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Exploration of active sites of DD-peptidases

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Introduction

Penicillin, discovered more than fifty years ago, has given rise to a flourishing family of drugs whose members (penicillins, cephalosporins and monobactams) are designated collectively as β-lactam antibiotics. The multiplicity of β-lactam molecules produced by the pharmaceutical industry has been a necessity dictated by the countermoves — tolerance, intrinsic resistance and, above all, β-lactamase production — made by the bacteria to control our most effective armoury of modern antibacterial chemotherapy. Resistance to β-lactams is a progressive process which is occurring before our eyes, and the need for new antibiotics remains urgent.

Problems of drug design depend on whether precise information regarding the drug binding site is available or not. The various approaches related to both types of problem can be illustrated by well-known examples. Enkephalinase (which cleaves the Gly^3-Phe^4 bond of the morphine-like enkephalins Tyr-Gly-Gly^3-Phe^4-Met and Tyr-Gly-Gly^3-Phe^4-Leu) and angiotensin converting enzyme (which cleaves the Phe^8-His^9 bond of angiotensin I, Asp-Arg-Val-Tyr-Ile-His-Pro-Phe^8-His^9-Leu, thus generating the powerful vasoconstrictor angiotensin II) are zinc proteases of considerable pharmaceutical interest. Although their three- dimensional structure is unknown, a detailed exploration of the active sites with inhibitors has led, in each case, to a single, specific geometrical arrangement of the groups postulated for enzyme-ligand interaction and catalysis and, on this basis, to the development of drugs with antihypertensive (Cushman *et al.*, 1977) or analgesic (Roques *et al.*, 1983) effects.

Drug design, however, is best addressed with visualization of the three-dimensional geometry and perception of the molecular interactions. Serine proteases and NADPH-dependent dihydrofolate reductases (DHFRs) of various origins have been characterized by X-ray diffraction at high resolution. Newly developed interactive graphics techniques have been extremely useful for the study of suicide inhibitors bound to serine proteases and the study of methotrexate and trimethoprin bound to DHFRs. The conformation of either partner and its surface can be varied interactively in real time, docking methods permit display of the energy of interaction as the drug is moved to its relevant binding site and global transformations (scalling, rotation, translation and clipping) may be performed to optimize the views.

In this laboratory, we are concerned with defining structure activity data concerning the bacterial DD-peptidases, and elucidating the geometry, mechanistic properties and functioning of their active sites.

Model DD-peptidases

The DD-peptidases are enzymes bound to the plasma membrane of the bacteria. They are specifically involved in the synthesis and control of extent of peptide crosslinking during wall peptidoglycan metabolism. Wall expansion, however, is not uniform among bacteria and, as a corollary, each bacterial species possesses its own assortment of DD-peptidases. In addition, the multiple DD-peptidases present in a single bacterial cell catalyse distinct reactions (related to initial incorporation of the newly synthesized peptidoglycan material and to maturation and remodelling of wall peptidoglycan during the life cycle), fulfil distinct cellular functions (related to cell elongation, shape maintenance, cell septation, etc.), seem not to exhibit the same degree of 'essentiality' and show widely varying sensitivity to β-lactams (Spratt, 1983; Waxman & Strominger, 1983). Bacterial DD-peptidases constitute a very large group of enzymes which differ from each other in their physical, molecular, mechanistic and enzymatic properties.

