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COMMENTARY

THE ENIGMATIC CATALYTIC MECHANISM OF ACTIVE-SITE SERINE β-LACTAMASES

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The success of β-lactam antibiotics in antibacterial chemotherapy rests on both their high efficiency and their specificity. They interfere with the last stage of peptidoglycan synthesis, a transacylation reaction, most often involving a D-alanyl-D-alanine terminated peptide. This reaction, catalysed by enzymes attached to the outer face of the cytoplasmic membrane, is unique to the bacterial world and has absolutely no equivalent in eucaryotic cells [1]. But bacteria have discovered various stratagems to escape the action of these compounds, and in consequence new molecules have to be progressively introduced in the chemotherapeutic arsenal, exhibiting structures that are increasingly different from those of the original penicillins (Fig. 1). As described at length in the 15 April, 1994 issue of *Science*, resistance phenomena have recently become increasingly widespread and worrisome. Three distinct mechanisms have been identified.

Intrinsic resistance is due to the synthesis of enzyme targets, the DD-transpeptidases, with a strongly decreased affinity for the antibiotics. These enzymes, also identified as PBPs§, are active-site serine enzymes, and their reaction with β-lactam antibiotics results in the formation of long-lived acylenzymes (Fig. 2) that are unable to fulfil their physiological roles, leading to the formation of non-functional peptidoglycan and, eventually, to cell death [2]. The resistant PBPs are very slowly acylated by the β-lactams, the k_2/K' values being as low as $10\,M^{-1}sec^{-1}$ versus up to $300{,}000\,M^{-1}sec^{-1}$ for penicillin-sensitive DD-peptidases [1, 2].

The diffusion of the antibiotic to its membrane-bound targets can also be impeded by the outer layers of the cell wall itself, the "outer membrane" in Gram-negative bacteria [3–5], and the mycolic acid layers of the Gram-positive mycobacteria. In the former, increased resistance has been attributed to modifications of the porins, proteins that form hydrophilic channels in the outer membrane and allow small, water-soluble molecules to penetrate into the periplasmic space [3].

Finally, bacteria can produce enzymes that chemically modify the antibiotics: acylases, esterases or β-lactamases (Fig. 1). Of these, only β-lactamases appear to be seriously involved in resistance phenomena. By contrast to those formed after the action of the two other types of hydrolytic enzymes, the products obtained by opening the β-lactam ring (Fig. 2) are completely devoid of significant antibacterial activity, and β-lactamases presently represent a major threat to the efficiency of the β-lactam family of antibiotics [6, 7].

Metallo and active-site serine β-lactamases

Some Zn^{2+}-containing metallo-β-lactamases have been described. Although only produced by a relatively small number of pathogenic strains of the *Bacteroides*, *Xhantomonas*, *Aeromonas*, *Pseudomonas* and *Serratia* genera [8], they represent a potential threat because they efficiently hydrolyse carbapenems, compounds that generally escape the activity of the more common active-site serine β-lactamases. It can be predicted that the increased utilization of this type of antibiotic will result in the spreading of genes coding for the Zn^{2+}-β-lactamases to an ever larger number of strains, and it would be advisable to develop the search for specific inhibitors of these enzymes.

However, the active-site serine β-lactamases represent a more immediate problem. They hydrolyse the antibiotics according to the acylation/deacylation pathway described by Fig. 2 [6]. Their efficiency can be really frightening. Some enzymes have indeed been described as "perfect catalysts" [9], with k_2/K' ratios close to the diffusion-limit values ($10^8\,M^{-1}sec^{-1}$) combined with very high k_3 values ($4000\,sec^{-1}$). Moreover, in constitutive mutants of Gram-negative strains, enormous amounts of enzyme are produced, yielding periplasmic β-lactamase concentrations up to 1 mM [10]. Under these conditions, a sensitive antibiotic has no chance of ever reaching its DD-peptidase target. A detailed understanding of the catalytic mechanism of these enzymes is thus essential to the design of new molecules that can escape their hydrolytic action.

The three classes of active-site serine β-lactamases

On the basis of their primary structures these

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§ Abbreviation: PBPs, penicillin-binding proteins.

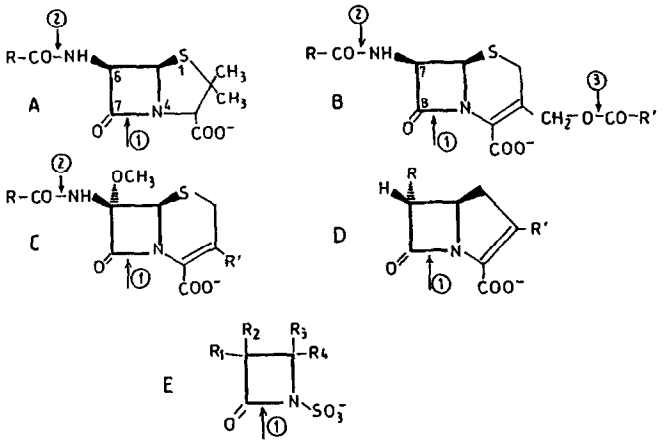

Fig. 1. Structures of penicillins (A), cephalosporins (B), cephamycins (C), carbapenems (D) and monobactams (E). The arrows indicate the sites of action of β-lactamases ①, acylases ② and esterases ③. Note that some cephalosporins do not exhibit an ester side-chain on C_3. The chemical structure common to all these compounds is the four-membered β-lactam ring, highlighted by heavier lines.

