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# THE ACTIVE SITES OF THE D-ALANYL-D-ALANINE-CLEAVING PEPTIDASES

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## Introduction

Thousands of β-lactam compounds have been isolated or made, characterized and tested as antibacterial agents. Those few which have found a place in medicine came primarily from the exploitation of large screening programs and chance observations. "The reason for this has been a lack of knowledge of the three-dimensional active sites of the various enzymes with which β-lactam antibiotics react" (Abraham, 1981). This, of course, applies to β-lactamases and D-alanyl-D-alanine-cleaving peptidases (in short DD-peptidases).

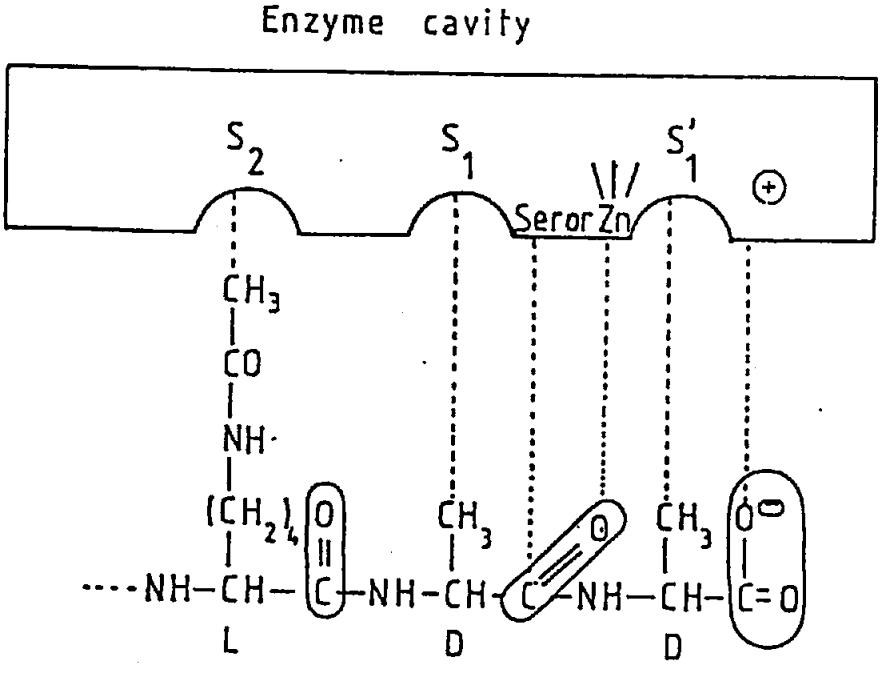
Despite progress in different areas of structural research, crystallography is still the only way to determine the spatial structure of proteins (enzymes) to atomic resolution. The DD-peptidases are membrane-bound and membrane proteins are inherently difficult to obtain in a suitable crystalline form. In cases where the DD-peptidases are merely anchored to the membrane by a hydrophobic protein tail, one strategy may be to use proteases that split the enzymes into a hydrophilic and a hydrophobic part. The hydrophilic part (with, hopefully, the intact active site) can then be submitted to crystallization like a water-soluble globular protein. Another strategy makes use of the fact that some bacteria, especially strains of

*Streptomyces* and *Actinomadura*, excrete water-soluble DD-peptidases during growth. The G, R61 and R39 DD-peptidases have been isolated from culture filtrates. The G and R61 DD-peptidases have been crystallized. A fourth model DD-peptidase (the K15 enzyme) is membrane-bound. It has been purified almost to protein homogeneity with the help of the detergent N-cetyl-NNN-trimethylammonium bromide. Altogether, these four model enzymes well represent the many variations found among the bacterial DD-peptidases.