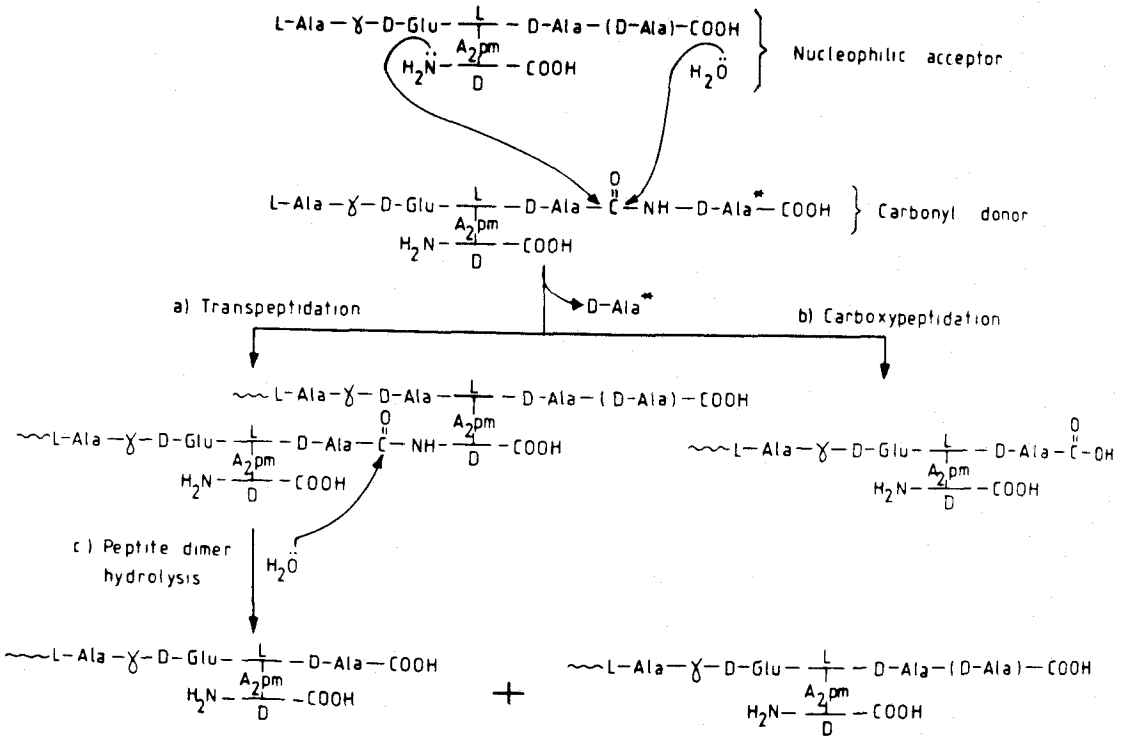

Figure 1 Reactions catalysed by DD-peptidases in *Escherichia coli*.

In spite of this diversity, the bacterial DD-peptidases catalyse essentially three types of reaction: transpeptidation (Figure 1a), carboxypeptidation (Figure 1b) and peptide dimer (oligomer) hydrolysis (Figure 1c), and have in common a unique optical specificity: the peptide bonds that they attack in the peptide carbonyl donors extend between two D centres, in α position to a free carboxylate. On this basis, DD-peptidases have been isolated which cleave with high efficiency, the D-Ala-D-Ala linkage in the synthetic tripeptide Ac_2-L-Lys-D-Ala-D-Ala, used as carbonyl donor analogue. With each of these DD-peptidases, initial recognition, binding energy, proper alignment of the scissile bond with regard to the enzyme active functional groups and catalytic efficiency (Figure 2) rely on charge pairing between an enzyme cationic group and the carboxylate of the substrate, and on the complementation of at least three enzyme subsites S_2, S_1 and S_1', by the corresponding lateral chains P_2, P_1 and P_1' (with the indicated configuration) of the carbonyl donor. In each case, enzyme activity: (i) strictly requires a D-Ala at position P_1; (ii) is decreased but not abolished when Gly or a D-amino acid other than D-Ala is at position P_1' (L-Ala, at this position, abolishes substrate activity); and (iii) requires a long side chain on the L-residue at position P_2.

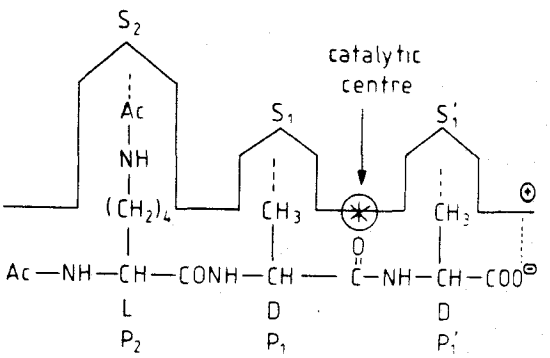

Figure 2 Positioning of the amide carbonyl donor Ac_2-L-Lys-D-Ala-D-Ala in the enzyme cavity of the DD-peptidases.

Four DD-peptidases of similar specificity profile for carbonyl donors have been selected for detailed study. They differ from each other with respect to their responses to β-lactam action, from extreme sensitivity (the R39 enzyme) to extreme resistance (the G enzyme), and with respect to the effects that the presence of amino compounds in the reaction mixture exert on the fate and rate of the consumption of

