Kinetic Features of L,D-Transpeptidase Inactivation Critical for β-Lactam Antibacterial Activity

Sébastien Triboulet1,2,3,9, Vincent Dubée1,2,3,9, Lauriane Lecoq4,5,6, Catherine Bougault4,5,6, Jean-Luc Mainardi1,2,3,7, Louis B. Rice8, Mélanie Ethève-Quelquejeu9,10, Laurent Gutmann1,2,3,7, Arul Marie11,12, Lionel Dubost11,12, Jean-Emmanuel Hugonnet1,2,3, Jean-Pierre Simorre4,5,6, Michel Arthur1,2,3

1 Centre de Recherche des Cordeliers, Equipe 12, Université Pierre et Marie Curie–Paris 6, UMR S 872, Paris, France, 2 INSERM, U872, Paris, France, 3 Université Paris Descartes, Sorbonne Paris Cité, UMR S 872, Paris, France, 4 CEA, DSV, Institut de Biologie Structurale (IBS), Grenoble, France, 5 CNRS, UMR 5075, Grenoble, France, 6 Université Joseph Fourier, Grenoble 1, France, 7 Assistance Publique-Hôpitaux de Paris, Hôpital Européen Georges Pompidou, Paris, France, 8 Rhode Island Hospital, Brown University, Providence, Rhode Island, 9 Laboratoire de Chimie et de Biochimie Pharmacologiques et Toxicologiques, Université Paris Descartes, UMR 8601, Paris, France, 10 CNRS, UMR 8601, Paris, France, 11 Muséum National d’Histoire Naturelle, USM0502, Plateforme de Spectrométrie de Masse et de Protéomique du Muséum, Paris, France, 12 CNRS, UMR8041, Paris, France

Abstract

Active-site serine D,D-transpeptidases belonging to the penicillin-binding protein family (PBPs) have been considered for a long time as essential for peptidoglycan cross-linking in all bacteria. However, bypass of the PBPs by an L,D-transpeptidase (Ldtfm) conveys high-level resistance to β-lactams of the penam class in Enterococcus faecium with a minimal inhibitory concentration (MIC) of ampicillin >2,000 μg/ml. Unexpectedly, Ldtfm does not confer resistance to β-lactams of the carbapenem class (imipenem MIC = 0.5 μg/ml) whereas cephalosporins display residual activity (ceftriaxone MIC = 128 μg/ml). Mass spectrometry, fluorescence kinetics, and NMR chemical shift perturbation experiments were performed to explore the basis for this specificity and identify β-lactam features that are critical for efficient L,D-transpeptidase inactivation. We show that imipenem, ceftriaxone, and ampicillin acylate Ldtfm by formation of a thioester bond between the active-site cysteine and the β-lactam-ring carbonyl. However, slow acylation and slow acylenzyme hydrolysis resulted in partial Ldtfm inactivation by ampicillin and ceftriaxone. For ampicillin, Ldtfm acylation was followed by rupture of the C5–C6 bond of the β-lactam ring and formation of a secondary acylenzyme prone to hydrolysis. The saturable step of the catalytic cycle was the reversible formation of a tetrahedral intermediate (oxyanion) without significant accumulation of a non-covalent complex. In agreement, a derivative of Ldtfm blocked in acylation bound ertapenem (a carbapenem), ceftriaxone, and ampicillin with similar low affinities. Thus, oxyanion and acylenzyme stabilization are both critical for rapid L,D-transpeptidase inactivation and antibacterial activity. These results pave the way for optimization of the β-lactam scaffold for L,D-transpeptidase-inactivation.

Introduction

Biosynthesis of peptidoglycan, the major constituent of bacterial cell-walls, is a preeminent target for antibiotics since the polymer is present and essential in nearly all bacterial species, with the exception of a few obligate intracellular parasites. Penicillin is the first antibiotic introduced for chemotherapy of bacterial infections and remains an essential in nearly all bacterial species, with the cell-walls, is a preeminent target for antibiotics since the polymer is necessary for bacterial cell-surface integrity and function, including peritrichous motility, osmotic stability, and autolytic degradation [1]. These features are essential for peptidoglycan cross-linking in all bacteria. However, bypass of the PBPs by an L,D-transpeptidase (Ldtfm) conveys high-level resistance to β-lactams of the penam class in Enterococcus faecium with a minimal inhibitory concentration (MIC) of ampicillin >2,000 μg/ml. Unexpectedly, Ldtfm does not confer resistance to β-lactams of the carbapenem class (imipenem MIC = 0.5 μg/ml) whereas cephalosporins display residual activity (ceftriaxone MIC = 128 μg/ml). Mass spectrometry, fluorescence kinetics, and NMR chemical shift perturbation experiments were performed to explore the basis for this specificity and identify β-lactam features that are critical for efficient L,D-transpeptidase inactivation. We show that imipenem, ceftriaxone, and ampicillin acylate Ldtfm by formation of a thioester bond between the active-site cysteine and the β-lactam-ring carbonyl. However, slow acylation and slow acylenzyme hydrolysis resulted in partial Ldtfm inactivation by ampicillin and ceftriaxone. For ampicillin, Ldtfm acylation was followed by rupture of the C5–C6 bond of the β-lactam ring and formation of a secondary acylenzyme prone to hydrolysis. The saturable step of the catalytic cycle was the reversible formation of a tetrahedral intermediate (oxyanion) without significant accumulation of a non-covalent complex. In agreement, a derivative of Ldtfm blocked in acylation bound ertapenem (a carbapenem), ceftriaxone, and ampicillin with similar low affinities. Thus, oxyanion and acylenzyme stabilization are both critical for rapid L,D-transpeptidase inactivation and antibacterial activity. These results pave the way for optimization of the β-lactam scaffold for L,D-transpeptidase-inactivation.
inactivation and antibacterial activity [1]. In contrast, the ester bond connecting the active-site serine to the carbonyl of D-Ala \(^4\) in the physiological substrate is readily attacked by the amine of the acyl acceptor substrate resulting in 4→3 cross-link formation and enzyme turnover.

