On the Origin of Bacterial Resistance to Penicillin: Comparison of a β-Lactamase and a Penicillin Target

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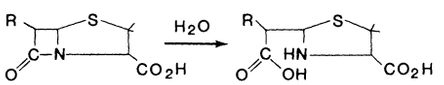
Structural data are now available for comparing a penicillin target enzyme, the D-alanyl-D-alanine-peptidase from *Streptomyces* R61, with a penicillin-hydrolyzing enzyme, the β-lactamase from *Bacillus licheniformis* 749/C. Although the two enzymes have distinct catalytic properties and lack relatedness in their overall amino acid sequences except near the active-site serine, the significant similarity found by x-ray crystallography in the spatial arrangement of the elements of secondary structure provides strong support for earlier hypotheses that β-lactamases arose from penicillin-sensitive D-alanyl-D-alanine-peptidases involved in bacterial wall peptidoglycan metabolism.

SINCE THE FIRST CURES ACHIEVED BY penicillin in 1941, an expanding family of β-lactam antibiotics has been developed to combat bacteria. As a result of gene transfer and recombination, an increasing number of bacterial species produce β-lactamases, enzymes that rapidly transform β-lactams into inactive metabolites by hydrolyzing the β-lactam bond:



The origin of the genes coding for β-lactamases has been much debated. In 1965, Tipper and Strominger proposed that β-lactamases might have arisen from an essential, penicillin-sensitive D-alanyl-D-alanine–cleaving peptidase (hereafter DD-peptidase) involved in the last stages of the bacterial wall peptidoglycan synthesis (*1, 2*). These cell wall synthetic enzymes are the targets of β-lactams (*3*). Others in the field have expressed doubt, however, that there is convincing evidence for common ancestry (*4, 5*).

All bacteria contain an assortment of membrane-bound penicillin-binding proteins (PBP's) that are related to such distinct cellular functions as elongation and septation. These proteins do not have the same degree of "essentiality" and show various amounts of sensitivity to β-lactam antibiotics. In spite of this diversity, the PBP's to which catalytic activity could be assigned have an active-site serine and function as DD-peptidases (*3, 6*). They catalyze transpeptidation reactions involving the COOH-terminal D-alanyl-D-alanine of the peptidoglycan precursor acting as carbonyl donor, and an amino group of the preexisting peptidoglycan acting as acceptor. Transpeptidation proceeds by conversion of the carbonyl donor to a short-lived acyl enzyme (with release of the COOH-terminal D-alanine) and subsequent attack on this acyl enzyme by the amino acceptor (with synthesis of a new interpeptide bond). The enzyme can become penicilloylated with a penicillin molecule instead of a D-alanine–terminated peptide, but the acyl enzyme thus formed is abnormally long-lived (*3*). Carboxypeptidation occurs when water is the acceptor.

More than 80 different β-lactamases have been identified and have been divided into three classes (A, B, and C). Most β-lactamases have been characterized as active-site serine enzymes belonging to classes A or C (*3, 7, 8*). Zinc-requiring β-lactamases constitute class B (*9*). One zinc DD-peptidase is also known; it catalyzes only carboxypeptidation reactions and is not a penicillin-binding protein (*10*). We now introduce three-dimensional structural data for a class A serine β-lactamase that will provide a new insight into the relatedness of serine members of the β-lactamase and DD-peptidase families.

The active-site serine DD-peptidase of *Streptomyces* R61 is excreted during growth in the form of a water-soluble PBP and has served as a model of the membrane-bound enzymes (*2, 3*). This PBP (37,400 daltons) has been crystallized, and its three-dimensional structure and penicillin binding site have been described at 2.8 Å resolution (*11*). Moreover, the structural gene has been cloned and sequenced (*12*).

In the β-lactamases of known sequence (*4, 8*), the active-site, penicillin-binding serine residue is flanked by a phenylalanine at the fourth position on its amino side and by a lysine at the third position on its carboxyl side, suggesting that these conserved phenylalanine and lysine residues are important for catalysis. The R61 DD-peptidase also possesses this important Phe-X-X-Ser-X-X-Lys sequence (Fig. 1). Moreover, when the comparison is broadened to a 25-amino-acid stretch that includes the active-site serine, the homology between the new sequence data for the R61 DD-peptidase and the *Bacillus licheniformis* (or *Bacillus cereus*) β-lactamase of class A (nine conserved amino acids), or between the R61 DD-peptidase and the *ampC* β-lactamase of class C (eight conserved amino acids), is found to be greater than the homology between the β-lactamases of class A and C (four or three conserved amino acids). The degree of homology, however, decreases when comparison extends away from the active site. For this reason, and because molecular mass differences between PBP's (40,000 to 90,000 daltons) and β-lactamases (30,000 daltons) are rather large, some authors believe evidence for common ancestry is weak (*13*).