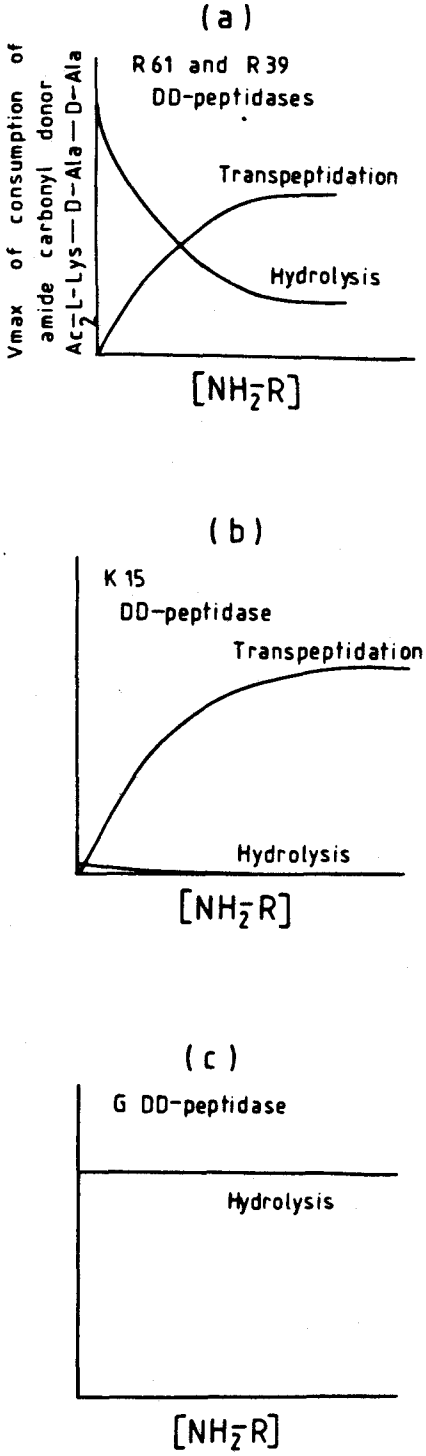

Figure 3 Effect of amino compounds (NH_2-R) on the consumption of the amide carbonyl donor Ac_2-L-Lys-D-Ala-D-Ala by the R61, R39, K15 and G DD-peptidases.

the carbonyl donor Ac_2-L-Lys-D-Ala-D-Ala (Figure 3). The R61 and R39 DD-peptidases catalyze concomitant hydrolysis and transpeptidation (Figure 3a), the K15 DD-peptidase functions as a strict transpeptidase (Figure 3b) and the G DD-peptidase functions as a strict hydrolase (Figure 3c). For recent reviews on these DD-peptidases, the reader is referred to Ghuysen *et al.* (1979, 1981, 1984) and Charlier *et al.* (1983).

The R61 and R39 DD-peptidases. Catalysed transfer reactions with the amide (ester) carbonyl donor Ac_2-L-Lys-D-Ala-D-Ala (Ac_2-L-Lys-D-Ala-D-Lac)

The R61 and R39 DD-peptidases catalyze transfer of the electrophilic group Ac_2-L-Lys-D-alanyl from the amide carbonyl donor Ac_2-L-Lys-D-Ala-D-Ala (and ester carbonyl donor Ac_2-L-Lys-D-Ala-D-lactate) via the transitory formation of an acyl enzyme. Strong experimental evidence shows that an active serine is involved in the process.

Giving E = DD-peptidase; C = carbonyl donor; E-C* = serine ester linked acyl enzyme (Ac_2-L-Lys-D-alanyl-E); P_1 = leaving group (D-Ala or D-lactate); HY = nucleophile; and P_2 = reaction product Ac_2-L-Lys-D-Ala-CO-Y, the reaction catalyzed is

$$E + C \overset{K}{\rightleftharpoons} E{\cdot}C \xrightarrow{k_{+2}} E\text{-}C^* \xrightarrow[HY]{k_{+3}} E + P_2 \quad (1)$$

where K = dissociation constant and k_{+2} and k_{+3} = first order rate constants.

When the reaction involves the ester carbonyl donor Ac_2-L-Lys-D-Ala-lactate and water as the only nucleophile,

$$Ac_2\text{-L-Lys-D-Ala-D-Lac} + H_2O \rightarrow Ac_2\text{-L-Lys-D-Ala-} + \text{D-Lac} \quad (2)$$

the acyl enzyme accumulates detectably at the steady state of the reaction and can be trapped and characterized.

Replacement of the ester donor substrate by the corresponding amide donor Ac_2-L-Lys-D-Ala-D-Ala

$$Ac_2\text{-L-Lys-D-Ala-D-Ala} + H_2O \rightarrow Ac_2\text{-L-Lys-D-Ala} + \text{D-Ala} \quad (3)$$

has, of course, no effect on the rate of enzyme deacylation k_{+3} (since the reaction proceeds through formation of the same acyl enzyme) but results in a decreased rate of enzyme acylation (k_{+2}). Under these conditions ($k_{+2} << k_{+3}$), $K_M = K$, $k_{cat} = k_{+2}$ and no acyl enzyme accumulates detectably. Nevertheless, addition of a suitable amino acceptor NH_2-R to the aqueous reaction mixture causes partitioning of the enzyme activity between the two nucleophiles (H_2O and NH_2-R). Hydrolysis and transpeptidation then occur simultaneously on a competitive basis

$$\begin{array}{l} Ac_2\text{-L-Lys-D-Ala-D-Ala} \\ H_2O \rightarrow Ac_2\text{-L-Lys-D-Ala} \\ \qquad\quad \rightarrow \text{D-Ala} \\ NH_2\text{-R} \rightarrow Ac_2\text{-L-Lys-D-Ala-CO-NH-R} \end{array} \quad (4)$$

