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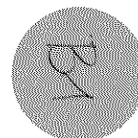

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On the Active Centers of Serine and Zn^{++} DD-Carboxypeptidases

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In spite of the great advances made over the past fifteen years, the mode of action of β-lactam antibiotics at the cellular and molecular levels remains a problem of enormous complexity. Multiple membrane-bound enzymes exhibiting antagonistic activities and widely varying sensitivities to β-lactam antibiotics are involved in the crosslinking step of cell wall biosynthesis. In parallel to this, bacteria possess multiple membrane-bound penicillin binding proteins exhibiting widely varying molecular weights and affinities to β-lactam antibiotics. Genetic and biochemical studies are being applied to define the in vivo function and enzymatic activity of these membrane-bound penicillin binding proteins and penicillin-sensitive enzymes, and to elucidate the mechanism(s) through which inactivation of one or several of these receptors causes inhibition of bacterial growth, induces cellular morphological abnormalities and results in killing and cellular lysis of the sensitive bacteria. In turn, biochemical and biophysical studies are also being applied to unravel the mechanistic properties and functioning of the membrane-bound enzymes (DD-carboxypeptidases/transpeptidases) that are the primary targets of the β-lactam antibiotics.

The DD-carboxypeptidases/transpeptidases catalyse attack, by a suitable exogenous nucleophile (HY), of the carbonyl carbon of the D-Ala-D-Ala amide bond of L-R-D-Ala-D-Ala terminated peptides (for example, the tripeptide Ac_2-L-Lys-D-Ala-D-Ala; Fig. 1). They also use, as carbonyl donor, the endocyclic amide bond which is exposed on the α face of the penicillins and Δ^3-cephalosporins in a position roughly equivalent to that of the D-Ala-D-Ala amide bond (Fig. 1). This latter reaction, however, stops, at least for some time, at an abortive level, thus causing enzyme inactivation.

In analogy with the usual proteases which can operate by four different mechanisms (the serine-, thiol-, acid- and metallo-proteases), the DD-carboxypeptidases fall into at least two classes of enzymes depending upon their distinctive mechanistic properties. The 38,000-Mr R61 (from Streptomyces R61)[1,2], the 53,000-Mr R39

(from Actinomadura R39)[3,4] and two 40,000-42,000-Mr Bacilli DD-carboxypeptidases[5] are serine-enzymes. The 18,000-Mr G DD-carboxypeptidase (from Streptomyces albus G)[6,7] is a metallo (Zn^{++}) enzyme. Preliminary data suggest that the 43,000-Mr DD-carboxypeptidase of Streptococcus faecalis ATCC 9790[8] and the 29,000-Mr DD-carboxypeptidase of B. coagulans[9] might be thiol-enzymes.

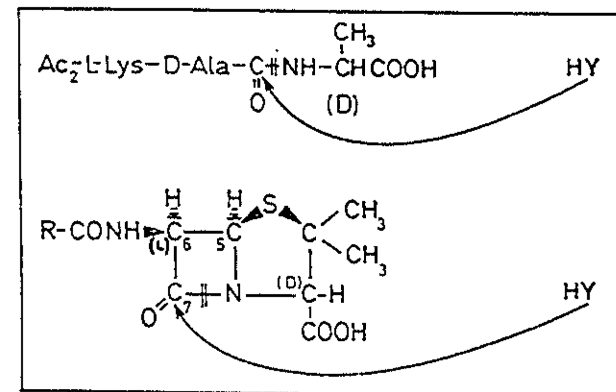
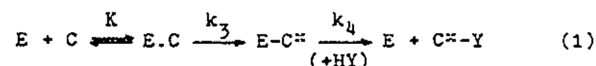


Figure 1

Obtaining the DD-carboxypeptidases in a truly water-soluble form, and from this in a crystalline state, is all the more important in order to understand the substrate — and drug — receptor systems at the molecular level and in terms of three-dimensional structures. DD-carboxypeptidases that are loosely bound to the plasma membranes can be solubilized by treating bacteria with the Ribi fractionator, by converting bacterial cells into protoplasts or by submitting the isolated membranes to osmotic shocks. Usually, DD-carboxypeptidases that are firmly bound to the membranes can be solubilized only with the help of detergents. In two cases, however, such enzymes have been converted by controlled proteolysis into shortened, water-soluble proteins without affecting the enzyme activity and the penicillin binding capacity of the parent molecules[8,10]. Treatment of membranes with 2,3-dimethylmaleic anhydride has also given rise to water-soluble and enzymatically active preparations[11]. Finally, various strains of Actinomycetes spontaneously release DD-carboxypeptidases in the external medium during growth. The R61, R39 and G DD-carboxypeptidases are secreted enzymes[12]. The mechanism(s) of enzyme excretion is under current study. At this time, the serine-R61 enzyme[13] and the metallo (Zn^{++}) G enzyme[14] are the only two DD-carboxypeptidases that are available in crystalline forms.

