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The Structures and Catalytic Mechanisms of Active-Site Serine β-Lactamases

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Introduction: DD-transpeptidases, β-lactamases and penicillin resistance

The success of β-lactam antibiotics, penicillins, cephalosporins and related compounds in antibacterial chemotherapy rests both on their high efficiency and on their specificity. They interfere with the last stage of peptidoglycan synthesis, a transacylation reaction, most often involving a D-alanyl–D-alanine terminated peptide (*Figure 1*). 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 eukaryotic cells (Frère *et al.*, 1992). The DD-transpeptidases are active-site serine enzymes (Frère and Joris, 1985) which perform their catalytic cycle according to an acylation/deacylation pathway, usually represented by the three-step model shown in the left branch of *Figure 2* and similar to the well- known mechanism of active-site serine proteases such as chymotrypsin and trypsin, although many DD-transpeptidases generally behave as more efficient transacylases than these latter enzymes. It is, however, rather clear that this model represents a simplification of the real situation (Jamin, Wilkin and Frère, 1993). The right branch of *Figure 2* depicts the interaction between β-lactam antibiotics and the DD-transpeptidases. By contrast to their catalytic equivalent, ES*, the adducts formed with the β-lactams are rather stable with k_{+3} values in the 10^{-4} to 10^{-6} s^{-1} range. Since these acylenzymes are unable to fulfil their physiological role, functionally impaired peptidoglycan is formed, leading eventually to cell death, a phenomenon in which the activation of cell autolysins also sometimes appears to be involved (Tomasz, 1979).

Abbreviations:CARB, carbenicillin-hydrolysing β-lactamases; EREF, enzymic rate enhancement factor; NMR, nuclear magnetic resonance; PBPs, penicillin-binding proteins; WT, wild-type.

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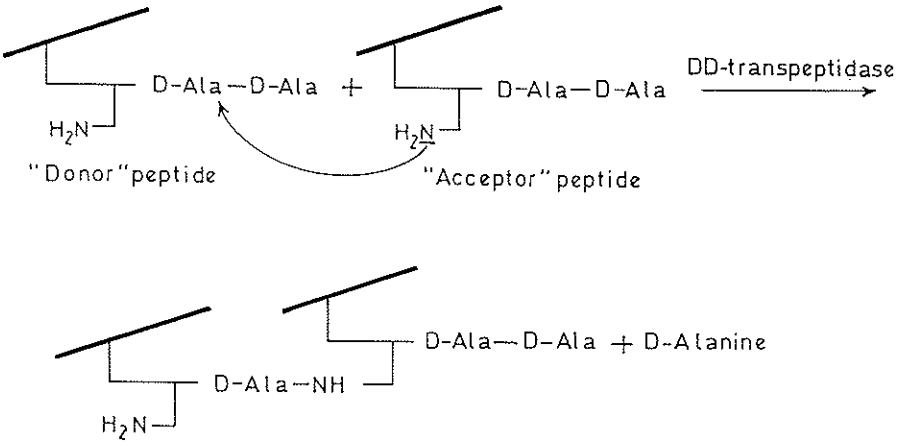

Figure 1. The transpeptidation reaction catalysed by the bacterial DD-peptidases. The heavy lines represent the glycan moiety of the nascent peptidoglycan network.

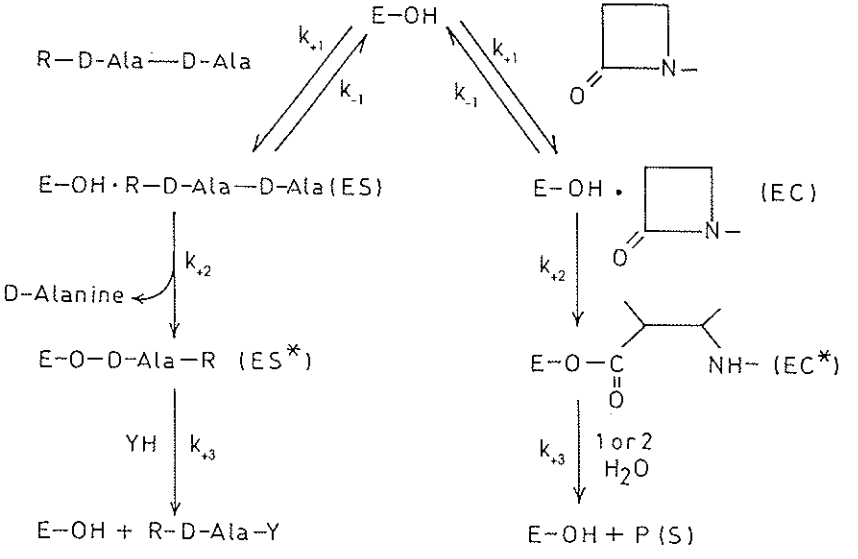

Figure 2. Interaction between the bacterial DD-transpeptidases (left branch) and their physiological substrates (R-DAla-DAla = donor substrate; YH = acceptor substrate; see *Figure 1*) and β-lactams (right branch). With the substrates, the values of k_{+2} and k_{+3} are high, thus ensuring a high catalytic turnover rate. By contrast, the efficiency of β-lactams as inactivators rests on high k_{+2}/K' values [where $K' = (k_{+2} + k_{-1})/k_{+1}$] and very low k_{+3} values, resulting in a nearly complete immobilization of the enzyme as the inactive adduct EC*. Some DD-carboxypeptidases have also been described. In this case, the acceptor is water (YH = H_2O). DD-trans- and carboxypeptidases form a family of DD-peptidases (Joris *et al.*, 1988). Deacylation of EC* can occur by the direct hydrolysis of the ester bond, or involve a rate-limiting hydrolysis of the penicillin C_5–C_6 bond, followed by rapid deacylation.

The DD-transpeptidases and the related DD-carboxypeptidases (see the legend to *Figure 2*), whose physiological role remains less well understood, have often been characterized by their ability to bind radiolabelled penicillins covalently and are therefore called penicillin-binding proteins (PBPs).

Bacteria have discovered various strategems to escape the lethal action of β-lactam compounds, and in consequence new molecules had to be introduced progressively in the chemotherapeutic arsenal, with structures increasingly different from those of the original penicillins (*Figure 3*). As described at length in the issue of *Science* dated 15 April 1994, resistance phenomena have recently become quite widespread and worrying.

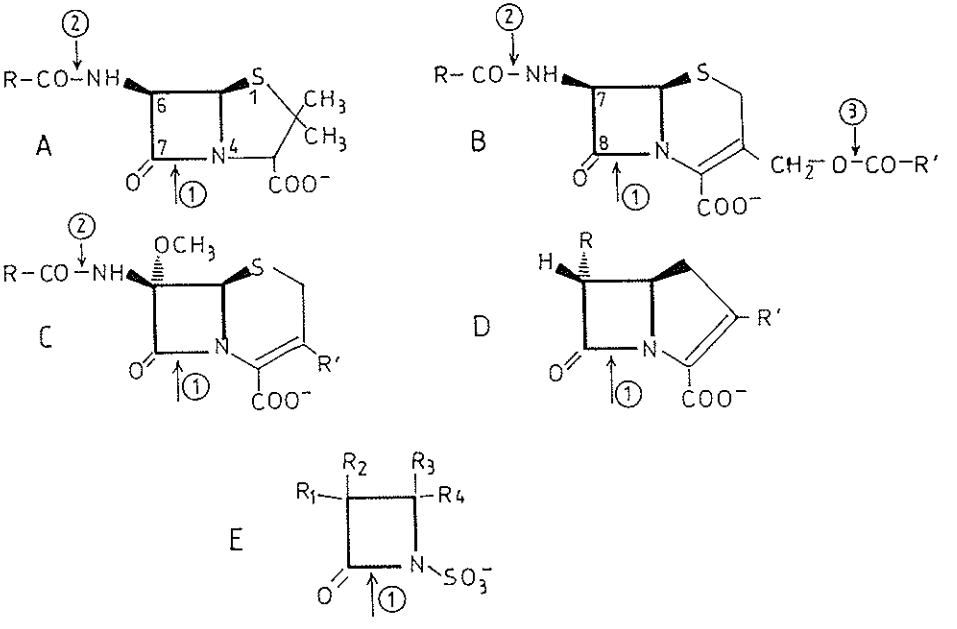

Figure 3. Structures of (A) penicillins; (B) cephalosporins; (C) cephamycins; (D) carbapenems; and (E) monobactams. The arrows indicate the sites of action of (1) β-lactamases, (2) acylases and (3) esterases. Note that some cephalosporins do not exhibit an ester side-chain on C_3. The chemical structure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

Three distinct mechanisms of resistance have been identified:

1. Intrinsic resistance is due to the synthesis of DD-transpeptidases with a strongly decreased affinity for the antibiotics. The resistant PBPs are very slowly acylated by the β-lactams, the k_{+2}/K' values being as low as 10 M^{-1} s^{-1} as against up to 300 000 M^{-1} s^{-1} for penicillin-sensitive DD-peptidases (Frère and Joris, 1985; Frère *et al.*, 1992). This intrinsic resistance to β-lactams is sometimes accompanied by resistance to other families of antibiotics, which can result in the emergence of deadly strains. A frightening example is the methicillin-resistant *Staphylococcus aureus* against which the only presently available efficient compounds are those of the vancomycin family.
2. The diffusion of the antibiotic to its membrane-bound targets can be impeded by the outer layers of the cell wall itself, the 'outer membrane' in Gram-negative

bacteria (Nikaido and Normark, 1987; Waley, 1987; Frère *et al.*, 1989), and the mycolic acid layers of the Gram-positive mycobacteria. In the former, increased resistance has been attributed to modifications of the porins, proteins which form hydrophilic channels in the outer membrane and allow small, water-soluble molecules to penetrate into the periplasmic space (Nikaido and Normark, 1987).

3. Last, but not least, bacteria can produce enzymes which chemically modify the antibiotics: acylases, esterases or β-lactamases (*Figures 3* and *4*). 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 the irreversible opening of the β-lactam ring 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 (Waley, 1992; Coyette *et al.*, 1994). Some strains have been isolated which produce two or even three different β-lactamases, exhibiting distinct specificity profiles. Thus, β-lactamases and DD-peptidases share a common ability to recognize compounds containing a β-lactam ring. As shown in the following sections, additional, even more striking similarities have been found between the latter enzymes and a large number of β-lactamases.

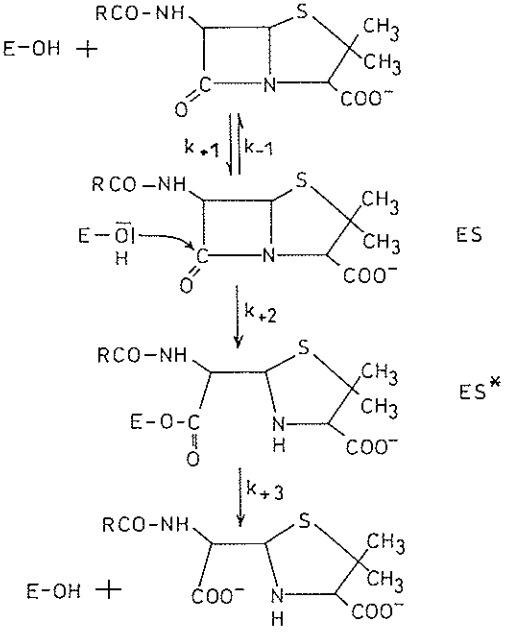

Figure 4. The acylenzyme mechanism of β-lactamases.

Metallo- and active-site serine β-lactamases

The hydrolytic properties of β-lactamases rest on two distinct catalytic mechanisms (Waley, 1992). Some enzymes are Zn^{2+}-containing metalloproteins, while the vast majority of known β-lactamases are active-site serine enzymes, just like the DD-peptidases.

For many years, only innocuous strains of *Bacillus cereus* were known to produce

Zn^{2+}-β-lactamases which were, in consequence, considered as mere biochemical curiosities. More recently, an increasing number of pathogenic strains of the *Bacteroides, Xanthomonas, Aeromonas, Enterobacter, Pseudomonas* and *Serratia* genera have been detected, characterized by a high resistance to carbapenems, compounds which generally escape the activity of the more common active-site serine β-lactamases. This resistance is due to the synthesis of Zn^{2+}-β-lactamases (Payne, 1993). It can be predicted that the increased utilization of carbapenems 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 for these enzymes. Most of them are monomeric, with *M*r values of about 23 000. The enzyme produced by *Xanthomonas maltophilia* is, however, tetrameric, but devoid of allosteric properties. The known sequences are clearly homologous (Felici *et al.*, 1993). In the *B. cereus* enzyme, three His residues are responsible for liganding the Zn^{2+}ion (Sutton *et al.*, 1987). These three His residues are found in equivalent positions in the other sequences, including that of the *Xanthomonas* monomer, but in the *Aeromonas* enzyme, one of them is replaced by Asn. A conserved Cys residue is also present, not far from the Zn^{2+} in the *B. cereus* enzyme structure, but its exact role remains mysterious, as well as the catalytic mechanism of these enzymes (for more details see Waley, 1992).

