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USE OF MODEL ENZYMES IN THE DETERMINATION OF THE MODE OF ACTION OF PENICILLINS AND Δ^3-CEPHALOSPORINS[1]

❖12003

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PERSPECTIVES AND SUMMARY

Studies carried out with exocellular penicillin-sensitive enzymes (PSEs) from various strains of actinomycetes (the only organisms known to excrete such enzymes during growth), show that:

[1]Abbreviations used are: PBP, penicillin-binding protein; PSE, penicillin-sensitive enzyme.

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(*a*) The penam or 3-cephem nuclei are the main portion of the antibiotic molecules concerned with the initial reversible binding to the enzyme. Binding to the enzyme active center is neither very selective nor very efficient but it has specific effects on the enzyme conformation. The sites thus induced in the enzyme react with suitably oriented and structured N_{14} substituents and force the β-lactam ring to gain a much increased chemical reactivity. As the β-lactam ring opens (with the help of electron attracting groups) and achieves acylation of the enzyme, the thiazolidine or dihydrothiazine ring interacts with a nearby site thus greatly increasing the stability of the acyl-enzyme complex.

(*b*) Elimination of the bound residue and regeneration of a free active enzyme by deacylation in the presence of a suitable exogenous nucleophile may proceed according to two different pathways. PSEs slowly overcome the stabilizing effect of the bound penicilloyl molecule by C_5–C_6 cleavage; deacylation of the N-acylglycyl-enzyme complex thus formed is then immediate whereas the other fragmentation product is further processed and eventually released as *N*-formyl-D-penicillamine. Alternatively, fragmentation does not occur and the bound residue is slowly eliminated as a whole. Irrespective of the pathway, the overall reactions between PSEs and β-lactams are characterized by very low turnover numbers.

(*c*) In some respects the above mechanism resembles that through which analogues of the natural substrates, L-*R*-D-Ala-D-Ala-terminated peptides (where *R* is an amino acid residue), interact with the PSEs. The D-Ala-D-Ala portion is mainly responsible for the initial binding whereas a suitable side chain in the preceding L residue is required to induce enzyme action on the bound peptide, which forces the D-Ala-D-Ala amide linkage to lose all double bond character. However, whether the enzyme functions as a catalyst template or forms a transitory acyl intermediate, the transfer of the L-*R*-D-Ala moiety to the exogenous nucleophile is rapid, and therefore the reactions are characterized by high turnover numbers.

(*d*) Depending upon the PSEs, the independence between the penicillin and L-*R*-D-Ala-D-Ala active centers is more or less pronounced. Indirect but strong evidence suggests that the physiological function of the β-lactam center in the PSEs may be regulatory rather than catalytic. By interacting with it, the β-lactam inactivates the specific catalytic center of the enzyme thus preventing action on natural substrate analogues.

Exocellular PSEs are probably not physiologically important whereas the membrane-bound components (or at least some of them) are likely to be crucial in mediating the antibacterial β-lactam effect. The relevance of the exocellular model systems to the actual problem of the mechanism of penicillin action has been examined by extending the above studies to several membrane-bound PSEs originating from taxonomically unrelated

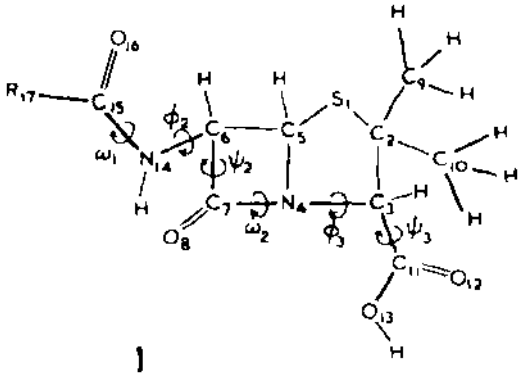
bacteria. The data suggest that they react with β-lactam antibiotics according to the same basic mechanism as that described above.

