Molecular evolution of bacterial β-lactam resistance

James R Knox[1], Paul C Moews[1] and Jean-Marie Frere[2]

Background: Two groups of penicillin-destroying enzymes, the class A and class C β-lactamases, may have evolved from bacterial transpeptidases that transfer x–D-Ala–D-Ala peptides to the growing peptidoglycan during cell wall synthesis. Both the transpeptidases and the β-lactamases are acylated by β-lactam antibiotics such as penicillin, which mimic the peptide, but breakdown and removal of the antibiotic is much faster in the β-lactamases, which lack the ability to process D-Ala–D-Ala peptides. Stereochemical factors driving this evolution in specificity are examined.

Results: We have compared the crystal structures of two classes of β-lactamases and a β-lactam-sensitive D-alanyl–D-alanine carboxypeptidase/transpeptidase (DD-peptidase). The class C β-lactamase is more similar to the DD-peptidase than to another β-lactamase of class A.

Conclusions: The two classes of β-lactamases appear to have developed from an ancestral protein along separate evolutionary paths. Structural differentiation of the β-lactamases from the DD-peptidases appears to follow differences in substrate shapes. The structure of the class A β-lactamase has been further optimized to exclude D-alanyl peptides and process penicillin substrates with near catalytic perfection.

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Introduction

Previous X-ray crystallographic results [1,2] indicated a possible ancestral link between a β-lactam-destroying class A β-lactamase and a β-lactam-sensitive D-alanyl–D-alanine carboxypeptidase/transpeptidase (DD-peptidase), which is responsible for catalyzing reactions of bacterial cell wall synthesis [3–5]. The two enzymes were shown to have homologous three-dimensional structures extending well beyond the β-lactam (penicillin and cephalosporin) binding site and to follow similar catalytic pathways. From these results, it seems likely that the β-lactamase evolved from an enzyme with DD-peptidase activity. The kinetic parameters of the first and second steps of catalysis for both enzymes are characterized by the k_{cat}/K_M (acylation) and k_3 (deacylation) kinetic parameters [6]. Although both of these serine-reactive enzymes interact with a common molecule, they differ significantly in the values of these kinetic parameters; the β-lactamase is highly efficient in the hydrolysis of β-lactam antibiotics (Fig. 1), yet has no significant DD-peptidase activity [7].

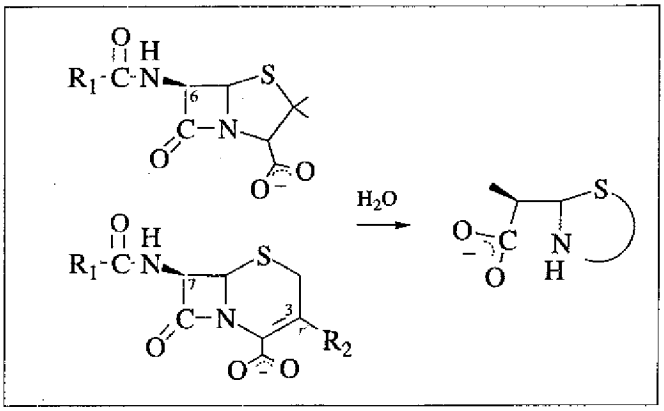
The structural comparison in 1986 [1,2] addressed a question posed over 20 years earlier [8] about the origin of the enzymes providing β-lactam resistance. More recently, the crystallographic structures of two class C β-lactamases [9,10] and, this year, a structure of a penicillin-binding protein (PBP-2x) [11] have become available. We are now in a position to ascertain whether the initial hypothesis about β-lactamase ancestry appears to hold for the whole family of enzymes by comparing the class C β-lactamase structures with that of the DD-peptidase, which has recently been refined at 1.6 Å resolution [12]. Using kinetic data on the reaction of these enzymes with early naturally-occurring β-lactams, we will extend discussions [10,13,14] of the evolution of substrate specificity from peptide to β-lactam within the penicillin-recognizing proteins. We also address the question of whether the two classes of β-lactamases developed sequentially from an ancestor of bacterial cell wall synthesis or diverged from the putative common ancestor along separate pathways.

Results

Global comparison

Figure 2 shows the tertiary structure of the three penicillin-recognizing enzymes. All have a mixed α + β motif including an α-helix domain on the left and an α/β domain on the right. The β-lactam binding site in all three enzymes, which is also the D-alanyl–D-alanine binding site in the DD-peptidase, lies in the center of each enzyme at the left edge of the five-stranded β-sheet. The reactive serine lies at the amino-terminal end of a central α-helix. The stable ($t_{1/2}$ measured in hours) acyl-serine intermediate resulting from the binding of cephalothin to the DD-peptidase in shown in Figure 3 [15]. For the two classes of β-lactamases, modeling [16,17] of the very unstable acyl intermediates based on the crystallographic structures of the free enzymes indicates similar binding geometries, in agreement with the structures of stabilized acylenzymes formed with substrates, inhibitors or transition state analogs of the class A enzyme [18–21] and class C enzyme [9,22].

