potential for these probes as substrates in studies of oligosaccharide recognition by enzymes: glycosyltransferases and glycosidases.

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Penicillin-recognizing enzymes

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Two families of enzymes recognize penicillins and other β-lactam antibiotics. The first family contains the DD-peptidases involved in the biosynthesis of the peptidoglycan of the bacterial cell wall. They are inactivated by β-lactams. The members of the second family, β-lactamases, efficiently hydrolyse the amide bond of the β-lactam nucleus, yielding products devoid of antibiotic activity. In short, penicillins inactivate DD-peptidases while β-lactamases inactivate penicillins. Although one might think that nothing could be more different, the two families of enzymes appear to share many characteristics and the qualitative difference can be well explained on the basis of the quantitative aspects of the various interactions.

In 1976, it was demonstrated that penicillin inactivated a penicillin-sensitive DD-peptidase by reacting with the hydroxyl group of a serine residue (Frère *et al.*, 1976). The finding of an essential serine residue in the active site of several β-lactamases followed within a few months (Knott-Hunziker *et al.*, 1979; Cartwright & Coulson, 1980).

If the major enzymic activity of β-lactamases, i.e. the hydrolysis of β-lactams, is easily measured, it is not so with the peptidase activity of most penicillin-sensitive enzymes. In fact, the vast majority of these latter enzymes were only identified via their penicillin-binding properties and became known as PBPs, penicillin-binding proteins. Their transpeptidase activity was seldom clearly demonstrated *in vitro*, and their role in the closing of interpeptide bridges during peptidoglycan biosynthesis was deduced by indirect methods. Moreover, several PBPs exhibit only a DD-carboxypeptidase activity and do not appear to be essential for the survival of the bacterium.

Although PBPs are generally membrane-bound proteins, some *Streptomyces* and *Actinomadura* strains readily excrete DD-peptidases in their growth medium. These soluble enzymes produced excellent models for the study of the catalytic and penicillin-binding properties of DD-peptidases (Frère & Joris, 1985).

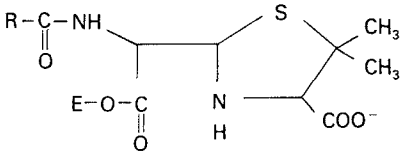
The results which have been accumulated at the present time show that penicillin-sensitive DD-peptidases and most β-lactamases appear to belong to one super-family of active serine enzymes. The only exceptions are a handful of Zn^{2+} β-lactamases which will not be thoroughly discussed here. In this contribution, we wish to compare the major kinetic and structural properties of the serine enzymes.

Kinetic aspects: interaction with β-lactams

The interaction between the two families of enzymes and β-lactams can be represented by the simple scheme

$$E + C \underset{}{\overset{K}{\rightleftharpoons}} EC \xrightarrow{k_2} EC^* \xrightarrow{k_3\,(H_2O)} E + P(s) \quad (1)$$

where E, C and EC are, respectively, the enzyme, the β-lactam and a non-covalent complex whose dissociation constant is K. The acyl-enzyme intermediate EC*:



is very stable if E is a DD-peptidase (k_3 generally $\leqslant 10^{-3}\ s^{-1}$) and exceedingly unstable with β-lactamases where k_3 can be larger than $10^3\ s^{-1}$. Such an enormous, but purely quantitative, difference translates in practice into a qualitative one for the casual observer, as underlined above. The range of variation of the constants is very large for the two families of enzymes. For the extracellular DD-peptidase of *Streptomyces* R61, k_2/K varies from less than $2 \times 10^{-3}\ M^{-1}\ s^{-1}$ with azteonam to more than $10^4\ M^{-1}\ s^{-1}$ with benzylpenicillin, and k_3 varies from the exceptionally 'high' value of $10^{-2}\ s^{-1}$ with some penems to $10^{-6}\ s^{-1}$ with some cephalosporins. Similarly, for β-lactamases, k_2/K values close to those characteristic of diffusion-limited processes ($10^8\ M^{-1}\ s^{-1}$) have been described, while cefoxitin does not seem to be recognized by the *Streptomyces albus* G β-lactamase. In the case of the

Abbreviation used: PBP, penicillin-binding protein.

