Tryptophan fluorescence quenching in β-lactam-interacting proteins is modulated by the structure of intermediates and final products of the acylation reaction

Sébastien Triboulet[a1], Zainab Edoo[a1], Fabrice Compain[a,b], Clément Ourghanlian[a], Adrian Dupuis[a], Vincent Dubée[a], Laetitia Sutterlin[a], Heiner Atze[a], Mélanie Ethève-Quelquejeu[c], Jean-Emmanuel Hugonnet[a2], and Michel Arthur[a2]

[1]Equal contribution
[2]Equal contribution and corresponding authors

[a] Dr. S Triboulet, Ms Z Edoo, Dr. F Compain, Dr. C Ourghanlian, Mr A Dupuis, Dr. V Dubée, Dr. L Sutterlin, Dr. H Atze, Dr. JE Hugonnet, Dr. M Arthur, Sorbonne Université, Sorbonne Paris Cité, Université Paris Descartes, Université Paris Diderot, INSERM, Centre de Recherche des Cordeliers, CRC, F-75006 Paris, France. E-mail: jean-emmanuel.hugonnet@crc.jussieu.fr; michel.arthur@crc.jussieu.fr

[b] Dr. F Compain, Service de Microbiologie, Assistance Publique-Hôpitaux de Paris, Hôpital Européen Georges Pompidou, Paris, France.

[c] Dr. M Ethève-Quelquejeu, Laboratoire de Chimie et de Biochimie Pharmacologiques et Toxicologiques, Université Paris Descartes, UMR 8601, Paris, F-75005 France; CNRS UMR 8601, Paris, F-75006 France.

Key words: acylenzyme, β-lactamase, fluorescence quenching, L,D-transpeptidase, tryptophan fluorescence.

In most bacteria, β-lactam antibiotics inhibit the last cross-linking step of peptidoglycan synthesis by acylation of the active-site Ser of D,D-transpeptidases belonging to the penicillin-binding protein (PBP) family. In mycobacteria, cross-linking is mainly ensured by L,D-transpeptidases (LDTs), which are promising targets for the development of β-lactam-based therapies for multidrug-resistant tuberculosis. For this purpose, fluorescence spectroscopy is used to investigate the efficacy of LDT inactivation by β-lactams but the basis for fluorescence quenching during enzyme acylation remains unknown. In contrast to what has been reported for PBPs, we show here using a model L,D-transpeptidase (Ldt_{fm}) that fluorescence quenching of Trp residues does not depend upon direct hydrophobic interaction between Trp residues and β-lactams. Rather, Trp fluorescence was quenched by the drug covalently bound to the active-site Cys residue of Ldt_{fm}. Fluorescence quenching was not quantitatively determined by the size of the drug and was not specific of the thioester link connecting the β-lactam carbonyl to the catalytic Cys as quenching was also observed for acylation of the active-site Ser of β-lactamase BlaC from *M. tuberculosis*. Fluorescence quenching was extensive for reaction intermediates containing an amine anion and for acylenzymes containing an imine stabilized by mesomeric effect, but not for acylenzymes containing a protonated β-lactam nitrogen. Together, these results indicate that the extent of fluorescence quenching is determined by the status of the β-lactam nitrogen. Thus, fluorescence kinetics can provide information not only on the efficacy of enzyme inactivation but also on the structure of the covalent adducts responsible for enzyme inactivation.

INTRODUCTION

Peptidoglycan is an essential constituent of bacterial cell walls since it prevents cell swelling and lysis by mechanically sustaining the osmotic pressure of the cytoplasm (1). This protective function depends upon synthesis and maintenance during the entire cell cycle of the net-like peptidoglycan macromolecule, which completely surrounds the bacterial cell. Peptidoglycan is made of glycan strands cross-linked by short peptide stems. In most bacteria, the cross-linking step is performed by D,D-transpeptidases, which are the essential targets of β-lactam antibiotics and are often referred to as penicillin-binding proteins (PBPs) (2). In mycobacteria (3, 4) and in *Clostridium difficile* (5, 6), the cross-links found are mainly (70% to 80%) formed by a second class of enzymes, the L,D-transpeptidases (LDTs). Since LDTs are not inhibited by β-lactams belonging to the penam class, such as ampicillin, (7, 8), these enzymes are responsible for high-level resistance to these drugs in mutants of *Enterococcus faecium* (9, 10) and *Escherichia coli* (11) selected in laboratory conditions.

PBPs and LDTs are structurally unrelated (12-15) and proceed through different catalytic mechanisms for activation of Ser and Cys nucleophiles (16), which are part of Lys-Ser (17) and Cys-His-Asp (18) catalytic diad and triad, respectively. PBPs and LDTs also differ by the structure of the stem peptide used as an acyl donor, a pentapeptide for PBPs (17) and a tetrapeptide for LDTs (16), except for *Enterococcus faecalis* Ldt_{fs} (19). PBPs cleave the D-Ala^4-D-Ala^5 peptide bond of the acyl donor, hence the D,D designation for these transpeptidases, and link the carbonyl of D-Ala^4 to the side-chain amino group of the 3rd residue thereby generating 4→3 cross-links (17) (Supplementary Fig. S1). In contrast, LDTs cleave the L-Lys^3-D-Ala^4 bond of the donor (L,D designation) and form 3→3 cross-links (16).

The first substrate of the transpeptidation reaction, the acyl donor, forms an acylenzyme intermediate that subsequently reacts with the second substrate, the acceptor, leading to the final cross-linked product. For PBPs, nucleophilic attack of the carbonyl of D-Ala^4 by the active-site Ser results in an acylenzyme containing an ester bond and release of D-Ala^5 (17). For the acylenzyme

formed by LDTs, a thioester bond connects the carbonyl of L-Lys[3] to the γ sulfur of the active-site Cys (16). β-lactams are structure analogues of the D-Ala[4]-D-Ala[5] extremity of stem pentapeptides and act as suicide substrates of the PBPs (20). Nucleophilic attack of the carbonyl of the β-lactam ring by the active-site Ser of PBPs leads to formation of an acylenzyme, which is only hydrolyzed very slowly, leading in practice to "irreversible" inactivation in the time scale of a bacterial generation (Supplementary Fig. S1) (2). LDTs are also acylated by β-lactams although efficacious enzyme inactivation and antibacterial activity occur only for a single class of β-lactams, the carbapenems, such as imipenem (7, 8). β-lactams of the cephem (cephalosporin) class, such as ceftriaxone, are only active at high concentrations since acylation is slower and the resulting thioester bond is prone to hydrolysis (7). The kinetic parameters are even less favorable for penams (ampicillin and penicillin) leading to equilibrium between the acylated and unacylated (functional) forms, which accounts for the lack of antibacterial activity (7).