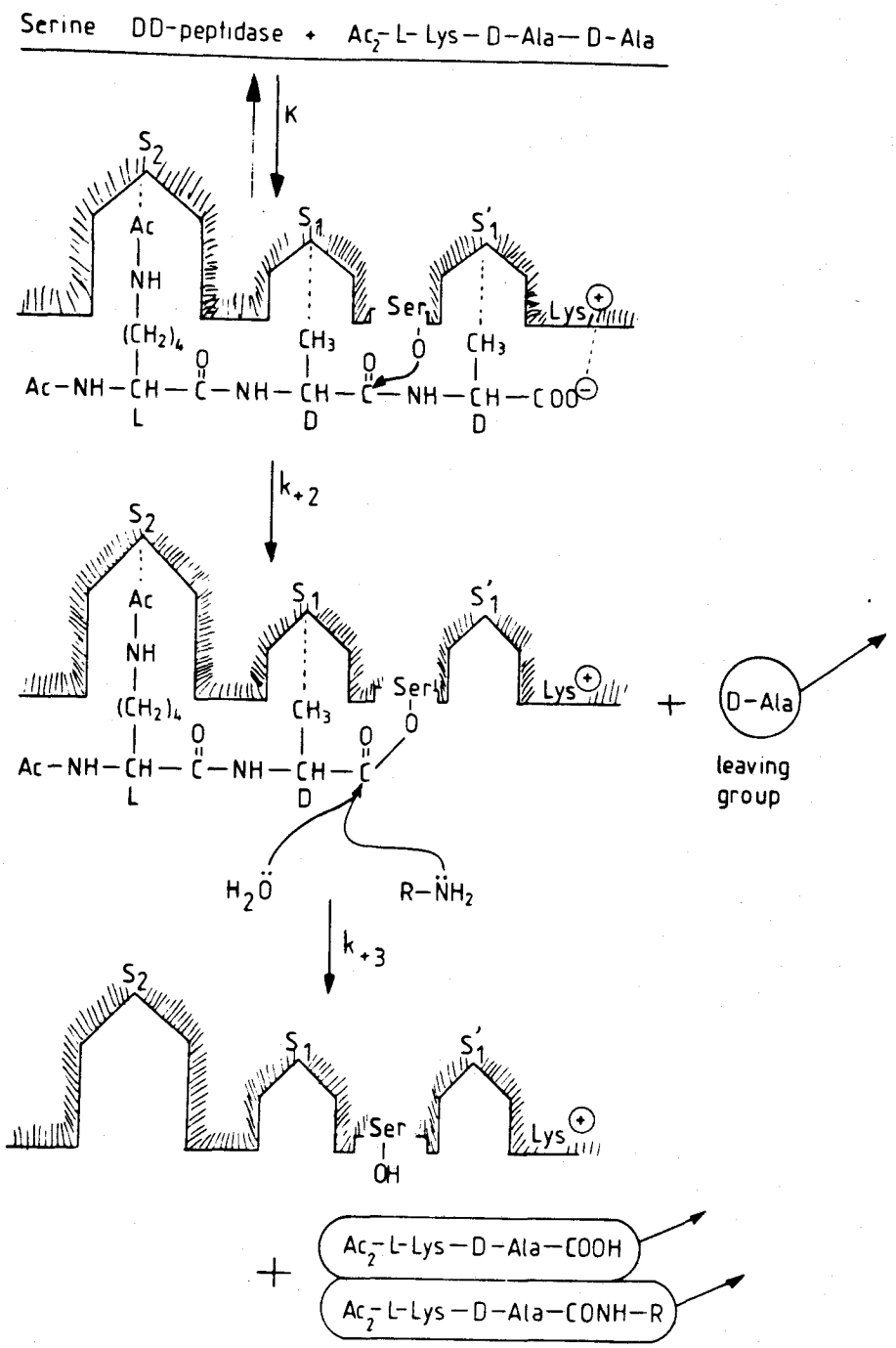

Figure 4 Covalent catalysis by the R61 and R39 serine DD-peptidases

but, under no circumstances, does the amino acceptor increase the maximal rate of carbonyl donor consumption (or the rate of enzyme acylation since $k_{cat} = k_{+2}$). It thus follows that the amino acceptor behaves as an alternate nucleophile that exerts its effect only at the level of the acyl enzyme. In this respect, mechanistically (Figure 4), the R61 and R39 DD-peptidases can be regarded as the counterparts of the mammalian serine proteases. However, they possess highly structured amino acceptor sites with distinct specificity profiles and these differences reflect distinct structural features in the wall peptidoglycans of the relevant producing strains (*Streptomyces* R61 and *Actinomadura* R39).

