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Review

# Contribution of mutant analysis to the understanding of enzyme catalysis: The case of class A β-lactamases ☆

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## Contents



☆ The authors wish to dedicate this review to the memory of their old friend Stephen G. Waley who died December 6th, 1993. His contribution is one of the major cornerstones to our present understanding of the catalytic mechanism of β-lactamases. He was also a very kind and lovable person who is deeply missed by his numerous friends. May his dear wife Mary also find in this dedication a token of our lasting affection.

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## 1. Summary

Class A β-lactamases represent a family of well studied enzymes. They are responsible for many antibiotic resistance phenomena and thus for numerous failures in clinical chemotherapy. Despite the facts that five structures are known at high resolution and that detailed analyses of enzymes modified by site-directed mutagenesis have been performed, their exact catalytic mechanism remains controversial. This review attempts to summarize and to discuss the many available data.


## 2. Introduction

The synthesis of one or several β-lactamases represents the most widespread and often the most efficient mechanism devised by bacteria to escape the lethal action of β-lactam antibiotics. These enzymes which can be chromosome or plasmid encoded and produced in a constitutive or inducible manner, are secreted into the periplasmic space by Gram-negative strains or in the outer medium by their Gram-positive counterparts.

Two distinct catalytic mechanisms have been presently identified. A small group of enzymes contain an essential $Zn^{2+}$ ion. For historical reasons, they are known as class B β-lactamases. Although they represent a potential clinical problem, since they have recently been found in pathogenic genera such as *Aeromonas*, *Pseudomonas*, *Serratia* and *Bacteroides*, they are still vastly outnumbered by the active-site serine β-lactamases which, on the basis of their primary structures, are divided into three molecular classes A, C and D [1–4]. All these serine enzymes catalyse the hydrolysis of the β-lactam amide bond according to a three-step mechanism, involving the transient formation of an acyl-enzyme (Fig. 1) in which the serine hydroxyl group is esterified by the carbonyl group of the antibiotic moiety [5–7].

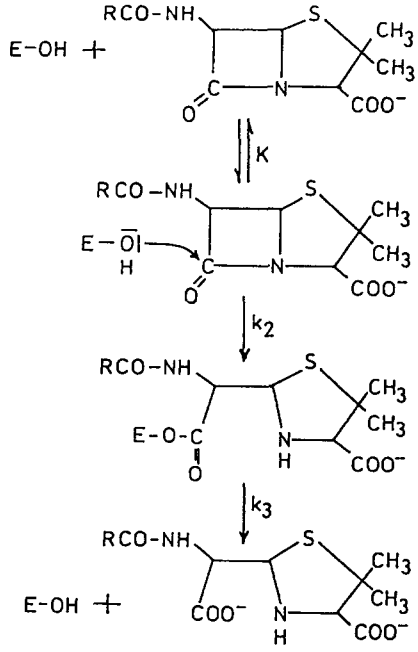


Fig. 1. General catalytic pathway of active-site serine β-lactamases. $K'$ is a dissociation constant and equals $(k_{-1}+k_2)/k_{+1}$; $k_2$ and $k_3$ are first-order rate constants.

Interestingly, a similar adduct is formed when β-lactams interact with their physiological targets, the DD-peptidases responsible for the synthesis and remodelling of the bacterial cell-wall peptidoglycan. But in this case, the adduct is very stable and the quasi-irreversible inactivation of the enzyme results in the synthesis of a functionally impaired peptidoglycan and eventually, in cell death [8]. In addition to this 'mechanistic' similarity, X-ray diffraction studies have underlined structural analogies between a penicillin-sensitive DD-peptidase and β-lactamases of classes A and C [9–11]. The proteins comprise one all-α and one α/β domains with the active site situated at the interface between the two domains. The active serine lies at the amino-terminus of the rather long, hydrophobic first helix of the all-α domain ($\alpha_2$-helix).

Several conserved elements have been identified in the vicinity of the active site serine which appear to be directly or indirectly involved in the catalytic and substrate recognition processes (Fig. 2). With the help of these structural data, the same elements have been identified in all active site serine penicillin-recognizing enzymes by careful sequence comparisons [4,12,13].

The first element contains the active serine residue (Ser*-70) and, one helix turn downstream, a lysine (Lys-73) whose side chain also points into the active site (Ser*-Xaa-Xaa-Lys sequence).

The second element, on a loop in the all-α domain, consists of Tyr-Xaa-Asn (β-lactamases of classes C and D, some penicillin-sensitive enzymes) or Ser-Xaa-Asn (β-lactamases of class A, most penicillin-sensitive enzymes) sequences.

Facing this triad, the third element, on a piece of β-strand, forms the opposite wall of the cavity. It is generally a Lys-Thr-Gly sequence, but Lys is replaced by His or Arg in a few exceptional cases and Thr by Ser in several class A β-lactamases. A positive side chain followed by one bearing a hydroxyl group appears to be universally conserved.

A fourth element, containing a negatively charged residue, has been tentatively identified in all enzymes, but it seems to play a functional role only in class A β-lactamases where it is situated on an 16-residue loop ($Arg^{164}$-$Asp^{179}$) usually referred to as the Ω-loop.

Of all the penicillin-recognizing enzymes, class A β-lactamases are those which have been most widely studied. A large number of enzymes have been described and more than 25 sequences determined. The 3D structures of five enzymes are known at high resolution [10,14–18,20–22] and the function of numerous residues has been probed by

site-directed mutagenesis. Nevertheless, their mechanism remains controversial and it is still difficult to establish correlations between the primary structures and the highly variable substrate profiles. This review is centred on the mechanism of class A β-lactamases and the results obtained by mutants analysis are discussed in depth. The numbering of the residues is that proposed by Ambler et al. [40] to facilitate comparisons between these homologous proteins (ABL scheme).

## 3. Substrate specificity and the conserved elements

Class A β-lactamases exhibit an amazing spectrum of specificity profiles [19]. They were initially considered as 'penicillinases' by opposition to the class C enzymes, which were referred to as 'cephalosporinases'. Indeed, in the fight against resistance phenomena, pharmaceutical companies isolated from natural sources or synthesized new β-lactams which progressively became increasingly

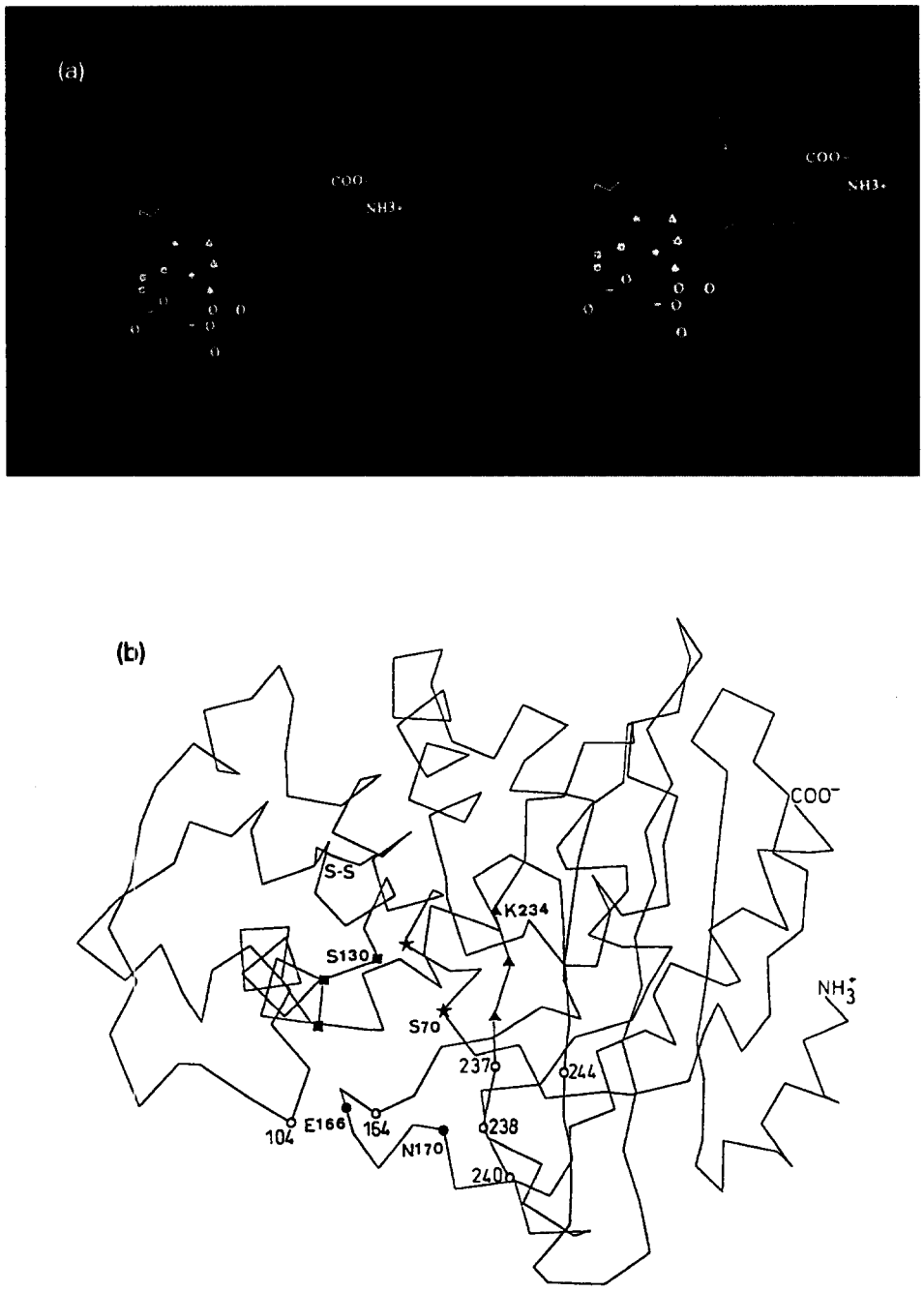


Fig. 2. (a) α-Carbon trace of the TEM-1 β-lactamases highlighting the conserved elements. (●) Element 1, (■) element 2, (▲) element 3, and (–) element 4. Some other residues discussed in the text are also identified (○). (b) The black-and-white drawing shows a similar view with clearer labelling of the α-carbon atoms where (★) element 1, and (●) element 4.