Serine-active D,D-transpeptidases have long been considered as essential for peptidoglycan cross-linking [4]. However, these enzymes can be bypassed by an unrelated enzyme family, the \(\text{L}_{\text{D}}\)-transpeptidases (LDts), in \(\beta\)-lactam-resistant mutants of \textit{Enterococcus faecium} selected in vitro [5]. LD-transpeptidases were subsequently identified as the main peptidoglycan cross-linking enzymes in wild-type strains of \textit{Mycobacterium tuberculosis} [6], \textit{Myxobacterium abscessus} [7], and \textit{Clastidium difficile} [8].

The enzymes generate 3→3 cross-links as they cleave the peptide bond connecting the 3\(^{\text{rd}}\) and 4\(^{\text{th}}\) residues of the acyl donor and link the carbonyl of the 4\(^{\text{th}}\) residue to the acceptor [9].

Classical PBP s and LD-transpeptidases (LDts) use different acyl donor substrates (stem pentapeptide versus tetrapeptide, respectively) and cleave peptide bonds of D- D and L- D configurations (D-Ala\(^4\)-D-Ala\(^5\) versus the L-Lys\(^5\)-D-Ala\(^4\), respectively) [9]. These differences were initially proposed to account for the lack of inhibition of \(E. \text{faecium}\) LD-transpeptidase by ampicillin because of the aforementioned structural analogy between the \(\beta\)-lactam scaffold and the D-Ala\(^4\)-D-Ala\(^5\) extermity of peptidoglycan precursors [5]. However, this explanation has been challenged by further analyses that unexpectedly revealed in vitro inactivation of Ldtfm by \(\beta\)-lactams of the carbenem class such as imipenem [10]. Activation of the LD-transpeptidation pathway in \(E. \text{faecium}\) resulted in high-level resistance to \(\beta\)-lactams of the penam class, with a minimal inhibitory concentration of ampicillin (MIC) greater than 2,000 \(\mu\text{g/ml}\). In contrast, carbenepens are active at low concentrations ([imipenem MIC = 0.5 \(\mu\text{g/ml}\), whereas \(\beta\)-lactams of the cephem class have a low residual activity (ceftriaxone MIC = 128 \(\mu\text{g/ml}\)). The molecular basis for this >4,000-fold difference in antibiotic activity is not understood.

Here we have developed novel assays to investigate the lack of significant inhibition of Ldtfm by penams. Several possibilities have been envisaged including low affinity for the drug, slow acylation, and hydrolysis of the acylenzyme that can account, alone or in combination, for inefficient target inactivation.

**Materials and Methods**

**Chemicals**

Imipenem was a gift from Merck. Ceftriaxone and ampicillin were purchased from Teva and Euromedex, respectively.

**Production and Purification of Ldtfm**

We have previously described the construction of a derivative of vector pET28l8 encoding domains I and II of Ldtfm (residues 216 to 466) fused to a C-terminal 6-histidine Tag (GSH6) [11]. Since pET28l8 encodes a \(\beta\)-lactamase, the insert encoding recombinant Ldtfm was subcloned into vector pET28a, which confers kanamycin resistance. Ldtfm was produced in \textit{Escherichia coli} BL21 and purified by metal-affinity and size-exclusion chromatographies as previously described [12] except for the presence of kanamycin (50 \(\mu\text{g/ml}\) in the culture medium. Protein concentration was determined by the Bradford method (Biorad Protein Assay) with BSA as a standard.