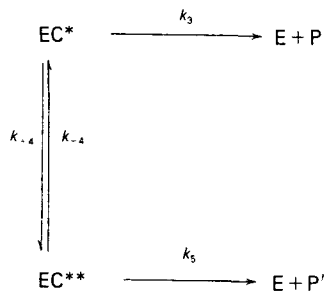
hydrolysis of benzylpenicillin by class A β-lactamases, $K_{cat.}$ values are larger than 1000 s^{-1} and the rate-limiting step appears to be the formation of the acyl-enzyme (k_2 step), which indicates even larger values for k_3. At the other extreme, the k_3 value for the hydrolysis of aztreonam by class C β-lactamases can be as low as 10^{-4} s^{-1} which makes a PBP out of the β-lactamase, since the k_2/K values remain larger than 10^4 M^{-1} s^{-1}.

In all these interactions, the exact structures of the ring which is fused to the β-lactam, and of the various side-chains, are of paramount importance. But no general structure–activity relationships can be established. For instance, the removal of the phenylacetyl side-chain of benzylpenicillin to yield 6-aminopenicillanic acid generally yields a nearly completely inefficient PBP inactivator. In contrast, 6-aminopenicillanic acid is a very good, a mediocre and a very poor substrate for the β-lactamases of *Streptomyces albus* G, *Bacillus licheniformis* and *Enterobacter cloacae*, respectively.

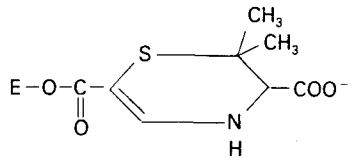
With some DD-peptidases, the k_3 step can lead to the breakdown of the penicilloyl moiety into two fragments (Frère *et al.*, 1975), a reaction which has never been observed with β-lactamases. However, the nature of the products obtained with PBPs has seldom been investigated, because the low k_3 values make it difficult to accumulate reasonable amounts of product(s).

Besides water, other nucleophiles can attack the acyl-enzyme. Hydroxylamine has been routinely used to accelerate the spontaneous reactivation of penicillin-bound PBPs. Methanolysis of the benzylpenicilloyl-enzyme occurs with class C and not with class A β-lactamases (Knott-Hunziker *et al.*, 1982), and the $k_{cat.}$ value increases indicating a rate-limiting k_3. The soluble DD-peptidase of *Streptomyces* R61 performs the transfer of the phenylacetylglycyl moiety of benzylpenicillin on glycylglycine, thus mimicking the reaction it catalyses with its usual peptide substrate (Marquet *et al.*, 1979). It is, however, difficult to decide whether such transfer reactions suggest the existence of a specific binding site for the nucleophile, or if they just reflect the random action of the latter compounds.

With some substrates and mechanism-based inactivators of β-lactamases, a branch must be added to the simple scheme depicted by scheme (1):



The k_4 step implies the rearrangement of the enzyme or of the penicilloyl moiety. The many different possibilities which arise from variations of the relative values of k_3, k_{+4}, k_{-4} or k_5 have recently been analysed by Pratt (1988) and will not be discussed here. We have, for instance, performed extended studies with β-iodopenicillanate, two DD-peptidases and 13 serine β-lactamases (De Meester *et al.*, 1986). The values of k_{-4} and k_5 are zero or negligible in all cases and a k_3 step has been detected with only four β-lactamases. In all cases, the k_{+4} step involves the very rapid rearrangement of the penicilloyl moiety into a u.v.-absorbing dihydrothiazine chromophore:



Again, the similarities between β-lactamases and DD-peptidases are striking.

Unfortunately, a detailed discussion of the interaction with most membrane-bound PBPs is made difficult by the absence of reliable kinetic data concerning these enzymes. Although there are a few interesting and well-detailed studies (Coyette *et al.*, 1978), they remain exceptional and most of the people who work with PBPs appear to have a quasi-philosophical objection against proper and rigorous studies of the PBP–β-lactam interactions.