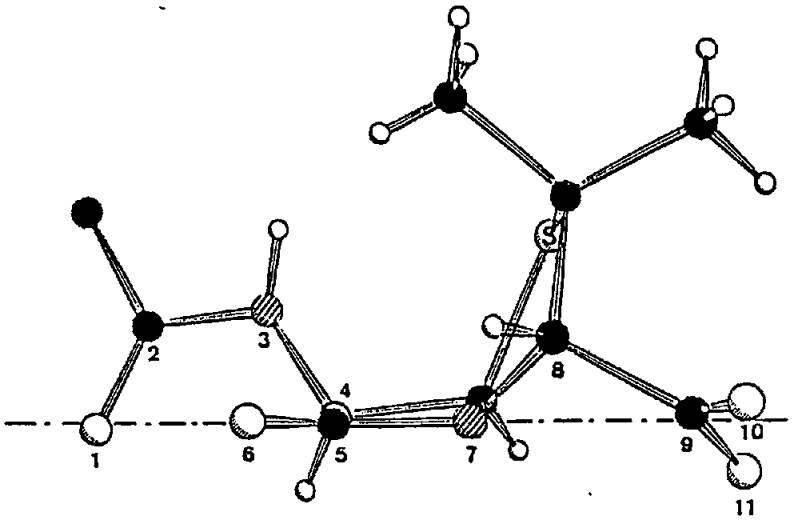
Enzymes are invariably able to distinguish between isomers. Like any peptidase, the DD-peptidases catalyse transfer of the electrophilic group $R-\overset{O}{\overset{\|}{C}}$ of aminoacyl amide ($R-\overset{O}{\overset{\|}{C}}-NH-R'$) or aminoacyl ester ($R-\overset{O}{\overset{\|}{C}}-O-R'$) substrates to a nucleophile HY. But the DD-peptidases are unique in that the scissile amide (ester) bond extends between two D centres and is in α position to a free carboxylate. These DD-peptidases also cleave the endocyclic amide bond of penicillins, cephalosporins, monobactams and other related compounds. However, the reaction flux stops at an abortive level, immobilizing the enzymes in an inactive form. β-Lactams are active site-directed inactivating reagents (or suicide substrates) of the DD-peptidases, but the inactivating efficacy very widely varies depending on the enzymes and the β-lactams. Low sensitivity of a DD-peptidase to β-lactam action is called intrinsic resistance.

Peptidase-catalysed cleavage of peptide (ester) $\overset{O}{\overset{\|}{C}}-N(O)$ bonds requires the concerted action of i) an electrophile (anion hole) which polarizes the bond C=O ; ii) a nucleophile which performs attack of the carbon atom; and iii) a proton donor which achieves proton donation to the nitrogen (oxygen) atom. These events can be achieved by different mechanisms. One metallo ($Zn^{++}$) and several serine DD-peptidases are known.

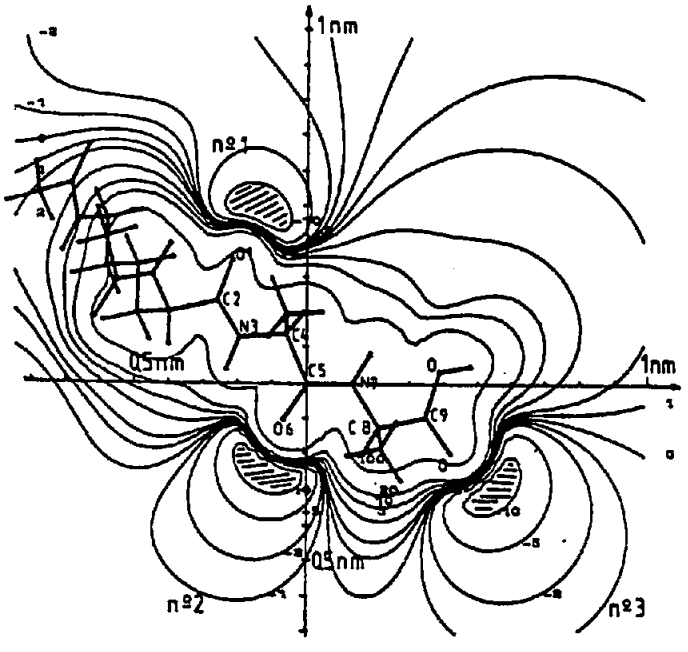
Initial Binding of Carbonyl Donor Substrates and β-Lactams to the DD-Peptidase Active Sites




Initial recognition of the carbonyl donor substrate by a DD-peptidase, binding energy and proper alignment of the scissile bond with regard to the enzyme catalytically active functional groups rely on the complementation of at least three enzyme binding subsites. Subsites $S_1'$ and $S_1$ accommodate the two methyl groups in the D configuration that occur on both sides of the scissile bond. In addition, subsite $S_1'$ contains a cationic side chain involved in charge pairing with the C-terminal carboxylate of the substrate. Subsite $S_2$ accommodates a long side chain that protrudes from an L centre of the substrate backbone. Experimental evidence suggests that complementation of $S_2$ may not be essential for initial binding but is crucial for subsequent catalysis. Subsite $S_2$ is highly species-specific, a property which relates to the many structural variations found at the level of the L centre among the bacterial peptidoglycans.




Model of penicillin, $C_3$ puckered conformer (BZPENK, Cambridge Data bank)
In this molecule, atoms $O_1$, $O_6$, $C_5$, $N_7$, $C_9$ and $O_{10}$ of the backbone $\overset{\overset{O_1}{\|}}{C_2}-N_3-C_4-\overset{\overset{O_6}{\|}}{C_5}-N_7-C_8-\overset{\overset{O_{10}\ominus}{\|}}{C_9}-O_{11}$ are virtually coplanar (α face).




Potential electrostatic map of $Ac_2$-L-Lys-D-Ala-D-Ala.
The map shows contour of equipotential values and localized electrostatic potential wells (marks 1, 2 and 3). A well gives the energy charge, in Kcal/mol, that would occur if a proton was placed at this position. The pattern refers to a section made through the scissile bond $\overset{\overset{O}{\|}}{C}-N$ .