In order to investigate the affinity of Ldtfm for \(\beta\)-lactams by NMR, we used a recombinant protein of smaller size, which only consisted in the catalytic domain of Ldtfm and harbored a substitution of catalytic Cys by Ala in order to block acylation. Briefly, the pET28a derivative used for protein production encoded an N-terminal polyhistidine tag followed by a TEV protease cleavage site [MHHHHHHHENLYFQGHM] fused to residues 341 to 466 of Ldtfm. Oligonucleotides 5′-ACCCCGGCTTGACACGCGCCCGATCAACACCACAAAG-3′ and 5′-CTTGGTGCGGGGTGGTGGTGGTGGTGTTGCGCGGTGTGAGACC CGGGGT-3′ were used to introduce a Cys to Ala substitution at position 442 by site-directed mutagenesis. The protein was produced and purified as described above except that bacteria were grown in M9 minimal media containing \(^{15}\text{C}\)glucose and \(^{15}\text{NH}_4\text{Cl}.\) The purified protein was cleaved with 6His-labeled TEV protease. The polylhistidine tag [MHHHHHHHENLYFQ] and the TEV protease were removed using NiNTA affinity resin generating recombinant enzyme consisting of residues GHM fused to residues 341 to 466 of Ldtfm.

**Spectrophotometry**

Kinetics were performed at 20°C with a stopped-flow apparatus RX-2000 (Applied Photophysics) coupled to a Cary 100 spectrophotometer (Varian SA) in 100 mM sodium phosphate (pH 6.0). The variation in the molar extinction coefficient resulting from opening of the \(\beta\)-lactam ring of imipenem (7,100 M\(^{-1}\) cm\(^{-1}\) at 299 nm), ceftriaxone (9,600 M\(^{-1}\) cm\(^{-1}\) at 263 nm), and ampicillin (7,000 M\(^{-1}\) cm\(^{-1}\) at 240 nm) were determined after alkaline hydrolysis (imipenem and ampicillin) or enzymatic hydrolysis with \textit{Mycobacterium abscessus} \(\beta\)-lactamase.

**Spectrofluorometry**

Fluorescence kinetic data were acquired with a stopped-flow apparatus RX-2000 (Applied Biophysics) coupled to a spectrofluorometer (Cary Eclipse; Varian) in 100 mM sodium phosphate (pH 6.0) at 20°C. The Trp residues were excited at 224 nm with a 5 nm slit and a 2 nm optical path length. Fluorescence emission was determined at 355 nm with a 5 nm slit and a 10 nm optical path length.

**Mass Spectrometry Analyses**

The formation of drug-enzyme adducts was tested by incubating Ldtfm with \(\beta\)-lactams at 20°C in water. Five microliters of acetonitrile and 1 \(\mu\text{l}\) of 1% formic acid were extemporaneously added, and the reaction mixture was injected directly into the mass spectrometer (Qstar Pulsar I; Applied Biosystem) at a flow rate of 0.05 ml/min (acetonitrile, 50%, water, 49.5%, and formic acid, 0.5%; per volume). Spectra were acquired in the positive mode as previously described [10].

**NMR Titrations**

Increasing molar ratios of ertapenem, ampicillin, and ceftriaxone (up to 2,016, 2,058, and 1,025 equivalents, respectively) were added to a 150 \(\mu\text{M}\) solution of \(^{15}\text{N}\)- and \(^{13}\text{C}\)-labeled Ldtfm C442A catalytic domain in 100 mM sodium phosphate (pH 6.4) containing 300 mM NaCl. Chemical shift perturbations (CSPs) were monitored at 25°C through the comparison of 2D \[^1\text{H},^1\text{N}\]-HSQC spectra recorded at 600 MHz proton frequency and were calculated using the equation 1,

\[
\Delta\delta(ppm) = \sqrt{\left(\Delta\delta_H\right)^2 + \left(\Delta\gamma_N/\gamma_H\right)^2}
\]

where \(\Delta\delta_H\) and \(\Delta\gamma_N\) are the variations of chemical shifts in the proton and nitrogen dimensions, respectively, and \(\gamma_H\) and \(\gamma_N\) are the gyromagnetic ratio of these two nuclei. CSPs were then analyzed to extract structural and thermodynamics binding information. Peaks showing chemical shift changes greater than
0.03 ppm after addition of ca. 500 molar equivalents of each antibiotic were simultaneously used to calculate a dissociation constant ($K_D$) that could be obtained from a non-linear least-square fit with equation 2,

$$\Delta \delta = \frac{\Delta \delta_{\text{max}}}{2} \left[ 1 + \left[ \frac{L}{P} \right]_0 + \frac{K_D}{\left[ P \right]_0} \right] - \sqrt{\left[ 1 + \left[ \frac{L}{P} \right]_0 + \frac{K_D}{\left[ P \right]_0} \right] - 4 \frac{\left[ L \right]_0}{\left[ P \right]_0}}$$

