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Novel fragments of clavulanate observed in the structure of the class A β-lactamase from *Bacillus licheniformis* BS3

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Objectives: Our aim was to unravel the inactivation pathway of the class A β-lactamase produced by *Bacillus licheniformis* BS3 (BS3) by clavulanate.

Methods: The interaction between clavulanate and BS3 was studied by X-ray crystallography, pre-steady-state kinetics and mass spectrometry.

Results: The analysis of the X-ray structure of the complex yielded by the reaction between clavulanate and BS3 indicates that the transient inactivated form, namely the *cis-trans* enamine complex, is hydrolysed to an ethane-imine ester covalently linked to the active site serine and a pentan-3-one-5-ol acid. It is the first time that this mechanism has been observed in an inactivated β-lactamase. Furthermore, the ionic interactions made by the carboxylic group of pentan-3-one-5-ol may provide an understanding of the decarboxylation process of the *trans*-enamine observed in the non-productive complex observed for the interaction between clavulanate and SHV-1 and *Mycobacterium tuberculosis* β-lactamase (Mtu).

Conclusions: This work provides a comprehensive clavulanate hydrolysis pathway accounting for the observed acyl-enzyme structures of class A β-lactamase/clavulanate adducts.

Keywords: bacterial resistance, clavulanate, β-lactamase inactivation

Introduction

β-Lactam antibiotics still represent the most used group of antimicrobial agents in medical practice. They have a high selective toxicity due to the inhibition of an essential step in the biosynthesis of a unique bacterial structure, the peptidoglycan.[1] This inhibition results from their covalent binding to essential penicillin-binding proteins (PBPs), leading to the irreversible blockade of peptidoglycan synthesis and cell death.[2] Nevertheless, since their very early use in clinical settings, significant problems have arisen with resistance, which is mainly mediated by enzymes—β-lactamases (EC 3.5.2.6)—hydrolysing the β-lactam bond and rendering the drug biologically ineffective.[3–5]

Among the strategies for circumventing the β-lactamase-mediated bacterial resistance, several compounds—such as clavulanate, sulbactam and tazobactam—that are able to efficiently and specifically inhibit the broad- and extended-spectrum active site serine β-lactamases have been developed. Clavulanate, a naturally occurring β-lactam, shows weak antibacterial activity, but displays potent β-lactamase inhibitory properties. It is characterized by an oxazolidine ring, the absence of an acylamino side chain and an unusual substituent on C2. Its combination with β-lactam antibiotics (e.g. amoxicillin) that are able to inactivate PBPs is among one of the major successes of chemotherapy and is still one of the most effective therapies used in hospitals against bacterial infections.[6]

Different pathways have been proposed to explain the inhibition mechanism of class A β-lactamases by clavulanate (Figure 1). They all involve the attack of the active serine residue on the carbonyl carbon atom of clavulanate to form a labile tetrahedral intermediate, and cleavage of the C–N bond in the β-lactam ring of clavulanate to form an acyl enzyme. The latter can be hydrolysed and lead to the regeneration of free and active enzyme, or its rearrangement can lead to the accumulation of non-active complexes and the complete inactivation of the enzyme.[7–9]

Spectroscopy and crystallography studies on the clavulanate inhibition of the class A β-lactamase from *Staphylococcus aureus* PC1 suggested that the Ser-70-attached *cis*-enamine is stabilized to the *trans*-enamine and further decarboxylated.[10,11]

