MECHANISTIC PROPERTIES AND FUNCTIONING OF DD-CARBOXYPEPTIDASES

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The bacterial target of β-lactam antibiotics consists of a set of multiple receptors which are localized within the plasma membrane. Fluorography of polyacrylamide gel electrophoreses in SDS of membranes radioactively labeled by reaction with [^{14}C]benzylpenicillin permits detection of the receptors, or at least some of them, as penicillin binding proteins (*52*). Their apparent molecular weights range between 25 and 140 Kdaltons. Some of these proteins have been characterized as penicillin-sensitive enzymes; they are DD-carboxypeptidases, transpeptidases, and endopeptidases. The affinities of the β-lactam antibiotics for their receptors vary widely depending upon the protein or the enzyme under consideration, the organism, and the nature of the β-lactam compound. Wide variations also occur with respect to the number of copies (from 20 to several thousands) of penicillin-binding proteins present in one given bacterial cell.

Obtaining DD-carboxypeptidases in a truly water-soluble form and devising peptides with substrate activities for these isolated enzymes are all the more important for a detailed understanding of the functioning of these penicillin receptors.

Assays have been developed that employ synthetic and/or natural peptides functioning directly as donors and, for transpeptidation reactions, as amino acceptors of the DD-carboxypeptidases (*33*). In addition, truly water-soluble DD-carboxypeptidases can be obtained in a number of ways. For example, the 43-Kdalton DD-carboxypeptidase 4 of various gram-negative bacteria is solubilized by treating the cells with a Ribi fractionator or by submitting the isolated membranes to hypertonic conditions (*53*). Conversion of the cells of various *Streptomyces* strains into protoplasts is accompanied by the release of a water-soluble 40-Kdalton DD-car-

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boxypeptidase (*43*). Removal by trypsin action of a 2–3-Kdalton fragment from the C-terminal of membrane-bound DD-carboxypeptidases of *Bacilli* yields slightly shortened and water-soluble enzymes (*56*). Similarly, the membrane-bound, 43-K dalton DD-carboxypeptidase of *S. faecalis* is converted under trypsin action into a water-soluble 30-Kdalton protein (*6*). Although one third of the original molecule is eliminated, this 30-Kdalton protein still functions perfectly as a DD-carboxypeptidase and penicillin-binding protein. During incubation of the isolated membranes of *S. faecalis* at 37°C and at alkaline pH, the 80-Kdalton penicillin-binding protein (of unknown enzyme activity) undergoes rapid and quantitative conversion (probably as a result of some endogenous proteolytic activity) into a water-soluble 73-Kdalton penicillin binding protein (*6*). Finally, various strains of *Actinomycetes* have the property, apparently unique, to excrete DD-carboxypeptidases in the external medium during growth. The release mechanism is under study. These organisms have been widely used as sources of water-soluble DD-carboxypeptidases. The R61 enzyme (from *Streptomyces* R61) (*24*), the G enzyme (from *S. albus* G) (*14*), and the R39 enzyme (from *Actinomadura* R39) (*30*) have been purified to protein homogeneity. Both the R61 (*41*) and G enzymes (*12*) have been crystallized.

In this paper, the current state of our knowledge of the mechanistic properties and functioning of the enzyme active centers of the DD-carboxypeptidases is presented.

THE REACTIONS CATALYZED

The DD-carboxypeptidases are involved in the last stages of wall peptidoglycan synthesis. Like the proteases, they catalyze the opening of amide bonds and transfer the carbonyl carbon to an exogenous nucleophile (HY):

$$-\overset{\overset{O}{\|}}{C}-N< + HY \rightarrow -\overset{\overset{O}{\|}}{C}-Y + HN< .$$

The DD-carboxypeptidases are specifically designed to operate on the amide bond of the D-Ala-D-Ala dipeptide of L-R-D-Ala-D-Ala terminated peptides (where R is most often a diamino acid residue). In addition, β-lactam antibiotics are also used in a manner analogous to the natural carbonyl donors. The endocyclic amide bond is exposed on the α face of the β-lactam antibiotics in a position roughly equivalent to that of the amide bond of D-Ala-D-Ala (Fig. 1).

Depending on the nucleophile that serves as acceptor of the L-R-D-alanyl moiety, the enzymes function as hydrolases (HY=H_2O) or transpeptidases (HY=a suitable amino compound). Hydrolysis and transpeptidation may occur concomitantly; they compete with each other and the channelling of the enzyme activity in either pathway depends on the microenvironmental conditions (*25*, *38*).