The Serine DD-Carboxypeptidases

The simplest model which expresses the reactions catalysed by the serine DD-carboxypeptidases (E) on a given carbonyl donor (C) and in the presence of a suitable exogenous nucleophile (HY) is :



$$E + C \overset{K}{\rightleftharpoons} E.C \xrightarrow{k_3} E\text{-}C^* \xrightarrow[(+HY)]{k_4} E + C^*\text{-}Y \qquad (1)$$

with E.C = first stoichiometric complex; $E\text{-}C^*$ = acyl-enzyme intermediate; $C^*\text{-}Y$ = reaction products; K = dissociation constant; k_3 and k_4 = first order rate constants. Reaction between the carbonyl donor and the enzyme active serine residue leads to the formation of a covalently ester-linked L-R-D-alanyl- or penicilloyl (cephalosporoyl)-enzyme complex $E\text{-}C^*$; in turn, transfer of the acyl group to HY leads to enzyme regeneration and release of the reaction products.

L-R-D-Ala-D-Ala terminated peptides that have high substrate activities characterize themselves by high k_3/K and high k_4 values (Fig. 2). With HY = H_2O , the reaction products are D-Ala and a shortened L-R-D-Ala peptide. With HY = NH_2-R', the reaction products are D-Ala and a modified L-R-D-Ala-CONH-R' peptide. The two reactions may occur concomitantly and compete with each other[12].

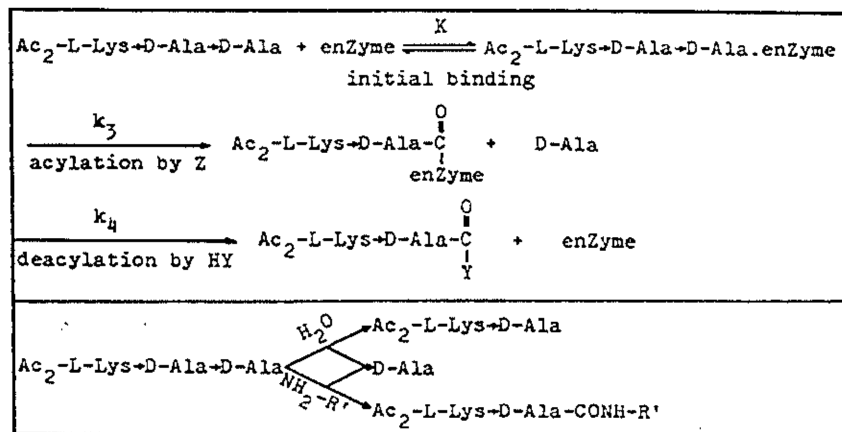


Figure 2

In turn, β-lactam antibiotics that are good enzyme inactivators characterize themselves by high k_3/K and low k_4 values (Fig. 3). When used at a 10 μM concentration (which is usually lower than the K value), a β-lactam compound that exhibits, for a given enzyme, a k_3/K value of 1,000 $M^{-1}s^{-1}$ or more and a k_4 value of $1 \times 10^{-4} s^{-1}$ or less, immobilizes 99 %, or more, of the enzyme at the steady state and the time required for the reaction to reach 95 % of the steady state is about 5 min or less.

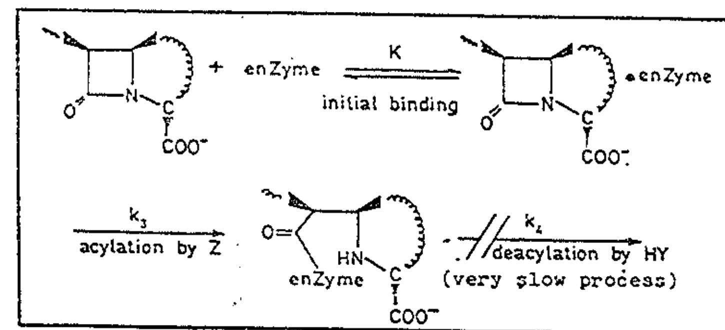


Figure 3

The amino-acid sequences around the active serine residue have been established by submitting isolated [^{14}C]benzylpenicilloyl-enzyme complexes to proteolytic degradations. Study of L-R-D-alanyl-enzyme complexes has been made possible by using as carbonyl donor, the depsipeptide Ac_2-L-Lys-D-Ala-D-lactate (instead of the usual Ac_2-L-Lys-D-Ala-D-Ala tripeptide), under which conditions the k_3 term is markedly increased and becomes higher than the k_4 term so that the Ac_2-L-Lys-D-alanyl-enzyme complexes accumulate. With the two *Bacilli*[15] and the R61[16] DD-carboxypeptidases, the penicilloyl and Ac_2-L-Lys-D-alanyl groups were shown to be covalently bound to the same enzyme serine residue.

