Unexpected Inhibition of Peptidoglycan LD-Transpeptidase from Enterococcus faecium by the β-Lactam Imipenem*

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The β-lactam antibiotics mimic the d-alanyl4-d-alanine5 extremity of peptidoglycan precursors and act as “suicide” substrates of the DD-transpeptidases that catalyze the last cross-linking step of peptidoglycan synthesis. We have previously shown that bypass of the DD-transpeptidases by the LD-transpeptidase of Enterococcus faecium (Ldtfm) leads to high level resistance to ampicillin. Ldtfm is specific for the l-lysyl3-d-alanine4 bond of peptidoglycan precursors containing a tetrapeptide stem lacking d-alanine5. This specificity was proposed to account for resistance, because the substrate of Ldtfm does not mimic β-lactams in contrast to the d-alanyl4-d-alanine5 extremity of pentapeptide stems used by the DD-transpeptidases. Here, we unexpectedly show that imipenem, a β-lactam antibiotic, totally inhibited Ldtfm at a low drug concentration that was sufficient to inhibit growth of the bacteria. Peptidoglycan cross-linking was also inhibited, indicating that Ldtfm is the in vivo target of imipenem. Stoichiometric and covalent modification of Ldtfm by imipenem was detected by mass spectrometry. The modification was mapped into the trypsin fragment of Ldtfm containing the catalytic Cys residue, and the Cys to Ala substitution prevented imipenem binding. The mass increment matched the mass of imipenem, indicating that inactivation of Ldtfm is likely to involve rupture of the β-lactam ring and acylation of the catalytic Cys residue. Thus, the spectrum of activity of β-lactams is not restricted to transpeptidases of the DD-specificity, as previously thought. Combination therapy with imipenem and ampicillin could therefore be active against E. faecium strains having the dual capacity to manufacture peptidoglycan with transpeptidases of the LD- and DD-specificities.

The peptidoglycan of Enterococcus faecium is generated by polymerization of a disaccharide-peptide subunit composed of β1–4-linked N-acetylglucosamine and N-acetylmuramic acid, a linear stem pentapeptide (L-Ala1-D-iGln2-L-Lys3-D-Ala4-D-Ala5) linked to the lactoyl group of N-acetylmuramic acid by an amide bond, and a side-chain d-isooasparagynyl or d-isooaspartyl (d-iAsx5) residue linked to the e amino group of l-Lys3 (1, 2). The final steps of peptidoglycan synthesis involve polymerization of the glycan strands by glycosyltransferases (3) and cross-linking of peptide stems by DD-transpeptidases (4). The peptide bond formed by the latter enzyme links the carbonyl of d-Ala4 to the amino group of d-iAsx. The two-step reaction for the formation of these d-Ala4–d-iAsx–l-Lys3 cross-links starts by the nucelophilic attack of the carbonyl of d-Ala4 of the acyl donor substrate by the γ oxygen of the catalytic serine leading to the release of d-Ala5 and to the formation of a covalent adduct (acyl enzyme) (5, 6). In the second step, the carbonyl of d-Ala4 is attacked by the α amino group of d-iAsx of the acceptor substrate leading to formation of the β-Ala4–d-iAsx–l-Lys3 cross-link and to the release of the DD-transpeptidase. The β-lactam antibiotics are structural analogues of the d-Ala4–d-Ala5 extremity of the pentapeptide stem and act as “suicide” substrates of the DD-transpeptidases (see Fig. 1). Opening of the β-lactam ring, which mimics the rupture of the d-Ala4–d-Ala5 peptide bond in the first step of the DD-transpeptidation reaction, leads to inactivation of the DD-transpeptidases. Because the acyl enzymes typically have half-lives in the order of several hours, inactivation of the DD-transpeptidases by β-lactams is essentially irreversible in the scale of the generation time of bacteria. The DD-transpeptidases, which are highly redundant enzymes, are collectively the killing target of β-lactams, because peptidoglycan cross-linking is essential to the integrity of the cell wall. These enzymes belong to a large family of active-site serine acyltransferases that bind penicillin covalently and are thus referred to as penicillin-binding proteins (PBPs) (4).

The β-lactams are one of the oldest and still the most broadly used class of antibiotics for the treatment of severe infections despite the development of several resistance mechanisms.