Figure 1

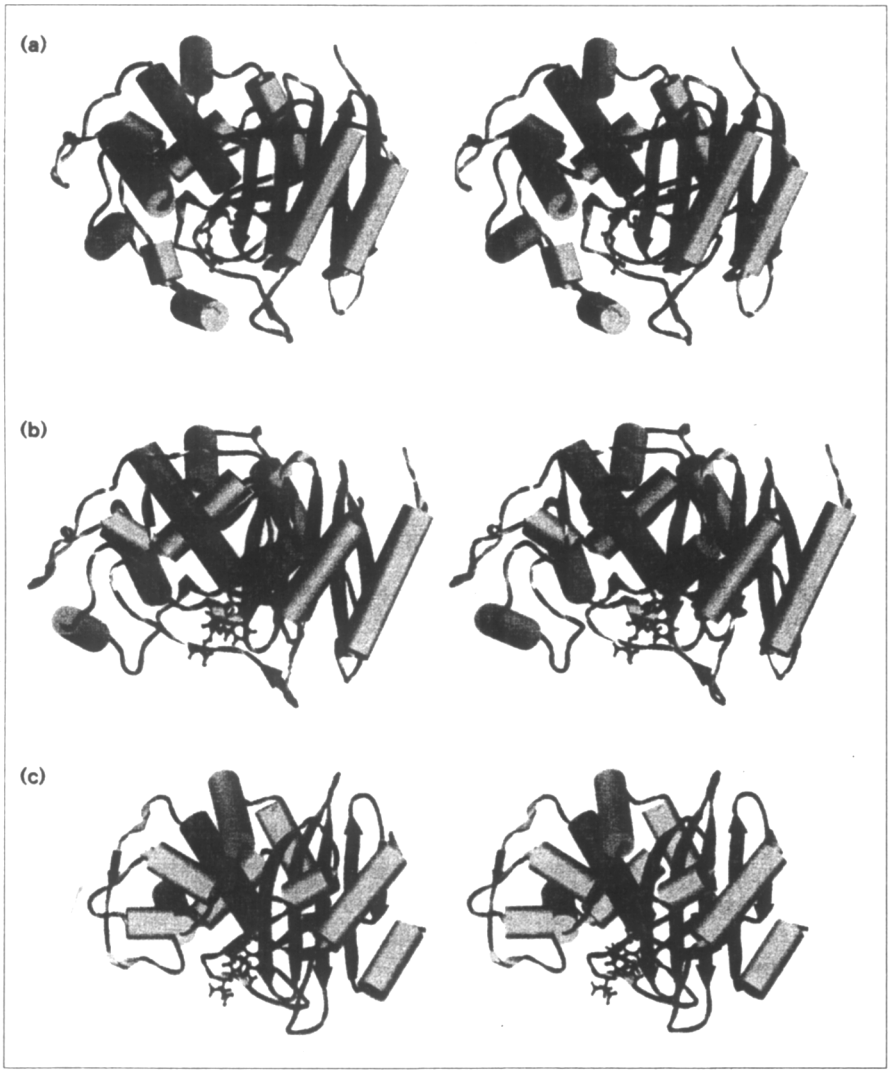


Hydrolysis of β-lactam antibiotics (penicillin and cephalosporin type).

Pairwise overlays of the polypeptide backbones of the three molecules are shown in Figure 4. The degree of similarity of the backbone structures was quantitated with DEJAVU [23], and the results are given in Table 1. For several combinations of secondary structure elements, it was found that the DD-peptidase motif more closely matches the class C β-lactamase than any of several class A β-lactamases.

DEJAVU was also used to confirm that when DD-peptidase is used as a search molecule only β-lactamases were found in a crystallographic database. Two principal parameters, the minimum number of residues in each element, and the minimum number of elements to match, were varied. When the number of match elements was eight or greater, the search process uniquely selected β-lactamases from the database.

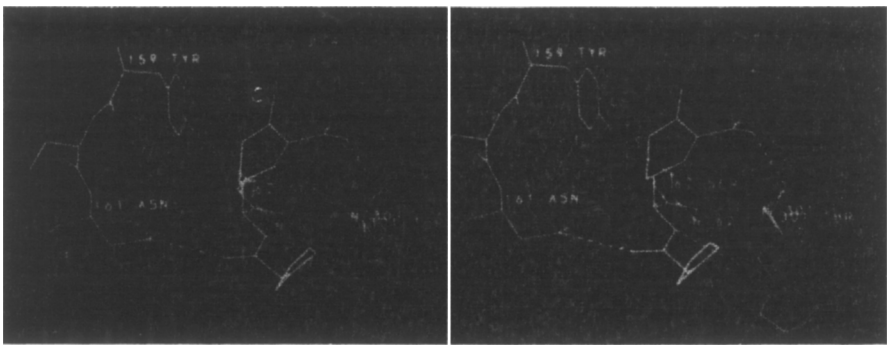
Figure 2



Stereoviews of **(a)** D-ala–D-ala carboxypeptidase/transpeptidase (37.4 kDa) of *Streptomyces* sp. R61 [12], **(b)** 39.0 kDa class C β-lactamase of *Enterobacter cloacae* P99 [10], and **(c)** 29.5 kDa class A β-lactamase of *Bacillus licheniformis* [26]. Cylinders and arrows represent α helices and β strands, respectively. The reactive serine is at the amino-terminus of the central helix H2 (orange). The Ω-loop lies at lower center of each molecule.

Figure 3

Crystallographic structure at 1.8 Å resolution of the acyl intermediate of cephalothin bound to Ser62 of the S. R61 DD-peptidase [15]. View angle as in Fig. 2a. Hydrogen bonds (dashed) to amide groups in the oxyanion pocket and on the B3 β strand are shown. Hydrogen atoms are omitted.



Binding site comparison

The Ω-loop

The chain direction of a 20- to 40-residue length of polypeptide at the bottom of the binding sites helps to distinguish the class A β-lactamase from both the class C β-lactamase and the DD-peptidase. This so-called Ω-loop is generally composed of residues 160–181 in the class A β-lactamases, residues 187–225 in the class C β-lactamases,

Figure 4

Stereoviews of pairwise overlays of backbone chains of the three β-lactam-recognizing enzymes. **(a)** DD-peptidase (red) and the class C β-lactamase (yellow), **(b)** DD-peptidase and the class A β-lactamase (green), **(c)** class C and class A β-lactamase. Pairs are overlaid based on least-squares fitting of the seven common atoms listed in Table 3.