The interaction between penicillin and the β-lactamase I of *B. cereus* and that of *Escherichia coli* RTEM also involves a serine residue, and this serine residue is conserved in the β-lactamases (of known sequences) of *Staphylococcus aureus* and *B. licheniformis*[17]. The serine β-lactamases and the serine DD-carboxypeptidases differ from each other at least in two respects. The β-lactamases do not use L-R-D-Ala-D-Ala terminated peptides as carbonyl donors and the k_4 term of the reaction on good β-lactam substrates is very high. Whether or not the DD-carboxypeptidases and the β-lactamases are structurally related enzymes will remain debated as long as the primary sequences of the DD-carboxypeptidases are not known. At this time, alignment of the active serine residue in the four DD-carboxypeptidases and the four β-lactamases shows an obvious homology only between the β-lactamases or between the two *Bacilli* DD-carboxypeptidases (Table 1).

Table 1. Amino-acid sequences around the serine active residue.

Enzyme	Sequence
DD-Carboxypeptidases from	
Streptomyces R61[2]	v g [s]
Actinomadura R39[4]	l p a [s] n g v
B. stearothermophilus[5]	p i a [s] m
B. subtilis[5]	g i a [s] m t k
β-Lactamases from	
S. aureus[17]	a y a [s] t s k
B. cereus 569/H (I)[17]	a f a [s] t y k
B. licheniformis 749/C[17]	a f a [s] t i k
E. coli RTEM[17]	p m n [s] t f k

X-Ray structure analyses at 5 Å resolution of the R61 DD-carboxypeptidase[18] and the RTEM[19] and other serine β-lactamases have been, or are close to be, completed. As long as the X-ray analyses have not been done at a higher resolution, the results presently available unfortunately reveal little of biochemical interest. One should mention, however, that the β-lactam o-iodophenylpenicillin has been successfully diffused in a native crystal of the R61 enzyme. On the basis of the difference Fourier map, the site of interaction is well visualized as a 22 Å segment elongated in the y direction of the map[18]. Interestingly, another peak is observed at similar x and y coordinates but at a lower z coordinate; it is most likely associated with a conformational change induced in the native structure upon interaction with this penicillin.

The 38,000-Mr R61 and 53,000-Mr R39 DD-carboxypeptidases characterize themselves by a very uneven distribution of the basic (Arg + Lys) amino-acid residues (non-published results). As a consequence, trypsin proteolysis releases from each enzyme three large segments (or core peptides) of about 50-60 , 75-80 and 100-120 residues, and a series of short peptides which altogether comprise virtually all the potential points of attack by trypsin (but represent only 30 % of the R61 protein and 53 % of the R39 protein). To all appearances, the active serine residue is released as part of a small peptide in the case of the R39 enzyme and remains part of one of the core peptides in the case of the R61 enzyme. Although the R61 and R39 enzymes have similar turnover numbers (1050 and 3300 min^{-1}, respectively) on the standard tripeptide Ac_2-L-Lys-D-Ala-D-Ala, they show variations in their specificity profiles for carbonyl donor peptides. Moreover, they differ from each other with respect to their requirements for complex amino compounds as nucleophilic acceptors in transpeptidation reactions, and their

sensitivities to β-lactam antibiotics[12]. In particular, the R39 enzyme is much more sensitive to the action of β-lactam antibiotics than the R61 enzyme.

An examination of the kinetic parameters of the reactions catalysed by the R61 and R39 enzymes on various peptide substrates and β-lactam inactivators has been of great assistance in interpreting the underlying functioning of the enzyme active centers[12,20-22]. With β-lactam antibiotics, not only the k_4 term of the reaction, but the K and k_3 parameters (or at least the k_3/K ratio) could be measured directly. With peptide substrates, it was assumed that the initial binding was a rapid equilibrium process and that enzyme acylation was the rate limiting step of the reaction. Under these conditions, Km and Vmax become equivalent to K and $k_3 \times E_o$, respectively. Within the limits of these approximations, the experimental data led to the conclusions discussed below. The same conclusions probably apply to the Bacilli DD-carboxypeptidases[23], and, as shown by extensive studies[24-26], to the presumed SH DD-carboxypeptidase of S. faecalis.

Acylation of the Enzyme Active Serine Residue (Enzyme Binding Site n°1). As shown in Table 2, 6-aminopenicillanic acid and 7-aminocephalosporanic acid have low proprensities to acylate the active serine residue of the enzymes (low k_3/K values). With 6(7)β substituted penicillins and Δ^3-cephalosporins, the k_3/K ratio values widely vary depending on both the antibiotic and the enzyme under consideration. (Note that irrespective of the antibiotics, the k_3/K ratio values are always higher or much higher with the R39 enzyme than with the R61 enzyme.) In all cases, however (Table 3),

Table 2. Enzyme acylation : $E + C \xrightarrow{k_3/K} E\text{-}C^*$

R61 enzyme +	k_3/K $M^{-1}s^{-1}$	R39 enzyme +	k_3/K $M^{-1}s^{-1}$
6-APA	0.2	7-ACA (20°C)	200
Cephalexin	4	6-APA	900
7-ACA	14	Carbenicillin (20°C)	3,000
Cloxacillin	30	Cephalexin (20°C)	3,000
Nitrocefin (10°C)	460	Cloxacillin	15,000
Carbenicillin	800	Cephalothin	> 70,000
Cephalothin	3,000	Benzylpenicillin	300,000
Benzylpenicillin (25°C)	14,000	Nitrocefin (10°C)	≈ 3,000,000

(At pH 7.5 and 37°C unless otherwise indicated.)