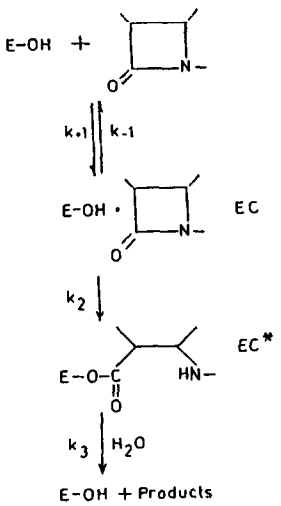

Fig. 2. Interaction between active-site serine penicillin-recognizing enzymes (E-OH) and a β-lactam. Formation of a non-covalent complex, EC, is followed by the acylation of the active-site serine residue side-chain. The acylenzyme EC* subsequently undergoes hydrolysis, regenerating the active enzyme. With DD-peptidases, the value of k_3 is very low, generally smaller than 10^{-3}sec^{-1}. The inactivating efficiency of a β-lactam is then characterized by the k_2/K' ratio [where $K' = (k_{-1} + k_2)/k_{+1}$]. With β-lactamases, k_3 is very high, up to several thousand sec^{-1}, which results in a rapid turn-over of the antibiotic.

enzymes have been divided into three molecular classes: A, C and D (for historical reasons, class B contains the metallo-β-lactamases). Within each class, the catalytic properties can exhibit strong variations, but the sequences are clearly homologous and new enzymes are easily distributed among these classes [6].

By contrast, computer-aided sequence comparisons fail to reveal significant isology between members of different classes [11]. However, the tertiary structures of various enzymes of classes A and C have been established by X-ray crystallography [12–16], and striking similarities in the general fold of the proteins have been detected (Fig. 3). They comprise an all-α and an α/β domain, with the active serine situated in a depression between the two domains, at the N-terminus of the long α-2 hydrophobic helix mostly buried in the core of the all-α domain. Moreover, these studies have demonstrated the existence of three structural and functional elements, located in equivalent positions in the three-dimensional structures [17] and containing residues with identical or chemically similar side-chains (Table 1).

The first element contains the active serine. After two variable residues, whose side-chains point away from the active site, a lysine is invariably found. Due to the helical structure of this element, the lysine side-chain lies in the active site, where it forms a hydrogen bond with the active serine hydroxyl group.

The second element is situated on a loop in the all-α domain. The side-chains of the first and third residues border the active site, while that of the second is in the core of the protein. The first residue (Ser or Tyr) bears a hydroxyl group and the third is nearly always an asparagine, with one single exception in a class A enzyme (Ser).

The third element on the innermost strand of the β-pleated sheet forms the opposite wall of the catalytic cavity. Although some variations are observed in class A enzymes, the first residue is positively charged (Lys, sometimes Arg), the second is hydroxylated (Thr or Ser), and the third is always Gly. In fact, any side-chain in this latter position would protrude into the active site and sterically hinder the interaction between most substrates and the active serine.