The abbreviations used are: d-iAsx, d-isooasparagynyl or d-isooaspartyl; PBP, penicillin-binding protein; MIC, minimum inhibitory concentration; rp-HPLC, reversed-phase high-performance liquid chromatography; Ldtfm LD-transpeptidase from E. faecium.

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including the enzymatic detoxification of the drug by β-lactamases (7), the modification of the transport of the antibiotic in Gram-negative bacteria (8), and modifications of the target, the DD-transpeptidases, that result in reduced acylation of these enzymes by β-lactams (4). In E. faecium, intrinsic resistance to moderate levels of ampicillin involves the production of one such low affinity PBP, designated PBPSfm (9, 10). Acquisition of higher levels of resistance to this drug in clinical isolates results from overproduction of PBPSfm and from amino acid substitutions in the DD-transpeptidase domain of the protein that further decrease the interaction of the protein with ampicillin (11, 12). We have previously described a novel mechanism of β-lactam resistance in a mutant of E. faecium selected in vitro in five consecutive steps on increasing concentrations of ampicillin (13, 14). Analysis of the peptidoglycan structure of the resulting mutant, E. faecium M512, indicated that ampicillin resistance was due to target substitution, because the D-Ala4→D-iAsx-L-Lys3 cross-links generated by the DD-transpeptidases (PBPs) were replaced by L-Lys3→D-iAsx-L-Lys3 cross-links generated by an LD-transpeptidase (13). The “LD” designation refers to cleavage of a peptide bond between amino acids of the L and D configurations prior to peptide bond formation, as opposed to DD-transpeptidases (PBPs) that act on the D-Ala4-D-Ala5 bond of the donor (Fig. 1A). The DD- and LD-transpeptidases use the same amino group in the acyl acceptor for peptide bond formation, corresponding to the α-amino group of D-iAsx in E. faecium (15).

The LD-transpeptidase from E. faecium, Ldtfm, is the first functionally characterized representative of a conserved family of active site cysteine peptidase (16, 17) that include enzymes involved in peptidoglycan cross-linking (15) and in the anchoring of lipoproteins to the peptidoglycan of Escherichia coli (18). Ldtfm exclusively uses donor substrate carrying a tetrapeptide stem (16). Production of a DD-carboxypeptidase, which cleaves the D-Ala4-D-Ala5 peptide bond of peptidoglycan precursors, is critical to the activation of the alternate pathway of transpeptidation, because it provides the essential donor substrate of Ldtfm (14, 16). Full elimination of pentapeptide stems ending in D-Ala5 by this DD-carboxypeptidase leads to high level cross-resistance to a second family of antibiotics, the glycopeptides, that bind to the peptidyl-D-Ala4-D-Ala5 extremity of peptidoglycan precursors (19).

The β-lactam antibiotics are thought to have a unique type of target consisting of members of the active-site serine acyltransferase family of proteins characterized by a common fold and the presence of conserved amino acid motifs, SXXK, SXN (or analogue), and KTG (or analogue) (4). Because Ldtfm functions on a different peptide bond (L-Lys3→D-iAsx-L-Lys3, respectively).
Ldt<sub>fm</sub>, LD-Transpeptidase Inhibition by Imipenem

