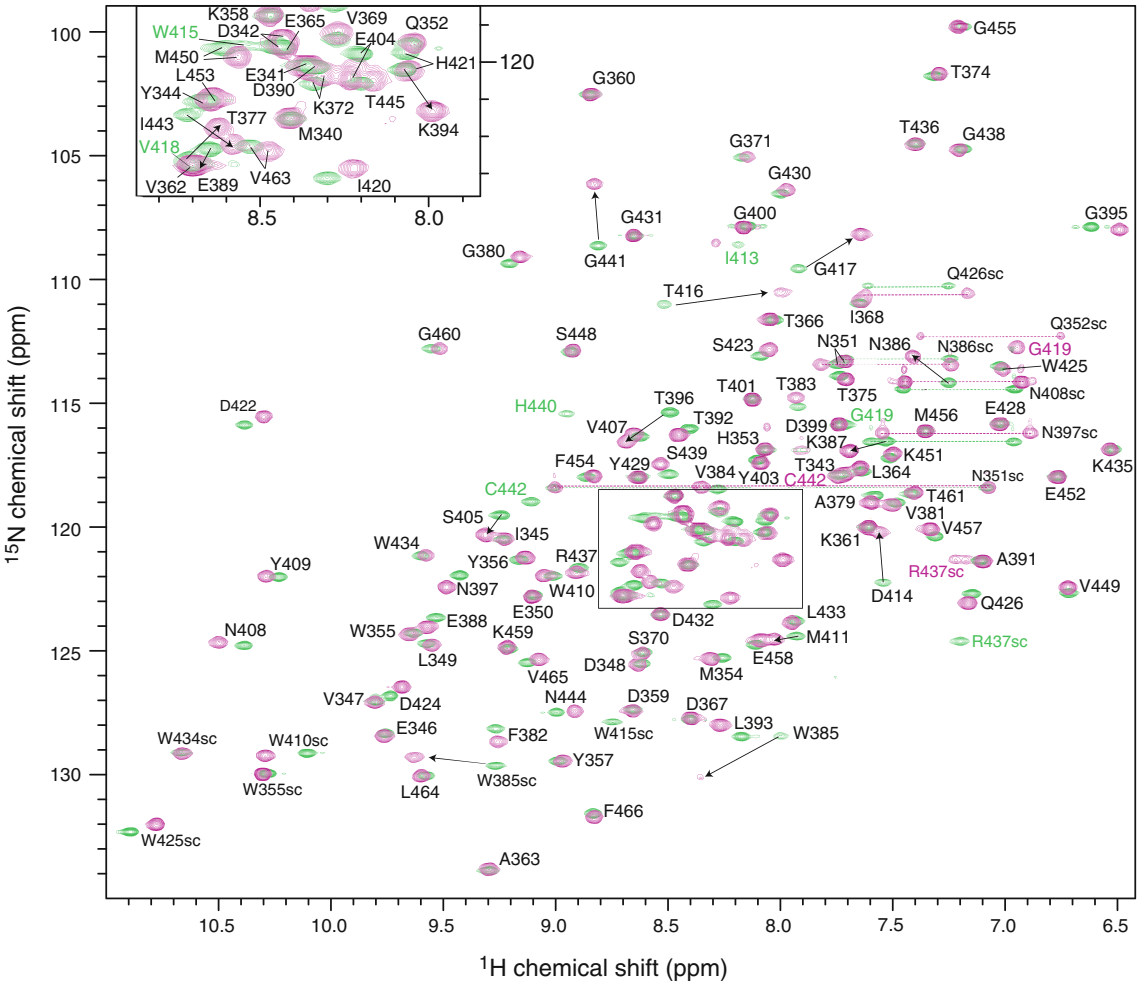

Fig. 1 Superposition of 2D [^{1}H,^{15}N]-HSQC spectra of a 0.9 mM Ldt$_{fm}$ sample in 100 mM sodium phosphate buffer, 300 mM NaCl, pH 6.4, collected at 298 K before (*green*) and after (*purple*) acylation with ertapenem. Each backbone amide resonance is labeled with the amino acid type and position in the sequence. Amine groups from tryptophan (Hε1-Nε1) and arginine (Hε-Nε) side-chains are labeled with "sc". Assigned amide groups from glutamine and asparagine side-chains are outlined with *horizontal lines* and labeled with "sc". Amide resonances with insignificant to moderate shifts upon acylation are labeled in *black*. Larger chemical shift changes are indicated with an *arrow*, unless impractical for the graphical representation as for C442, G419 or R437sc. Amide resonances from residues I413, W415, V418 and H440 identified only before acylation are labeled in *green*