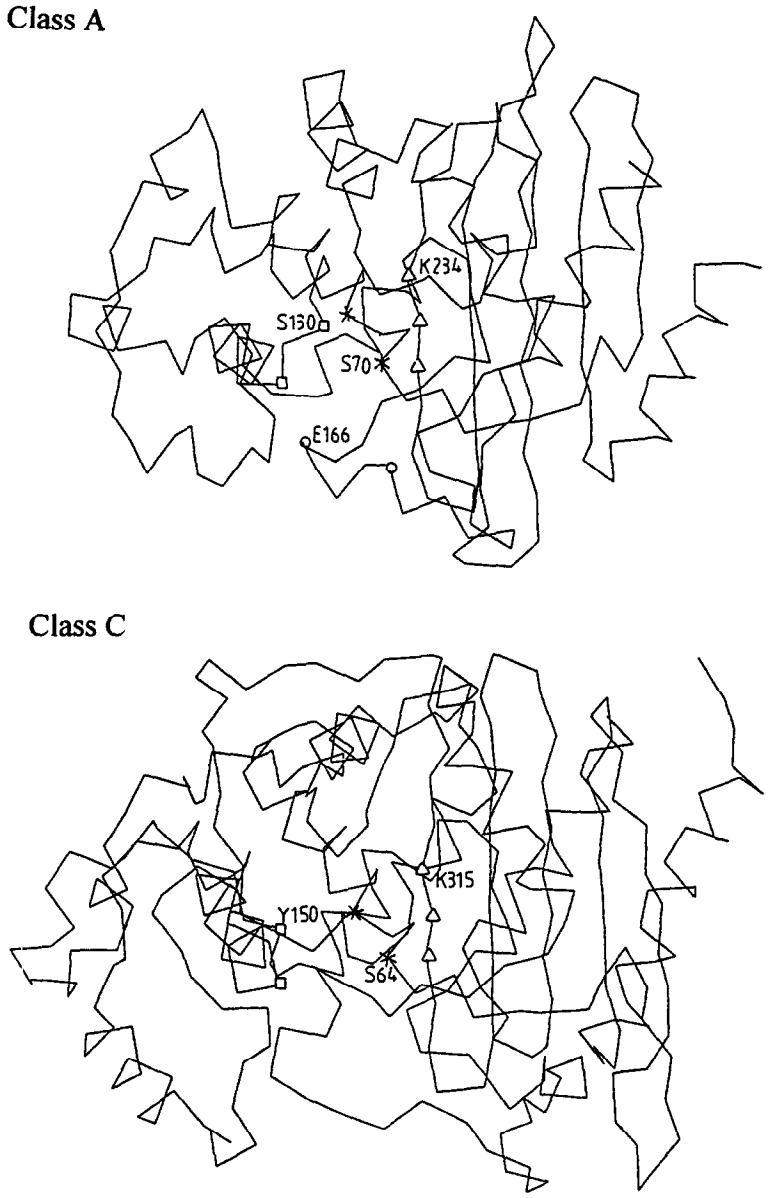

Fig. 3. Tertiary structures, represented by the α-carbon traces, of the *Streptomyces albus* G (class A) and the *Enterobacter cloacae* P99 (class C) β-lactamases. The active-site serine (*) and the residues of the equivalent structural elements are labelled [element 1 (*); element 2 (□); and element 3 (△)].

Site-directed mutagenesis experiments have shown that replacement of the active serine by a cysteine can yield active enzymes [18, 19], but naturally occurring cysteine β-lactamases remain to be found.

No class D β-lactamase tertiary structure has been determined, but sequence alignments allow the identification of the three structural elements [17]. Moreover, the elucidation of the *Streptomyces* R61 DD-peptidase structure reveals the presence of equivalent elements that nicely superimpose on those of the class A and C β-lactamases, results that underline close relationships between the two families of penicillin-recognizing enzymes [16, 20]. The three elements can be identified in the sequences of all PBPs, irrespective of the exact affinity that these enzymes exhibit for the β-lactam antibiotics.

Mechanistic considerations

The catalytic mechanism of other active-site serine amidases and esterases has been studied in detail [21, 22]. In many of these, a histidine residue acting as a general base activates the serine during the acylation process and the hydrolytic water molecule during the deacylation. The oxyanion, which is expected to be formed at the level of the tetrahedral intermediate, is also stabilized by two hydrogen

Table 1. Three equivalent functional elements of active-site serine β-lactamases and penicillin-sensitive DD-peptidases

	Element 1	Element 2	Element 3
	70	130	234
Class A	S*-X-X-K	S-D-N	K-T-G
		S-D-S	K-S-G
			R-S-G
	64	150	315
Class C	S*-X-X-K	Y-A-N	K-T-G
	70	144	214
Class D	S*-X-X-K	Y-G-N	K-T-G
	62	159	298
S. R61 DD-peptidase	S*-X-X-K	Y-S-N	H-T-G
Other known PBPs	S*-X-X-K	S-X-N	K-T-G
		S-X-C	
		Y-G-N	

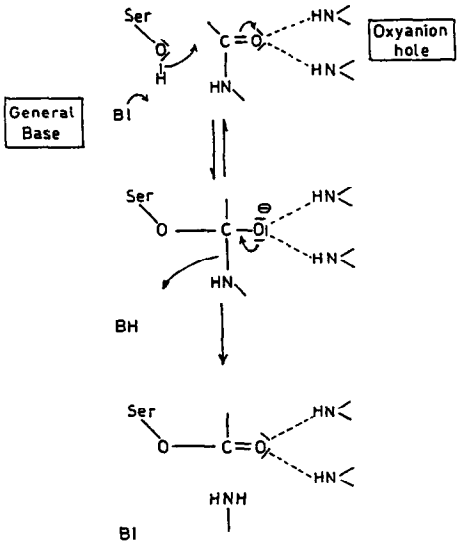

Fig. 4. Formation of the tetrahedral intermediate during the hydrolysis of a peptide bond by an active-site serine enzyme, showing the combined actions of a general base (B1) and of the oxyanion hole and collapse of the tetrahedral intermediate to yield the acylenzyme.

bonds, with the main chain NH-groups of Ser 195 and Gly 193 in the case of chymotrypsin (Fig. 4). Docking of the penicillin molecule in the active sites of various β-lactamases and of the *S.* R61 DD-peptidase indicates that similar "oxyanion holes" can be located in all of the structures, comprising the main chain NH-groups of Ser 70 and Ala 237 in the class A enzymes, Ser 64 and Ser/Ala 318 in class C, and Ser 62 and Thr 301 in the DD-peptidase [12, 13, 15, 16, 20]. Conversely, no conserved histidine is found in β-lactamases and PBPs. The *Streptomyces albus* G class A β-lactamase does not