The enzyme requirements for a D-amino acid residue at the C-terminal position of the peptide substrates are not strictly restricted to D-alanine, which can be replaced at this position by other D-amino acids or Gly, although most often at the expense

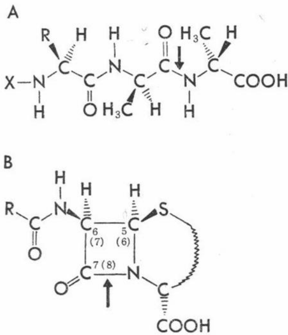
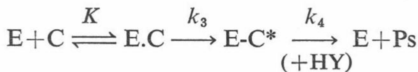


FIG. 1. Amide bonds (arrows) attacked by the DD-carboxypeptidases.
A: L-R-D-Ala-D-Ala terminated peptides. The N-terminal amino group is substituted by X. B: β-lactam antibiotics. The carbon atoms in the β-lactam ring are numbered 5, 6, 7 in the penicillins and (6), (7), (8) in the Δ^3-cephalosporins.

of substrate activity. The DD-carboxypeptidases vary widely in this respect. Some of them have a high propensity to hydrolyze D-Ala-D-X linkages where the D-center of the C-terminal X residue is substituted by very bulky groups (*44*), as for example, the D-Ala-(D)-*meso*-diaminopimelic interpeptide linkages which in the peptidoglycans of many gram-negative bacteria occur in the α-position to a free carboxyl group. Such enzymes may function as powerful endopeptidases.

The general equation of the reaction for both L-R-D-Ala-D-Ala terminated peptides and β-lactam antibiotics is:



$$E + C \overset{K}{\rightleftharpoons} E.C \xrightarrow{k_3} E\text{-}C^* \underset{(+HY)}{\xrightarrow{k_4}} E + Ps$$

where E=enzyme; C=carbonyl donor; E. C=first stoichiometric complex; E-C*= modified complex; Ps=reaction products; K=dissociation constant of complex E.C; and k_3 and k_4=first-order rate constants. The interaction between enzyme, peptide, and β-lactam drug is a competition between substrates (*22, 23*). However, while k_4 is high with the L-R-D-Ala-D-Ala terminated peptides (high turnover numbers), it is very low with the β-lactam antibiotics. β-Lactam drugs are suicide substrates, which explains their potency as enzyme inactivators. Although somewhat arbitrarily, a good β-lactam inactivator of a given enzyme should have a k_3/K value of 1,000 M^{-1} sec^{-1} or more, and a k_4 value of 1×10^{-4} sec^{-1} or less. Under these conditions, at a β-lactam drug concentration smaller than the dissociation constant K, 99% of the enzyme is inactivated in the steady state and the time required for the reaction to reach 95% of the steady state is about 5 min (*36*).

MECHANISTIC PROPERTIES

Central to the problem are the following questions: 1) Are the complexes E-C* formed with L-R-D-Ala-D-Ala terminated peptides or β-lactam antibiotics covalently bound to acyl-enzyme intermediates? 2) Assuming that such acyl-enzyme complexes are formed, what are the enzyme amino acid residues involved in the linkages?

Proteases operate by at least four different mechanisms. The serine proteases (trypsin) and the thiol proteases (papain), on the one hand, catalyze the hydrolysis of sensitive amide bonds *via* the transitory formation of covalently ester or thiolester-linked acyl-enzyme complexes, The acid proteases (pepsin) and the metalloproteases (carboxypeptidase A), on the other hand, are thought not to form acyl-enzyme complexes. Recent progress has established that the DD-carboxypeptidases also fall into several classes depending upon their distinctive mechanistic properties.

1. The Serine R61 DD-Carboxypeptidase

The water-soluble, 38-K dalton R61 enzyme has been crystallized (Fig. 2) (*41*). X-ray diffraction photographs of well-formed octahedral crystals show orthorhombic 222 Laue symmetry. The unit cell dimensions are: a=51.1 Å, b=67.4 Å, and c= 102.9 Å. The space group is $P2_12_12_1$. With four molecules of molecular weight 38,000,

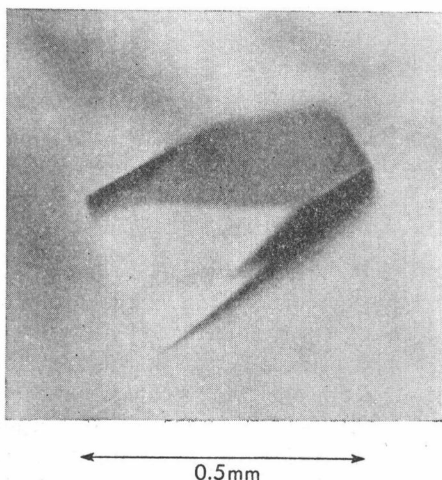


FIG. 2. Orthorhombic crystal of the R61 serine DD-carboxypeptidase.
The enzyme was crystallized from polyethylene glycol (MW 6,000–7,500) solution at pH 7.6. Well-formed octahedral crystals developed from a solution containing 14 g of polyethylene glycol per 100 ml of 0.05 M imidazole-HCl buffer. The final protein concentration was 13.3 mg/ml. After centrifugation, the protein solution was slowly concentrated over a reservoir of 28% polyethylene glycol solution by vapor diffusion at 10°C.