SCOPE AND LIMITATION

The damages caused by penicillins and Δ^3-cephalosporins to growing bacteria are related to the ability of these antibiotics to bind to several proteins (penicillin-binding proteins or PBPs) and to inactivate several enzymes (penicillin-sensitive enzymes or PSEs). With few exceptions, PBPs and PSEs are membrane-bound and in some cases, PBPs and PSEs are known to be synonymous. The understanding of the mechanism through which the β-lactams exert their antibacterial effects requires a combination of studies. They include: (*a*) the pore properties of those proteins (porins) which facilitate the permeation of the β-lactams through the outer membrane of the gram-negative bacteria (1–3); (*b*) the identification and genetic analysis of the PBPs and PSEs and the roles that they fulfil in vivo (4–7); (*c*) the physiological and biochemical events that follow the inactivation of the PBPs/PSEs and frequently culminate in cell death and lysis (8); and (*d*) the interaction at the molecular level between the β-lactams and the PBPs/PSEs (9–11).

Penicillins and Δ^3-cephalosporins (Figure 1) are stereochemically similar, not because of their detailed dimensions and conformations, but because the N–CO bond of the β-lactam ring does not have the normal character of a free amide. On the basis of X-ray data in the solid state, the dihedral angles about the pyramidal β-lactam nitrogen (ω_2) are 135° for penicillins and 155° for Δ^3-cephalosporins (16). Among the two sets of dihedral angles (ϕ_2, ψ_2) and (ϕ_3, ψ_3), ψ_2 and ϕ_3 are fixed to specific values by the lactam and the thiazolidine or dihydrothiazine rings. Hence the conformation depends on the two variable angles ϕ_2 and ψ_3 which determine the orientations of the aminoacyl substituent and the carboxyl grouping, respectively. A review on the functional modifications and nuclear analogues of β-lactam antibiotics can be found in (17, 18). Antibacterial activity (*a*) requires a β-lactam ring of sufficient strain and possibilities for electron delocalization outside the lactam ring; (*b*) appears to be associated with ϕ_2 rotational angles ranging, in solution, between – 180° and – 160°, i.e. with a ϕ_2 region termed region A (12); and (*c*) is considerably enhanced by the occurrence of suitable substituents on N_{14} and the presence of a carbonyl group in α position to the nitrogen atom of the lactam amide bond.

The present work is restricted to the roles played by the various portions of penicillins and Δ^3-cephalosporins and by the various residues of well-defined peptides used as natural substrate analogues, in their interactions with several model PBPs/PSEs.



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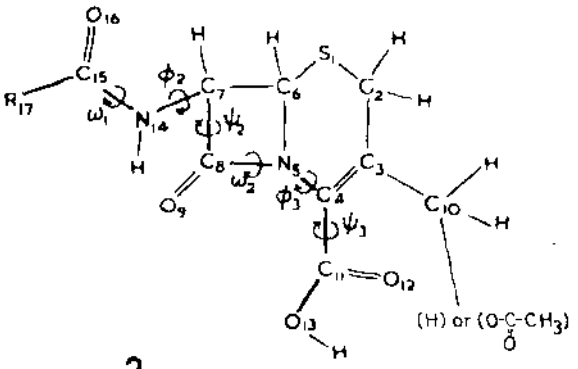

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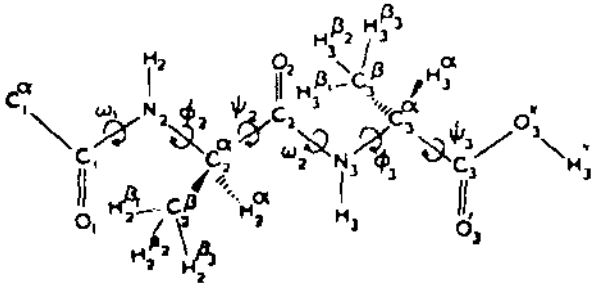

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MODEL ENZYMES

PSEs and PBPs are members of the bacterial wall peptidoglycan cross-linking system which performs attachment of the nascent peptidoglycan strands to the preexisting wall peptidoglycan, controls the final extent of cross-linkages, and imparts to the bacterial wall its shape and physical strength (19). The main known reactions catalyzed by the PSEs are nucleophilic attacks of glycan-substituted pentapeptides.