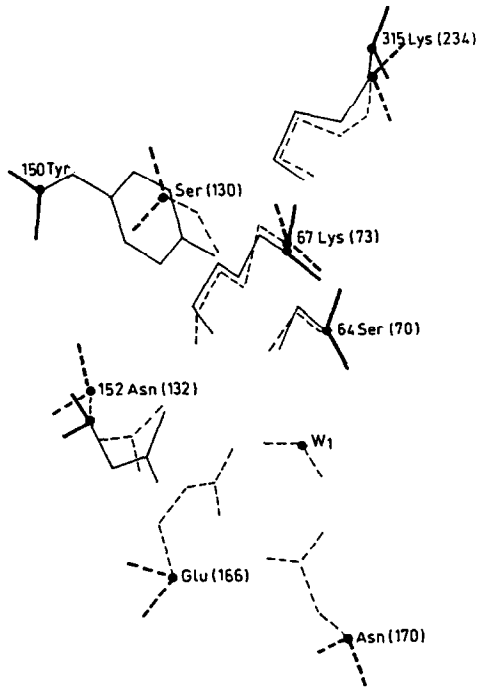

Fig. 5. Superposition of the active-site residues of a class A β-lactamase (dashed lines) with the corresponding residues of a class C enzyme (redrawn from Ref. 16). Heavy lines represent the backbone bonds and the α-carbons are labelled by dots. The residue numbers for the class A enzyme are given between parentheses.

contain even one such residue [23]. In consequence, the search for a general base in β-lactamases has generated a large number of studies and strong controversy. If one assumes that a unique mechanism is valid for all active-serine β-lactamases, the sole likely candidate is the Lys residue of the first element, which should accordingly exhibit a strongly lowered pK_a. An alternative hypothesis, recognizing that the β-lactam amide is intrinsically less stable than its peptide counterpart, assumes that the generally positive environment of the active site, with two Lys side-chains and the dipole of the α-helix, might be sufficient to activate the serine hydroxyl group [13].

In class A β-lactamases, a third possibility involves a glutamate residue, which is conspicuously absent in the other enzymes, and whose modification results in a very serious impairment of the catalytic process. The possible mechanisms are now discussed on the basis of the known three-dimensional structures and in the light of the results obtained by site-directed mutagenesis.

Glu 166 in class A enzymes. This residue is situated on an Ω loop, which borders the catalytic cavity [12–14, 24]. Its side-chain takes part in a dense hydrogen bond network within the active site. No corresponding residue is found in that position in the class C enzyme or in the *S.* R61 DD-peptidase structure (Fig. 5). Its importance in the catalytic process was revealed by

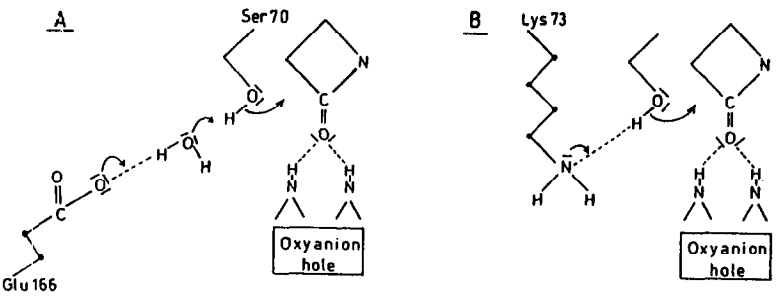

Fig. 6. Alternative activations of the active-site serine in class A β-lactamases (A) by Glu 166 via the conserved water molecule, and (B) by a deprotonated Lys 73.

the study of a natural variant of the *Staphylococcus aureus* and of the Glu166Asp mutant of the *Bacillus cereus* I enzymes [25, 26]. The detailed study of this latter protein obtained by site-directed mutagenesis indicated that both the k_2 and k_3 first-order rate constants were decreased by the same, 2000-fold factor, results that suggested a similar role for this residue in both acylation and deacylation processes. However, the structures show that the distance between its carboxylate group and the active serine hydroxyl is too long to allow a direct proton transfer between the two side-chains. But the crystallographic and modelling data [24] clearly show that a conserved water molecule may serve as a relay in this proton transfer (Fig. 6). This hypothesis is strengthened by modelling results obtained by "docking" cefoxitin in the enzyme active site. This molecule, which contains a methoxy group on C7 (Fig. 1), acylates the active serine with exceedingly poor efficiency. It does, nevertheless, easily fit into the active site, but the 7-methoxy group displaces the water molecule [27], which can explain why no further reaction occurs after the formation of the non-covalent EC complex. Studies performed with the class A enzyme from *Staphylococcus aureus* PC1 and a covalently bound phosphonate acting as a transient state analog seemed to confirm this hypothesis [28].

The behaviour of other mutants where Glu 166 has been replaced by other residues is, however, not very consistent. In some of these, both acylation and deacylation are impaired, in agreement with a pivotal role of Glu 166 in both processes [26, 29, 30], but in others acylation appears to occur readily, while deacylation is extremely slow [31, 32]. A surprising observation was that the same mutation, Glu166Ala, in two very similar enzymes from *Bacillus cereus* [30] and *Bacillus licheniformis* [31] more strongly affected the acylation rate with the first enzyme than with the second, whereas the inverse situation prevailed for deacylation. In consequence, it is generally accepted that Glu 166 acts as a general base in the deacylation reaction by activating the hydrolytic water molecule, but its exact role in the acylation process remains much more poorly understood.