The K15 DD-peptidase. Catalysed transfer reactions with the amide (ester) carbonyl donor Ac_2-L-Lys-D-Ala-D-Ala (Ac_2-L-Lys-D-Ala-D-Lac)

The K15 DD-peptidase (from *Streptomyces* K15) also performs covalent catalysis (whether or not, an active serine is involved in the transitory formation of the acyl enzyme is not yet known). But catalysis is peculiar (Nguyen-Distèche *et al.*, 1982; and unpublished data).

When water is the only nucleophile present, acyl enzyme accumulation occurs with both ester and amide carbonyl donors. From measurements of the levels of accumulated acyl enzyme for varying concentrations of each of the carbonyl donors and on the basis of the k_{cat} value for the ester substrate (from Lineweaver-Burk plots 1/v *versus* 1/[Ac_2-L-Lys-D-Ala-D-Lac]), the constants K, k_{+2} and k_{+3} involved in the reactions have been estimated. Although these values express high catalytic efficiency on Ac_2-L-Lys-D-Ala-D-Lac and, to a lesser extent, on Ac_2-L-Lys-D-Ala-D-Ala, actual hydrolysis proceeds as expected only with the ester carbonyl donor. Hydrolysis of the amide carbonyl donor is, in fact, negligible. The reason for this surprising behaviour is that the enzyme-catalysed hydrolysis of the ester donor is not affected by the endogenously released D-lactate whereas D-Ala, as it is released from the amide donor, is effectively reutilized by the enzyme in a transpeptidation reaction.

$$Ac_2\text{-L-Lys-D-Ala-D-Ala} + \text{D-Ala} \rightarrow Ac_2\text{-L-Lys-D-Ala-D-Ala} + \text{D-Ala} \quad (5)$$

which maintains the concentration of the carbonyl donor at a constant level.

Effective consumption of the amide carbonyl donor can be achieved by supplementing the reaction mixture with a suitable amino compound structurally related to wall peptidoglycan and able to successfully compete with the released D-Ala. Under these conditions, the electrophilic group Ac_2-L-Lys-D-alanyl is channelled and transferred to the exogenous amino acceptor, the acyl enzyme no longer accumulates at the steady state, the DD-peptidase functions as a strict transpeptidase and the kinetic parameters can be estimated on the basis of the general initial rate equation for an enzyme-catalysed bimolecular reaction ($S_1 + S_2 \rightleftarrows S_1' + S_2'$). These determinations show that the amino acceptor acts not only as an alternate nucleophile at the level of the acyl enzyme but, in addition, enhances the rate of enzyme acylation (k_{+2}) by the amide carbonyl donor. Basically, the mechanism of covalent catalysis by the K15 DD-peptidase may be similar to that of the R61 and R39 DD-peptidases. But, the observed effects caused by the amino acceptor and the capability of the K15 DD-peptidase of preventing the amide carbonyl bound to the enzyme donor site from being hydrolysed are unique features. In these respects, the K15 DD-peptidase appears to be without equivalent among the known (serine or thiol) proteases.

The R61, R39 and K15 DD-peptidases. Catalysed transfer reactions with β-lactam amide carbonyl donors

β-Lactams are suicide inhibitors of the R61, R39 and K15 DD-peptidases. The β-lactams are recognized and taken up as carbonyl donors, and processed according to reaction (1). However, because of the endocyclic nature of the scissile bond, the 'leaving group' produced during enzyme acylation (k_{+2}) does not leave the enzyme site which therefore remains occupied. As a consequence, the reaction flux stops, at least for some time, at the level of the acyl enzyme which thus shows high stability (low k_{+3}) (Figure 5).

Amino compounds, known to have high acceptor activities in transfer reactions involving the amide (ester) carbonyl donors Ac_2-L-Lys-D-Ala-D-Ala (D-Lac), do not labilize the acyl enzyme formed with β-lactams, suggesting that the amino acceptor site in the enzyme cavity is blocked by the bound penicilloyl (cephalosporoyl, etc.) moiety.

Trapping of the DD-peptidases by β-lactams can be overcome either by a slow transfer of the acyl moiety to water (in which case, the DD-peptidase functions as a β-lactamase of very weak efficiency) or by the slow rupture of the C_5–C_6 linkage (using the nomenclature of penicillins) of the bound penicilloyl moiety. As a result of this latter process, the 'leaving' group (previously generated during enzyme acylation) can now leave the enzyme site while the newly formed acyl enzyme (phenylacetylglycyl enzyme in the case of benzylpenicillin) is immediately susceptible to attack by water and, if present, by a suitable amino compound.