(2)

where $\Delta \delta$ is the chemical shift perturbation at each titration point, $\Delta \delta_{\text{max}}$ is the chemical shift difference between the free and bound forms of the protein, and $[P]_0$ and $[L]_0$ are the total concentrations of protein and ligand, respectively. Error on the CSP ($d\Delta \delta$) were estimated using equation 3,

$$d\Delta \delta_{\text{ppm}} = \frac{1}{2\Delta \delta} \left[ 2\Delta \delta_{H}d\delta_{H} + 2 \left( \frac{\Delta \delta_{N}}{\Delta \delta_{H}} \right)^2 \Delta \delta_{N} d\delta_{N} \right]$$

(3)

where $d\delta_{H}$ and $d\delta_{N}$ are the estimated absolute values of the errors committed on the determination of chemical shifts in the $^1$H and $^{15}$N dimensions, respectively (here $d\delta_{H}=0.005$ ppm and $d\delta_{N}=0.02$ ppm).

**Results**

Ldtfm Displays Similar Low Affinity for β-lactams of the Carbapenem (Imipenem and Ertapenem), Cephem (Ceftriaxone), and Penam (Ampicillin) Classes

In order to evaluate non-covalent binding of the drugs to Ldtfm, we blocked the acylation step of the reaction by replacing the catalytic cysteine by alanine. The Ldtfm variant harboring the C442A substitution was totally inert when incubated with carbapenems, cephems, and penams since representatives of these classes could not be detected by mass spectrometry. The C442A substitution was totally inert when incubated with three carbapenems, cephems, and penams since representatives of these classes could not account for differences in the antibacterial activity of these classes.

Acylation of Ldtfm by β-lactams Results in the Formation of Various Adducts

Acylation of Ldtfm by imipenem, ceftriaxone, and ampicillin was investigated by mass spectrometry (Table 1) and the deduced reaction schemes are presented in Fig. 2. As previously described [10], incubation of Ldtfm with imipenem resulted in the formation of a single adduct, EI*, with a mass increment corresponding to the mass of the antibiotic. This adduct is generated by formation of a thioester bond between the sulphydryl group of the Ldtfm active-site cysteine and the carbonyl group of the imipenem β-lactam ring (Fig. 2A) [10]. The mass of the acylenzyme obtained with ceftriaxone (EI*) indicated that a portion of one of the two drug side chains was lost upon acylation (Table 1 and Fig. 2B). Acylation of the active-site cysteine and loss of the drug side chain may occur in a single step since evidence for formation of the complete acylenzyme was not obtained. The same inactivation scheme was recently reported for inactivation of L,D-transpeptidase LdtM from M. tuberculosis [13]. The Ldtfm variant form two acylenzymes with ampicillin (EI* and EII*, Fig. 2C). The mass of EII* corresponds to the complete acylenzyme as found for imipenem. The second acylenzyme, EI**, was generated by additional cleavage of the C–C ester bond of the β-lactam ring (Fig. 2C). Thus, ampicillin covalently binds to Ldtfm although this does not lead to antibacterial activity.

Partial Acylation of Ldtfm by Ceftriaxone and Ampicillin

Kinetics of Ldtfm acylation were analyzed by mass spectrometry (Fig. 3). Acylation of Ldtfm by imipenem was too rapid to be kinetically analyzed as acylenzyme EI* was the only Ldtfm detectable form after 0.3 min of incubation (Fig. 3A). Acylation of Ldtfm by ceftriaxone was slower (Fig. 3B). After 5 min, acylenzyme EI* reached a maximum, ca. 88% of total enzyme based on peak height, and this percentage remained stable between 5 and 20 min. The mass deduced from the remaining peaks (12%) corresponded to the mass of the apoenzyme. These peaks may originate from the presence of both free enzyme (E) and the oxanion (EI**) in the reaction mixture since EI** may dissociate upon injection in the mass spectrometer and electrospray ionization. For ampicillin, the relative abundance of acylenzymes EI* and EII** reached equilibrium at 5 min (Fig. 3C). The two acylated forms accounted for ca. 56% of total enzyme. Kinetics indicated that EI* is an intermediate in the formation of EII**, as indicated in Fig. 2C, since the two enzyme forms accumulated sequentially.

A second assay was developed to independently evaluate the extent of Ldtfm acylation by ampicillin. The assay relies on rapid acylation of the Ldtfm free form by imipenem, which results in an absorbance decrease at 299 nm due to rupture of the carbapenem β-lactam ring. Ldtfm (20 μM) was incubated with ampicillin (200 μM) and the enzyme free form was titrated at various time intervals (0 to 500 min) based on addition of imipenem to reaction samples. The concentration of free Ldtfm decreased during the first 6 min of incubation with ampicillin to reach 52% of total enzyme (Fig. 4A). Thereafter, the concentration of free enzyme remained stable for 100 min indicating that the different enzyme forms were in equilibrium. The concentration of free Ldtfm, at equilibrium, was determined for various ampicillin concentrations using the same
Acylenzyme Hydrolysis Accounts for Partial Acylation of Ldtfm by Ceftriaxone and Ampicillin

