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Acyl acceptor recognition by *Enterococcus faecium* $_{L,D}$-transpeptidase Ldt_{fm}

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Summary

In *Mycobacterium tuberculosis* and ampicillin-resistant mutants of *Enterococcus faecium*, the classical target of β-lactam antibiotics is bypassed by $_{L,D}$-transpeptidases that form unusual 3→3 peptidoglycan cross-links. β-lactams of the carbapenem class, such as ertapenem, are mimics of the acyl donor substrate and inactivate $_{L,D}$-transpeptidases by acylation of their catalytic cysteine. We have blocked the acyl donor site of *E. faecium* $_{L,D}$-transpeptidase Ldt_{fm} by ertapenem and identified the acyl acceptor site based on analyses of chemical shift perturbations induced by binding of peptidoglycan fragments to the resulting acylenzyme. An NMR-driven docking structure of the complex revealed key hydrogen interactions between the acyl acceptor and Ldt_{fm} that were evaluated by site-directed mutagenesis and development of a cross-linking assay. Three residues are reported as critical for stabilization of the acceptor in the Ldt_{fm} active site and proper orientation of the nucleophilic nitrogen for the attack of the acylenzyme carbonyl. Identification of the catalytic pocket dedicated to the acceptor substrate opens new perspectives for the design of inhibitors with an original mode of action that could act alone or in synergy with β-lactams.

Keywords

Acyl acceptor; β-lactam; *Enterococcus faecium*; $_{L,D}$-transpeptidase; peptidoglycan

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Introduction

The peptidoglycan polymer (PG) is a giant macromolecule that surrounds the cytoplasmic membrane to protect the bacterium from bursting due to the osmotic pressure of the cytoplasm (Typas *et al.*, 2011). The PG also determines cell shape, intimately participates in cell division, and serves as a scaffold for anchoring various surface polymers. PG is assembled from a disaccharide-pentapeptide subunit, which is polymerized by glycosyltransferases (GTs) (Ostash and Walker, 2005) and cross-linked by transpeptidases (TPs) (Sauvage *et al.*, 2008).

β-lactams are one of the most effective and broadly used family of antibiotics. These drugs inhibit the $_{D,D}$-transpeptidase activity of enzymes, commonly referred to as penicillin-binding proteins (PBPs), which are responsible for the formation of the most prevalent type of peptidoglycan cross-links (4→3) (Sauvage *et al.*, 2008). A second type of cross-links, the 3→3 cross-links, are formed by $_{L,D}$-transpeptidases (Ldt) that by-pass the classical PBPs (Mainardi *et al.*, 2000). In *Mycobacterium tuberculosis*, 80% of the peptidoglycan layer is cross-linked by $_{L,D}$-transpeptidases (Lavollay *et al.*, 2008) and these enzymes are attractive targets for the development of new drugs for the treatment of multidrug-resistant tuberculosis (Mainardi *et al.*, 2008, Mainardi *et al.*, 2011, Dhar *et al.*, 2015, Hugonnet *et al.*, 2009).

PG cross-linking by Ldts is a two-step reaction (Fig. 1A) (Mainardi *et al.*, 2005). In the first step, the $_{L,D}$-transpeptidase cleaves the $_{L}$-Lys^{3}-$_{D}$-Ala^{4} peptide bond of the first substrate, the acyl donor, and forms a thioester bond linking the catalytic cysteine residue to the carbonyl of $_{L}$-Lys^{3}. In the second step, nucleophilic attack of the resulting acylenzyme by the side-chain nitrogen at the third position of the second substrate, the acyl acceptor, leads to formation of a 3→3 cross-link. In *Enterococcus faecium*, the nucleophilic nitrogen is carried by a $_{D}$-*iso*-asparaginyl ($_{D}$-iAsn) residue leading to formation of $_{L}$-Lys^{3}→$_{D}$-iAsn-$_{L}$-Lys^{3} cross-links. The $_{L,D}$-transpeptidase of *E. faecium* (Ldt$_{fm}$) is highly specific for acyl donors containing a stem tetrapeptide ending in $_{L}$-Lys^{3}-$_{D}$-Ala^{4} and for acyl acceptors containing a $_{D}$-iAsn-substituted $_{L}$-Lys^{3} at the third position of the stem peptide (Fig. 1B) (Mainardi *et al.*, 2005, Magnet *et al.*, 2007).

β-lactams are mimics of the acyl donor substrate of PBPs that act as suicide substrates and inactivate the enzymes by acylation of their catalytic serine (Tipper and Strominger, 1965). β-lactams of the carbapenem class similarly inactivate $_{L,D}$-transpeptidases by acylation of their active site cysteine (Fig. 1C) (Mainardi *et al.*, 2007, Triboulet *et al.*, 2011, Triboulet *et al.*, 2013). Recent NMR and X-ray structures of carbapenem-Ldt acylenzymes have allowed identifying the β-lactam binding pocket and hence the putative acyl donor site in three Ldts including *E. faecium* Ldt$_{fm}$ (PDB code 3ZGP) (Lecoq *et al.*, 2013) and *M. tuberculosis* Ldt$_{Mt1}$ (PDB code 4JMX) (Correale *et al.*, 2013) and Ldt$_{Mt2}$ [PDB code 4GSU (Kim *et al.*, 2013) and 3VYP (Li *et al.*, 2013)]. These structures revealed that the catalytic cysteine is located at the entrance of a narrow tunnel connecting two cavities, which are accessible to the solvent (Pockets 1 and 2; Fig. 2). The carbapenem gets access to the cysteine from Pocket 1 and the β-lactam carbonyl interacts with the backbone amide of the catalytic cysteine. For all $_{L,D}$-transpeptidases except Ldt$_{Mt1}$, the β-lactam nitrogen interacts with a

tyrosine hydroxyl group located in the β-sheet separating the two cavities. In some cases, the drug carboxylate group interacts with residues in Pocket 2 but this interaction is more versatile and depends on the nature of the carbapenem side-chain. Together, these data indicate that Pocket 1 is most likely the binding site for the donor stem peptide of the cross-linking reaction catalyzed by $_{L,D}$-transpeptidases (Lecoq *et al.*, 2013). In contrast, the identity of the binding site for the acyl acceptor is controversial. Biarrotte-Sorin and co-workers proposed that the two paths to the catalytic cysteine are used in $_{L,D}$-transpeptidases, one for the acyl-donor (Pocket 1) and the other for the acyl acceptor (Pocket 2) (Biarrotte-Sorin *et al.*, 2006). On the contrary, Erdemli and co-workers proposed that both substrates reach the catalytic cysteine from Pocket 1 (Erdemli *et al.*, 2012). Here, we report identification of the determinants for recognition of the acceptor muropeptide within Pocket 2. This information is a key step for the design of new inhibitors active on the $_{L,D}$-transpeptidases of multidrug-resistant mycobacteria.