The Lys residue of the first element

The Glu166Asn mutant of the TEM class A β-lactamase is one of those proteins where acylation readily occurs but which fails to deacylate efficiently [32]. To explain these results, it has been proposed that the deprotonated side-chain of Lys 73 might accept the serine proton in acylation, while the role of Glu 166 would be restricted to the deacylation step. This is only possible if the enzyme active site environment decreases the pK of the ammonium group of Lys 73 by 5–6 pH units.

According to the authors, such an exceptionally great effect could be due to a very positive electric field created by the α-2 helix dipole, the ammonium group of Lys 234 (element 3) and, possibly, some other residues, such as Arg 244 and Arg 164, located somewhat further away from the active-site serine. However, this hypothesis, which rests on electrostatic factors, is clearly undermined by the fact that the carboxylate group of Glu 166 is much closer to the ε-NH_2/NH_3^+ group of Lys 73 than is any positively charged side-chain [13, 24], and, in fact, one would even expect the elimination of the negatively charged carboxylate to *increase* the nucleophilicity of the Lys 73 side-chain. Moreover, as shown by Gibson *et al.* [26], the Lys73Arg mutation in the *B. cereus* enzyme decreases the k_2 value only 70-fold, i.e. significantly less than the Glu166Asp mutation in the same enzyme, a result that seems to indicate a more crucial role for this latter residue [26].

The positive environment around the active serine

As discussed above, the results of the modifications of the Glu 166 side-chain appear to contradict this hypothesis. In addition, modification of Asn 170 in the *B. licheniformis* enzyme, a residue that helps to maintain both the water molecule and the side-chain of Glu 166 in their observed positions (Fig. 5), also results in decreased acylation rates.*

The same hypothesis, however, appears more likely to be valid for class C β-lactamases since the absence of a residue equivalent to the class A Glu 166 results in a distinctly more positive active site in the former enzymes (and also possibly in class D β-lactamases). However, by superimposing the active serine side-chain and the two oxyanion hole NH-groups of the *Citrobacter freundii* class C enzyme on the corresponding group of chymotrypsin, Oefner *et al.* [15] observed that the Tyr 150 oxygen atom of

* Fink AL, personal communication. Cited with permission.

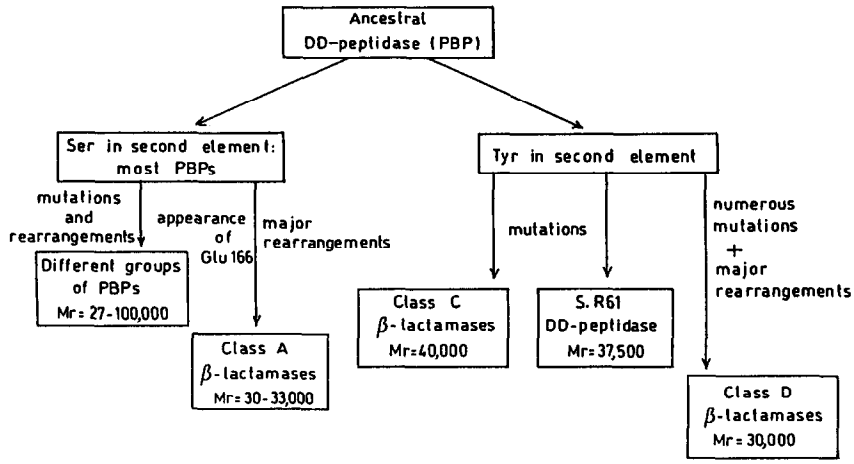

Fig. 7. Tentative phylogeny of DD-peptidases and β-lactamases. As shown by the M_r values, the rearrangements often involve the loss of large parts of the polypeptide chains.

the former was in a position equivalent to the proton-accepting imidazole nitrogen of His 57 in the latter, thus making Tyr 150 a possible candidate for the role of general base. But again, the pK of this residue should be very low, around 5–6, to account for the pH–activity profile of the enzyme, a value that might be explained by electrostatic considerations. Crystallographic results obtained with a phosphonate monoester inhibitor and another class C enzyme from *Enterobacter cloacae* confirmed this hypothesis [33]. Accordingly, the activity of the Tyr150Phe mutant was generally decreased in a very significant way [34], and the properties of the Lys315His and Lys315Gln mutants seemed to indicate that this latter residue was mainly responsible for increasing the nucleophilic properties of another side-chain [35]. However, the Tyr150Phe mutant retained most of its activity on poor substrates, suggesting a different, more complex role for the Tyr 150 side-chain [34]. Although modifications of Lys 67 of the *E. cloacae* enzyme also resulted in lowered acylation rates, this seemed to be due to an increase of the K' value rather than to a decrease of k_2, at least with poor substrates [36]. The interpretation of the experimental data is very often made quite hazardous by the fact that, for technical reasons, it is not possible to determine the individual values of k_2 and K' for good substrates, and, in consequence, the effects of the various mutations become very difficult to assess.