Acylenzyme stability was evaluated by determining the rate of hydrolysis of imipenem, ceftriaxone, and ampicillin by Ldtfm (Fig. 5). Enzyme turnover was not detected with imipenem (<4 × 10^{-4} min^{-1}) indicating that the acylenzyme formed with this drug is stable. Ceftriaxone was slowly hydrolyzed by Ldtfm and the turnover number did not vary with the drug concentration (0.027 ± 0.003 min^{-1}). The rate of ampicillin hydrolysis increased with the drug concentration in the 25 to 1,200 μM range. The maximum turnover number was 0.18 ± 0.01 min^{-1} and half of this value was reached at an ampicillin concentration of 370 ± 30 μM. The turnover number was higher for ampicillin than for imipenem and ceftriaxone, at least 450 and 6.7 fold, respectively, if a saturating concentration of ampicillin is considered for the comparisons. These results indicate that acylenzyme hydrolysis accounts for the partial acylation of Ldtfm detected by mass spectrometry (Fig. 3) and titration with imipenem (Fig. 4). Equal rates of Ldtfm acylation and acylenzyme hydrolysis lead to equilibrium between the various enzyme forms, which may include sufficient active L,D-transpeptidase to prevent inhibition of peptidoglycan cross-linking by ceftriaxone and ampicillin in vivo.

Slow Acylation of Ldtfm by Ceftriaxone and Ampicillin Contributes to Partial Enzyme Inactivation

Ldtfm inactivation by β-lactams was investigated by stopped-flow fluorescence spectroscopy as previously described [12]. Progress curves obtained with imipenem were biphasic (Fig. 6A). The initial rapid fluorescence quenching of Ldtfm Trp residues results from reversible binding of the drug to the enzyme. Formation of the acylenzyme subsequently leads to a fluorescence increase as quenching is less important for EI* than for EI++. This biphasic behavior was used to determine the association rate constant k_1 (359 ± 4 mM^{-1} min^{-1}) and the rate constant of the chemical step of the reaction k_2 (11.8 ± 0.1 min^{-1}), whereas k_{-1} was too low (<0.1 min^{-1}) to be determined (Fig. 6B). These values, obtained at 20°C, were slightly higher than those previously reported for assays performed at 10°C [12] (k_1 = 65 mM^{-1} min^{-1}; k_2 = 4.3 min^{-1}; k_{-1} <0.1 min^{-1}).

Since kinetics of fluorescence quenching were monophasic for ceftriaxone (Fig. 6C) and ampicillin (Fig. 6D), rate constants for binding (k_1) and inactivation (k_2) could not be determined. Fluorescence decreases were fitted to exponential decays and the resulting rate constants (k_{\text{inva}}) were determined for various drug concentrations (Fig. 6E). The rate constant k_{\text{inva}} increased linearly with the drug concentration and the slope was used as an estimate of the overall efficiency of the acylation reaction (Fig. 6F), as previously described [13]. Based on these estimates, ceftriaxone and ampicillin acylated Ldtfm 150 and 840 fold less efficiently than imipenem.

Discussion

We have previously showed that activation of a cryptic locus encoding a D,D-carboxypeptidase is the key event that results in activation of the L,D-transpeptidation pathway and high-level
resistance to ampicillin in E. faecium [14]. The D,D-carboxypeptidase generates the tetrapeptide substrate of the D,L-transpeptidase resulting in mutants that rely exclusively on Ldtfm for peptidoglycan cross-linking [5,10]. Bypass of PBPs by Ldtfm results in high-level resistance to ampicillin (MIC >2,000 μg/ml) and moderate resistance to ceftriaxone (MIC =128 μg/ml) whereas imipenem remains active (MIC =0.5 μg/ml) [10]. The latter drug was found to inactivate Ldtfm in vitro and to block peptidoglycan cross-linking by this enzyme in vivo [10]. Here we show that the three drugs acylate Ldtfm in vitro (Fig. 3) despite the difference in antibacterial activity.

Two approaches identified partial Ldtfm inactivation at all ampicillin concentrations as the basis for the lack of antibacterial activity. First, mass spectrometry indicated that only one half of the enzyme was acylated by ampicillin at 200 μM, a proportion that remained stable upon prolonged incubation (Fig. 3). Second, partial inactivation was detected using an independent assay based on determination of the proportion of Ldtfm that remained able to react with imipenem (Fig. 4). Even at high ampicillin concentration, Ldtfm inactivation was incomplete leading to the persistence of functional enzyme, peptidoglycan cross linking, and high-level drug resistance. The lack of full enzyme inactivation resulted from a combination of acyl enzyme instability (Fig. 5) and slow enzyme acylation (Fig. 6). For ceftriaxone, higher acylenzyme stability and acylation rate account for residual activity. Conversely, the excellent antibacterial activity of ampicillin results both from the absence of detectable acyl enzyme hydrolysis and efficient acylation.