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Table 1. Kinetic parameters of the interactions between the DD-peptidase of *Streptomyces* R61 and β-lactamase of *B. licheniformis* and various carbonyl donors.

Carbonyl donor*	*Streptomyces* R61 DD-peptidase				*B. licheniformis* β-lactamase		
	K_m (M)	k_{cat} (sec^{-1})	k_{+2}/K ($M^{-1}sec^{-1}$)	k_{+3} (sec^{-1})	K_m (M)	k_{cat} (sec^{-1})	k_{+2}/K ($M^{-1}sec^{-1}$)
Benzylpenicillin	1×10^{-8}	1.4×10^{-4}	14,000	1.4×10^{-4}	5×10^{-5}	2,600	53×10^{6}
Ac_2-L-Lys-D-Ala-D-Ala	1×10^{-2}	55	5,500	>55		No substrate activity	
Ac_2-L-Lys-D-Ala-D-Lac	4×10^{-2}	160	4,000†	>160		No substrate activity	

*H_2O is the nucleophilic acceptor of the transfer reactions. †Determined as described in (*9*).

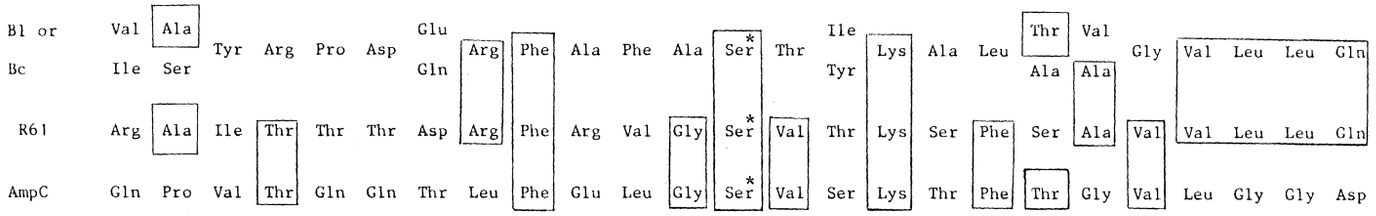

Fig. 1. Amino acid sequences around the active-site serine residue (Ser*). B1, Bc, and AmpC: β-lactamases of *B. licheniformis*, *B. cereus*, and *Escherichia coli* (chromosome-coded) (*4, 8*). R61: DD-peptidase of *Streptomyces* R61 from the gene sequence. Conserved residues are boxed.

If one looks to substrate specificity for confirmation of the active-site sequence homology, it is not apparent. Table 1 gives the kinetic parameters that govern the interaction between the two enzymes (E) and three types of carbonyl donor (D) in the following scheme:

$$E + D \overset{K}{\rightleftharpoons} E \cdot D \xrightarrow{k_{+2}} E - D^* \xrightarrow[HY]{k_{+3}} E + P$$

where K is the dissociation constant, k_{+2} and k_{+3} are first-order rate constants, E · D is the Michaelis complex, E − D* is the acyl (R-D-alanyl or penicilloyl) enzyme, and HY is an amino acceptor or water. The k_{+2}/K or k_{cat}/K_m is the second-order rate constant of enzyme acylation by the carbonyl donor. Depending on whether the carbonyl donor is a peptide, a depsipeptide, or benzylpenicillin, the leaving group of the enzyme acylation step k_{+2} is D-alanine, D-lactate, or remains part of the acyl enzyme. Moreover, depending on whether the enzyme is a DD-peptidase or a β-lactamase, the acyl enzyme is, in principle, long-lived or short-lived, respectively. The very low value of k_{+3} for the interaction of the DD-peptidase with benzylpenicillin expresses the high stability of the corresponding acyl enzyme. The *B. licheniformis* β-lactamase is devoid of peptidase activity and does not recognize the noncyclic amide or ester carbonyl donors.

The tertiary structure determinations of several active-site serine β-lactamases have been the goal of laboratories throughout the world for many years (*14*). Yet, results beyond 4 Å resolution have not been available for any of them. We report here the calculation of an electron density map at 3.5 Å resolution for the class A β-lactamase of *B. licheniformis* 749/C (29,500 daltons). The map provides a clear image of the β-lactamase folding. A novel graphics algorithm for protein mapping called GRINCH (*15*) is used to trace the path of the polypeptide chain through the elements of secondary structure. The availability of crystallographic maps for the *Streptomyces* R61 and *B. licheniformis* enzymes thus allows comparison of a DD-peptidase and β-lactamase not only in terms of functionality but also in terms of new primary and three-dimensional structures.