Fluorescence kinetics revealed that the acylation reaction of LDTs by β-lactams of the carbapenem class comprises two limiting steps (Fig. 1A and B). In the first step, nucleophilic attack of the β-lactam carbonyl by the negatively charged sulfur atom of the catalytic Cys was thought to lead to formation of a covalent sulfur-carbon bond and to the development of a negative charge on the drug (21). Initially, this negative charge was proposed to be located on the β-lactam oxygen (21). This hypothesis was based on analogies with PBPs, which contain an oxyanion hole for stabilization of the negative charge developing on the β-lactam oxygen (17). However, hybrid potential simulation of the acylation reaction indicated that the path to an oxyanion is energetically disfavored in the case of LDTs and that an oxyanion could not correspond to a stabilized reaction intermediate (22). Rather, hybrid potential simulation indicated that the most energetically favored reaction path involves a concerted mechanism leading to the concomitant formation of the thioester bond, rupture of C-N β-lactam bond, and formation of an amine anion (22). In the second step, the final acylenzyme is formed by protonation of the amine anion. The origin of this proton remains elusive (18, 22). Formation of a non-covalent complex is not considered in this reaction scheme as the affinity of the Ldt_{fm} for carbapenems is very low, in the order of 60 to 80 mM (7).

Fluorescence kinetics proved useful to assess the efficacy of inactivation of LDTs from *Mycobacterium tuberculosis* (23-25) and contributed to the choice of the drugs that were tested in a phase II clinical trial (26). The assays were also useful in evaluating series of synthetic carbapenems (27) and non-β-lactam LDT inhibitors (28). However, the basis for the variation in fluorescence intensity detected during the two steps of the acylation reaction remained elusive. Initially, we developed fluorescence kinetics since determination of the crystal structure of Ldt_{fm} revealed a Trp residue at the entrance of the catalytic cavity (15), which appeared ideally located for fluorescence quenching upon drug binding due to changes in its environment (21). However, this naïve explanation was subsequently found to be insufficient to account for the various behaviors observed for acylation of LDTs by representatives of the β-lactam classes (7, 22-25). This prompted us to investigate here the basis for variations in fluorescence intensity occurring upon acylation of LDTs using the well-characterized L,D-transpeptidase Ldt_{fm} from *E. faecium* as a model. We show that variations in fluorescence intensity are not due to modification of the environment of Trp residues but to the formation of a β-lactam-derived fluorescence quencher during the acylation reaction.

RESULTS

119 **Trp to Phe and His to Ala substitutions do not abolish the biphasic behavior of fluorescence kinetics**
120 **observed for Ldt_{fm} inactivation by imipenem.** We have previously proposed that the acylation of Ldt_{fm}
121 by carbapenems is a two-step reaction involving reversible formation of an amine anion (EI^{An}, step 1)
122 followed by irreversible formation of an acylenzyme (EI*, step 2) (Fig. 1A) (21, 22). Fluorescence
123 kinetics also display two phases enabling the determination of kinetic parameters for the two steps of
124 the reaction (Fig. 1B) (21). In the first phase, fluorescence intensity decreases because EI^{An} is rapidly
125 formed to the detriment of the free enzyme (E) and the fluorescence intensity of EI^{An} is lower than that
126 of E. In the second phase, fluorescence intensity increases since the acylenzyme (EI*) is formed to the
127 detriment of EI^{An} (acylation step) and the fluorescence intensity of EI* is greater than that of EI^{An}. Our
128 first aim was to determine whether variations in the fluorescence intensity could be correlated to the
129 modification of the environment of a specific Trp residue of Ldt_{fm}. To address this question, Trp
130 residues of the catalytic domain of Ldt_{fm} were replaced by Phe residues in various combinations. For
131 the sake of simplicity, the six Trp residues at positions 355, 385, 410, 415, 425, and 434 of Ldt_{fm} were
132 designated as residues a to f, respectively. Fluorescence kinetics were determined for Ldt_{fm} derivatives
133 lacking 2 to 5 Trp residues (Fig. 1C). Fluorescence kinetics remained bi-phasic in all cases, except for
134 the Ldt_{fm} derivative only retaining the "a" residue (*i.e*. Trp^{355}). Thus, the variations in the fluorescence
135 intensity associated with the two steps of the acylation reaction do not depend upon the modification
136 of the environment of a specific Trp residue of Ldt_{fm}. This prompted us to investigate fluorescence
137 quenching by His residues of Ldt_{fm}. Fluorescence kinetics obtained with the $His^{353}Ala$ and $His^{440}Ala$
138 derivatives of Ldt_{fm} indicated that neither residue is essential for biphasic fluorescence kinetics (Fig.
139 1C). His at position 421 could not be analyzed since this residue is part of the catalytic triad and is
140 essential for Ldt_{fm} activity.

141 **Impact of Trp to Phe and His to Ala substitutions on the efficacy of the inactivation reaction and on**
142 **the relative fluorescence intensity of the three enzyme forms.** Fitting simulations to experimental
143 data for four concentrations of imipenem was used to determine the inactivation kinetic parameters
144 (Supplementary Fig. S2). None of the Trp residues was essential for the acylation of Ldt_{fm} by imipenem
145 although important variations were observed in the k_1 (formation of the amine anion) and k_2 (acylation
146 step) parameters (Fig. 2A and 2B, respectively). The Trp to Phe substitutions produced large variations
147 in the intrinsic fluorescence of the three forms of the enzyme (apo Ldt_{fm}, amine anion, and acylenzyme;
148 Fig. 2C). The formation of the acylenzyme (EI*) was confirmed by mass spectrometry for all Ldt_{fm}
149 derivatives (mass increment of 299.3 Da; data not shown).