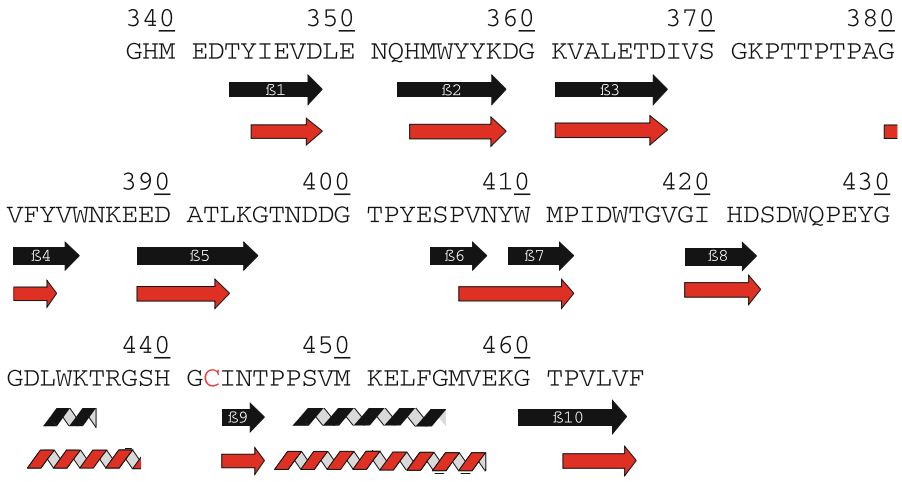

Fig. 2 Amino acid sequence and secondary structure of Ldt_{fm} in the absence of ertapenem. The secondary structure elements predicted by TALOS+ using the NMR chemical shifts are reported in black and are compared to those from the X-ray structure (PDB code: 1ZAT), indicated in *red*. The first three residues G338 to M340 are unique to the NMR sample and originate from the engineered histidine tag. *Helices* and *arrows* sketch α-helices and β-strand secondary structure elements, respectively

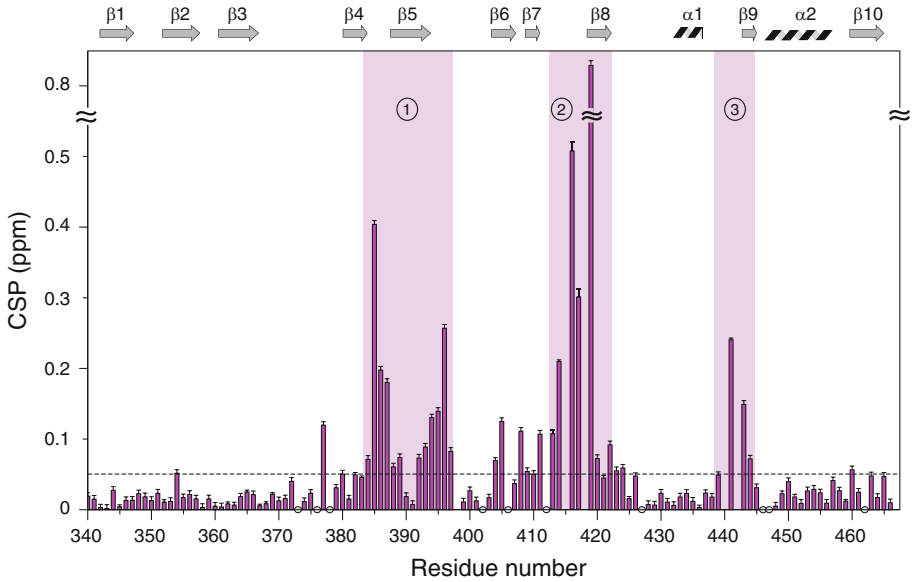

Fig. 3 Amide chemical shift perturbations (CSPs) induced by the acylation of Ldt_{fm} catalytic cysteine with ertapenem. CSPs are calculated as $CSP = \sqrt{(\delta_{H,apo} - \delta_{H,acyl})^2 + \left(\left(\frac{\gamma_N}{\gamma_H}\right)(\delta_{N,apo} - \delta_{N,acyl})\right)^2}$, where $\delta_{i,apo}$ and $\delta_{i,acyl}$ stand for the observed chemical shift of nuclei i before and after acylation of Ldt_{fm} with ertapenem, respectively, and γ_i is the gyromagnetic ratio of nucleus i. The first residues reminiscent from the histidine-tag are omitted. *Grey circles* on the *horizontal axis* indicate proline residues. *Error bars* on each CSP value are reported. Amide resonances remain undetected for residues D398, I413, W415, V418 and H440 in ertapenem-acylated Ldt_{fm}. Three main regions are specifically impacted by the acylation: from residues 384 to 389 (region 1), 413–423 (region 2), and 439–443 (region 3). The secondary structure elements predicted from NMR chemical shifts before acylation are reported on the upper *horizontal axis*