Results

Preparation of a stable Ldt_{fm}-ertapenem acylenzyme

Ldt_{fm} catalyzes *in vitro* the transpeptidation of peptidoglycan fragments (Fig. 1A) (Mainardi *et al.*, 2005, Magnet *et al.*, 2007). This reaction is too fast to capture the acyl acceptor bound to the acylenzyme for NMR studies. In contrast, adducts resulting from acylation of Ldt_{fm} by β-lactams of the carbapenem class, such as ertapenem (Fig. 1C), are stable for more than 24 hours (Triboulet *et al.*, 2011, Triboulet *et al.*, 2013). We therefore used Ldt_{fm} acylated by ertapenem to investigate the acyl acceptor binding site, as this β-lactam is thought to be a muropeptide mimetic. The initial formation of the acylenzyme was probed by recording 2D ^{1}H-^{15}N HSQC spectra of the ^{15}N-labeled catalytic domain of Ldt_{fm} before and after incubation with 2.2 molar equivalent of ertapenem. Acylation occurred in less than a minute and was clearly established from the characteristic pattern of the amide resonances from residues close to the active site in comparison to the signature of the apoenzyme (Fig. 3) (Lecoq *et al.*, 2014). The stability of the spectroscopic signature over 48 hours confirmed the stability of the acylenzyme over this period (data not shown and Lecoq *et al.*, 2013).

Binding of muropeptides to the Ldt_{fm}-ertapenem acylenzyme

To identify the acyl-acceptor binding site, disaccharide-peptides (muropeptides), obtained from the enzymatic digestion of *E. faecium* M512 peptidoglycan by muramidases (Mainardi *et al.*, 2000), were progressively added to a freshly prepared 100 μM ertapenem-Ldt_{fm} acylenzyme solution, up to a ~300 molar excess. The acylenzyme remained stable for at least 24 hours after the addition of the muropeptide substrates as shown by its characteristic ^{1}H-^{15}N HSQC signature (Supplementary Fig. S1). However, small but definite chemical shift perturbations (CSPs) were detected in the ^{1}H-^{15}N HSQC spectra collected at different muropeptide:protein ratios (Fig. 4A). These results clearly establish the formation of a non-covalent complex between the acylenzyme and the muropeptides. Furthermore, superimposition of the different spectra showed a gradual shift of a few resonances in response to the increase in muropeptide:protein ratio, suggesting a fast exchange regime between the acylenzyme and the muropeptide:acylenzyme complex with a dissociation constant in the millimolar range.

Identification of the acceptor binding site and mapping of residues in contact with the substrate

To localize the muropeptide acyl-acceptor binding site, the combined $^{1}H_{N}$ and ^{15}N CSP of each amide resonance was calculated for the largest muropeptide:protein ratio and reported in the histogram showing the perturbation induced by muropeptides along the protein sequence (Fig. 4B). Protein residues, that showed a combined CSP greater than two standard deviations above the mean (red line in Fig. 4B), were considered as significantly affected by the interaction with muropeptides and were sketched on the structure of the acylenzyme (Fig. 4C). These residues were found to mainly concentrate in the vicinity of the catalytic cysteine and in Pocket 2.

Construction of a model for the interaction of the acyl acceptor with the ertapenem-Ldt_{fm} acylenzyme

An NMR-driven structural model of the Ldt_{fm} acylenzyme:muropeptide complex was calculated with the CNS structure calculation software (Brunger, 2007), in which the NMR experimental data (CSPs) were introduced as ambiguous interaction restraints using the HADDOCK protocol (de Vries *et al.*, 2010). In this approach, the structure of a disaccharide-tetrapeptide acyl-acceptor generated *in silico* (see *Experimental Procedures*) is docked onto the NMR structure of the acylenzyme (Lecoq *et al.*, 2013), considering the two biomolecules as rigid bodies. During the initial minimization process, residues of the protein, identified as perturbed by the muropeptides in the CSP analysis (Fig. 4B and 4C), are considered to potentially interact with all atoms of the acyl acceptor. Structures of the complex were further refined using online HADDOCK parameters. Even though the muropeptide always localized in Pocket 2 of Ldt_{fm} in a well-defined position, introduction of a 2.5 Å-distance restraint between the sulfur atom of the catalytic cysteine and the amino nitrogen of $_{D}$-iAsn improved the initial convergence. This constraint, which is consistent with the $_{L,D}$-transpeptidation mechanism, was kept in the final calculation, that led to a cluster of 40 structures (Supplementary Table S1). A representative model from the cluster, corresponding to the structure of lowest energy, is depicted in Fig. 5A and 5B.

In order to confirm the specificity of Pockets 1 and 2 for the acyl donor and acceptor, respectively, modelling was also performed with two disaccharide-tetrapeptides substituted by a $_{D}$-iAsn residue (Fig. 6A). This muropeptide can act both as a donor and an acceptor. Introduction of spatial constraints specific of the Ldt_{fm}-donor (C442-Lys^{3}) and Ldt_{fm}-acceptor (C442-$_{D}$-iAsn) interactions showed that the acyl donor approaches the catalytic cysteine from Pocket 1, whereas the acyl acceptor binds to Pocket 2 (see Supplementary Table S2 for statistical convergence). These results confirmed the specificity of Pockets 1 and 2 for the donor and for the acceptor in the absence of ertapenem. Altogether, the *ab initio* model depicted in the lower part of Fig. 6A and the experimentally-derived model with ertapenem (Fig. 5 and Fig. 6B) also show that the steric hindrance caused by the positioning of the donor in Pocket 1 prevents the acceptor from accessing to the catalytic cysteine by the same pocket. Set side-by-side, the two models evidence the diminished occupancy of Pocket 1 with the antibiotic in comparison to its occupancy with the muropeptide. The latter observation indicates that binding of ertapenem in Pocket 1 is unlikely to impair binding of the acyl acceptor in Pocket 2.

Candidate interactions stabilizing the acyl acceptor within the Ldt_{fm} catalytic cavity

In the model with ertapenem depicted in details in Fig. 5, the peptide stem of the acceptor is mainly stabilized in Pocket 2 by a series of hydrogen bonds (Fig. 5C). The relevance of these interactions in the stabilization of the complex was analyzed based on their persistence in the 5 structures of lower energy (Fig. 5D). In Pocket 2, W425 is likely to critically contribute to the orientation of the nucleophilic nitrogen of the acceptor by establishing a hydrogen bond with the oxygen of the carboxamide of $_{D}$-iAsn. S439 and N444 may be of assistance to this orientation process. Additionally, K372 and R437 form several hydrogen bonds with the γ-carbonyl and α-carboxamide of $_{D}$-iGln, respectively. These interactions are likely to stabilize the conformation of the tetrapeptide stem within Pocket 2. Together, these results led to the identification of the acceptor binding site of Ldt_{fm} and of candidate enzyme residues potentially involved in binding of the acceptor substrate.