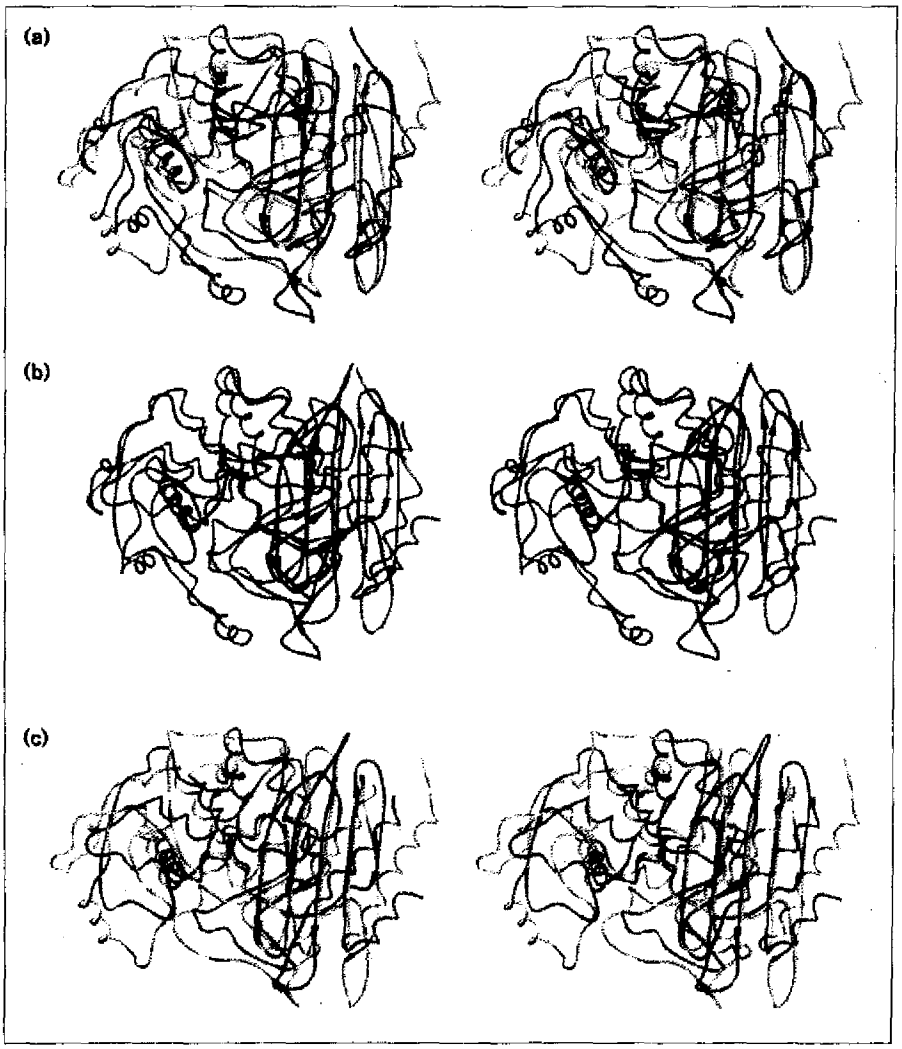


Table 1

Three-dimensional fit of motif of DD-peptidase to motifs of class C and class A β-lactamases.*

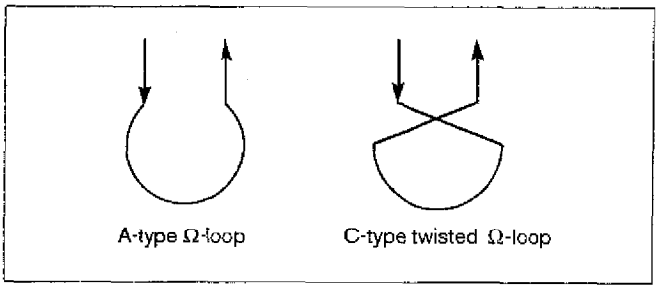
Minimum number elements (α,β)	Separation (rms) of element centers, Å		Number of matches found other than β-lactamase
	class C	class A†	
14 (7,7)	2.2	>6Å	0
10 (5,5)	1.1	>5	0
8 (4,4)	0.7	3.8–4.7	0
7 (3,4)	0.6	3.2–4.4	12

*Fitting was done with DEJAVU [23]. At least three residues were included in each secondary structure element. †Includes β-lactamases from *Bacillus licheniformis* 749/C, *Staphyloccocus aureus* PC1, and the TEM plasmid.

and residues 197–242 in the DD-peptidase. A twisting of the Ω-loop in the class C β-lactamase and DD-peptidase reverses the loop direction relative to that in the class A β-lactamase (Fig. 5).

The functional consequence of this difference in direction is the introduction of a catalytic base, Glu166, into the class A β-lactamase [10]. The position of this acidic sidechain in the class A catalytic site in relation to the class C β-lactamase and DD-peptidase sites is shown in Figure 6. Glu166 and its hydrogen-bonded water molecule are strategically positioned to be actively involved in both acylation [24] and deacylation [18,25–27].