The active-site serine β-lactamases, however, represent a more immediate problem. They hydrolyse the antibiotics according to the acylation/deacylation pathway described by *Figure 4* and characterized by the following values of the steady-state kinetic parameters (Waley, 1992):

$$k_{cat} = \frac{k_{+2} \cdot k_{+3}}{k_{+2} + k_{+3}}$$

$$K_m = \frac{k_{+3} \cdot K'}{k_{+2} + k_{+3}}$$

$$\frac{k_{cat}}{K_m} = \frac{k_{+2}}{K'} \text{ where } K' = \frac{k_{+2} + k_{-1}}{k_{+1}}.$$

The efficiency of these enzymes can be really frightening. Some of them have indeed been described as 'perfect catalysts' (Christensen, Martin and Waley, 1990), with k_{+2}/K' ratios close to the diffusion-limit values (10^8 M^{-1} s^{-1}) combined with very high k_{+3} values (4000 s^{-1}). Moreover, in constitutive mutants of some Gram-negative strains, enormous amounts of enzyme are produced, yielding periplasmic β-lactamase concentrations up to 1 mM (Hechler *et al.*, 1989). 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 which can escape their hydrolytic action.

The interactions between β-lactams and both the active-site serine β-lactamases and DD-peptidases thus occur according to identical or very similar reaction pathways (compare *Figures 2* and *4*) with a major difference at the level of the k_{+3} value, which is usually very high with the former and very low with the latter. Practically, this quantitative difference translates into qualitatively distinct behaviours: β-lactams inactivate DD-peptidases while β-lactamases destroy the same molecules.

Genetic data and the three classes of active-site serine β-lactamases

On the basis of their primary structures these 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 newly discovered enzymes are easily distributed among these classes (Waley, 1992; Joris *et al.*, 1991).

Table 1. Molecular classification of β-lactamases and genetic characteristics

Class	Examples	Type of bacteria	Gene location	Induction
A	TEM	Gram –	Plasmid	C
	SHV	Gram –	Plasmid	C
	CARB	Gram –	Plasmid	C
	Bacillus licheniformis	Gram +	Chromosome	I[a]
	Bacillus cereus I	Gram +	Chromosome	I[a]
	Staphylococcus aureus	Gram +	Chromosome or plasmid	I[b]
	Streptomyces albus G	Gram +	Chromosome	C
	Streptomyces cacaoi ULG	Gram +	Chromosome	I
	Actinomadura R 39	Gram +	Chromosome	C
B	*Bacillus cereus* II	Gram +	Chromosome	C[a]
	Aeromonas hydrophila	Gram –	Chromosome	C
	Xanthomonas maltophilia	Gram –	Chromosome	I
	Bacteroides fragilis	Gram –	Chromosome	C[c]
	Serratia marcescens *Enterobacter cloacae* *Pseudomonas aeruginosa*	Gram –	Chromosome or plasmid	C[d]
	Pseudomonas aeruginosa	Gram –	Plasmid	C
C	*Escherichia coli* K 12	Gram –	Chromosome	C
	Citrobacter freundii	Gram –	Chromosome	I
	Serratia marcescens	Gram –	Chromosome	I[a]
	Enterobacter cloacae	Gram –	Chromosome	I[a]
	Pseudomonas aeruginosa	Gram –	Chromosome	I
	BIL-1, MIR-1	Gram –	Plasmid	C
D	OXA	Gram –	Plasmid	C
	PSE-2	Gram –	Plasmid	C

C, Constitutive; I, inducible.
[a] Constitutive mutants (*Bacillus licheniformis* 749/C, *Enterobacter cloacae* P99 and 908R) are deregulated and produce large amounts of enzyme.
[b] Constitutive production in some strains.
[c] Inducible in other *Bacteroides* strains or species.
[d] Probably the same or very similar protein.
An alternative classification, based on the catalytic profiles, has been proposed by Bush (1989a). Detailed references about the listed enzymes can be found in the same article.
CARB, carbenicillin-hydrolysing β-lactamases.

The structural β-lactamase genes are found both on the chromosome and on plasmids (*Table 1*). In this latter case, the danger of rapid spreading through an initially penicillin-sensitive population is evident. Gram-positive bacteria only produce class A and class B β-lactamases which are most often chromosome-encoded. In Gram-negative bacteria, class B and class C enzymes are usually chromosome-encoded (but some exceptions are known) and class D β-lactamases are always plasmid-encoded. The Gram-negative class A enzymes are usually plasmid-encoded, but in *Klebsiella* and *Proteus* the gene is on the chromosome, probably on a transposon. The most widespread plasmids code for the TEM and SHV β-lactamases

which are consequently responsible for many clinical problems. The production of the enzymes is either constitutive or inducible. In *Staphylococci*, the β-lactamase regulation system seems to share some elements with that of the penicillin-resistant PBP 2' (or 2a) (Joris, Hardt and Ghuysen, 1994). Synthesis of the class C β-lactamase is inducible in most Enterobacteria, with the notable exception of *Escherichia coli*. The strong selective pressure exerted in the hospital environment results in the emergence, with a high frequency, of constitutive overproducers of a class C enzyme. This is due to the inactivation of the *amp*D gene, whose product, a 21 000 M_r protein, is involved in the cytoplasmic recycling of peptidoglycan fragments. In *amp*D mutants, one of these fragments accumulates and triggers a continuous production of the enzyme by binding to the AmpR protein, which in turn acts as a transcription activator (Jacobs *et al.*, 1994a, 1994b).

Interestingly, a very similar regulation system, involving an AmpD and an AmpR-like protein also prevails in *Proteus vulgaris*, but the β-lactamase is a class A enzyme (Datz *et al.*, 1994). Similarly, an *amp*R gene, controlling the production of a class A enzyme, has been identified in the Gram-positive *Streptomyces cacaoi* (Lenzini *et al.*, 1992).

Sequence and structure comparisons

A large number of class A β-lactamase sequences are known and they exhibit a high degree of diversity, reflecting a similar diversity in the catalytic profiles (Matagne *et al.*, 1990; Ambler *et al.*, 1991). Only nine residues are strictly conserved and, even if highly conservative substitutions such as Leu → Ile are neglected, this number does not increase above 25, out of a total chain length of 270–290 residues. Nevertheless, superposition of the peptide backbones of the four class A enzymes whose structures have been established by X-ray diffraction highlights a strongly conserved general architecture (Dideberg *et al.*, 1987; Herzberg, 1991; Jelsch *et al.*, 1992; Lamotte-Brasseur *et al.*, 1991; Moews *et al.*, 1990; Knox and Moews, 1991), with the exception of a short stretch (residues 80–105) where insertions occur in several enzymes. The molecules present mixed α–β structures, with an all-helical domain on the left (*Figure 5a*) and a mixed α/β domain on the right, composed of five antiparallel β-strands covered by three helices. The helices and β-sheet comprise about 45% and 25% of the polypeptide, respectively. The active-site serine is situated in a depression between the two domains, at the N-terminus of the eleven residue α_2 hydrophobic helix mostly buried in the core of the all-α domain. Despite the fact that computer-aided sequence comparisons fail to reveal significant isology between the members of class A and class C, striking similarities in the general folding of the proteins have been detected when the structures of two class C enzymes were established (Oefner *et al.*, 1990; Lobkovsky *et al.*, 1993). A similar, two-domain organization was found (*Figure 5b*), with an identical positioning of the active-site serine between the two domains at the N-terminus of the α_2 helix. In the class C enzymes, four additional β-strands form a small antiparallel sheet, which is twisted with respect to the major, five-stranded sheet common to both classes of enzymes, while helix α_{10} is antiparallel to α_{11}, rather than parallel. The most important difference occurs at the bottom of the active site, where loops are found in both structures, but running antiparallel to each other (*Figure 6*). In class A enzymes, this loop is referred to as the Ω-loop and contains one turn of helical structure, helix α_7.

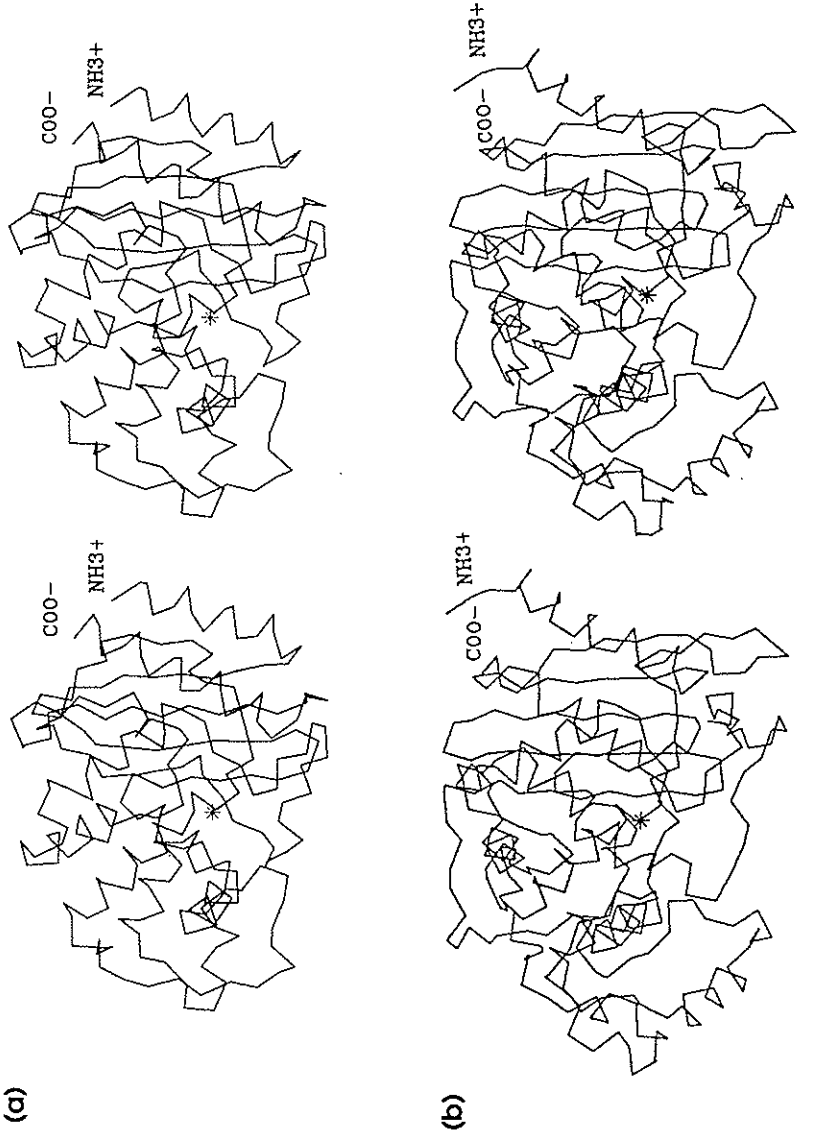

Figure 5. Tertiary structures, represented by the α-carbon traces of (a) the *Streptomyces albus* G (class A) and (b) the *Enterobacter cloacae* P99 (class C) β-lactamases. The active-site serine is labelled (*).

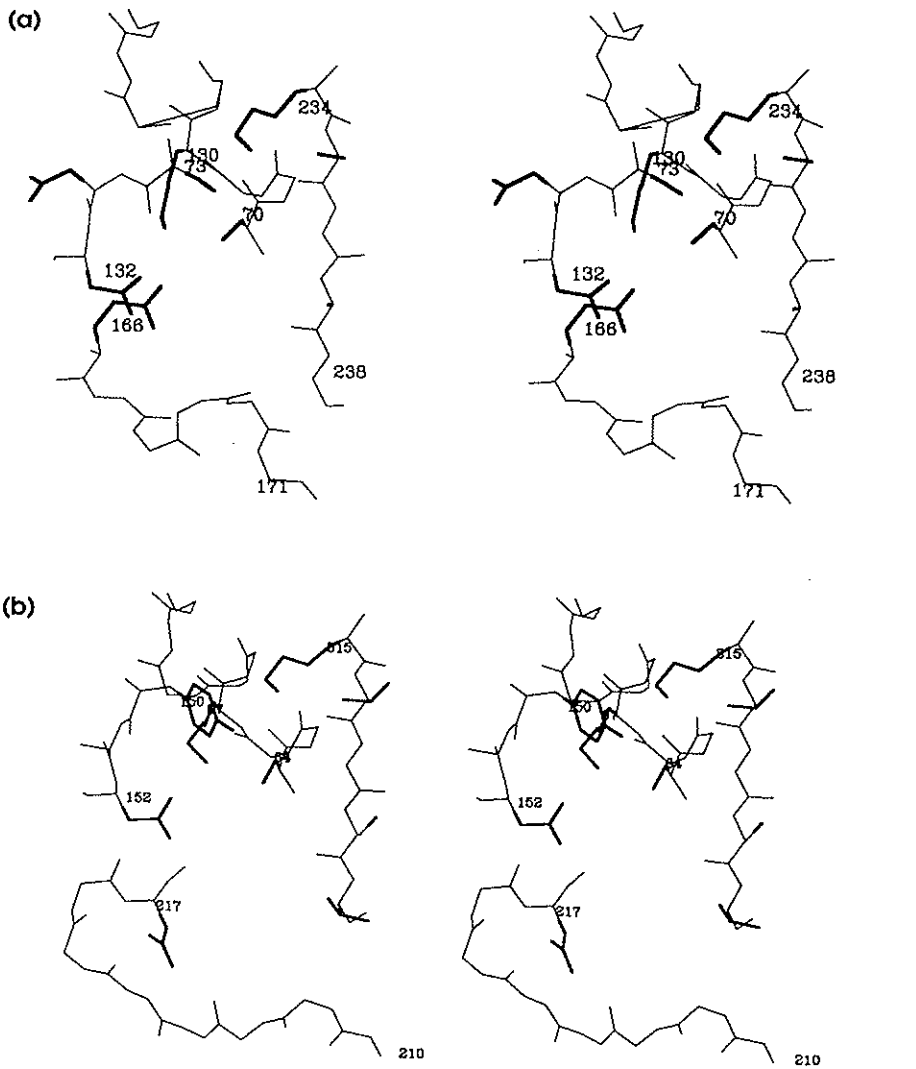

Figure 6. Simplified stereo views of the active sites of (a) class A (TEM1) and (b) class C (*E. cloacae* P99) β-lactamases. The similar relative positions of the first element Ser and Lys side-chains and of the third element Lys are quite striking. The hydroxyl groups of Ser130 in class A and Tyr150 in class C can also be superimposed. By contrast, the very different orientations of the Glu166 (class A) and Asp217 (class C) side-chains are easily visualized. The antiparallel directions of the backbone traces bearing these residues are highlighted by the numbering of the α-carbon atoms.