Assay of the cross-linking activity of Ldt_{fm} and derivatives obtained by site-directed mutagenesis

The role of Ldt_{fm} residues inferred from the structural model was assessed by determining the cross-linking activity of derivatives obtained by site-directed mutagenesis. Chemical shift perturbation assays were used to show that impaired enzyme activity was not due to important modification of the protein conformation (Fig. 3). A linear tetrapeptide (DS-Tetra) and a branched tripeptide [DS-Tri(Asn)] were used as substrates since these muropeptides are exclusively used as acyl donor and acceptor, respectively (Fig. 1A and 1B). This led to formation of a single peptidoglycan dimer [DS-Tri(Asn)-DS-Tri], which was not further polymerized. The only side reaction was the hydrolysis of the $_{L}$-Lys^{3}-$_{D}$-Ala^{4} peptide bond of the acyl donor to form a tripeptide (DS-Tri) (Fig. 1A).

In the presence of equimolar concentrations (30 μM) of the acyl donor and acceptor, wild type Ldt_{fm} catalyzed formation of the muropeptide dimer ($_{L,D}$-transpeptidase activity) and of DS-Tri ($_{L,D}$-carboxypeptidase activity) with similar efficiencies (Table 1). The ratio of the two activities increased linearly with the concentration of the acceptor in the 10 to 90 μM range. The observed absence of saturation of the enzyme by the acyl acceptor at 90 μM is commensurate with the relatively high dissociation constant (mM range) reported above for NMR-based analyses of the binding of the acceptor to the Ldt_{fm}-ertapenem acylenzyme (Supplementary Fig. S2). Thus, binding of ertapenem to Pocket 1 does not appear to artefactually impair binding of the acyl acceptor in Pocket 2 in agreement with the results of the modelling experiments presented above (Fig. 6).

The K372A and R437A substitutions, alone or in combination, had little impact on the $_{L,D}$-carboxypeptidase activity (relative activities ranging from 45 to 140%). In contrast, substitution K372A led to a 14-fold reduction in the $_{L,D}$-transpeptidase activity (from 100 to 7%). A further 4-fold decrease was observed for the combination of substitutions K372A and R437A (from 7 to 2%). Alone, the latter substitution resulted in a similar 4-fold decrease (from 100 to 26%). These results indicate that R372A, and to a lesser extent R437A, impaired interaction of Ldt_{fm} with the acceptor, without interfering with formation of the acylenzyme. In agreement, our structural model (Fig. 5) predicts that these residues are located remotely from the catalytic C442 in the acceptor pocket and should therefore not

participate in the hydrolytic pathway. Residue W425 is located at the bottom of the acceptor pocket, closer to C442 than K372 and R437. The W425A substitution almost completely abolished the $_{L,D}$-transpeptidase activity but produced a much more moderate effect on the $_{L,D}$-carboxypeptidase activity (0.04% versus 18% residual activities). This single substitution leads to a 500-fold decrease in the $_{L,D}$-transpeptidase to $_{L,D}$-carboxypeptidase turnover ratio indicating that the substitution preferentially impaired the $_{L,D}$-transpeptidase activity. In the model depicted in Fig. 5C, W425 establishes a hydrogen bond with the $_{D}$-iAsn residue that carries the nucleophilic amine of the cross-linking reaction. This interaction may directly participate in the positioning of this group for catalysis. A minor impact on the first step of the reaction may be accounted for by the close proximity of W425 and the catalytic C442 residue.

Discussion

Peptidoglycan transpeptidases are attractive and validated targets for antibacterial drug development, as demonstrated by successful use of antibiotics of the β-lactam family in the past seven decades. These targets include the classical PBPs in most bacterial pathogens (Zapun *et al.*, 2008). These targets also include $_{L,D}$-transpeptidases, which are the predominant cross-linking enzymes in *M. tuberculosis* (Lavollay *et al.*, 2008, Kumar *et al.*, 2012), *Mycobacterium abscessus* (Lavollay *et al.*, 2011) and *Clostridium difficile* (Peltier *et al.*, 2011). In addition, one of the five $_{L,D}$-transpeptidase paralogues of *M. tuberculosis* has a critical role in virulence (Gupta *et al.*, 2010) and inhibition of these enzymes by certain β-lactams results in rapid cytolysis and elimination of persisters (Dhar *et al.*, 2015).

Most structural studies of PBPs and $_{L,D}$-transpeptidases have focused on the interaction of β-lactams with the acyl donor site (Sauvage *et al.*, 2008, Zapun *et al.*, 2008). Consequently, little is known on the acceptor site. The specificity of PBPs for the acceptor substrate has only been indirectly assessed *in vivo* based on genetic manipulation of the structure of peptidoglycan precursors (Arbeloa *et al.*, 2004, Bellais *et al.*, 2006). These studies led to the conclusion that PBPs tolerate important variations in the structure of both the acyl acceptor and the acyl donor. In contrast, *in vitro* cross-linking assays showed that $_{L,D}$-transpeptidases tolerate little variation in the donor and essentially no variation in the acceptor (Magnet *et al.*, 2007). Here we show that the acceptor site of transpeptidases is amenable to structural analyses by NMR based on irreversible acylation of the enzyme by a β-lactam, which occupies the donor site, and titration of the acceptor site by muropeptides. The approach is applicable to both PBPs and $_{L,D}$-transpeptidases.

Using *E. faecium* Ldt_{fm} as a model, we show that the acylenzyme formed with carbapenem remains stable upon muropeptide addition. This allowed mapping the chemical shift perturbations specifically induced by the binding of muropeptides to the acceptor site. Based on these structural data we propose a model in which the acyl acceptor bind to Pocket 2 which is distant from the antibiotic binding cavity (Fig. 4 and 5). Interestingly, a model built independently from the chemical shift perturbations led to the identification of the same binding Pockets for the acyl donor and acyl acceptor (Fig. 6). The interaction model of the acyl acceptor with Ldt_{fm} was validated by site-directed mutagenesis of candidate residues and assay of the residual cross-linking activity of the resulting Ldt_{fm} derivatives. Of note,

our $_{L,D}$-transpeptidase assay relies on determination of the $_{L,D}$-carboxypeptidase activity of Ldt_{fm}, a side reaction which provides an internal control for the efficacy of the first step of the cross-linking reaction (*i.e.* formation of the acylenzyme). Thus, any decrease in the $_{L,D}$-transpeptidase to $_{L,D}$-carboxypeptidase ratio of activities identifies impaired interactions with the acyl acceptor in the second step of the reaction. Using these approaches, we identified amino acid substitutions that exclusively (R372A and R437A) or preferentially (W425A) impaired the $_{L,D}$-transpeptidase activity of Ldt_{fm} (Table 1). The latter substitution had the largest impact on Ldt_{fm} activity and led to a 500-fold decrease in the $_{L,D}$-transpeptidase to $_{L,D}$-carboxypeptidase ratio. Based on these data K372 and R437 are predicted to stabilize the conformation of the acyl acceptor by forming hydrogen bonds with the γ-carbonyl and α-carboxamide of $_{D}$-iGln at the 2nd position of the stem peptide, respectively (Fig. 5C). Residue W425 forms a hydrogen bond with the α-carboxamide of $_{D}$-iAsn and is predicted to orientate the α-amino group of $_{D}$-iAsn for nucleophilic attack of the acylenzyme carbonyl.