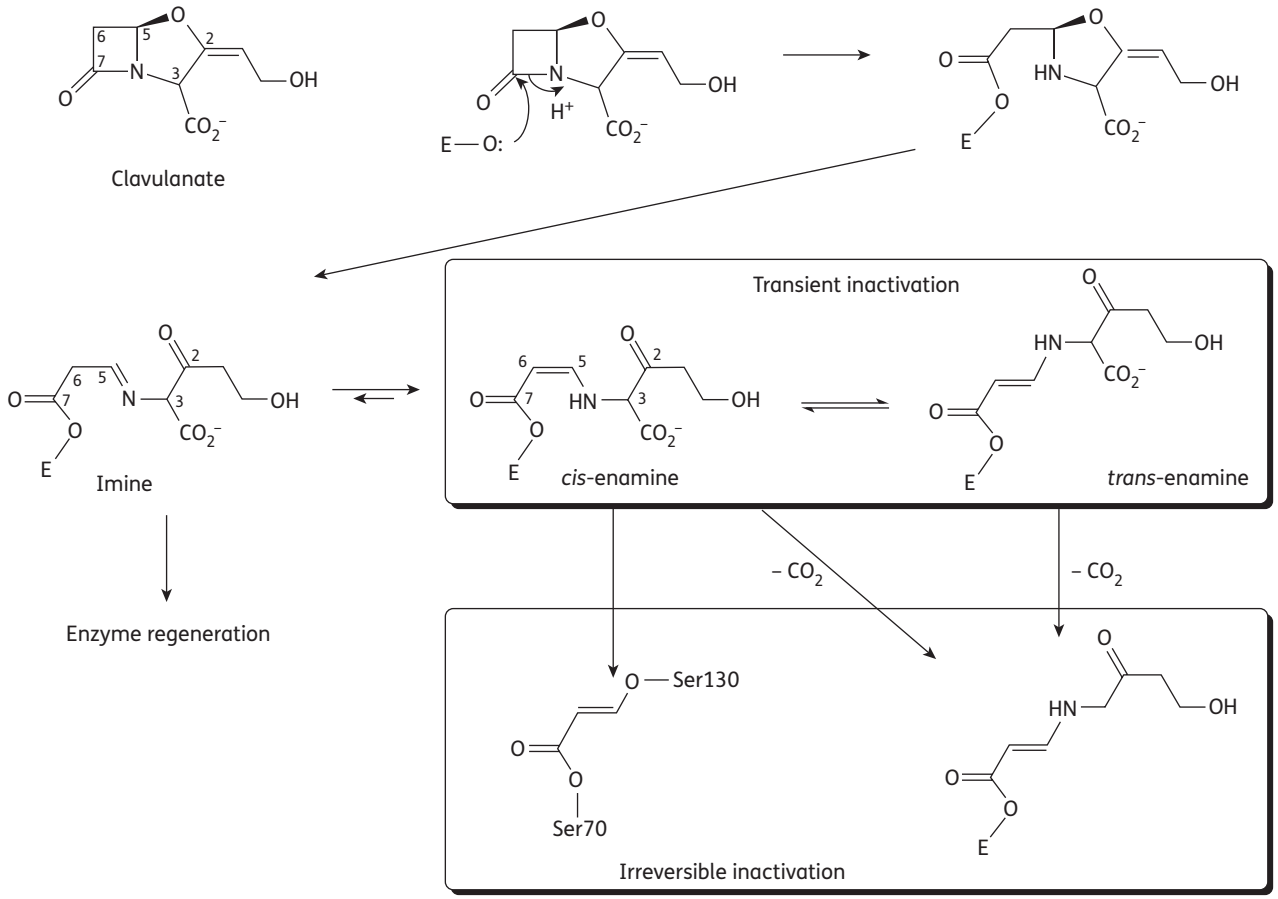

Figure 1. Structure of clavulanate and combined scheme of proposed mechanisms for the inactivation of class A β-lactamase by clavulanate.

These studies also proposed Lys-73 as being properly positioned for making a cross-link with the inhibitor molecule.[12] In opposition with this model, the resolution of the structure of *Bacillus licheniformis* 749/C apo-β-lactamase led to the proposal of Ser-130, instead of Lys-73, as the candidate for establishing a cross-linking with the clavulanate moiety, rendering an ether with Ser-130 also compatible with previous spectroscopy analyses.[13,14] Using electrospray ionization mass spectrometry, Brown *et al.*[15] later supported this model for *Escherichia coli* TEM-2 and proposed Ser-130 as having the nucleophilic role needed for yielding the formation of ether and effective inactivation of the enzyme.[15]

In this study we report the 2 Å resolution crystal structure of the adduct of the class A β-lactamase from *B. licheniformis* BS3 with clavulanate, and provide clues about the pathway of class A β-lactamase inhibition by clavulanate.

Materials and methods

Enzymes and chemicals

The expression and purification of the BS3 enzyme were performed as described previously.[16] Clavulanate (BRL 14151) was a gift from GSK (Brentford, UK), and nitrocefin was purchased from ProGenosis (Sart-Tilman, Liège, Belgium).

Kinetics

Kinetic experiments were performed on a Specord 50 Analytikjena spectrophotometer connected to a personal computer via an RS232C interface. Enzyme and inhibitors were diluted in 25 mM phosphate buffer (pH 7.5). BSA (20 mg/L) was added to the diluted solutions of β-lactamase to prevent enzyme denaturation. The reactions were followed by monitoring the hydrolysis of a reporter substrate in the presence of various concentrations of the inactivator. The BS3 β-lactamase (2.5 nM) was added to a solution of 100 μM nitrocefin containing increasing concentrations of clavulanate (from 400 nM to 8 μM). Typically the reactions were performed at 30°C in a final volume of 500 μL. The values of the pseudo-first-order inactivation constant rate k_i were calculated from the hydrolysis curves. The ratio between the number of productive turnovers and those leading to enzyme inactivation was calculated from experiments in which the ratio between the initial inactivator concentration and enzyme concentration was such that incomplete inactivation occurred. This was done by monitoring the residual activity after incubating the enzyme (50 nM) with increasing concentrations of clavulanate (50 nM–50 μM).

BS3 β-lactamase crystallography

Crystals were grown at 20°C by hanging-drop vapour diffusion with drops containing 5 μL of BS3 solution (38 mg/mL) and 2 μL of 20% PEG 6000 in 100 mM sodium citrate buffer (pH 3.4) equilibrated against 1 mL of the latter solution at 20°C. The BS3/clavulanate adduct was obtained by

diffusing into the crystal, increasing concentrations of clavulanate over the course of 20 min at room temperature. A fresh solution of clavulanate was continuously added up to a final concentration of 25 mM.