Table 3. Enzyme acylation : $E + C \underset{}{\overset{K}{\rightleftharpoons}} E.C \xrightarrow{k_3} E\text{-}C^{*}$

Enzyme	Antibiotic	K (mM)	k_3 (s^{-1})
R61	6-APA	1	0.0002
	Carbenicillin	0.11	0.09
	Ampicillin	7	0.8
	Benzylpenicillin	13 (25°C)	180 (25°C)
R39	Cephalosporin C	0.19 (20°C)	13 (20°C)

(At pH 7.5 and 37°C unless otherwise stated.)

binding occurs with the same poor efficiency (K = 0.1 - 13 mM). The structure of the 6(7)β substituent has thus little influence on the initial binding to the enzymes while that part of the molecule which is mainly involved in the process is the bicyclic fused ring system. The occurrence of an intact β-lactam ring is an essential structural feature but thiazolidine or dihydrothiazine may occur at the C-terminal position of the β-lactam molecules.

As shown in Table 4, Ac-D-Ala-D-Ala is a very poor substrate of the R39 and R61 enzymes. In fact, this dipeptide behaves as an inhibitor for the hydrolysis of Ac_2-L-Lys-D-Ala-D-Ala (the standard substrate) by the R61 enzyme. Substrate activity entirely depends on the occurrence at the L-center of L-R-D-Ala-D-Ala terminated peptides of a side chain exhibiting the proper size, charge and conformation. Whether the peptide has high or low substrate activity (Table 5), enzyme binding occurs with the same poor efficiency (Km = 10-35 mM with the R61 enzyme; 0.2-2.5 mM with the R39 enzyme; assuming Km is equivalent to K). Hence, the side chain at the L center of the peptide has little influence on the initial binding to the enzymes while that part of the molecule which is mainly involved in the process is the C-terminal D-Ala-D-Ala dipeptide. The occurrence of a D-Ala residue at the penultimate position

Table 4. Efficacy of hydrolysis.

Peptides	Activity as substrate (%)	
	R61 enzyme	R39 enzyme
Ac-D-Ala→D-Ala	nonsubstrate inhibitor	< 1
Ac_2-L-Ala → D-Ala→D-Ala	1.4	< 1
Ac_2-L-A_2bu→D-Ala→D-Ala	8	15
Ac_2-L-Orn → D-Ala→D-Ala	50	83
Ac_2-L-Lys → D-Ala→D-Ala	100	100

(At pH 7.5 and 37°C, and at a 0.5 mM peptide concentration.)

Table 5. Efficacy of hydrolysis.

Peptides	R61 enzyme		R39 enzyme	
	Km (mM)	Turnover number (min^{-1})	Km (mM)	Turnover number (min^{-1})
Ac_2-L-Lys→D-Ala→D-Ala	12	3,300	0.8	1,050
Ac_2-L-Lys→D-Ala→D-Leu	10	200	0.7	700
Ac_2-L-Lys→D-Ala→Gly	35	750	2.5	300
Ac_2-L-Lys→D-Ala→L-Ala	nonsubstrate		nonsubstrate	
Ac_2-L-Lys→D-Leu→D-Ala	nonsubstrate		nonsubstrate	
Ac_2-L-Lys→Gly→D-Ala	15	6	nonsubstrate	
Ac-L-Lys→D-Ala→D-Ala (↓c H)	15	15	0.2	2,100

(At pH 7.5 and 37°C).

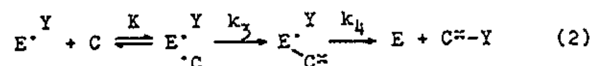
is an essential structural feature but D-amino-acids other than D-alanine or glycine can occur at the C-terminal position of the peptide (although often at the expense of substrate activity).

Conversion of the bound peptide or the bound penicillin (Δ^3-cephalosporin) into a reactive serine directed reagent thus requires the occurrence of a suitable lateral chain at the L-center of the peptide, or the 6(7)β position of the β-lactam ring. The simplest interpretation is that by interacting with some specific amino-acid groupings (enzyme binding sites n°2), such side chains induce specific conformational changes in the protein which in turn cause a much increased electrophilic character of the carbonyl carbon of the penultimate D-Ala or the β-lactam ring[22]. Hence both efficacy and specificity in enzyme acylation are mainly governed by the side chains under consideration. That conformational changes occur during enzyme acylation is supported by the observations that penicilloylation of the R61 enzyme causes fluorescence quenching and alterations in the CD spectrum of the protein[27]. X-Ray crystallographic studies[18] (see above) lead to the same conclusions.

In spite of the aforementioned similarities, enzyme acylation by L-R-D-Ala-D-Ala terminated peptides and enzyme acylation by penicillins and Δ^3-cephalosporins differ from each other in, at least, two respects : i) the D-Ala-D-Ala amide bond is, of course, planar while the β-lactam nitrogen is pyramidal. Non-planarity can be expressed by the distance h between the apex and the base of the trigonal pyramid, where N is at the apex and its three carbon substituents at the corners of the base. In Δ^3-cephalosporins and penicillins, h is about 0.24-0.32 and 0.38-0.40 Å, respectively.