These differences notwithstanding, comparisons of the primary and tertiary structures have demonstrated the existence of three structural and functional elements, located in equivalent positions in the three-dimensional structures (Joris *et al.*, 1991) and containing residues with identical or chemically similar side-chains (*Table 2*).

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.

Table 2. The three equivalent functional elements of active-site serine β-lactamases and penicillin-sensitive DD-peptidases

	Element 1	Element 2	Element 3
Class A	70	130	234
	S*–X–X–K	S–D–N	K–T–G
		S–D–S	K–S–G
			R–S–G
			R–T–G
Class C	64	150	314
	S*–X–X–K	Y–A–N	K–T–G
Class D	70	144	214
	S*–X–X–K	Y–G–N	K–T–G
S. R61 DD-peptidase	62	159	298
	S*–V–T–K	Y–S–N	H–T–G
Other known PBPs	S*–X–X–K	S–X–N	K–T–G
		S–X–C	K–S–G
		Y–G–N	

The second element is situated on a loop in the all-α domain. The side-chains of the first and third residues in this sequence 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, forming a hydrogen bond with the Lys of the first element, with one single exception in a class A enzyme, where Ser replaces Asn.

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 of this conserved sequence 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. The side-chain of the Lys residue forms a hydrogen bond with the hydroxyl group of the Ser/Tyr residue in the second element.

In addition, in class A enzymes, the Ω-loop contains a strictly conserved Glu residue (E166) whose carboxylate forms a salt-bridge with the alkylammonium group of Lys73. In most cases, the loop contains the E166 X E X N sequence. An acidic residue (Asp217) is also observed in the corresponding, but antiparallel loop of class C β-lactamases, but its side-chain points *away* from the active site (*Figure 6*).

No class D β-lactamase tertiary structure has been presently determined, but sequence alignments allow the identification of the three structural elements (Joris *et al.*, 1991). Moreover, the elucidation of the *Streptomyces* R61 DD-peptidase structure reveals the same two-domain organization as in β-lactamases, with a similar position of the active-site serine and the presence of structural elements which nicely superimpose on those of the class A and C β-lactamases, results which underline the close relationships between the two families of penicillin-recognizing enzymes (Kelly *et al.*, 1989). The three elements can be identified in the sequences of all penicillin-binding proteins, irrespective of the exact affinity that these enzymes exhibit for the β-lactam antibiotics (*Table 2*).

In the high-molecular-mass penicillin-binding proteins, whose M_r value can be as high as 100 000, the penicillin-binding and putative transpeptidase domain which

contains these elements is located in the C-terminal portion of the protein, whereas the N-terminal part supplies the membrane-anchoring segment and, at least in some cases, a polypeptide chain catalysing the transglycosylation, the reaction which immediately precedes transpeptidation in the peptidoglycan biosynthesis (Ghuysen, 1991).

It is interesting to note that the fold of class C β-lactamases is much closer to that of the *S*.R61 DD-peptidase than is the fold of the class A enzymes (Lobovsky *et al.*, 1993). The class C and *S*.R61 enzymes also exhibit a tyrosine as the first residue of the second element, in contrast to the class A β-lactamases and most PBPs where this residue is a serine.

The similarity between β-lactamases and DD-peptidases is further underlined by the finding that various compounds behave as substrates of both types of enzymes. Indeed, even if the β-lactamases fail to recognize the peptide substrates, both types of enzymes hydrolyse linear esters, thiolesters and an aziridine (*Table 3*). The class C β-lactamases even catalyse transacylation reactions with ester and thiolester carbonyl donors and D-amino acids as acceptors (Pratt and Govardhan, 1984; Pazhanisamy, Govardhan and Pratt, 1989; Adam *et al.*, 1990; Jamin *et al.*, 1991).

Table 3. Linear substrates of DD-peptidases and β-lactamases

Substrate	k_{cat}/K_m value (M^{-1} s^{-1})		
	S.R61 DD-peptidase	Class A β-lactamase	Class C β-lactamase
Peptide I	4000	0	0
Ac_2-LLys-DAla-DAla	Tp:+		
Ester II[a]			
C_6H_5-CO-Gly-O-CH-COOH CH_2-C_6H_5	5500 Tp:+	80	20 000 Tp:+
Thiolester III	100 000	4500	7000
C_6H_5-CO-Gly-S-CH_2-COOH	Tp:+		Tp:+
Thiolester IV			
C_6H_5-CO-DAla-S-CH_2-COOH	400 000 Tp:+	<10	180
Aziridine V	600	120[b]	130
C_6H_5-CH_2-CO-Gly-N-CH-COOH		1900[c]	

The class A and class C β-lactamases are those of *Bacillus licheniformis* 749/C and of *Enterobacter cloacae* P99, respectively.
Tp, Transpeptidation or transacylation.
[a] The phenyllactate has a *D* configuration.
[b] and [c] Results obtained by Murphy and Pratt (1991) with other class A enzymes: [b] TEM1 and [c] *Bacillus cereus* I.

At this point, it is interesting to compare some kinetic parameters of the different enzymes. *Table 4* highlights the very low k_{+3} values of the DD-peptidase and striking differences in the rates of acylation which are generally much higher with the β-lactamases, although cefoxitin acylates the *S* R61 DD-peptidase more readily than the class A enzymes. The relative resistance of some compounds to hydrolysis by several class A enzymes is often due to low acylation rates (cefuroxime, cefotaxime and TEM-1) while in class C, this is most often due to slow deacylation (ampicillin, cefuroxime, cefotaxime and cefoxitin). The wide diversity in the catalytic properties of class A enzymes has been discussed by Matagne *et al.* (1990) and Raquet *et al.* (1994). The class C β-lactamases seem to form a more homogeneous group (Galleni,

Table 4. Some kinetic parameters characterizing the interactions between penicillin-recognizing enzymes and their substrates or inactivators

Substrate	β-lactamases								*Streptomyces* R61 DD-peptidase	
	Class A						Class C			
	Bacillus licheniformis		TEM1		*Staphylococcus aureus* PC1		*Enterobacter cloacae* P99			
	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}\ s^{-1}$)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}\ s^{-1}$)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}\ s^{-1}$)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}\ s^{-1}$)	k_{+3} (s^{-1})	k_{+2}/K_1 ($mM^{-1}\ s^{-1}$)
Benzylpenicillin	2200	29000	1600	84000	400	80000	15	35000	1.4×10^{-4}	17
Ampicillin	1500	11000	1000	33000	750	50000	1	1200	1.4×10^{-4}	0.11
Carbenicillin	400	8000	220	25000	180	70	0.003	220	1.4×10^{-4}	0.17
Cephaloridin	630	5000	1500	2200	0.07	100	700	10000	-	0.15
Cephalothin	50	2500	160	650	0.006	8	200	20000	3×10^{-6}	0.8
Cefuroxime	16	170	6	6	0.07	1.2	0.05	3000	4×10^{-6}	0.8
Cefotaxime[a]	7	30	9	1.5	Not detectable		0.015	1500	$<4 \times 10^{-6}$	0.016
Cefoxitin	-	0.02	0.004	0.006	Not detectable		0.06	2500	5×10^{-5}	1.5
References	Matagne *et al.* (1990)		Raquet *et al.* (1994)		Richard Virden, personal communication; Matagne and Frère, unpublished		Galleni, Amicosante and Frère (1988)		Frère and Joris (1985)	

a Various TEM variants hydrolyse this compound much more efficiently.
(-) Not determined.
Note that $k_{cat}/K_m = k_{+2}/K_1$.

Amicosante and Frère, 1988) but these results concern a much smaller number of enzymes.

Mechanistic considerations

The catalytic mechanism of other active-site serine amidases and esterases has been studied in detail (see for instance Fersht, Blow and Fastrez, 1973; Brady *et al.*, 1990). 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 bonds, with the main chain-NH-groups of Ser195 and Gly193 in the case of chymotrypsin (*Figure 7*). 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 the structures, comprising the main chain -NH-groups of Ser70 and Ala237 in the class A enzymes, Ser64 and Ser/Ala318 in class C (*Figure 8*) and Ser62 and Thr301 in the DD-peptidase (Kelly *et al.*, 1989; Moews *et al.*, 1990; Oefner *et al.*, 1990; Herzberg, 1991; Jelsch *et al.*, 1992; Lobkovsky *et al.*, 1993). Conversely, no conserved histidine is found in β-lactamases and PBPs. The *Streptomyces albus* G class A β-lactamase does not even contain one such residue (Dehottay *et al.*, 1987). In consequence, the search

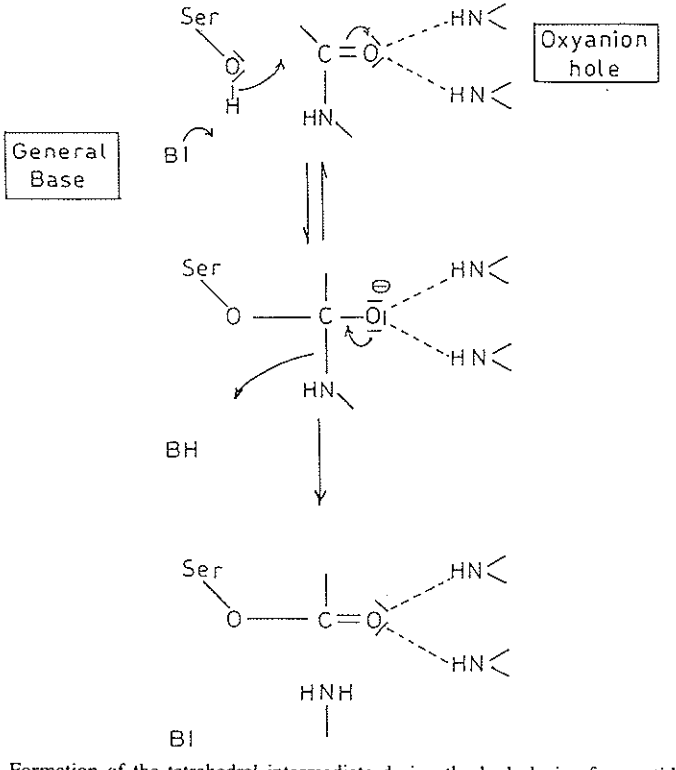

Figure 7. Formation of the tetrahedral intermediate during the hydrolysis of a peptide bond by an active-site serine enzyme, showing the combined actions of a general base (BI, His57 in chymotrypsin) and of the oxyanion hole and collapse of the tetrahedral intermediate to yield the acylenzyme.