FIG. 3. Fourier map of the native R61 enzyme at 5-Å resolution phased by uranyl derivative.
The x-axis is perpendicular to the plane of the figure. One-sixth of the sections of the x-axis is included in the figure.

the $Å^3$/dalton ratio for the cell is 2.33. Data have been collected to 5-Å resolution for both the native crystal and a very isomorphous uranyl derivative ($K_3UO_2F_5$). Figure 3 shows a portion of the Fourier map of the native enzyme, phased by the uranyl derivative (figure of merit, 0.70). The radius of gyration of the native enzyme, as measured by small angle X-ray scattering, is 20.8 ± 0.5 Å (in 10 mM phosphate buffer, pH 7.8, and for a protein concentration of 16 mg/ml). Assuming that the molecule is spherical, this value corresponds to a molecular diameter of 52 Å, a value close to that for the crystallographic a unit cell dimension.

The characterization of the R61 enzyme as a serine DD-carboxypeptidase has been established by various procedures carried out on both β-lactam antibiotics and L-R-D-Ala-D-Ala-terminated peptides. Because of the very low k_4 value of the reaction, the complexes E-C* formed with various β-lactam antibiotics are stable enough to be easily isolated (*29*). NMR studies of complex E-C* formed with benzylpenicillin showed that the penicilloyl moiety is covalently bound in the denatured complex, with the penicilloyl group able to epimerize while still attached to the protein (*9*).

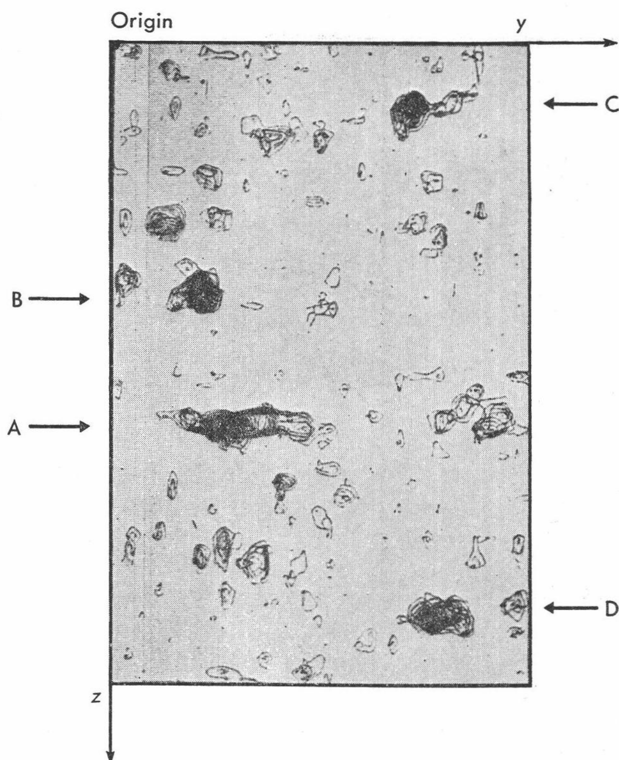


FIG. 4. Native R61 enzyme—*ortho*-iodo-phenyl penicillin difference map.
The *x*-axis is perpendicular to the plane of the figure. One-third of the sections of the *x*-axis is included in the figure. Peaks A and B: see text. The two peaks C and D on the right of the figure are symmetry equivalents of the two peaks on the left. This second set of peaks is centered on an *x*-level just above those sections included in the photograph.

Degradation of complex E-C* formed with [^{14}C]benzylpenicillin generated a tripeptide, Val-Gly-Ser, with the radioactive penicilloyl group ester-linked to the serine residue (*18*). The β-lactam *ortho*-iodo-phenylpenicillin was successfully diffused into a native crystal, and the difference Fourier map at 5-Å resolution was calculated (*10*). In Fig. 4, the site of interaction is well visualized as the 22-Å segment of difference density elongated in the *y* direction of the map (peak A). Also observed in Fig. 4 is a peak at similar *x* and *y* coordinates but at a lower *z* coordinate. This peak B is probably associated with a conformational change induced in the native structure upon interaction with this penicillin.

The isolation and characterization of complex E-C* from L-R-D-Ala-D-Ala-terminated peptides was made possible by using as a substrate the depsipeptide Ac_2-

L-Lys-D-Ala-D-lactate instead of the standard tripeptide Ac_2-L-Lys-D-Ala-D-Ala. As first observed with the *Bacilli* DD-carboxypeptidases, the k_3 term of the reaction is then markedly increased and becomes higher than the k_4 term so that complex E-C* accumulates (*51*). On this basis, the reaction with the R61 enzyme was shown by Yocum and Strominger (*59*) to proceed through an acyl-enzyme complex. In addition, the Ac_2-L-Lys-D-alanyl and penicilloyl moieties were shown to be covalently bound to the same enzyme serine residue (*59*).

The effects of serine-containing active site directed reagents were studied. Phenylmethanesulfonyl fluoride had no effect on the enzyme activity nor on its ability to bind penicillin (*18*), but methanesulfonyl fluoride and diisopropylphosphofluoridate inhibited both the enzyme activity on substrate analogs and benzylpenicillin binding with second order rate constants of 0.5 and 1.2 M^{-1} sec^{-1} (*32*). The inactivation rate with diisopropylphosphofluoridate, however, is considerably smaller than that observed with the serine proteases, suggesting inefficient binding.