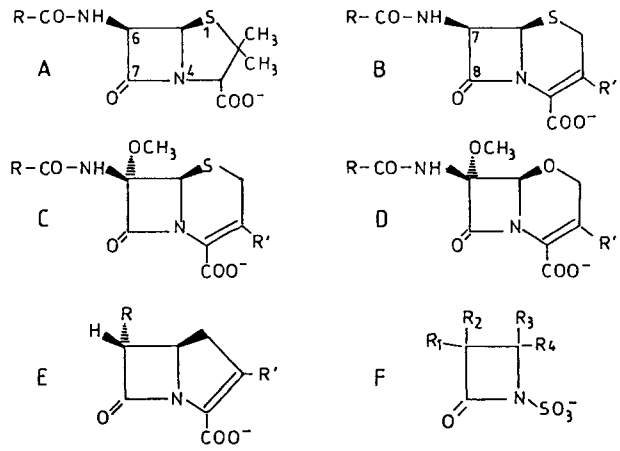


Fig. 3. Structures of some β-lactam antibiotics. (A) Penams (benzylpenicillin, ampicillin), (B) cephems (cephalosporins), (C) cephamycins (cefoxitin), (D) oxocephamycins (moxalactam), (E) carbapenems (imipenem), and (F) monobactams (aztreonam).

different from the original penicillins. The main families of compounds are shown in Fig. 3. The exact structure of the side chain on C-6 of penicillins and C-7 of cephalosporins is also a major factor in determining the sensitivity of the antibiotic to a given β-lactamase, methicillin and oximino β-lactams (e.g., third-generation cephalosporins and aztreonam, Fig. 4) being poorly hydrolysed by the *Staphylococcus aureus* and TEM-1/2 β-lactamases, respectively. However, the introduction of these compounds was usually followed by the emergence of resistant strains producing enzymes which exhibited an increased efficiency against the 'resistant' compounds (see Section 5.7).

Table 1
Comparison of the $k_{cat}/K_m$ values ($mM^{-1}s^{-1}$) for the hydrolysis of some characteristic β-lactams by various class A enzymes

| Mutant | Benzylpenicillin | Oxacillin | Cephalothin | Cefazolin | Cefuroxime | Cefotaxime | Ceftazidime |
|---|---|---|---|---|---|---|---|
| *Staphylococcus aureus* PC1 | 40000 (100) | 1 (< 0.1) | 8 (< 0.1) | 100 (0.25) | 1 (< 0.1) | ≪ 1 | ≪ 1 |
| *Bacillus licheniformis* | 29000 (100) | 1200 (4) | 2500 (8) | 25000 (86) | 170 (0.6) | 33 (0.1) | 9 (< 0.1) |
| *Streptomyces albus* G | 2800 (100) | 630 (23) | 370 (13) | 560 (19) | 25 (0.9) | 1 (< 0.1) | ≪ 1 |
| *Actinomadura* R39 | 7500 (100) | 5900 (79) | 3700 (49) | 3700 (49) | 640 (9) | 400 (5) | 13 (0.2) |
| *Citrobacter diversus* | 8300 (100) | 900 (11) | 1300 (16) | 8300 (100) | 770 (9) | ND | ND |
| TEM 1/2 | 80000 (100) | 18000 (23) | 650 (0.8) | 400 (0.5) | 5 (< 0.1) | 2 (< 0.1) | 0.1 (< 0.1) |
| TEM 7 | 13000 (100) | 10000 (77) | 250 (2.0) | 26 (0.2) | 12 (0.1) | 16 (0.1) | 9 (0.1) |
| TEM 10 | 25000 (100) | 2200 (9) | 160 (0.6) | 30 (0.1) | 20 (0.1) | 10 (< 0.1) | 28 (0.1) |
| TEM 19 | 10000 (100) | 4200 (42) | 2300 (23) | 500 (5) | 40 (0.4) | 230 (2.3) | 5 (< 0.1) |
| *Mycobacterium fortuitum* | 1000 (100) | ND | 1900 (190) | 1200 (120) | 200 (20) | 120 (12) | < 0.05 |
| *Proteus vulgaris* | 500 (100) | ND | 1800 (360) | 670 (130) | 500 (100) | 230 (23) | ND |

The values between parentheses are relative values with those for benzylpenicillin arbitrarily fixed at 100. Substrates are penams (benzylpenicillin, oxacillin), first (cephalothin, cefazolin), second (cefuroxime) or third (cefotaxime, ceftazidime) generation cephalosporins. The data are from Matagne et al. [19] (*B. licheniformis*, *S. albus* G, *A.* R39), Amicosante et al. [109] (*M. fortuitum*) or unpublished data from the authors' laboratory. ND, not determined.

Table 2
Comparison of the $k_{cat}/K_m$ values ($mM^{-1}\ s^{-1}$) for the hydrolysis of 6-aminopenicillanic acid (a penam devoid of R-CO side-chain), imipenem (a carbapenem) and aztreonam (a monobactam) by some class A enzymes

| Mutant | Benzylpenicillin | 6-aminopenicillanic acid | Imipenem | Aztreonam |
|---|---|---|---|---|
| *Staphylococcus aureus* PC1 | 40000 (100) | 65 (0.1) | ND | ND |
| *Streptomyces albus* G | 2800 (100) | 3700 (130) | 0.32 (≪ 0.1) | 0.4 (≪ 0.1) |
| *Actinomadura* R39 | 7500 (100) | 8000 (110) | 9 (0.1) | 76 (1) |
| TEM 1/2 | 80000 (100) | 8000 (10) | 1.5 (≪ 0.1) | 0.25 (≪ 0.1) |
| *Enterobacter cloacae* NOR1 | ND (100) | ND | ND (50) | ND (17) |

The values for benzylpenicillin are also shown. The results are from Matagne et al. [19,100] (*S. albus* G, *A.* R39), Sowek et al. [96] (TEM and aztreonam), Zafaralla et al. [80] (imipenem and the R244S mutant of TEM-1, but the value for the wild-type protein should not be different), Nordmann et al. [110] (*E. cloacae*) and unpublished data from the authors' laboratory (*S. aureus* PC1, TEM and 6-aminopenicillanic acid). In the case of the *E. cloacae* enzyme, only relative values were given. ND, not determined.

Although additional mechanisms were sometimes involved, such as the appearance of a target of extremely low sensitivity in the methicillin-resistant *Staphylococcus aureus* (MRSA) strains and in enterococci, bacteria most often utilized two strategies:

(i) The appearance of new enzymes only distantly related to the previously identified proteins.

(ii) The modification of the substrate profile of an already identified enzyme by a very limited number (1 to 3) of point mutations.

These observations are well illustrated by the $k_{cat}/K_m$ values for several enzymes and characteristic β-lactam antibiotics (Tables 1 and 2).

In all these enzymes, the structural elements described above remained nearly unchanged. The Ser$^{*70}$-Xaa-Xaa-Lys (element 1), Ser$^{130}$-Asp-Asn (element 2) and Lys$^{234}$-Thr(Ser)-Gly (element 3) were nearly absolutely conserved, the only exceptions being a Ser-Asp-Ser element 2 in one enzyme and Arg-Ser-Gly elements 3 in some others. Residue Glu-166 (element 4) was most often followed by Xaa-Glu-Xaa-Asn, but more variations were found in this part of the Ω-loop. This high degree of conservation and the positions of the residues constituting the 4 conserved elements in the vicinity of the active serine (Fig. 2) suggest that they probably play catalytic roles as opposed to being responsible for the exact specificity of these enzymes. A few additional conserved residues have also been identified, but they are further away from the active site and appear to be mostly involved in the general architecture of the proteins. Moreover, their number seems to decrease as new enzymes are sequenced. At the present stage, not more than 15 such residues are still considered as strictly conserved. The diversity of the specificity profiles of class A β-lactamases is thus not surprising and some of the available structure–function relationships are summarized below.

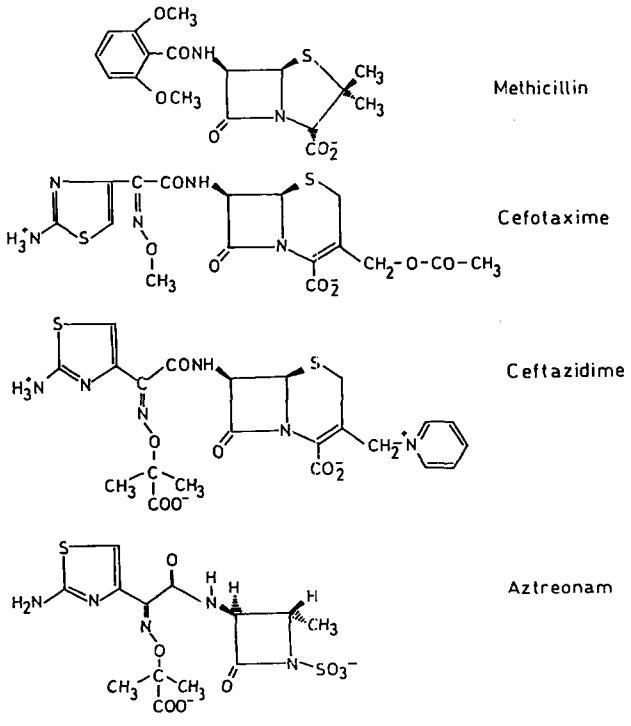


Fig. 4. Structures of methicillin (penam), cefotaxime and ceftazidime (third generation cephalosporins) and aztreonam (monobactam).