Potency of a β-lactam as a DD-peptidase inactivator, is best expressed by the values of the constants which govern the interaction. The higher the bimolecular rate constant of enzyme acylation (k_{+2}/K) and the smaller the rate of constant of enzyme deacylation (k_{+3}), the more potent is the β-lactam. The inactivating efficacy of the β-lactams is enzyme

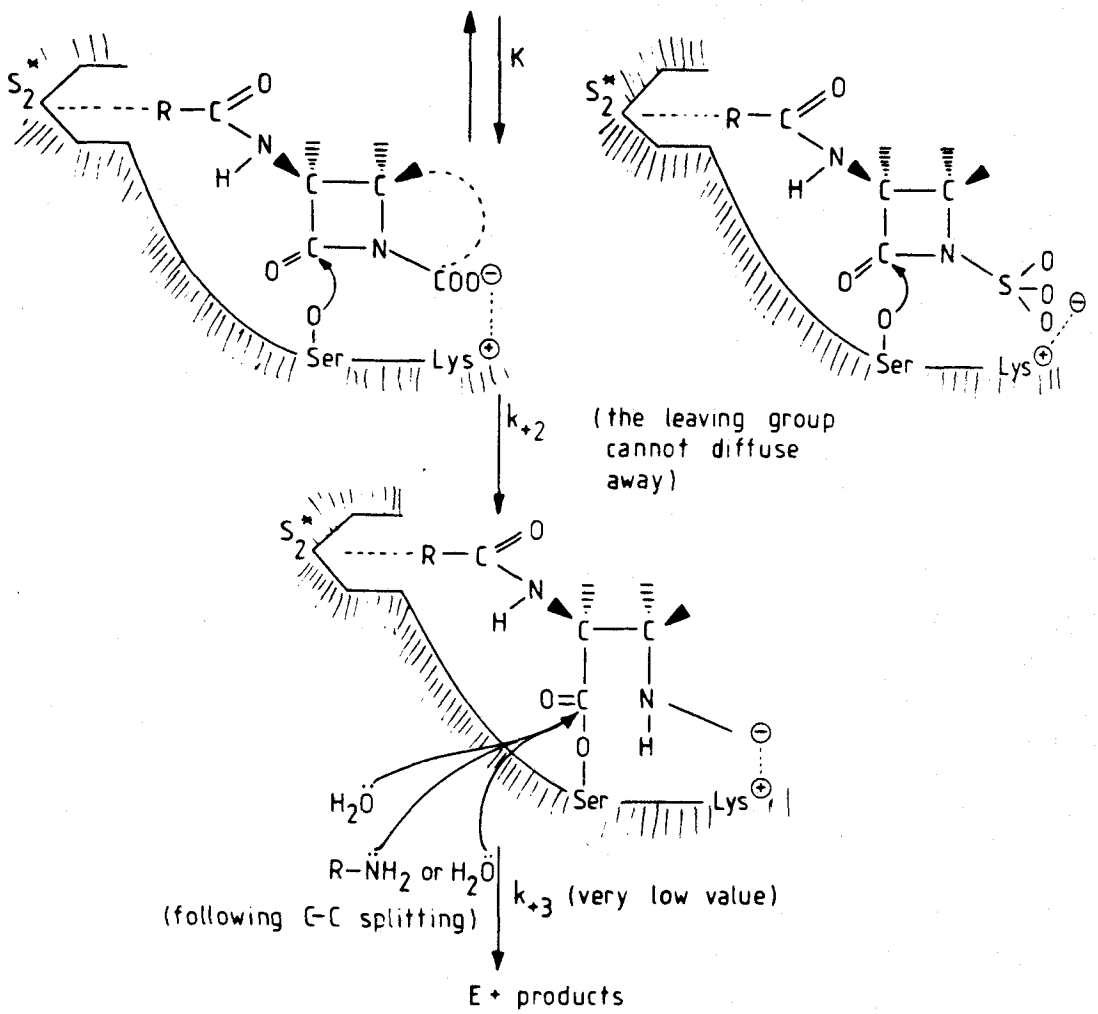

Figure 5 Inactivation of the R61 and R39 serine DD-peptidases by β-lactams. The amino acid sequence around the active serine are Val-Gly-Ser-Val-Thr-Lys in the R61 DD-peptidases and Leu-Pro-Ala-Ser-Asn-Gly-Val in the R39 DD-peptidase.

specific. Thus with benzylpenicillin, the k_{+2}/K values are 150, 13,000 and 300,000 $M^{-1}s^{-1}$ (and the k_{+3} values are 1×10^{-4}, 1.4×10^{-4} and $3 \times 10^{-6} s^{-1}$) for the K15, R61 and R39 DD-peptidases, respectively. In addition, the efficacy of enzyme acylation may be extremely dependent on the structure of the β-lactam. Thus, with the R39 DD-peptidase, the k_{+2}/K values range from 200 $M^{-1}s^{-1}$ (7-ACA) to 2,600,000 $M^{-1}s^{-1}$ (nitrocefin). Large series of β-lactams (penams, Δ^2-cephems, Δ^3-cephems, cephamycins, penems, carbapenems and monobactams) have been examined. For more details, the reader is referred to Ghuysen *et al.* (1984).