Data were collected at 100°K on an ADSC Q315r CCD detector at a wavelength of 0.9763 Å on beamline BM30A at the European Synchrotron Radiation Facility (ESRF, Grenoble, France). X-ray diffraction experiments were carried out under cryogenic conditions (100°K) after transferring the crystals into 33% glycerol and 33% PEG6000. Indexing and integration were carried out using Mosflm,[17] and the scaling of the intensity data was accomplished with SCALA of the CCP4 program suite.[18] Refinement was carried out using REFMAC5,[19] TLS[20] and Coot.[21] The structure of the BS3 β-lactamase bound to clavulanate was refined to 2 Å. Data statistics and refinement are given in Table 1.

Mass spectrometry

Mass spectrometry spectra were acquired for BS3 and BS3/clavulanate. For the latter, 2 μL of 0.5 M Li-clavulanate was mixed with 3.2 μL of 26 mg/mL BS3 in 10 mM Tris, pH 7.2/50 mM NaCl. The reaction was stopped by the addition of 5 μL of 12% (v/v) formic acid after 30 min. The volume was decreased to 10 μL by ultrafiltration using Microcon YM-10 (Millipore). Sample preparation was done by two additional ultrafiltration steps with 500 μL of 25 mM ammonium acetate. The sample was concentrated to 30 μL and mixed with 50% (v/v) acetonitrile and 0.1% (v/v) formic acid. Mass spectrometry analysis was then conducted using ESI-Q-TOF micro mass (Waters) operated in positive mode, with a capillary voltage of 3.0 kV, a sample cone voltage of 100 V and a temperature of 100°C, RF lens 1 set at 100 V and a 500 L/h desolvation gas flow. Sample was injected into the source for desolvation at 4 μL/min flow.

Table 1. X-ray data collection and refinement statistics for BS3 β-lactamase

Crystal	BS3/clavulanate
PDB code	2y91
Data collection	
Resolution range (Å)[a]	34.60–2.00 (2.11–2.00)
No. of unique reflections	40601
R_{merge} (%)[a]	7.9 (53.4)
Redundancy[a]	3.2 (3.1)
Completeness (%)[a]	99.3 (98.9)
I/σI[a]	9.2 (2.2)
Refinement	
Resolution range	34.1–2.00
No. of protein atoms	4336
No. of water molecules	254
R_{cryst} (%)	17.8
R_{free} (%)	21.9
Root-mean-square deviations from ideal stereochemistry	
Bond lengths (Å)	0.010
Bond angles (°)	1.21
Mean B factor (all atoms) (Å^2)	34.9
Mean B factor (clavulanate CL1) (Å^2)	28.5[b]
Mean B factor (clavulanate CL2) (Å^2)	36.3[b]
Ramachandran plot	
Favoured region (%)	98.2
Allowed regions (%)	1.8
Outlier regions (%)	0.0
Root-mean-square difference of Cα atoms with native structure (Å)	0.32[b]

[a]Statistics for the highest resolution shell are given in parentheses.
[b]Monomer A.

Results

Kinetics

The reaction of the *B. licheniformis* β-lactamase BS3 with clavulanate proceeds along a branched pathway. At a clavulanate/BS3 concentration ratio lower than 10, we did not observe a complete recovery of the β-lactamase activity even after a long incubation of 36 h at room temperature. In addition, when the ratio was higher than 40, we observed a complete and irreversible inactivation of the class A β-lactamase. These data indicated that no transient inactivation process of BS3 could be observed and are in good agreement with the model of interaction already proposed by Charnas and Knowles[7] for TEM-1.

We also observed a hyperbolic dependence of the k_i values versus clavulanate concentration from which we could compute the individual values of $k_{i\ lim}=8\times10^{-3}\ s^{-1}$, $K_m=0.6\ \mu M$ and $k_{i\ lim}/K_m=1.33\times10^{4}\ M^{-1}\ s^{-1}$ (Figure 2).

Crystallography

The crystal structure of BS3 in complex with clavulanate was solved at 2 Å resolution. The electron density in the BS3/clavulanate crystal is well defined for both monomers present in the asymmetric unit, from Lys-30 to Asn-291. The root-mean-square deviation between the equivalent backbone atoms in both monomers is 0.32 Å for the Cα atoms, and no significant

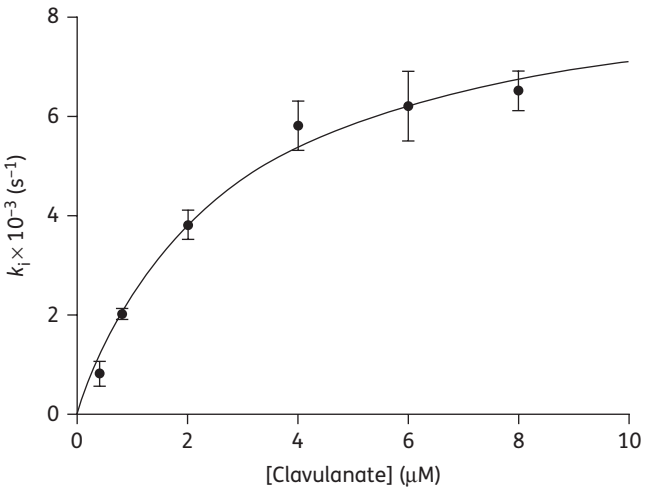

Figure 2. Evolution of k_i values as a function of clavulanate concentration. The curve was fitted, by Sigma plot version 12, with the help of the following equation: $k_i = k_{i\ lim}[C]/K(K_m + [S])/K_m + [C]$, where k_i, $k_{i\ lim}$ and C represent the pseudo-first-order inactivation constant, the limit value of k_i and the clavulanate concentration, respectively. K_m and S represent the Michaelis–Menten constant (38 μM) and the concentration of nitrocefin (100 μM), respectively.