For more information, see Lamotte-Brasseur *et al.* (1983).

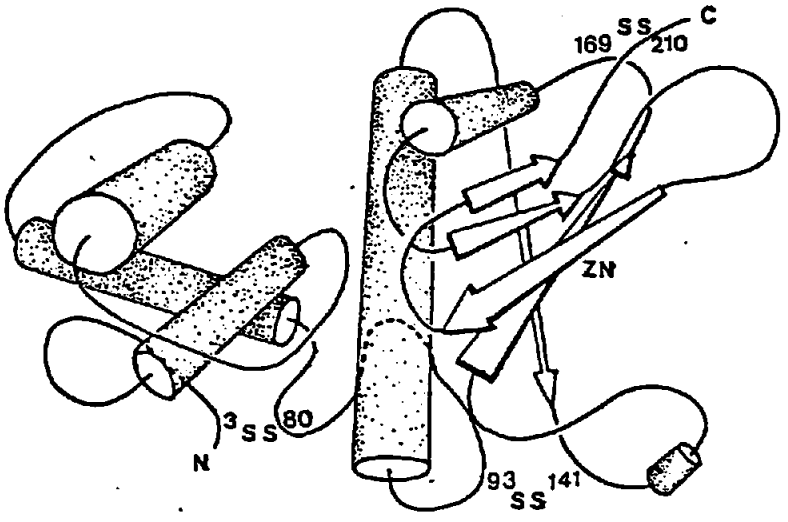
D-Ala-D-Ala terminated peptides and β-lactams lack isosterism at least when the same sequences of atoms are compared. Yet, the D-Ala-D-Ala peptide bond in the carbonyl donor substrates and the endocyclic amide bond in the β-lactam inactivators are functionally equivalent in that they are predisposed to attack by the enzyme active site functional groups.

Looking at models, one sees the scissile $\overset{\overset{O}{\|}}{C}-N$ bond at a central position, flanked on one side by the C-terminal carboxylate and on the other, by another $\overset{\overset{O}{\|}}{C}-N$ amide bond. These three functional groups are well exposed on the α face of the molecules. They create, around the common backbone $\overset{\overset{O}{\|}}{C}-N-C-\overset{\overset{O}{\|}}{C}-N-C-\overset{\overset{O}{\|}}{C}-OH$, zones of positive and negative electrostatic potentials whose relative spatial disposition and strength must be important for the reactivity and orientation of the whole molecule within the enzyme active site. Examination of a set of peptide and β-lactam conformers shows common reactive properties around the carbonyl of the scissile bond. But, depending on the conformation of the peptide backbone, the presence of bulky side chains, the type of bicyclic framework in the β-lactams, the presence of ionized or electron-withdrawing substituents, etc., there are important variations in the electrostatic environments of the molecules. These variations suggest enzyme-ligand associations of widely varying complementarity and productiveness (whether the ligand is a carbonyl donor peptide or β-lactam).

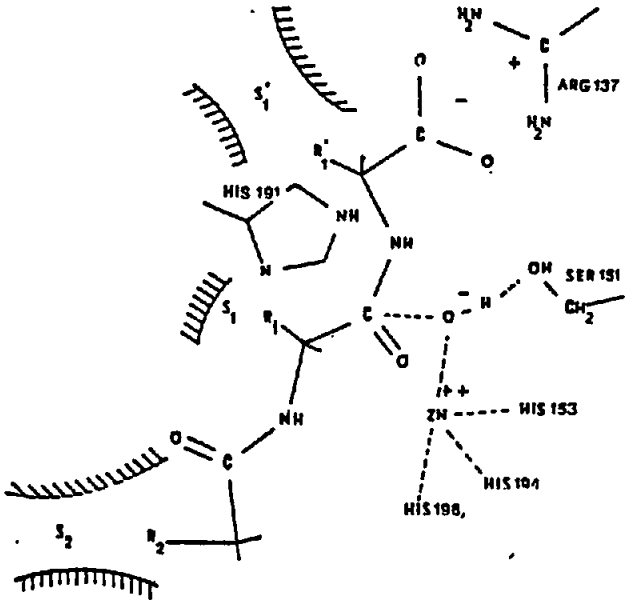
## Liganding Catalysis by the G DD-Carboxypeptidase

### 1. Geometry of the active site

The G DD-peptidase is a metallo ($Zn^{++}$) enzyme. Its chemical make-up has been elucidated. The molecule consists of one single polypeptide of 212 amino acid residues (or 213 if a Trp not detected in the sequence occurs between $Arg^{52}$ and $Phe^{53}$ as seen by X-ray diffraction analysis) with internal crosslinks




Schematic drawing of the model of the G DD-peptidase (from *Streptomyces albus* G) (Dideberg *et al.*, 1982).
The numbering is that proposed by Joris *et al.* (1983).