The R61 DD-peptidase has been crystallized and its three-dimensional structure provisionally established (Kelly *et al.*, 1982). Using the GRINCH system, developed by M. Pique and Chapel Hill, North Carolina, which combines Carroll Johnson's ridge line representations of electron density maps with appropriate interactive tools, J. Kelly from Connecticut University has been able to trace the folding of the main polypeptide chain and to place provisionally side chain atoms (Kelly, 1983). Although not yet completed, this work demonstrates that the R61 DD-peptidase has no three-dimensional relatedness with any known serine protease. Diffusion of a cephalosporin and a desazapenicillin in the protein crystal has permitted visualization of the active site with the hydroxyl group of a serine residue pointing towards the α face of the β-lactam molecule. Fourier synthesis

of the complex formed with di-isopropylfluorophosphonate (an active site directed reagent of serine proteases) shows only one peak located in the enzyme active site (J. Kelly, personal communication).

The R61, R39 and K15 DD-peptidases. Geometrical arrangement of groups postulated for enzyme ligand association during interaction with peptide (ester) and β-lactam carbonyl donors

The D-Ala-D-Ala peptide bond (or D-Ala-D-Lac ester bond) and the β-lactam amide bond are predisposed to attack by the same enzyme active groups, that is, are functionally equivalent (Figures 4 and 5). Hence, contrary to a long belief professed by chemists, non-planarity of the nitrogen atom of the β-lactam ring in the bicyclic β-lactams, which causes 'suppressed amide resonance' and 'increased intrinsic reactivity', is not the essential feature which governs the efficacy of enzyme acylation (and hence enzyme inactivation) by the antibiotics. The discovery of active monobactams where all of the atoms attached to the β-lactam linkage are essentially coplanar, has eventually settled the problem.

In both types of carbonyl donors (peptide substrates and β-lactam inactivators), the scissile CO-N link is flanked on one side by a C-terminal carboxylate (or SO_3^- group in monobactams) and on the other by another CO-N amide bond. Examination of the most probable conformers of a series of peptides (which altogether cover 90% of the whole conformational space) or varying substrate activity and of a series of β-lactams (crystal structures, Cambridge Data Bank) of varying inactivating potency shows that the geometrical arrangement (spanning distance and relative orientation) of these three functional groups does not differ drastically (Lamotte-Brasseur *et al.*, 1984). However, the patterns of the molecular electrostatic potentials that they generate around the molecules largely vary depending on the conformation of the peptide backbone, the presence and structure of the side chains, the type of bicyclic or monocyclic framework in the β-lactams and the presence of ionized or electron-withdrawing substituents. Since the strength and spatial disposition of these positive and negative electrostatic potentials must be important for initial recognition, orientation and reactivity of the whole molecules in the enzyme active sites, the observed variations suggest enzyme-ligand association of widely varying complementarity and productiveness.

The significance of this conclusion is also well illustrated by the fact that the lateral chain at the L-centre of the L-R-D-Ala-D-Ala terminated peptides and the acyl substituent of (most) β-lactams largely dictate the efficacy of enzyme acylation (k_{+2}). Effective side chains are DD-peptidase specific and — for a given DD-peptidase — may have quite different structures depending on the carbonyl donor (peptide, penam, cephem, cephamycin, carbapenem, monobactam, etc.) which is to be bio-activated. Hence, multiple productive modes of binding with the active site (serine) residue and alternative binding modes for the transition state and acyl intermediates probably occur depending on both the carbonyl donors and the DD-peptidases involved in the interactions.

The G DD-peptidase. Catalysed transfer reactions with the amide carbonyl donor Ac_2-L-Lys-D-Ala-D-Ala. Intrinsic resistance to β-lactams.

In contrast to the R61, R39 and K15 DD-peptidases, the G DD-peptidase (from *Streptomyces albus* G) performs liganding catalysis through an essential Zn^{++} cofactor and exhibits high intrinsic resistance to all β-lactams. The Zn^{++} DD-peptidase utilizes only H_2O as acceptor of the transfer reaction and hence is unable to catalyse transpeptidation. This Zn^{++} DD-peptidase is a strict hydrolase. It has no amino acid sequence (Joris *et al.*, 1983) nor three-dimensional relatedness (Dideberg *et al.*, 1983) with any known metalloprotease but, mechanistically, it may be regarded as the counterpart of the Zn^{++} proteases, thermolysine and carboxypeptidase A.