D-Ala<sup>4</sup> instead of D-Ala<sup>4</sup>·D-Ala<sup>5</sup>) and is structurally unrelated to the PBPs (16, 17), it was expected that the lack of inhibition by β-lactams, as shown for ampicillin (16), should also concern all drugs of this family. In this report, we have examined this notion in a critical manner and unexpectedly found that a specific class of β-lactams, the carbapenems (Fig. 1D), is highly active against the mutant M512. We show that Ldt<sub>fm</sub> is the target of the carbapenems in vivo and that these drugs inactivate the enzyme by acylation of the catalytic cysteine. These results extend the diversity of the targets of β-lactams to include active-site cysteine peptidase of the LD-specificity.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Growth Conditions, and β-Lactam Susceptibility Testing—**E. faecium D344S derives from D344, a clinical isolate of E. faecium (10), by a spontaneous deletion of pbp5 encoding the low affinity PBP5. E. faecium M512 is a spontaneous mutant of D344S obtained by five serial selection steps on agar containing increasing concentrations of ampicillin (14). All cultures were performed at 37 °C in brain heart infusion broth or agar (Difco Laboratories). Antibiotic susceptibility was tested by the disk-diffusion assay for representatives of the four main classes of β-lactams, including penams (penicillin, ampicillin, amoxicillin, amoxicillin plus clavulane, piperacillin, and oxacillin), cephs (cefamandole, cefepime, cefixime, cefoperazone, cefotiam, cefoxitin, cefsludin, cefzadime, ceftriaxone, and cefuroxime), monobactams (aztreonam), and carbapenems (imipenem, meropenem, and ertapenem). Minimal inhibitory concentrations (MICs) were determined by the agar dilution method (13) for a representative antibiotic of each class of β-lactams, including penams (penicillin, ampicillin, and oxacillin), cephs (cefamandole, cefepime, and cefuroxime) with 2-fold dilutions of ampicillin, ceftriaxone, and imipenem (1

**Detection of Ldt<sub>fm</sub>-Carbapenem Adducts by Electrospray Mass Spectrometry—**Ldt<sub>fm</sub> (34 µM) was incubated with imipenem (0, 3.4, 6.8, 17, 27.2, and 92 µM) for 1 h at 37 °C in 50 mM Tris-HCl (pH 7.5). The reaction mixture was dialyzed against water for 30 min and conserved at −20 °C. Formation of adducts between Ldt<sub>fm</sub> and meropenem or ertapenem was tested in the same conditions. For electrospray mass spectrometry, 5 µl of the reaction mixture was mixed extemporaneously with 5 µl of acetonitrile and 1 µl of 1% formic acid. The mixture (11 µl) was directly injected into the mass spectrometer (Qstar Pulsar I, Applied Biosystems) using rp-HPLC pumps at a flow rate of 0.05 ml/min (acetonitrile 50%, water 49.5%, formic acid 0.5%, per volume). The data were acquired in the positive mode with a capillary voltage of 5200 V and a declustering potential of 20 V. The mass scan range was from m/z 400–2500, and the scan cycle was 1 s. The average mass of proteins and protein-carbapenem adducts was deduced from a total of nine pairs of peaks obtained in three independent experiments, and results were expressed as means and standard deviations. Digestion with trypsin (Proteomics Grade, Sigma-Aldrich) was performed for 18 h at 37 °C at a trypsin to Ldt<sub>fm</sub> ratio of 0.05 per mass. Fragments of Ldt<sub>fm</sub> were detected by nanospray mass spectrometry.

**RESULTS**

β-Lactam Resistance Profile Resulting from Activation of the LD-Transpeptidation Pathway in E. faecium M512—Because ampicillin was the only β-lactam studied in previous reports (14, 16), antibiotic susceptibility testing was performed with 22
β-lactams belonging to the four main classes of β-lactams by the disk-diffusion assay and with representatives of each class by the agar dilution method (Fig. 2A and data not shown). Unexpectedly, activation of LD-transpeptidation pathway in E. faecium M512 did not result in resistance to imipenem, and the other carbapenems (meropenem and ertapenem) retained substantial residual activity. The mutant M512 was resistant to all other β-lactams, although residual activity was observed for third generation cephalosporins such as ceftriaxone (MIC 128 μg/ml). Finally, M512 remained resistant to the monobactam aztreonam, which has no anti-enterococcal activity (data not shown).

In Vitro Inhibition of the LD-Transpeptidase Activity of Ldt fm by β-Lactams—The effective concentration of imipenem inhibiting Ldt fm by 50% (EC50) was 0.077 ± 0.003 μg/ml, and full inhibition was detected at 0.5 μg/ml (Fig. 2B). Thus, inhibition of Ldt fm by imipenem could account for the antimicrobial activity of this drug (MIC 0.5 μg/ml). The low antibacterial activity of ceftriaxone against M512 (MIC 128 μg/ml) also correlates with inhibition of Ldt fm, because the EC50 of ceftriaxone was 19 ± 7 μg/ml, and complete inhibition of the enzyme was obtained with 200 μg/ml. The EC50 of ampicillin was >3200 μg/ml in agreement with the lack of antibacterial activity of this antibiotic (MIC > 2000 μg/ml).