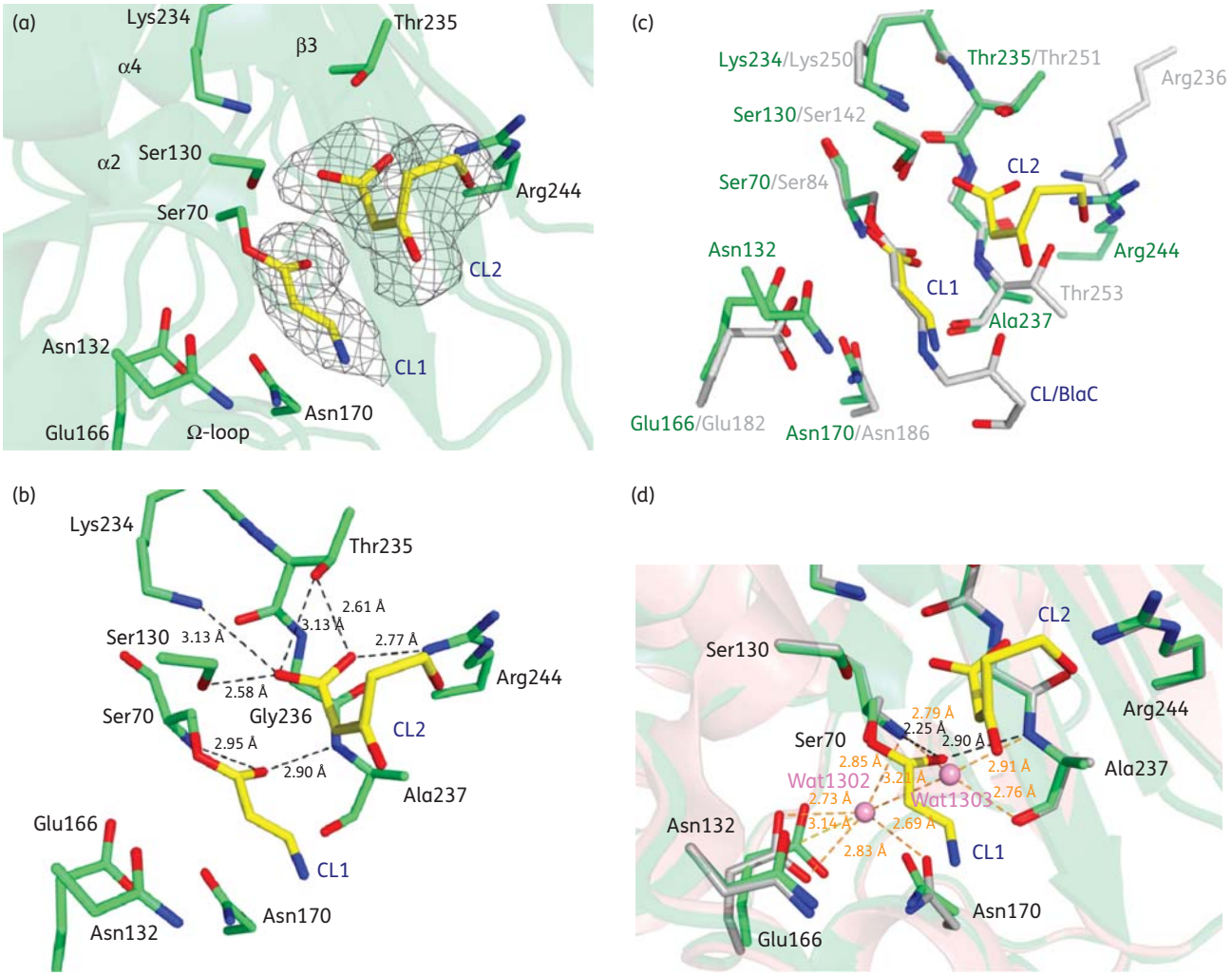

Figure 3. Detailed view of the structure of the active site of *B. licheniformis* BS3 β-lactamase in complex with clavulanate. (a) F_O-F_c map contoured at 2.5σ is shown in grey around clavulanate derivatives (in yellow). CL1, ethane-imine ester; CL2, pentan-3-one-5-ol acid. Important amino acid residues are shown in green. (b) Main hydrogen bonds implicated in the stabilization of CL1 and CL2, along with the corresponding distances (black broken lines). Other colour references: oxygen, red; nitrogen, blue; and sulphur, green. (c) Comparison of BS3 β-lactamase (in green) in complex with CL1/CL2 (yellow) and *M. tuberculosis* BlaC (3CG5) in complex with decarboxylated *trans*-enamine (in grey). Names of residues in green are those corresponding to BS3, following class A β-lactamase nomenclature, and the equivalent residues for *M. tuberculosis* BlaC are shown in grey, according to the numbering in the PDB structure (3CG5). (d) Comparison of BS3 β-lactamase (in green) in complex with CL1/CL2 (yellow) with the structure of BS3 apo-β-lactamase (PDB 1I2S, in grey). Wat1302 and Wat1303 belong to the BS3 apo-enzyme structure. Hydrogen bonds are shown as black broken lines for the complex and orange broken lines for the apo enzyme. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

difference is found between the two active sites. The following discussion will refer to monomer A.