Representations of the three-dimensional structures of each enzyme (Fig. 2) show the spatial arrangement of the ensemble of secondary structure elements of the *B. licheniformis* β-lactamase and R61 DD-peptidase from our x-ray crystallographic analyses. The matching of the helices and strands of the β-sheet is so marked between the two structures that it implies a close relationship. Each structure contains a β-sheet composed of five strands of similar length protected on the front face by two helices and on the back face by one helix. When the β-strands of the two enzymes are overlaid on a graphics system, seven of the eight helices of the DD-peptidase structure are identified with seven helices in the β-lactamase structure. All helices in each enzyme are at similar angles and distances with respect to each other and with respect to the β-sheet. Differences, however, are expected as the molecular weights differ by 8000 daltons, and the catalytic properties are not identical (Table 1). For example, the eighth helix G of the DD-peptidase does not have a corresponding helix in the smaller β-lactamase, though the β-lactamase chain passes through the equivalent G region. Moreover, there is a well-defined helix X in the β-lactamase that lies at the top of the helical cluster and crosses the edge of the β-sheet. The DD-peptidase has one turn of the polypeptide chain in the corresponding X position. In both enzymes the active-site serine is near the NH_2-terminus [residue 44 in the β-lactamase and residue 62 in the DD-peptidase (Fig. 1)]. The spatial location of the β-lactam binding site in the DD-peptidase is shown in Fig. 2. A comparable location would be expected in the β-lactamase.

Ancestral origin is difficult or impossible to establish with certainty. Amino acid sequences change rather rapidly on the genetic time scale, and they are therefore intrinsically less informational than the more time-stable three-dimensional foldings that are maintained in accordance with external entropic and energetic limitations. The DD-peptidase and β-lactamase discussed here are from different species and lack close relatedness in their overall primary structures. Yet, they share the same basic catalytic mechanism, they recognize the same β-lactam compounds, they contain several conserved amino acids in their active sites, and they exhibit clear similarity in their three-dimensional structures. The analogous positioning of secondary structure elements, even well away from the active site, suggests that these

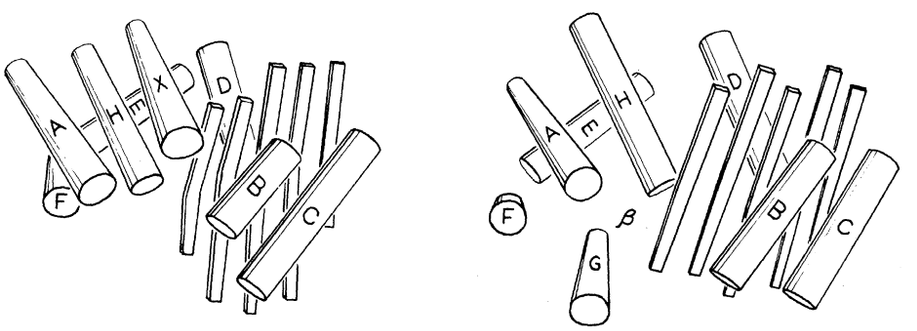

Fig. 2. Secondary structure elements in *B. licheniformis* β-lactamase (left) and *Streptomyces* R61 DD-peptidase (right). Cylinders are α-helices and ribbons are β-strands. The two drawings are the result of overlaying computer graphics plots of crystallographically determined polypeptide folding and are to the same scale. The known site of β-lactam binding in the DD-peptidase from x-ray crystallographic studies (*11*) is indicated by β. The crystallographic structure of the β-lactamase in space group $P2_1$ was determined by the isomorphous method. Results from heavy atom refinement and phasing, based on K_2PtCl_4 and $K_3UO_2F_5$ derivatives with anomalous differences, are rms ϵ (lack-of-closure)/f (heavy atom) = 0.53 and 0.59; R (on derivative F) = 0.20 and 0.13, respectively. A solvent-leveled map with a mean figure-of-merit equal to 0.76 was calculated from 5225 unique diffractometer data to 3.5 Å resolution.

enzymes may be another example of divergent evolution where structural changes required for their distinctive functions have appeared only in the relatively compact catalytic area. Deletions in the DD-peptidase sequence could have produced a smaller β-lactamase molecule, but the overall structural scaffolding of the penicillin-binding proteins has been maintained in spite of primary sequence changes. It may also be hypothesized that the primary response of soil bacteria, like *Streptomyces* spp., to exposure to β-lactam compounds produced by other microorganisms was to develop an excretion mechanism permitting release of a membrane-bound PBP. Subsequently, improvement of this mode of detoxification resulted in the transformation of this water-soluble penicillin-binding enzyme into a penicillin-hydrolyzing enzyme.

Detailed modeling of the DD-peptidase structure to 1.6 Å resolution and extension of the β-lactamase structure with x-ray diffraction data to 2.0 Å may explain why β-lactam antibiotics are potent inhibitors of the target enzymes but are rapidly hydrolyzed substrates of the defensive β-lactamases.