Mapping of the acceptor site opens new avenues for the design of inhibitors with an original mode of action. Such drugs are unlikely to be affected by modifications of the target that confers resistance to β-lactams since distinct sites are involved in the binding of the donor and acceptor substrates. Furthermore, drugs targeting the acceptor site may act in synergy with β-lactams.

Experimental Procedures

Production and Purification of Ldt_{fm}

A ^{13}C,^{15}N–labeled protein containing the catalytic domain of Ldt_{fm} (residues 341 to 466) was produced in *Escherichia coli* BL21 (DE3) cells harbouring plasmid pETTEVΩ*ldt*$_{fm}$ and purified, as previously described (Lecoq *et al.*, 2014, Lecoq *et al.*, 2013).

Muropeptides preparation and $_{L,D}$-transpeptidase assay

The disaccharide-peptides used as the acyl donor (DS-Tetra) and acceptor [DS-Tri(Asn)] in the transpeptidase assay were purified (Arbeloa *et al.*, 2004) from the peptidoglycan of *Enterococcus faecalis* BM4314 (Bouhss *et al.*, 2002) and *E. faecium* M512 (Mainardi *et al.*, 2000), respectively. The concentration of the disaccharide-peptides was determined by amino acid analysis after acidic hydrolysis with a Hitachi autoanalyser (Mengin-Lecreulx *et al.*, 1999). Formation of 3→3 cross-links was tested in 60 μL of phosphate buffer (15 mM, pH 7.0) containing Ldt_{fm} (2.5 to 50 μM), (DS-Tetra) (30 μM), and [DS-Tri(Asn)] (30 μM). The reaction was allowed to proceed at 37 °C, aliquots (10 μL) were withdrawn at various times (15 min to 2 h), and the reaction was stopped by the addition of 2 μL of 10% trichloroacetic acid. Muropeptides were desalted (ZipTipC_{18}, Millipore) and analyzed by electrospray mass spectrometry in the positive mode (Qstar Pulsar I, Applied Biosystem), as previously described (Arbeloa *et al.*, 2004). Muropeptides used in the NMR interaction experiment were produced from unlabeled *E. faecium* M512 peptidoglycan (Mainardi *et al.*, 2000).

NMR Spectroscopy

A 100 μM $^{13}C,^{15}N$–Ldt_{fm} sample in 100 mM sodium phosphate buffer containing 100 mM NaCl at pH 6.4 (buffer A) and 2.2 molar equivalent of ertapenem (INVANZ) was used to prepare the acylenzyme for the NMR study. One aliquot of 10 μL (~25 molar equivalent with respect with the protein), 4 aliquots of 20 μL, and a last aliquot of 15 μL of the unlabeled muropeptides stock-solution (155 mg/mL in buffer A) were successively added to the protein sample. ^{1}H-^{15}N HSQC spectra were recorded at 25 °C on a 600 MHz Agilent Direct Drive spectrometer after each muropeptide addition using a triple resonance cryogenic probe. Data were processed with NMRPipe and analyzed with CcpNMR in which the published acylenzyme assignments (Lecoq *et al.*, 2014, Lecoq *et al.*, 2013) were initially transferred.

Ldt_{fm}/ muropeptide docking

Models of muropeptide docked onto Ldt_{fm} were built with “The HADDOCK web server for data-driven biomolecular docking” of HADDOCK2.1 (de Vries *et al.*, 2010) using CNS1.2 (Brunger, 2007) for the structure calculations. The initial structural model for muropeptide DS-Tetra(Asn) was built from a disaccharide and a modified tetrapeptide motif generated within the $GlyC_aNS$ (http://haddock.chem.uu.nl/glycans/) and PRODRG softwares, respectively. A patch for the topology and parameter files was used to connect the two fragments through the lactoyl group of MurNAc leading to the initial PDB structure for the muropeptide.

For the NMR data-driven modelling of the muropeptide acceptor into the ertapenem Ldt_{fm} acylenzyme, the coordinates of the acylenzyme were taken from the NMR structure [PDB code 3ZGP (Lecoq *et al.*, 2013)]. The docking was performed and analyzed with default HADDOCK parameters except a clustering cutoff of 3.0 Å and random removal of restraints turned off. Protein residues E350, G395, T396, N397, N408, I420, Q426, R437, G438, S439, G441 and I443 that were identified through CSP analysis were declared as ambiguous interaction restraints. Non-bonded interactions were calculated with the OPLS force field using a cutoff at 6.5 Å. The HADDOCK score was used to rank the generated models, which were further analyzed within the Pymol software. The model of lowest energy was examined with LigPlot+v.1.4 to extract the interaction map between the muropeptide and the protein.

For modelling of the complex containing two $_D$-iAsn-substituted disaccharide-tetrapeptide substrates in the Ldt_{fm}, catalytic cavity, the coordinates of the apoenzyme were taken from the X-ray structure [PDB code 1ZAT (Biarrotte-Sorin *et al.*, 2006)]. The docking was performed and analyzed with default HADDOCK parameters. In order to position the reactive groups of the acyl donor and acceptor of the transpeptidation reaction, two independent distance restraints of 2 ± 1 Å were introduced between (i) the sulfur of C442 and the carbonyl carbon of Lys^3 and (ii) the sulfur of C442 and the amino nitrogen of $_D$-iAsn. No experimental restraints were used in this calculation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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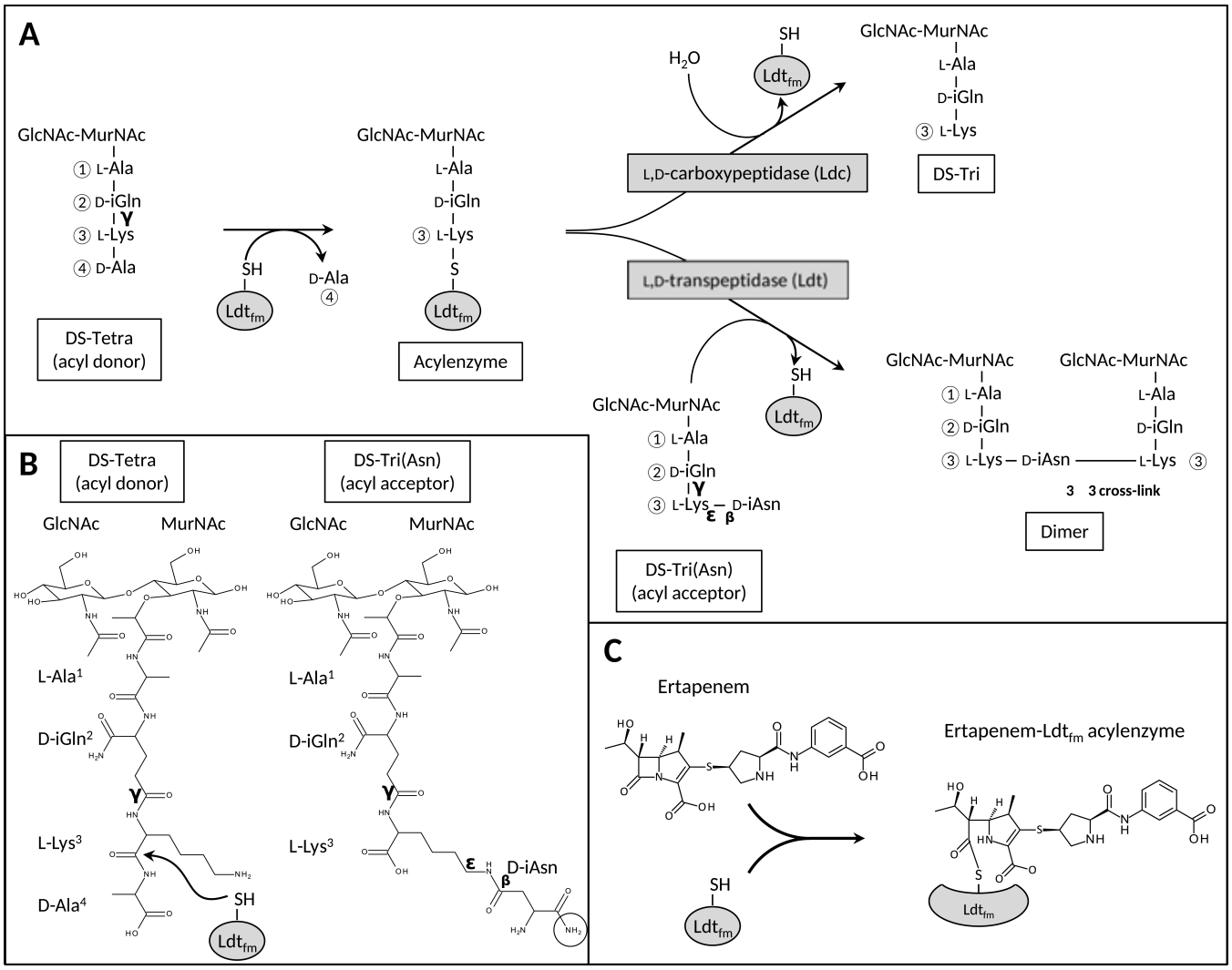