The overall fold of the BS3/clavulanate complex is similar to the previously reported BS3 structure (PDB: 1I2S).[22] As other class A β-lactamases, the active site motifs are located in the interface between the 'all α' and 'α/β' domains, defined as 'Ser-Thr-Ile-Lys' (motif 1, carrying the nucleophile serine), 'Ser-Asp-Asn' (motif 2, in the loop between α4 and α5), and 'Lys-Thr-Gly' (motif 3, on strand β3), linked by the Ω-loop bearing α7 (Phe-165 to Glu-171).[22]

In the active site of both monomers we observed electron densities compatible with two different molecules derived from clavulanate hydrolysis, an ethane-imine ester (called CL1), and a pentan-3-one-5-ol acid (called CL2) (Figure 3a). CL1 is covalently linked to the active site serine. The β-lactam-derived ester carbonyl oxygen is connected by hydrogen bonds to backbone Ser-70 and Ala-237 nitrogen atoms, which define the oxyanion hole (Figure 3b). The distances between the carbonyl oxygen of CL1 and the backbone amide groups of the residues Ser-70 and Ala-237 are 2.95 and 2.90 Å, respectively, as described for other class A β-lactamases.[23]

The other density can be filled by a CL2 molecule, which is hydrogen bonded to the Ser-130 hydroxyl group (from the second motif), Lys-234Nζ, Thr-235Oγ (both part of the third motif) and Arg-244 through CL2 carboxyl-mediated hydrogen bonds (Figure 3b). For *Mycobacterium tuberculosis* BlaC

β-lactamase (PDB: 3CG5), a decarboxylated *trans*-enamine seems to be the major tautomer attached to the active site (Figure 3c), but, like BS3 β-lactamase, no evidence of a Ser-130 adduct was found.[24] In the BlaC structure, the decarboxylated clavulanate-derived enamine seems to be stabilized by several water molecules, which are not present in equivalent positions in the BS3/clavulanate structure (not shown).

The BS3/clavulanate adduct is solvated by 307 ordered water molecules. Compared with the structure of BS3 apo-β-lactamase, the absence in the structure of BS3 clavulanate adduct of the hydrolytic class A conserved water molecule implicated in the deacylation step explains the stability of the acyl-enzyme. A water molecule was reported in the BlaC/clavulanate structure, although slightly displaced from its position in the apo-enzyme (Figure 3d). The absence of water is accompanied by a slight displacement of the Ω-loop.

We compared the structures of BS3 in complex with clavulanate and other inhibitors such as lactivicin (PDB 2X71; Figure 4a), 6-β-iodopenicillanate (PDB 2WKO; Figure 4b), and cefoxitin (PDB 1I2W; Figure 4c). The overall structure of the BS3 backbone in complex with all these compounds is superimposable with that of BS3/clavulanate (not shown). Noteworthy is the superimposition between CL1 (linked to the active site serine) and part of the backbone of the lactivicin, 6-β-iodopenicillanate and cefoxitin molecules, and CL2 with lactivicin and cefoxitin. On the other hand, the Ω-loop motif (Phe165-Glu171) of the BS3/clavulanate backbone shows a slight deviation when compared with those from the other complexes (up to 0.848 Å).

Mass spectrometry

The presence of a covalent adduct between BS3 and CL1 has been confirmed by mass spectrometry. The major peak was observed for the apo-enzyme. The mass increment between BS3 (29478 ± 3 Da) and the most prominent minor peak in the spectrum of BS3 with clavulanate (295548 ± 3 Da) was 70, consistent with the formation of the covalently attached CL1 moiety.

Discussion

As in other class A β-lactamases, BS3 is inhibited by clavulanate following a complex scheme. Previous mass spectrometry and Raman crystallography studies on class A β-lactamases have pointed to a scheme involving hydrolysis of clavulanate, transient inhibition as well as irreversible enzyme inactivation.[9,15,25,26] The biphasic curves that we have observed with BS3 are in agreement with this complex scheme. Biphasic curves mean that the initial turnover rate of the enzyme is progressively reduced by the accumulation of one or more acyl-enzyme complexes that inactivate the enzyme. Biphasic curves are usually observed in the hydrolysis of clavulanate by class A β-lactamases, pointing to a branched pathway in which one path corresponds to the hydrolysis of clavulanate and the other refers to transient or irreversible inactivation.[27] The species involved in transient and irreversible inactivation are acyl-enzymes resulting from the opening of both the lactam and the oxazilidinone rings, and different fragments have been observed covalently bonded to the active serine.