Ldt fm is the Target of Imipenem in Vivo—Inhibition of the formation of the l-Lys3→D-iAsx-L-Lys3 cross-links by Ldt fm could not be tested in E. faecium M512, because this mutant does not produce low affinity PBPs, and cannot therefore be grown in the presence of imipenem. To circumvent this problem, we introduced in M512 plasmid pAA20, which encodes PBP5 fm and allowed growth of the mutant in the presence of 8 μg/ml of imipenem. In the absence of β-lactam, peptidoglycan manufactured by the resulting strain, M512/pAA20(pbp5 fm), contained both D-Ala3→D-iAsx-L-Lys3 cross-links generated by DD-transpeptidation and l-Lys3→D-iAsx-L-Lys3 cross-links generated by LD-transpeptidation (Table 1 and Fig. 3A). In the presence of ampicillin (128 μg/ml), all multimers contained L-Lys3→D-iAsx-L-Lys3 cross-links generated by Ldt fm, indicating that all PBPs, including PBP5 fm, were inhibited by this high drug concentration (Table 1 and Fig. 3B). In the presence of imipenem (8 μg/ml), cross-links were exclusively generated by the DD-transpeptidase activity of PBP5 fm (Table 1 and Fig. 3C). These results indicate that the susceptibility of M512 to imipenem is due to inhibition of the cross-linking activity of Ldt fm by this antibiotic.

Synergy between Imipenem and Ampicillin—The MIC of ampicillin decreased from >2000 to 16 μg/ml upon addition of a low concentration of imipenem (2 μg/ml). This strong synergistic effect indicates that the dual capacity of E. faecium M512/pAA20(pbp5 fm) to manufacture peptidoglycan containing L-Lys3→D-iAsx-L-Lys3 and D-Ala3→D-iAsx-L-Lys3 cross-links accounts for growth of the mutant in the presence of either ampicillin or imipenem, respectively, because PBP5 fm and Ldt fm provide alternative modes of transpeptidation. However, the combination of ampicillin and imipenem inhibited growth of E. faecium M512/pAA20(pbp5 fm), because neither transpeptidase was functional in the presence of both drugs.

Mechanism of Inhibition of Ldt fm by Carbapenems—Binding of imipenem to Ldt fm was tested with a fixed concentration of enzyme (34 μM) and different drug concentrations by electrospray mass spectrometry (Fig. 4). Formation of an adduct was detected at the lowest concentration of imipenem that was tested (3.4 μM). The relative intensity of the peaks corresponding to the adduct and to the native enzyme matched the molar ratio of imipenem to Ldt fm very closely. Thus, the bulk of the added antibiotic was trapped into the adduct up to the saturation of the protein.

To purify the adduct, Ldt fm was incubated with a 10-fold molar excess of imipenem and purified by size-exclusion chromatography. The adduct and the native protein eluted both as monomers. Electrospray mass spectrometry revealed that the major protein peak corresponded to the protein adduct with only minor
**Ldt₉₉, LD-Transpeptidase Inhibition by Imipenem**

**TABLE 1**

Composition of the peptidoglycan manufactured by *E. faecium* M512/pAA20(pbpS₉₉) in the presence or absence of β-lactams

Ampicillin and imipenem were added to the culture medium at a concentration of 128 and 8 μg/ml to inhibit the LD-transpeptidase activity of the PBPs and the LD-transpeptidase activity of Ldt₉₉, respectively. The lactoyl-peptides purified by rp-HPLC were identified by mass spectrometry and sequenced by tandem mass spectrometry as previously described (20).

| Peak | Proposed structure | Monoisotopic mass | Amounts | Ampicillin | Imipenem | None |
|------|--------------------|-------------------|---------|------------|----------|------|
|      |                    |                   | %       |            |          |      |
| **Monomers** |                |                   |         |            |          |      |
| I    | Tri                | 417.2             | 7.0     | 1.4        | 10.1     |
| II   | Tetra              | 488.3             | 0.5     | 2.2        | 1.2      |
| III  | Tri(Asp)           | 523.3             | 18.4    | 4.5        | 18.8     |
| IV   | Penta              | 559.3             | 1.3     | 2.8        | ND*      |
| V    | Tetra(Asp)         | 603.3             | 10.6    | 3.0        | 8.6      |
| VI   | Penta(Asp)         | 674.3             | 3.7     | 5.4        | ND       |
| **Total** |                |                   | 41.5    | 19.3       | 38.7     |