150 **Conservation of Trp residues in Ldt_{fm} homologues.** The Trp residues were poorly conserved in L,D-
151 transpeptidases from Gram-positive and Gram-negative bacteria (Supplementary Fig. S3), as expected
152 from the substantial high residual acylation activity of Ldt_{fm} derivatives containing Trp to Phe
153 substitutions (above, Fig. 2A and B). However, residue e (Trp^{425}) was relatively conserved in L,D-
154 transpeptidases from *M. tuberculosis*, being present in the Mt1, Mt2, Mt3, and Mt4 enzymes as well
155 as in the corresponding orthologues of *M. abscessus*. These enzymes are known to display bi-phasic
156 fluorescence kinetics upon inactivation by carbapenems (23, 24, 29) and (unpublished results). Since
157 Trp^{425} was the only conserved Trp residue in the LDTs from mycobacteria we investigated the impact
158 of a Trp to Phe substitution at this position in Ldt_{fm}. As shown in Supplementary Fig. S4A and B,
159 derivatives of Ldt_{fm} lacking all Trp residues except Trp^{425} or lacking only this residue displayed biphasic
160 kinetics. These results indicated that the biphasic nature of fluorescence kinetics does not depend
161 upon any conserved Trp residue in the L,D-transpeptidase protein family.

162 **Fluorescence kinetics with a single ectopic Trp residue at position 383.** All six Trp residues of Ldt_{fm}
163 were replaced by Phe residues. As expected, the variant devoid of Trp residues was very weakly
164 fluorescent (data not shown). No modification of this weak fluorescence was observed upon acylation
165 by imipenem. This observation ruled out the formal possibility that the catalytic Cys acylated by
166 imipenem could be a fluorophore. Replacement of Tyr at position 383 by a Trp residue in this Ldt_{fm}
167 derivative restored biphasic fluorescence kinetics (Supplementary Fig. S4C). This result confirmed that
168 biphasic fluorescence kinetics are not dependent upon the presence of any of the six Trp residues of
169 Ldt_{fm} in their original positions.

170 **Investigation of the opened β-lactam ring linked to the catalytic Cys residue as a quencher.** Since the
171 variations in the fluorescence observed during the two steps of the acylation reaction could not be
172 accounted for by modification of the environment of any specific Trp residue, our next objective was
173 to investigate whether quenching could result from energy transfer from Trp residues to the Cys-β-
174 lactam covalent adduct generated upon enzyme inactivation. To investigate this possibility, we first
175 examined acylation of Ldt_{fm} by the chromogenic cephalosporin nitrocefin. Fluorescence kinetics with
176 this compound displayed a monophasic behavior with a large decrease (72%) in the fluorescence
177 intensity (Fig. 3A). In the Ldt_{fm}-nitrocefin acylenzyme, the negative charge developing on the β-lactam
178 nitrogen is stabilized by a mesomeric effect that prevents protonation of the amine (30). Thus, the
179 amine anion formed as an end product with nitrocefin (Fig. 3A) or as an intermediate with imipenem
180 (Fig. 1) was associated with extensive fluorescence quenching. As found for nitrocefin, acylation of
181 Ldt_{fm} by ceftriaxone led to a monophasic decrease in the fluorescence intensity (Fig. 3B). Acylation of
182 Ldt_{fm} by ceftriaxone involves a concerted mechanism leading to the elimination of the side chain and
183 formation of a conjugated imine (7). The extent of fluorescence quenching observed for the
184 acylenzyme formed with ceftriaxone (40%; imine) or by the intermediate formed with imipenem (45%;
185 amine anion) were more important than that observed for the final Ldt_{fm}-imipenem adduct (20%)
186 suggesting that quenching was decreased by protonation of the β-lactam nitrogen.

187 **Fluorescence quenching associated with inactivation of β-lactamases by β-lactamase inhibitors.** In
188 order to enrich the structural diversity of molecules investigated in the fluorescence quenching assay,
189 we aimed to test β-lactamase inhibitors. Most β-lactams could not be tested with β-lactamases since
190 the fluorescence assay requires relatively high enzyme concentrations (typically 10 µM) leading to
191 rapid hydrolysis of the drugs even if the turnovers are low. For this reason, we focused on inactivation
192 of BlaC from *Mycobacterium tuberculosis* by clavulanate since the corresponding acylenzyme is not
193 prone to hydrolysis at the timescale of our experiments (31). Acylation of BlaC by clavulanate led to a
194 large decrease in the fluorescence intensity (37%) (Fig. 3C). Previous studies have shown that acylation
195 of BlaC by clavulanate leads to formation of imine and *trans*-enamine tautomers (depicted in Fig. 3C)
196 following drug decarboxylation (32). Since Ldt_{fm} and BlaC contain Cys and Ser as the active-site
197 nucleophile, respectively, these results indicate that fluorescence quenching is observed with
198 acylenzymes containing both thioester and ester bonds.
199 Avibactam offered the possibility to test a non-β-lactam inhibitor that inhibits BlaC by formation
200 of a carbamoylenzyme (33). Formation of the adduct (Supplementary Fig. S5) was not associated with
201 any significant modification of the fluorescence intensity of the enzyme (Fig. 3D). Protonation of the
202 amino sulfate nitrogen of avibactam in the avibactam-BlaC adduct was thus associated with limited
203 quenching as found for the protonation of the β-lactam nitrogen in the final Ldt_{fm}-imipenem and BlaC-
204 clavulanate adducts (Fig. 1 and 3C).