Figure 5

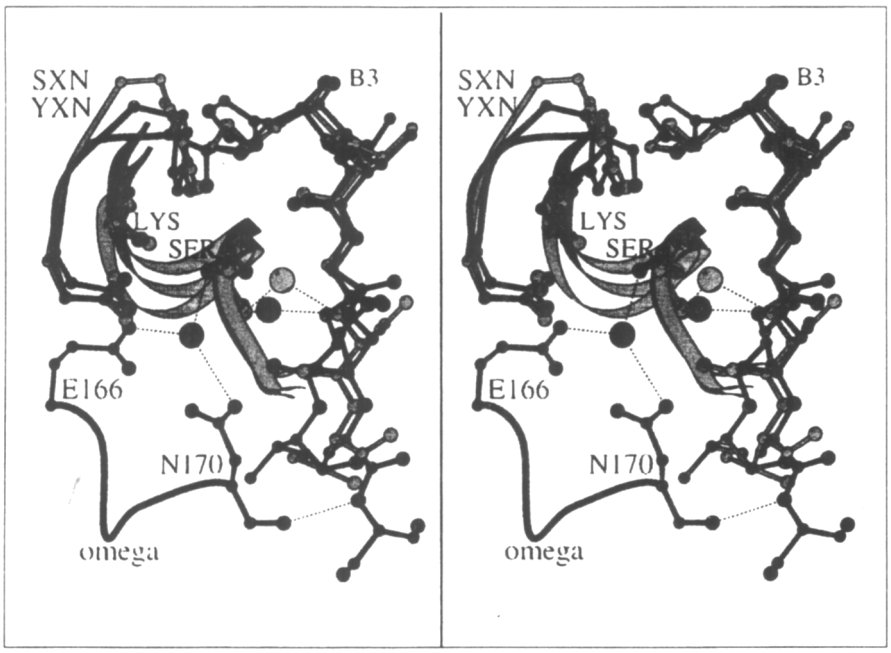


The Ω-loop twisting is shown. It is generally composed of residues 160–181 in the class A β-lactamases, residues 187–225 in the class C β-lactamases and residues 197–242 in the DD-peptidase.

The SXN and YXN sequences

The class A β-lactamase has a serine at position 130 of a conserved SXN sequence (Fig. 6), whereas the class C β-lactamase and the DD-peptidase have a tyrosine here at position 150 or 159, respectively (the YXN motif). It has been suggested that the phenolate ion of the tyrosine might act as a general base [5,9], thus compensating for the absence of the class A Glu166 in the two enzymes. Note, however, that sequence alignments show that most other DD-peptidases carry the SXN sequence in this position. The identity, or even the presence, of a general base in these DD-peptidases, which are devoid of the equivalents of both Glu166 (in class A β-lactamase) and Tyr150/159 (in the class C β-lactamase or R61 DD-peptidase) remains unknown [7].

Figure 6



Overlay of active site groups in the three β-lactam-recognizing enzymes, with DD-peptidase in red, the class C β-lactamase in yellow and the class A β-lactamase in green. The overlay was made by optimizing the fit of the seven common atoms listed in Table 3. Water molecules are hydrogen bonded in the oxyanion hole of the two β-lactamases. The Glu166 sidechain on the Ω-loop and the hydrogen-bonded water molecule occur only in the class A β-lactamases.

Table 2

Selected interatom distances (Å) in the β-lactam-binding sites of the DD-peptidase and β-lactamases of class C and class A.*

	DD-peptidase	β-lactamases		
		class C†	class A‡	class A(ave.)§
A	4.15	3.10	2.65	2.70
B	2.75	2.95	3.20	3.55
C	3.75	3.20	3.20	3.25
D	4.70	3.05	2.90	2.90
E	2.85	2.60	2.55	2.70
F	3.70	3.75	4.55	4.70
G	5.30	4.75	4.55	4.55
L1	#	3.60	3.05	3.25
L2	#	2.85	2.80	2.85

*Letters denoting distances are diagrammed in Figure 7. Values are given to nearest 0.05 Å. Estimated uncertainty is 0.15 Å, based on rms deviation of distances in five copies of three different class A enzymes (see note §). †From *Enterobacter cloacae* P99. ‡From *Bacillus licheniformis* 749/C. Average values are listed for this and the P99 β-lactamase because two independent molecules exist in the crystal structures. §Averaged values, using β-lactamases of *B. licheniformis* [26], TEM-1 [43,44], and *Staphloccocus aureus* [25]. #Water is absent from the oxyanion hole.

Interatomic distances and spatial positions.

Distances involving the binding site atoms common to all three enzymes are listed in Table 2 and in Figure 7. The distances A and B, of importance in several proposed catalytic mechanisms, vary in concert from DD-peptidase to the class C β-lactamase to class A β-lactamase. The DD-peptidase is distinguishable from both β-lactamases in having a much greater A distance. A notable parameter separating the class A enzyme from the other two enzymes is the F distance across the oxyanion pocket formed by the backbone amide groups; here the class A

Table 3

Pairwise comparison of active site architectures in the DD-peptidase and two β-lactamases.

	DD-peptidase	β-lactamases	
		class C	class A
DD-peptidase	0	0.48 (0.13)	0.67 (0.12)
class C	0.62 (0.19)	0	0.29 (0.12)
class A	0.74 (0.14)	0.48 (0.14)	0

Values above the diagonal are the average differences (Å) in all possible interatom distances between seven atoms common to both molecules. Values below the diagonal are the average differences (Å) in position of the seven atoms. Average values are listed for each β-lactamase because two independent molecules exist in the crystal structures. Differences for the two independent molecules are given in parentheses. The common atoms used in the fitting are pictured in Figure 7 and are Oγ and N(H) in Ser62/64/70 (from the DD-peptidase/classC/classA enzymes, respectively), Nε in Lys65/67/73, Nε in His298/Lys315/234, N(H) in Xaa301/318/237, O(H) in Tyr159/150/Ser130, and Oδ in Asn161/152/132.

