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OVERVIEW

Evolution of class C β-lactamases: factors influencing their hydrolysis and recognition mechanisms

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Abstract The most common bacterial resistance mechanism to β-lactam antibiotics is the production of β-lactamases. So far, β-lactamases have been classified into four different classes, three of them (A, C and D) have a serine in the active site as the nucleophilic group, which attacks to lactam antibiotic. Despite the large number of kinetic and theoretical studies and many native and complexed β-lactamases crystal structures, the mechanism by which they act is not well understood. The aim of this review is to show the different hypotheses which have been proposed to explain the hydrolysis mechanisms for class A and C lactamases and to cast light onto the interactions between the antibiotic and the *Enterobacter cloacae* P99 (a class C β-lactamase) in the Henry-Michaelis complex formed previous to the serine attack. Knowledge of these crucial points is essential for obtaining new β-lactam antibiotics not vulnerable to β-lactamases in order to minimize bacterial resistance.

Keywords β-lactamases · Hydrolysis mechanism · Preacylation complex

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1 Background

β-Lactams constitute one of the three largest antibiotic classes besides macrolides and fluoroquinolones [1,2], and are the most widely used antimicrobials on account of their efficacy, broad spectra and low toxicity. These substances inhibit bacterial penicillin-binding proteins (PBPs), which are involved in the final cross-linking process of the polymer layers of peptidoglycan, which constitute the bacterial cell wall. Consequently bacterial cell walls are weakened and bacterial autolysis occurs [3]. However, the efficacy of these antibiotics has fallen over the past 30 years due to the emergence of drug-resistant bacterial strains resulting from evolutionary responses to the widespread overuse and abuse of antibiotics in clinical and agricultural practice. An urgent need has thus arisen to understand the mechanism of bacterial resistance in order to develop new and more effective drugs [4].

The most common and effective resistance mechanism for bacteria involves the production of β-lactamases, which inactivate β-lactam antibiotics by hydrolysing the C-N bond in the β-lactam ring [4–6]. In Gram-negative bacteria, β-lactamases are constitutively expressed and restricted to the periplasmic space; on the other hand, in Gram-positive bacteria they are inducible and exocellular, even though they probably bind to cell wall via electrostatic interactions. Plasmid-mediated β-lactamases can be rapidly transferred between bacteria, thereby compromising the efficacy of β-lactam antibiotics.

Among other criteria, β-lactamases can be classified according to function (e.g., by their substrate profile or sensitivity towards specific inhibitors), physical properties (e.g., molecular weight), primary structure and amino acid homology. Also, they can be classified as serine β-lactamases and metallo-β-lactamases, depending on the nature of the

main component of their active site [7]. Serine β-lactamases belong to one of three different classes: A, C or D.

Class A enzymes are also called penicillinases on account of their high hydrolytic activity on penicillins. They comprise a large number of well-studied enzymes among which are TEM and SHV. The massive widespread use of β-lactam antibiotics has caused the parent enzymes TEM-1 and SHV-1 to undergo mutations, which have yielded more than one hundred variants of the former and more than twenty of the latter. Most variants exhibit a high catalytic efficiency and broader spectral activity; for this reason, they have been named Extended Spectrum β-Lactamases (ESBLs).

Class D enzymes are essentially OXA type enzymes, which have been frequently detected in *Pseudomonas aeruginosa*. Like TEM and SHV, these enzymes have evolved into at least thirty variants, some of which have an extended spectrum profile.

Class C β-lactamases constitute the second most common class of β-lactamase hydrolysing enzymes. These enzymes were originally termed cephalosporinases on account of their preference for cephalosporins as substrates, even though they exhibit a high hydrolytic activity on penicillins. They typically occur in Gram-negative bacteria and are chromosomally encoded [8]. The significance of class C β-lactamases has grown in parallel with their presence in organisms commonly found in hospital and community settings (e.g., *Klebsiella pneumoniae* and *Escherichia coli*) [9].

Classes A and C enzymes have been the most extensively studied so far for their ability to hydrolyse the two most important families of β-lactam antibiotics: penicillins (I in Fig. 1) and cephalosporins (II in Fig. 1). The hydrolytic efficiency of individual enzymes in these two classes depends on the nature of their active sites. An accurate knowledge of such sites and the role of each amino acid in the process are therefore crucial in deciphering the mechanism behind the molecular recognition of substrates and their hydrolysis. In addition, such knowledge can be useful to design more effective drugs against pathogenic bacteria.

In recent years, a large number of native and complexed β-lactamases have been obtained in crystallized form. Related information can be retrieved from the Protein Data Bank [10–22]. Such information revealed significant differences between the X-ray structures of class C and class A β-lactamases, particularly in the arrangement of the secondary structural elements. However, the superimposition of class C P99 enzyme and a representative class A enzyme has revealed the presence of a few active site residues which occupy similar positions in both enzymes (class C/class A), namely: Ser64/Ser70, Lys67/Lys73, Lys315/Lys234, Tyr150/Ser130, Thr316/Ser235, Ser318/Ala237, Asn152/Asn132 and Gln120/Asn104.