205 **Acylation of Ldt_{fm} and BlaC by faropenem.** Faropenem, a β-lactam belonging to the penem class, is
206 poorly hydrolyzed by BlaC and rapidly inactivates L,D-transpeptidases (24). Thus, faropenem provided
207 the opportunity to study the acylation of the two enzyme types, *i.e*. a β-lactamase (BlaC) and an L,D-
208 transpeptidase (Ldt_{fm}), by the same β-lactam. Acylation of BlaC by faropenem led to extensive
209 fluorescence quenching (44%) (Fig. 3E). The resulting acylenzyme contained an unprotonated β-lactam
210 nitrogen. In contrast, acylation of Ldt_{fm} by faropenem, which resulted in the rupture of the C^5-C^6 bond
211 and the loss of a large portion of the drug including the β-lactam nitrogen (24), was associated with a
212 moderate decrease in the fluorescence intensity (17%) (Fig. 3F).
213
214 **Acylation of BlaC by the meropenem carbapenem.** Although BlaC displays moderate carbapenemase
215 activity, previous studies have shown that a BlaC-meropenem adduct is the predominant enzyme form
216 upon incubation of the enzyme with low drug concentrations (34, 35). As expected, the control
217 experiment depicted in Supplementary Fig. S6 shows that the concentration of meropenem remains
218 saturating for at least 80 min during hydrolysis of meropenem (40 – 200 µM) by BlaC (10 µM).
219 Fluorescence kinetics at a shorter time scale (6 min) showed a transitory decrease in fluorescence
220 intensity (40%) (Fig. 3G). This observation suggests that formation of an amine anion initially leads to
221 an important fluorescence quenching. Then, fluorescence returned to the initial level suggesting that
222 protonation of the amine anion fully suppressed quenching. Thus, formation of an amine anion
223 following nucleophilic attack of the carbonyl carbon of carbapenems by Ldt_{fm} (Fig. 1) and BlaC (Fig. 3G)
224 similarly led to extensive fluorescence quenching despite the presence of different catalytic residues
225 (Cys *versus* Ser) and different modes of activation of these nucleophiles involving His and Lys residues.
226
227 **DISCUSSION**
228
229 The main aim of the present study was to determine the basis for the variations in the fluorescence
230 intensity observed upon acylation of LDTs by β-lactam antibiotics. Previous analyses of the D,D-
231 transpeptidase R61 (PBP family) have shown that acylation of the enzyme by penicillin G leads to a
232 fluorescence quenching, which specifically depends upon one of two Trp residues of the protein (36,
233 37). This residue, W^{233}, is essential for the D,D-carboxypeptidase activity of the protein, as determined
234 by the release of the terminal D-Ala residue from a model peptide ending in D-Ala-D-Ala (37). The crystal
235 structure of R61 in complex with a peptide mimicking the acyl donor revealed hydrophobic interactions
236 between the side-chain of W^{233} and methylene groups of this substrate (38). Likewise, the structure of
237 a penicillin-R61 complex revealed that W^{233} is in hydrophobic interaction with the phenylacetyl side
238 chain of the drug (39) indicating that fluorescence quenching may depend upon this direct interaction.
239 In contrast, we show here that the variations in fluorescence during acylation of LDTs by β-lactam
240 antibiotics require at least one Trp residue but that the position of this residue is not determinant. This
241 was established by deleting Trp residues from Ldt_{fm} in various combinations (Fig. 1 and 2), by
242 introducing a single Trp residue in an ectopic position of Ldt_{fm} (Fig. S4), and by showing that large
243 variations in fluorescence intensity occur in distantly related LDTs and unrelated β-lactamases that do
244 not share any conserved Trp residue (Fig. S3). Since fluorescence quenching was not mainly due to
245 changes in the environment of Trp residues, we investigated the possibility that the nucleophilic attack
246 of the β-lactam ring could itself generate a fluorescence quencher. If this is the case, the fluorescence
247 profile should depend upon the structural features of the drug bound to the active-site residue.
248 Accordingly, extensive quenching was not mainly determined by the size of the side chains of β-lactams
249 or by the nature of the enzyme nucleophile (Cys *versus* Ser in Ldt_{fm} and BlaC, respectively) (Fig .3).

250 Acylation of Ser or Cys *per se* was not sufficient for fluorescence quenching since a modest or no
251 decrease in fluorescence intensity was observed for the final acylation products of Ldt_{fm} by faropenem,
252 of BlaC by avibactam, and of both enzymes by carbapenems. By elimination, these results pointed to
253 the status of the β-lactam nitrogen as the only source of variation that could account for the main
254 variations in fluorescence intensity. A large fluorescence quenching occurred if the β-lactam nitrogen
255 was negatively charged, as transiently found during acylation of Ldt_{fm} and BlaC by carbapenems. A
256 large fluorescence quenching also occurred if the nitrogen atom was engaged in a double bond as
257 observed for the acylenzymes formed by Ldt_{fm} with ceftriaxone or nitrocefin and by BlaC with
258 faropenem or clavulanate. In agreement with the critical role of the double bond, fluorescence
259 quenching was not observed for acylation of BlaC by avibactam, which results in the formation of a
260 carbamoyl-enzyme with a protonated nitrogen, or for acylation of Ldt_{fm} by faropenem, which results
261 in the formation of an acylenzyme lacking the nitrogen atom following rupture of the C^5-C^6 bond. These
262 results indicate that fluorescence kinetics can provide information not only on the efficacy of enzyme
263 inactivation but also on the structure of the covalent adducts, including for example the presence of
264 Δ1- or Δ2-pyrroline ring in enzyme-carbapenem adducts. Our results also indicate that nitrocefin
265 provides a very sensitive assay to titrate the active site of enzymes that do not hydrolyze this
266 cephalosporin. This property could be exploited to identify β-lactams and non-β-lactam inhibitors that
267 reversibly or irreversibly bind to LDTs.
268
269 **MATERIALS AND METHODS**
270
271 **Enzyme production and purification.** The catalytic domain of Ldt_{fm} (residues 341 to 466) and soluble
272 BlaC (residues 39 to 306) were produced in *E. coli* and purified by metal affinity and size exclusion
273 chromatography as previously described (30). Synthetic genes were purchased for production of Ldt_{fm}
274 derivatives with Trp to Phe and His to Ala substitutions (GeneCust). All experiments were performed
275 with a fixed concentration of Ldt_{fm} and BlaC (10 µM).
276
277 **Spectrofluorimetry.** Fluorescence kinetics were studied in 100 mM sodium phosphate (pH 6.0) at 20°C
278 by using a stopped-flow apparatus (RX-2000, Applied Biophysics) coupled to a spectrofluorimeter (Cary
279 Eclipse; Varian). Excitation was performed at 224 nm with a 5 nm slit and a 2 mm optical path.
280 Fluorescence emission was determined at 335 nm with a 5 nm slit and a 10 mm optical path.
281 Fluorescence data were collected exactly in the same conditions for comparison of the relative
282 fluorescence of wild-type Ldt_{fm} and derivatives with Trp to Phe and His to Ala substitutions. These
283 conditions included the photomultiplier voltage, which was set to 600 V.
284
285 **Mass spectrometry.** Formation of the proposed Ldt_{fm}-β-lactam and BlaC-β-lactam adducts in the
286 conditions described in the text was systematically checked by mass spectrometry (data not shown).
287 Enzymes and β-lactams were incubated for appropriate time periods at 20°C. Five µl of acetonitrile
288 and 1 µl of 1% formic acid were extemporaneously added. Injection in the mass spectrometer (Qstar
289 Pulsar I; Applied Biosystem) was performed at a flow rate of 0.05 ml/min (acetonitrile, 50%, water,
290 49.5%, and formic acid, 0.5%; per volume). Spectra were acquired in the positive mode, as previously
291 described (8). Detailed analyses of the mass spectra of the β-lactam adducts considered in the current
292 study have been previously published (7, 8, 24, 29, 40).