Protonation of the leaving group

In chymotrypsin catalysis, protonation is thought to occur via the back-delivery of the proton first accepted by the general base (His 57, Fig. 4). In class C, if Tyr 150 is indeed the general base (but see above), a similar mechanism could prevail. However, in class A enzymes, this is certainly not so. Crystallographic and modelling studies of the structure of the acylenzyme indicate that the hydroxyl group of Ser 130 might be ideally located to fulfil this function [24, 32], which is only possible if a proton is simultaneously transferred onto the same side-chain, since the instability of the alcoholate ion precludes its formation as an end product. The dense array of hydrogen bonds within the active site would make this exchange possible, with the proton originating from Glu 166 or Lys 73, depending upon the identity of the general base.

Conclusions

Despite the striking similarities found in their tertiary structures and the equivalent positions occupied by chemically identical or similar groups, it seems difficult to propose a unique mechanism for the hydrolysis of β-lactams by class A and class C enzymes. Since with the latter, deacylation is often the rate-limiting step in the reaction pathway, it is tempting to hypothesize that Glu 166 was "invented" by class A enzymes to accelerate this final step. It would subsequently have been "recruited" for playing a similar role in acylation. But, as stated above, its identification as the general base in the acylation process is not supported by all the experimental evidence unless, in some mutants where Glu 166 is replaced by an uncharged residue and for which the electrostatic argumentation might appear more convincing, a deprotonated Lys 73 could replace it as the general base. Moreover, slow deacylation is not an absolute property of class C enzymes: with some substrates, this occurs at very respectable rates [37], an observation that is also valid for the less well-studied class D enzymes [38], which also lack an equivalent of Glu 166, but like their class C counterparts, contain a Tyr residue in the second element.

When the comparison is extended to the DD-peptidases, further difficulties arise. If the intrinsic reactivity of the β-lactam amide could be considered as partly responsible for the relative efficiency of the active serine acylation by penicillins, it is not so with the physiological peptide substrates. As in class C enzymes, the Tyr residue of the second element may participate in the activation of the serine, but most

PBPs contain a serine in the equivalent position, and it seems unlikely that the peptide bond might be broken without the participation of additional side-chains that remain to be identified.

If one assumes that all active-site serine penicillin-recognizing enzymes share a common ancestor, an early divergence must have occurred at the level of the second element between the "tyrosine" and the "serine" enzymes (Fig. 7). Additional mutations then occurred in "R61-like DD-peptidases" to yield the class C and class D enzymes. Similarly, the "serine" PBPs would have evolved into class A β-lactamases, a process in which the most significant step would have been a major rearrangement leading to the formation of the Ω loop, with the essential Glu 166 [16], thus ensuring an efficient deacylation. Moreover, it should be stressed that, in both evolutionary pathways, the mutations leading to the β-lactamases also increase the acylation efficiencies by β-lactams and result in the complete loss of peptidase activities. However, both β-lactamases and DD-peptidases recognize depsipeptide substrates, where the NH-group of the scissile bond has been replaced by O or S [39, 40]. As suggested by Murphy and Pratt [41], β-lactamases have lost the ability of acting on the relatively unreactive, planar peptide bond, but can readily attack the more reactive and flexible ester and thiolester bonds.

We are presently witnessing a continuation of this evolutionary process. Two strategies have been utilized in fighting β-lactamase-producing bacteria: the introduction of "β-lactamase stable" β-lactams and the simultaneous administration of a β-lactamase inactivator with a classical, β-lactamase-sensitive compound. Bacteria have efficiently countered both strategies. In the first case, enzymes have emerged that efficiently hydrolyse the stable compounds. These were either completely new enzymes, initially produced by little-studied strains [42], or mutants of well-known proteins, differing from the parent enzymes by only a few mutations [7]. Such point mutations have also been involved recently in the appearance of inactivator-resistant enzymes [43, 44], but at least one novel enzyme has also been described on which one of the most widely utilized inactivators became rather inefficient [45]. The struggle against pathogenic bacteria is still far from over, and the need for a better understanding of the exact mechanism of β-lactamase catalysis is thus more crucial that ever.