Multidimensional NMR spectroscopy revealed similar high $k_D$ values for non-covalent binding of ertapenem (50 mM), ceftriaxone (44 mM), and ampicillin (79 mM) to LdtfmC442A, which cannot be acylated (Fig. 1). These results indicate that the substrate specificity of Ldtfm for β-lactams is not determined at a non-covalent binding step of the reaction that would precede nucleophilic attack of the β-lactam carbonyl by the active-site cysteine. The ceftriaxone and ampicillin concentrations for half saturation of the β-lactamase activity of Ldtfm were $<0.025$ and $0.37 \pm 0.03$ mM, respectively (Fig. 5). These values are $2 \times 3$ orders of magnitudes lower that the $k_D$ values determined by NMR for LdtfmC442A (Fig. 1). Thus, incubation of Ldtfm with submicromolar concentrations of β-lactams, as used for kinetic analyses, cannot result in significant accumulation of a non-covalent complex. The saturable step observed in kinetic analyses of β-lactamase activity was therefore assigned to irreversible formation of the oxyanion (Fig. 2).

**Table 1. Average mass of acylenzymes (mass increment)**

| β-lactam (average mass) | EI*     | EI**     |
|-------------------------|---------|----------|
| Imipenem (299.4)        | 29,309.7 (300.4) | NA       |
| Ceftriaxone (554.6)     | 29,406.2 (396.9) | NA       |
| Ampicillin (349.4)      | 29,359.2 (349.9) | 29,200.6 (191.3) |

The mass increment was calculated by subtracting the mass of the native enzyme (29,009.3) from the mass of acylenzymes. NA, not applicable.

doi:10.1371/journal.pone.0067831.t001

In order to identify the limiting step in acylation of Ldtfm by ampicillin, data generated by mass spectrometry (Fig. 3) and titration with imipenem (Fig. 4) were combined to roughly estimate the relative concentrations of the four enzyme forms (E, EI*, oxyanion; EI* and EI**), acylenzymes. Sh, sulphhydryl of the catalytic cysteine.

doi:10.1371/journal.pone.0067831.g002

Figure 2. Inactivation of E. faecium D,L-transpeptidase (Ldtfm) by β-lactams. Reaction schemes for Ldtfm inactivation by β-lactams of the carbapenem (imipenem), cephem (ceftriaxone), and penam (ampicillin) classes. E, free form of the enzyme; EI*, oxyanion; EI* and EI**, acylenzymes. Sh, sulphhydryl of the catalytic cysteine.

**Table 2.** Average mass of acylenzymes (mass increment).
irreversibly inactivates the M. tuberculosis β-lactamase [18,19] have raised considerable interest in using carbapenems for treatment of extensively drug resistant tuberculosis [19–21]. Development of an oral drug is needed for tuberculosis therapy as approved carbapenems are only administrable by the parenteral route, which is not broadly applicable in clinical settings in which

![Figure 3](image-url)  
**Figure 3.** Mass spectrometry analysis of kinetics of Ldtfm inactivation by β-lactams. Ldtfm (20 μM) was incubated with 200 μM of β-lactams. Left panels, representative mass spectra obtained after 0.3, 5, and 10 min of incubation of Ldtfm with indicated β-lactams. Pair of peaks labeled with the same letter are [M+32H]^{32+} and [M+31H]^{31+} ions. (A) imipenem, peaks a and a' at m/z 916.93 and 946.48 correspond to acylenzyme EI*. (B) Ceftriaxone, peaks a and a' at m/z 907.58 and 936.84 correspond to free enzyme. Peaks b and b' at m/z 919.95 and 946.60 correspond to acylenzyme EI*. (C) Ampicillin, peaks a and a' (m/z 907.52 and 936.79), b and b' (m/z 918.52 and 948.08), and c and c' (m/z 913.51 and 942.95) correspond to free enzyme, EI*, and EI**, respectively. Right panels, kinetics of Ldtfm-β-lactam adducts formation. Relative intensities were deduced from peak heights. Blue diamond, free enzyme; Red square EI*; Green triangle EI**.

![Figure 4](image-url)  
**Figure 4.** Determination of ampicillin-free Ldtfm using rapid inactivation by imipenem. (A) Ldtfm (20 μM) was incubated with ampicillin (200 μM) for indicated time and imipenem was used to determine the concentration of free enzyme by using stopped-flow spectrophotometry at 299 nm. The concentration of free Ldtfm reached a plateau revealing equilibrium between the various enzyme forms. The concentration of free Ldtfm slowly increased after 100 min due to a decrease in ampicillin concentration. (B) Concentration of free Ldtfm at equilibrium as a function of ampicillin concentration. Data are mean ± SD of 3 experiments.