| **Multimers containing 1-Lys₃→D-Asp→1-Lys₃ cross-links generated by LD-transpeptidation** |
|--------------------------------------------------------------------------------------------|
| 1    | Tri(Asp)/Tri(Asp) | 931.5             | 2.8     | ND         | 0.7      |
| 2    | Tri(Asp)/Tri(Asp) | 1046.5            | 18.7    | 7.2        |
| 3    | Tri(Asp)/Tetra    | 1002.5            | 3.7     | ND         | ND       |
| 4    | Tri(Asp)/Tetra(Asp) | 1117.5         | 6.8     | ND         | ND       |
| 5    | Tri(Asp)/Penta    | 1073.5            | 6.8     | ND         | ND       |
| 6    | Tri(Asp)/Penta(Asp) | 1188.6        | 6.1     | ND         | ND       |
| 7    | Tri(Asp)/Tri(Asp)/Tri(Asp) | 1560.7     | 6.1     | ND         | 2.6      |
| 8    | Tri(Asp)/Tri(Asp)/Tetra | 1516.7     | 3.0     | ND         | 0.6      |
| 9    | Tri(Asp)/Tri(Asp)/Tetra(Asp) | 1631.8     | 2.7     | ND         | 4.2      |
| 10   | Tri(Asp)/Tri(Asp)/Penta(Asp) | 1702.8 | 1.8     | ND         | ND       |
| **Total** |                |                   | 58.5    | 0.0        | 15.3     |

| **Multimers containing D-Ala₄→D-Asp→1-Lys₃ cross-links generated by DD-transpeptidation** |
|------------------------------------------------------------------------------------------|
| A    | Tetra(Asp)/Tri(Asp) | 1002.5            | ND      | 0.9        | 5.5      |
| B    | Tetra(Asp)/Tri(Asp) | 1117.5            | ND      | 23.8       | 18.8     |
| C    | Tetra(Asp)/Tetra    | 1073.5            | ND      | 1.7        | 7.1      |
| D    | Tetra(Asp)/Penta    | 1144.6            | ND      | 0.8        | ND       |
| E    | Tetra(Asp)/Tetra(Asp) | 1188.5         | ND      | 12.0       | 5.5      |
| F    | Tetra(Asp)/Penta(Asp) | 1259.6        | ND      | 11.9       | ND       |
| G    | Tetra(Asp)/Penta(Asp)/Tri(Asp) | 1702.8     | ND      | 24.7       | 9.1      |
| H    | Tetra(Asp)/Tetra(Asp)/Tetra(Asp) | 1773.8     | ND      | 1.6        | ND       |
| I    | Tetra(Asp)/Tetra(Asp)/Penta(Asp) | 1844.9 | ND      | 3.3        | ND       |
| **Total** |                |                   | 0.0     | 80.7       | 46.0     |

*ND, not detected.*

amounts of native protein (relative intensity 7%). The latter peak was not increased upon further incubation of the purified protein adduct for 2, 7, 24, and 48 h. These results indicate that the link between imipenem and Ldt₉₉ is stable in solution.

The average mass of the Ldt₉₉-imipenem adduct matched the average mass of the protein incremented by the average mass of imipenem (Table 2). Adducts matching increments of the average mass of meropenem and ertapenem were also detected. For each of the three carbapenems, binding of a single molecule of drug to Ldt₉₉ was observed up to a drug to protein ratio of 10 that corresponds to the highest ratio that was tested. The observed mass increments are compatible with non-covalent binding of the carbapenems to Ldt₉₉ or with formation of a covalent bond involving opening of the β-lactam ring (Fig. 1E). To distinguish between these two possibilities, Ldt₉₉ was incubated with carbapenems and digested with trypsin, and the fragments were analyzed by nano-spray mass spectrometry. Digestion of Ldt₉₉ was expected to generate 14 fragments greater than three residues that were all detected in the trypsin digest of the native protein. Upon addition of imipenem and meropenem, the fragment containing the catalytic Cys residue of Ldt₉₉ (GSH-GCINTPPSVMK) was replaced by fragments incremented by the monoisotopic mass of the corresponding carbapenems (Table 3). These results establish that imipenem and meropenem bind covalently to the same fragment of Ldt₉₉. The fact that the observed mass increments are equal to the mass of the drugs indicates that formation of the covalent adduct involves opening of the β-lactam ring. Substitution of the catalytic Cys⁴⁴² by Ala (16) totally abolished drug binding. Thus, inactivation of the LD-transpeptidase by carbapenems required a catalytically active protein and is likely to involve formation of a thioester with the catalytic Cys residue (Fig. 1E).