Based on the structural similarities between clavulanate and benzylpenicillin, and the observed superposition of the BS3/clavulanate structure with the acyl-enzyme TEM-1-benzylpenicillin (PDB: 1FQG) (Figure 5a), a mechanism describing the inhibition of class A β-lactamases by clavulanate at the atomic level can be proposed (Figures 5 and 6). As a first step,

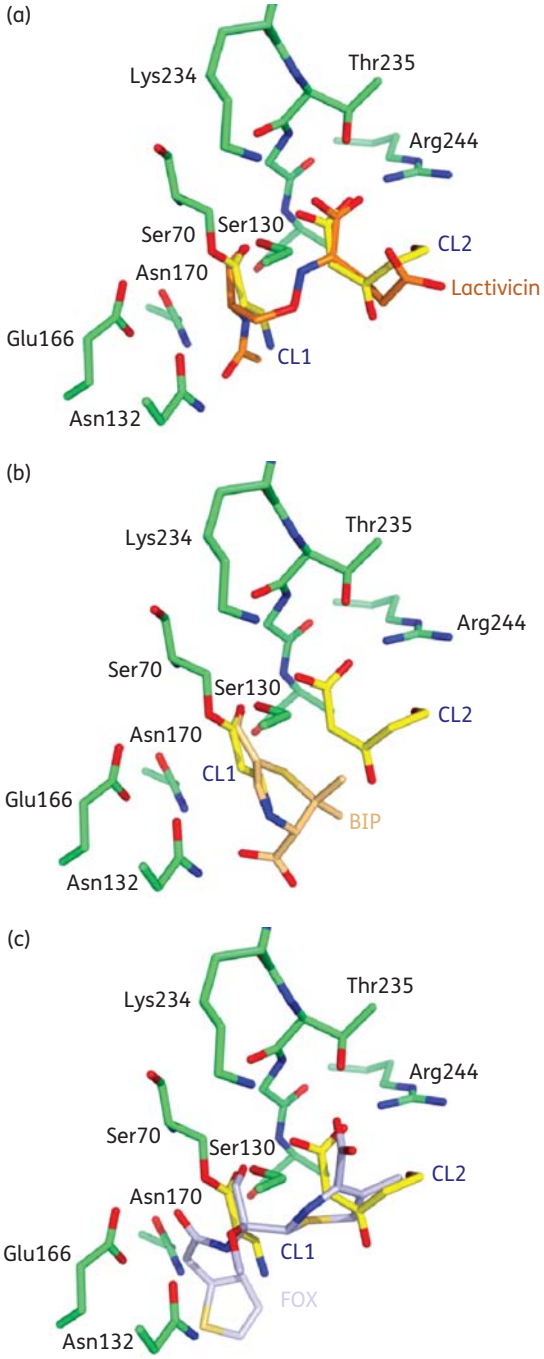

Figure 4. Comparison of the overall structure of the active site of the BS3/clavulanate complex (BS3 backbone in green; CL1 and CL2 in yellow) with equivalent adducts of BS3 in complex with (a) lactivicin (LTV; in orange), (b) 6-β-iodopenicillanate (BIP; in light orange) and (c) cefoxitin (FOX; in light blue). This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