Kinetic aspects: peptides and ester substrates

As outlined above, it is not always easy to demonstrate *in vitro* the DD-peptidase activity of penicillin-sensitive enzymes. Some utilize short synthetic D-Ala-D-Ala-terminated peptides (Fig. 1*a*) in hydrolysis and/or aminolysis reactions. If the peptide contains a suitably situated amino group, polymerization reactions can be observed. In other cases, the natural pentapeptide (Fig. 1*b*) is hydrolysed and/or polymerized. The high-molecular mass PBPs (Fig. 1 *a* and *b*) of *Escherichia coli* catalyse both a transglycosylation and a transpeptidation reaction starting with the 'lipid-bound intermediate' (Fig. 1*c*). Moreover, the transglycosylation seems to be a prerequisite for the transpeptidation: the latter reaction does not occur if the former is blocked by moenomycin (Ishino *et al.*, 1980). For many other PBPs, no activity *in vitro* could be found.

Under these circumstances, it is probably not surprising that the first attempts to show an interaction between β-lactamases and DD-peptidase substrates (the simple peptides of Fig. 1*a*) were a complete failure. However, Pratt & Govardhan (1984) succeeded in showing that some relatively simple esters (Fig. 1*d*) could act as substrates for some β-lactamases and the soluble DD-peptidase of *Streptomyces* R61. Moreover, transfer reactions on acceptor molecules such as D-phenylglycine were also observed. This led to the paradoxical situation, where β-lactamases could exhibit a transferase activity *in vitro*, while many PBPs could not. Table 1 presents a brief summary of the observations made with a few enzymes, a complete discussion being outside the scope of this review.

Structural aspects: primary structures

Until 1980, one did not know more than the complete sequences of four relatively similar β-lactamases (Ambler, 1980) and pieces of some DD-peptidases either at the *N*-terminus or near the active serine residue: for instance, the identification of the serine residue of the *Streptomyces* R61 DD-peptidase (Frère *et al.*, 1976) rested on the sequence of the tripeptide Val-Gly-Ser, which hardly changed: thanks to the development of gene-sequencing techniques, 27 complete sequences are presently (April, 1988) known, 17 β-lactamases and 10 PBPs (when enzymes differ only by a few residues, such as the TEM-1, 2- and 3-β-lactamases or the three chromosome-encoded β-lactamases produced by different strains of *E. cloacae*, only one is included in the total), and the number appears to be growing every month! Peptides containing the active serine have also been isolated and sequenced for nine additional proteins (five β-lactamases and four PBPs). On the basis of the available data, five broad groups might be distinguished: low-molecular-mass PBPs