293 **ACKNOWLEDGEMENTS**

294 We thank L. Dubost and A. Marie for technical assistance in the collection of mass spectra. This work
295 was supported by the French National Research Agency (grant MycWall, ANR-17-CE18-0010) and the
296 Fondation pour la Recherche Médicale (grant ECO20160736080 to ZE).

297

298 **REFERENCES**

299 1. **Turner RD, Vollmer W, Foster SJ.** 2014. Different walls for rods and balls: the diversity of
300 peptidoglycan. Mol Microbiol **91:**862-874.
301 2. **Zapun A, Contreras-Martel C, Vernet T.** 2008. Penicillin-binding proteins and beta-lactam
302 resistance. FEMS Microbiol Rev **32:**361-385.
303 3. **Lavollay M, Arthur M, Fourgeaud M, Dubost L, Marie A, Veziris N, Blanot D, Gutmann L,**
304 **Mainardi JL.** 2008. The peptidoglycan of stationary-phase *Mycobacterium tuberculosis*
305 predominantly contains cross-links generated by L,D-transpeptidation. J Bacteriol **190:**4360-
306 4366.
307 4. **Lavollay M, Fourgeaud M, Herrmann JL, Dubost L, Marie A, Gutmann L, Arthur M, Mainardi**
308 **JL.** 2011. The peptidoglycan of *Mycobacterium abscessus* is predominantly cross-linked by L,D-
309 transpeptidases. J Bacteriol **193:**778-782.
310 5. **Peltier J, Courtin P, El Meouche I, Lemee L, Chapot-Chartier MP, Pons JL.** 2011. *Clostridium*
311 *difficile* has an original peptidoglycan structure with a high level of *N*-acetylglucosamine
312 deacetylation and mainly 3-3 cross-links. J Biol Chem **286:**29053-29062.
313 6. **Sutterlin L, Edoo Z, Hugonnet JE, Mainardi JL, Arthur M.** 2018. Peptidoglycan cross-linking
314 activity of L,D-transpeptidases from *Clostridium difficile* and inactivation of these enzymes by
315 beta-lactams. Antimicrob Agents Chemother **62**.
316 7. **Triboulet S, Dubee V, Lecoq L, Bougault C, Mainardi JL, Rice LB, Etheve-Quelquejeu M,**
317 **Gutmann L, Marie A, Dubost L, Hugonnet JE, Simorre JP, Arthur M.** 2013. Kinetic features of
318 L,D-transpeptidase inactivation critical for beta-lactam antibacterial activity. PLoS One
319 **8:**e67831.
320 8. **Mainardi JL, Hugonnet JE, Rusconi F, Fourgeaud M, Dubost L, Moumi AN, Delfosse V, Mayer**
321 **C, Gutmann L, Rice LB, Arthur M.** 2007. Unexpected inhibition of peptidoglycan LD-
322 transpeptidase from *Enterococcus faecium* by the beta-lactam imipenem. J Biol Chem
323 **282:**30414-30422.
324 9. **Mainardi JL, Legrand R, Arthur M, Schoot B, van Heijenoort J, Gutmann L.** 2000. Novel
325 mechanism of beta-lactam resistance due to bypass of DD-transpeptidation in *Enterococcus*
326 *faecium*. J Biol Chem **275:**16490-16496.
327 10. **Mainardi JL, Morel V, Fourgeaud M, Cremniter J, Blanot D, Legrand R, Frehel C, Arthur M,**
328 **Van Heijenoort J, Gutmann L.** 2002. Balance between two transpeptidation mechanisms
329 determines the expression of beta-lactam resistance in *Enterococcus faecium*. J Biol Chem
330 **277:**35801-35807.
331 11. **Hugonnet JE, Mengin-Lecreulx D, Monton A, den Blaauwen T, Carbonnelle E, Veckerle C,**
332 **Brun YV, van Nieuwenhze M, Bouchier C, Tu K, Rice LB, Arthur M.** 2016. Factors essential for
333 L,D-transpeptidase-mediated peptidoglycan cross-linking and beta-lactam resistance in
334 *Escherichia coli*. Elife **5:**e19469.
335 12. **Both D, Steiner EM, Stadler D, Lindqvist Y, Schnell R, Schneider G.** 2013. Structure of LdtMt2,
336 an L,D-transpeptidase from *Mycobacterium tuberculosis*. Acta Crystallogr D Biol Crystallogr
337 **69:**432-441.
338 13. **Correale S, Ruggiero A, Capparelli R, Pedone E, Berisio R.** 2013. Structures of free and
339 inhibited forms of the L,D-transpeptidase Ldt_{Mt1} from *Mycobacterium tuberculosis*. Acta
340 Crystallogr D Biol Crystallogr **69:**1697-1706.