It thus appears that the peptides overcome a much higher barrier to reaction with the enzymes than the penicillins or Δ^3-cephalosporins; ii) Effective side chains in the peptides or in penicillins and Δ^3-cephalosporins are completely structurally unrelated. These two observations suggest that the serine DD-carboxypeptidases may have two distinct binding sites n°2, for bound peptides and bound β-lactam compounds, respectively.

Enzyme Deacylation. Deacylation of L-R-D-alanyl-enzyme complexes is a rapid process; it is assumed not to be rate limiting and water can serve as a non-specific acceptor. The serine DD-carboxypeptidases (at least the R61 and R39 enzymes), however, possess highly structured amino acceptor sites which enable them to perform specific transpeptidase activities[29-32]. Steady state kinetic studies suggest that the transpeptidation reactions follow an ordered pathway in which binding of the exogenous amino nucleophile HY occurs before binding of the carbonyl carbon donor[33] so that reaction (1) is probably an oversimplification and should be replaced by :



$$E^{\cdot Y} + C \overset{K}{\rightleftharpoons} E^{\cdot Y}_{\cdot C} \xrightarrow{k_3} E^{\cdot Y}_{C^*} \xrightarrow{k_4} E + C^*\text{-}Y \qquad (2)$$

In aqueous media and with D-Ala or Gly as amino acceptors, hydrolysis and transpeptidation interfere with each other on a simple competitive basis. In the presence of increasing concentrations of more complex amino compounds related to wall peptidoglycan, not only is hydrolysis progressively inhibited but transpeptidation, after rising to a maximum, is in turn progressively inhibited so that, eventually, the enzymes can be frozen in a non-operational state[30,33]. Complex amino compounds may thus act as modulators of the enzyme activity, a property which may be of biological significance.

Deacylation of the penicilloyl (cephalosporoyl)-enzyme complexes is a slow process and, as a consequence, β-lactam antibiotics

Table 6. Stability of complexes.

Enzyme	Antibiotic	Half-life (min)	k_4 (s^{-1})
R61	Benzylpenicillin	80	1.4×10^{-4}
	7-ACA	3	4.4×10^{-3}
	Cephalosporin C	10,000	1×10^{-6}
R39	Benzylpenicillin	4,100	2.8×10^{-6}
	7-ACA	11,600	1.1×10^{-6}
	Cephalosporin C	38,000	3×10^{-7}

(At pH 7.5 and 37°C).

behave as suicide substrates (Table 6). (Note that the complexes formed with the R39 enzyme are always more or much more stable than those formed with the R61 enzyme.) It has been proposed[22] that formation of the ester linkage between C(7) [C(8)] of the penicillin (cephalosporin) molecule and the active serine residue (binding site n°1) not only requires interaction between the 6(7)β substituent and the enzyme binding site n°2, but also involves interaction between the monocyclic thiazolidine (dihydrothiazine) ring and another enzyme binding site n°3. The three dimensional disposition of these three enzyme sites appears to be an important parameter that governs the stability of the complex and the fate of the bound metabolite. Such a model well explains the wide variations in complex stability that are observed depending on the enzymes and the β-lactam compounds (Table 6) and the fact that, depending on the enzymes and the procedures used, denaturation of the penicilloyl-enzyme complexes may increase or decrease their half-lives (Table 7). In all cases, however, breakdown of such denatured complexes results in the release of penicilloate.

Table 7. Half-lives of acyl-enzyme complexes. Nature of the breakdown products.

Penicilloyl-R61 enzyme complex	Native	80 min	PAG + FPA
	Denatured by :		
	- heat (100°)	80 hrs	penicilloate
	- heat + trypsin	35 hrs	penicilloate
	- 6 M guanidine-HCl in water	several days	penicilloate
	- 6 M guanidine-HCl in methanol	100 min	penicilloate (α-methyl ester)
Penicilloyl-R39 enzyme complex	Native	70 hrs	PAG (+ FPA ?)
	Denatured by :		
	- heat (100°)	10 hrs	penicilloate
	- heat + trypsin	very labile	penicilloate

PAG = phenylacetylglycine; FPA = N-formyl-D-penicillamine

The "enzyme catalysed" fragmentation of the penicillin molecule is carried out by C(5)-C(6) cleavage and protonation at C(6) of the enzyme-bound penicilloyl moiety[34-38] (Fig. 4). The mechanism of the reaction is unknown, but it has been established that : i) fixation of one hydrogen atom on C(6) is made at the expense of H_2O; ii) the primary fragment which arises from the thiazolidine part of the molecule is an unidentified compound Z which, with a half-life