## 4. Mechanisms and modelling

On the basis of the crystal structures of several class A active sites and molecular modelling studies, functional roles for some of the conserved residues and a plausible positioning of the substrate in the cavity were proposed (Fig. 5; [15,20–22]). The most detailed study has been performed with the *Streptomyces albus* G β-lactamase [22]. With this enzyme, the various stable complexes occurring along the hydrolysis pathways of two good substrates (benzylpenicillin and cephalosporin C), namely the Henri–Michaelis complex, the two tetrahedral intermediates, the acyl-enzyme and the enzyme-product complex, were optimized by energy minimization. In this model, five residues (Ser-70, Ser-130, Asn-132, Thr-235 and Ala-237) were involved in ligand binding and four (Ser-70, Lys-73, Ser-130 and Glu-166), together with three water molecules (W1, W'1 and W2), in the hydrolysis of the bound β-lactam compound.

On the basis of the widely studied mechanism of serine proteinases [23], one would expect a general base to increase the nucleophilicity of the β-lactamase Ser-70 residue. But in contrast to the former families of enzymes, there is no conserved histidine in class A β-lactamases. The enzyme of *Streptomyces albus* G does not even contain a single such residue. In the mechanism proposed for this latter enzyme [22] and depicted in Fig. 6, the conserved Glu-166 was suggested to be the general base which firstly increases the nucleophilicity of the Ser-70 residue leading to protein acylation and which secondly activates the hydrolytic water molecule in the deacylation process.

The crystallographic data however indicated that, in the free enzyme, the Glu carboxylate was too far away to directly accept the Ser hydroxyl proton. But, in all refined structures, a water molecule (W1) was found which formed a bridge between these two residues and could in consequence act as an intermediate in the proton transfer. This hypothesis has received a strong support from data obtained with cefoxitin and other compounds, bearing a methoxy group on C-7 ([24]; cephamycins and oxocephamycins, Fig. 3). These β-lactams acylate class A β-lactamases very slowly or not at all. Computer-assisted docking of cefoxitin and of the very similar cephalothin in the active-site of the *S. albus* G enzyme showed that the only important difference at the level of the Henri–Michaelis complex was that the C-7 methoxy group of the

former completely displaced the water molecule, thus rendering the proton transfer impossible. With cephalothin, the position of the water molecule remained unchanged, which nicely explained the behaviour of this compound as a fair substrate (Table 1).

According to this mechanism, the proton would be back-delivered to the leaving nitrogen atom via a network of hydrogen bonds involving Lys-73, a second water molecule (W2) and the hydroxyl group of Ser-130 which, at the level of the tetrahedral intermediate, would be well positioned to form a hydrogen bond with the β-lactam nitrogen (Fig. 6). Hydrolysis of the acyl-enzyme would subsequently occur according to a symmetrical mechanism, where Glu-166 would activate the hydrolytic water molecule (W1) for attack on the carbonyl carbon and insure back-delivery of a proton to the oxygen atom of Ser-70, after re-entry of a third water molecule (W'1, replacing W1).

The negative charge which appears on the β-lactam carbonyl oxygen when tetrahedral intermediates are formed both during acylation and deacylation (Fig. 6), is stabilized by hydrogen-bonding interactions with the backbone NH groups of Ser-70 and Ala-237. These interactions also help to polarize the carbonyl group of the scissile β-lactam amide bond, thus favouring the nucleophilic attack by Ser-70. Experimental evidence for the existence of this 'oxyanion hole' similar to that found in serine proteinases has been reported for both class A and class C β-lactamases [7,11,17,20,25].

A different hypothesis assumes an 'asymmetrical' mechanism in which the acylation and deacylation steps are not catalysed by the same general base, the ε-amino group of Lys-73 acting in the first and the carboxylate of Glu-166 in the second [17]. The first step would involve back-delivery of the proton to the leaving group along a hydrogen bond network similar to that described above.

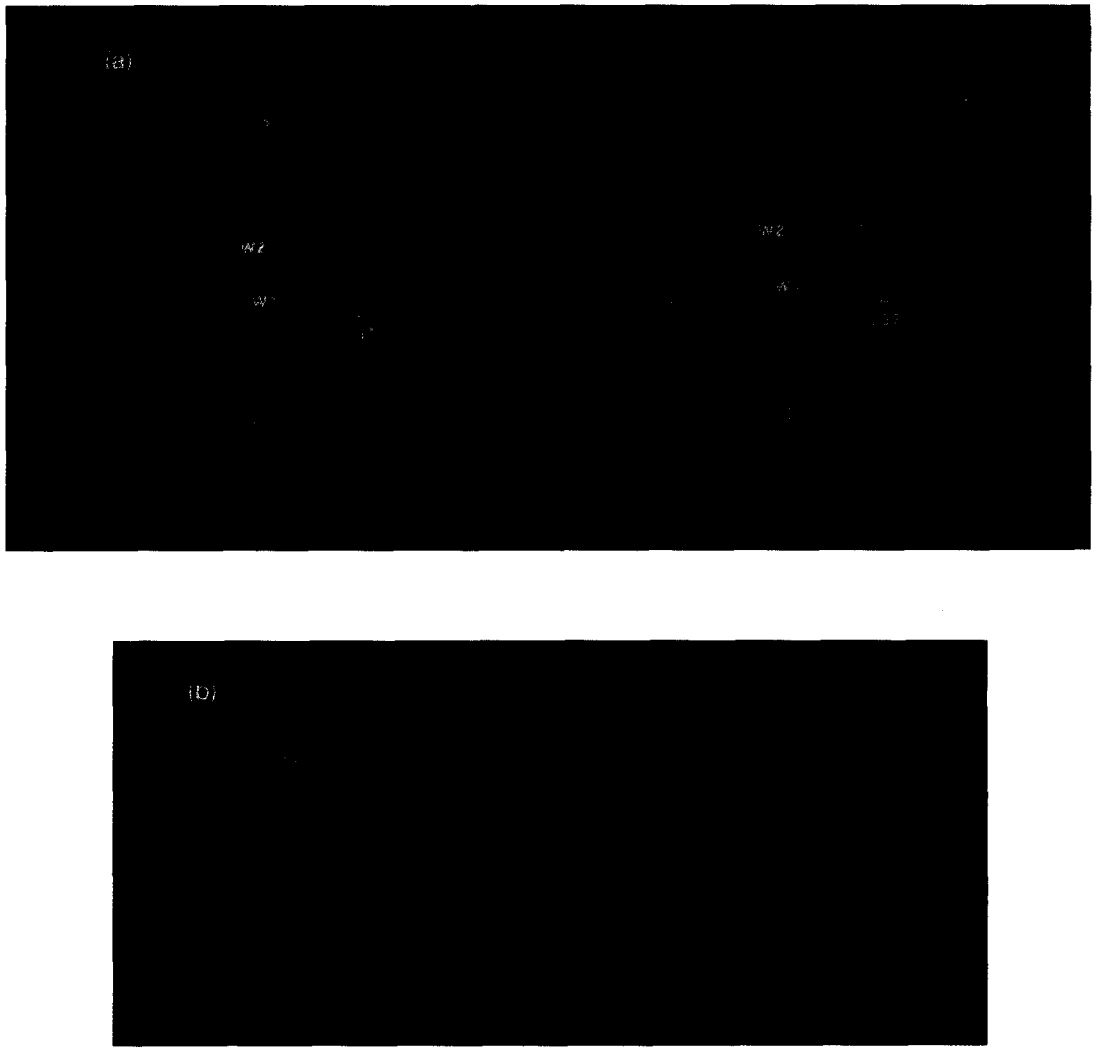


Fig. 5. Stereo view of the hydrogen bond networks in the active site of the *Streptomyces albus* G β-lactamase (a) and of the Henri–Michaelis complex formed with Benzylpenicillin (b). The peptide backbone bonds are in magenta, the side chains in blue-green and the conserved water molecules W1 and W2 (found in the same positions both in the crystal structure and by computer modelling) in red. The important H-bonds are shown as dashed green lines. In (b), the Benzylpenicillin molecule is in blue and only the residues which are thought to directly interact with the substrate are represented.

Finally, a third, more simple hypothesis [16] assumes that the helix dipole, combined with the intrinsic instability of the β-lactam ring, would be sufficient to allow an efficient acylation process. Again, Glu-166 would be necessary for the deacylation reaction.

In conclusion, if Glu-166 appears to be recognized as the essential general base in the hydrolysis of the acyl-enzyme, the exact mechanism of the acylation step remains highly controversial. The site-directed mutagenesis studies discussed below attempt to shed some light on this problem but also raise a number of additional questions.

## 5. Function of the active-site residues

### 5.1. Active-site serine (Ser-70)

Among the various residues conserved in penicillin-recognizing enzymes, the active- site serine is certainly the only one for which the catalytic function has been unambiguously established. Various class A [27–30], class C [31,32] and class D [33] β-lactamases and various PBPs [34–39] were reacted with specific β-lactam compounds acting as poor substrates or inactivators. In all cases, the labelled residue was a serine, identified as Ser-70 in class A β-lactamases, Ser-64 in class C and Ser-70 in class D.