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REFERENCES

1. Frère J-M, Nguyen-Distèche M, Coyette J and Joris B, Mode of action: Interaction with the penicillin binding proteins. In: *The Chemistry of β-lactams* (Ed. Page MI), pp. 148–195. Blackie Academic & Professional, an imprint of Chapman & Hall, Glasgow, 1992.
2. Frère J-M and Joris B, Penicillin-sensitive enzymes in peptidoglycan biosynthesis. *CRC Crit Rev Microbiol* **11**: 299–396, 1985.
3. Nikaido H and Normark S, Sensitivity of *Escherichia coli* to various β-lactams is determined by the interplay of outer membrane permeability and degradation by periplasmic β-lactamase: A quantitative predictive treatment. *Mol Microbiol* **1**: 29–36, 1987.
4. Waley SG, An explicit model for bacterial resistance: Application to beta-lactam antibiotics. *Microbiol Sci* **4**: 143–146, 1987.
5. Frère J-M, Joris B, Crine M and Martin HH, Quantitative relationship between sensitivity to β-lactam antibiotics and β-lactamase production in Gram-negative bacteria—II. Non-steady-state treatment and progress curves. *Biochem Pharmacol* **38**: 1427–1433, 1989.
6. Waley SG, β-lactamase: Mechanism of action. In: *The Chemistry of β-lactams* (Ed. Page MI), pp. 198–228. Blackie Academic & Professional, an imprint of Chapman & Hall, Glasgow, 1992.
7. Coyette J, Nguyen-Distèche M, Lamotte-Brasseur J, Joris B, Fonzé E and Frère J-M, Molecular adaptations in resistance to penicillins and other β-lactam antibiotics. *Adv Comp Environ Physiol* **20**: 233–267, 1994.
8. Payne DJ, Metallo-β-lactamases—a new therapeutic challenge. *J Med Microbiol* **39**: 93–99, 1993.
9. Christensen H, Martin MT and Waley SG, β-Lactamases as fully efficient enzymes. Determination of all the rate constants in the acyl-enzyme mechanism. *Biochem J* **266**: 853–861, 1990.
10. Hechler U, Van Den Weghe M, Martin HH and Frère J-M, Overproduced β-lactamase and the outer membrane barrier as resistance factors in *Serratia marcescens* highly resistant to β-lactamase stable β-lactam antibiotics. *J Gen Microbiol* **135**: 1275–1290, 1989.
11. Joris B, Ghuysen J-M, Dive G, Renard A, Dideberg O, Charlier P, Frère J-M, Kelly J, Boyington JC, Moews PC and Knox JR, The active-site-serine penicillin-recognizing enzymes as members of the *Streptomyces* R61 DD-peptidase family. *Biochem J* **250**: 313–324, 1988.
12. Herzberg O, Refined crystal structure of β-lactamase from *Staphylococcus aureus* PC1 at 2.0 Å resolution. *J Mol Biol* **217**: 701–719, 1991.
13. Moews PC, Knox JR, Dideberg O, Charlier P and Frère J-M, β-Lactamase of *Bacilus licheniformis* 749/C at 2 Å resolution. *Proteins Struct Funct Genet* **7**: 156–171, 1990.
14. Jelsch C, Lenfant F, Masson JM and Samama JP, β-Lactamase TEM1 of *E. coli*. Crystal structure determination at 2.5 Å resolution. *FEBS Lett* **299**: 135–142, 1992.
15. Oefner C, D'Arcy A, Daly JJ, Gubernator K, Charnas RL, Heinze I, Hubschwerlen C and Winkler FK, Refined crystal structure of β-lactamase from *Citrobacter freundii* indicates a mechanism for β-lactam hydrolysis. *Nature* **343**: 284–288, 1990.
16. Lobkovsky E, Moews PC, Liu H, Zhao H, Frère J-M and Knox JR, Evolution of an enzyme activity: Crystallographic structure at 2-Å resolution of cephalosporinase from the *amp* C gene of *Enterobacter cloacae* P99 and comparison with a class A penicillinase. *Proc Natl Acad Sci USA* **90**: 11257–11261, 1994.
17. Joris B, Ledent P, Dideberg O, Fonzé, Lamotte-Brasseur J, Kelly JA, Ghuysen JM and Frère J-M, Comparison of the sequences of class-A β-lactamases and of the secondary structure elements of penicillin-recognizing proteins. *Antimicrob Agents Chemother* **35**: 2294–2301, 1991.
18. Sigal IS, Harwood BG and Arentzen R, Thiol-β-lactamase: Replacement of the active-site serine of