14. **Li WJ, Li DF, Hu YL, Zhang XE, Bi LJ, Wang DC.** 2013. Crystal structure of L,D-transpeptidase Ldt_{Mt2} in complex with meropenem reveals the mechanism of carbapenem against *Mycobacterium tuberculosis*. Cell Res **23:**728-731.
15. **Biarrotte-Sorin S, Hugonnet JE, Delfosse V, Mainardi JL, Gutmann L, Arthur M, Mayer C.** 2006. Crystal structure of a novel beta-lactam-insensitive peptidoglycan transpeptidase. J Mol Biol **359:**533-538.
16. **Mainardi JL, Fourgeaud M, Hugonnet JE, Dubost L, Brouard JP, Ouazzani J, Rice LB, Gutmann L, Arthur M.** 2005. A novel peptidoglycan cross-linking enzyme for a beta-lactam-resistant transpeptidation pathway. J Biol Chem **280:**38146-38152.
17. **Goffin C, Ghuysen JM.** 2002. Biochemistry and comparative genomics of SxxK superfamily acyltransferases offer a clue to the mycobacterial paradox: presence of penicillin-susceptible target proteins versus lack of efficiency of penicillin as therapeutic agent. Microbiol Mol Biol Rev **66:**702-738.
18. **Lecoq L, Dubee V, Triboulet S, Bougault C, Hugonnet JE, Arthur M, Simorre JP.** 2013. Structure of *Enterococcus faecium* L,D-transpeptidase acylated by ertapenem provides insight into the inactivation mechanism. ACS Chem Biol **8:**1140-1146.
19. **Magnet S, Arbeloa A, Mainardi JL, Hugonnet JE, Fourgeaud M, Dubost L, Marie A, Delfosse V, Mayer C, Rice LB, Arthur M.** 2007. Specificity of L,D-transpeptidases from gram-positive bacteria producing different peptidoglycan chemotypes. J Biol Chem **282:**13151-13159.
20. **Tipper 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.
21. **Triboulet S, Arthur M, Mainardi JL, Veckerle C, Dubee V, Nguekam-Nouri A, Gutmann L, Rice LB, Hugonnet JE.** 2011. Inactivation kinetics of a new target of beta-lactam antibiotics. J Biol Chem **286:**22777-22784.
22. **Bhattacharjee N, Field MJ, Simorre JP, Arthur M, Bougault CM.** 2016. Hybrid potential simulation of the acylation of *Enterococcus faecium* L,D-transpeptidase by carbapenems. J Phys Chem B **120:**4767-4781.
23. **Dubee V, Triboulet S, Mainardi JL, Etheve-Quelquejeu M, Gutmann L, Marie A, Dubost L, Hugonnet JE, Arthur M.** 2012. Inactivation of *Mycobacterium tuberculosis* L,D-transpeptidase LdtMt(1) by carbapenems and cephalosporins. Antimicrob Agents Chemother **56:**4189-4195.
24. **Dhar N, Dubée V, Ballell L, Cuinet G, Hugonnet J-L, Signorino-Gelo F, Barros D, Arthur M, McKinney J.** 2015. Rapid cytolysis of *Mycobacterium tuberculosis* by faropenem, an orally bioavailable beta-lactam antibiotic. Antimicrob Agents Chemother **59:**1308-1319.
25. **Gupta R, Lavollay M, Mainardi JL, Arthur M, Bishai WR, Lamichhane G.** 2010. The *Mycobacterium tuberculosis* protein Ldt(Mt2) is a nonclassical transpeptidase required for virulence and resistance to amoxicillin. Nat Med **16:**466-469.
26. **Diacon AH, van der Merwe L, Barnard M, von Groote-Bidlingmaier F, Lange C, Garcia-Basteiro AL, Sevene E, Ballell L, Barros-Aguirre D.** 2016. beta-lactams against tuberculosis-new trick for an old dog? N Engl J Med **375:**393-394.
27. **Iannazzo L, Soroka D, Triboulet S, Fonvielle M, Compain F, Dubee V, Mainardi JL, Hugonnet JE, Braud E, Arthur M, Etheve-Quelquejeu M.** 2016. Routes of synthesis of carbapenems for optimizing both the inactivation of L,D-transpeptidase Ldt_{Mt1} of *Mycobacterium tuberculosis* and the stability toward hydrolysis by beta-Lactamase BlaC. J Med Chem **59:**3427-3438.
28. **Edoo Z, Iannazzo L, Compain F, Li de la Sierra Gallay I, van Tilbeurgh H, Fonvielle M, Bouchet F, Le Run E, Mainardi JL, Arthur M, Etheve-Quelquejeu M, Hugonnet JE.** 2018. Synthesis of avibactam derivatives and activity on beta-lactamases and peptidoglycan biosynthesis enzymes of mycobacteria. Chemistry doi:10.1002/chem.201800923.
29. **Cordillot M, Dubee V, Triboulet S, Dubost L, Marie A, Hugonnet JE, Arthur M, Mainardi JL.** 2013. In vitro cross-linking of *Mycobacterium tuberculosis* peptidoglycan by L,D-transpeptidases and inactivation of these enzymes by carbapenems. Antimicrob Agents Chemother **57:**5940-5945.

392 30. **Edoo Z, Arthur M, Hugonnet JE.** 2017. Reversible inactivation of a peptidoglycan
393 transpeptidase by a beta-lactam antibiotic mediated by beta-lactam-ring recyclization in the
394 enzyme active site. Sci Rep **7:**9136.
395 31. **Hugonnet JE, Blanchard JS.** 2007. Irreversible inhibition of the *Mycobacterium tuberculosis*
396 beta-lactamase by clavulanate. Biochemistry **46:**11998-12004.
397 32. **Tremblay LW, Hugonnet JE, Blanchard JS.** 2008. Structure of the covalent adduct formed
398 between *Mycobacterium tuberculosis* beta-lactamase and clavulanate. Biochemistry **47:**5312-
399 5316.
400 33. **Xu H, Hazra S, Blanchard JS.** 2012. NXL104 irreversibly inhibits the beta-lactamase from
401 *Mycobacterium tuberculosis*. Biochemistry **51:**4551-4557.
402 34. **Hugonnet JE, Tremblay LW, Boshoff HI, Barry CE, 3rd, Blanchard JS.** 2009. Meropenem-
403 clavulanate is effective against extensively drug-resistant *Mycobacterium tuberculosis*. Science
404 **323:**1215-1218.
405 35. **Tremblay LW, Fan F, Blanchard JS.** 2010. Biochemical and structural characterization of
406 *Mycobacterium tuberculosis* beta-lactamase with the carbapenems ertapenem and
407 doripenem. Biochemistry **49:**3766-3773.
408 36. **Frere JM, Ghuysen JM, Iwatsubo M.** 1975. Kinetics of interaction between the exocellular DD-
409 carboxypeptidase-transpeptidase from *Streptomyces* R61 and beta-lactam antibiotics. A
410 choice of models. Eur J Biochem **57:**343-351.
411 37. **Bourguignon-Bellefroid C, Wilkin JM, Joris B, Aplin RT, Houssier C, Prendergast FG, Van**
412 **Beeumen J, Ghuysen JM, Frere JM.** 1992. Importance of the two tryptophan residues in the
413 *Streptomyces* R61 exocellular DD-peptidase. Biochem J **282 (Pt 2):**361-367.
414 38. **McDonough MA, Anderson JW, Silvaggi NR, Pratt RF, Knox JR, Kelly JA.** 2002. Structures of
415 two kinetic intermediates reveal species specificity of penicillin-binding proteins. J Mol Biol
416 **322:**111-122.
417 39. **Silvaggi NR, Josephine HR, Kuzin AP, Nagarajan R, Pratt RF, Kelly JA.** 2005. Crystal structures
418 of complexes between the R61 DD-peptidase and peptidoglycan-mimetic beta-lactams: a non-
419 covalent complex with a "perfect penicillin". J Mol Biol **345:**521-533.
420 40. **Soroka D, Ourghanlian C, Compain F, Fichini M, Dubée V, Mainardi J, Hugonnet J, Arthur M.**
421 2017. Inhibition of β-lactamases of mycobacteria by avibactam and clavulanate. J Antimicrob
422 Chemother **72:**1081-1088.
423 41. **Chow C, Xu H, Blanchard JS.** 2013. Kinetic characterization of hydrolysis of nitrocefin, cefoxitin,
424 and meropenem by beta-lactamase from M*ycobacterium tuberculosis*. Biochemistry **52:**4097-
425 4104.