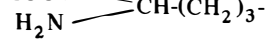
L-Ala-D-Glu ⌐ L-X-D-Ala-D-Ala
|ω
(H)

where X is a diamino acid residue whose lateral chain is either free or extended by one or several additional amino acids. Attacks occur on the carbonyl carbon of the penultimate D-Ala. The nucleophile may be either water causing the formation of tetrapeptides (DD-carboxypeptidase activity; reaction *1* in Figure 2), or an amino compound (NH_2-R) causing the formation of

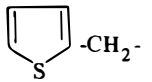
L-Ala-D-Glu ⌐ L-X-D-Ala-CONH-R
|ω
(H)

products (DD-transpeptidase activity; reaction *2* in Figure 2). Free D-Ala is produced by either pathway. DD-transpeptidase action on the same pep-

Figure 1 (*1*) Structure of penicillins (*2*) Δ^3-cephalosporins; and (*3*) X-D-Ala-D-Ala showing the backbone dihedral angles (ω_i, ϕ_i, ψ_i). The β-lactam ring is fused to another ring, either thiazolidine forming the penam nucleus of penicillins, or dihydrothiazine forming the 3-cephem nucleus of Δ^3-cephalosporins. With penicillins, $\omega_2 = 0$ when the bond C_7–N_4 eclipses the bond C_6–N_{14}, and $\phi_2 = 0$ when the bond C_6–C_7 eclipses the bond N_{14}–C_{15}; clockwise rotation is considered as positive (12). All penicillins are derivatives of 6-aminopenicillanic acid (a condensed L-cysteinyl-D-valine dipeptide); R_{17} is C_6H_5-CH_2- in benzylpenicillin, C_6H_5-O-CH_2- in phenoxymethylpenicillin, C_6H_5-CH(NH_2)- in ampicillin, and C_6H_5-CH(COOH)- in carbenicillin. Cephalosporins with a 3'-acetoxy side chain are derivatives of 7-aminocephalosporinic acid; R_{17} is C_6H_5-CH(NH_2)- in cephaloglycine,



(i.e. D-α-aminoadipic acid) in cephalosporin C, and



in cephalothin. Cephalexin is a Δ^3-cephalosporin with a 3-methyl side chain (instead of an acetoxy) and with R_{17} = C_6H_5-CH(NH_2)-. Antibacterial β-lactams are known that either do not contain a sulphur atom in their ring system, such as thienamycine (13) and clavulanic acid (14), or are not fused to another ring, such as the monocyclic nocardicins (15). [Adapted from (12) with the permission of Munksgaard, Copenhagen.]

tide acting both as carbonyl donor and as nucleophilic acceptor leads to peptide dimer formation (reaction *2* in Figure 2, middle part). DD-carboxypeptidases can act on interpeptide bonds extending between D-Ala and another D-center in α-position to a free carboxyl group (reaction *5* in Figure 2); such DD-carboxypeptidases behave as endopeptidases, performing hydrolysis of those peptide dimers previously formed by DD-transpeptidase action. Finally, the peptidoglycan cross-linking system also performs LD-carboxypeptidase (reaction *3* in Figure 2) and LD-transpeptidase (reaction *4* in Figure 2) activities. Model PSEs catalyzing these latter reactions are not discussed in this work.