Using site-directed mutagenesis, Ser-70 was substituted by residues exhibiting little steric hindrance (Ala or Gly) or retaining some nucleophilic properties (Cys or Thr). All these experiments confirmed the essential role played by Ser-70 in the catalytic process [41–48]. The S70C mutant of the *S. albus* G and TEM β-lactamases retained up to 2% of the wild-type activity [42,48,49]. More surprisingly, the S70A mutant of the *S. albus* G β-lactamase exhibited about 0.01% of the activity of the wild-type, indicating that other groups in the active site could somehow activate a water molecule to perform a direct attack on the β-lactam amide bond [48].

### 5.2. Lysine-73

The side-chain amino group of Lys-73 is intimately involved in the hydrogen-bonding network within the active site (Fig. 5). The proximity of the Ser-70 and Lys-73 side chains, which are hydrogen-bonded, indicated the

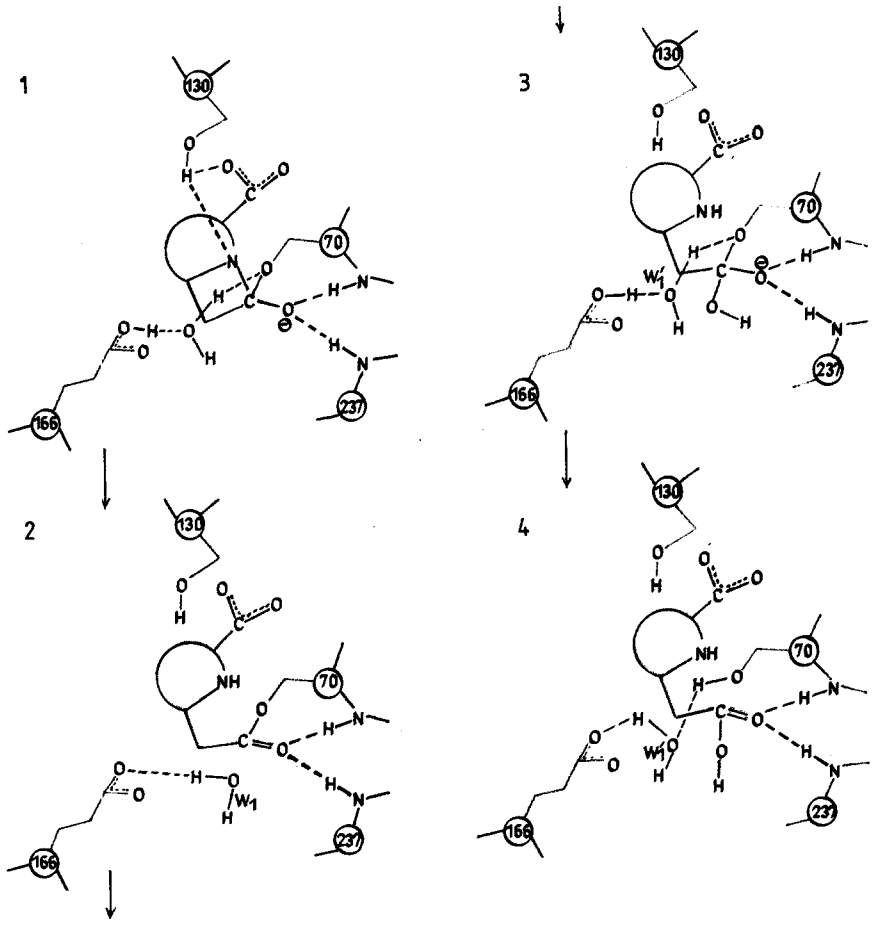


Fig. 6. Intermediates in the catalytic pathway of class A β-lactamases. (1) First tetrahedral intermediate, (2) acyl-enzyme, (3) second tetrahedral intermediate, and (4) enzyme-product complex. The colour code is as above for the backbone and the water. The side-chains are green and the H-bonds are black dashed lines.

likely implication of the Lys side-chain amino group in the catalytic process. Herzberg and Moult [20] first suggested that Lys-73 contributed to orienting the proton of the Ser-70 hydroxyl group and facilitating its transfer to the β-lactam nitrogen atom. Knox and Moews [16] proposed that Lys-73 induced the polarisation and even the deprotonation of the Ser-70 hydroxyl group. More recently, Strynadka et al. [17] argued that at neutral pH, the amino group of Lys-73 is deprotonated both in the free enzyme and in the Henri–Michaelis complex. This residue would thus play a major role in the acylation process, acting as a general base in the activation of Ser-70.

Conversion of Lys-73 into Arg (K73R) in β-lactamase I from *Bacillus cereus* 569/H resulted in a 100-fold decrease of the ampicillin-resistance of *Escherichia coli* TG1 after replacement of the wild-type by the mutant gene on the same plasmid [50]. A kinetic study of the purified enzyme [26] indicated that the $K_m$ values were not significantly altered whereas the $k_{cat}$ values were decreased 20 to 200-fold (note that unfortunately the columns describing the K73R and the E166D mutants were inverted in Table 2 of the original paper (Rosemary Gibson, personal communication)). A more careful analysis showed that for the hydrolysis of benzylpenicillin, acylation became the rate-limiting step of the reaction catalysed by the K73R mutant, whereas with the wild-type enzyme, the rate constants for acylation and deacylation were of the same order of magnitude. The values of the rate constants for the formation and dissociation of the non-covalent enzyme-substrate complex were also lowered but the resulting equilibrium constant $K$ $(=k_{-1}/k_{+1})$, which characterizes substrate binding to the enzyme active site, was not significantly modified. These results clearly demonstrated the important role played by Lys-73 in the catalytic process, especially in the acylation step, in agreement with the various functions proposed for this residue [17,20,22].

The K73A mutant of the *Bacillus licheniformis* β-lactamase [47] lost not only its catalytic activity but also its substrate binding ability, indicating the necessity of a lysine side chain at this position, either for providing a positive charge or acting as a general base.

Recently, it was attempted to estimate the $pK_a$ of Lys-73 by experiments combining site-directed mutagenesis, chemical modification and $^{15}$N-NMR [51]. The K73C mutant was produced, yielding an inactive enzyme which, when reacted with $^{15}N$-bromoethylamine (giving $^{15}N$-aminoethylcysteine, a lysine homologue), regained activity to near wild-type levels. A $pK_a$ 'significantly lower than 10' was found for the amino group so engineered at position 73, indicating that it might function as a general base, in agreement with the mechanism proposed by Strynadka et al. [17]. This would imply a strongly decreased $pK_a$ value for the Lys-73 primary amine, resulting from the very positive electrostatic environment due to the $\alpha_2$-helix dipole and the Lys-234 ammonium. However, the K73R mutant of the *Bacillus cereus* 569/H β-lactamase I was significantly less impaired than its E166D counterpart ([26], see below), an observation which seems to underline a more important role for Glu-166 than for Lys-73. Moreover, in the wild-type TEM-1 enzyme, one would expect the Glu-166 negative carboxylate group to decrease the general positive electrostatic potential of the active site. Only the direct determination of the Lys-73 ε-amino group $pK_a$ in the original enzyme will shed some light on this problem but this is not easy, since the enzyme contains a total of 11 lysine residues.

### 5.3. The $Ser^{130}$-$Asp^{131}$-$Asn^{132}$ motif

This motif (SDN loop, ABL 130–132), which is nearly invariant in all class A β-lactamases (among the 20 sequences aligned by Ambler et al., [40], the only exception is the N132S substitution in the *B. cereus* III β-lactamase), is situated between helices 4 and 5 in the all-α domain and forms one side of the cavity (Fig. 2). The crystallographic data indicate that the side chains of Ser-130 and Asn-132 point into the active site cleft and are thus ideally positioned to take part in the catalytic process, whereas that of Asp-131 is buried in the protein core and involved in several H-bonds, probably fulfilling an important structural role. The corresponding triads YAN and YSN are found in class C β-lactamases and in the *Streptomyces* R61 DD-peptidase, respectively [4].

#### 5.3.1. Serine-130

Ser-130 was replaced by Ala, Asp and Gly in the *S. albus* G β-lactamase. A detailed kinetic study [52] combined with a molecular modelling analysis [53] of the mutants allowed the assignment of a double role to this residue. Firstly, Ser-130 might be involved in the proton shuttle carrying the proton from the hydroxyl group of Ser-70 to the nitrogen atom of the β-lactam ring. Secondly, this residue participates in the complex hydrogen-bonding network within the cavity (Fig. 5) and thus contributes to maintaining a competent active site. The crystallographic data indicate that the side chains of Ser-130 and Lys-234 are hydrogen-bonded, thus crosslinking the all-α and the α/β domains and stabilizing the enzyme active site [21].

In addition, the hydroxyl group of Ser-130 interacts with the carboxylate of penicillins and is thus involved in ligand binding [22]. Study of the corresponding residue in another class A β-lactamase (ROB-1 [54]) confirmed both the structural and the functional roles played by Ser-130 in class A enzymes.

#### 5.3.2. Aspartate-131

The important structural role played by this residue was clearly demonstrated for the *S. albus* G β-lactamase. Even the conservative D131E mutation yielded a very unstable protein. This probably explains the strict conservation of this residue in class A β-lactamases [52].