Fig. 1. Reactions catalyzed by the L,D-transpeptidase Ldt_{fm}

A. Two-step cross-linking reaction catalyzed by Ldt_{fm}. In the first step, the active site Cys residue (C442) of Ldt_{fm} attacks the carbonyl of L-Lys^3 in the stem tetrapeptide of the acyl donor. This step results in the release of D-Ala^4 and the formation of a thioester bond between C442 and the carbonyl of L-Lys^3. The resulting acylenzyme is a common intermediate for the L,D-transpeptidation and L,D-carboxypeptidation reactions. In the former reaction, the thioester bond of the intermediate is attacked by the D-iAsn amino group of the acyl acceptor leading to the formation of a muropeptide dimer and release of the enzyme. In the latter reaction, the acylenzyme is hydrolyzed leading to formation of a tripeptide. The two reactions occur in competition.

B. Structure of the acyl donor and acceptor. The arrow indicates the position of nucleophilic attack by catalytic C442. The nucleophile of the acyl acceptor is circled. The muropeptides (peptidoglycan fragments) contain a disaccharide (DS) composed of β-1,4-linked *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc). Of note, DS-Tetra is only used as acyl donor since it lacks the side-chain D-iAsn residue (Magnet *et al.*, 2007).

Conversely, DS-Tri(Asn) is only used as acyl acceptor since it lacks the C-terminal $_{D}$-Ala^{4} residue (Mainardi *et al.*, 2005). Tetra, tetrapeptide, Tri, tripeptide.

C. Acylation of Ldt_{fm} by ertapenem, a β-lactam of the carbapenem class.

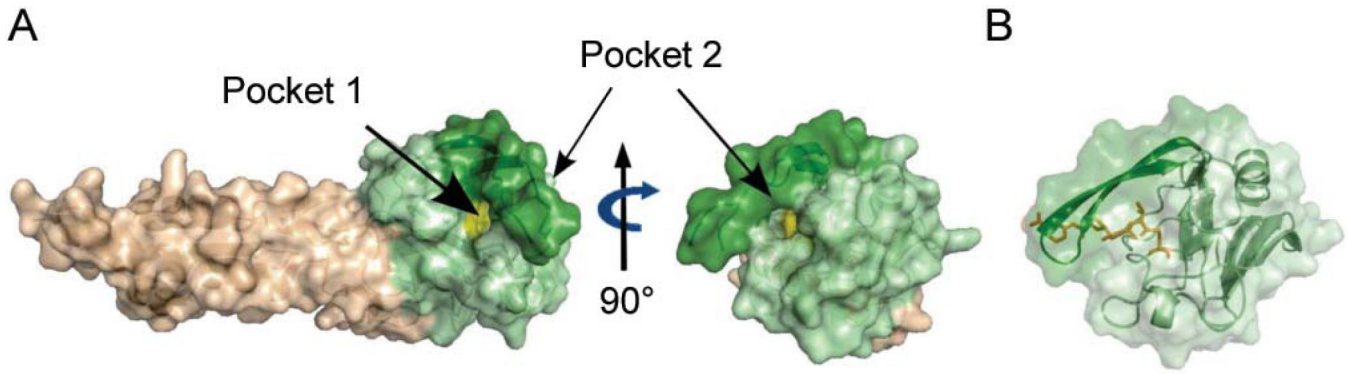

Fig. 2. Surface representations of the L,D-transpeptidase Ldt_{fm}

A. Access to the catalytic cysteine (C442) in the Ldt_{fm} apoenzyme (PDB code 1ZAT). The representation of the catalytic domain of Ldt_{fm} (green) highlights the access to C442 (yellow) from Pockets 1 and 2. The tunnel between Pockets 1 and 2 is formed by a loop that can act like a flap (dark green). The surface representation of the mixed alpha-beta N-terminal domain is shown in beige.

B. Surface and ribbon representation of Ldt_{fm} acylenzyme catalytic domain (PDB code 3ZGP) emphasizing the structure of the flap. Small structural reorganization of a limited number of side chains and/or a limited flexibility of the backbone double-stranded β-sheet is sufficient to open the tunnel between Pockets 1 and 2. This is expected to allow for the release of the cross-linked reaction product without any major structural change.