The large number of studies conducted on class A β-lactamases helped not only to elucidate the role in the hydrolysis of most amino acid residues at the active site, but also to assign the same roles to the residues occupying equivalent positions in class C β-lactamases. However, some residues can act differently depending on the particular enzyme class. A deep knowledge of the Henry-Michaelis complex and its interactions is crucial to elucidate the different hydrolysis mechanisms of β-lactamases.

In this review, we examine the state of the art for the Henry-Michaelis complexes of the class C enzyme β-lactamase P99 with various substrates. The close relationship of such complexes with the hydrolysis mechanism of β-lactamases led us to review the different schemes proposed for both class A and class C enzymes. A deep knowledge of these factors is essential in accomplishing one of today's most interesting scientific challenges: obtaining new lactam antibiotics that are not vulnerable to β-lactamases in order to minimize bacterial resistance.

2 Hydrolysis mechanism

The hydrolysis mechanism for class A and class C β-lactamases involves acylation (steps 1–3 in Fig. 2) and subsequent deacylation of the substrate (steps 4–6). During acylation, the nucleophilic serine is activated by having its hydroxyl proton abstracted by a base (step 1). Then, the β-lactam carbonyl of the antibiotic undergoes nucleophilic attack and a tetrahedral intermediate is formed (step 2), which evolves to an acyl-enzyme intermediate by cleavage of the C-N bond and protonation of the β-lactam nitrogen aton (step 3). During deacylation, the acyl-enzyme intermediate is hydrolysed. Following the nucleophilic attack by a water molecule previously activated by the acceptor base (step 4), the formed tetrahedral intermediate evolves to the hydrolysis end-product (step 5) and the enzyme is regenerated (step 6).

2.1 Hydrolysis mechanism for class A enzymes

There is wide agreement that the conserved Glu166 residue in class A enzymes activates the water molecule for the attack on the carbonyl (step 4) [23]. Recent QM/MM calculations on TEM-1 docked with benzylpenicillin [24] and the evidence that mutant Glu166Ala abolishes deacylation [25] support this mechanism. However, the acylation mechanism is controversial. Thus, the general base that activates Ser70 remains unknown and various acylation mechanisms involving different candidates have been proposed.

2.1.1 Hypothesis A

Oefner et al. [12] proposed Lys73 as the activating base. In fact, the results of some mutational studies and electrostatic analysis of X-ray structures suggest that this residue