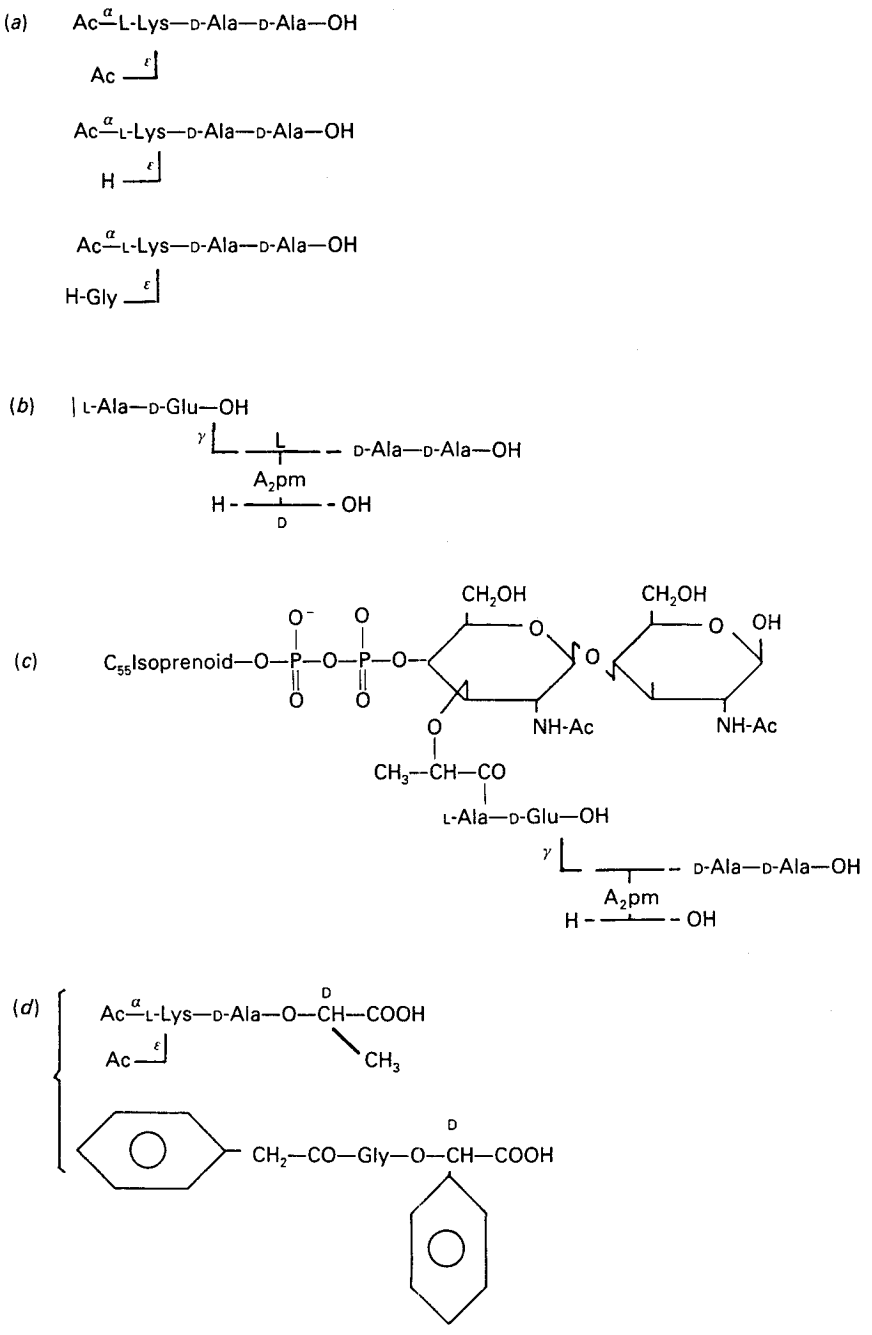


Fig. 1. *Substrates for the* DD*-peptidases*

(*a*) Synthetic peptides. The free *n*-group on the glycine residue of the tetrapeptide is utilized as carbonyl acceptor by the *Streptomyces* R61 DD-peptidase, which leads to polymerization. (*b*) Natural pentapeptide of *E. coli* or *Actinomadura* R39. The free amino group on the D-carbon of the *meso*-diaminopimelyl residue (A_2pm) can be utilized as acceptor by the DD-peptidase of *Actinomadura* R39 and probably PBP4 of *E. coli*. (*c*) 'Lipid' intermediate in the biosynthesis of peptidoglycan which is utilized by PBPs 1, 2 and 3 of *E. coli* to form cross-linked peptidoglycan. (*d*) Depsipeptides which can be substrates of the DD-peptidases and of the β-lactamases.

exhibiting DD-carboxypeptidase activity *in vitro*, high-molecular-mass PBPs and three classes, A, C and D, of β-lactamases (class B contains the few Zn^{2+}-β-lactamases). Moreover, the soluble DD-peptidase of *Streptomyces* R61 appears to be in a class by itself. At first sight, little relationship could be found between the different groups, but an unexpectedly high number of residue identities was found when the DD-peptidase of *Streptomyces* R61 was compared with class C β-lactamases. In contrast, classes A and C appeared to be unrelated, but the DD-peptidase might represent a link between them, although much closer to class C (Joris *et al.*, 1988).