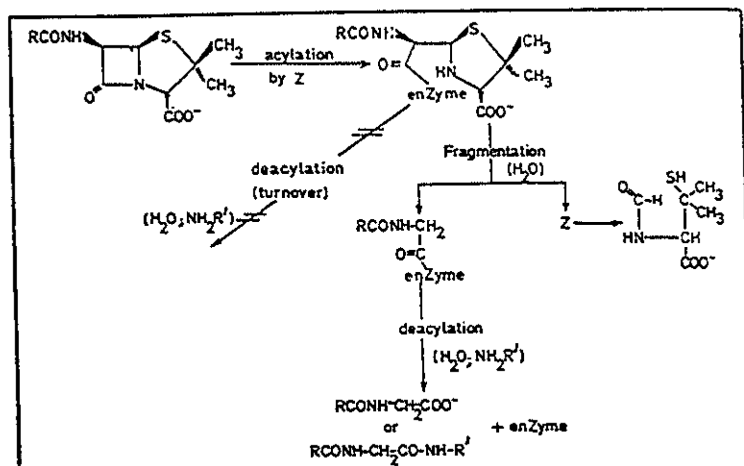
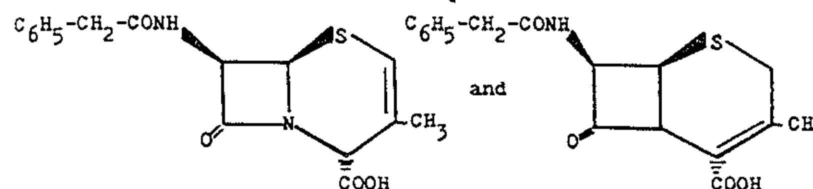


Figure 4

of 10-15 min at 37°C and pH 7.5, is further processed to give rise to free N-formyl-D-penicillamine; and iii) the fragmentation reaction is the rate limiting step of complex breakdown.

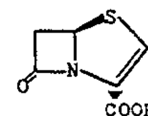
Since the N-acylglycyl-enzyme complex, once formed by C(5)-C(6) cleavage, is susceptible to immediate attack by H_2O or a suitable NH_2-R' amino nucleophile (with release of N-acylglycine or both N-acylglycine and an N-acylglycyl-CONH-R' compound, on a competitive basis), it follows that the thiazolidine (dihydrothiazine) ring must play an important role in stabilizing the original penicilloyl (cephalosporoyl) complex. This suicide mechanism may be extremely efficient (high k_3/K and low k_4 values) and may occur in the virtual absence of normal turnover. Few β-lactam compounds are also suicide substrates of the serine β-lactamases[39]. Even in the most favorable cases, β-lactamase inactivation is a rather rare event which occurs concomitantly with normal turnover.

Reaction with β-Lactam Compounds Other than Penicillins and Δ^3-Cephalosporins. The antibacterial inactivity of the carba-1-penems in which the β-lactam nitrogen is extremely pyramidal (h = 0.54 Å), is probably due to their high propensity "to react with any nucleophile they may encounter with the result that they never reach the enzyme target"[28]. Conversely, one may hypothesize that the virtual antibacterial inactivity of the Δ^2-cephalosporins which have less ring strain (h = 0.06-0.10 Å) than the Δ^3-cephalosporins, is due to their inability to overcome the height of the barrier to reaction with the enzyme. However, both the following Δ^2- and Δ^3-cephalosporins (they are gifts from Prof. H. Vanderhaeghe, Rega



Institute, Leuven) react with the R61 enzyme (at pH 7.5 and 37°C) with the same (rather low) k_3/K value (60-70 $M^{-1}s^{-1}$) but the k_4 value is considerably higher with the Δ^2-derivative ($3 \times 10^{-3}s^{-1}$; half-life of the complex : 230 s) than with the Δ^3-derivative ($5 \times 10^{-6}s^{-1}$; half-life of the complex : 40 hrs). Hence, in this case, the poor inactivating property of the Δ^2-cephalosporin (when compared with the corresponding Δ^3-cephalosporin) is due to a non-negligible turnover of the molecule.

Some of the recently discovered 2-penems and 1-carba-2-penems with no or very simple side chains, exhibit antibacterial activities. Since the non-planarity of their β-lactam nitrogen (h = 0.43-0.50) is considerably more pronounced than in penicillins and Δ^3-cephalosporins (but less pronounced than in carba-1-penems), one could also hypothesize that binding of the nucleus itself to at least some enzyme receptors may cause rapid acylation of the enzyme active centers. Interestingly, and as observed by using the iodine test, the following 2-penem



(a gift from Drs I. Ernest and H.R. Pfaendler from the Woodward Research Institute, Basel)

behaves as a substrate of the R61 enzyme (Km_{app} = 85 μM ; k_{cat} = 2.7 min^{-1}) and the R39 enzyme (Km_{app} = 4 μM ; k_{cat} = 0.42 min^{-1}). Note the low Km_{app} values. At this time, it is not known whether enzyme acylation or enzyme deacylation is the rate limiting step of the reaction.