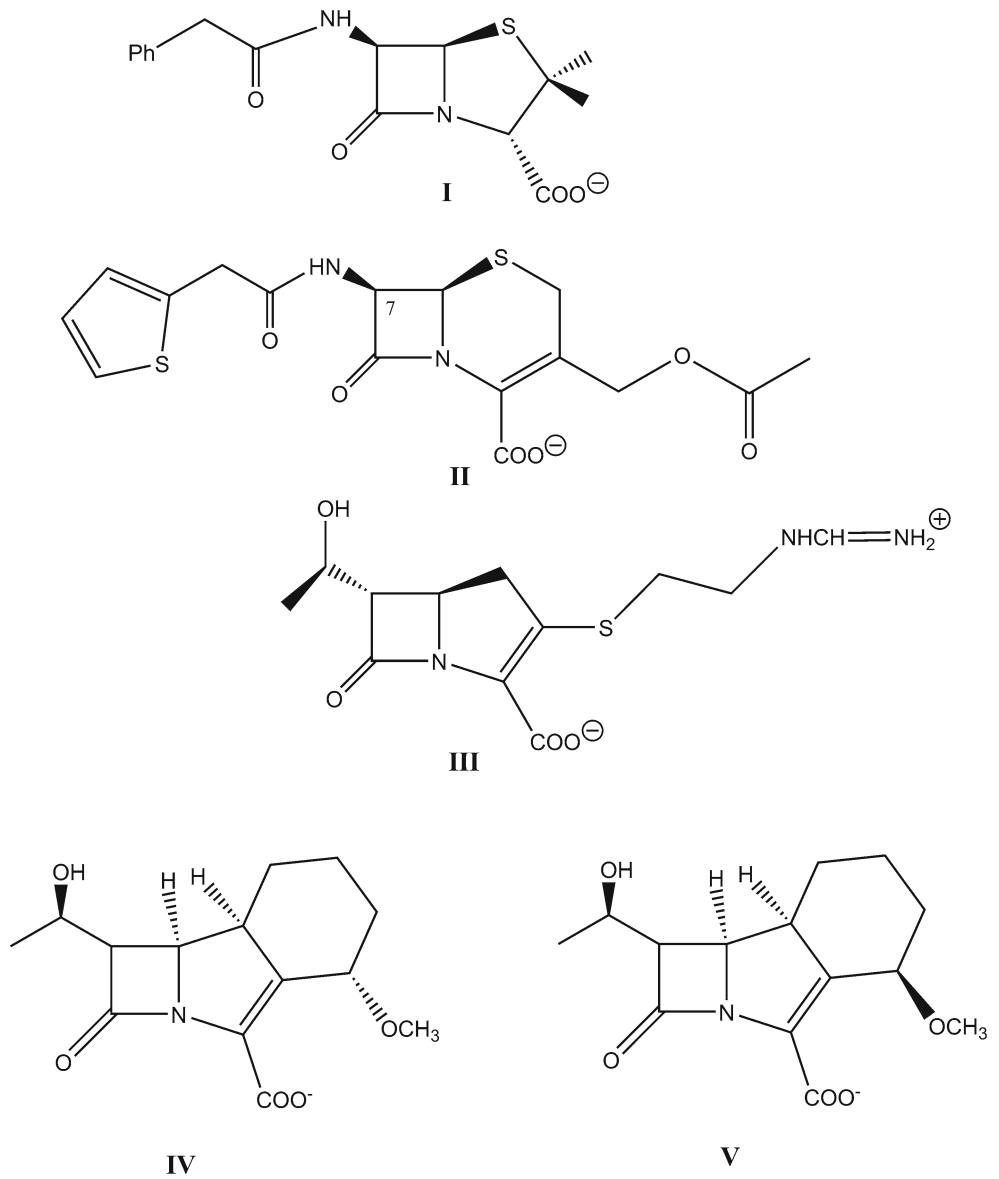

Fig. 1 Molecular structure of β-lactam compounds: penicillin G (I), cephalothin (II), imipenem (III), sanfetrinem (IV) and 4β-methoxy-trinem (V)

plays a prominent role in the structure and catalytic action of the enzymes and that the unusual neutral state of Lys73 is stabilized by its electrostatic environment at the active site and by substrate effects [26,27]. Using QM/MM calculations, Pitarch et al. [28] modelled the mechanism proposed by Strynadka et al. [27]. This mechanism would explain both the deprotonation of Ser70 and the nitrogen protonation by means of a sequence of proton transfers involving Ser70, Lys73 and Ser130. In any case, the results of other experimental and theoretical studies suggest that Lys73 is more likely to be protonated than neutral under physiological conditions [29–33]. In addition, Lys73 is associated with all major catalytic residues; therefore it plays a central role in the hydrogen-bonding network of the active site, thereby stabilizing the tetrahedral intermediate formed in the hydrolysis process.

2.1.2 Hypothesis B

One alternative mechanism involves Glu166 as the activating base via a water molecule. The protonated status of the residues at the active site of the enzyme suggests that Ser70 activation occurs via a double proton transfer involving Glu166, a water molecule and the serine residue itself. This mechanism is supported by some authors. By QM/MM calculations, Hermann et al. [34,35] proposed a concerted mechanism involving the simultaneous activation of Ser70