*5.3.3. Asparagine-132*

As already suggested before on the basis of the crystallographic data [20,21], Asn-132 might act as an hydrogen bond donor to the carbonyl group of the antibiotic side chain (Fig. 5b). This hydrogen bond would contribute to the adequate positioning of the substrate for the nucleophilic attack by the active serine oxygen side chain, with a main effect on the stabilization of the transition state related to the acylation step. Interestingly, this entropic effect appeared to be much more important for the reaction with cephalosporins than for that with penicillins. Indeed, substitution of Asn-132 by Ser in the *S. albus* G β-lactamase yielded an enzyme with a nearly unchanged activity towards penicillins, but with a greatly decreased activity towards cephalosporins. The docking attempts indicated that cephalosporins required a more subtle adjustment to the active site geometry. With the mutant, very large structural modifications became necessary, explaining the important loss of activity observed towards those substrates [52,53,55].

Finally, Asn-132, although significantly involved in the hydrogen-bonding network within the active site, appears to be of lower structural importance than Ser-130.

Instead of modifying the enzyme active site, Pratt and coworkers [56] chose the complementary approach of altering the substrate. The carbonyl group of the substrate side chain was changed to a thiocarbonyl. Two side chain-thionated β-lactams (a penicillin and a cephalosporin) were tested and found not to be significantly poorer substrates of both class A and class C β-lactamases than their oxo analogues. Consequently, the authors argued that the supposed hydrogen bond, if any, was not essential for catalysis and that the substrate side chain did not require a strict positioning: the structure of the enzyme would be rather flexible and might accommodate a large number of amide side chains thus resulting in a low substrate specificity for this part of the substrate. In contrast to this hypothesis, in Table 1 some dramatic influences are found of this side chain on the rates of acylation, but the direct involvement of Asn-132 in this selectivity remains to be demonstrated. Another interesting and possibly related observation is that 6-aminopenicillanic acid, which is devoid of acyl side-chain, can be a fair or poor substrate, depending upon the studied class A β-lactamase (Table 2).

The molecular modelling studies highlight the importance of the dense hydrogen-bonding network within the active site of the *S.albus* G β-lactamase. Local modifications which cause the disappearance or weakening of any hydrogen bond may propagate their effects far from the mutated amino acid and modify the entire configuration of the cavity. Such a damaged active site may regain functionality upon binding of a properly structured β-lactam compound, either by readopting a hydrogen-bonding configuration similar to that of the wild-type enzyme or by utilizing an alternative route of proton shuttle from Glu-166 to the nitrogen atom of the β-lactam ring [53]. These perturbations, which could be responsible for the discrepancies between the results obtained with modified enzymes and modified substrates make the interpretation of the kinetic data particularly hazardous. This underlines the necessity of using different methods for the study of modified enzymes, such as molecular modelling or X-ray diffraction techniques.

*5.4. Glutamate-166*

Glu-166 is strictly conserved in class A β-lactamases [40]. Its carboxylate side chain lies in the active site and interacts with several other residues and with a water molecule (Fig. 5). Its strategic position, close to the active site serine side chain makes it an ideal candidate for a critical role in the catalytic mechanism. Herzberg and Moult [20] first suggested that Glu-166 deprotonates the attacking water molecule in the hydrolysis of the acyl-enzyme, thus acting as a general base (proton abstractor) in the deacylation step. This suggestion, resting upon the refined structure of the *S. aureus* PC1 β-lactamase, has been widely accepted. The role of this residue in acylation is still highly controversial. However, the high number of site-directed mutagenesis experiments performed on Glu-166 have so far failed to supply a definitive picture.

Madgwick and Waley [50] first demonstrated that the conversion of Glu-166 into Gln (E166Q) resulted in a mutant that failed to confer ampicillin-resistance to *E. coli*. They also suggested that this residue deprotonated the serine hydroxyl group in acylation, so that its role was analogous in acylation and in deacylation. Subsequently, Waley and collaborators [26] carefully analysed the kinetic properties of the *B.cereus* β-lactamase I E166D mutant. They observed that this mutant had greatly reduced activity and that the rate constants for both acylation and deacylation were reduced about 2000-fold. This result clearly demonstrated that the mutation similarly affected both processes, which seemed to be in perfect agreement with the proposal that Glu-166 was the general base in both acylation and deacylation. It should also be noted that both rate constants $k_2$ and $k_3$, measured for the hydrolysis of benzylpenicillin by β-lactamase I, displayed similar pH-dependences, which argues for the involvement of the same side chains in both steps [57].

A different view arose from the study of mutants of the TEM-1 β-lactamase (E166A, D, Q, N) by Adachi et al. [58]. These authors observed that all these mutants could bind [$^{14}$C]benzylpenicillin forming relatively long-lived acyl-enzymes. They concluded that Glu-166 was expendable for acylation but acted as an essential catalyst for deacylation. However, although the mutations actually caused conversion of the penicillin-hydrolysing TEM β-lactamase into penicillin-binding proteins, thus implying that the $k_3$ values for the mutant enzymes were similar to or smaller than $k_2$ and had very small absolute values, the individual values of $k_2$ and $k_3$ were not determined and it

cannot be claimed that the conversion of the β-lactamase into a penicillin-binding protein is sufficient to demonstrate that the mutation selectively affected $k_3$. Indeed, the rate constant for acylation could have been reduced by a large factor without completely impeding the formation of the acyl-enzyme. It should also be noted that these authors reported that the $K_m$ value obtained with benzylpenicillin and the E166D mutant, for which a slight hydrolytic activity was retained, was very similar to that of the wild-type. This observation, in agreement with the results of Gibson et al. [26], is inconsistent with a selective decrease of $k_3$. Thus, it cannot be concluded that the acylation step of the β-lactamase catalysed reaction is Glu-166 independent.

By informational suppression, Delaire et al. [59] observed that substitution of Glu-166 by a Tyr (E166Y) in the tEM-1 β-lactamase unexpectedly yielded an enzyme with a significant activity. Kinetic studies of that mutant enzyme led to the suggestion that Glu-166 played a major part in defining the substrate profile of class A β-lactamases. However, this relied on the study of only four substrates and should thus be considered with caution. Nevertheless, it is interesting to note that the apparent second-order rate constant for the hydrolysis of the studied substrates ($k_{cat}/K_m$; i.e., $k_2/K'$) was decreased 50 to 10 000-fold, which clearly indicated that the acylation step was affected by the mutation.

Substitution of Glu-166 by Ala was also performed in the *B. licheniformis* β-lactamase [60,61]. The modified protein was studied with nitrocefin and 6β-furylacryloylamido-penicillanate as substrates and, in both cases, rapid accumulation of a normally transient acyl-enzyme intermediate was shown to occur. The apparent $k_{+3}$ values which were observed were $10^4$-fold lower than those obtained by Waley and colleagues [26,63] with the same mutant of the closely related *B. cereus* enzyme. However, these low apparent $k_{+3}$ values might be due to the isomerization of the acyl-enzyme into a more stable conformation [61] and the authors also showed that the acidic limb of the pH-activity profile of the wild-type enzyme might indeed reflect the protonation state of Glu-166. If the acyl-enzyme intermediate formed with the E166A mutant and the two considered substrates could be observed by HPLC, a similar adduct could not be detected by X-ray diffraction when the mutant was co-crystallized with benzylpenicillin [62] in contrast to the results obtained with the E166N mutant of the TEM-1 β-lactamase [17].

Two additional Glu-166 mutants of the *B. cereus* 569/H β-lactamase I were recently constructed [63]: one (E166Cmc) with a lengthened 'artificial' side chain where the sulfhydryl group of the E166C mutant was transformed into a *S*-carboxymethyl function by reaction with iodoacetate and the other with a short non-polar side chain (E166A). With good penicillin substrates, the values of the kinetic parameters of the two conservative E166Cmc and E166D mutants bearing the carboxylate functional group were similar to those of the non-conservative E166A mutant, suggesting that optimal catalytic activity required the exact length of the Glu side-chain to bring the carboxylate in a strategic situation [63]. Similarly, replacement of the highly conserved Asp-179 by Asn in the *S. aureus* PC1 β-lactamase disorganized the Ω-loop, resulting in a bad orientation of the Glu-166 carboxylate group and in a lowered deacylation rate for the mutant enzyme [64].

The acyl-enzyme intermediate of the E166A mutant of β-lactamase I formed with the good substrate phenoxymethylpenicillin was trapped by acid-quench and characterized by electrospray ionization mass spectrometry. Although the $k_2$ and $k_3$ values could not be individually determined, these experiments indicated that both rate constants were dramatically decreased when compared to those of the wild-type, with $k_3$ being more affected than $k_2$ [63]. Again, nitrocefin gave rise to unusual kinetic behaviours with all the mutants and in addition no accumulation of the acyl-enzyme could be observed when the E166A mutant of β-lactamase I acted on nitrocefin or 6β-furylacryloylamido-penicillanate, a result which strongly contrasted with what was observed with the corresponding mutant of the very similar *B. licheniformis* enzyme [60].

The numerous studies related above clearly demonstrate the critical role played by residue Glu-166. However, if all results agree with a major role in deacylation, its function in acylation is obviously less clear.

### 5.5. The $Lys^{234}$-$Thr(Ser)^{235}$-$Gly^{236}$ motif

The Lys-Thr(Ser)-Gly triad, where the second residue (ABL 235) is indifferently a Ser or a Thr, is situated on the β3 strand of the β-sheet in class A β-lactamases (in which this highly conserved structural feature also encompasses residue Asp-233) forming one side of the cavity and facing the Ser-Asp-Asn loop (Fig 2). Similar Lys-Thr-Gly and His-Thr-Gly sequences were found on corresponding β strands in the 3D structures of class C β-lactamases [11,108] and the *S.* R61 DD-peptidase [65], respectively and can be located in similar positions in the sequences of all PBPs and β-lactamases [4].