Comparisons of the three-dimensional structures (see below) indicated an unambiguous similarity between the

Table 1. *DD-peptidase and esterase activities*

H, Hydrolysis (DD-carboxypeptidase); T, aminolysis; Pol, peptide polymerization; ND, not done; (+), very weak activity. For more details, see Frère & Joris (1985).

	Synthetic peptides A*			Synthetic esters D*		Natural peptides B*		Transglycosylase + transpeptidase C*
	H	T	Pol	H	T	H	Pol	
Soluble DD-peptidases								
Streptomyces R61	+	+	+	+	+	+	ND	—
Actinomadura R39	+	+	+	+	ND	+	+	—
Membrane-bound PBPs								
Streptomyces K15	(+)	+	—	+	+	ND	ND	—
E. coli 5-6	+	+	—	+	+	+	—	—
E. coli 4	+	+	+	ND	ND	+	+	—
E. coli 1-3	—	—	—	—	—	—	—	+
β-Lactamases								
Class A		—		+	(+)	—	—	
Class C		—		+	+	—	—	—
Class D		ND			ND	ND		

*The letters refer to the structures in Fig. 1.

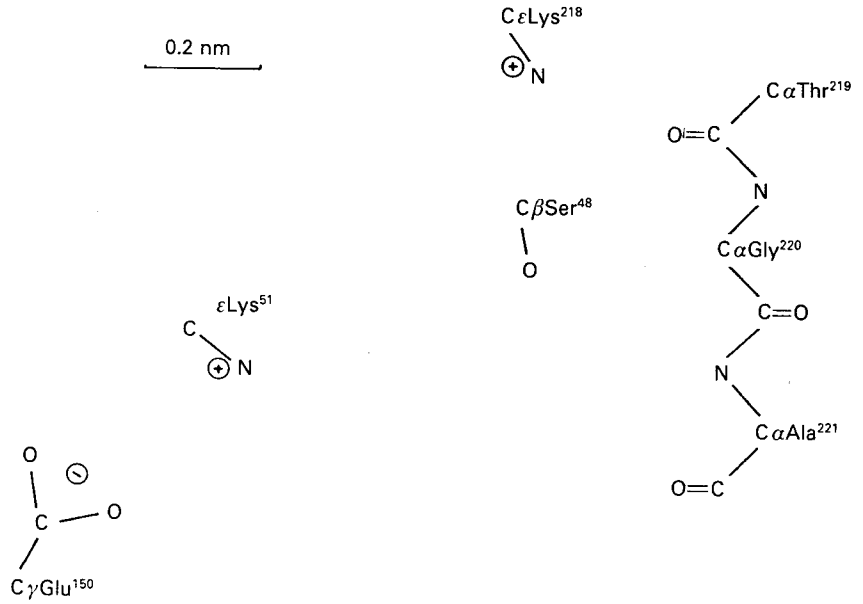


Fig. 2. *Simplified scheme of some of the important features of the active site of the β-lactamase of S. albus G*

The hydrogen atoms are not represented. The oxygen of the essential serine residue (Ser^{48}) is not far from Gly^{220}, which is part of the exterior strand of the β-pleated sheet and the last residue of the very conserved Lys-Thr-Gly sequence. Also shown are the carboxylate of Glu^{150} and the ε-amino groups of Lys^{51} and Lys^{218}. In the active site of the DD-peptidase of *Streptomyces* R61, the ε-group of Lys^{65} (corresponding to Lys^{51}) is not clearly seen in the maps, Asp^{225} replaces Glu^{150} and His^{300} replaces Lys^{218}, but their relative positions remain the same. The role of the *N*-group of Lys^{51} remains mysterious. According to Herzberg & Moult (1987), it could facilitate proton transfer between Ser^{48} and the β-lactam nitrogen. However, binding of both β-lactam and peptide substrates to the *Streptomyces* R61 DD-peptidase seems to depend upon the protonated form of a group of apparent $pK_a \cong 9$–9.5 (Varetto *et al.*, 1987). In this latter case, the only reasonable candidate seems to be the ε-group of Lys^{65}.

folding of the DD-peptidase and of several class A β-lactamases, showing that similar geometries could be obtained with very distant primary structures.