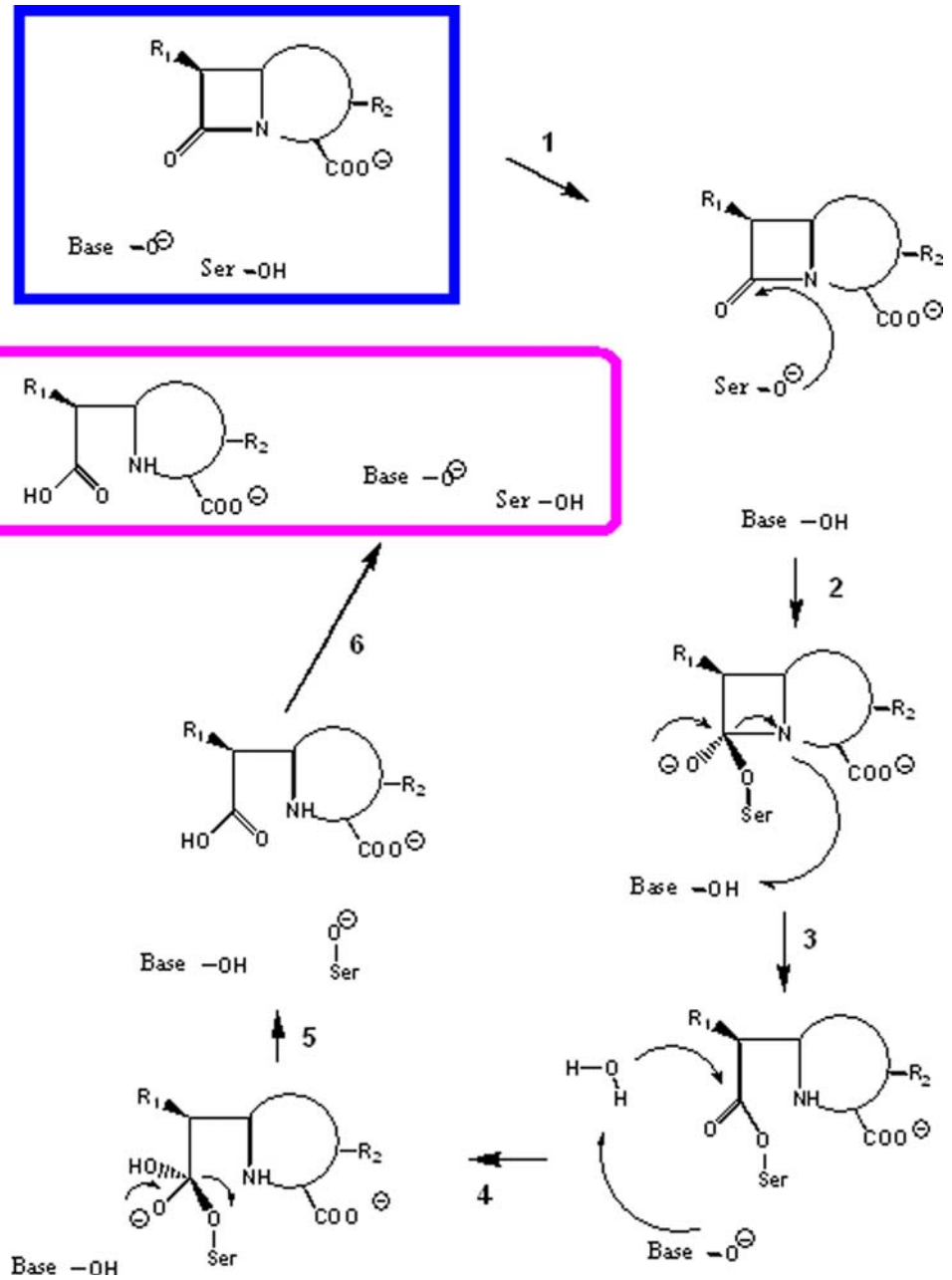

Fig. 2 General hydrolysis mechanism for class A and class C β-lactamases. Steps 1–3 represent acylation and steps 4–6 deacylation process

by Glu166 via a water molecule and the nucleophilic attack, the energy barrier for the process being 26 kcal/mol. Also, Glu166 was found to be protonated in one of the crystal structures analyzed by ultrahigh-resolution X-ray spectroscopy [36,37]. According to Díaz et al. [38], the Lys73→Glu166 proton transfer leading to an unprotonated Lys73 is energetically disfavored and, therefore, Lys73 is predicted to not be the general base in the acylation process and suggested that Glu166, via a water molecule, could abstract a proton from Ser70.

It has been also suggested that the replacement of Glu166 by a non-charged residue such as Asn in TEM-1 decreases its acylation rate constant by two orders of magnitude relative to the wild-type enzyme. This mutation yields mutants forming stable acyl-enzyme intermediates, the structure of which has been elucidated by X-ray crystallography [25,27]. These results confirm that Glu166 has a non-negligible effect on TEM-1 acylation; however, it does not prove that Glu166 has a clear-cut role in the acylation process. Recent results by Chen et al. [39] derived from the high-resolution CTX-M X-ray structure are consistent with this hypothesis.

Meroueh et al. [40] performed ONIOM calculations for TEM-1 that were consistent with a concerted Lys73 general base pathway for the formation of the tetrahedral

intermediate. This would compete favourably with an alternative pathway requiring an activation energy only 4 kcal/mol higher and involving the Glu166 as the general base. These results account for the apparently conflicting findings of some mutagenicity studies and reconcile the duality for Glu166 and Lys73 in serine activation. In any case, activation of Ser70 by Glu166 invariably occurs via a water molecule [25,40,41].

2.1.3 Hypothesis C

Diaz et al. [38] proposed a new mechanism where the Ser130 hydroxyl group and the substrate carboxylate group can also play an active kinetic role through a Ser130-OH→-OOC-benzylpenicillin proton transfer followed by a Ser70-OH→-O-Ser130 process. This mechanism and the reported previously, as hypothesis B, constitute competitive pathways for activating the hydroxyl group of Ser70 in the class A β-lactamases. However, this last mechanism has not been supported by any *ab initio* calculations regarding its energy feasibility.

The second step in the acylation process is the protonation of the β-lactam nitrogen, which is commonly accepted to be produced by Ser130 [40,42,43]. The results obtained by Meroueh et al. [40] and Hermann et al. [34] from QM/MM calculations suggest the involvement of Glu166 and Lys73 in the protonation of the β-lactam nitrogen; however, the former authors deemed Lys73 neutral and Glu166 deprotonated, whereas the latter suggested that both residues are protonated. Clearly, protonation of the β-lactam nitrogen is assisted by both Glu166 and Lys73.

2.2 Hydrolysis mechanism for class C enzymes

Unlike class A β-lactamases, there is no general agreement on which residue acts as the general base in activating Ser64 during acylation and a water molecule during deacylation. However, there is unanimous consensus on the significance of Tyr150 for both steps and its central role in the catalytic mechanism.

2.2.1 Acylation step

Studies on class C β-lactamases have suggested that Tyr150 may be deprotonated at physiological pH and may act as the general base activating Ser64 [12–14]. Once Ser64 is activated, Tyr150 donates the proton back to the β-lactam nitrogen in order to allow the collapse of the tetrahedral intermediate. The feasibility of this proposal was questioned by calculations based on the Poisson-Boltzmann methodology, which predicted an unusually low pK_a value (8.3) for Tyr150 in *Enterobacter cloacae* P99 [44], which does not support deprotonation of the hydroxyl. This is consistent with NMR and site mutagenesis studies. ^{13}C-NMR results have shown that the chemical shifts for Tyr150 remain unchanged up to pH 11 [45]; consequently the residue in the substrate-free enzyme must be neutral. The mutagenesis study by Dubus et al. [46] on the β-lactamase AmpC revealed that replacing Tyr150 in this enzyme with a Phe residue had little effect on its steady-state kinetics. These results are inconsistent with Tyr150 activating Ser64.