#### 5.5.1. Lysine-234

In class A β-lactamases, Lys-234 is conserved in most known sequences, but an Arg residue is found in the CARB enzymes which efficiently hydrolyse α-carboxypenicillins [66,67]. Crystallographic data indicate that the side chain of Lys-234 points into the active site and Herzberg and Moult [20] first suggested that its protonated amino group participated in binding the substrate ground state through the formation of a salt-bridge with the free carboxylate on C-3 of penicillins or C-4 of cephalosporins ('electrostatic anchor').

In the *S. albus* G enzyme [69], replacement of Lys-234 by His yielded an enzyme whose pH-dependence was

characterized by the appearance of a novel $pK_a$ of about 6.4 for both the $k_{cat}$ and $k_{cat}/K_m$ parameters. This $pK_a$ value could be attributed to the newly introduced imidazole side-chain by direct NMR titration. Under acidic conditions (pH < 6), the $k_{cat}$ value for benzylpenicillin was as high as 50% of that of the wild-type enzyme, demonstrating that a positively charged His could successfully replace the original Lys side-chain and that an efficient active site was maintained. The $k_{cat}/K_m$ ratio dramatically decreased above pH 6.0, but this parallelled the decrease of $k_{cat}$ and could not be attributed to larger $K_m$ values. These results suggested that the enzyme was nearly fully functional as long as the positive charge was present on the side chain of residue 234, and this electrostatic feature appeared to be more essential for transition-state stabilization than for initial recognition of the substrate, a conclusion which was strengthened by the study of Lys-234 mutants of the *B. licheniformis* [68] and TEM-1 [70] enzymes. In contrast, in the corresponding position of a class C enzyme, a protonated His side-chain could not efficiently replace the Lys-315 ammonium group [71].

It should also be noted that, in the acyl-enzyme formed by the E166N mutant of the TEM-1 β-lactamase with benzylpenicillin, the distance between the ammonium group of Lys-234 and the substrate carboxylate was 3.5 Å, suggesting a poor electrostatic contribution of Lys-234 at the level of this intermediate [17].

More recently, Page and colleagues [72] studied the interaction of the *B. cereus* 569/H β-lactamase I with modified substrates. Replacement of the C-3 carboxylate in phenoxymethylpenicillin by a hydroxymethyl group and of the C-4 carboxylate in cephalosporins by both a lactone and an aldehyde yielded derivatives which were still fair substrates for the enzyme. These results seem to preclude any direct electrostatic interaction between the substrate carboxylate and the Lys-234 side chain. These authors also argue that the 'alkaline' $pK_a$ of class A β-lactamases (≅ 8.5–10) cannot be attributed to Lys-234. However, the behaviour of the K234H mutant of the *S. albus* G enzyme suggested that deprotonation of Lys-234 was likely to be, at least in part, responsible for the activity decrease observed at high pH values and thus this residue should contribute, possibly with others, to the $pK_{a2}$ value observed in the pH-dependence curves of both $k_{cat}$ and $k_{cat}/K_m$.

Lys-234 was substituted by Arg in the TEM-1 β-lactamase [70]. The kinetic behaviour of the resulting mutant was virtually identical to that of the wild-type, highlighting the importance of a positively charged side-chain at this position, and even exhibited higher turn-over numbers for carbenicillin (6-fold increase), ticarcillin (17-fold) and piperacillin (7-fold). However, the corresponding $K_m$ values were increased by similar factors and in consequence the corresponding $k_{cat}/K_m$ values were not significantly modified by the mutation. These results suggested that the higher activities observed against carbenicillin and related substrates with the CARB enzymes was partly due to the K234R mutation but also to other substitutions responsible for increasing the affinity for these substrates [70].

Careful sequence alignments of these CARB enzymes revealed some specific substitutions occurring in the highly conserved 'boxes' found by Joris et al. [13], namely M68L, E104T, E240G and K234R with the exception of PSE-3 (where the substitution W165R was found instead of K234R), strongly suggesting that these residues are associated with the carbenicillinase activities [73]. All these mutations were individually introduced in the TEM-1 β-lactamase and the catalytic properties of the resulting enzymes were thoroughly studied [73]. Although the important role played by residues 234 and 240 could be clearly demonstrated, the potential role of the various CARB-specific amino-acid residues in determining the substrate profile of these enzymes could hardly be defined. This study confirmed that the properties of the CARB enzymes probably result from subtle adjustments in the active site topology and not only from single point-mutations. These can undoubtedly change the substrate profile but complementary modifications are required to optimize the activity of the new enzymes [73].

### 5.5.2. *Threonine- or serine-235*

The residue at position 235 is invariably a hydroxylated residue, either Ser or Thr. It was suggested that this potent hydrogen bond donor interacts with the carboxylate moiety of the β-lactam molecule [17,21,22,74]. Random substitutions of this residue (Ser-235) in the TEM-1 β-lactamase yielded mutant enzymes with reduced activity compared to the wild-type, even in the case of the conservative S235T substitution [75]. More recently, Ser-235 was selectively substituted by Ala in the TEM-1 β-lactamase, resulting in a modified enzyme with surprising kinetic properties. Indeed, the disappearance of the hydroxyl group had little impact on the penicillinase activity of the enzyme but the cephalosporinase activity was much more affected. Evolutionary considerations were advanced to tentatively explain these observations [76,77] but the exact role of a hydroxylated residue at position 235 and the reasons for its absolute conservation remain poorly understood.

### 5.5.3. *Glycine-236*

The third member of the Lys-Thr(Ser)-Gly triad is devoid of side chain. This Gly is strictly conserved in the superfamily of active site serine penicillin-recognizing enzymes [13]. This conservation can be attributed to purely steric factors. Indeed, Gly-236 is situated near the active site serine and any side chain at this position would severely impair the geometry of the active site (collision with the Ser-70 side-chain [15]) and the approach of the substrate. Palzkill and Botstein [75] clearly demonstrated that any mutation at position 236 resulted in an enzyme with strongly reduced activity. However, several substitu-

tions were found to confer significant but low levels of ampicillin resistance to *E. coli*.

### 5.6. Arginine-244 and arginine-220

An arginine is present at position 244 in 15 of the 20 class A sequences aligned by Ambler et al. [40]. This residue is located on the β4 strand of the α/β domain. Although its α-carbon is rather distant from the active site, the long side chain of Arg-244 points in the direction of the cavity and the guanidinium group is actually positioned close to the β-strand containing the Lys-Thr(Ser)-Gly sequence (Fig. 7).

Surprisingly no Arg was found at position 244 in the *S. albus* G β-lactamase but a careful comparison of the three-dimensional structures of the *S. albus* G (Asn-244) and of the *B. licheniformis* (Arg-244) β-lactamases indicated that the guanidinium group of Arg-220 in the former lied in a position equivalent to that of Arg-244 in the latter (Fig. 7). This probably represents an example of compensatory mutations within a family of homologous proteins which result in similar geometries for similar groups supplied by residues situated in different positions in the sequence [74]. A survey of the sequences aligned by Ambler et al. [40] indicates that a similar situation is likely to prevail for the *Streptomyces lavendulae* and the *Streptomyces fradiae* (Arg-220 and Asn-244) β-lactamases. Enzymes from *Streptomyces aureofaciens* (Thr-220 and Ala-244) and *Klebsiella oxytoca* (Ser-220 and Thr-244) seem to be somewhat different but errors in the gene sequences remain possible.

Arg-220 was found to be important for the acylation of the *S. albus* G β-lactamase by classical substrates [74]. Indeed, with the R220L mutant and most of the substrates tested, the $k_{cat}/K_m$ ratio was decreased by two to three orders of magnitude. Strikingly, the hydrolysis of two compounds devoid of a carboxylic group (benzylpenicillin methyl ester and deacetycephalosporin C lactone) was barely affected; they appeared to be even better substrates for the mutant than for the wild-type enzymes. These results suggested a possible involvement of Arg-220 in the binding of the substrate carboxylate. However, molecular