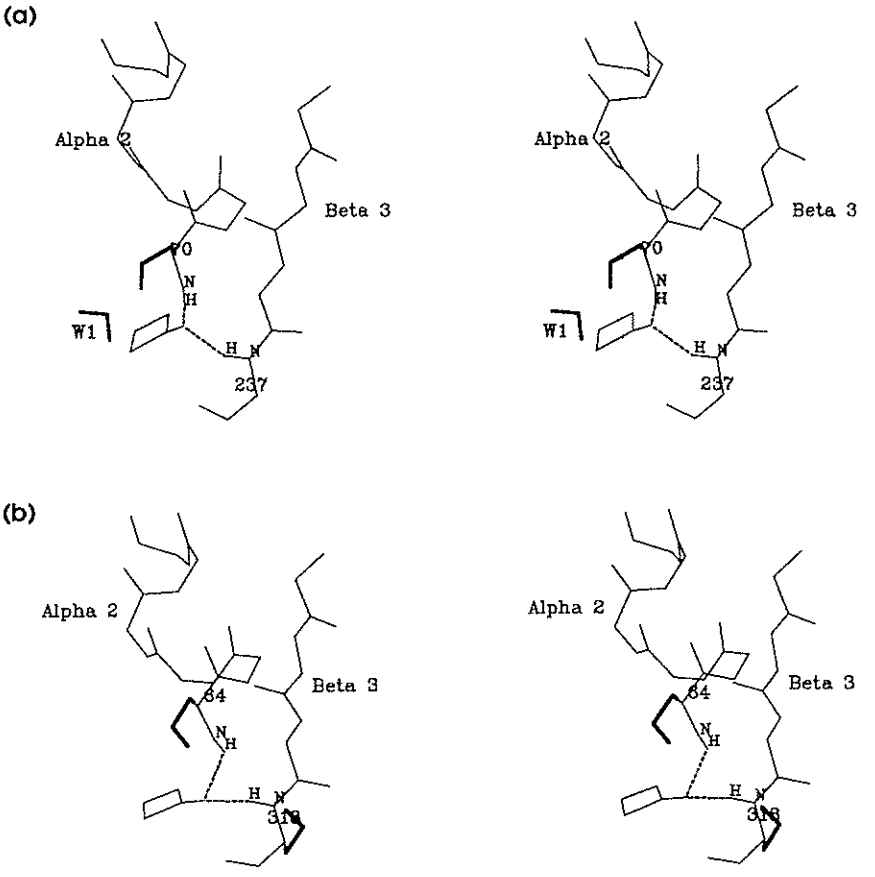

Figure 8. The oxyanion holes in (a) class A and (b) class C β-lactamases. A β-lactam ring has been positioned in the active sites by molecular modelling. The hydrogen bonds formed between the β-lactam carbonyl and the two backbone amide groups forming the oxyanion holes are shown.

for a general base in β-lactamases has generated a large number of studies in which the properties of numerous modified proteins obtained by site-directed mutagenesis have been analysed. In the following paragraphs, these results will be summarized, together with those involving the other residues of the conserved structural elements. For a more exhaustive review of site-directed experiments performed with class A enzymes see Matagne and Frère (1994).

To facilitate the discussion, the few cases in which the individual rate constants have been determined will be presented first.

Individual rate constants

In many cases the high turn-over numbers of β-lactamases have unfortunately precluded the determination of the individual rate constants. Waley and his co-workers (Christensen, Martin and Waley, 1990; Gibson, Christensen and Waley, 1990) have, however, succeeded in obtaining information with some class A enzymes and penicillin substrates (*Tables 5* and *6*). The interactions are characterized by very

high k_{cat}/K_m values, close to the diffusion limit. The values of k_{+2} and k_{+3} are similar so that, at the steady-state and at substrate saturation, the acylenzyme represents about 50% of the total enzyme. This might not be true for some cephalosporins and the *B. cereus* β-lactamase I, in which case k_{+2} is much smaller than k_{+3}, in contrast with the situation with nitrocefin, a cephalosporin with an unusually reactive β-lactam ring, where $k_{+2}/k_{+3} = 0.4$.

Table 5. Individual rate constants for the hydrolysis of benzylpenicillin at 20°C

Rate constant (units)	*B. cereus* β-lactamase I	*S. aureus* PC1	TEM1
k_{+1} (μM^{-1} s^{-1})	40	22	120
k_{-1} (s^{-1})	2300	200	12000
k_{+2} (s^{-1})	4100	170	2800
k_{+3} (s^{-1})	2000	100	1500
K' (μM)	160	17	120

Data from Christensen, Martin and Waley (1990).

Table 6. Values of k_{+2} and k_{+3} for some class A enzymes (at 20°C)

Enzyme	Substrate	$k_{+2}(s^{-1})$	$k_{+3}(s^{-1})$
Bacillus cereus β-lactamase I	Phenoxymethyl penicillin	5000	4500
	Phenethicillin	6500	2700
	Carbenicillin	1100	730
	Nitrocefin	30	71
Staphylococcus aureus PC1	Phenoxymethyl penicillin	130	100
	Phenethicillin	110	50

Data from Christensen, Martin and Waley (1990).

With class C β-lactamases, deacylation is generally rate-limiting, as shown by the increased reaction rates observed upon addition of methanol (Knott-Hunziker *et al.*, 1982). Values of k_{+2} could only be determined for some poor substrates, since those of good substrates and of many poor substrates were too high to allow estimation by stopped-flow methods (*Table 7*; Monnaie, Virden and Frère, 1992).

Table 7. Kinetic parameters for some substrates of the class C *Enterobacter cloacae* 908R β-lactamase (at 30°C)

Substrate	$k_{+2}(s^{-1})$	$k_{+3}(s^{-1})$	k_{+2}/k_{+3}	K'(μM)
Carbenicillin	3.3	0.003	2500	15
Ampicillin	120	1	120	100
Benzylpenicillin	>150	15	>10	>5
Cloxacillin	>150	0.006	>40000	>15
Cefotaxime	>150	0.006	>25000	>100
Imipenem	>150	0.003	>50000	>1000
Aztreonam	>150	0.0004	>700000	>800

Data from Monnaie, Virden and Frère (1992).

Site-directed mutagenesis

The major results are listed in *Tables 8–10*. A critical analysis of these data highlights a few clear conclusions but also underlines unexpected, distinct behaviours of the different enzymes, which makes it difficult to propose a 'unified' mechanism, not only for the β-lactamases and DD-peptidases, but also for the β-lactamases of different classes.

FIRST ELEMENT: THE ACTIVE-SITE SERINE

The replacement of the active Ser by Cys yields significantly poorer β-lactamases, with altered substrate profiles (Sigal *et al.*, 1984; Jacob, Joris and Frère, 1991). The acylation of the class C enzyme appears to be specifically impaired, while the k_{+3} value is barely modified (Dubus *et al.*, 1993). This indicates that the nucleophilic attack on the substrate carbonyl probably requires a more stringent geometry than the hydrolysis of the acylenzyme, which might be facilitated by the more pronounced reactivity of the thiolester. Accordingly, the equivalent mutant of the *S.* R61 peptidase completely loses both activity and penicillin-binding capacity (Hadonou *et al.*, 1992a), also suggesting defective acylation processes. In this respect, the fact that no active-cysteine β-lactamase or DD-peptidase has ever been found is worth mentioning. Surprisingly, the S70A mutant of the *S. albus* G β-lactamase retains a very low, but significant, activity, with a $kcat/K_m$ for benzyl penicillin of 200 M^{-1} s^{-1}, i.e. 0.01% of the wild-type (WT) protein. Other groups in the catalytic site somehow directly activate a hydrolytic water molecule, but the initial binding of the substrate also seems to be deficient (Jacob, Joris and Frère, 1991).

FIRST ELEMENT: THE LYSINE RESIDUE

The individual rate constants for the hydrolysis of benzylpenicillin by the K73R mutant of the *B. cereus* class A β-lactamase were determined by Gibson, Christensen and Waley (1990), who showed that k_{+2} was decreased 70-fold and k_{+3} less than 15-fold. A similarly impaired acylation step appears to prevail for the corresponding K67R and K65R mutants of the class C β-lactamase (Monnaie, Dubus and Frère, 1994) and of the DD-peptidase (Hadonou *et al.*, 1992a). With the former, this seems to be due to an increase of K rather than to a decrease of k_{+2}, a result which underlines the fact that similar effects on the steady-state parameters can result from variations of different rate constants. The K67Q mutant of the class C enzyme is much more severly affected and this might not only suggest an electrostatic role for the positive charge on the 67 side-chain, but also highlight the central role of the dense array of hydrogen bonds in the active site, which may be partially conserved with an Arg, but not with a Gln side-chain.

With the DD-peptidase, the K65R is the only mutant where the modification of a conserved residue results in a smaller decrease of the enzymic activity than of the penicillin acylation rate, thus yielding a poor, but essentially pencillin-insensitive DD-peptidase.

Table 8. Kinetic characteristics of class A β-lactamase mutants

		Benzylpenicillin		Cephalosporin C		Cephalothin		Cephaloridine		Nitrocefin		Enzymes and references
		Relative values[a] of		Relative values[a] of		Relative values[a] of		Relative values[a] of		Relative values[a] of		
		k_{cat}	k_{cat}/K_m	k_{cat}	k_{cat}/K_m	k_{cat}	k_{cat}/K_m	k_{cat}	k_{cat}/K_m	k_{cat}	k_{cat}/K_m	
S70C		80	130	-	-	-	-	850	1500	-	-	TEM1, Sigal *et al.* (1984)
		-	6000b	-	>10000	-	100000	-	25000	-	-	*S. albus* G, Jacob, Joris and Frère (1991)
K73R		40	60	20	35	-	-	-	-	170	400	*B. cereus*, Gibson, Christensen and Waley (1990)
S130A		40	40	-	500	700	1000	-	3300	-	12	*S. albus* G,
S130G		>1	6	-	500	800	1000	-	800	-	4	Jacob *et al.* (1990a,b)
N132S		2	2	-	300	500	2000	430	1000	-	2000	*S. albus* G,
N132A		-	300	-	-	-	>100000	-	>10000	–	10000	Jacob *et al.* (1990b)
K234H,	pH=5.5[c]	2	11	-	50	-	80	-	-	4	6	*S. albus*, G,
	pH=7.0	10	63	-	-	-	440	-	-	-	48	Brannigan *et al.* (1991)
K234R		1	10	-	-	1.5	1.2	1.2	1.5	-	-	TEM1,
K234T		50	2000	-	-	>300	-	-	-	2.5	-	Lenfant, Labia and Masson (1991)
K234A		-	10000	-	-	-	-	-	-	-	7000	*B. licheniformis*,
K234E		50	3000	-	-	-	-	-	-	40	3500	Ellerby *et al.* (1990)
S235A		3	4	100	250	-	-	14	130	-	-	TEM1, Imtiaz *et al.* (1993a)
		4	3	10	260	7	270	45	300	<5	100	TEM1, Dubus *et al.* (1994a)
E166D		3300	500	200	180	-	-	-	-	170	230	*B. cereus*, Gibson, Christensen and Waley (1990)
E166A		3000	600	-	-	-	-	-	-	3000	200	*B. cereus*,
E166C		6000	1200	-	-	-	-	-	-	5000	300	Leung *et al.* (1994)
E166A		>10^7	-	-	-	-	-	-	-	>10^7	-	*B. licheniformis*, Escobar, Tan and Fink (1991)
E166Y		28000	8000	-	-	4300	80	2000	50	-	-	TEM1, Delaire *et al.* (1991)
E166N		>10^7	-	-	-	-	-	-	-	-	-	TEM1, Adachi, Ohta and Matsuzawa (1991)
R220L[d]		-	60	-	600	-	800	-	6	-	14	*S. albus* G, Jacob-Dubuisson *et al.* (1991)
R244K		0.9	1.5	-	-	-	-	1	1	1	1.3	TEM1,
R244S		1	8	-	-	-	-	1	1.6	1.5	2.5	Zafaralla *et al.* (1992)
R244T		6	60	-	-	240	300	140	1300	-	-	TEM1,
R244Q		2	20	-	-	240	300	70	700	-	-	Delaire *et al.* (1992)
M69L		1.6	2.5	-	–	5	5	4	4	-	-	TEM1, Delaire *et al.* (1992)

[a] Relative values = (parameter) wild type/(parameter) mutant; [b] Result obtained with ampicillin; [c] At pH > 8, k_{cat} is close to zero; [d] k_{cat}/K_m values increase with the methyl ester of benzylpenicillin and the lactone of cephalosporin C.

Table 9. Kinetic characteristics of class C β-lactamase mutants

	Benzylpenicillin		Cephalothin		Cephaloridine		Nitrocefin		Cefotaxime		Enzymes and references
	Relative values of		Relative values of		Relative values of		Relative values of		Relative values of		
	k_{cat}	k_{cat}/K_m	k_{cat}	k_{cat}/K_m	k_{cat}	k_{cat}/K_m	k_{cat}	k_{cat}/K_m	k_{cat}	k_{cat}/K_m	
S64C	1.5	5000	25000	100000	5000	7000	<50	2000	0.4	3000	*E. cloacae* 908R, Dubus *et al.* (1993)
K67R	1	1000	1.5	25	3	20	<4	500	0.1	250	*E. cloacae* 908R
K67Q	–	–	2500	45000	–	–	–	–	–	–	Monnaie, Dubus and Frère (1994)
Y150S	35	25	90	400	540	400	200	55	1	1.3	
Y150F	15	9	1500	6000	270	400	100	2	0.6	18	*E. coli* K12,Dubus *et al.* (1994b)
Y150E	60	150	120	6000	1800	860	800	1000	2.5	12	
Y150S	9	150	20000	7000	3800	15000	300	10	14	10000	*E. coli* K12,
Y150F	400	90	10000	350	4400	90	1800	17	100	–	Dubus and Frère, unpublished
K315H[a]	20	1400	300	300	–	–	<8	100	–	–	*E. cloacae* 908R,
K315Q	560	5600	1400	1200	–	–	<70	800	–	–	Monnaie *et al.* (1994)
T316A[a]	0.5	1.5	1.7	37	2	20	1	1.3	2	15	*E. coli* K12,
T316V	1.2	3	6	4000	<10	120	20	40	4	160	Dubus *et al.* (1994)

a Loss of transacylation properties.
–, Not determined.