At this point, we must consider the possibility of Lys67 acting as the general base for Ser64 activation. This was first suggested by Strynadka et al. [27] for class A β-lactamases and subsequently supported by other authors. Tsukamoto et al. [47] noted the significance of a basic group at this position; the replacement of Lys67 with an Arg residue [47–50] was not found to alter the deacylating activity of the enzyme even though it resulted in dramatically reduced acylation. In any case, Lys67 should have an unusually low pK_a to act as a base in this mechanism; this contradicts the results by Damblon et al. [31] and Lamotte-Brasseur et al. [44], who obtained a pK_a close to 11 for Lys67. In the last years, new methods for calculations of pK_a have emerged and should be examined in order to determine the protonation state of these residues which it is essential to suggest a reaction mechanism.

In cephalothin and penicillin G complexes, Shoichet and co. [50] proposed the carboxylate of the substrate to accept a hydrogen bond from Tyr150, which accepts a hydrogen bond from Ser64, activating the serine for nucleophilic attack.

Kato-Toma et al. [45] proposed a mechanism where an OH^- from the solvent abstracts a proton from Tyr150. The cationic Lys67 (or Lys315), which are hydrogen-bonded to the phenolic oxygen atom, may neutralize this group. Since Lys67 is not exposed to the aqueous phase, the proton of Tyr150 is expected to be completely buried from the solvent. Although the previous results are inconclusive as regards the identity of the base catalyst, they introduce the widely accepted notion of cooperative action by Tyr150 and Lys67. In order to know the protonation configuration of the native form of class C enzymes, mixed QM/MM calculations on the acyl-enzyme intermediate formed by class C P99 enzyme with cephalothin suggest that a state with an anionic Tyr150 could be slightly more stable than one with a neutral Lys67. However, the computed energy difference between the two configurations (1.8 kcal/mol) was within the error range of the methodology [51]. Recently, Diaz et al. [52] analysed the protonation configurations of the native form of *C. freundii* class C β-lactamase and the acyl-enzime with aztreonam by using MD simulations and QM free-energy calculations. They obtained a very small energy difference between two protonation states (neutral Lys67 and protonated Tyr150; and protonated Lys67 and unprotonated Tyr150). Therefore, these results do not permit the identification of the protonation configuration in the native enzyme. However, the most favourable protonation configuration for the complex of P99

with aztreonam has a neutral Lys67 and a protonated Tyr150, therefore the authors suggest the mechanistic role of the neutral Lys67 is to be a proton acceptor from Ser64 during acylation of the enzyme.

2.2.2 *Deacylation step*

The deacylation step is also controversial. In fact, both the specific residue which activates the hydrolytic water molecule in this step, and the hydrolysis mechanism have been the subjects of various hypotheses.

One hypothesis has been formulated by Oefner et al. [12] and Lobkovsky et al. [13,18] and assumes deprotonated Tyr150 to act as the base activating the hydrolytic water molecule (W402). A study of the X-ray structure of the acyl-enzyme intermediate formed by the Tyr150Glu mutant AmpC and the loracarbef (a synthetic β-lactam antibiotic of the carbacephem class) revealed the significance of a hydroxyl group in that position. Moreover, the replacement of the hydroxyl group by a water molecule caused by the mutation in the native structure eventually establishes the same interactions as Tyr150, although the hydrolytic activity of AmpC decreases [14]. A recent theoretical study by Hata et al. [53] involving the deacylation of a class C β-lactamase provides support for this mechanism. These authors proposed Tyr150 as a water molecule activator with an activation energy of 30.5 kcal/mol. The role of Lys67 and Lys315 would be to stabilize the unprotonated form of Tyr150 during the process; this is consistent with the results by Monnaie et al. [48,49] that emphasized the role of Lys67 in maintaining electrostatic balance at the active site. However, the feasibility of this mechanism relies on the presence of unprotonated Tyr150, which is impossible judging from the results by Kato-Toma et al. [45] and Díaz et al. [52].

A second mechanism has been proposed by Mobashery and co. [54] from their analysis of the hydrolytic activity of the β-lactamase *Enterobacter cloacae* 908R on one compound with no β-lactam nitrogen. These authors found such a compound to undergo acylation, but not deacylation, and to act as an inhibitor ($k_{\text{inact}}/K_{\text{I}} = 34 \pm 5\,\text{M}^{-1}\text{s}^{-1}$), concluding that a substrate-assisted catalysis applies to this system. The analyses of the crystallographic structure of AmpC in a complex with boronic acid deacylation transition state analogue, as well as the crystallographic structure of the acyl-enzyme intermediate formed by AmpC with moxalactam [14] are consistent with this mechanism. Recent studies, done by Shoichet and co. [55], favour a model where the phenol form of Tyr150 stabilizes the tetrahedral deacylation transition state in conjunction with the lactam nitrogen atom of the substrate. In all cases, the authors emphasized the importance of the orientation of the hydrolytic water molecule, which allows the involvement of the ring nitrogen atom in the hydrolysis step. This is consistent with the pK_{a} value (5–6) assigned to this nitrogen atom [56].

Because the energy barrier for the activation of the water molecule by the β-lactam nitrogen has never been calculated, it is impossible to state whether the water is activated via Tyr150 or the nitrogen atom. This stresses the significance of theoretical calculations, either molecular dynamics or hybrid QM/MM, as useful and necessary supplements for studying these biological systems.