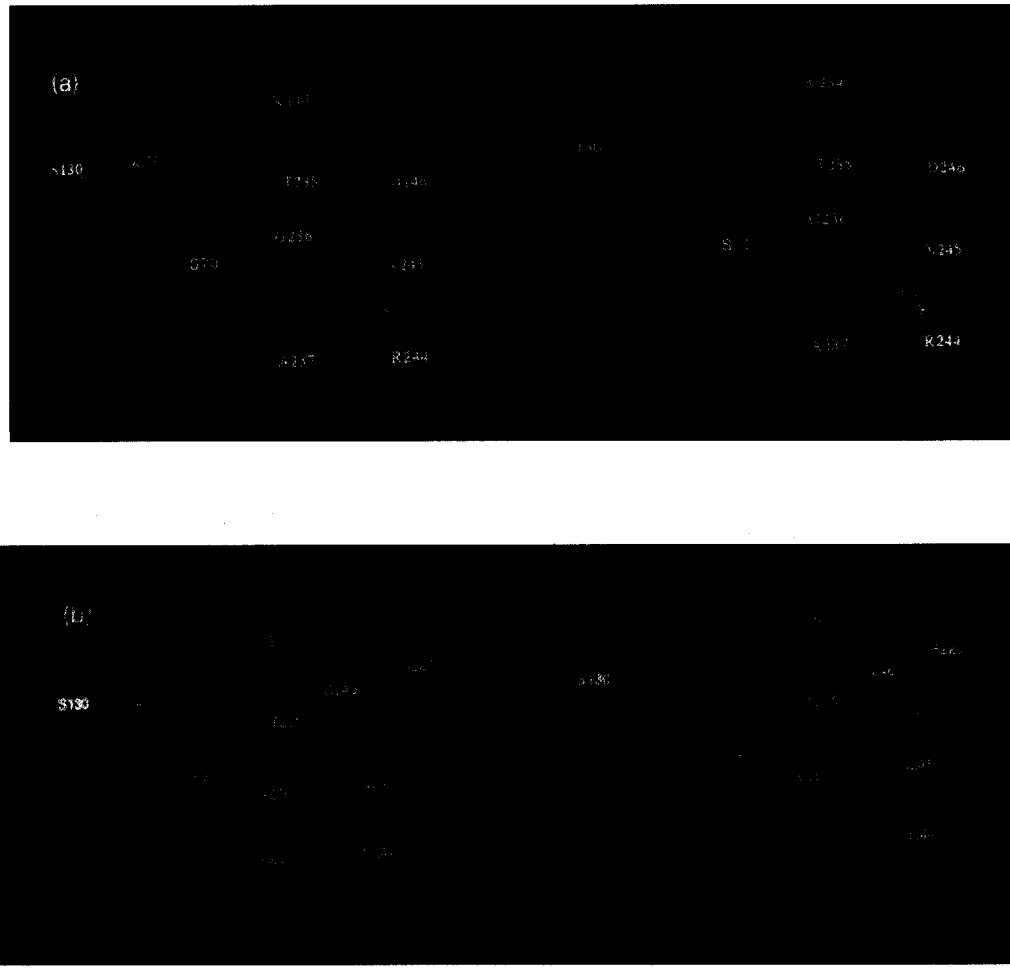


Fig. 7. Equivalent positions of the Arg-244 (a) and Arg-220 side chains (b) in the structures of the *Bacillus licheniformis* and *Streptomyces albus* G enzymes, respectively. The TEM-1 β-lactamase contains an Arg-244 residue and is thus similar to that of *B. licheniformis* in that respect. The colour code is as in Fig. 5 but the two Arg side chains are in yellow.

modelling studies did not support a direct ionic interaction between this anion and the positively charged guanidinium group of Arg-220. Nevertheless, it could participate in the proper positioning of the antibiotic by forming together with Arg-274, Lys-234 and the dipole of the $\alpha_2$-helix a large positive field which attracts the substrate negative charge to that side of the cavity [74]. A similar electrostatic role was suggested for Arg-244 in the *B. licheniformis* β-lactamase, on the basis of crystallographic and molecular modelling data [21].

Replacement of Arg-220 by Leu in the *S. albus* G β-lactamase not only modified the electrostatic features of the active site cavity but also strongly affected the dense hydrogen-bonding network in which this side chain appears to be involved. It was proposed that the substitution of Arg-220 modified the exact position of the hydroxyl of Thr-235, which would be responsible for the proper orientation of other functional groups in the cavity and for increasing the affinity for the substrate ground state through the formation of an hydrogen bond with the carboxylate of both penicillins and cephalosporins [74]. In the R220L mutant, the Thr-235 side chain is no longer maintained in an efficient orientation and in consequence the $K_m$ values for all classical substrates (penicillins as well as cephalosporins) were drastically increased. However, the elimination of the corresponding Ser-235 hydroxyl group barely affected the penicillinase activity of the TEM-1 β-lactamase but a much stronger effect was observed with cephalosporins [76,77], results which contrast with the properties of the *S. albus* G R220L mutant where the acylation step was severely impaired with both cephalosporins and penicillins. One might be tempted to conclude that the mechanisms of the TEM-1 and *S. albus* G enzymes might be somewhat different and that the roles of the similarly positioned guanidinium groups might not be completely equivalent. However, the R244T and R244Q mutants of the TEM-1 enzyme were also severely impaired, exhibiting $k_{cat}/K_m$ values two to three orders of magnitude lower than those of the wild-type protein [78], a result similar to that observed with the R220L mutant of the *S. albus* G β-lactamase. The activity decrease seemed to be less drastic for the R244S mutant, especially with cephalosporins [79]. However, differences were not very large and the behaviours of the various mutants lacking the positive charge at position 244 or 220 can be regarded as similar. Nevertheless, Zafaralla et al. [79] favoured a different interpretation, implying the involvement of a long (and thus weak) hydrogen bond between Arg-244 and the substrate carboxylate, both in the ground and transition states.

By contrast, Delaire et al. [78] rejected the possibility of a direct interaction between the guanidinium group of Arg-244 and the substrate's carboxylate and attributed the observed effect to structural modifications in the enzyme active site. These authors also suggested that Arg-244 could help to destabilize the enzyme-product complex and was thus to be responsible for the high turnover rate of the enzyme. However, it must be stressed that nothing seems to indicate that the dissociation of the enzyme-product complex might be the rate-limiting step of the reaction. In fact, the determination of all the individual rate constants by Waley and colleagues [7] appears to invalidate this hypothesis.

It should also be remembered that in the acyl-enzyme formed between the E166N mutant of the TEM-1 β-lactamase and benzylpenicillin, strong hydrogen bonds appeared to be formed between the substrate carboxylate and the side chains of residues Arg-244 and Ser-235 [17].

Another interesting feature arising from the study of Arg-244 is its possible implication in the mechanism of β-lactamase inactivation by suicide substrates such as clavulanate. Interestingly, Delaire et al. [78] observed that replacement of Arg-244 by most other amino acids resulted in increased resistance to inactivation by clavulanate and sulbactam. Similarly, Zafaralla and Mobashery [80] showed that the progressive inactivation observed when the TEM-1 β-lactamase reacts with imipenem could be eliminated by replacing Arg-244 by Ser. These authors suggested Arg-244 as the essential proton source for the $\Delta^2 \rightarrow \Delta^1$ pyrroline tautomerization of carbapenem antibiotics, which is the main event in β-lactamase inactivation by these compounds [81]. More recently, a revised mechanism, resting mainly on molecular modelling computations, was proposed for the inactivation of class A β-lactamases by clavulanic acid [82]. In this mechanism Arg-244 would serve as an 'electrostatic anchor' for a structurally conserved water molecule which would play a critical role as a source of proton in the inactivation mechanism. Interestingly, the transamination reaction proposed by Knowles [81] as the crucial event leading to enzyme irreversible inactivation, was suggested to involve the hydroxyl group of Ser-130 instead of the amino group of an unidentified Lys side chain. Nevertheless, more direct experimental evidence is needed to further support this model.

Other inactivation experiments indicated that residue Met-69 of the SHV-1 and TEM-1 β-lactamases might also be involved in the resistance of these enzymes to mechanism-based inactivators [78]. This residue, which is not conserved in all class A enzymes, is situated on the substrate-side of the $\alpha_2$-helix [21].

It was recently shown [78,83,84] that the M69I, M69L and M69V mutations in the TEM-1 and M69I in the OHIO-1 (which exhibits 97% identity with SHV-1) β-lactamases resulted in enzymes with decreased sensitivities to clavulanate or sulbactam. A more careful analysis of the M69L (TEM) and M69I (SHV) mutants indicated that their substrate profile was also significantly altered.

Residues at positions 244 (Arg) and 69 (Met) of the SHV and TEM β-lactamases are likely to play an important role in resistance to the inactivators of class A β-lactamases. Recently, new natural TEM variants were

found which conferred resistance to the amoxycillin/clavulanate combination [85,86] and where residue Arg-244 appeared to be substituted by Ser or Cys (Belaaouaj, Ph.D. thesis, Université de Paris VII, 1992).

### 5.7. Extended-spectrum β-lactamases

In the past ten years, a high number of undesirable naturally occurring mutants were isolated around the world [87–89]. These extended-spectrum β-lactamases are plasmid-mediated enzymes that confer resistance to β-lactam antibiotics such as cefotaxime, ceftazidime and aztreonam characterized by β-acyl side chains containing an oximino group (Fig. 4). These so-called β-lactamase-stable compounds were initially very effective against strains producing most known plasmid-mediated β-lactamases but their extensive clinical utilization since 1980 has probably been responsible for the selection of the extended-spectrum β-lactamases and consequently for the emergence of novel resistances. The most common of these new enzymes belong to the SHV and TEM families, the parent enzymes, TEM-1 and SHV-1, exhibiting 68% of sequence identity [90]. To date, more than 4 SHV- and 20 TEM-like enzymes have been described, all exhibiting increased activities against the oximino β-lactams [91,92].

The mutations responsible for the new activity profiles of these enzymes seem to be restricted to a very low number of residues, namely Glu-104 (TEM), Arg-164 (TEM), Arg-205 (SHV), Ala-237 (TEM), Gly-238 and Glu-240 (both in TEM and SHV). Note that residues 238 and 240 are adjacent in the TEM and SHV sequences, an insertion occurring at this level in some homologous class A enzymes. Not surprisingly, if one excepts Arg-205, all these residues were found to border the active site cavity. Substitutions were also found at position 168 in the TEM-1 β-lactamase (E168G and E168A) that increased the enzyme activity towards third-generation cephalosporins [93]. However, to our knowledge, these mutations have not been found in any of the naturally occurring variants characterized so far.

It is particularly striking that none of the side-chains modified in the TEM and SHV variants appeared to be involved in the catalytic machinery of class A β-lactamases. It is also interesting to note that the acquisition of extended substrate profiles was often concomitant with decreased activities against the classical, good substrates of the parents enzymes ([94–96], see Tables 1 and 3).