2. Serine DD-Carboxypeptidases Other than the R61 Enzyme

With the membrane-bound 40-K dalton DD-carboxypeptidases of *B. subtilis* and *B. stearothermophilus* (which can be converted into water-soluble forms by trypsin action), it has been shown that the Ac_2-L-Lys-D-alanyl (from Ac_2-L-Lys-D-Ala-D-lactate) and penicilloyl moieties are covalently linked to the same enzyme serine residue (*60*). The sequences of the NH_2-terminal 40 amino acid residues of the two *Bacilli* enzymes are known, and in both cases the active serine occurs at the 36-position (*57*). By aligning these sequences with those of the serine β-lactamases at the active serine, DD-carboxypeptidases and β-lactamases might exhibit some degree of homology, at least in this region of the molecules.

The water-soluble 57-Kdalton R39 enzyme is another serine DD-carboxypeptidase. When covalently bound to this enzyme, cephaloglycine, cephalexin, and cephalosporin C have their ε_{260} decreased to the same extent as that obtained after β-lactamase action (*31*) and nitrocefin has a $\varepsilon_{482}/\varepsilon_{386}$ ratio of 2.40, which is also that obtained after β-lactamase action. Moreover, degradation of complex E-C* formed with [^{14}C]benzylpenicillin yielded a heptapeptide Leu-Pro-Ala-Ser-Asn-Gly-Val with the radioactive penicilloyl moiety ester-linked to the serine residue (*15*).

3. The Metallo G DD-Carboxypeptidase

The water-soluble 18-Kdalton G DD-carboxypeptidase effectively hydrolyzes R-D-Ala-D-Ala terminated peptides with a turnover number one order of magnitude lower than those of the R61 or R39 serine DD-carboxypeptidases (*14*, *44*). However, the G enzyme differs drastically from the latter enzymes in several respects: i) the G enzyme utilizes only H_2O as a nucleophile and is unable to catalyze transpeptidation reactions, suggesting that it lacks a structured acceptor site for amino compounds (*50*); ii) it exhibits high endopeptidase activities (*44*); and iii) it has a low propensity to react with β-lactam antibiotics, especially with the penicillins. The k_3/K values which govern the formation of complexes E-C* are very low: 9×10^{-3} M^{-1} sec^{-1} for

benzylpenicillin and 6×10^{-2} M^{-1} sec^{-1} for cephalosporin C (*19*). For comparison, the corresponding values for the same antibiotics are 14,000 and 1,150 M^{-1} sec^{-1} with the R61 enzyme (*22*) and >90,000 and 67,000 M^{-1} sec^{-1} with the R39 enzyme (*31*). Because of these very low k_3/K values, detection of the G enzyme as a penicillin binding protein requires high [^{14}C]benzylpenicillin concentrations and prolonged periods of incubation. Although low, these k_3/K values are nevertheless 10- to 100-fold higher than those observed with proteins such as lysozyme or insulin (*3*) which are completely devoid of DD-carboxypeptidase activity.

As shown by proton-induced X-ray emission studies, the native G enzyme possesses one Zn^{2+} ion per molecule (*13*). The affinity of the Zn^{2+} ion for the apoprotein expressed by an association constant of about 1×10^{13} M^{-1}, is of the same order of magnitude as for EDTA. The Zn^{2+} cofactor, or its Co^{2+} substitute, is required for activity on Ac_2-L-Lys-D-Ala-D-Ala and, apparently, for penicillin binding (high concentrations of EDTA cause a 10-fold decrease in the k_3/K value) (*13*). The Zn^{2+} (Co^{2+}) ligands have not been characterized and the nature of the complexes E-C* formed with the G enzyme is unknown. With the Zn^{2+} β-lactamase II of *B. cereus*, the reaction that this enzyme catalyzes on its substrate, penicillin, probably proceeds through a random mechanism in which the ternary complex enzyme-metal-penicillin may be reached by alternative pathways (*39*). The Zn^{2+} (Co^{2+}) ligands in this β-lactamase II are three histidine residues and the solitary cysteine residue (*39*).

The G enzyme has been crystallized (*12*) (Fig. 5). Precession X-ray photographs of well-formed prismatic crystals show that the crystals belong to the $P2_1$ space group, with unit cell dimensions a=51.1 Å, b=49.7 Å, c=38.7 Å, β=100.6 Å, and

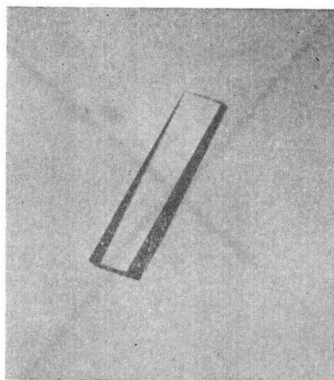


FIG. 5. Crystal of the Zn^{2+} G DD-carboxypeptidase.
The enzyme was crystallized using the vapor diffusion method. Droplets (10 μl) of the enzyme preparation (2% protein, final concentration) in 50 mM Tris-HCl, 5 mM $MgCl_2$, 10 mM NaN_3, 6% (w/v) polyethylene glycol 6000 were equilibrated against 12% (w/v) polyethylene glycol solution. The crystals grew in a few weeks, with a prismatic shape, to a maximum size of $1.4\times0.6\times0.6$ mm^3.