doi:10.1371/journal.pone.0067831.g003
doi:10.1371/journal.pone.0067831.g004
Figure 5. Determination of turnover numbers for full catalytic cycles leading to hydrolysis of β-lactams by Ldt<sub>tm</sub>. Turnover numbers were determined for hydrolysis of ceftriaxone (A) and ampicillin (B) by Ldt<sub>tm</sub> (5 μM).

doi:10.1371/journal.pone.0067831.g005

Figure 6. Kinetics of Ldt<sub>tm</sub> inactivation by imipenem, ceftriaxone, and ampicillin. Fluorescence kinetic data were acquired with a stopped-flow apparatus. Trp residues of Ldt<sub>tm</sub> were excited at 224 nm and fluorescence emission was determined at 335 nm to monitor quenching upon β-lactam binding. Kinetics were biphasic for imipenem (A) providing estimates of catalytic constants k<sub>1</sub>, k<sub>-1</sub>, and k<sub>2</sub>(B). See Supplementary methods in File S1 for the iterative fitting method and Supplementary Fig. S2 in File S1 for the complete set of data. Monophasic fluorescence decreases observed for ceftriaxone (C) and ampicillin (D) were fitted to exponential decays (representative plots are shown). Regression analysis was performed with equation F<sub>t</sub> = F<sub>eq</sub> + ΔF e<sup>-k<sub>obs</sub>t</sup> in which F<sub>eq</sub> and F<sub>t</sub> are the fluorescence intensities at equilibrium and at time t, respectively, ΔF is the difference between fluorescence intensity at time = 0 and at equilibrium, t is time, and k<sub>obs</sub> is a constant. The resulting rate constants (k<sub>obs</sub>) increased linearly with the drug concentration (E) and the slope provided an estimate of the efficiency of enzyme acylation (F).

doi:10.1371/journal.pone.0067831.g006
extensively drug-resistant tuberculosis is prevalent [20,22]. Our analysis has revealed key features of efficient β-lactams that will be critical for drug development, namely oxygenation stabilization, which was identified as the limiting step for efficient acylation, and absence of acylenzyme hydrolysis, which was identified as essential for full enzyme inactivation. The former feature mainly depends upon the reactivity of the β-lactam ring, which appears to be determined by the conjugated ring rather than by the drug side chains, as previously concluded from NMR-based analyses of the structure and dynamics of the model L,D-transpeptidase from Bacillus subtilis [23] and Enterococcus faecium [24]. The latter feature is modulated by secondary catalytic reactions that lead to elimination of a portion of the drug molecules prior to hydrolysis of the thioester bond. These kinetic analyses in combination with recent determination of the structures of E. faecium LdtM1 [24] and M. tuberculosis LdtM2 [25,26] acylated by a carbapenem or in complex with a peptidoglycan fragment [13] will pave the way for optimization of the β-lactam scaffold.

**Supporting Information**

File S1 (DOCX)

**Author Contributions**

Conceived and designed the experiments: ST VD LL CB ME-Q J-EH J-PS MA. Performed the experiments: ST VD LL CB J-EH AM LD. Analyzed the data: ST VD LL CB J-LM LBR ME-Q LG J-EH J-PS MA. Wrote the paper: ST VD CB ME-Q J-EH J-PS MA.