Table 10. Kinetic characteristics of the *S.* R61 DD-peptidase mutants

	Peptide I		Ester II		Thiolester III			Benzyl-penicillin	Cephalosporin C,	Cefuroxime,	References
	Relative values of		Relative values of		Relative values of		Relative	Relative	relative	relative	
	k_{cat}	k_{cat}/K_m	k_{cat}	k_{cat}/K_m	k_{cat}	k_{cat}/K_m	T/H	k_{+2}/K	k_{+2}/K	k_{+2}/K	
K65R	–	200	–	–	<1	1500	–	20000	–	–	Hadonou *et al.* (1992a)
Y159S	2500	3000	250	400	16	1.6	>50	1.7	3	0.5	Wilkin *et al.* (1993a)
Y159F	7500	8000	–	>5000	600	1000	>50	300	100	>70	
N161S	30	30	15	20	15	2.5	4	1	3	0.3	Wilkin *et al.* (1993b)
N161A	350	300	40	200	2.5	30	7	70	45	1	
H298K	<50	130	–	–	5	2.5	4	60	12	–	Hadonou *et al.* (1992b)
H298Q	100	180	–	–	4	10	22	100	60	–	
T299V	550	900	–	>5000	3	12	50	25	>15000	>2000	Wilkin *et al.* (1994)

Relative T/H is the $\frac{(\text{transpeptidation/hydrolysis})_{WT}}{(\text{transpeptidation/hydrolysis})_{mutant}}$ ratio under the same conditions of acceptor and donor substrate concentrations (WT = wild type).

Replacement of the following residues by uncharged side-chains did not significantly alter the activity: E172, E182, D195, D225, E228, D246, D248 (Hadonou *et al.*, 1992a; Wilkin and Frère, unpublished).

SECOND ELEMENT: THE SERINE/TYROSINE RESIDUE

Replacement of Ser130 by Gln in the *S. albus* G β-lactamase yields a nearly inactive protein since this bulky side-chain does not allow an adequate binding of the substrate. In the S130A and S130G mutants, the loss of the important 130–234 hydrogen bond results in a considerable destabilization, but the proteins retain some activity against benzylpenicillin (2.5 and 17%), methicillin (10 and 35%) and nitrocefin (8 and 25%). With all other substrates, the activity is below 1%. The *k*cat/*K*m values are generally more affected for cephalosporins (Jacob *et al.*, 1990a,b).

The analysis of the Y150F, Y150S and Y150E mutants of the *E. coli* K12 class C enzyme yields a particularly incoherent picture (Dubus *et al.*, 1994b) and significantly different results have been obtained in different laboratories (Dubus and Frère, unpublished). However, the general trend shows large decreases (100-fold or more) of *k*cat and *k*cat/*K*m for good substrates, while some poor substrates seem to be less affected. Apparently, the presence of slowly isomerizing forms of the free enzyme seriously complicates the analysis (Page, 1993).

The activity of both the Y159S and the Y159F mutants of the *S.* R61 DD-peptidase on the peptide and ester substrates is strongly impaired but, surprisingly, acylation of the former by the thiolester and β-lactams is barely modified – it is even increased with cefuroxime (Wilkin *et al.*, 1993a). The mutants are totally unable to catalyse the transpeptidation reaction.

SECOND ELEMENT: THE ASPARAGINE RESIDUE

The N132S mutant of the *S. albus* G enzyme exhibits a spectacular modification of its substrate profile: while retaining most of the penicillinase activity of the wild-type protein, it becomes a very poor cephalosporinase (Jacob *et al.*, 1990a), properties similar to those of the *S. aureus* β-lactamase, where residue 132 is, however, the usual Asn. A serine residue in position 132 is found in the *Bacillus cereus* β-lactamase III, an enzyme which is not specifically more active on penicillins than on cephalosporins. These results underline the difficulties encountered when one attempts to generalize the behaviour of even closely related proteins. Although more impaired, the behaviour of the N132A mutant of the *S. albus* G enzyme parallels that of its N132S counterpart.

The peptidase and esterase activities of the *S.* R61 DD-peptidase are also more affected by the N161A than by the N161S mutations and, strikingly, acylation of the latter by the thiolester and β-lactams are unaffected (Wilkin *et al.*, 1993b).

Preliminary results (A. Dubus, personal communication) indicate very impaired activities for the N152D, E, L and H mutants of a class C enzyme.

THIRD ELEMENT: THE LYSINE RESIDUE

In class A, the main function of this side-chain mainly appears to be to supply a positive charge. The K234R and, at low pH, the K234H mutants are fully active. Moreover, an R234 side-chain naturally occurs in the CARB class A enzymes (Lachapelle, Dufresne and Levesque, 1991; Couture, Lachapelle and Levesque, 1992) which are especially active against carbenicillin (Labia, Guionie and Barthélémy,

1981), a compound bearing a double negative charge at neutral pH (R = C6H5-CH(COO^-)–, *Figure 3A*). The other mutants, with a neutral or negative 234 side-chain are three orders of magnitude less active. Some of the results suggest that the positive charge might enhance both initial recognition and transition state stabilization (Lenfant, Labia and Masson, 1991), but the *k*cat (and *not* the *K*m) value of the K234H mutant closely parallels the titration of the imidazole group (Brannigan *et al.*, 1991), indicating a major role for the second factor.

The pH-dependency of the class C enzyme K315H mutant is quite different and the activity remains poor even at pH 5.0 (Monnaie *et al.*, 1994). Although this mutation increased its similarity to the R61 DD-peptidase, it resulted in a loss of the transacylation properties of the *E. cloacae* enzyme. Conversely, the H298K and H298Q mutants of the peptidase were significantly affected, but less so than the class C mutants, with an additional, specific impairment of the transpeptidation properties (Hadonou *et al.*, 1992b). Optimization of the respective activities of the two enzymes has thus been attained by the utilization of different side-chains. In this respect, it is also worth remembering that the *S.* R61 enzyme is the only known DD-peptidase with a His residue in the third element, all the other ones exhibiting β-lactamase-like KTG or KSG sequences.

THIRD ELEMENT: THE THREONINE/SERINE RESIDUE

The hydroxyl group of this residue is one of the very few functionalities to be strictly conserved in all the active-site serine penicillin-recognizing proteins, and its elimination was expected to result in dramatic decreases of the enzyme activities. Surprisingly, with the β-lactamases, this is only so for the cephalosporinase properties, suggesting that the 'natural' selective pressure responsible for the conservation of the hydroxyl group was due to cephalosporins rather than penicillins (Imtiaz *et al.*, 1993a; Dubus *et al.*, 1994a). The parallel strict conservation of a Ser or Thr in DD-peptidases is more easy to rationalize, since the T299V mutant of the *S.* R61 enzyme becomes a poor peptidase, practically devoid of transpeptidation properties, and consequently unable to efficiently participate in peptidoglycan biosynthesis (Wilkin *et al.*, 1994).

THE GLUTAMATE166 RESIDUE OF CLASS A β-LACTAMASES

This is certainly the active-site residue whose role in the catalytic process has been the subject of the most heated controversies. The experimental results also appear to be quite incoherent and difficult to reconcile. In several cases, substitutions by other residues (including the conservative Glu→Asp mutation) decrease both the *k*cat and *k*cat/*K*m parameters, with rather minor effects on the *K*m values. The E166D mutant of the *B. cereus* enzyme has been particularly well studied by Gibson, Christensen and Waley (1990), who observed similar, 2000-fold decreases of k_{+2} and k_{+3}. By contrast, two other mutants, E166A of *B. licheniformis* (Escobar, Tan and Fink, 1991) and E166N of TEM (Adachi, Ohta and Matsuzawa, 1991) are nearly completely unable to catalyse the deacylation step. The most surprising discrepancy is the enormous difference in the behaviours of the identical E166A mutants of the closely related *B. cereus* and *B. licheniformis* enzymes. The most recent data suggest, however, that the apparent k_{+3} value for the latter might be lowered by an isomerization of the

acylenzyme into a non-productive form (Escobar *et al.*, 1994) and, in the same contribution, the authors confirm that the acid limb of the *k*cat/*K*m pH profile indeed reflects the protonation state of Glu166.

Catalytic mechanisms

THE GENERAL BASE

The nucleophilic attack of the substrate carbonyl group by an active-site serine side-chain is expected to be facilitated by a general base to which the proton of the serine hydroxyl group is transferred during this process. As noted above, this function is often fulfilled by a histidine residue in other active-site serine enzymes. The pH-dependencies of the *k*cat/*K*m values of class A β-lactamases suggest the involvement of a more acidic group, with a pK of 4.5–5.5 (Waley, 1975; Brannigan *et al.*, 1991). The properties of the E166D mutant of the *B. cereus* enzyme underline the central role of the Glu166 side-chain in both the acylation and deacylation steps.

GLU166 IN CLASS A ENZYMES

The side-chain of this residue takes part in a dense hydrogen-bond network within the active site. As seen above, no corresponding residue is found in that position in the class C enzymes or in the *S.* R61 DD-peptidase structure (*Figure 6*). Its importance in the catalytic process was suggested by the study of a natural variant of the *Staphylococcus aureus* enzyme (Herzberg *et al.*, 1991). The structures show, however, that the distance between the Glu166 carboxylate group and the active serine hydroxyl is too long to allow a direct proton transfer between the two side-chains. On the other hand, the crystallographic and modelling data (Lamotte-Brasseur *et al.*, 1991) clearly show that a conserved water molecule might serve as a relay in this proton transfer (*Figure 9*). 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 (*Figure 3*) acylates the active serine of class A enzymes with an exceedingly poor efficiency (*Table 4*). It does, nevertheless, easily fit into the active site, but the 7-methoxy group displaces the water molecule (Matagne *et al.*, 1993), which can explain why no further reaction occurs after the formation of the non-covalent ES complex (*Figure 10*). Studies performed with the class A enzyme from *Staphylococcus aureus* PC1 and a covalently bound phosphonate acting as a transient state analogue seem to confirm this hypothesis (Rahil and Pratt, 1994).

By contrast, with the D166N mutant of the TEM β-lactamase, acylation appears to occur readily, while deacylation is extremely slow (Adachi, Ohta and Matsuzawa, 1991; Strynadka *et al.*, 1992). To explain these results, it has been proposed that the deprotonated side-chain of Lys73 might accept the serine proton in acylation, while the role of Glu166 would be restricted to the deacylation step. This is only possible if the enzyme active-site environment decreases the pK of the alkylammonium group of Lys73 by 5–6 pH units.

According to the authors, such an exceptionally large effect could be due to a very positive electric field created by the α2-helix dipole, the alkylammonium group of Lys234 (element 3) and, possibly, some other residues, such as Arg244 and Arg164

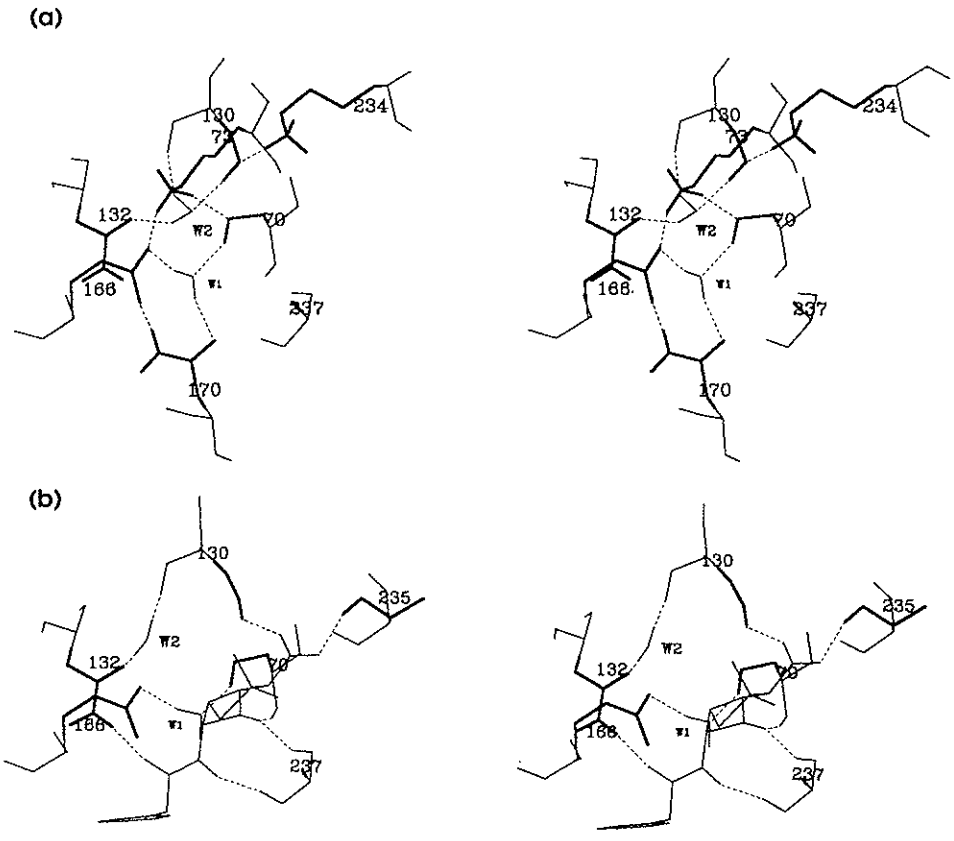

Figure 9. The dense array of hydrogen bonds in the active site of a class A β-lactamase. The two conserved water molecules, detected by high-resolution X-ray crystallography are labelled W1 and W2. W1 is hypothesized to act as a relay in the transfer of the Ser70 proton to Glu166. In (a) the active site is empty; in (b) a benzylpenicillin molecule has been docked by molecular modelling. The side-chains of the enzyme residues are shown as heavy lines.