3 Formation of the Henry-Michaelis complex

We must examine the formation of the Henry-Michaelis complex upon the recognition of the substrate by the enzyme, before assigning a hydrolysis mechanism [57]. At this point theoretical simulations are very useful as the high reactivity of this complex makes its isolation and crystallization nearly impossible.

Over the past decade, our group has conducted part of the research on Henry-Michaelis complexes formed between various β-lactam compounds and class A *Staphylococcus aureus* PC1 [58–60] and class C *Enterobacter cloacae* P99 β-lactamases [60–63]. Following, we analyse the most important interactions of the Henry Michaelis complexes formed with *Enterobacter cloacae* P99 in order to help in the determination of the mechanism by which the enzyme can discriminate between penicillins and cephalosporins.

First, we should identify the specific residues of the active site that are directly involved in the substrate recognition process and establish a complete hydrogen-bonding recognition network for penicillins and cephalosporins. The interactions formed by each substrate can be grouped into three different subsets, corresponding to different regions of the complex: (1) the β-lactam carbonyl with the hydroxyl group of Ser64 and Ser318 (A, B in Fig. 3); C distance corresponds to the distance between the nucleophilic oxygen in Ser64 and the carbon of the carbonyl group in β-lactam compound; (2) the β-lactam carboxyl group with the hydroxyl groups of Tyr150, Thr316 and Ser318, and the amino groups of Lys315 and Asn346 (D, L, I, H, E and K in Fig. 3); (3) the β-lactam side chain with the amino groups of Asn152 and Gln120, and the carbonyl group of Ser318 (G, J and F in Fig. 3).

In all these complexes, we have considered the influence of the protonation state of some residues on the efficiency of the substrate recognition process. In fact, our Henry-Michaelis complexes are consistent with the two principal acylation mechanisms described above; therefore, once Ser64 is activated, both Tyr150 and Lys67 are in protonated form.

Based on the results, carboxyl and carbonyl groups in penicillins and cephalosporins exhibit rather different binding patterns. Our results indicate that O_1 in the carboxyl group of penicillins and cephalosporins are similarly oriented

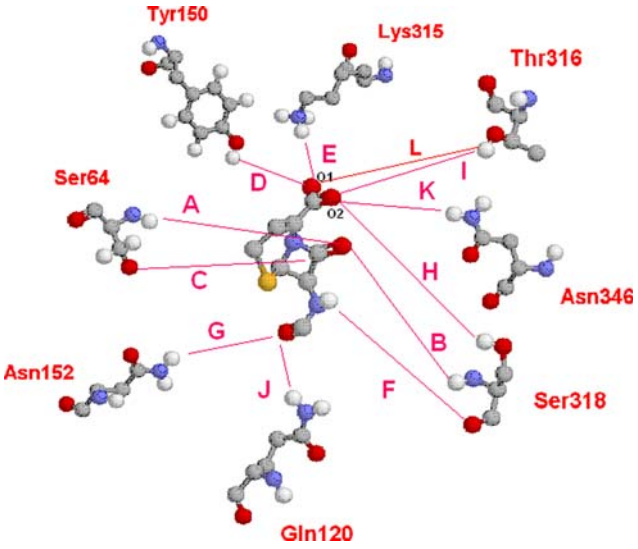

Fig. 3 Schematic representation of the interactions between the active site amino acids in class C β-lactamase P99 and a lactam compound

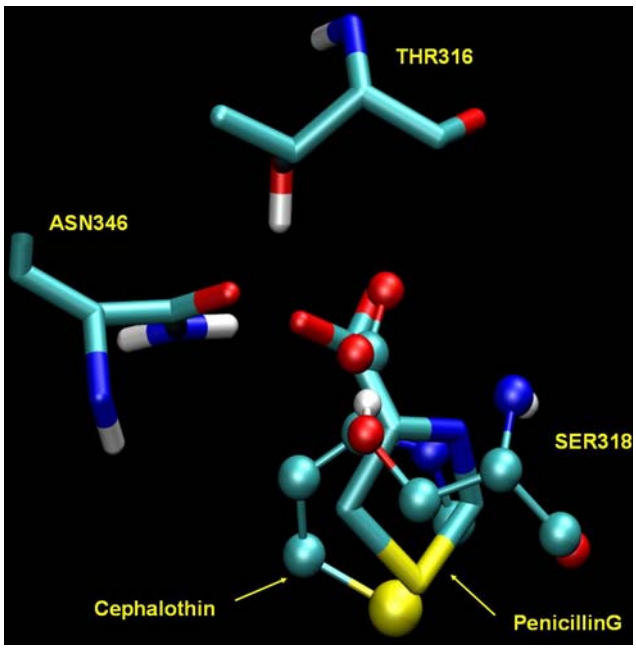

Fig. 4 Representation of the different orientation of carboxylic group in penicillin G and cephalothin in the Henry-Michaelis obtained with P99 β-lactamase. The specific spatial disposition of one of the oxygen atoms in the carboxyl group allows the interaction with Thr316 and Asn346 (penicillin G) or with Ser 318 (cephalothin)

showing always two hydrogen bond interactions (D and E). Moreover, atom O_2 in penicillins can interact with Asn346 and Thr316 (K and I), whereas O_2 in cephalosporins can only interact with Ser318 (H). This behaviour could be related to the different spatial orientation of the carboxyl group in penicillins and cephalosporins (Fig. 4).