**References**

1. Zapun A, Contreras-Marel C, Vernet T (2008) Penicillin-binding proteins and beta-lactam resistance. FEMS Microbiol Rev 32: 361–385.
2. Sauvage E, Keff F, Terrak M, Ayala JA, Chartier F (2008) The penicillin-binding proteins: structure and role in peptidoglycan biosynthesis. FEMS Microbiol Rev 32: 234–250.
3. Tüpper DJ, Strominger JL (1965) Mechanism of action of penicillins: a proposal based on their structural similarity to acyl-D-alanyl-D-alanine. Proc Natl Acad Sci U S A 54: 1133–1141.
4. Mainardi JL, Villet R, Bugg TD, Mayer C, Arthur M (2008) Evolution of peptidoglycan biosynthesis under the selective pressure of antibiotics in Gram-positive bacteria. FEMS Microbiol Rev 32: 380–408.
5. Mainardi JL, Legrand R, Arthur M, Schoot B, van Heijenoort J, et al. (2000) Novel mechanism of beta-lactam resistance due to bypass of DD-transpeptidation in Enterococcus faecium. J Biol Chem 275: 16490–16496.
6. Lavialle M, Arthur M, Fourgeaud M, Dubost I, Marie A, et al. (2008) The peptidoglycan of stationary-phase Mycobacterium tuberculosis predominantly contains cross-links generated by L,D-transpeptidation. J Bacteriol 190: 4360–4366.
7. Lavialle M, Fourgeaud M, Herrmann JL, Dubost I, Marie A, et al. (2011) The peptidoglycan of Mycobacterium bovis bacille Calmette-Guérin is predominantly cross-linked by L,D-transpeptidases. J Bacteriol 193: 776–782.
8. Pellet J, Courtois P, El Messaoudi I, Lecerf M, Chaop-Chartier MP, et al. (2011) Clostridium difficile has an original peptidoglycan structure with a high level of N-acetylglucosamine deacetylation and mainly 3-3 cross-links. J Biol Chem 286: 29053–29062.
9. Mainardi JL, Fourgeaud M, Hugonnet JE, Dubost I, Brouard JP, et al. (2005) A novel peptidoglycan cross-linking enzyme for a beta-lactam-resistant transpeptidation pathway. J Biol Chem 280: 38146–38152.
10. Mainardi JL, Hugonnet JE, Rousset F, Fourgeaud M, Dubost I, et al. (2007) Unexpected inhibition of peptidoglycan L,D-transpeptidase from Enterococcus faecium by the beta-lactam imipenem. J Biol Chem 282: 30414–30422.
11. Briarrot-Sorn S, Hugonnet JE, Delvoise V, Mainardi JL, Gutmann L, et al. (2006) Crystal structure of a novel beta-lactam-insensitive peptidoglycan transpeptidase. J Mol Biol 359: 533–538.
12. Triboutel S, Arthur M, Mainardi JL, Veckerle C, Dubessy V, et al. (2011) Inactivation kinetics of a new target of beta-lactam antibiotics. J Biol Chem 286: 22777–22784.
13. Delly V, Triboutel S, Mainardi JL, Ezélie-Quagateju M, Marie A, et al. (2012) Inactivation of Mycobacterium tuberculosis L,D-transpeptidase LdhMt by carbapenems and cephalosporins. Antimicrob Agents Chemother 56: 4189–4195.
14. Mainardi JL, Morel V, Fourgeaud M, Cremminter J, Blanot D, et al. (2002) Balance between two transpeptidation mechanisms determines the expression of beta-lactam resistance in Enterococcus faecium. J Biol Chem 277: 33801–33807.
15. Erdemli SB, Gupta R, Bishai WR, Lamischhane G, Amzel LM, et al. (2012) Targeting the cell wall of Mycobacterium tuberculosis: structure and mechanism of L,D-transpeptidase 2. Structure 20: 2103–2115.
16. Free J, Ghuyes J, Vanderhaeghe H, Adriani P, Degele JG, et al. (1976) Fate of thiazolidine ring during fragmentation of penicillin by exocellular DD-carboxypeptidase-transpeptidase of Staphylococcus R61. Nature 260: 451–454.
17. Marquet A, Free J, Ghuyes JM, Loffet A (1979) Effects of nucleophiles on the breakdown of the benzylpenicilloyl-enzyme complex EI formed between benzylpenicillin and the exocellular DD-carboxypeptidase-transpeptidase of Staphylococcus strain R61. Biochem J 177: 909–916.
18. Hugonnet JE, Blanchard JS (2005) Irreversible inhibition of the Mycobacterium tuberculosis beta-lactamase by clavulanate. Biochemistry 44: 11998–12004.
19. Hugonnet JE, Trensblay LV, Bodhoff HJ, Barry CE, Ihrle P, Blanchard JS (2009) Meropenem-clavulanate is effective against extensively drug-resistant Mycobacterium tuberculosis. Science 323: 1215–1218.
20. Mainardi JL, Hugonnet JE, Gutmann L, Arthur M (2011) Fighting resistant tuberculosis with old compounds: the carbapenem paradigm. Clin Microbiol Infect 17: 1755–1756.
21. Gupta R, Lavialle M, Mainardi JL, Arthur M, Bishai WR, et al. (2010) The Mycobacterium tuberculosis protein LdhM2 is a nonclassical transpeptidase required for virulence and resistance to amoxicillin. Nat Med 16: 466–469.
22. Anonymous. (2012) Tuberculosis 2011 WHO report.
23. Lecoq L, Bougault C, Hugonnet JE, Veckerle C, Pessey O, et al. (2012) Dynamics induced by beta-lactam antibiotics in the active site of Bacillus subtilis L,D-transpeptidase. Structure 20: 850–861.
24. Lecoq I, Dubessy V, Triboutel S, Bougault C, Hugonnet JE, et al. (2013) Structure of Enterococcus faecium L,D-transpeptidase acylated by etrapenem provides insight into the inactivation mechanism. ACS Chem Biol. In press DOI: 10.1021/cb4001603.
25. Kim HS, Kim J, Im HN, Youn JY, An DR, et al. (2013) Structural basis for the inhibition of Mycobacterium tuberculosis L,D-transpeptidase by meropenem, a drug effective against extensively drug-resistant strains. Acta Crystallogr D Biol Crystallogr 69: 420–431.
26. Li WJ, Li DF, Hu YL, Zhang XE, Bi LJ, et al. (2013) Crystal structure of L,D-transpeptidase LdhMt in complex with meropenem reveals the mechanism of carbapenem against Mycobacterium tuberculosis. Cell Res 23: 726–731.