**DISCUSSION**

Bypass of the DD-transpeptidase activity of the PBPs by the LD-transpeptidase activity of Ldt₉₉ was originally detected in an ampicillin-resistant mutant of *E. faecium* designated M512 (13). Activation of the LD-transpeptidation pathway was expected to confer broad spectrum resistance to all β-lactams, because Ldt₉₉ functions with a donor substrate containing a tetrapeptide stem (16) in contrast to the pentapeptide substrate of the PBPs (6) that mimics the β-lactam (22). Unexpectedly, screening of a large panel of β-lactams revealed in this study that carbapenems remain active against M512, although this mutant has the capacity to manufacture peptidoglycan independently from the DD-transpeptidase activity of the PBPs. To explore this intriguing phenotype, we first showed that the LD-transpeptidase activity of Ldt₉₉ is inhibited by low concentrations of imipenem (EC⁵⁰ = 0.077 ± 0.003 μg/ml (Fig. 2)). Ldt₉₉ was fully inhibited *in vitro* by imipenem at a concentration corresponding to the MIC of the drug (0.5 μg/ml) indicating that the LD-transpeptidase activity of Ldt₉₉ could be the target of imipenem *in vivo*. To establish this point, it was necessary to
FIGURE 3. rp-HPLC profiles of lactoyl-peptides from *E. faecium* M512 pNJ2(pbpm).

Peptidoglycan was extracted from bacteria grown in the absence of β-lactam (A) and in presence of ampicillin (128 μg/ml) (B) or imipenem (8 μg/ml) (C). Peptidoglycan was digested with muramidases and treated with ammonium hydroxide, and the resulting lactoyl-peptides were separated by rp-HPLC; mAU, absorbance unit × 10³ at 210 nm. Peaks labeled with roman numbers are uncross-linked monomers. Peaks labeled with letters are multimers generated by the Dd-transpeptidase activity of PBPs. Peaks labeled with Arabic numbers are multimers generated by the LD-transpeptidase activity of Ldtfm. D and E, structure of major dimers generated by Dd- and LD-transpeptidation, respectively.
show that imipenem fully inhibits the formation of the L-Lys$_3$$^3$D-iAsx-L-Lys$_3$ cross-links in the peptidoglycan of E. faecium M512. Toward this aim, we have expressed in M512 the pbp5fm gene encoding low affinity PBP5fm responsible for intrinsic resistance to moderate levels of $\beta$-lactams in natural isolates of E. faecium. PBP5fm acted as a surrogate of Ldtfm in the resulting strain, M512/pAA20(pbpm), because the peptidoglycan manufactured in the presence of imipenem or ampicillin exclusively contained D-Ala$_4$$^3$D-iAsx-L-Lys$_3$ or L-Lys$_3$$^3$D-iAsx-L-Lys$_3$ cross-links, respectively (Table 1 and Fig. 3). Thus, the dual capacity of M512/pAA20(pbpm) to synthesize peptidoglycan with either Ldtfm or PBP5fm as the lone functional transpeptidase allowed to establish that Ldtfm is the target of imipenem in vivo. Interestingly, Ldtfm and PBP5fm conferred resistance to imipenem and ampicillin if either drug was present in the culture medium although M512/pAA20(pbpm) remained susceptible to the combination of imipenem and ampicillin. Combination therapy could therefore be effective in the treatment of bacteria endowed with the alternate modes of transpeptidation.
Investigation of the mechanism of inhibition of Ldtfm by imipenem showed that the enzyme bound a stoichiometric amount of the drug (Fig. 4). The Ldtfm-imipenem adduct was stable, because it was purified by size-exclusion chromatography. Furthermore, bound imipenem could be recovered in a specific tryptic fragment of Ldtfm that contained the catalytic cysteine residue of the enzyme (Cys442). These results also show that imipenem is covalently bound to Ldtfm.