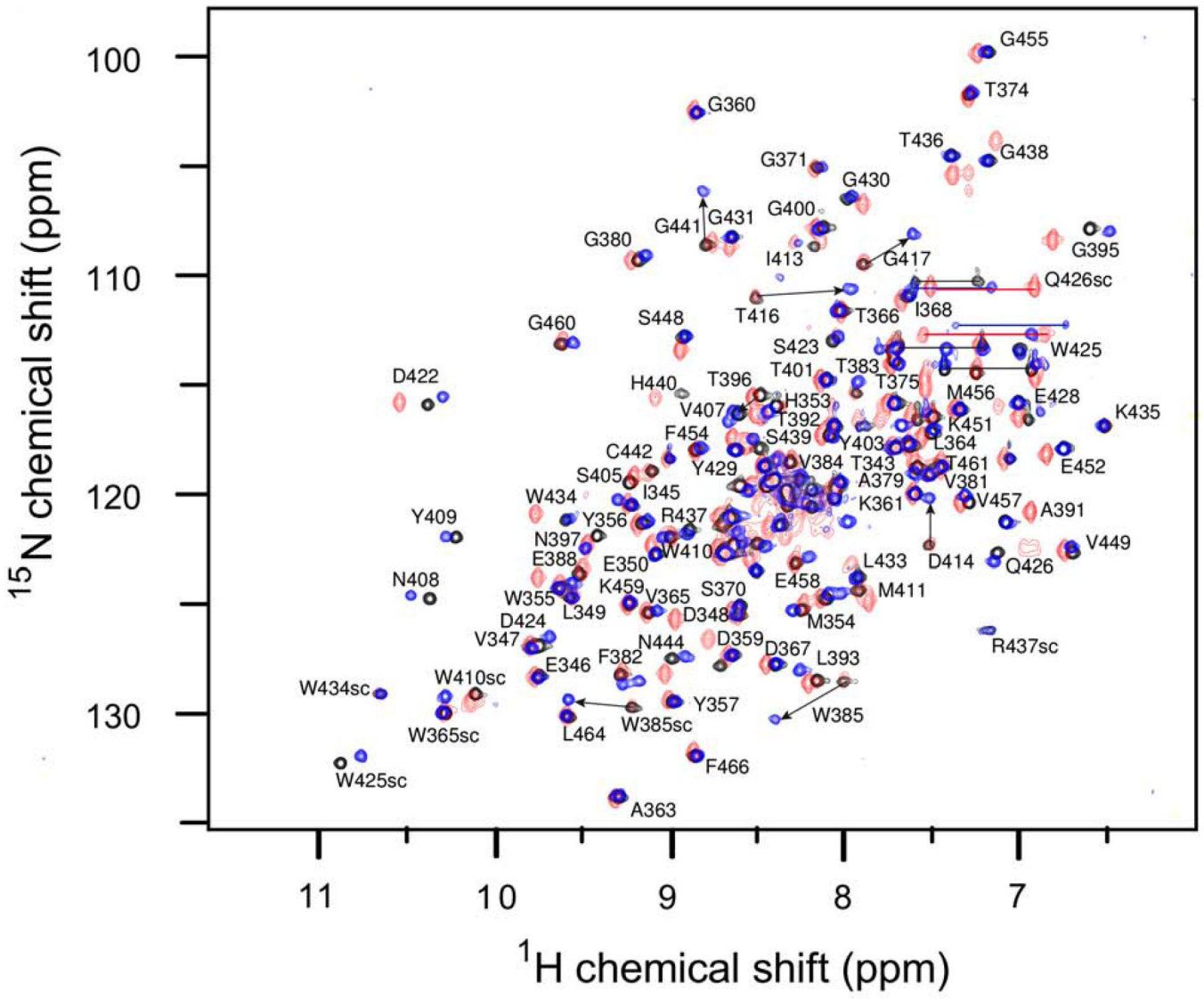

Fig. 3.

Superimposition of the ^{1}H-^{15}N HSQC spectra of wild-type apoenzyme (black), wild-type Ldt_{fm} acylated with ertapenem (blue) and W425A R437A apoenzyme (red). The NMR spectra were collected on a 100 μM solution of ^{13}C,^{15}N-labeled protein in 100 mM sodium buffer containing 300 mM NaCl at pH 6.4 and 25 °C. The overall fingerprint of the protein is not affected by the substitution of amino acids W425 and R437 by Ala indicating that the protein structure remains unchanged. The substituted residues are shown in red. sc indicates side-chain ^{1}H-^{15}N correlations. Correlations are labeled except in the center of the spectrum due to overlaps.