Three exocellular PSEs have been used. The R39 (M_r 53,000), R61 (M_r 38,000), and G (M_r 20,000) enzymes are excreted by *Actinomadura* R39, *Streptomyces* R61, and *Streptomyces albus* G, respectively. They have been purified to homogeneity (20–22) and their chemical, physical, and immunological properties have been studied extensively (20–25). The three enzymes give rise to different peptide maps (after proteolysis with trypsin; C. Duez and J. M. Frere, unpublished). Both R39 and R61 enzymes probably consist of a single polypeptide chain but the G enzyme might consist of two chains

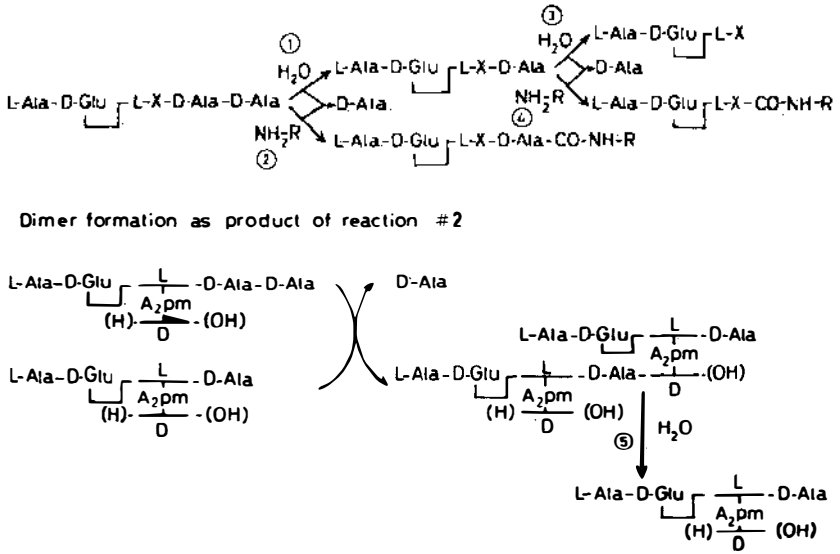


Figure 2 Schematic representation of the enzyme activities performed by the peptidoglycan cross-linking enzyme system. (*1*) DD-carboxypeptidase activity; (*2*) DD-transpeptidase activity; (*3*) LD-carboxypeptidase activity; (*4*) LD-transpeptidase activity; (*5*) DD-endopeptidase activity. In the natural substrates, the L-Ala residues substitute the glycan strands through *N*-acetylmuramyl-L-Ala linkages.

of similar size. Ac_2-L-Lys-D-Ala-D-Ala is an analogue of the natural carbonyl donor substrate. The general equation for the enzyme-catalyzed nucleophilic attacks (HY = the nucleophile) is Ac_2-L-Lys-D-Ala-D-Ala + HY $\rightarrow$ Ac_2-L-Lys-D-Ala-Y + D-Ala. With HY = H_2O (hydrolysis pathway), the reaction products are Ac_2-L-Lys-D-Ala and free D-Ala. With HY = NH_2-*R* (transpeptidation pathway), the reaction products are the transpeptidated derivative Ac_2-L-Lys-D-Ala-CONH-*R* and free D-Ala. In DD-carboxypeptidase assays, the turnover number of the R61 and R39 enzymes are high (3300 and 1050, respectively). In the presence of suitable amino nucleophiles, both enzymes also function as DD-transpeptidases; they catalyze transpeptidation reactions occurring concomitantly with the hydrolysis of the peptide donor (26–30). The G enzyme is a DD-carboxypeptidase that has a relatively low turnover number of 150. It does not function as a transpeptidase (31) but it is the only one of the three enzymes under consideration that exhibits a high endopeptidase activity and solubilizes isolated bacterial walls where the peptidoglycan subunits are cross-linked through C-terminal N^{α}-(D-Ala-D) linkages (32, 33) (reaction *5* in Figure 2).

Bacteria possess from three to eight or more membrane-bound PBPs. PBPs of low molecular weight (20,000–50,000) have been identified as PSEs. Like the exocellular PSEs, they act on simple carbonyl donor-nucleophilic acceptor systems. The possible enzymic functions of the PBPs of high molecular weight (50,000 to 100,000 or more) is unknown. The three following membrane-bound PBPs/PSEs are especially relevant to this work but others also receive attention.