426

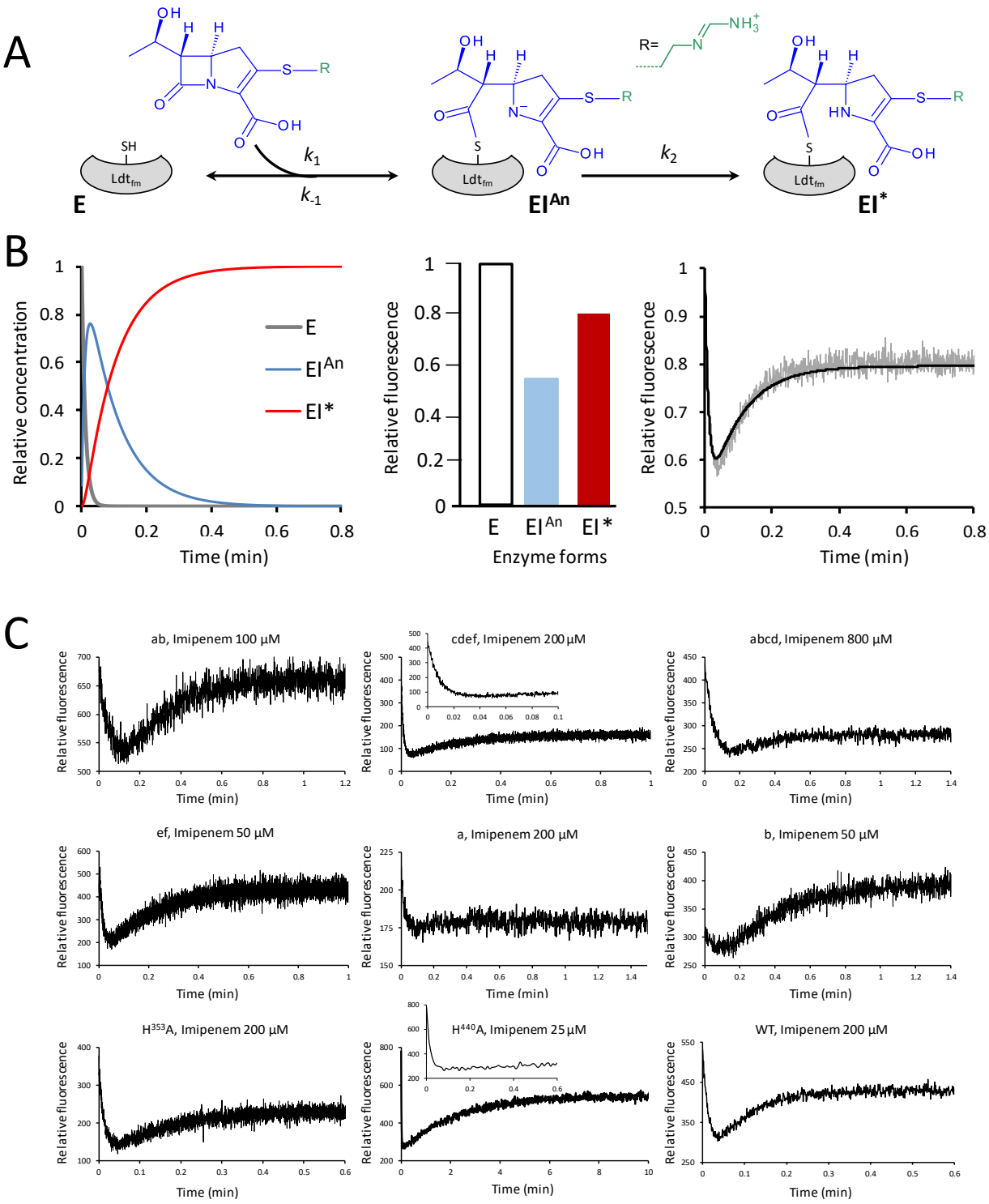

Figure 1. Impact of amino acid substitutions on fluorescence kinetics. (**A**) Inactivation reaction scheme. E, free Ldt_{fm}; EI^{An}, amine anion; EI*, acylenzyme; I, imipenem. (**B**) Fluorescence variations during Ldt_{fm} inactivation. The charts show the variations in the relative concentrations of the three enzyme forms (left panel) and the relative fluorescence intensity of the three enzyme forms (middle panel), which account for the observed fluorescence kinetics (right panel). (**C**) Fluorescence kinetics of Ldt_{fm} inactivation by imipenem. Trp residues were replaced by Phe residues in various combinations. Designation of the remaining Trp residues: a, Trp^{355}; b, Trp^{385}; c, Trp^{410}; d, Trp^{415}; e, Trp^{425}; f, Trp^{434}. Insets present the initial decreases of fluorescence over a short timescale.