A detailed analysis of the possible relationships between the various groups was performed by Joris *et al.* (1988). Here, we will only describe some of the most striking features of the possible analogies.

A characteristic sequence Phe^{44}-Xaa-Xaa-Xaa-Ser^{48}-Xaa-Xaa-Lys^{51}, where Ser is the active serine (the residue numbering is that of the *S. albus* G β-lactamase), was found in

β-lactamases of classes A and C, in the *Streptomyces* R61 DD-peptidase and in PBP3 of *E. coli* (i.e. 16 proteins out of 27). In the others, variations occur at the level of the Phe residue, but the Ser-Xaa-Xaa-Lys sequence is conserved in the 27 proteins. The active serine is always close to the *N*-terminus (position 36–70) of β-lactamases and of low-molecular-mass PBPs. It is also not far from the beginning of what is thought to be the 'transpeptidase domain' of high-molecular-mass PBPs. Much further away in the direction of the *C*-terminus (136–350 residues), a Lys^{218}-Thr-Gly^{220} or Lys-Ser-Gly triad is found in all the proteins, with the sole exception of the *Streptomyces* R61 DD-peptidase, where Lys is replaced by His. The importance of that area was underlined recently when a mutation described a long time ago by Hall & Knowles (1976), and which altered the substrate specificity of the RTEM β-lactamase, was found to correspond to the substitution of an Ala by a Thr residue just after that sequence.

Structural aspects: three-dimensional structures

Good X-ray diffraction data have been obtained for three class A β-lactamases (from *Bacillus cereus, Staphylococcus aureus* and *S. albus* G). As expected, the three structures were closely related (Samraoui *et al.*, 1986; Kelly *et al.*, 1986; Herzberg & Moult, 1987; Dideberg *et al.*, 1987), but the relative positions of the secondary structure elements and of the penicillin-binding site were also very similar to those observed in the *Streptomyces* R61 DD-peptidase. As mentioned above, the number of identical residues found in the same positions in the three β-lactamases and in the DD-peptidase was not statistically meaningful. However, around the active sites, similar residues appeared in equivalent positions (Fig. 2). The active serine residue (Ser^{48}) lies near the *N*-terminus of a rather long α-helix. The conserved Lys^{51} is at the end of the next turn and its side-chain points into the putative binding site. The side chain of Phe^{44} is buried in the core of the proteins. Its substitution by another residue might disturb the packing of the molecule. An acidic side-chain (Glu in the β-lactamases, Asp in the DD-peptidase) about 0.8 nm away from the oxygen of the serine lies in a very conserved position and certainly plays an important, although as yet undetermined, role. Finally, the Lys-Thr-Gly (Lys-Ser-Gly or His-Thr-Gly) sequence is part of the last strand of the sole β-pleated sheet of the enzymes and borders the catalytic cavity, the side-chain of Lys (or His) pointing to the cavity, and that of Thr outside. Any residue larger than Gly in the third position would have its side-chain pointing into the cavity and would result in impaired accessibility. Mutations of the residues which immediately follow the triad might modify its orientation and, in consequence, the general shape of the binding site, inducing variations of specificity. Unfortunately, the limited space available for the present contribution does not make it possible to discuss further the structures about which more details can be found in the papers of Herzberg & Moult (987), Dideberg *et al.* (1987) and Joris *et al.* (1988). We still do not have any indication concerning the major question: why do β-lactamases get rid of the penicilloyl moiety so efficiently and DD-peptidases so slowly? It is quite interesting to demonstrate similarities, but the differences are even more important. Refinement of the crystal structures, and the detailed analyses of n.m.r. spectra in synergy with the utilization of site-directed mutagenesis will hopefully supply the answers, which should be of the utmost value, not only for the future of antibacterial chemotherapy, but also for the understanding of enzyme catalytic phenomena.

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