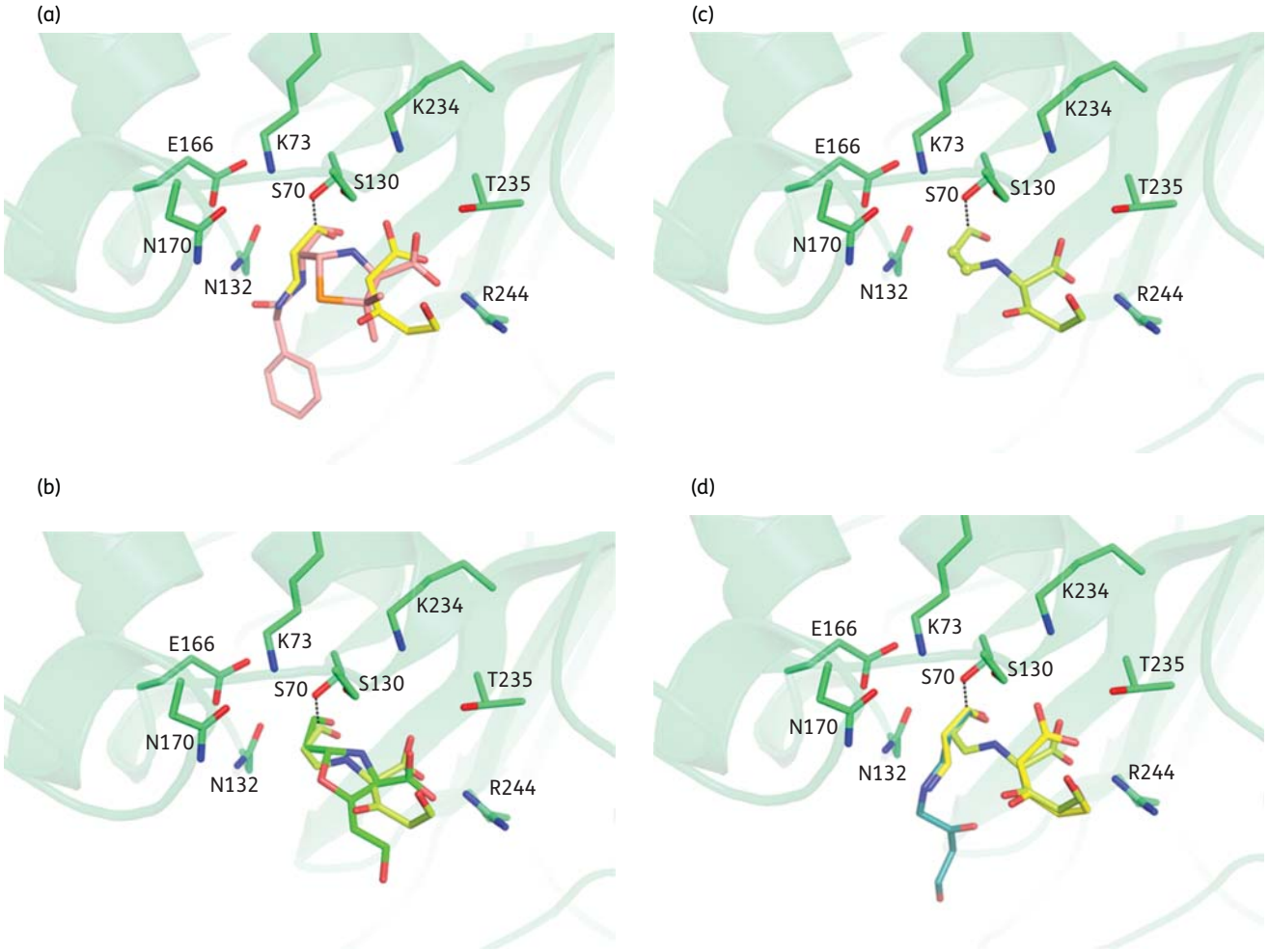

Figure 5. Illustration of the proposed mechanism for clavulanate-mediated inhibition of BS3 β-lactamase. (a) Comparison of BS3 β-lactamase (in green) in complex with CL1/CL2 (in yellow) and the acyl-enzyme TEM-1-benzylpenicillin (pink). (b) β-Lactam ring opening, followed by oxazolidine ring opening. (c) *cis* conformer of clavulanate. (d) Comparison between the *cis* conformer model of clavulanate and the fragments observed in BS3 (yellow) and Mtu (cyan). This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

one can assume that clavulanate enters the active site as penicillins do and that, upon acylation, β-lactam ring opening is immediately followed either by deacylation or by oxazolidine ring opening (Figure 5b and c). Upon oxazolidine ring opening, the C5–C6 bond becomes a double bond that can adopt a *cis* or a *trans* conformation. In the structure described in this article the C5–C6 bond adopts the *trans* conformation, as previously observed in PC1, *M. tuberculosis* β-lactamase (Mtu) and SHV-1, whereas the *cis* conformation has only been observed in PC1 β-lactamase. The *trans* conformation is associated with electronic delocalization on the C6–C7 bond that forces N4, C5, C6, C7, O8 and Ser-70Oγ atoms to be coplanar, as observed in all the structures with fragments of clavulanate in the *trans*-enamine configuration (Figure 5d). The clavulanate carboxylate is in strong interaction with Ser-130, Lys-234, Thr-235 and Arg-244, and the *trans*-enamination induces important stresses between the carboxylate and the carbonyl linked to the active serine. The weakening of the bonds around the C3 atom eventually results in loss of the carboxylate (as observed in PC1, Mtu and SHV-1) or the breaking of the C3–N4 bond, producing the two fragments observed in BS3.

The *cis*-enamine carboxylated species observed in PC1 can possibly undergo decarboxylation to give the *trans*-enamine decarboxylated species, but the contrary is unlikely. Moreover, the *cis* conformer may undergo deacylation, whereas the *trans* conformer is stabilized over deacylation by partial delocalization of the C5–C6 double bond on C6–C7, rendering C7 less susceptible to the attack of the nucleophilic water molecule. The *cis* conformer may thus be responsible for the transient inhibition, whereas the *trans* conformer should be responsible for irreversible inactivation. As suggested by Charnas *et al.*,[28] a direct enamine intermediate can be obtained by deprotonation of C7. The proximity between Glu-166 and C7, and the absence of water between Glu-166 and Ser-70 in the BS3/clavulanate structure argue in favour of Glu-166 as a good base candidate to achieve this deprotonation, in which case the imine intermediate is not necessary (Figure 6). Mass spectrometry experiments performed on TEM-1, SHV-1 and Mtu suggest that both forms of the