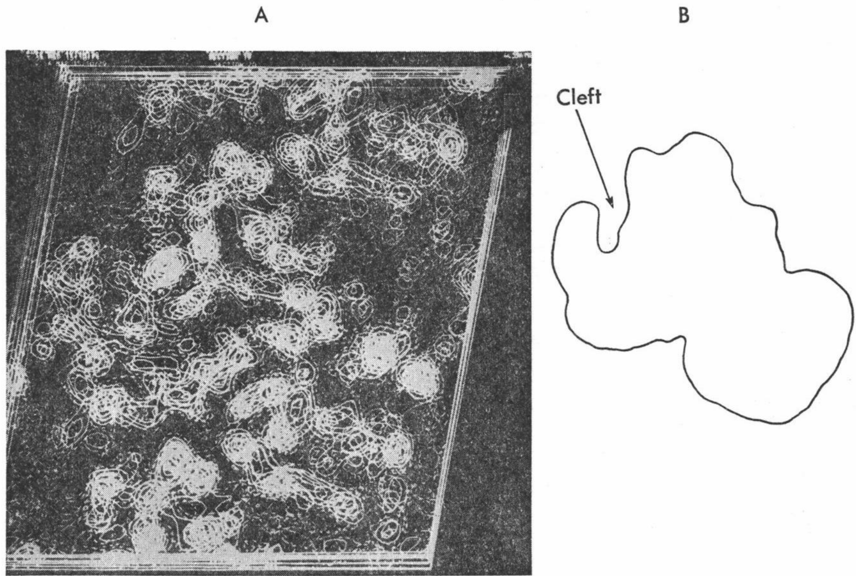


FIG. 6. A: Portion of Fourier map of the native G enzyme at 4.5-Å resolution phased by three heavy atom derivatives. The map is viewed down to the *y*-axis. B: Schematic representation of the molecular envelope as devised from A.

one molecule in the asymmetric unit. Data have been collected to 4.5-Å resolution for the native crystal and three heavy atom derivatives, K_2PtCl_4, $NaUO_2(CH_3COO)_3$, and $K_2Pt(C_2O_4)_2$, respectively (*11*) (Figs. 6 and 7). The Fourier map of the native enzyme phased by these three derivatives (figure of merit, 0.86) reveals the presence of two enzyme molecules per unit cell, surrounded by a very low electron density region. Each enzyme molecule can be inscribed in a 48 Å × 34 Å × 28 Å ellipsoid, and consists of two globular domains connected by three strands of electron density. The largest domain has a deep cleft (20 Å × 6 Å × 6 Å) in the vicinity of which the map shows a prominent peak, presumed to be the Zn^{2+} ion cofactor.

Crystallographic studies have also permitted the visualization of the enzyme active center (*11*). The dipeptide Ac-D-Ala-D-Glu (a competitive inhibitor of the hydrolysis of Ac_2-L-Lys-D-Ala-D-Ala) and the β-lactam *para*-iodo-7-β-phenylacetylaminocephalosporanic acid were successfully diffused in native crystals and the difference Fourier maps calculated at 4.5 Å. The difference Fourier synthesis for the enzyme-dipeptide inhibitor complex gave one peak which was three times higher than any other feature in the map. The site of interaction is well 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^{2+} ion site. Although the difference Fourier synthesis for the enzyme-β-lactam complex was much more noisy, the highest peak found

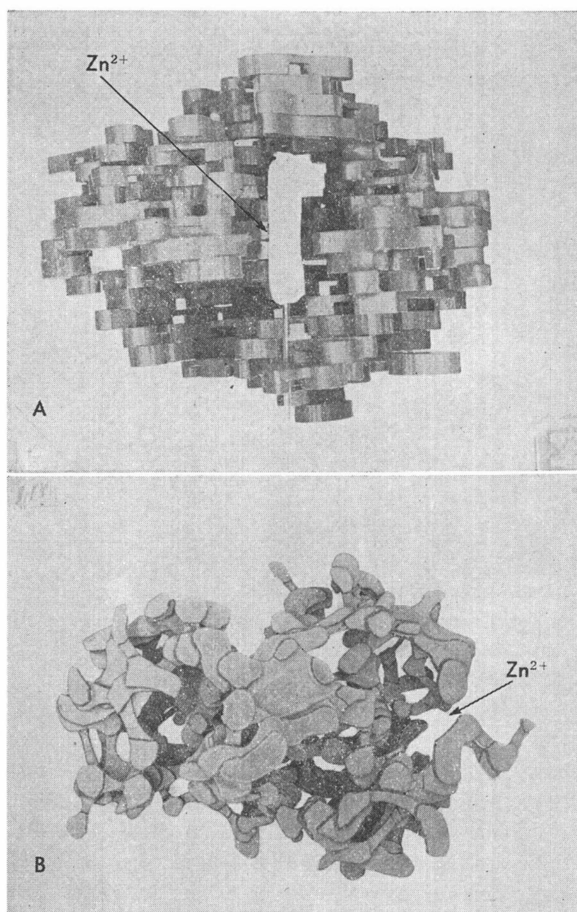


FIG. 7. A: Model of the G enzyme molecule looking into the cleft of the large domain. Arrow marks the Zn^{2+} ion site. B: Model of the G enzyme molecule viewed along the *y*-axis and showing the two domains. Arrow marks the Zn^{2+} ion site. The difference density observed with the peptide inhibitor is represented in white, inside the cleft.