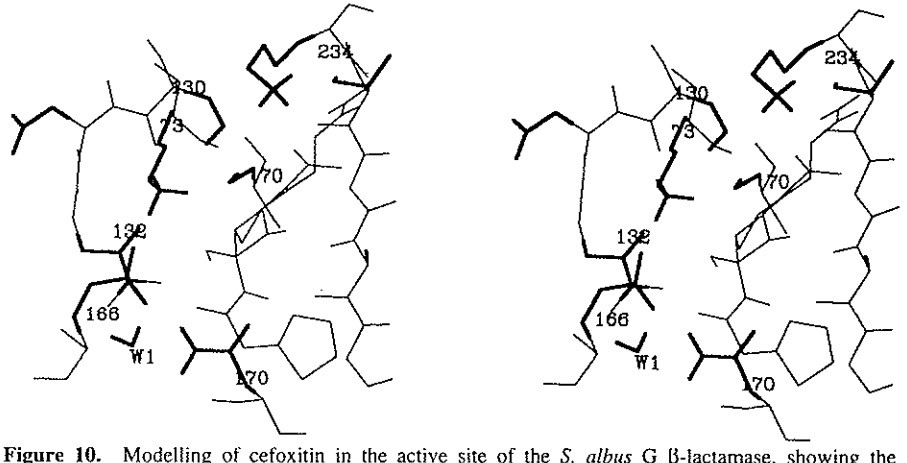

Figure 10. Modelling of cefoxitin in the active site of the *S. albus* G β-lactamase, showing the displacement of W1 by the methoxy group of the substrate. The side-chains of the residues are shown as heavy lines.

located somewhat further away from the active-site serine. The major advantage of this hypothesis is that it allows a uniform mechanism to be proposed for all active-site serine penicillin-recognizing enzymes. Indeed, the Lys of the first element is the only strictly conserved residue with acid/base properties in all these proteins, and its replacement by another residue results in significant impairment in all cases. However, for the class C enzyme the decreased acylation rate of the K67R mutant seemed to be due to an increased K' value, whereas a specific effect on k_{+2} would be expected if Lys67 were the general base. Moreover, in the case of the class A enzymes, this hypothesis, which rests on electrostatic factors, is clearly undermined by the fact that the carboxylate group of Glu166 is much closer to the ε-NH2/NH3$^+$ group of Lys73 than is any positively charged side-chain (Moews *et al.*, 1990; Lamotte-Brasseur *et al.*, 1991) and, in fact, one would even expect the elimination of the negatively charged carboxylate to *increase* the nucleophilicity of the Lys73 side-chain. As shown by Gibson, Christensen and Waley (1990) 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 which indicates a more crucial role for this latter residue. Finally, nuclear magnetic resonance (NMR) titration of the 13C-labelled lysine residues in the TEM enzyme failed to reveal that one of these side-chains exhibited an abnormally low pK (Raquet, Damblon and Frère, unpublished). In addition, modification of Asn170 in the *B. licheniformis* enzyme, a residue which helps maintain both the water molecule and the side-chain of Glu166 in their observed positions (*Figure 9*), also results in decreased acylation rates (Fink, personal communication).

The hypothesis of Strynadka *et al.* (1992) might, however, remain valid in the case of the mutant that they have studied. Indeed, as suggested above, their electrostatic argument would be more convincing if no Glu166 was present, but one may then wonder why a deprotonated Lys73 cannot also replace Glu166 in the deacylation step.

Similarly, in class C β-lactamases, the absence of a residue equivalent to the class A Glu166 results in a distinctly more positive active site, and the same situation might possibly prevail in class D β-lactamases. By superimposing the active serine side-chain and the two oxyanion hole NH groups of the *Citrobacter freundii* class C enzyme on the corresponding groups of chymotrypsin, Oefner *et al.* (1990) observed that the Tyr150 oxygen atom of the former was in a position equivalent to the proton-accepting imidazole nitrogen of His57 in the latter, thus making Tyr150 a possible candidate for the role of general base. Again, the pK of this residue should be very low, at around 5–6 to account for the pH-activity profile of the enzyme, a value which might now be explained by electrostatic considerations. Crystallographic results obtained with a phosphonate monoester inhibitor and another class C enzyme from *E. cloacae* confirmed this hypothesis (Lobkovsky *et al.*, 1994). Accordingly, the activity of the *Y150F* mutant was generally decreased in a very significant way (Dubus *et al.*, 1994b) and the properties of the K315H and K315Q mutants seemed to indicate that this latter residue was mainly responsible for increasing the nucleophilic properties of another side-chain (Monnaie *et al.*, 1994). However, the interpretation of the experimental data is made quite hazardous by the discrepancies in the results obtained with the poor substrates and the Tyr150 mutants and by the fact that, for technical reasons, it is not possible to determine the individual values of k_{+2} and K' for

good substrates. In consequence, the effects of the various mutations become very difficult to assess.

PROTONATION OF THE LEAVING GROUP

In chymotrypsin catalysis, this is thought to occur via the back-delivery of the proton first accepted by the general base (His57, *Figure 7*). In class C, if Tyr150 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 Ser130 might be ideally located to fulfil this function (Lamotte-Brasseur *et al.*, 1991; Strynadka *et al.*, 1992), which is only possible if a proton is simultaneously transferred on to 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 Glu166 and travelling to the β-lactam nitrogen via Lys73, a second conserved water molecule (W2, *Figure 9*) and, finally, Ser130.

Despite the striking similarities found in their tertiary structures and the equivalent positions occupied by chemically identical or similar groups, it thus 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 Glu166 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 slow deacylation is not an absolute property of class C enzymes: with some substrates, this occurs at very respectable rates (Galleni, Amicosante and Frère, 1988), an observation which is also valid for the less well-studied class D enzymes (Ledent *et al.*, 1993) which also lack an equivalent of Glu166, but, like their class C counterparts, contain a Tyr residue in the second element.

On the basis of the data that are presently available, it seems reasonable to assume that two distinct mechanisms prevail in the hydrolytic pathways of class A and class C β-lactamases. *Figures 11* and *12* illustrate the various possible intermediates in these pathways. It should be emphasized that these schemes are probably too simplistic and that other side-chains in or near the active sites might also play secondary, but yet important, roles in these phenomena (see below the examples of the Lys residue of the third element and of Arg244/Arg220 in the class A enzymes).

AND THE DD-PEPTIDASES?

When the comparison is extended to the DD-peptidases, further difficulties arise. If the intrinsic reactivity of the β-lactam amide can 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. In the *S.* R61 DD-peptidase the Tyr residue of the second element might participate in the activation of the serine acting as the general base, and back-deliver the proton to the leaving group, just as in class C enzymes. The barely affected k_{cat}/K_m value of the thiolester (which has a much better leaving group than the peptide) with the Y159S mutant would support this hypothesis. Similarly, the

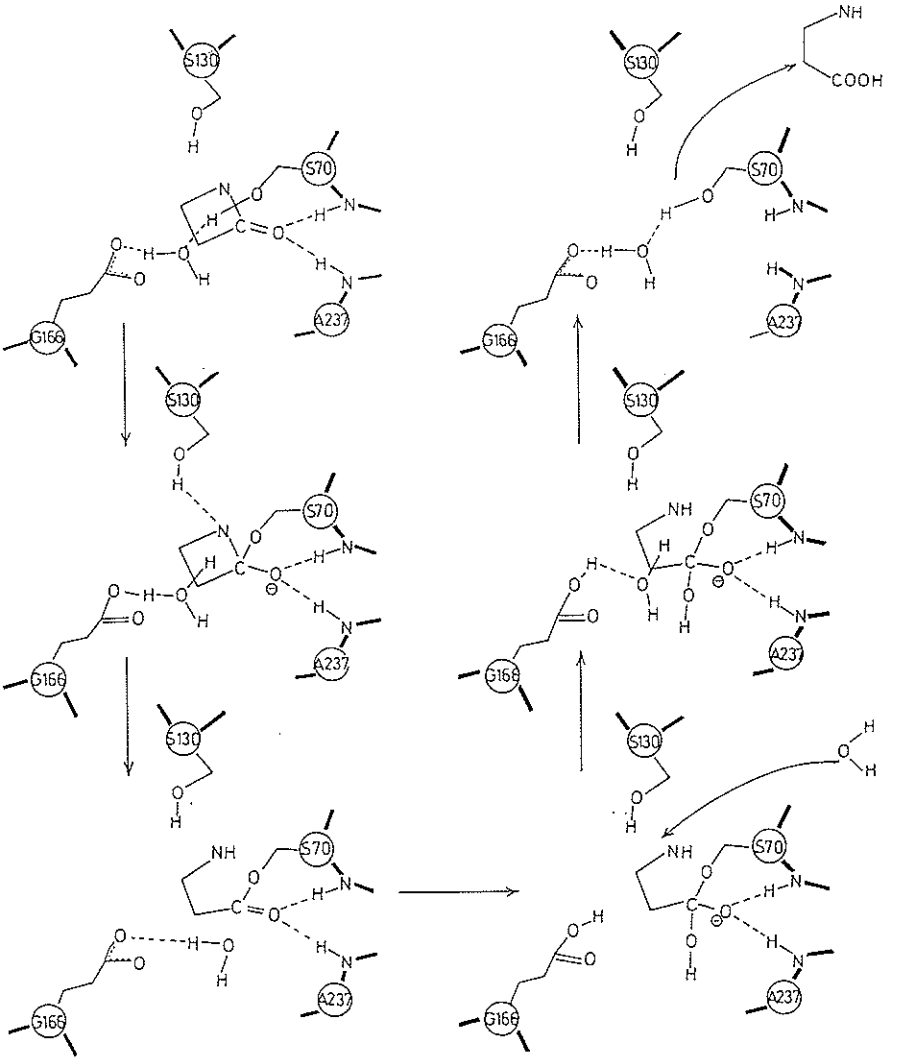

Figure 11. Hypothetical intermediates in the hydrolysis of a β-lactam by a class A enzyme.

acylation of the peptidase by β-lactams is very slow when compared to that of the β-lactamases and this relatively inefficient process is not impaired by the same mutation. A water molecule, loosely bound to the Ser159 side-chain might be sufficient to protonate the leaving group, an alternative mechanism that would not be possible with the bulky side-chain of the Y159F mutant.

One must finally remember that most DD-peptidases and PBPs contain a Ser residue in the second element, in contrast to the Tyr of the *S.* R61 enzyme. Despite this apparent handicap, the *Actinomadura* R39 DD-peptidase is more efficiently acylated by the peptide (k_{cat}/K_m = 50 000 M^{-1} s^{-1}) and β-lactams (k_2/K' = 300 000 M^{-1} s^{-1} with benzylpenicillin) than is the R61 enzyme. It seems unlikely that such high rates might be attained without the participation of additional side-chains which remain to be identified.

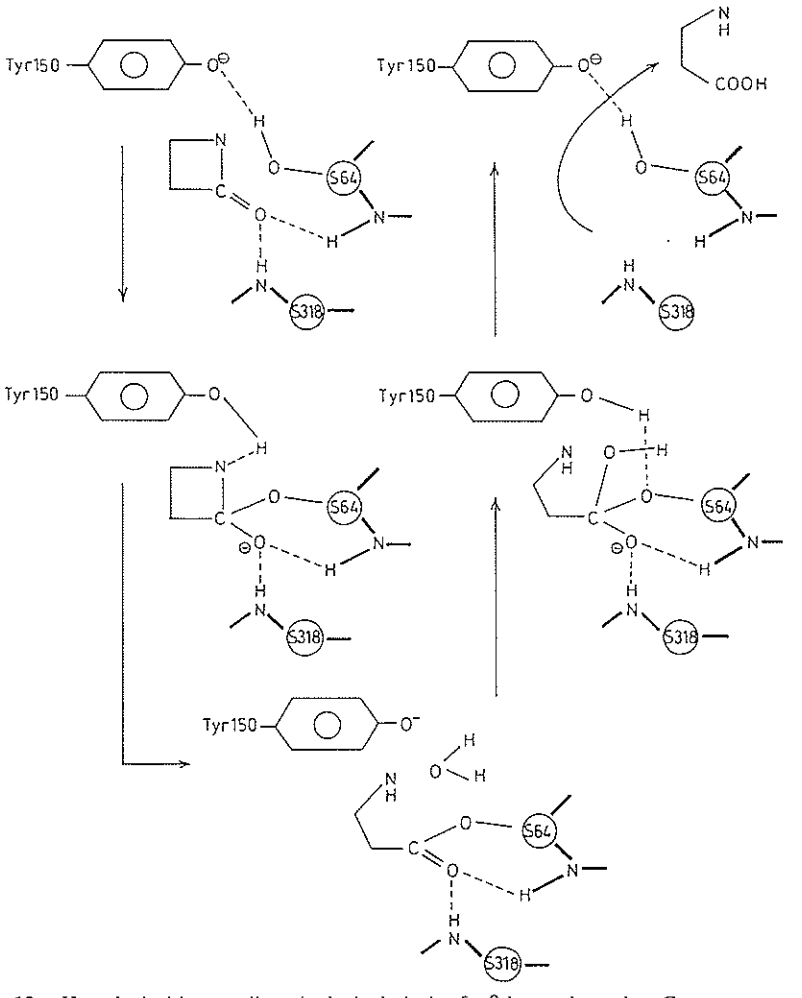

Figure 12. Hypothetical intermediates in the hydrolysis of a β-lactam by a class C enzyme.