Schematic drawing of the active site of the G DD-peptidase showing the relative disposition of the bound peptide substrate and the enzyme functional groups.

occurring in three places through S-S bridges. Its three-dimensional structure has been determined at 1.8 Å resolution, thus permitting comparison with the well-known zinc-containing thermolysin and carboxypeptidase A.

Thermolysin, carboxypeptidase A and the G DD-peptidase show complete lack of amino acid sequence homology and overall structural relatedness. Carboxypeptidase A is a one-domain protein of the α/β type structure; its dominant feature is one eight-stranded wall of β structure which constitutes the core of the molecule; its active site is a shallow depression on the surface of the domain. Thermolysin has two domains of the type α+β and all β, respectively; an α-helix runs through the centre of the molecule and the active site is located in the cleft at the junction between the two domains. The G DD-peptidase has two globular domains connected by a single link. The small N-terminal domain has three helices. The large C-terminal domain is of the α/β type structure and has three helices (the longest one being at the junction between the two domains) and five β strands. Remarkably, the enzyme active site (containing $Zn^{++}$) is located in the C-terminal domain; it appears as an open cleft, one side of which is a mixed sheet formed by the five β-strands; it has been identified as the binding site of the dipeptide Ac-D-Ala-D-Glu (a competitive inhibitor of the G DD-peptidase).

Models show that accommodation of the tripeptide substrate $Ac_2$-L-Lys-D-Ala-D-Ala in the enzyme active site leads to a close interaction between the C-terminal carboxylate and the guanidinium side chain of $Arg^{137}$ involved in charge pairing and, simultaneously, to a positioning of the scissile $\overset{O}{\overset{\|}{C}}$-N bond such that the oxygen atom is oriented toward the zinc atom while the nitrogen atom is oriented toward the imidazole ring of $His^{191}$ (apparently ready to donate a proton).

When the tripeptide is thus aligned, then the methyl group of the C-terminal D-Ala points to a large subsite $S_1'$. This

subsite can accommodate side chains much larger than a methyl group, a property which is compatible with the peptidoglycan hydrolase activity of the G DD-peptidase. Changing the D configuration of the methyl group to an L configuration, however, suppresses all possible interaction with the enzyme cavity.

The methyl group of the penultimate D-alanine finds its place in a subsite $S_1$ of small size. Large side chains cannot be accommodated and a methyl group with an L configuration is excluded since it would collide with the zinc ligands and other residues of the active site. This property well explains the strict requirement shown by this DD-peptidase for the occurrence of a D-alanine at this position.

Finally, the side chain at the L centre of the carbonyl donor peptide complements subsite $S_2$. Subsite $S_2$ is a largely open cavity and $Arg^{203}$, located on the edge of the protein, offers a possible site of interaction. As already stated, substrate activity greatly depends on the interaction between a suitable L side chain of the bound substrate and subsite $S_2$.

2. Intrinsic resistance to β-lactams

Penicillins bind very loosely to the G DD-peptidase (dissociation constant $K \simeq 150$ mM for phenoxymethylpenicillin). Cephalosporins have a somewhat more favorable K value (1-10 mM). Irrespective of the type of bicyclic framework and the nature of 6(7)substituent, the first order rate constant of enzyme inactivation is always very small ($\simeq 1\text{-}5 \times 10^{-4} s^{-1}$ or less).

Kinetically, cephalothin and cephalosporin C act as noncompetitive inhibitors. Cephalosporin C destroys the crystal structure thus preventing any study by X-ray diffraction. Cephaloglycine and β-iodo-benzyl-7-amino-cephalosporanate act as competitive inhibitors and the crystal enzyme derivative formed with this latter β-lactam shows satisfactory isomorphism. Study at 4.5 Å resolution has provided direct evidence that the β-lactam binds to the enzyme active site.

With penicillins and cephalosporins, models show that it is not possible to align simultaneously both the β-lactam amide bond with the zinc ion and the C-terminal carboxylate with $Arg^{137}$ because the fused ring system would collide with $His^{191}$. Intrinsic resistance to β-lactams can thus be seen before our eyes as being caused by the enzyme active site geometry which permits correct alignment and enzyme ligand association of high productiveness, only with carbonyl donor peptides.

6-β-Iodopenicillanate has a very short 6-β-substituent. When used at a high [6-β-iodopenicillanate]/[enzyme] molar ratio of about 8,000 , it also causes slow and irreversible inactivation of the G DD-peptidase (first order rate constant : $7 \times 10^{-4} s^{-1}$). The crystal enzyme derivative thus obtained is perfectly isomorphous. The difference Fourier synthesis at 2.8 Å shows that the inactivator molecule is located just in front of the zinc ion and superimposes $His^{191}$ which has slightly moved toward the exterior of the cavity. This disposition suggests that, in the inactivation process, $His^{191}$ acts as a nucleophile and undergoes alkylation by 6-β-iodopenicillanate with loss of the iodine.