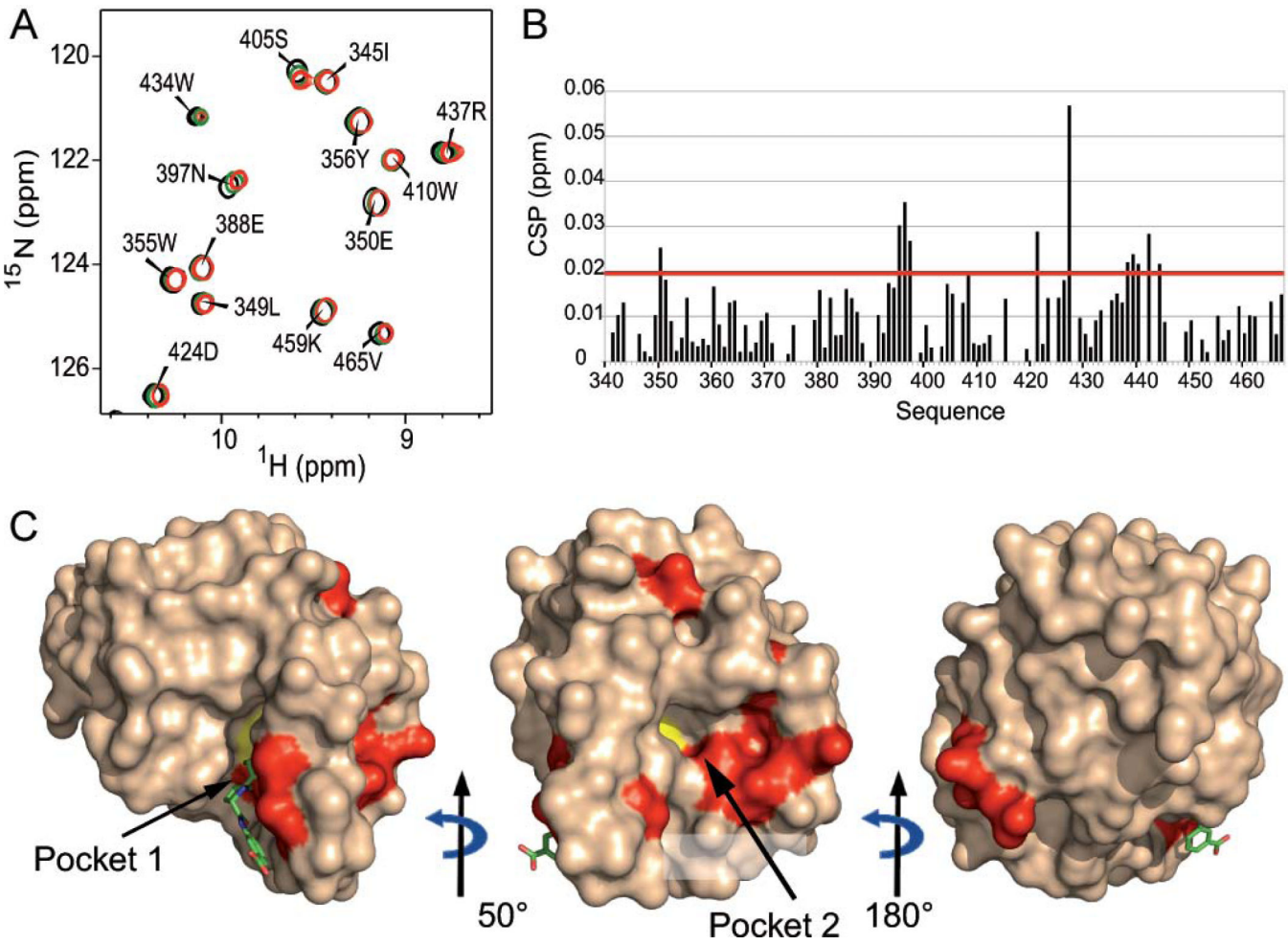

Fig. 4. Muropeptide titration of Ldt_{fm} acylated with ertapenem

A. ^{1}H-^{15}N HSQC spectra recorded at 25°C for muropeptide:acylenzyme molar ratios of 0 (black), ~25 (green), and ~300 (red). The small amplitude of the chemical shift perturbations suggests that there is no major structural reorganization of the protein and, in particular, of the flap, upon addition of the muropeptide acceptor.

B. Combined $^{1}H_{N}$ and ^{15}N chemical shift perturbations (CSPs) measured after addition of ~300 molar equivalents of muropeptides. The red line corresponds to two standard deviations above the mean, calculated over all residues with assigned backbone resonances.

C. Surface representations of the ertapenem-Ldt_{fm} acylenzyme structure (PDB code 3ZGP) (Lecoq *et al.*, 2013). Residues with significant CSP (greater than two standard deviations above the mean) are coloured in red (E350, G395, T396, N397, N408, I420, Q426, R437, G438, S439, G441 and I443). Ertapenem is displayed with green sticks and the catalytic cysteine (C442) is coloured in yellow. Residues impacted by the interaction are localized in the vicinity of C442 in Pockets 1 and 2 and at a remote distance of C442 only in Pocket 2.

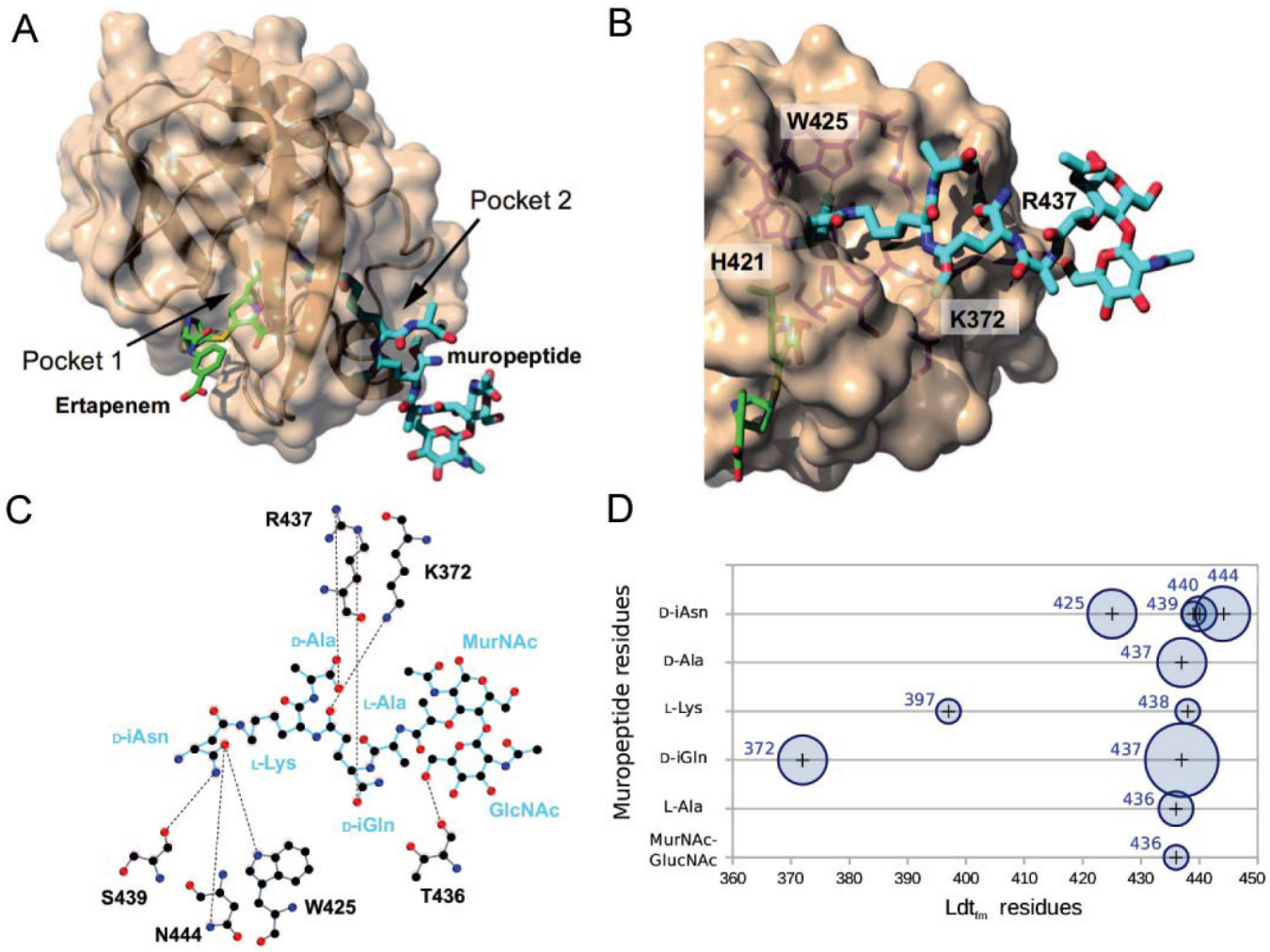

Fig. 5. Binding of the acyl acceptor to Pocket 2 of Ldt_{fm}

A. Lowest energy structure obtained by NMR data-driven docking of the muropeptide DS-Tetra(D-iAsn) into the ertapenem-Ldt_{fm} acylenzyme structure (PDB code 3ZGP) (Lecoq *et al.*, 2013). Ertapenem mimicking the acyl donor and the acceptor muropeptide are shown in green and cyan, respectively. DS-Tetra(D-iAsn), GlcNAc-MurNAc-L-Ala^1-D-$iGln^2$-L-Lys^3(D-iAsn)-D-Ala^4.

B. Surface representation highlighting the acyl acceptor in Pocket 2 (cyan, in sticks) and contacts with protein residues.

C. Schematic representation of the H-bond network (dashed lines) in the ertapenem-Ldt_{fm}:acyl acceptor complex established with LigPlot+.