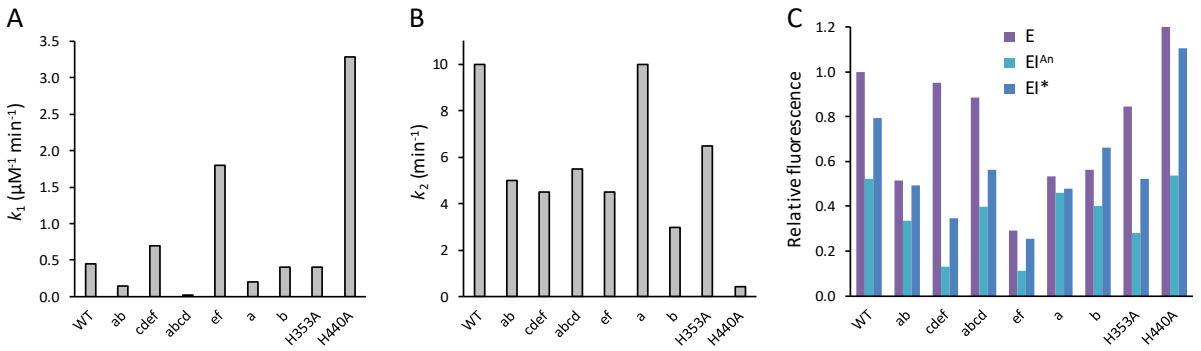

Figure 2. Inactivation efficacy and relative fluorescence intensity of Ldt_{fm} and derivatives with Trp to Phe and His to Ala substitutions. (**A**) Kinetic parameter k_1 for formation of the amine anion. (**B**) Kinetic parameter k_2 for the acylation step. (**C**) Fluorescence intensity of the three enzyme forms relative to that of the free form of the wild-type enzyme. E, Ldt_{fm}; EI^{An}, amine anion; EI*, acylenzyme. All, presence of the full complement of Trp residues (a, d, c, d, e, and f). Trp residues were replaced by Phe residues in various combinations. Designation of the remaining Trp residues: a, Trp^{355}; b, Trp^{385}; c, Trp^{410}; d, Trp^{415}; e, Trp^{425}; f, Trp^{434}.

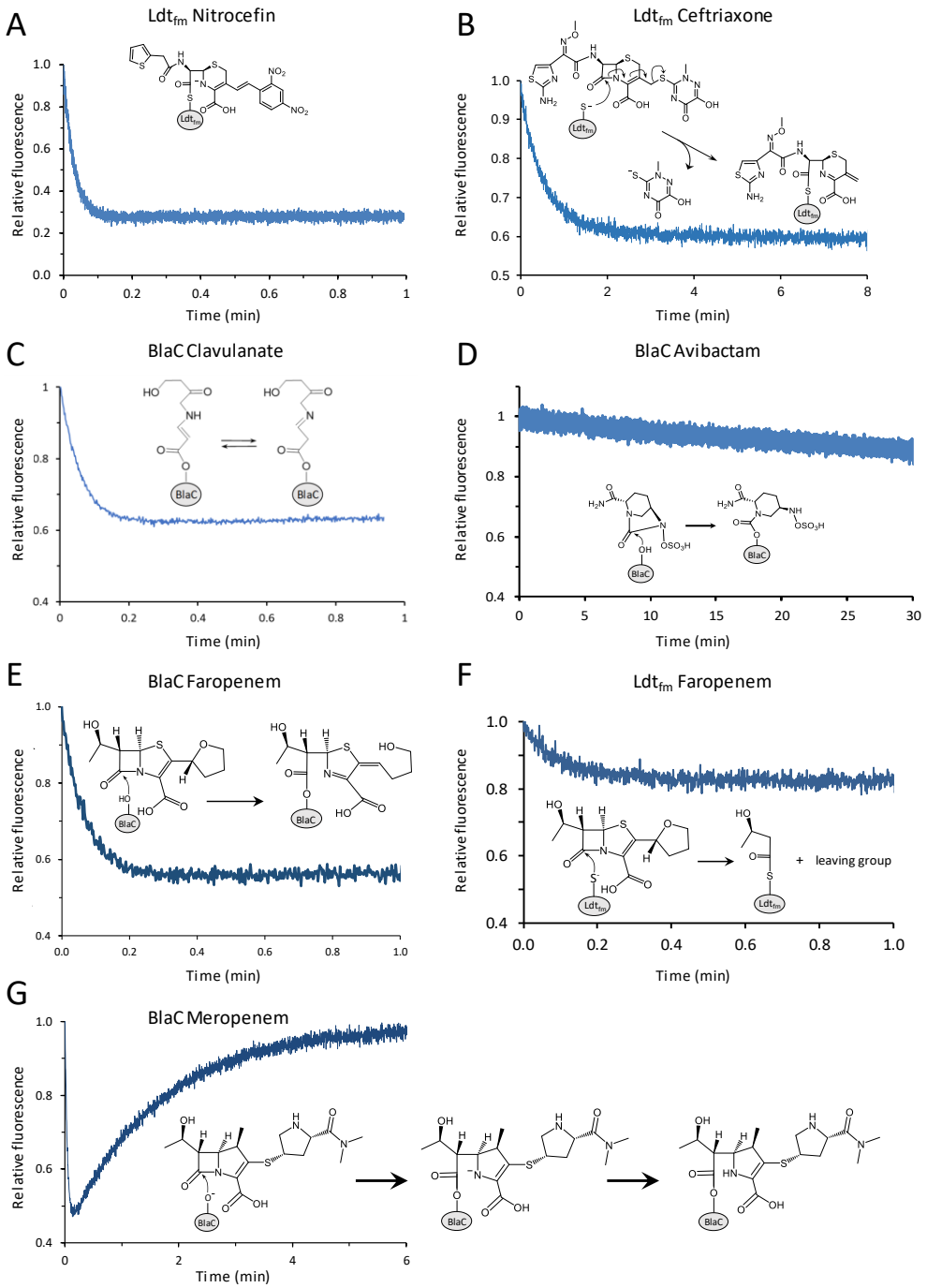

Figure 3. Variations in fluorescence intensity resulting from acylation of L,D-transpeptidase Ldt_{fm} and β-lactamase BlaC by various β-lactams and β-lactamase inhibitors. Stopped-flow fluorescence kinetics were obtained with final concentrations of 10 µM for the enzymes and 100 µM for β-lactams and β-lactamase inhibitors (**A**) The negative charge developing on the amine nitrogen upon acylation of Ldt_{fm} by nitrocefin is stabilized by a mesomeric effect involving the electron withdrawing dinitrobenzene (30, 41). (**B**) Transfer of the negative charge to a leaving group in the concerted mechanism leading to acylation of Ldt_{fm} by ceftriaxone (7). (**C**) Tautomeric forms of the acylenzyme resulting from acylation of BlaC by clavulanate and decarboxylation of the β-lactamase inhibitor (32, 40). (**D**) BlaC-avibactam carbamoylenzyme (33). (**E** and **F**) Acylation of BlaC and Ldt_{fm} by faropenem, respectively (24). (**G**) Accumulation of an amine anion in the onset of the reaction of hydrolysis of meropenem by BlaC ($BlaC^{An}$). This is followed by protonation of the β-lactam nitrogen (35).