As the carboxyl group is the first to interact with the residues in the active site, the different interactions established by this group in both types of substrates will dictate the subsequent relative orientation of the rest of the antibiotic and hence, those of the other groups in the β-lactam. As a matter of fact, the carbonyl group in penicillin G forms two hydrogen bonds with the residues in the oxyanion hole (A with Ser64 and B with Ser318), whereas the corresponding carbonyl group in cephalosporin forms just one (B, with Ser318). Finally, the amido group in the side chain of both penicillins and cephalosporins establishes the same interactions; this is a result of side chain intrinsic mobility, which allows the molecule to rotate in order to adopt a favourable conformation for interacting with Ser318, Asn152 and Gln120 (F, G and J, respectively).

This specific arrangement of Ser318, Thr316 and Asn346 allows the enzyme to discriminate between penicillins and cephalosporins [61,62]. If the lactam compound interacts with the hydroxyl group of Ser318, the enzyme recognizes the substrate as a cephalosporin, whereas if the interactions are with Thr316 and Asn346, it recognizes it as a penicillin.

The binding patterns proposed by our group can explain an important number of experimental facts such as why Ala237Thr and Ala237Asn mutations in TEM1 (position 237 in class A β-lactamases is equivalent to 318 in class C β-lactamases) increase the activity of the enzyme against cephalosporins by up to 380% [64–67] or why Ser237Ala and Thr237Ala mutations on class A K1, CTX-M-4, Sme1 and PER1 reduce their activity against cephalosporins up to 4 times [68–71]. The mutations performed in TEM1 introduce a functional group capable of establishing H interactions; as a result, the enzyme can recognize cephalosporins as substrates. On the other hand, the mutations in K1, CTX-M-4, Sme1 and PER1 remove such a group and the enzymes can no longer recognize cephalosporins as substrates. On the other hand, Thr316Ala and Thr316Val mutations in AmpC slightly reduce its hydrolytic activity on penicillins [27,72]. In fact, based on our binding pattern, the enzyme loses the I interaction but can still establish K interactions with the carboxyl group in penicillins and recognize them as substrates. As a consequence, molecular dynamics of the complex between P99 and penicillin G, where Thr316 and Asn346 have been mutated to Ala, show the gradual destabilization of the complex through the loss of the enzyme ability to recognize penicillins as substrates [62]. Although no experimental data is available to confirm it so far, this hypothesis illustrates the usefulness of molecular dynamics calculations in driving or supplementing experimental work.

The ability of the P99 to discriminate between penicillins and cephalosporins at the substrate-recognition stage is mainly the result of the disparate spatial orientation of the carboxyl groups. Going deeper into this subject requires knowledge of the behaviour of a new family of β-lactams:

carbapenems (III in Fig. 1). These compounds are structurally similar to penicillins (they also contain a five-membered ring fused to the β-lactam ring), but their carboxyl group is oriented as in cephalosporins. In the modelled Henry-Michaelis complex between P99 and the imipenem (a member of the carbapenem family), O_1 atom in imipenem shows the interaction with Lys315 (E) and other new interaction with Thr316 (L), but it is 3.45 Å away from Tyr150. Also, atom O_2 interacts with Ser318 (H), as in cephalosporins, but not with Thr316 or Asn346 [63].

These results show a different role of the carboxyl group of the antibiotic in the binding of the substrate to the active site and can induce a new acylation mechanism. In cephalothin and penicillin G complexes, the presence of the carboxylate in the active site decreases the distance between the phenol O-atom in Tyr150 and the amine N-atom in Lys315 (3.58 Å in the native enzyme and 2.85 and 2.92 Å in penicillin G and cephalothin, respectively) and slightly increases that one between the phenol O-atom in Tyr150 and the amine N-atom in Lys67 (2.86 Å in the native enzyme and 2.69 and 3.12 in penicillin G and cephalothin, respectively). This rearrangement agrees with the hypothesis of Shoichet and co. who assigned the acid group to activate the Tyr150 [50]. In the case of the imipenem, the special arrangement of the carboxylate group and the presence of a hydrophobic methyl group in C(6) increases the distance between Tyr150 and Ser64, hindering the proton exchange between Ser64 and Tyr150 [63].

The proposed recognition patterns for penicillins, cephalosporins and carbapenems [61–63] are crucial for determining whether the enzyme can recognize other compounds as substrates, depending on the particular interactions with their carboxyl groups. In fact, the substrate must be appropriately oriented in the binding pocket in order to react with the enzyme upon recognition.