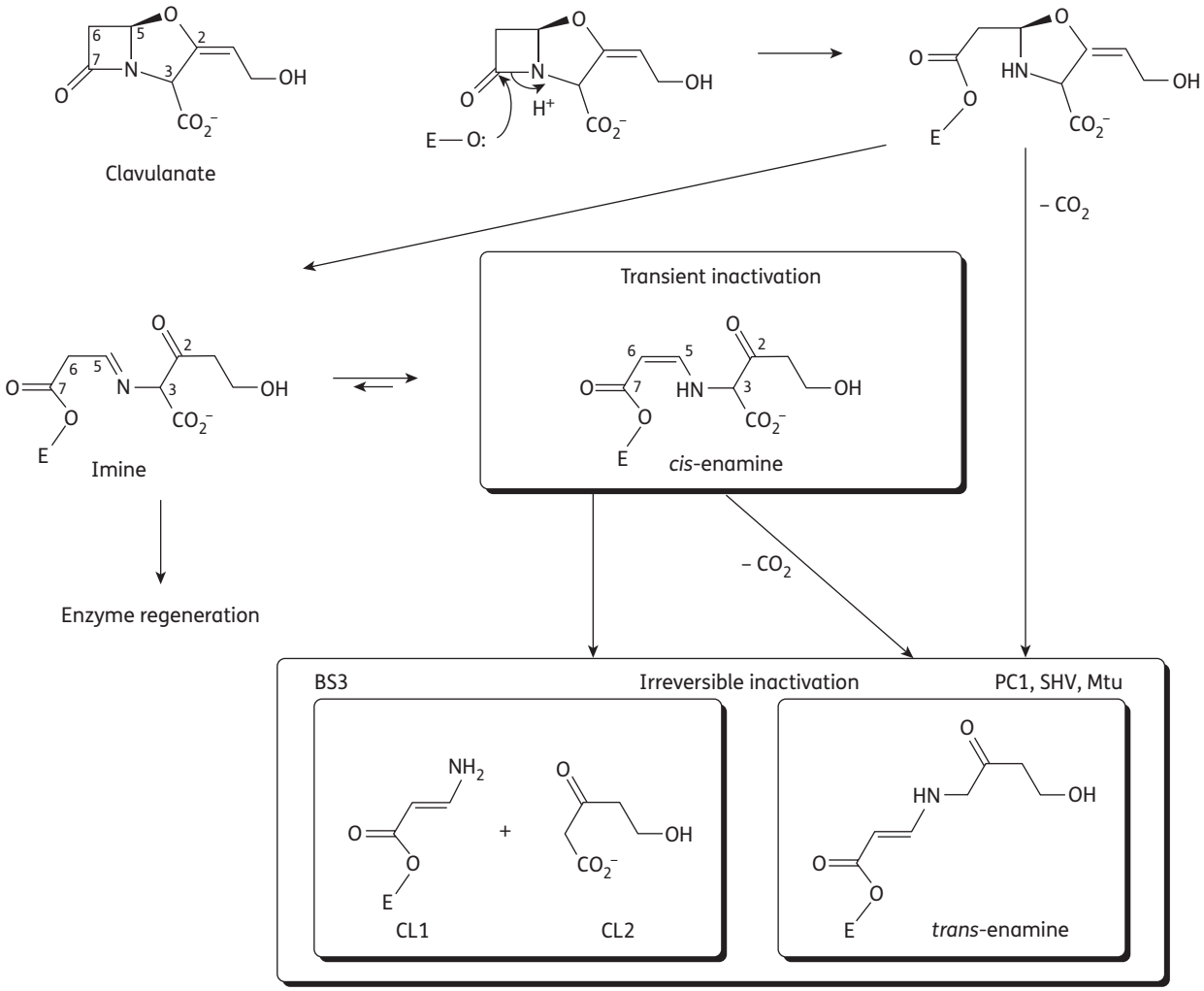

Figure 6. Updated mechanisms for the inactivation of class A β-lactamase by clavulanate based on X-ray structures.

irreversible complex observed by crystallography can presumably be present in the active site of class A β-lactamases, but partition between both pathways of the irreversible inhibition cannot be predicted from the sequence. Although different fragments of clavulanate are observed in BS3, SHV-1 and PC1, most residues of their active site are identical, and those that are different have no direct contact with the fragments of clavulanate. Moreover, they all have an arginine at position 244, which represents the main difference with Mtu. Yet the adduct in Mtu is similar to that observed in SHV-1.

Rather than being related to the partition between the observed fragments of clavulanate, the position of the arginine in the sequence could tentatively be related to the amount of clavulanate needed for inhibition. Inhibition constants observed in enzymes with the arginine at position 244 (TEM-1, SHV-1, BS3 and PC1) generally show lower values than those with an arginine at position 220 (Mtu, *Streptomyces clavuligerus*[29] and *Streptomyces albus* G[30]) or 276 (CTX-M-9[31] and *Mycobacterium fortuitum*[32]). Nevertheless other residues close to the active site clearly have an influence on the inhibition, notably the amino acid at position 69[33,34] or 276.[35]

Finally, TEM variants resistant to clavulanate (IRT) show various mutations of Arg-244, suggesting that the interaction between Arg-244 and the clavulanate carboxylate is essential for clavulanate inhibitory properties.[36] Alternatively, although less common than the mutation of arginine, the Ser130Gly mutation encountered in IRT-17 (TEM-59) also confers bacterial resistance to clavulanate.[37] The reduced number of interactions made by the clavulanate carboxylate may favour the hydrolysis path over the inhibition path resulting from oxazolidine ring opening.

Conclusions

Unlike structural results obtained from crystals of the SHV-1 β-lactamase or the β-lactamases from *S. aureus* and *M. tuberculosis*, the structure of clavulanate observed after its reaction with the β-lactamase from *B. licheniformis* BS3 is made of two moieties. Hydrolysis of clavulanate in two fragments results from interactions of the carboxylate with active site residues.

The interactions of the carboxylate of the CL2 moiety with conserved residues of class A β-lactamases represent a clue not only for explaining the clavulanate hydrolysis observed in

BS3, but also for understanding the decarboxylation of clavulanate leading to the decarboxylated *trans*-enamine species observed in the active site of PC1, Mtu and SHV-1.

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Transparency declarations

None to declare.

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