Figure 7

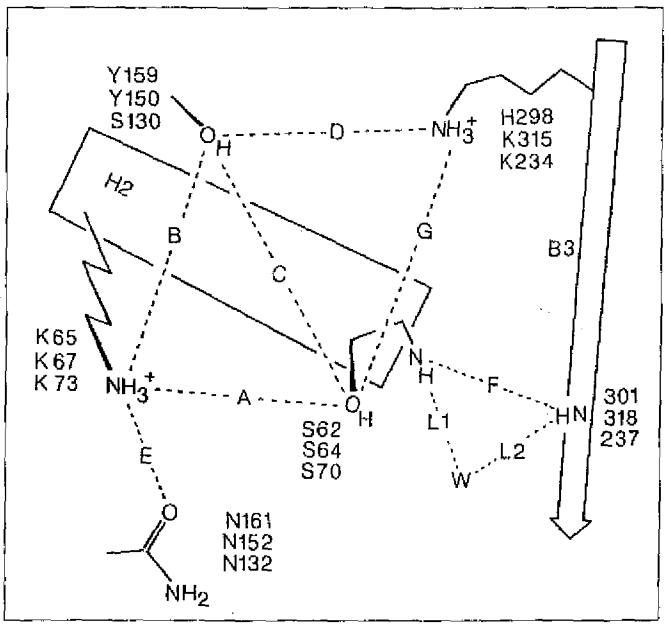


Diagram of interatom distances which are listed in Table 2. Corresponding amino acids in the three enzymes are numbered in the order DD-peptidase, class C β-lactamase and class A β-lactamase. W is the water molecule present in the oxyanion hole of the β-lactamases.

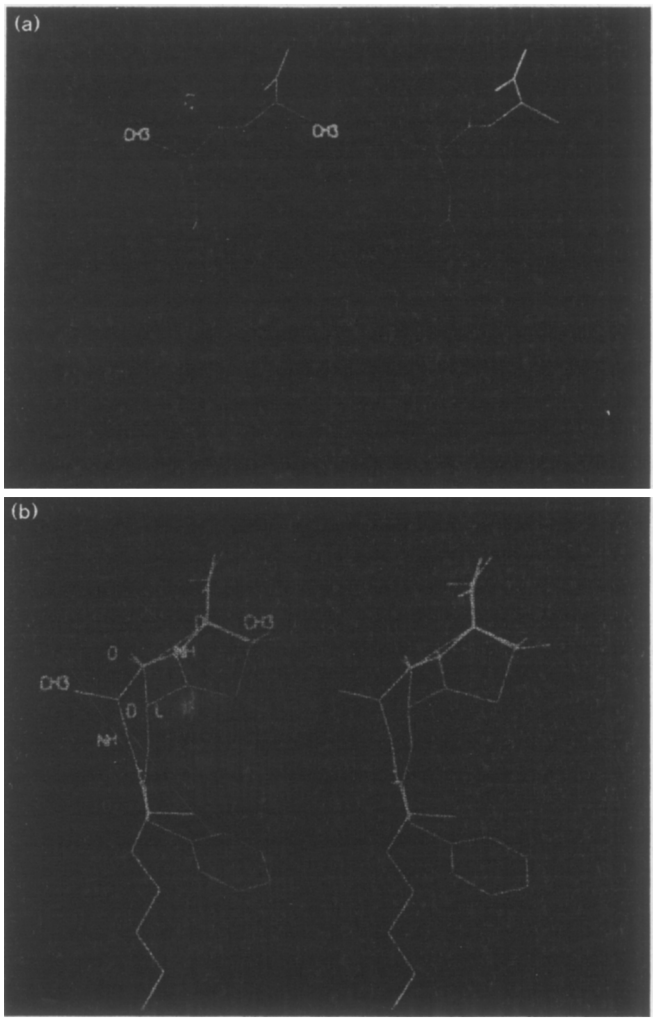
β-lactamase has a significantly wider gap by 0.9–1.0 Å. The F distance is determined in part by the contact of the B3 β-strand with the residue just prior to the reactive serine. We note that DD-peptidase and the class C β-lactamases have glycine here, and the PBPs have either glycine or alanine [28]. The class A β-lactamases, however, predominantly have side-chains equal to or larger than alanine preceding the serine [29,30].

We show in Table 3 pairwise differences in all possible interatom distances for seven atoms common to the three binding sites, and pairwise differences in their atomic positions. The class C β-lactamase fits more closely with the DD-peptidase than with the class A β-lactamase.

Electrophilicity of the oxyanion pocket

A feature of the active site unique to the two β-lactamases is the water molecule in the oxyanion pocket. The water is held by two hydrogen bonds (L1 and L2), the stronger of which is associated with the amide group of the B3 β-strand. The combined strength of both hydrogen bonds increases slightly from class C to class A (Table 2). This clearly parallels the rates of deacylation in the penicillins, but not in the cephalosporins (Table 4). A more elaborate discussion of the cephalosporins' behavior is made difficult by our ignorance of the actual values of k_3. Remarkably, in the high resolution (1.6 Å) structure of the DD-peptidase, no water molecule is seen in the oxyanion hole.

Figure 8



Stereoviews of **(a)** L-Lys–D-Ala–D-Ala peptide and **(b)** its overlay with benzylpenicillin G. The peptide has Φ/ψ of 130°/-100° at the penultimate α-carbon atom and 120°/30° at the carboxy-terminal α-carbon atom. Penicillin has the open 2β,3α-equatorial conformation [54] found in solution by NMR [55]. Hydrogen atoms are omitted.

Binding mode of peptide and β-lactam

Figure 8a shows the cell wall peptide L-Lys–D-Ala–D-Ala in a conformation as near as possible to that of benzylpenicillin G, a presumed transition state analog [8,31]. The resulting peptide conformation, with a ϕ/ψ of 130°/–100° at the penultimate α-carbon atom and 120°/30° at the carboxy-terminal α-carbon atom, is near to the calculated conformation [32,33] and a conformation modelled to fit the DD-peptidase [34]. A superposition of the modelled peptide and benzylpenicillin G is shown in Figure 8b.

Figure 9 shows how the two molecules could interact with the oxyanion pockets of the three enzymes, superimposed as in Figure 6. The position of the penicillin is from crystallographic mapping of β-lactamoyl complexes with the DD-peptidase [15,34] and TEM class A β-lactamase [18,21]. It is noteworthy that the orientation of the C6 exocyclic acylamido bond of penicillin differs from the corresponding peptide bond of the peptide, such that better hydrogen bonding of the β-lactam to the β-lactamases is possible. Figure 10 indicates how the peptide and penicillin might bind to the DD-peptidase and class A β-lactamase, respectively. The greater tilt of the B3 β-strand in the class A enzyme is observed in the superposition of Figure 6.