in the map was also located in the same cavity. These data suggest that the enzyme active center, where binding of the dipeptide and the *para*-iodo-cephalosporin inhibitors occurs, is situated in the vicinity of the Zn^{2+} ion site. In agreement with this conclusion, the inhibition of the enzyme activity on Ac_2-L-Lys-D-Ala-D-Ala by 7-aminocephalosporanic acid was found to be competitive, under conditions where inhibition was due exclusively to the formation of the first complex E. C between enzyme and the β-lactam compound (*40*). Surprisingly, under similar conditions,

cephalosporin C behaved as a noncompetitive inhibitor (*19*). The reason why the two β-lactam compounds behave differently is not understood.

4. Other Possible Classes of DD-Carboxypeptidases

The *S. faecalis* membrane-bound 43-Kdalton DD-carboxypeptidase and its water-soluble 30-Kdalton derivative differ from the aforedescribed DD-carboxypeptidases in that their enzyme activities and penicillin-binding abilities are inhibited by low dose levels of *para*-chloromercuribenzoate, and dinitro 5,5′-dithiobis-(2-nitrobenzoate) (*6*), suggesting that this enzyme might be a thiol DD-carboxypeptidase. Further experiments, however, are required to confirm this possibility since the effects of chemical reagents of this type and others may be caused by reaction with some satellite amino acid residues which operate in conjunction with, for example, the serine residue or the Zn^{2+} cofactor and its ligands, in the active centers of the corresponding enzymes.

Low concentrations of *para*-chloromercuribenzoate inhibit the enzyme activity of the *E. coli* DD-carboxypeptidase 1A but not its penicillin-binding ability (*8*). The interpretation has been that one enzyme SH group, not involved in the formation of the presumed acyl-enzyme complexes, is essential for the deacylation processes (k_4), which are therefore prevented from occurring in the presence of the thiol reagent. With the serine R61 enzyme, the use of *ortho*-methylisourea or 2,4-dinitrobenzene suggests that one ε-amino group of lysine is probably involved in both enzyme activity and penicillin binding (*2*). Methylglyoxal, 2,3-butanedione, and phenylglyoxal are also inhibitors of the R61 enzyme activity on the peptide substrates, suggesting the involvement of an arginine residue (*32*). The same α-dicarbonyls inhibit benzylpenicillin binding but to a much lesser degree, depending on the size of the α-dicarbonyl side chain (*32*). Characterization of these satellite amino acid residues is a topic for future research.

FUNCTIONING

One approach to an understanding of the functioning of the enzyme active centers has been to study the effects that each residue of the tripeptide Ac_2-L-Lys-D-Ala-D-Ala or each portion of the β-lactam antibiotic molecules exerts on the various kinetic parameters of the corresponding reactions. Since the carbonyl carbon that is transferred to the exogenous nucleophile belongs to the amide bond of D-Ala-D-Ala or the β-lactam ring, the fused bicyclic ring and the 6(7)-β-substituent in the penicillins or Δ^3-cephalosporins are regarded as the counterparts of the D-Ala-D-Ala sequence and the preceding L-residue in the tripeptide substrate, respectively (Fig. 1).

With the β-lactam compounds and because of the high stability of the corresponding acyl-enzyme complexes, the k_4 term, k_3/K ratio, and, in some cases, the K and k_3 terms of the reaction can be determined directly (*17, 22, 23, 26, 29, 31*). With the peptide substrates and because of the very low stability of the corresponding acyl-enzyme complexes, the terms of the reaction that could be measured were K_m

and V_{max} (*42, 44, 45*). As a first approximation, K_m and V_{max} were considered as equivalent to K and k_3. Note that with Ac$_2$-L-Lys-D-Ala-D-Ala, $k_4 > k_3$ and therefore $V_{max} = k_3[E_o]$. If, in addition, $k_2 \gg k_3$ ($E + C \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E.C$; with $k_2/k_1 = K$), then $K_m = K$.

1. The Serine DD-Carboxypeptidases

The R61 and R39 enzymes have been studied extensively and the results have been reviewed in detail elsewhere (*17, 33–36*). The main conclusions are presented here. From the data so far available, the same conclusions probably apply to the serine DD-carboxypeptidases of *Bacilli* (*55*) as well as to the presumed thiol DD-carboxypeptidase of *S. faecalis* (*4–7*).