proline. This leads to 98.3, 99.2, and 89.9 % of assignment for amide protons, alpha-carbons and carbonyl-carbons in the backbone, respectively, while 98.4 % of the proton resonances and 81.3 % of the heteroatom resonances were identified in the side-chains.

Resonance assignment of protein nuclei in the covalent adduct of Ldt_{fm} with ertapenem is slightly less complete with 89.8 % overall assignment of all ^{1}H, ^{13}C, and ^{15}N resonances. In the [^{1}H,^{15}N]-HSQC spectrum, backbone amide signals from residues D398, I413, W415, V418 and H440 are missing, although other nuclei in these residues were mostly assigned. The four latter residues are all located in regions that are clearly affected by the addition of the antibiotic, which might induce dynamics as previously shown in the *B. subtilis* enzyme (Lecoq et al. 2012). 90.7, 96.1, and 82.2 % of the amide protons, alpha-carbons and carbonyl-carbons were unambiguously assigned in the backbone of ertapenem-acylated Ldt_{fm}, respectively. 97.1 % of the proton resonances and 78.6 % of the heteroatom resonances were also identified in the protein side-chains. In addition 11 out of the 15 non-exchangeable protons of the unlabeled ertapenem drug moiety were

unambiguously assigned in the covalent Ldt_{fm} adduct with the help of the $^{13}C,^{15}N$-filtered 2D NOESY experiment.

The reported X-ray structure of a longer Ldt_{fm} protein construct (residues 217–466, PDB code 1ZAT) (Biarrotte-Sorin et al. 2006) reveals a catalytic domain with nine β-sheets and two α-helices. Analysis of the backbone ^{1}H, ^{13}C and ^{15}N chemical shifts with TALOS+ (http://spin.niddk.nih.gov/NMRPipe/talos/) shows that the predicted secondary structure elements in the catalytic domain alone in solution are highly similar to the elements in the larger crystal structure (Fig. 2). The only marked differences are the fusion of the β6 and β7 strands into a single element in the X-ray structure, and the lengthening of the two α-helices by a few residues. These differences are attributed to small structural variations between the solution and the crystal rather than to the presence of the additional residues 217–340.

Unusual chemical shifts have been observed in Ldt_{fm}, before and after addition of the antibiotics, for E346 Hα, H353 Hδ2, Y409 Hδs, D422 H_N, S423 Hβs, K435 Hγs, K451 Hα and among the N351 Hδs, S370 Hβs, L393 Hδs, Pro412 Hγs and Hδs, S439 Hβs, P446 Hγ. A major part of these shifts, that show a standard deviation of 0.2–0.5 ppm upfield or downfield of the expected chemical shifts, can be explained by ring current effects from vicinal aromatic residues (including residues W355, Y403, Y409, W410, Y429, W434, F454, and F466) on the basis of the reported 1ZAT crystal structure of the apoenzyme. Similarly the I368 Cγ2 resonance shows a 1.36 ppm downfield shift from its expected position due to the proximity of Y409 within 5 Å. The exceptionally high number of unusual chemical shifts in this protein can be accounted for its high content in aromatic residues (15.5 %).

The chemical shift perturbations induced by the acylation of Ldt_{fm} by ertapenem are reported in Fig. 3. Affected residues are located within three regions. Region 1, extending from residues 384–397, includes the β5-strand. Region 2, including residues 413–423 and the β8-strand, is localized on the opposite side of the cavity in which stand the catalytic cysteine C442 and residues S439 and H440 that are also part of region 3.

All ^{1}H, ^{13}C and ^{15}N chemical shifts for Ldt_{fm} before and after acylation of the catalytic cysteine with ertapenem have been deposited in the BioMagResBank (http://www.bmrb.wisc.edu) under the accession numbers 18900 and 18911, respectively.

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Conflict of interest The authors declare that they have no conflict of interest.

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