To determine whether formation of the adduct is catalyzed by Ldtfm, imipenem was incubated with the same concentration of enzyme or of a synthetic peptide with the same sequence ([35S]GSHGCFINTPPSVMK[35S]) as the tryptic fragment containing the catalytic Cys442 residue (underlined). Mass spectrometry analyses (data not shown) indicated that full modification of Ldtfm occurred in 30 min, whereas no modification of the synthetic peptide was observed in 60 min under the same conditions. Thus, formation of the covalent bond between Ldtfm and imipenem was catalyzed by the enzyme.

The mass of the Ldtfm-imipenem adduct was equal to the mass of the free antibiotic plus the mass of the free enzyme (Tables 2 and 3). Opening of the β-lactam ring is therefore the most likely mechanism for formation of the covalent adduct (Fig. 1E), because formation of bonds involving amines, hydroxyls, or carboxyls would lead to the elimination of a water molecule. Site-directed mutagenesis identified the γ sulfur atom of the catalytic Cys442 residue as the nucleophile for attack of the carbonyl of the β-lactam ring, because the Cys442 → Ala substitution totally abolished formation of the adduct. Thus, Ldtfm commits suicide by forming a thioester between Cys442 and carbapenems in a reaction similar to the acylation of the active site serine of DD-transpeptidase by β-lactams (4, 5).

Because the initial proposal of Tipper and Strominger (22), β-lactams are regarded as molecular mimics of the N-acetyl-d-alanyl-d-alanine extremity of peptidoglycan precursors, which accounts for the fact that they act as suicide substrates of the DD-transpeptidase module of high molecular weight penicillin-binding proteins. However, this could not be confirmed by direct co-crystallization of the DD-transpeptidases with their natural substrates or with the β-lactams, because co-crystals contained the reaction products (23). For this reason, the analogy is only supported by modeling of the conformation of the two types of substrates (6). As discussed by Goffin and Ghysen (6), the structural analogy relies on the spatial disposition of the carbonyl and carboxylic acid groups, but the C–CON–C–COOH bonds are far from being isosteric in the β-lactams and in the terminal d-Ala5-d-Ala2 dipeptide. Considering the classic alignment of the d-Ala5-d-Ala2 backbone with β-lactams (Fig. 1), the Cα of d-Ala5 at the C terminus of the natural substrate (Fig. 1A) has a d configuration as the analogous carbon atoms of penams (ampicillin, Fig. 1B). The corresponding carbon is not tetrahedral in cephems (ceftriaxone, Fig. 1C) and carbapenems (Fig. 1D). The carbon atoms mimicking the Cα of d-Ala5 have opposite configuration in carbapenems and in all other β-lactams. This chiral center can be superimposed to an amino acid of the l configuration in ampicillin and ceftriaxone but to an amino acid of the d configuration in carbapenems. Despite the configuration inversion in this carbon, the DD-transpeptidase activity of the PBPs is inhibited by all β-lactams, including carbapenems. Thus, for the PBPs, the structural analogy between the suicide substrates (the β-lactams) and the natural substrates (the peptidoglycan precursors ending D-Ala4-D-Ala5) does not depend upon the configuration of the carbon mimicking the α carbon of D-Ala5. For the LD-transpeptidase, the analogy should be analyzed by aligning the β-lactams with the l-Lys3-D-Ala5 backbone. Paradoxically, the Cα of l-Lys3 can be superimposed to an amino acid of the d configuration in carbapenems and of the β configuration in other β-lactams, despite the fact that only the former antibiotics inactivate Ldtfm. In conclusion, the reactivity of β-lactams for transpeptidases of the LD- and DD-specificities is not principally determined by the configuration of carbons in the antibiotics that mimic the Cα carbons in the l-Lys3-D-Ala5-D-Ala5 extremity of peptidoglycan precursors. It is therefore not surprising that imipenem was reported not to inhibit the LD-carboxypeptidase of E. coli (24).

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