The Metallo (Zn^{++}) DD-Carboxypeptidase

The 18,000-Mr G DD-carboxypeptidase effectively hydrolyses Ac_2-L-Lys-D-Ala-D-Ala, although with a turnover number one order of magnitude smaller than those of the R61 and R39 serine DD-carboxypeptidases (Table 8). In marked contrast with the R61 and R39 enzymes, however, the G enzyme i) utilizes only H_2O as nucleophilic acceptor and is thus unable to catalyse transpeptidation reactions[40]; ii) exhibits high endopeptidase activities[41]; iii) is highly resistant to inactivation by β-lactam antibiotics[42 43]; and iv) possesses one

Table 8. Hydrolytic activity on Ac_2-L-Lys-D-Ala-D-Ala.

Enzymes	Km (mM)	Turnover number (min^{-1})
53,000-Mr R39	0.8	1,050
38,000-Mr R61	10	3,300
18,000-Mr G	0.3	150

(At pH 7.5 and 37°C).

equiv. of bound Zn^{++} ion[7]. The apoprotein binds the Zn^{++} ion with an association constant of about 2×10^{14} M^{-1} (a value close to that found with EDTA) and this Zn^{++} ion cofactor is required for enzyme activity on Ac_2-L-Lys-D-Ala-D-Ala. The Co^{++} ion also binds stoichiometrically to the G apoenzyme, a feature which should permit study by NMR of the amino-acid residues that must serve as ligands of the cofactor ion. The G DD-carboxypeptidase may be the counterpart of the metallo (Zn^{++}) β-lactamase II of B. cereus[44,45]. Hopefully, the amino acid sequences of the G DD-carboxypeptidase (B. Joris; in preparation) and the β-lactamase II (R.P. Ambler; personal communication) will be known in a near future.

The 4.5 Å resolution structure analysis of the G enzyme[46] has revealed that the enzyme molecule can be inscribed in a 48 Å × 34 Å × 28 Å ellipsoid and consists of two globular domains. The largest domain possesses a region of very high electron density, most likely the Zn^{++} ion cofactor, in the vicinity of which occurs a deep cleft (20 Å × 6 Å × 6 Å) which roughly cuts the domain in two parts. Although at this time the mechanistic properties of the G enzyme are unknown (metalloproteases operate on sensitive substrates without forming transitory acyl-enzyme intermediates), crystallographic studies have permitted visualization of the enzyme active center. The dipeptide Ac-D-Ala-D-Glu (a competitive inhibitor of the hydrolysis of Ac_2-L-Lys-D-Ala-D-Ala)[47] was successfully diffused in a native enzyme crystal and the difference Fourier map calculated at 4.5 Å . The site of interaction is visualized as a 12 Å segment of difference density elongated in the y direction of the map, inside the cavity and close to the Zn^{++} ion site (Fig. 5).

The G enzyme has the same general substrate requirements for L-R-D-Ala-D-Ala terminated peptides as the R61 and R39 serine DD-carboxypeptidases. In particular, the activity of the G enzyme is modulated by the structure of the side chain at the L position of the peptide substrate (Table 9), suggesting that the G enzyme possesses an effective binding site n°2 for bound peptides.

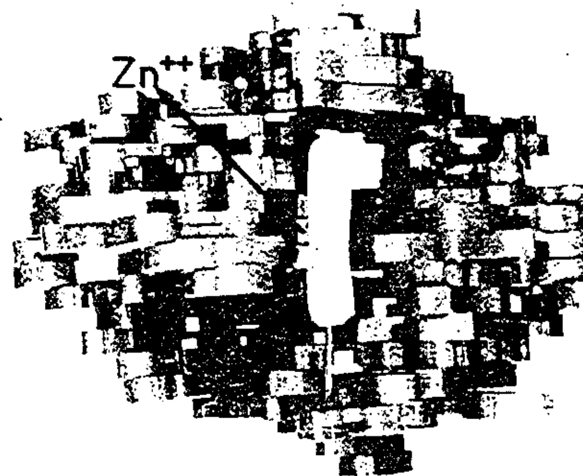


Figure 5
A view of the model of the G enzyme, with the active center in white and as constructed from the electron density map.

Table 9. Efficacy of hydrolysis by the Zn^{++} G DD-carboxypeptidase.

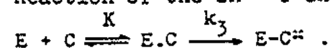
Peptides	Km (mM)	Turnover number (min^{-1})
Ac_2-L-Lys→D-Ala→D-Ala	0.3	150
Ac_2-L-Lys→D-Ala→D-Leu	0.3	50
Ac_2-L-Lys→D-Ala→Gly	2.5	90
Ac_2-L-Lys→D-Ala→L-Ala	nonsubstrate	
Ac_2-L-Lys→D-Leu→D-Ala	nonsubstrate	
Ac_2-L-Lys→Gly→D-Ala	15	150
Ac_2-L-A_2bu→D-Ala→D-Ala	0.6	38
Ac-L-Ala→D-Ala→D-Ala	3.3	0.6
Ac-Gly→D-Ala→D-Ala	1.1	1.7
Ac-D-Ala→D-Ala	nonsubstrate	inhibitor
Ac-L-Lys→D-Ala→D-Ala (+ε H)	6.0	18

(At pH 7.5 and 37°C).