Three of the positions mentioned above were studied by site-directed mutagenesis in the TEM-1 β-lactamase (Glu-104, Arg-164 and Glu-240 [96]). This thorough study pointed out the major role of the R164S substitution in the emergence of the new phenotype. It also highlighted the spectacular synergistic effect obtained by combining two or three mutations (Table 3). Subtle modifications in the geometry of the active site pocket and in the interactions between the substrates and the modified side-chains have been proposed as the molecular origins of the new properties of the variants [91,93,96–99]. However, all these interpretations should be considered cautiously since they do not rely on an accurate knowledge of the three dimensional structures and moreover, most of the various members of the SHV and the TEM families have not been submitted to detailed kinetic studies.

Table 3
Synergy between mutations for the hydrolysis of third generation cephalosporins and aztreonam by TEM mutants

| Mutant | $k_{cat}/K_m$ ($mM^{-1}s^{-1}$) | | |
|---|---|---|---|
| | Cefotaxime | Ceftazidime | Aztreonam |
| TEM-1 | 0.56 (1) | 0.02 (1) | 0.25 (1) |
| E104K | 5.3 (10) | 0.45 (23) | 3.1 (12) |
| E240K | 4.7 (8) | 0.61 (31) | 2.0 (8) |
| R164S | 10 (18) | 13 (650) | 3.8 (15) |
| E104K/R164S | 24 (43) | 380 (19,000) | 160 (640) |
| R164S/E240K | 12 (21) | 89 (4,500) | 81 (320) |

The data are from Sowek et al. [96]. The values between parentheses are relative values with those for the wild-type enzyme arbitrarily set to 1.0.

Finally some other class A β-lactamases, which do not exhibit any of the substitutions described here, but whose sequences differ greatly from those of the TEM or SHV enzymes, are also capable of hydrolysing so-called β-lactamase-stable antibiotics [100,101]. This observation shows that the same result can be obtained by apparently unrelated variations on the same original theme (Table 1).

Mutations at positions 237, 238 and 240 have received much attention and are discussed in the following section.

### 5.8. Residues situated downstream of the KT(S)G triad

The known three-dimensional structures of class A β-lactamases show that the residues which immediately follow the conserved $Lys^{234}$-Thr(Ser)$^{235}$-$Gly^{236}$ triad on the β3 strand lie at the edge of the cavity where the substrate side chain binds (Fig. 2). These non-conserved residues are most often Ala-237 (found in 55% of the sequences) and Gly-238 (85% of the sequences) but more variations are found at position 240 [40]. Structural data from crystallographic and molecular modelling studies indicate that the main chain NH group of residue 237 interacts with the oxygen of the β-lactam carbonyl, thus contributing to the formation of oxyanion hole. A second hydrogen bond is formed between the main chain carbonyl group of residue 237 and the amide NH group of the substrate side chain (Fig. 5b).

In a pioneering attempt to alter the catalytic properties of an enzyme, Hall and Knowles [102] applied random chemical mutagenesis and direct selective pressure to the TEM-2 β-lactamase (the TEM-1 and TEM-2 β-lactamases differ only by one residue, K/Q39, and cannot be distinguished on the basis of their catalytic properties). Selection of strains with increased resistance to cephalosporin C yielded the A237T mutant [103] exhibiting an increased

activity towards this compound but a decreased activity towards penicillins.

More than ten years later, Healey et al. [103] used site-saturation mutagenesis to assess the role of the same residue in the TEM-1 enzyme. The A237N mutant displayed the same modified activity profile as the A237T mutant.

The two cephalosporins tested by Healey et al. [103] (cephalothin and cephalosporin C) were not 'oximino' cephalosporins. The residue at position 237 does not appear to play an important role in resistance to these latter compounds. Indeed, among the TEM and SHV variants characterized to date, only TEM-5 contains the A237T mutation [98] and mutations R164S and E240K, which seem to play a more crucial role, are also found in that variant. Nevertheless, it would be interesting to test the sensitivity of second- and third-generation cephalosporins to both the A237T and A237N mutants.

Lee et al. [99] presented a kinetic study of both the SHV-1 and SHV-2 β-lactamases, which only differ by a mutation at position 238 [104]. In this β-lactamase, replacement of Gly-238 (SHV-1) by Ser (SHV-2) resulted in resistance of strains producing the SHV-2 enzyme to third-generation cephalosporins like cefotaxime and ceftizoxime. Accordingly, the SHV-2 β-lactamase showed a significantly increased activity towards cefotaxime. The activity profile of SHV-2 towards three penicillins and three cephalosporins also seemed to be slightly altered, with somewhat lower $K_m$ values and $k_{cat}$ values which were either slightly increased or decreased.

Shlaes and Currie-McCumber [105] used direct selective pressure to isolate mutants of the very similar OHIO-1 β-lactamase capable of hydrolysing various oximino β-lactams (e.g., cefotaxime, ceftriaxone, ceftazidime and aztreonam) that are normally resistant to the wild-type enzyme. The spontaneous mutations which increased the resistance of *E. coli* to these antibiotics were G238S, G238C and G238V or G242C. More recently, the role of Ser-238 and Lys-240 in the hydrolysis of third generation cephalosporins by the SHV family of β-lactamases was reexamined by site-directed mutagenesis and modelling [106]. A good activity on cefotaxime was confirmed to be due to the sole G238S mutation, which also resulted in a slight activity on ceftazidime but a high activity towards this compound was only observed when both G238S and E240K mutations were present. Accordingly, these two positions have been found to be modified in several extended-spectrum SHV-like enzymes [87,107]. Docking of cefotaxime and ceftazidime in the SHV binding site allowed the assignment of a structural role to residue 238, while that of residue 240 would be based on its electrostatic properties [106].

All these experiments clearly indicated that the residues situated downstream the Lys-Thr(Ser)-Gly triad play a significant part in β-lactamase specificity, correlated to the structure of the substrate acyl side chain. The dominant feature in the behaviour of these mutants would consist in rather subtle reorganizations of the active site resulting in a slightly different positioning of the substrate in the enzyme catalytic cavity, thus explaining the observed changes in specificity. Side-chains in this part of the enzyme could play either an indirect, structural or a direct substrate-binding role. Some residues at position 238 could distort the β3 strand, so that the β-lactam would be maintained in a more favourable position. Alternatively, new residues which are potential hydrogen-bond donors (Ser or Cys-238) or possess appropriate electrostatic properties (Lys-240) could form new bonds with the substrate, thus ensuring a more stable binding and a position in the active site more favourable to the hydrolysis of the bound β-lactam compound. These two possibilities, which are obviously not mutually exclusive [105], are likely to explain the appearance of the numerous mutations involving residues downstream of the Lys-Thr(Ser)-Gly triad, found in the TEM or SHV families. However, it should again be stressed that an accurate knowledge of the three-dimensional structures of these mutant enzymes is a prerequisite to a better understanding of the exact implications of the substitutions observed in that part of the cavity.

## 6. Conclusions

Class A β-lactamases constitute a fascinating group of enzymes and represent an apparently ideal case for structure–activity relationship studies. A large number of substrates are available and many different enzymes have been isolated and characterized. The proteins can easily be modified by site-directed mutagenesis but bacteria, which were submitted to strong selective pressures, have also been busy performing their own protein engineering experiments. Even when they could not increase the activity of the enzymes in a sufficient manner, they increased their synthesis rate and some Gram-negative strains also decreased the permeability of the outer membrane.

The side chains of the residues forming the four conserved elements are essential components of the catalytic machinery. At this level, only a very limited number of conservative mutations seem to be allowed (N132S, K234R and T235S). One of the most surprising results of the site-directed mutagenesis experiments involves residue 235. The strict conservation of the hydroxyl side-chain appears to be only important for the hydrolysis of cephalosporins and it can be tentatively concluded that the natural selective pressure exerted on β-lactamases after they diverged from their putative common ancestor was due to these latter compounds and not to penicillins [76,77]. Indeed, the surprising diversity of primary structures and the very small number of strictly conserved residues indicate a very ancient divergence and the presence of chromosome-encoded β-lactamases in bacteria which are not exposed to β-lactams in their natural environment remains a complete and baffling mystery.

Many residues appear to be involved in the determination of the enzymatic specificity profiles, including some in the conserved elements. If the pressure exerted by the increasingly diverse chemotherapeutic arsenal resulted in the recent emergence of new enzymes with a small number of modified residues, very different proteins were also found which could hydrolyse the newly introduced antibiotics and which certainly already existed well before these compounds were synthesized and utilized as antibacterial agents. Although the selection of these latter proteins can be easily rationalized, their very existence is much more difficult to explain.

The structures of five class A β-lactamases have now been solved to high resolution but our present understanding of both the catalytic process and the specificity profiles remain, at best, very imperfect. The mechanism by which the active-site serine hydroxyl is activated remains a subject of controversy. By contrast, the role of Glu-166 in the deacylation step appears to be well established and it could be argued that, if its absence really affects deacylation more severely than acylation, the contribution of structural element 4 might represent an efficient solution to the deacylation problem, 'invented' by class A β-lactamases. Indeed, this element is absent or non-functional in both the DD-peptidases, where deacylation is always exceedingly slow, and in class C β-lactamases where deacylation is generally the rate-limiting step at substrate saturation. But this explanation might be a little too simple. In fact, with some substrates of class C enzymes, deacylation can occur quite readily, with a $k_3$ value up to 1000 $s^{-1}$.

These considerations underline the extent of the gap which remains presently unbridged between the highly detailed but static pictures obtained by X-ray crystallography and the very rapid phenomena of enzyme catalysis. Hopefully, modelling based on molecular mechanics can supply a partial solution to the problem by visualizing energy-minimized structures of transient reaction intermediates, but a complete description of the reaction pathways must rest on more elaborate and complex quantum mechanical calculations.

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