Catalytic comparison

The values of k_{cat} and k_{cat}/K_M for the three enzymes and selected substrates are shown in Table 4. Only naturally-occurring β-lactams are considered because the semi-synthetic β-lactams have been developed for their greater resistance, relative to benzylpenicillin G, to breakdown and removal by β-lactamases. They are therefore inappropriate for an analysis of enzymic evolution, presumably driven by ancient substrates such as benzylpenicillin G. The rates and catalytic efficiency for the turnover of benzylpenicillin G increase from the DD-peptidase through class C to the class A β-lactamase.

Acylation step

The second order rate constant characterizing the acylation reaction is k_{cat}/K_M [6]. With benzylpenicillin G (and this is true for most penicillins), both β-lactamases have rate constants that are three orders of magnitude faster than that of the DD-peptidase. A similar situation prevails with cephalosporin C. Only the DD-peptidase recognizes the cell wall-like peptide, but the class C β-lactamase exhibits significant activity against the ester analog, while the class A enzyme is completely inactive. Finally, the synthetic ester benzoyl-Gly–D-phenylactate acylates the class A enzyme very slowly, but reacts more readily with the other two enzymes; it acylates the class C β-lactamase even more efficiently than it acylates the DD-peptidase.

Deacylation step

With the β-lactams, the β-lactamases are many orders of magnitude (five or more) more efficient than the DD-peptidase. With benzylpenicillin G, the class A β-lactamase deacylates significantly faster than its class C counterpart, an observation which can be extended to many other penicillins [35]. With cephalosporin C, however, and several other cephalosporins [36] the deacylation rate of the class C enzyme is much larger than with penicillins. For the rates of hydrolysis of the diacetyl-L-Lys–D-Ala-enzyme and benzoyl-Gly-enzyme adducts, only minimum values have been obtained. Note, however, that these adducts are easily hydrolysed. Thus, it can be tentatively concluded that the inefficiency of β-lactamases when processing the peptide substrates is mainly due to their near total inability to acylate these compounds.

Table 4

Kinetic parameters for reactions of the DD-peptidase and two β-lactamases.*

Substrate	DD-peptidase	β-lactamases	
		class C†	class A‡
Benzylpenicillin G			
k_{cat} (k_3) (s^{-1})	0.14×10^{-3} (0.14×10^{-3})	14 (14)§	2200 (7100)#
k_{cat}/K_M ($mM^{-1}\ s^{-1}$)	17	23 000	29 000
Cephalosporin C			
k_{cat} (k_3)	$1x10^{-6}$ (1×10^{-6})	1100 (>1000)	14
k_{cat}/K_M	1.5	2700	150
Diacetyl-L-Lys-D-Ala-D-Ala			
k_{cat} (k_3)	50 (>50)	ND	ND
k_{cat}/K_M	4.6	ND	ND
Diacetyl-L-Lys-D-Ala-D-Lactate			
k_{cat} (k_3)	32 (>32)**	19 (>19)††	ND
k_{cat}/K_M	0.8	0.2	ND
Benzoyl-Gly-D-phenyl-lactate			
k_{cat} (k_3)	>3 (>3)	> 30 (>30)	> 0.1 (> 0.1)
k_{cat}/K_M	2.6	20	0.08

*All data are from [36,49,50] unless noted otherwise. †From *Enterobacter cloacae* P99. ‡From *Bacillus licheniformis* 749/C. §[51]. #A Matagne, SG Waley and JMF, unpublished data. ND = No detectable activity. **[52]. ††[53].

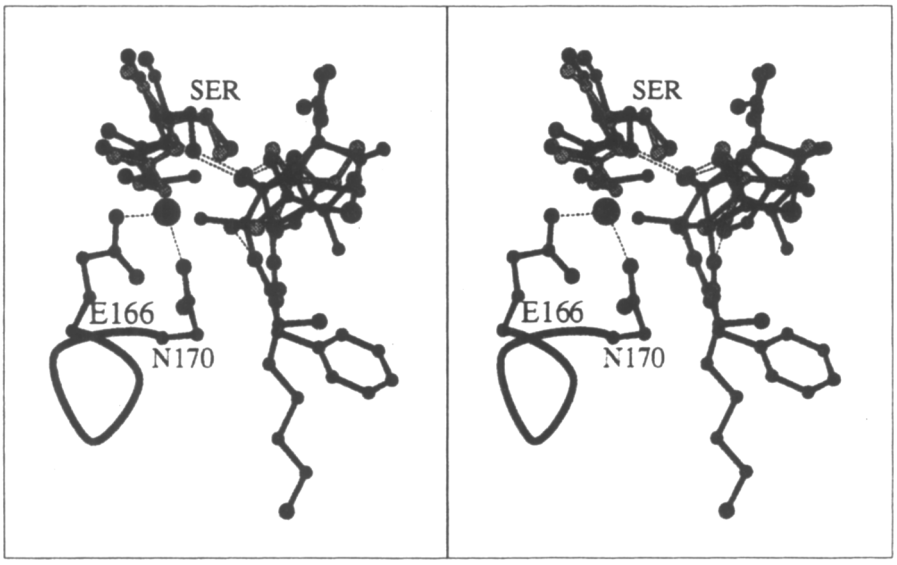
Discussion

The above analysis suggests that the structural factors influencing the development of β-lactamase activity include the following:

β-Lactam shape

It has been said that the evolution of a DD-peptidase into a β-lactamase might have been driven by the shape of the new substrate, the β-lactam [13]. A β-lactam resembles the normal substrate of the DD-peptidase in having a D-configuration of a carboxylated carbon atom and a peptide-like β-lactam bond [8,31]. But several important differences which exist between the two molecules (Fig. 8) must be accommodated by the new binding site: bonds about the nitrogen atom of the β-lactam ring are nonplanar; the C6 β-acylamido group of the β-lactam ring

Figure 9



Fitting of the L-Lys-D-Ala-D-Ala peptide (purple) and benzylpenicillin G (blue) to residues forming the oxyanion hole in the three overlaid β-lactam-recognizing enzymes. View from the left side of Figure 6. The orientation of the C6 exocyclic acylamido bond of penicillin differs from the corresponding peptide bond of the tripeptide, such that better hydrogen bonding of the β-lactam to the β-lactamases is possible.