1) Initial binding (K)

Three distinct features characterize the first step of the reaction. i) Neither the L-residue of the peptide nor the 6(7)-β-substituent of the β-lactam is involved, at least substantially, in initial binding. On the contrary, that part of the relevant molecules which is mainly responsible for binding to the enzyme binding site n°1 is the C-terminal D-Ala-D-Ala sequence or the equivalent portion of the bicyclic fused ring system. ii) There is a strict requirement for the occurrence of a D-Ala residue at the penultimate position or an intact β-lactam ring. Conversely, the enzymes have a less strict requirement for a D-Ala at the C-terminal position, and, similarly, the β-lactam ring may be fused to various cyclic compounds (thiazolidine in the penicillins, dihydrothiazine in the Δ^3-cephalosporins, *etc.*). iii) Whether the peptides have high or poor substrate activities, or are nonsubstrate inhibitors (*48*), and whether the β-lactam compounds have high or poor inactivating properties, the K term of the reaction is always rather high (0.1–10 mM). One may thus conclude that initial binding is not very selective nor very efficient.

2) Enzyme acylation (k_3)

The second step of the reaction consists of a series of connected events. Once the peptide or the antibiotic is bound to the enzyme, a suitable lateral chain on the L-residue or a suitable 6(7)-β-substituent on the β-lactam ring interacts with some specific enzyme groupings (binding sites n°2), inducing conformational changes in the protein. As a result, the enzyme center thus made catalytically active is then able to considerably increase the electrophilic character of the carbonyl carbon of the penultimate D-Ala or the β-lactam ring, so that, eventually, enzyme acylation is achieved. Induced conformational changes are suggested by X-ray crystallographic studies of the complex formed between the R61 enzyme and *para*-iodo-phenylpenicillin (see above). They are also reflected by the fluorescence quenching and alterations in the CD spectrum observed as a result of penicilloylation of this enzyme (*47*).

The interaction between a suitable side chain at the positions under consideration and the enzyme binding sites n°2 is remarkable in several respects as follow:

i) The interaction may be extremely effective. Thus, with the R61 enzyme, the k_3 value is about 200 sec^{-1} for benzylpenicillin, 1 sec^{-1} for ampicillin, and 2×10^{-4}

sec^{-1} for the 6-(β)unsubstituted aminopenicillanic acid. Similarly, the turnover number on Ac_2-L-Lys-D-Ala-D-Ala is 3,300; it is decreased by 15% with Ac_2-L-A_2bu-D-Ala-D-Ala and by 99% for Ac-D-Ala-D-Ala, which in fact, becomes an inhibitor of the enzyme activity on Ac_2-L-Lys-D-Ala-D-Ala. Similar observations have been made with the R39 enzyme.

ii) The interaction may be extremely specific. Thus, although a long side chain at the L-position in the peptide is a general requirement for substrate activity, the occurrence of charged groups at this terminal position may exert very specific effects. Succinylation instead of acetylation of the ε-amino group of L-lysine, lack of substitution of this amino group, or replacement by other charged groups may, depending on the enzyme, increase, decrease, or suppress substrate activity. Similarly, the effects of a given side chain on the 6(7)-β-position of the β-lactam antibiotics, in particular its degree of steric hindrance and rigidity, the occurrence of charged groups, *etc.*, vary widely depending on the enzymes.

iii) Effective side chains on the L-residue of the peptide or at the 6(7)-β-position in the antibiotic molecule are (apparently) completely unrelated, suggesting the occurrence, in a given enzyme, of two binding sites n°2, for the bound peptide and the bound β-lactam compound, respectively.

iv) In its ground state conformation, the amide nitrogen of the β-lactam ring of penicillins and Δ^3-cephalosporins, on the one hand, is pyramidal, and must be so for the reaction to occur effectively. Δ^2-Cephalosporins, in which the nonplanarity of the β-lactam nitrogen is less pronounced, and monocyclic β-lactam compounds are weak acylating agents and poor enzyme inactivators. The amide nitrogen of D-Ala-D-Ala, on the other hand, is planar and, in spite of this lack of reactivity, hydrolysis of sensitive peptides may occur at very high rates. Thus, there are considerable differences in the heights of the barrier to reaction with the enzyme that are overcome as a result of the interaction of the lateral side chain of the bound peptide or the 6(7)-β-substituent of the bound β-lactam molecule, with the relevant enzyme binding sites n°2. Some of the recently discovered Δ^2-penems and 1-carba-Δ^2-penems, with no or very simple side chains, exhibit antibacterial activities. The nonplanarity of their β-lactam nitrogen is considerably more pronounced than in penicillins and Δ^3-cephalosporins (*58*) so that binding of the nucleus itself to at least some enzyme receptors may be sufficient to cause rapid opening of the β-lactam ring and acylation of the enzyme active centers.