## 3. Catalysis

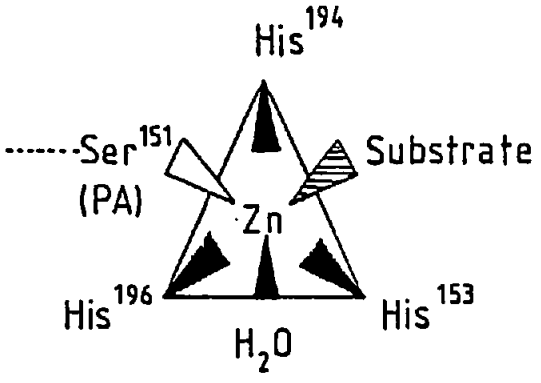
The coordination around $Zn^{++}$ involves two histidine residues and one glutamic acid residue in both thermolysin and carboxypeptidase A. Note, however, that $His^{142}$, $His^{146}$ and $Glu^{166}$ in thermolysin align with $His^{196}$, $Glu^{72}$ and $His^{69}$ in carboxypeptidase A. The fourth ligand is a water molecule.

Near $Zn^{++}$, there are two catalytic residues. $Glu^{143}$ in thermolysin (or $Glu^{270}$ in carboxypeptidase A) is thought to act as proton abstractor heightening the nucleophilicity of the zinc-bound water molecule. In turn, $His^{231}$ in thermolysin (or $Tyr^{248}$ in carboxypeptidase A) is thought to facilitate proton donation to the nitrogen atom at the level of the tetrahedral intermediate.

Following initial binding to thermolysin or carboxypeptidase A, the carbonyl donor substrate becomes the fifth ligand of $Zn^{++}$ in a transient pentagonal complex in which $Zn^{++}$ itself plays the role of an anion hole. Collapse of the intermediate by proton donation and re-entry of a water molecule permits release of the reaction products. At any time during the process, no covalent intermediate is formed and partitioning of the enzyme activity between alternate nucleophiles ($H_2O$ and an amino compound) cannot occur. Thermolysin and carboxypeptidase A do not catalyse transpeptidation reactions; they are strict hydrolases.

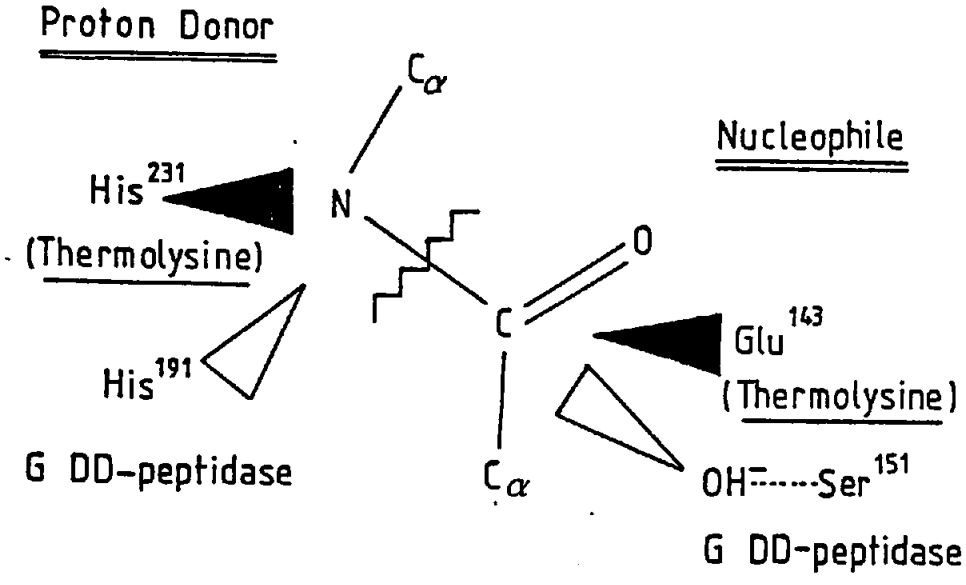
The G DD-peptidase is also a strict hydrolase (DD-carboxypeptidase). Basically, its mode of action may be similar to that of thermolysin (or carboxypeptidase A), but there are marked differences between them. In the G DD-peptidase, the zinc protein ligands are three histidine residues ($His^{194}$, $His^{196}$ and $His^{153}$). Moreover, to all appearances, $His^{191}$ acts as proton donor while $Ser^{151}$ would be somehow involved in water activation, a situation reminiscent to that found in the zinc-containing alcohol dehydrogenase (where a similar role is played by the dyad $His^{31}...Ser^{48}$).