Figure 10

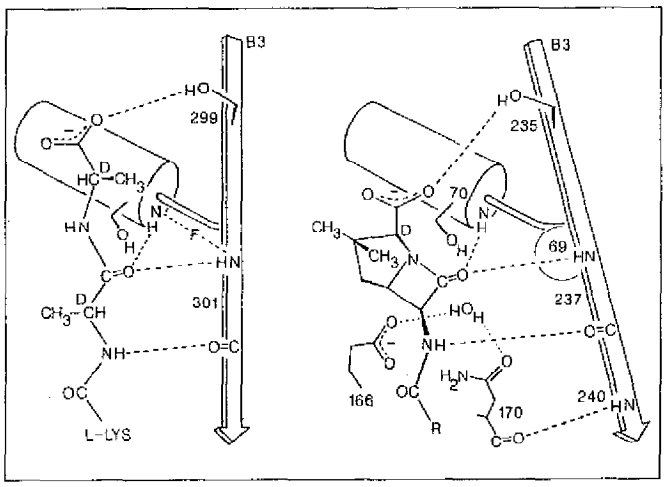


Diagram of possible binding of **(a)** the L-Lys–D-Ala–D-Ala peptide to DD-peptidase and **(b)** a penicillin to class A β-lactamase. The longer F distance and the greater tilt of the B3 β-strand in the β-lactamase may be related to the size of the substituent at position 69 and to the hydrogen bond from B3 to Asn170 in the Ω-loop. D-configured carbon atoms are indicated.

is quite apart in direction from the corresponding peptide group of the DD-peptide; and the β-lactam lacks a methyl group corresponding to the penultimate D-methyl group of the DD-peptide.

β-Lactam binding

Crystallographic complexes of the DD-peptidase with β-lactams [15,34] have indicated that it is possible for the DD-peptide, like the β-lactam in Figure 3, to form primary interactions with the enzyme's B3 β-strand via at least three groups on the peptide: the carboxy-terminal carboxylic acid group, the carbonyl group of the carboxy-terminal peptide bond, and the amido group of the penultimate peptide bond (Fig. 10). If in the DD-peptidase the DD-peptide is replaced by a β-lactam, the first two of these interactions could easily be maintained but the third would be less favourable. A better interaction between the C6 β-acylamido group of a β-lactam and the β-strand would result, however, if the strand were tilted outward to improve the hydrogen bonding between the acylamide and the carbonyl group on the β-strand. Figures 6 and 9 show that while the B3 strand in the primitive class C β-lactamase has the needed tilt with respect to the β-strand in the DD-peptidase, the β-strand in the class A β-lactamase has a greater tilt. The data in Table 2 show that the F distance is significantly expanded from the 3.7 Å observed in the DD-peptidase to 4.6–4.7 Å in the class A β-lactamases. Such a change in architecture of the catalytic site would seem to favor binding of β-lactams over DD-peptides.

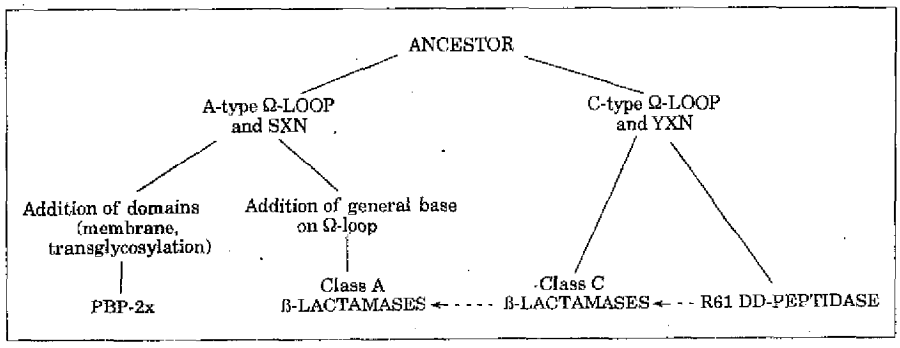
β-Lactam hydrolysis

It is not clear whether the accentuated tilt of the B3 strand in the β-lactamases would facilitate hydrolysis of β-lactams. Perhaps the strand movement accommodates another important change elsewhere. For example, only in the class A β-lactamases does the Ω-loop protrude deeply into the active site. Figure 6 shows that the Ω-loop contacts the B3 strand. The backbone carbonyl group of Asn170 forms a hydrogen bond to the amide of residue 240 on the β-strand. This interaction (and the residue 69 contact mentioned in Results) may produce an expanded F distance which favors initial binding and positioning of the β-lactam for hydrolysis. In contrast, the Ω-loops of the DD-peptidase and class C β-lactamase do not contact the β-strand this closely. These two enzymes also have a small glycine residue preceding the reactive serine, considerably shortening their F distances (Table 2).

Oxyanion pockets and rates of β-lactam turnover

The slow turnover of the β-lactamoyl intermediate by the DD-peptidase makes the antibiotic a very effective inhibitor of cell wall synthesis. The fact that the oxyanion pocket of the (ligand-free) DD-peptidase has no hydrogen-bonded water [12] is evidence that the two backbone NH groups are less able to stabilize the oxyanion of β-lactam tetrahedral intermediates than those in β-lactamases.