3) Deacylation (k_4) of L-R-D-alanyl-enzyme complexes

Enzyme deacylation is the process through which the serine ester linkage formed during enzyme acylation is, in turn, broken down with concomitant transfer of the acyl moiety to an exogenous nucleophile. With the peptide substrates, deacylation is a rapid process and water can serve as a nonspecific acceptor (DD-carboxypeptidase activity). The serine enzymes, however (at least the R61 and R39 enzymes), also possess structured amino acceptor sites which confer on them very specific transpeptidase activities (*28, 37, 38, 49, 61*). Thus by using peptide monomers such as

Ac-L-Lys-D-Ala-D-Ala or L-Ala-D-Glu-(L)-A_2pm-(D)-D-Ala-D-Ala
Gly

where the N-terminal glycine or the N-terminal group of the D center of meso-diaminopimelic acid, fits the acceptor sites of the R61 and R39 enzymes, respectively, peptide dimers can be synthesized *in vitro* that are identical or very similar to those synthesized *in vivo* during wall peptidoglycan synthesis in the corresponding bacteria. Steady-state kinetic studies suggest that transpeptidation follows an ordered pathway in which binding of the amino nucleophile to the enzyme occurs before binding of the carbonyl carbon donor (*25*). In aqueous media and with simple amino compounds such as D-Ala or Gly, hydrolysis and transpeptidation interfere with each other on a simple competitive basis. With complex amino nucleophiles related to wall peptidoglycan synthesis, the phenomenon is more complicated (*25, 38*). In the presence of increasing concentrations of such amino compounds, not only is hydrolysis progressively inhibited but transpeptidation, after rising to a maximum, is in turn progressively inhibited so that, eventually, the enzyme may be frozen in a nonoperational state. Hence, these amino nucleophiles not only serve as acceptors of the L-R-D-Ala-D-Ala moiety but may also act as modulators of enzyme activity. Crystallographic studies should contribute, in the near future, to a better understanding of these phenomena.

4) Deacylation (k_4) of penicilloyl (cephalosporoyl)-enzyme complexes

With the β-lactam antibiotics, enzyme deacylation is a low or very low process with k_4 values ranging, for instance, from 2.8×10^{-4} sec^{-1} for the interaction between the R61 enzyme and phenoxymethylpenicillin, and 3×10^{-7} sec^{-1} for the interaction between the R39 enzyme and cephalosporin C. The stability of the corresponding acyl-enzyme complexes has been attributed to a specific interaction between the monocyclic thiazolidine (dihydrothiazine) ring and a specific enzyme binding site n°3, conferring on the acyl-enzyme complex a conformation that is not suitable for attack by an exogenous nucleophile (*34–36*). This interpretation rests upon the observation that with the penicilloyl-R61 enzyme complex, once the stabilization effect has been eliminated by C-5–C-6 cleavage of the penicilloyl moiety and protonation at C-6 (Fig. 1), the serine ester-linked N-acylglycyl fragment thus produced is then susceptible to immediate nucleophilic attack (by water or a suitable amino nucleophile) (*20, 21, 27, 46*). Fragmentation is the rate limiting step of the deacylation process. The mechanism is not yet understood. It might proceed *via* an oxazolinone-thiazolidine derivative (*54*). It is known that the thiazolidine part of the penicilloyl moiety is converted into as yet an unidentified compound Z which is further processed to give rise to N-formyl-D-penicillamine (*1*).

Enzyme deacylation may also (slowly) proceed by elimination, without fragmentation, of the bound metabolite. Thus the product of the reaction between the R61 enzyme and nitrocefin is the corresponding cephalosporoic acid (unpublished results). This latter pathway may also occur with other Δ^3-cephalosporins but the characterization of the product(s) thus formed is a difficult problem, since it is known that

hydrolysis of the β-lactam amide bond in the Δ^3-cephalosporins (by β-lactamase action) is usually accompanied by a series of further changes in the molecules.

From the foregoing, it appears that the thiazolidine (dihydrothiazine) part of the β-lactam molecule fulfils antagonistic functions. By distorting the β-lactam ring, it forces the β-lactam nitrogen to adopt a pyramidal character, a feature which is essential for enzyme action. By interreacting with the enzyme binding site n°3, the same thiazolidine (dihydrothiazine) forces the reaction to stop at the abortive level of the acyl-enzyme intermediate.

2. The Metallo G Enzyme

Although mechanistically different from the serine R61 and R39 DD-carboxypeptidases, the Zn^{2+} G enzyme is a DD-carboxypeptidase of comparable efficiency and with similar substrate requirements for L-R-D-Ala-D-Ala-terminated peptides. Moreover, and in complete analogy with the serine enzymes, the conformation of the Zn^{2+}-containing active center in the G enzyme is modulated by the structure of the side chain at the L position of the peptide substrates (*44*). In marked contrast, the G enzyme has an extremely low propensity to react with β-lactam antibiotics (see above), not that binding (K) is less efficient, at least with Δ^3-cephalosporins, but because, irrespective of the structure of the 6(7)-β-substituent, the k_3 term of the reaction is always extremely low ($\geq 1 \times 10^{-3}$ sec^{-1}) (*19*). Phenotypically, the G enzyme, which has an effective binding site n°2 for bound peptides, lacks the binding site n°2 for bound β-lactam compounds. Fianlly, breakdown of complex E-C* (whatever its structure) formed with benzylpenicillin (at high concentrations and after prolonged incubation) releases benzylpenicilloate ($k_4=0.6 \times 10^{-4}$ sec^{-1}) (*16*). The G enzyme behaves as a penicillinase of very low efficiency.

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