The free carboxylate of β-lactam antibiotics

The presence of a negatively charged carboxylate on C3 of penicillins or C4 of cephalosporins is a prerequisite for antibiotic activity. In monolactams, this is replaced by a sulphonate group, which, thanks to its larger size, occupies a similar position relatively to the scissile amide bond.

Accordingly, the methylester and the amide of benzylpenicillin acylate both the DD-peptidase and the class A β-lactamases with a very poor efficiency (*Table 11*). Surprisingly, it is not so for the class C β-lactamases, which readily hydrolyse the methylester. To avoid the complications due to the different intrinsic reactivities of the various compounds, Laws and Page (1989) have introduced the enzymic rate enhancement factor (EREF), the ratio of the enzyme k_{cat}/K_m to the second-order rate constant of hydrolysis by OH^- ions, and *Table 11* compares these EREF values for pairs of compounds containing, or devoid of, a free carboxylate.

Table 11. Ratios of the EREF values for pairs of compounds with and without a free carboxylate

Enzyme			BP/BPMe	BP/BPamide	PV/PV-CH_2OH	CP/lactone	References
Peptidase	*S.* R61	WT	12700	>100000	–	28	Varetto *et al.* (1991)
		T299V	9200	–	–	–	Wilkin *et al.* (1994)
Class A	TEM1	WT	170000	–	860	–	Dubus *et al.* (1994a)
		S225A	33000	–	425	–	
	S. albus G	WT	50000	–	–	6.0	Jacob-Dubuisson *et al.* (1991)
		R220L	110	–	–	0.5	
	B. licheniformis	WT	500000	5000	–	3	Varetto *et al.* (1991)
	B. cereus	WT	100000	–	170	2.5	Laws *et al.* (1993)
Class C	*E. coli* K12	WT	6.6	–	2.5	260	
		T315A	4.1	–	5	80	Dubus *et al.* (1994a)
		T315V	1.8	–	1.8	110	
	E. cloacae 908R	WT	110	500	–	800	Varetto *et al.* (1991)

WT, Wild type; EREF, enzyme rate enhancement factor (see text); BP, benzylpenicillin; BPMe, BPmethylester; PV, phenoxymethylpenicillin; PV-CH_2OH, 3-hydroxymethyl-PV; CP, cephalosporin; lactone, corresponding lactone.

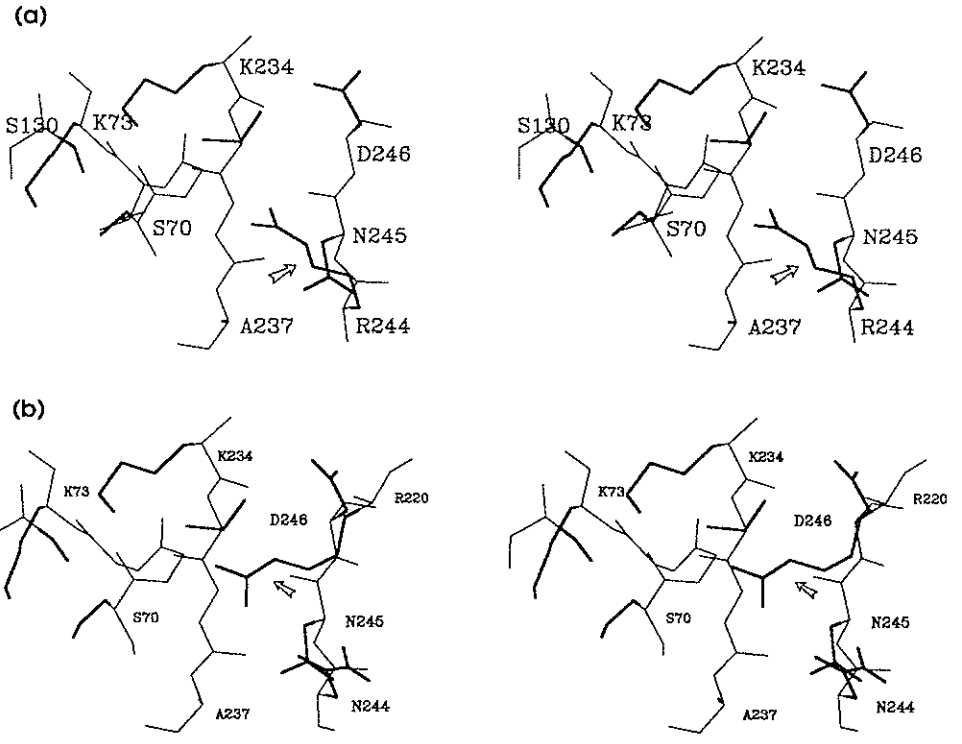

Figure 13. Similar positions of the guanidinium groups (arrows) of (a) Arg244 (*B. licheniformis* β-lactamase) and (b) Arg220 (*Streptomyces albus* G β-lactamase) near the backbone of the β3-strands.

Docking the β-lactam antibiotics in the active sites of the various enzymes always orients the negatively charged carboxylate (or sulphonate) to the side-chains of the first, hydroxylated residue of the second and the two first residues of the third elements. In addition, in the *B. licheniformis* class A β-lactamase, the guanidinium group of Arg244, whose C_α is in the β4-strand, is positioned rather close to the active site, and it was hypothesized that it could form a long ionic bond with the penicillin carboxylate. Arg244 is not conserved in all class A enzymes (Ambler *et al.*, 1991) but when it is absent, the guanidinium group of Arg220 is found lying in the same position, although the C_α of this residue is located on a different part of the protein backbone (*Figure 13*). The residues which might, directly or indirectly, interact with the negative charge of the substrate are thus Ser130, Lys234, Thr/Ser235 and Arg220/Arg244 in class A β-lactamases; Tyr150, Lys315 and Thr316 in class C; and Tyr159, His298 and Thr299 in the DD-peptidase. Unfortunately, the mutants have not been systematically studied with the methylester or with the cephalosporin lactones, other derivatives devoid of a net negative charge (*Figure 14*).

The high-pH limbs of the pH-activity profiles of class A enzymes reflect the titration of a group of pK 9.0–10.0. The results obtained with the K234H mutant of the *S. albus* G β-lactamase indicate that the titrated side-chain might be that of Lys234 (Brannigan *et al.*, 1991), although other explanations have been proposed (Laws *et al.*, 1993). Computer-aided docking of β-lactams in the active site of class A β-lactamases has often been performed with the assumption that a salt-bridge was

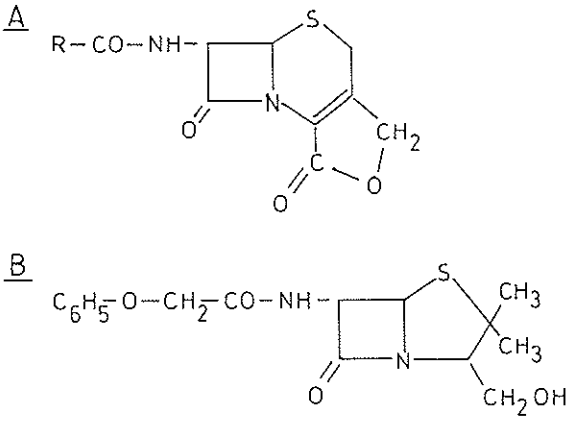

Figure 14. Structure (a) of a cephalosporin lactone, and (b) of 3-hydroxymethyl-phenoxymethylpenicillin.

formed between the alkylammonium of Lys234 and the substrate carboxylate. The nearly unchanged activities of the K234R mutant of the TEM1 and, at low pH, of the K234H mutant of the *S. albus* G enzymes might, at first sight, appear to corroborate this hypothesis but, in the latter case, the deprotonation of the imidazole side-chain modifies the *k*cat much more than the *K*m values. It is thus clear that a positive charge on residue 234 is necessary for a high activity, but its exact role is less evident. The same conclusion appears to prevail for the class C enzymes, but here a positive histidine is unable to replace the Lys315 alkylammonium, which further underlines fundamental differences in the mechanisms of both classes of enzymes. In other docking attempts, hydrogen bonds, rather than salt-bridges, are formed between the substrate carboxylate and the S130/Y150 and T(S)235/T316 hydroxyl groups, the first hydrogen bond being significantly weaker with cephalosporins (Lamotte-Brasseur *et al.*, 1991; Juteau *et al.*, 1992). This seems to be confirmed by the distinctly more pronounced effect of the disappearance of the T(S)235/T316 hydroxyl on the rates of acylation by cephalosporins when compared to those of penicillins, and this conclusion can be extended to the peptidase.

Analysis of *Table 11* also shows that, for class C enzymes, the interactions between the penicillin carboxylate and the enzymes are much weaker than for class A and for the peptidase. Conversely, lactonization of the cephalosporins has a greater effect in class C, although much less spectacular than the decreased activity of class A enzymes on the methyl ester. One may wonder if steric problems do not arise with this compound, since the differences are not so large with the hydroxymethyl derivative of penicillin (*Figure 14*). It has also been noted (Laws and Page, 1989) that the lactone oxygens bear partial negative charges, but the electrostatic potential maps of the penicillin ester and amide also show deep negative wells centred on the C3' carbonyl oxygen(s) (Varetto *et al.*, 1991). The elimination of the hydroxyl group of the third element's second residue never significantly modifies the EREF ratios, which is somewhat surprising. In fact, these ratios are only significantly modified in the R220L mutant of the *S. albus* G enzyme, indicating a possible, direct or indirect interaction between Arg220 and the substrate carboxylate. Accordingly, this mutant exhibits decreased acylation rates by all the 'normal' substrates,

with the exception of cephaloridine. Since this compound bears a positively charged side-chain on C3, the lesser effect of the mutation might result from a 'substrate assisted catalysis' (Jacob-Dubuisson *et al.*, 1991).

In agreement with the structural data illustrated by *Figure 12*, the R244T and R244Q mutants of the TEM1 β-lactamase are impaired by factors quite similar to those of the R220L mutant of *S. albus* G, but here, the very different behaviour of the R244S mutant remains unexplained.

Evolution under our eyes

RESISTANCE TO MECHANISM-BASED INHIBITORS

The discovery of clavulanic acid (*Figure 15*) supplied a new chemotherapeutic tool in fighting the β-lactamase-producing bacteria. This compound, acting as a suicide substrate, efficiently acylates most class A β-lactamases. The acylenzyme can then undergo both hydrolysis and rearrangement(s) leading to more stable adducts. In consequence, and although clavulanic acid itself fails to inactivate the DD-peptidases, it has been widely utilized to vastly increase the efficiency of classical, β-lactamase-sensitive compounds, such as amoxycillin, against the TEM- and SHV-producing strains. This selective pressure has recently resulted in the spontaneous appearance of TEM mutants exhibiting a decreased sensitivity to this mechanism-based inactivator. The mutations were identified as R244S or R244C (Belaaouaj, 1992; Vedel *et al.*, 1992) and site-directed mutagenesis experiments confirmed the involvement of Arg244 in the inactivation phenomenon (Delaire *et al.*, 1992; Imtiaz *et al.*, 1993b). Its guanidinium group anchors a structurally conserved water molecule which serves as the source of a critical proton in the stepwise rearrangement of the clavulanate moiety. The M69L mutant of the same enzyme is also rather resistant to the same inactivator and remains quite efficient against the 'normal' substrates, observations which have been explained by a slight modification of the active-site structure (Delaire *et al.*, 1992). Class C enzymes are not sensitive to clavulanic acid and this seems to be mainly due to a poor recognition, resulting in an inefficient acylation (Monnaie and Frère, 1992), although an additional impairment of the rearrangement process could also be relevant (Lobkovsky *et al.*, 1993).

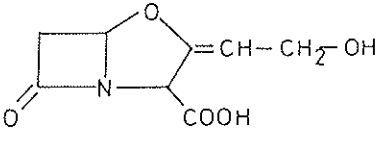

Figure 15. Structure of clavulanic acid.

A detailed review of the highly diversified mechanisms of β-lactamase inactivation has recently been presented (Pratt, 1992).

HYDROLYSIS OF THIRD-GENERATION CEPHALOSPORINS

Another strategy devised for fighting β-lactamase-producing pathogens was to try to avoid the activity of the enzymes by synthesizing 'β-lactamase stable' molecules. The

utilization of methicillin (*Figure 16*) against the β-lactamase-producing *Staphylococci* was the first successful example of this strategy.