D. Schematic representation of hydrogen-bond interactions in the 5 model complexes of lowest energy. These models are selected within the cluster with the best HADDOCK score. The horizontal and vertical axes sketch the protein sequence (residue numbers) and the muropeptide residues, respectively. Circles represent intermolecular interactions between residues of the protein and of the muropeptide. These interactions may involve one or several hydrogen bonds. The surface of the circles is proportional to the number of structures showing the interaction among the five structures with the best HADDOCK

scores. The number of structures showing interaction ranges from one (*e.g.* T436 with GlcNAc) to five (*e.g.* R437 with D-iGln).

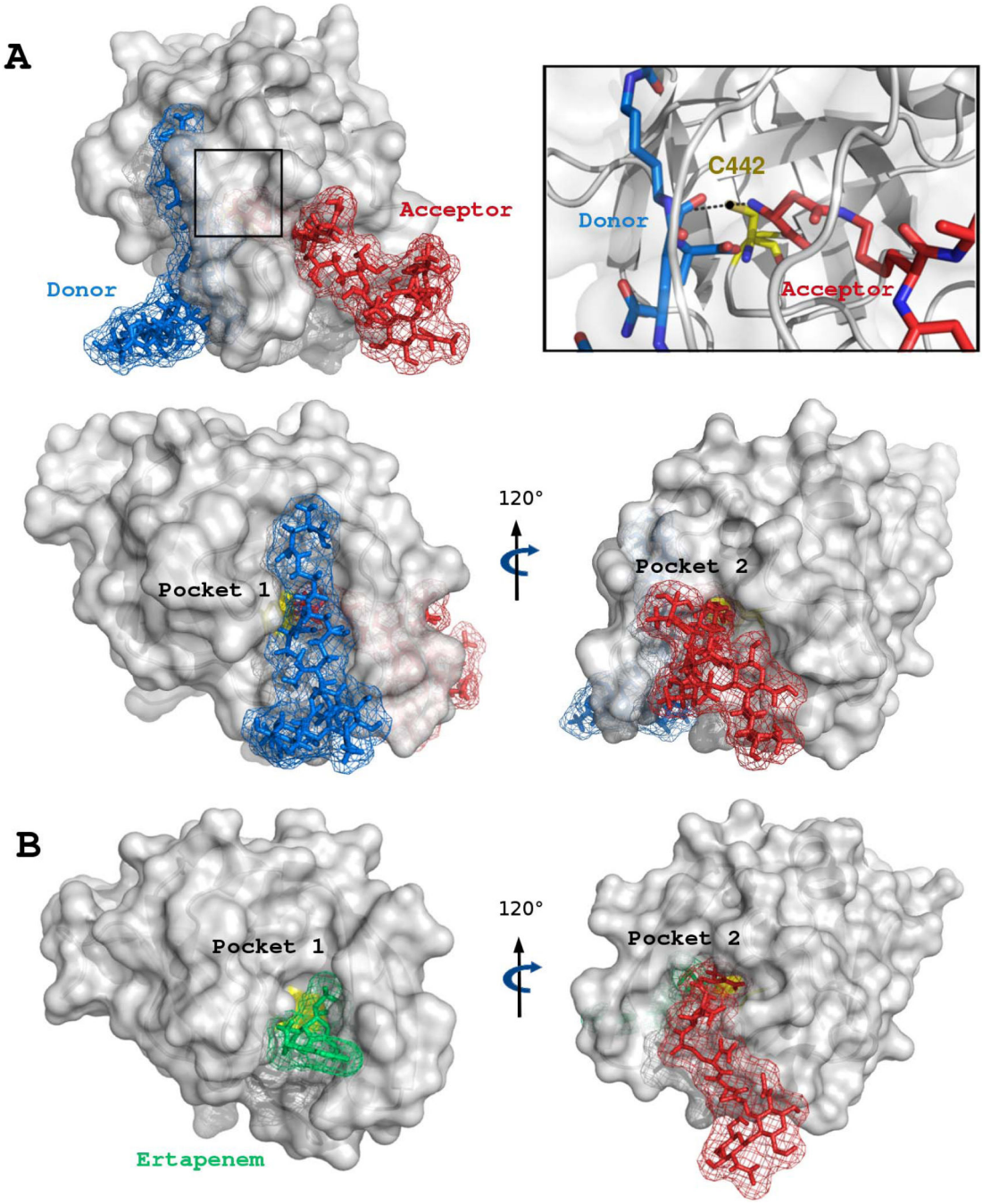

Fig. 6. Binding of the acyl donor and acceptor to Ldt_{fm}

A. Modelling of the complex formed by the binding of two DS-Tetra(D-iAsn) muropeptides to Ldt_{fm}. An energy minimization was run with the structures of Ldt_{fm} (PDB code 1ZAT) and of two identical muropeptides, DS-Tetra(D-iAsn), which can act as a donor and as an acceptor in the cross-linking reaction. In order to assign a donor role to one of the two muropeptides, a distance restraint was introduced between the sulfur of the catalytic cysteine (C442) and the carbonyl carbon of L-Lys[3]. In order to assign an acceptor role to the other muropeptide, a distance restraint was introduced between the sulfur of C442 and the

nitrogen of the amino group of D-iAsn. Following energy minimization, the distance constraint involving C442 and L-Lys^3 led to the localisation of the muropeptide into Pocket 1 (blue). Conversely, the C442-D-iAsn constraint led to the localisation of the muropeptide into Pocket 2 (red). The upper-left panel shows a front view of the two cavities separated by the flap. The upper-right panel shows an enlargement of the C442 environment with the distance restraints indicated by dotted lines. The occupancy of each individual pocket by the muropeptide is illustrated in the two lower panels.

B. NMR data-driven model of the DS-Tetra(D-iAsn) muropeptide docked onto the ertapenem-Ldt$_{fm}$ acylenzyme. The surface representation is shown in the same orientation as the lower part of panel A. These views show that ertapenem (in green) provides a smaller steric hindrance than the donor in Pocket 1.

DS-Tetra(D-iAsn), GlcNAc-MurNAc-L-Ala^1-D-iGln^2-L-Lys^3(D-iAsn)-D-Ala^4.

Table 1

Impact of amino acid substitutions on Ldt_{fm} activities[a].

Substitution	L,D-transpeptidase		L,D-carboxypeptidase		
	Turnover[b]	%[c]	Turnover[b]	%[c]	Ratio[d]
None	1,800 ± 620	100	2,400 ± 900	100	1.3
R437A	460 ± 82	26	3,400 ± 1,100	140	7
K372A	130 ± 16	7	1,800 ± 500	77	14
K372A R437A	37 ± 7	2	1,100 ± 200	45	29
W425A	≈ 0.69	≈ 0.04	430 ± 18	18	630

[a] Ldt_{fm} was incubated with muropeptides DS-Tetra (30 μM) and DS-Tri(Asn) (30 μM) to determine competitive formation of dimer (L,D-transpeptidase activity) and DS-Tri (L,D-carboxypeptidase activity).

[b] Mean ± SD from a minimum of three determinations ($\times 10^6$ s^{-1}).

[c] Relative to native Ldt_{fm}.

[d] Ratio of turnover numbers (L,D-carboxypeptidase over L,D-transpeptidase).