In order to check the recognition pattern proposed, we studied the Henry-Michaelis complexes between sanfetrinem (IV in Fig. 1) and 4β-methoxy-trinem (V in Fig. 1) with P99. The orientation of sanfetrinem within the active site of P99 was similar to that of cephalothin and only slightly different from that of imipenem. Therefore, sanfetrinem can be expected to bind to P99 active site and act either as a good substrate (similar to cephalothin) or as an inhibitor (similarly to imipenem). Atom O_2 in sanfetrinem only interacts with the hydroxyl group of Ser318 (H interaction), whereas O_1 interacts with Tyr150, Lys315 and Thr316 (D, E and L interactions) [60]. However, the similarities between sanfetrinem and cephalothin are not related to the carbonyl group, and sanfetrinem forms a binding pattern similar to that of imipenem, involving the formation of two strong hydrogen bonds with Ser64 and Ser318. The presence of a smaller side chain in sanfetrinem as compared to cephalothin allows the former to approach Ser64 more closely and the carbonyl group to interact with both Ser64 and Ser318. The methoxy group exhibits no steric hindrance or interactions with the enzyme in sanfetrinem or 4β-methoxy-trinem. Therefore, both are similarly orientated.

Another important issue concerning β-lactams is the presence in 7α (the carbon atom close to the carbonyl group of the four membered ring) of a methoxy group with a direct influence on the hydrolytic activity of the enzyme on these compounds. The position of the methoxy group prevents the compound from adopting an optimal orientation similar to those of penicillins and cephalosporins within the active site. In fact, our results show that the methyl group on the α-face of cefoxitin pushes the compound out of the active site, whereas that in cephalothin does not. As a result, neither the side chain nor the carbonyl group in cefoxitin can interact with the necessary enzyme residues and the attack distance exceeds 3 Å (3.6 Å) [61], which is consistent with the low activity of the enzyme towards this compound.

These results can indeed help to explain the way the active site recognizes lactams; in fact, recognition is the first step in the hydrolytic process, since the efficiency of the hydrolysis reaction depends on the substrate orientation. Understanding the mechanism by which these substrates are recognized can be very useful with a view to designing more effective drugs. Research should be focused on mutation studies on highly conserved residues in the active site of the enzyme, which can result in substrate selectivity changes [73], or on changing or inserting functional groups in the potential substrates, which can redirect the process towards hydrolysis or inhibition. Obviously, a deeper knowledge of the subject is required in order to completely elucidate the mechanism of action of β-lactamases. This can be a laborious process, where theoretical calculations based on molecular dynamics or hybrid methods can be very useful in supporting experimental results. However, the outcome can help us further approach the final goal: reducing the bacterial resistance induced by β-lactamases and lay the foundations for designing antibiotics with a higher resistance and broader antibacterial spectrum.

4 Theoretical methodology

Theoretical calculations have been widely used because are an useful complement to experimental results. All the references cited in this paper use basically molecular dynamics (MD) simulations in order to analyze the Henry Michaelis complexes and combined quantum mechanics/molecular (QM/MM) methods as a good approach to the investigation of enzyme-catalysed reaction mechanisms.

The conditions of MD have been changing during the years. Most of the simulations [38,40,52,60,62] used a constant pressure and temperature controlled by Berendsen's method, periodic boundary conditions to simulate a

continuous system, a time step of 1.5–2.0 fs and the SHAKE algorithm to constrain all bonds involving hydrogen atoms. The length of the trajectories has been increasing until the 2–3 ns considered in the last papers and the two force fields mainly used are AMBER and CHARMM. The protein atoms, as well as all the water molecules of the crystal structure are surrounded by a periodic box of TIP3P water molecules in most of the simulations [38,40,52]. On the other hand, most of the bond, angle and dihedral parameters of β-lactam compounds are available from the force field, however some structural data required to represent the equilibrium geometry of the β-lactam ring and of the acylamido side chain were extracted from the HF calculations of the optimized structure. Atomic charges are also computed using the RESP fitting procedure on the gas phase HF/6-31G* electrostatic potential.

Quantum/classical methods treat a reduced part of the system at a quantum mechanical level while the rest is described using molecular mechanics. The location of the stationary points, minima and transition state structures that characterize a reaction mechanism must be carried out taking into account the coupling between the classical and quantum subsystems. Generally four o five link atoms are introduced in the interface between the QM and the MM regions. Main differences arise from the QM/MM level. Different groups [24,28,34,35] used a semiempirical AM1-CHARMM QM/MM level to optimize the structures, though high level energy corrections (B3LYP/6-31G+(d)//AM1-CHARM22) were later applied by Hermann et al. [24,34] to obtain more reliable reaction energetics. Gherman et al. [51] used a B3LYP/6-31G*-OPLS-AA level to optimize the structures, followed by a single point calculations using the cc-pVTZ(-f) correlation consistent basis set of Dunning and coworkers [74]. They also use an adiabatic approach to optimization in which the MM region is fully optimized after each QM step, resulting in a very large reductions in computational effort as compare to carrying out a QM gradient evaluation at each geometry step. Meroueh et al. [40] used a HF3-21G/AMBER scheme for optimization, followed of single point energy calculation using MP2/6-31+G*. Most of these calculations have a large QM region which contains the entire substrate, the catalytic water molecule and the sidechain atoms of the main aminoacids (Ser70, Lys73, Ser130, Lys315 and Glu166 for class A β-lactamases and the analogues for class C enzymes). Finally, Hata et al. [53] built a QM model (HF/6-31G**) of the system in which the enzyme was soaked in thousands of TIP3P water molecules.

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