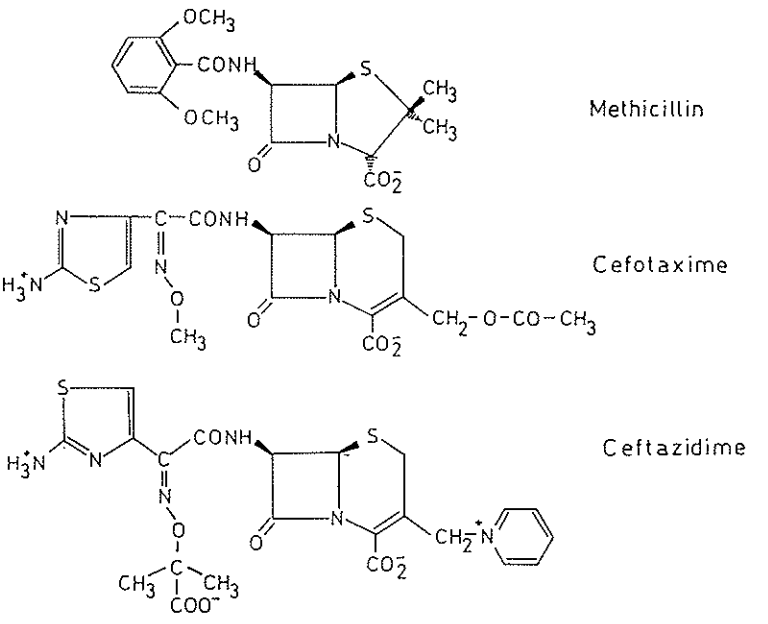

Figure 16. Structures of methicillin and of the oxyimino cephalosporins, cefotaxime and ceftazidime.

More recently, cefotaxime and ceftazidime (*Figure 16*), third-generation cephalosporins which are hydrolysed orders of magnitude more slowly than many β-lactams by the prevalent TEM and SHV enzymes, became very popular in the hospital environment. Maybe too popular. Indeed, their widespread, and sometimes abusive, utilization was responsible for the emergence of strains producing variants of these enzymes which exhibited increased hydrolytic activities against the two compounds. At least five SHV and 25 TEM variants have now been identified and were found to differ from the parent enzymes by a very limited number of amino acid substitutions (Sougakoff *et al.*, 1988; Collatz, Labia and Gutmann, 1990; Jacoby and Medeiros, 1991; Coyette *et al.*, 1994). Not surprisingly, none of the residues in the conserved elements were involved, but examination of the wild-type TEM1 structure shows that all the modified residues are close to the active site (*Figure 17*). *Table 12* summarizes the properties of some of these new enzymes and identifies the mutations.

Several authors (Lee *et al.*, 1991; Sowek *et al.*, 1991; Huletsky, Knox and Levesque, 1993) have tried to rationalize the observed alterations of substrate profiles on the basis of the structural variations, relying on the three-dimensional structures of related enzymes, generally that of the *B. licheniformis* β-lactamase. More recently, Raquet *et al.* (1994) have performed a detailed kinetic study of six TEM variants and analysed their properties on the basis of the newly determined structure of the TEM1, 'wild-type' enzyme, since no data are presently available about the structures of the variants (Jelsch *et al.*, 1992; Strynadka *et al.*, 1992; Fonzé *et al.*, in press). The data shown in *Table 12* are mostly taken from this work and although a detailed

Table 12. Properties of some TEM mutants and of other enzymes which hydrolyse third-generation cephalosporins (Raquet *et al.*, 1994)

	Sequences				Kinetic parameters					
					Absolute values[a]			Relative values		
	Residue 104	Omega loop	β3-strand		BP	CT	CR	CTX/CT	CAZ/CR	CTX/CAZ
		171	234 240							
TEM1	E	RWEPELNE	KSGAGE	k_{cat}/K_m	84	0.65	2.2	0.002	30×10^{-6}	20
				k_{cat}	1600	160	1500	0.06	0.0002	30
TEM11	E	*H*WEPELNE	KSGAGE	k_{cat}/K_m	14	0.36	1.3	0.064 (32)	0.011 (350)	1.7
				k_{cat}	85	110	160	0.025 (.4)	0.11 (550)	0.15
TEM10	E	*S*WEPELNE	KSGAG*K*	k_{cat}/K_m	25	0.16	0.12	0.062 (31)	0.23 (8000)	0.35
				k_{cat}	51	7	6	0.04 (.7)	2.8 (14000)	0.02
TEM19	E	RWEPELNE	KSGA*S*E	k_{cat}/K_m	10	2.3	2.2	0.1 (50)	0.002 (70)	46
				k_{cat}	62	34	105	1.9 (30)	0.25 (1250)	2.5
TEM3	*K*	RWEPELNE	KSGA*S*E	k_{cat}/K_m	9.5	1.9	1.1	0.53 (250)	0.036 (1200)	25
				k_{cat}	38	14	33	3.6 (60)	0.2 (1000)	73
M. fort	P	RWEVEL NS	KTGAGD	k_{cat}/K_m	1	1.9	0.7	0.05	–	–
PER1	T	ANEAQMHA	KTGTSG	k_{cat}/K_m	40[b]	68[b]	22[b]	0.85	0.77	3.4

BP, Benzylpenicillin; CT, cephalothin; CR, cephaloridine; CTX, cefotaxime; CAZ, ceftazidime.
The values between parentheses give the factor of increase when compared to TEM1.
[a] Absolute values: k_{cat}/K_m in $\mu M^{-1} s^{-1}$, k_{cat} in s^{-1}.
M. fort = *Mycobacterium fortuitum* (Amicosante *et al.*, 1990).
PER1: [b] = relative values (Nordmann *et al.*, 1993).

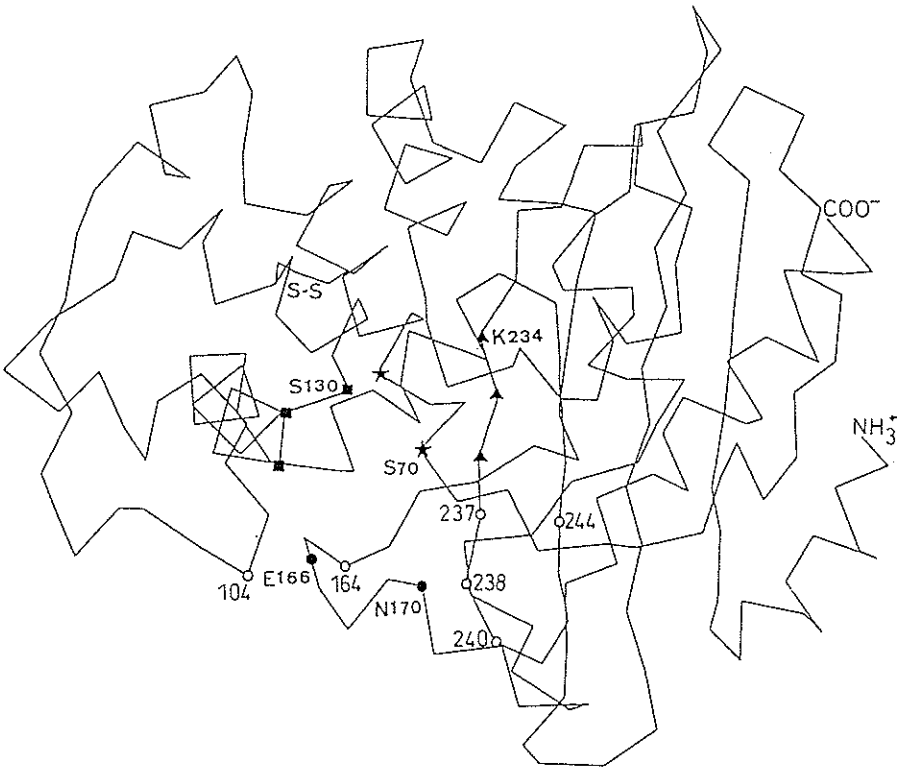

Figure 17. Location of the α-carbons of Arg244 and of the residues modified in the TEM variants which hydrolyse third-generation cephalosporins (o). The conserved elements are also labelled.

discussion of these data is beyond the scope of the present review, the following points deserve attention.

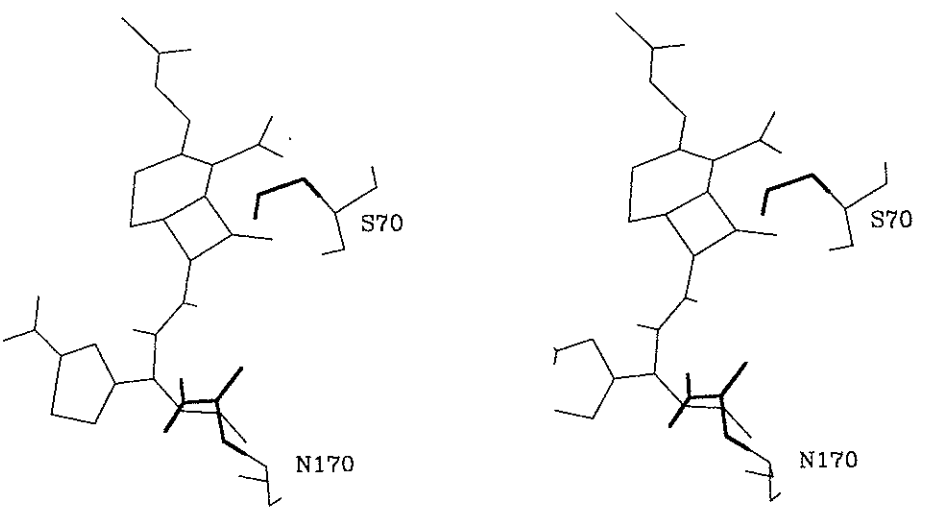

Figure 18. Docking of cefotaxime in the active site of TEM1, showing the contacts with the side-chain of Asn170.

1. Docking the optimized structures of cefotaxime and ceftazidime in the TEM1 active site leads to short contacts between the oxime substituents and the side-chain of Asn170 (*Figure 18*), resulting in a displacement of the catalytic Glu166. Moreover, with ceftazidime, the position of the catalytic water molecule W1 is also modified, which explains the even poorer activity of the enzyme against this substrate.
2. In TEM1, residue Arg164 forms a salt-bridge with Asp179, thus stabilizing the Ω loop. Substitution of Arg164 by uncharged residues in TEM10 and TEM11 makes the loop more mobile, which somewhat decreases the activity against the best substrates. By contrast, this allows more freedom for the oxime carboxylate of ceftazidime, which no longer disturbs the water molecule. This explains the increased efficiency with this substrate. The effects of the mutation on the rate of cefotaxime hydrolysis result from a negative factor, the perturbation of the Ω loop (hence, the *decreased* kcat value) and a larger positive effect, the disappearance of the short contacts with Asn170, mainly reflecting on the K' value. These considerations account for the larger increase in the activity of these mutants against ceftazidime, reflected in the lowered CTX/CAZ ratios in *Table 12*.
3. In the absence of three-dimensional structures for these proteins, modelling of the mutants with new Lys side-chains (TEM10 and TEM3) is made difficult by the large number of possible conformations of the long alkyl moiety of this residue.
4. The Ser238 side-chains of TEM19 and TEM3 might form a new H-bond with the oxime oxygen of cefotaxime. To avoid short contacts with the hydroxymethyl group, the side-chain of cefazidime must be oriented away from the active site, allowing the return of W1 to its efficient position.
5. The high activity of TEM3 against ceftazidime might be explained by the preferential binding of a minor conformer of the antibiotic.
6. Other enzymes have been described (*Table 12*), which exhibit relatively high activities against third-generation cephalosporins, often with significantly reduced activities against penicillins. A striking example is the enzyme produced by *Mycobacterium fortiutum* (*Table 12*). Mutations of the residues defined as strategic on the basis of the sequences of the TEM variants are sometimes found in these enzymes, but different modifications also appear to yield similar specificity profiles. The catalytic properties of class A β-lactamases thus present examples of converging evolution within the generally divergent sequence differentiations. The utilization of new compounds can, in consequence, result in the appearance of enzymes which destroy them, by the selection of strains producing either 'original' enzymes, quite different from those which are already well recognized, or variants derived from the latter by a few point mutations.

Conclusions

Our understanding of the catalytic mechanisms of β-lactamases has been significantly improved by the determination of several structures at high resolution. However, it is much more difficult to account accurately for the values of the kinetic parameters which, *in fine* determine the exact difficulties that a given enzyme might be responsible for in a clinical situation. The high turnover numbers of β-lactamases make the isolation and detailed study of intermediates quite problematic, although

some low-temperature studies have been performed with success (Cartwright and Waley, 1987). Alternatively, computer modelling has also succeeded in rationalizing the behaviour of some enzyme–substrate pairs (Lamotte-Brasseur *et al.*, 1992; Matagne, Lamotte-Brasseur and Frère, 1993). However, even when a high-resolution structure is available for the parent enzyme, the quantitative interpretation of the catalytic properties of mutants and of related proteins remains difficult when the reaction pathway involves several steps and includes the formation of covalent intermediates. Catalysis is indeed a complex process and these considerations underline the difficulties which can be encountered when attempting to deduce the properties of the protein from the gene sequence.

The past successes of antibacterial chemotherapy are currently challenged by many pathogenic strains which have efficiently countered the various strategies developed to keep them under control. A few years ago, the medical community relaxed in the security afforded by 'β-lactamase-stable' compounds (Bush, 1989b) and inactivators like clavulanic acid and sulbactam. Bacteria have exhibited extraordinary adaptation capacities and, in this respect, it is quite surprising that several pharmaceutical companies appear to have lost interest in the search for new antibacterial agents.

When a new compound is introduced in the clinical arsenal, the only prediction which can safely be made is that, given enough time, bacteria will find a way to escape its effects. Only a deeper understanding of the various physiological processes which govern bacterial multiplication can help us continue to be 'one compound ahead' of the pathogens.

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