Figure 11



Phylogeny scheme for penicillin-recognizing enzymes incorporating only enzymes of known crystallographic structure. The dashed arrows show an alternate path requiring a mutation in the YXN sequence and an untwisting of the Ω-loop [10]. Class D β-lactamases contain a YXN sequence and nearly all PBPs contain SXN. PBP-2x is a high molecular weight PBP classed together with PBPs 2 and 3 of *E. coli*, PBP-2′ of *Staphloccocus aureus*, and PBP-2 of *Neisseria meningiditis* and *N. gonorrhoeae*, and it is likely that all these proteins have similar structures.

)D-peptide hydrolysis

t has always intrigued enzymologists that the β-lactamses, presumably derived from cell-wall-synthesizing :nzymes, have completely lost their ancestral peptidase .ctivity. The comparative analysis presented here)rovides a possible explanation for the loss of peptidase .ctivity in the class A β-lactamase (although the argument s less clear for the class C β-lactamase). We propose that he DD-peptide may be prevented from hydrogen)onding to the B3 strand of β-lactamase by steric contact vith the Ω-loop, particularly Asn170 (Fig. 9). If the)eptide manages to bind, its penultimate D-methyl group nay displace the hydrolytic water molecule, as is thought :o be true for the 7α-methoxy group of cefoxitin [37,38]. Given that the glycyl–D-Ala peptide is not significantly ıydrolyzed by class A β-lactamases [N Rhazi and JMF, ınpublished data], it may be the steric contact with the Ω-loop, and not displacement of the water molecule, that s the more important factor in preventing reaction of the :lass A β-lactamases with the cell wall peptides.

An effort was reported to generate DD-peptidase activity in a class A β-lactamase by exchanging a 28-amino acid segment of the TEM β-lactamase for the homologous segment of PBP-5 of *Escherichia coli* [39]. The segment contained the 20 residues prior to the reactive serine residue. A small but detectable amount (1 %) of DD-peptide hydrolysis was observed. Our analysis indicates that the important change in this segment was the replacement of methionine with alanine at position 69, as the smaller alanine would bring the F distance closer to that seen in the DD-peptidase structure (Fig. 10). An increase in activity in the chimera might be expected if, in addition, the Asn170 of the Ω-loop were to be replaced with a smaller group.

Evolution and phylogeny

In terms of global structure, local active site architecture, catalytic behavior, and substrate specificity, the class C β-lactamase is significantly closer to the DD-peptidase than to the class A β-lactamase. Following the suggestion that the serine-reactive penicillin-recognizing enzymes might have arisen from a common ancestor [1,2,28], we show in Figure 11 a structure-based phylogenic branching of these enzymes. It is based on an analysis which divides the family according to the SXN or YXN motif in the conserved element 2 [6]. We base the initial divergence on the type of twist observed in the critical loop forming the bottom of the binding sites; this loop is either the A-type Ω-loop or the C-type twisted Ω-loop. In the known crystal structures, the SXN sequence is always accompanied by the A-type loop and the YXN sequence by the C-type loop. With this scheme, surprisingly, the class A and class C β-lactamases lie in separate branches, as do the curently known PBP (2x) and DD-peptidase (R61) structures [11,12]. An earlier hypothesis [10], that the class A β-lactamase derived directly from class C, can be accommodated in this scheme if one allows a Tyr to Ser mutation in the YXN motif and an untwisting of the Ω-loop.

Significance

We have shown that structural features of the three β-lactam binding sites are better optimized for penicillin turnover in the class A β-lactamase, which has become more effective in the binding and hydrolysis of β-lactam antibiotics and has completely lost its ancestral activity on D-Ala–D-Ala-peptides of nascent bacterial cell wall. The accentuated tilt of the B3 β-strand, the increased electrophilicity of the oxyanion hole and the prevention of peptide binding by the intrusion of the Ω loop, all combine with a well-placed general base to make the class A β-lactamase a highly efficient enzyme. Although the less-efficient class C β-lactamase falls between a D-Ala–D-Ala peptidase and the highly optimized class A β-lactamasein structural and kinetic properties, it is concluded that the two β-lactamases evolved inde-pendently in parallel paths rather than sequentially along the same path. Increasing clinical use of modern β-lactams and β-lactamase inhibitors is now driving β-lactamase evolution by producing annual mutations which will alter further the architecture of these troublesome enzymes [40–42].

Materials and methods

Crystallographic structures

The enzyme structures examined are: the D-alanyl–D-alanine-carboxypeptidase/transpeptidase of *Streptomyces* sp. R61, Protein Data Bank entry 3PTE [12], the class C β-lactamase of *Enterobacter cloacae* P99, entry 1BLT [10], and three class A β-lactamases: *Bacillus licheniformis* 749/C, entry 4BLM [26], *Staphylococcus aureus* PC1, entry 3BLM [25], and plasmid TEM-1, entry 1BTL [43,44]. In addition, complexes of the DD-peptidase with several β-lactams were examined, entries 1CEF and 1CEG [15, 34].

Comparison of structures

To fit sets of equivalent atoms in two identical molecules, a least-squares minimization algorithm of Nyburg [45] was used. The global comparison of structures with significant differences in size and chain folding required the use of the program DEJAVU [23], which matches secondary structure arrays without regard to unstructured and unmatched chain segments. DEJAVU can search for a particular secondary structure array in a database of crystal structures, or it can simply compare chosen structures. The program finds three-dimensional motifs, i.e. collections of α-helices and β-strands, that have approximately the same number of residues per element, are separated by similar center-to-center distances, and have comparable directions and connectivity. The overlay of the two motifs is then optimized. Structures were viewed on an Evans & Sutherland PS330 graphics system using FRODO [46] and on a Silicon Graphics IRIS Crimson/Elan using RIBBONS [47] and MOLSCRIPT [48].

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