A constant feature of the zinc-containing thermolysin, carboxypeptidases A and B, carbonic anhydrase and alcohol dehydrogenase, is that the hand and geometry of the zinc environment is invariant with regard to the protein ligands, water position, substrate binding site and proton abstractor (Argos *et al.*, 1978). The same disposition applies to the G DD-peptidase. In turn, the nucleophile in the environment of the carbon atom and the proton donor in the environment of the nitrogen atom of the scissile peptide bond determine the hand of the reactive intermediates (Argos *et al.*, 1978). In thermolysin and carboxypeptidase A, the nucleophile points to the carbonyl carbon from below the plane formed by the triad $\overset{O}{\overset{\|}{C}}-N$ while the proton donor points to the nitrogen atom from above that plane. Interestingly, a reverse disposition of these two catalytic groups occurs in the G DD-peptidase.




DD-Carboxypeptidase
S. albus G
(PD : His191)

Hand and geometry of the zinc environment of the G DD-peptidase. PD = proton donor. The same disposition occurs in the zinc-containing thermolysin, carboxypeptidases A and B, carbonic anhydrase and alcohol dehydrogenase (Argos *et al.*, 1978).



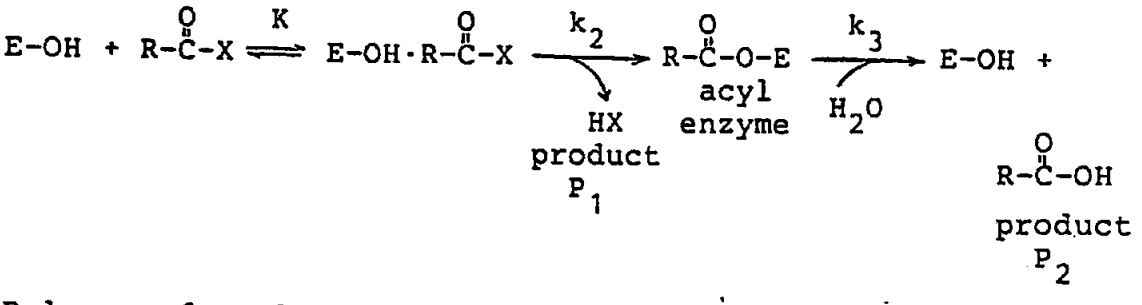

Disposition of the nucleophile and proton donor in thermolysin and the G DD-peptidase with respect to the carbon atom and nitrogen atom, respectively, of the scissile peptide bond.

The concept of bi-product analogues has led to the synthesis of specific and potent inhibitors of thermolysin, carboxypeptidases A and B, and the zinc-containing angiotensin converting enzyme (Ondetti *et al.*, 1981). Similarly, bifunctional compounds possessing both a C-terminal carboxylate function susceptible to interact with $Arg^{137}$ and either a thiol, hydroxamate or carboxylate function susceptible to bind the zinc atom, are inhibitors of the G DD-peptidase. Inhibition is competitive. β-Mercaptopropionic acid ($K_i = 5 \times 10^{-9}$ M) and the L isomer of mercaptoisobutyric acid ($K_i = 1 \times 10^{-8}$ M) are very effective. Curiously, the D isomer is less potent ($K_i = 6 \times 10^{-8}$ M). Such small truncated and flexible molecules do not offer the possibility of complementing subsites $S_1$ and $S_2$ and, as the above observations suggest, subsite $S_1'$ alone seems not to play any significant role in binding. Enzyme inhibition may be caused by bidentate interaction and formation of a penta-coordinate zinc atom. Obviously, specificity requires more elaborate structures that would be apt to interact with the $S_1$ and $S_2$ subsite targets.

## Covalent Catalysis by the R61, R39 and K15 DD-Peptidases

### The concept of acyl enzyme intermediate

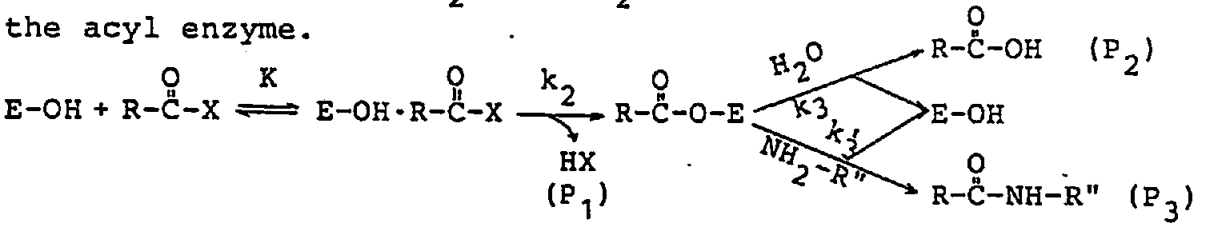
α-Chymotrypsin (or any other serine peptidase)-catalysed cleavage of peptide (ester) bond proceeds through formation of a serine ester-linked acyl enzyme intermediate. Giving E-OH = the enzyme active serine residue; X = NH-R' or O-R'; K = dissociation constant, $k_2$ and $k_3$ = first order rate constants, the reaction can be written :




Release of products $P_1$ and $P_2$ proceeds through a Ping-Pong mechanism.