RTEM β-lactamase by a cysteine residue. *Proc Natl Acad Sci USA* **79**: 7157–7160, 1982.
19. Jacob F, Joris B and Frère J-M, Active-site serine mutants of the *Streptomyces albus* G β-lactamase. *Biochem J* **277**: 647–652, 1991.
20. Kelly JA, Knox JR, Zhao H, Frère J-M and Ghuysen JM, Crystallographic mapping of β-lactams bound to a D-alanyl-D-alanine peptidase target enzyme. *J Mol Biol* **209**: 281–295, 1989.
21. Fersht AR, Blow DM and Fastrez J, Leaving group specificity in the chymotrypsin-catalysed hydrolysis of peptides. A stereochemical interpretation. *Biochemistry* **12**: 2035–2041, 1973.
22. Brady L, Brzozowski AM, Derewenda Z, Dodson E, Dodson G, Tolley S, Turkenburg JP, Christiansen L, Huge-Jensen B, Norskov L, Thim L and Menge U, A serine protease triad forms the catalytic centre of a triacylglycerol lipase. *Nature* **343**: 767–770, 1990.
23. Ambler RP, Coulson AFW, Frère J-M, Ghuysen J-M, Joris B, Levesque RC, Tiraby G and Waley SG, A standard numbering scheme for the class A β-lactamases. *Biochem J* **276**: 269–270, 1991.
24. Lamotte-Brasseur J, Dive G, Dideberg O, Charlier P, Frère J-M and Ghuysen JM, Mechanism of acyl transfer by the class A serine β-lactamase of *Streptomyces albus* G. *Biochem J* **279**: 213–221, 1991.
25. Herzberg O, Kapadia G, Blanco B, Smith TS and Coulson A, Structural basis for the inactivation of the P54 mutant of β-lactamase from *Staphylococcus aureus* PC1. *Biochemistry* **30**: 9503–9509, 1991.
26. Gibson RM, Christensen H and Waley SG, Site-directed mutagenesis of β-lactamase I. Single and double mutants of Glu-166 and Lys-73. *Biochem J* **272**: 613–619, 1990.
27. Matagne A, Lamotte-Brasseur J, Dive G, Knox JR and Frère J-M, Interactions between active-site serine β-lactamases and compounds bearing a methoxy side chain on the face of the β-lactam ring: Kinetic and molecular modeling studies. *Biochem J* **293**: 607–611, 1993.
28. Rahil J and Pratt RF, Characterization of covalently bound enzyme inhibitors as transition-state analogs by protein stability measurements: Phosphonate monoester inhibitors of a β-lactamase. *Biochemistry* **33**: 116–125, 1994.
29. Delaire M, Lenfant F, Labia R and Masson JM, Site-directed mutagenesis on TEM-1 beta-lactamase. Role of Glu 166 in catalysis and substrate binding. *Protein Eng* **4**: 805–810, 1991.
30. Leung Y-C, Robinson CV, Aplin RT and Waley SG, Site-directed mutagenesis of β-lactamase I: Role of Glu 166. *Biochem J* **299**: 671–678, 1994.
31. Escobar WA, Tan AK and Fink AL, Site-directed mutagenesis of β-lactamase leading to accumulation of a catalytic intermediate. *Biochemistry* **30**: 10783–10787, 1991.
32. Strynadka NCJ, Adachi H, Jensen SE, Johns K, Sielecki A, Betzel C, Sutoh K and James MNJ, Molecular structure of the acyl-enzyme intermediate in β-lactam hydrolysis at 1.7 Å resolution. *Nature* **359**: 700–705, 1992.
33. Lobkovsky E, Billings EM, Moews PC, Rahil J, Pratt RF and Knox JR, Crystallographic structure of a phosphonate derivative of the *Enterobacter cloacae* P99 cephalosporinase: Mechanistic interpretation of a β-lactamase transition state analog. *Biochemistry* **33**: 6762–6772, 1994.
34. Dubus A, Normark S, Kania M and Page MGP, The role of tyrosine 150 in catalysis of β-lactam hydrolysis by AmpC β-lactamase from *Escherichia coli* investigated by site-directed mutagenesis. *Biochemistry* **33**: 8577–8586, 1994.
35. Monnaie D, Dubus A, Cooke D, Marchand-Brynaert J, Normark S and Frère J-M, Role of residue Lys 315 in the mechanism of action of the *Enterobacter cloacae* 908R β-lactamase. *Biochemistry* **33**: 5193–5201, 1994.
36. Monnaie D, Dubus A and Frère J-M, The role of lysine-67 in class C β-lactamase is mainly electrostatic. *Biochem J* **302**: 1–4, 1994.
37. Galleni M, Amicosante G and Frère J-M, A survey of the kinetic parameters of class C β-lactamases. II. Cephalosporins and other β-lactam compounds. *Biochem J* **255**: 123–129, 1988.
38. Ledent P, Raquet X, Joris B, Van Beeumen J and Frere J-M, A comparative study of class D β-lactamases. *Biochem J* **292**: 555–562, 1993.
39. Pratt R and Govardhan P, β-Lactamase-catalysed hydrolysis of acyclic depsipeptides and acyl transfer to specific amino acid acceptors. *Proc Natl Acad Sci USA* **81**: 1302–1306, 1984.
40. Adam M, Damblon C, Plaitin B, Christiaens L and Frère J-M, Chromogenic depsipeptide substrates for β-lactamases and penicillin-sensitive DD-peptidases. *Biochem J* **270**: 525–529, 1990.
41. Murphy BP and Pratt RF, *N*-(Phenylacetyl)glycyl-D-aziridine-2-carboxylate, an acyclic amide substrate of β-lactamases. Importance of the shape of the substrate in β-lactamase evolution. *Biochemistry* **30**: 3640–3649, 1991.
42. Amicosante G, Franceschini N, Segatore B, Oratore A, Fattorini L, Orefici G, Van Beeumen J and Frère J-M, Characterization of a β-lactamase produced in *Mycobacterium fortuitum* D316. *Biochem J* **271**: 729–734, 1990.
43. Delaire M, Labia R, Samama JP and Masson JM, Site-directed mutagenesis at the active site of *Escherichia coli* TEM-1 β-lactamase. Suicide inhibitor-resistant mutants reveal the role of arginine 244 and methionine 69 in catalysis. *J Biol Chem* **267**: 20600–20606, 1992.
44. Imtiaz U, Billings E, Knox JR, Manavathu EK, Lerner SA and Mobashery S, Inactivation of class A β-lactamases by clavulanic acid: The role of arginine-244 in a proposed nonconcerted sequence of events. *J Am Chem Soc* **115**: 4435–4442, 1993.
45. Galleni M, Fransceschini N, Quinting B, Fattorini L, Orefici G, Oratore A, Frère J-M and Amicosante G, Use of the chromosomal class A β-lactamase of *Mycobacterium fortuitum* to study potentially poor substrates and inhibitory β-lactam compounds. *Antimicrob Agents Chemother* **38**: 1608–1614, 1994.