Kinetically, the interaction between the G enzyme and phenoxymethylpenicillin, cephalothin and cephalosporin C proceeds according to reaction (1)[43]. β-Lactam antibiotics, however, are extremely weak inactivators (Table 10), not that binding (K) is less efficient, at least with Δ^3-cephalosporins, than that measured with the serine DD-carboxypeptidases, but because, irrespective of the

structure of the 6(7)β substituent, the k_3 term of the reaction is very low. This very low ability of the G enzyme to convert the penicillins and Δ^3-cephalosporins into reactive enzyme center directed reagents suggests that this enzyme lacks binding site n°2 for β-lactam compounds. As a consequence, the propensity of benzylpenicillin to react with the G enzyme is only about 10-100 times higher than that observed with lysozyme or insulin[48] (and 10^6 to 10^7 times lower than that observed with the R61 and R39 enzymes respectively). Yet, removal of the Zn^{++} ion from the G enzyme results in an apoprotein which is as resistant to penicillin action as any common protein or large polypeptide[7,48].

Table 10. Reaction of the Zn^{++} G enzyme with β-lactam antibiotics:



$$E + C \overset{K}{\rightleftharpoons} E.C \xrightarrow{k_3} E\text{-}C^* .$$

Antibiotic	K (mM)	k_3 (s^{-1})	k_3/K ($M^{-1}s^{-1}$)
Phenoxymethylpenicillin	150	8×10^{-4}	0.005
Cephalosporin C	1.6	1×10^{-4}	0.06
Cephaloglycine	9.5	5×10^{-4}	0.06

(At pH 7.5 and 37°C).
k_3/K values smaller than 2×10^{-3} $M^{-1}s^{-1}$ have been found with 6-APA, benzylpenicillin, carbenicillin, methicillin, oxacillin, cloxacillin, 7-ACA, cephalothin, nitrocefin and cefoxitin.

The complexes E-C* formed under conditions of high antibiotic concentrations and prolonged incubation times at 37°C exhibit high stabilities (Table 11). Spontaneous breakdown, in water, of the native complex formed with benzylpenicillin causes the release of benzylpenicilloate[48], suggesting that in complex EI* the enzyme is penicilloylated. The G enzyme thus appears to behave as a β-lactamase of very low efficiency. Its turnover number on benzylpenicillin is about 5×10^6 fold lower than that observed with the true exocellular β-lactamase which is also excreted by *Streptomyces albus* G.

Table 11. Half-lives of complexes E-C*.

Antibiotic	Half-life (min)	k_4 (s^{-1})
Benzylpenicillin	180	6.4×10^{-5}
Phenoxymethylpenicillin	130	9×10^{-5}
Cephalothin	350	3.3×10^{-5}
Cephalosporin C	150	5×10^{-5}

(At pH 7.5 and 37°C).

Finally, formation of complex E-C* with the G enzyme (i.e. enzyme acylation ?) probably occurs at the enzyme active center. The β-lactam para-iodo-β-phenylacetylaminocephalosporanic acid was successfully diffused into a native enzyme crystal[46]. Although the difference Fourier synthesis at 4.5 Å resolution was rather noisy, the highest peak found in the map was also located in the enzyme cavity close to the Zn^{++} ion site. In agreement with this view, 7-aminocephalosporanic acid competitively inhibits the hydrolysis of Ac_2-L-Lys-D-Ala-D-Ala (J.A. Kelly; unpublished results). Surprisingly, however, inhibition by cephalosporin C is noncompetitive[43]. At this time, the reason why these two β-lactam compounds behave differently is not understood.

Other Possible Classes of DD-Carboxypeptidases

Low concentrations of para-chloromercuribenzoate inhibit the enzyme activity of the DD-carboxypeptidase 1A of *Escherichia coli* on L-D-D-Ala-D-Ala terminated peptides but not its ability to bind penicillin[49]. The interpretation has been that one enzyme SH group, not involved in the formation of the presumed acyl-enzyme complexes, is essential only for the deacylation processes which are thus selectively prevented from occurring in the presence of the tiol reagent. More recently, it has been observed that both enzyme activity and penicillin binding capacity of the membrane-bound 43,000-Mr DD-carboxypeptidase of *Streptococcus faecalis* ATCC 9790 and its 30,000-Mr water-soluble derivative obtained by trypsin treatment of the membranes, are inhibited by low dose levels of para-chloromercuribenzoate, suggesting that this DD-carboxypeptidase might be a thiol enzyme[8]. The 29,000-Mr DD-carboxypeptidase of *B. coagulans* might also belong to this class of enzymes[9]. Further experiments, however, are required to confirm this possibility since chemical reagents of this type might be directed towards some satellite amino-acid residues which operate in conjunction with the serine residue or the Zn^{++} cofactor and its ligands, in the enzyme active centers.

Conclusions

Knowledge of the active centers of the DD-carboxypeptidases, the primary targets of penicillin, has just emerged from darkness. The data obtained with the few enzymes examined show that mechanistically different DD-carboxypeptidases exist, and shed some light on the molecular mechanisms which underly the bewildering variations that the penicillin receptors exhibit with respect to their sensitivities to β-lactam antibiotics.

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