If acyl enzyme formation ($k_2$) is rate determining (as generally observed with amide substrates R-C(=O)-NH-R'), the acyl enzyme does not accumulate detectably at the steady state and cannot be trapped. If acyl enzyme breakdown ($k_3$) is rate determining (as generally observed with ester substrates R-C(=O)-O-R'), the acyl enzyme accumulates and can be trapped and isolated.

Assume the presence of an alternate amino nucleophile $NH_2$-R" which does not bind to the free enzyme nor to the Michaelis complex but does react only after the leaving group $P_1$ has diffused away. Then, partitioning of the enzyme activity between the two nucleophiles, $H_2O$ and $NH_2$-R", occurs at the level of the acyl enzyme.




If acyl enzyme formation ($k_2$) is rate determining, increasing concentrations of $NH_2$-R" cannot increase the maximal rate of carbonyl donor consumption (Vmax) but aminolysis occurs at the expense of hydrolysis on a competitive basis. If acyl enzyme breakdown ($k_3$) is rate determining, increasing concentrations of $NH_2$-R" may increase the rate of acyl enzyme breakdown and hence Vmax.

The R61 and R39 serine DD-peptidases

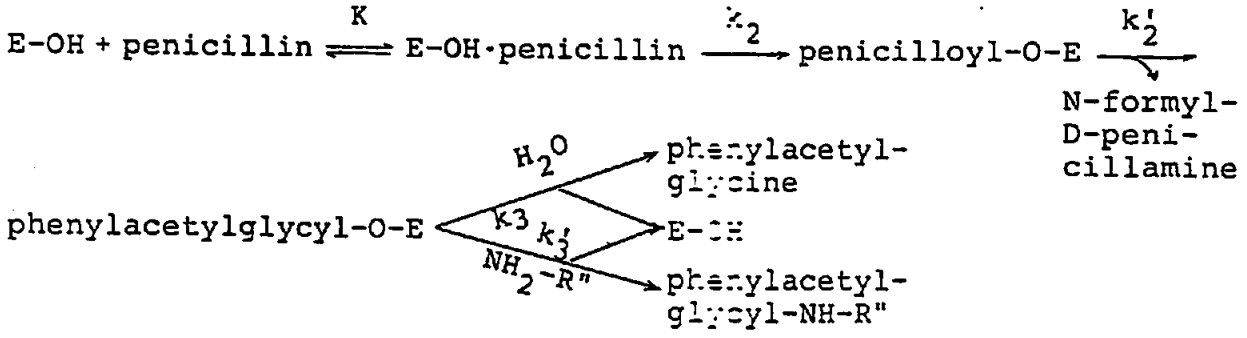
Although substantial progress in the determination of the three-dimensional structure of the R61 DD-peptidase is being made (Judith Kelly; this symposium), a precise picture of how this enzyme performs catalysis cannot yet be proposed. However, the enzyme catalyses concomitant hydrolysis and aminolysis of suitable carbonyl donor substrates (*i.e.* it functions both as carboxypeptidase and transpeptidase); with the ester substrate $Ac_2$-L-Lys-D-Ala-D-lactic acid (depsipeptide), an intermediate accumulates at the steady state; penicillin effectively immobilizes the enzyme in the form of a serine-ester linked penicilloyl derivative and experimental evidence strongly suggests that the

same serine residue is involved in the formation of the intermediate that can be trapped during hydrolysis of the depsipeptide. To all appearances, the R61 DD-peptidase, as well as the R39 DD-peptidase, are serine enzymes analogous to α-chymotrypsin.

Using $Ac_2$-L-Lys-D-Ala-D-Ala as carbonyl donor and simple amino acids as acceptors (D-Ala; *meso*-$A_2$pm), hydrolysis and transpeptidation proceed as expected for an amide carbonyl donor substrate : the increase in the rate of transpeptidation and the decrease in the rate of hydrolysis caused by increasing concentrations of the amino acceptor, are commensurate. However, with more complex amino acceptors related to wall peptidoglycan, the picture is more complicated. The observed increase of the rate of transpeptidation is less than can be accounted for by the decrease of the rate of hydrolysis. Furthermore, at high concentrations of amino acceptor, both hydrolysis and transpeptidation are inhibited so that, eventually, the enzyme can be frozen in a catalytically inactive state. Kinetic studies also suggest that transpeptidation of $Ac_2$-L-Lys-D-Ala-D-Ala is an ordered pathway where the amino acceptor binds first to the enzyme. In addition, as shown with the R61 enzyme and complex amino acceptors, the enzyme can bind more than one molecule of amino acceptor leading to the formation of an unproductive quaternary complex [enzyme-donor-$(acceptor)_2$]. Finally, the R39 DD-peptidase possesses an additional peptide binding site distinct from the carbonyl donor and amino acceptor sites. Simple peptides (Gly-Gly-Gly, for example) that do not function as acceptors may produce extensive inhibition of the transpeptidation reaction while hydrolysis proportionally increases. Inhibition is noncompetitive *versus* the acceptor, implying that these "allosteric" inhibitors are not binding to the enzyme active site.