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Tyr^{527} Is Phosphorylated in pp60$^{c\text{-}src}$: Implications for Regulation

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The Rous sarcoma virus oncogene product, pp60$^{v\text{-}src}$, transforms cultured fibroblasts but its corresponding proto-oncogene product, pp60$^{c\text{-}src}$, does not. Both proteins are known to be protein–tyrosine kinases. Published results suggest that the kinase activity of pp60$^{c\text{-}src}$ is inhibited relative to that of pp60$^{v\text{-}src}$, due perhaps to phosphorylation of a tyrosine in pp60$^{c\text{-}src}$ that is not phosphorylated in pp60$^{v\text{-}src}$. In this study, it was observed that the tyrosine phosphorylated in pp60$^{c\text{-}src}$ is Tyr^{527}, six residues from the COOH-terminus of the protein. The region of pp60$^{c\text{-}src}$ from residue 515 to the COOH-terminus, including Tyr^{527}, has been replaced with a different sequence in pp60$^{v\text{-}src}$. Thus, the increase in transforming ability and kinase activity that occurred in the genesis of pp60$^{v\text{-}src}$ may have resulted from the loss of a tyrosine involved in negative regulation.

THE CELLULAR PROTEIN PP60$^{c\text{-}src}$ IS the normal counterpart of the retroviral oncogene product, pp60$^{v\text{-}src}$ (*1*). Fibroblasts cannot be transformed by pp60$^{c\text{-}src}$ even when it is expressed at up to 15 times the normal level (*2–4*). In contrast, expression of pp60$^{v\text{-}src}$ at an equivalent level to endogenous pp60$^{c\text{-}src}$ induces transformation (*4, 5*). Both proteins are kinases that transfer phosphate to tyrosine residues in acceptor polypeptides, but when assayed in vitro with a variety of peptide substrates the specific activity of pp60$^{c\text{-}src}$ is about 2 to 10 percent of the activity of pp60$^{v\text{-}src}$ (*6, 7*). Comparison of their sequences shows that pp60$^{c\text{-}src}$ and pp60$^{v\text{-}src}$ differ in scattered point mutations and in their extreme COOH-termini: the last 19 residues of pp60$^{c\text{-}src}$ are replaced by an unrelated sequence of 12 residues in pp60$^{v\text{-}src}$ (*8–10*). Mutagenesis of c-*src* has shown that changes in either the body or the COOH-terminus of pp60$^{c\text{-}src}$ can cause transformation (*2*). Significantly, replacement of the unique COOH-terminal tail of pp60$^{c\text{-}src}$ by the pp60$^{v\text{-}src}$ sequence or by an arbitrary sequence is sufficient for transformation (*2, 3, 11*). Thus the COOH-terminal tail, in the context of the pp60$^{c\text{-}src}$ protein, apparently suppresses the transforming ability and protein kinase activity of pp60$^{c\text{-}src}$. How this occurs is unknown.

Phosphorylation of a tyrosine in pp60$^{c\text{-}src}$ appears to be important for regulation. In the cell, both pp60^{src}'s are phosphorylated at tyrosine and serine (*1*). Although the serines phosphorylated in pp60$^{c\text{-}src}$ and pp60$^{v\text{-}src}$ are the same, the major site of tyrosine phosphorylation is different (*12, 13*). The v-*src* protein is phosphorylated at Tyr^{416}, and pp60$^{c\text{-}src}$ is phosphorylated at an unknown tyrosine. In vitro, both pp60^{src}'s can autophosphorylate, principally at Tyr^{416} (*12*) and secondarily at one or more NH_2-terminal tyrosines (*14*). Thus, another protein kinase may phosphorylate the unknown tyrosine in pp60$^{c\text{-}src}$. Two pieces of evidence suggest that this phosphorylation may be inhibitory. Firstly, spontaneous mutations in c-*src* that activate its transforming ability encode proteins that are structurally very similar to pp60$^{c\text{-}src}$ but are not phosphorylated at the c-*src*–specific tyrosine (*6*). Secondly, activation of pp60$^{c\text{-}src}$ can occur after cell lysis because of the action of phosphatases that remove phosphate from the unique tyrosine (*15*). Here we report that pp60$^{c\text{-}src}$ is phosphorylated at Tyr^{527}, which lies in the COOH-terminal sequence that appears to suppress transformation. Phosphorylation of Tyr^{527} is probably critical for the regulation of pp60$^{c\text{-}src}$ kinase activity and transforming potential.

The tyrosine phosphorylation site in pp60$^{c\text{-}src}$ is known to lie in the COOH-terminal 26,000 daltons (*12*). For more precise mapping, we analyzed tryptic and chymotryptic peptides obtained from $^{32}P_i$-labeled pp60$^{c\text{-}src}$. Trypsin-digestion of pp60$^{c\text{-}src}$ from $^{32}P_i$-labeled mouse cells that express high levels of chicken pp60$^{c\text{-}src}$

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