Important catalytic groups for the hydrolysis of the D-Ala-D-Ala amide bond have been provisionally assigned (Charlier *et al.*, 1983) to the guanidinium side chain of Arg^{136} involved in charge pairing, the Zn^{++} ion acting as electrophile (and fixed in the enzyme cavity by the three protein ligands His^{112}, His^{193} and His^{196}), the imidazole ring of His^{190} involved in proton donation and the hydroxyl group of Ser^{151}, possibly acting as orientor or enhancing the nucleophilicity of a water molecule bound to the Zn^{++} ion. Productive binding of the amide carbonyl donor Ac_2-L-Lys-D-Ala-D-Ala to the enzyme cavity implies that the C-terminal carboxylate interacts with Arg^{136}, the carbonyl oxygen of the scissile bond is oriented towards the Zn^{++} ion and the nitrogen atom towards His^{190}. When the peptide is thus aligned, then the three side chains P_2, P_1 and P_1', in their respective L, D and D configuration, find their place in the enzyme subsites S_2, S_1 and S_1' (Charlier *et al.*, 1983).

In the conformation thus imposed by the geometry of the active site, the relative orientation of the C-terminal carboxylate and the central amide carbonyl falls well outside the limit values found for the corresponding functional groups in the β-lactams. This situation is consistent with the fact that β-lactams bound to the Zn^{++} DD-peptidase are neither substrates nor effective inactivators but, essentially, behave as reversible inhibitors of poor efficacy. Thus cephalosphorin C and cephalothin inhibit the Zn^{++} DD-peptidase non competitively, cause disruption of the protein crystal lattice and induce large

conformational changes, and most likely, aggregation of the enzyme molecules in solution (Labischinski *et al.*, 1984). In contrast, *p*-iodo-7-β-phenylacetamido cephalosporinate and 6-β-iodopenicillanate are competitive inhibitors; they give rise to isomorphous crystal enzyme derivatives and bind to the active site. But binding is unproductive (very low substrate activity) because the geometry of the active site makes it difficult for the oxygen atom of the β-lactam amide bond to align with the Zn^{++} ion and, simultaneously, for the C-terminal carboxylate to align with Arg^{136}. Moreover, enzyme inactivation does not occur (unless high β-lactam concentrations and prolonged incubations are used) because chances for the bound molecule to acylate a residue in the enzyme cavity are low. 6-β-Iodopenicillanate has been shown to bind just in front of the Zn^{++} ion, superimposing the proton donor His^{190}. Alkylation then probably occurs with concomitant loss of the iodine from the inactivator (limit value of the first order rate constant: $7 \times 10^{-4} s^{-1}$) (Charlier *et al.*, 1984).

In analogy with work carried out with the enkephalinase and the angiotensin converting enzyme, bifunctional compounds possessing both a C-terminal carboxylate function and, at the other end of the molecule, a thiol, hydroxamate or carboxylate function inactivate the Zn^{++} DD-peptidase. β-Mercaptopropionate (racemic) and β-mercaptoisobutyrate (L-isomer) have high potency with K_i values of 5-10 $\times 10^{-9}$ M (Charlier *et al.*, 1984).

Conclusions

Bacterial DD-peptidases constitute a very large family of enzymes which fall into structurally and mechanistically distinct classes and, most likely, have a polyphyletic origin. The four DD-peptidases studied in this laboratory are obtained in pure state and with their enzymatic activities fully preserved. They well illustrate the diversity found in the DD-peptidases family concerning the underlying (covalent and liganding) catalytic mechanisms, the types of reactions catalyzed on peptide carbonyl donors (strict transpeptidation, concomitant transpeptidation and carboxypeptidation and strict carboxypeptidation) and the varying responses to β-lactam inactivators (from extreme sensitivity to extreme resistance). Elucidation of the three-dimensional structure of the R61 serine DD-transpeptidase/carboxypeptidase and the G Zn^{++} DD-carboxypeptidase is well in progress. Before long, display programs for fitting ligands with active sites and performing interactive energy estimations might help for the design of potent and selective DD-peptidase inactivators with or without β-lactam nuclei.

The β-lactamases — which are the main lines of defense developed by the bacteria against β-lactam action — also constitute a very large group of enzymes. The only known representative of class B is a metallo (Zn^{++}) β-lactamase, the counterpart of the G Zn^{++} DD-peptidase. These two enzymes, however, have no amino acid sequence homology (Joris *et al.*, 1983 and R.P. Ambler, personal communication). β-Lactamases of classes A and C, although structurally unrelated to each other, operate by covalent catalysis according to reaction (1), possess an active serine residue, show transacylation activity (at least with suitable ester carbonyl donors such as phenylacetylglycyl-D-mandelate) (Pratt & Govardhan, 1984), and thus may be regarded as the counterparts of the R61 and R39 serine DD-peptidases. Serine DD-peptidases and serine β-lactamases, however, distinguish themselves by the highly stable and short-lived acyl enzyme intermediates, respectively, that they form with β-lactams. Several β-lactamases have been crystallized and are under investigation by X-ray diffraction. Unfortunately, establishment of their detailed three-dimensional structure seems to be a challenge.

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