All these observations show that the carboxypeptidase and transpeptidase activities of the serine DD-peptidases are susceptible to exquisite modulation. Similar mechanisms might be involved in the control of the peptide crosslinking of peptidoglycan in living bacteria.

Much has been written on the inactivation of the serine DD-peptidases by the penicillins, cephalosporins and monobactams. Essentially, the underlying mechanism is that the corresponding acyl enzyme "intermediate" has, in most cases, a very long half-life. Since the scissile amide bond of the β-lactam ring is endocyclic, what should be regarded as the leaving group ($P_1$) during acyl enzyme formation cannot leave the enzyme active site which thus remains occupied. Enzyme deacylation, however, may slowly occur. In some cases, rupture of the $C_5$-$C_6$ linkage in the enzyme-bound penicilloyl moiety with formation of phenylacetylglycyl enzyme is the rate determining step of enzyme deacylation. Once formed, this new intermediate is immediately attacked by water or a suitable amino compound (on a competitive basis) with regeneration of an active enzyme




Since $k_2'$ is rate determinant, $NH_2$-R" does not accelerate the rate of enzyme reactivation. In other cases, enzyme deacylation may slowly occur without prior fragmentation of the bound acyl moiety. When this occurs, the DD-peptidase functions as a classical β-lactamase of very weak efficiency.

The higher the bimolecular rate constant of enzyme acylation ($k_2/K$), the more potent is the β-lactam as enzyme inactivator. Various side chains in homologous series of β-lactams can be accommodated by a given DD-peptidase but the $k_2/K$ values thus generated may vary widely. The goodness of fit of the β-lactam molecule to the enzyme active site, rather than any other feature (intrinsic reactivity of the β-lactam ring, for example) is the primary parameter that governs the efficacy of enzyme inactivation.

The K15 DD-peptidase

The membrane-bound K15 DD-peptidase also performs covalent catalysis. Whether an active serine or another residue of the active site is involved in the process is not yet known. Whatever the case, the K15 enzyme strikingly differs from the R61 and R39 serine DD-peptidases. Indeed, the Vmax of consumption of the amide carbonyl donor $Ac_2$-L-Lys-D-Ala-D-Ala is very low in water (low carboxypeptidase activity). It is increased at least 30-fold in the presence of a suitable amino acceptor such as Gly-Gly (high transpeptidase activity). Hence, depending on whether the amino acceptor is present or not, the acyl enzyme, at the steady state, should be undetectable or should accumulate massively. Using [$^{14}$C]$Ac_2$-L-Lys-D-Ala-D-Ala at saturating conditions and SDS as trapping agent, attempts to estimate the acyl enzyme have been carried out by fluorography after gel electrophoresis in the presence of SDS. When Gly-Gly is present, no acyl enzyme can be trapped but, unexpectedly, when Gly-Gly is absent, no more than 10 % of the enzyme occurs in the form of acyl enzyme (indicating that the rate of acyl enzyme formation is much smaller than the rate of acyl enzyme breakdown). It thus seems that the effect of the amino acceptor on Vmax cannot be attributed to a simple partitioning at the level of the acyl enzyme but implies an acceleration of both acyl enzyme formation and breakdown.

This presumed effect of the amino acceptor on the rate of acyl enzyme formation is not observed when benzylpenicillin is used as carbonyl donor. Indeed, the $k_2/K$ value (150 $M^{-1}s^{-1}$) is the same whether Gly-Gly is present or not. Similarly, Gly-Gly has no effect on the rate constant $k_3$ ($1 \times 10^{-4}s^{-1}$) of breakdown of the penicilloyl-enzyme since $k_2'$ is rate determining (see above). It thus follows that, to all appearances, the K15 DD-peptidase does not require any "effector" to react with penicillin but reacts with a peptide carbonyl donor much more effectively in the presence of a suitable amino acceptor than in its absence. Such a behaviour may apply to the penicillin binding proteins (PBPs) 1A, 1B, 2 and 3 of *Escherichia coli*.

While the DD-peptidases R61 and R39 appear to be good models of the PBPs involved in secondary hydrolytic and transpeptidation reactions during wall peptidoglycan remodelling, the K15 DD-peptidase seems to be a good model of those PBPs involved in the primary transpeptidation reactions through which the nascent peptidoglycan undergoes attachment to the preexisting wall peptidoglycan.

Acknowledgement

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