1. *Streptomyces* R61 possesses a major PBP (or perhaps a triplet; M_r about 22,000) that has the following properties (34–37):

(*a*) It catalyzes nucleophilic attacks on Ac_2-L-Lys-D-Ala-D-Ala of suitable amino nucleophiles and functions primarily as a DD-transpeptidase.

(*b*) A temperature as low as – 30°C is necessary to prevent the membranes from catalyzing the reaction, which suggests that the enzyme functions in a lipid environment that remains remarkably fluid at low temperature.

(*c*) The enzyme can be solubilized with *N*-acetyl- or *N*-dodecyl-*N,N,N*-trimethyl-ammonium bromide or chloride. The solubilized and partially purified enzyme continues to function almost exclusively as a DD-transpeptidase but it is inactive in the frozen state.

(*d*) The enzyme is probably the lethal target of penicillins and cephalosporins in this bacterium.

2. *Streptococcus faecalis* ATCC 9790 possesses at least six PBPs (38). The 43,000 dalton PBP is a DD-carboxypeptidase-transpeptidase but its

ability to perform transpeptidation reactions in vitro (with Ac_2-L-Lys-D-Ala-D-Ala) is limited to simple amino nucleophiles such as D-amino acids and Gly-Gly (39). The enzyme can be solubilized with Genapol X-100 and has been highly purified (38). The isolated membranes also perform an LD-transpeptidase activity (Ac_2-L-Lys-D-Ala + NH_2-*R* → Ac_2-L-Lys-CONH-*R* + D-Ala) which is sensitive to various penicillins and Δ^3-cephalosporins (40, 41). This latter enzyme has not been isolated and its possible identification with one of the PBPs is unknown.

3. *Proteus mirabilis* strain 19 possesses six PBPs. It can grow as an unstable L-form in the presence of benzylpenicillin; the wall peptidoglycan made under these conditions is a round and fragile structure of apparently normal composition (42). Binding of [^{14}C]benzylpenicillin to the membranes isolated from these L-forms reveals the presence of PBPs 5 and 6. PBP5 (M_r: about 40,000) has been highly purified (43, 44). It is a DD-carboxypeptidase, active on L-Ala-D-Glu-(L)-*meso*-A_2pm-(L)-D-Ala-D-Ala. Whether or not it also functions as a DD-transpeptidase remains to be established.

INTERACTION BETWEEN PSEs AND β-LACTAM ANTIBIOTICS

The three exocellular R61, R39, and G enzymes have been of particular interest in this work. They react with β-lactams according to the same scheme (24, 45, 46)

$$E + I \overset{K}{\rightleftharpoons} EI \xrightarrow{k_3} EI^* \xrightarrow{k_4} E + \text{degraded antibiotic}$$

In the above equation E = enzyme and I = antibiotic. K is the dissociation constant of the stoichiometric complex EI; k_3 and k_4 are first order rate constants. The second and third steps are irreversible and the third step regenerates the free, active enzyme. Complex EI^* exhibits a rather high stability (low k_4); hence, enzyme regeneration is a slow or very slow process.

Formation of complex EI^* as a function of time is given by:

$$[EI^*]/[E_0] = k_a(1-e^{-(k_4 + k_a)t})/(k_4 + k_a) \text{ or}$$

$$t = -\ln(1-(k_4 + k_a)[\mathrm{EI}^*]/k_a \cdot E_0)/(k_4 + \mathrm{k_a})$$

where $k_a = k_3/(1 + K/[I])$.

The time at which $[EI^*]/E_0$ is 95% of the value at the steady state is $t_{0.95} = 3/(k_4 + k_a)$. At the steady state (*ss*) $[EI^*]_{ss}/E_0 = k_a/(k_4 + k_a) = 1/(1 + (k_4/k_3) + k_4K/k_3[\mathrm{I}])$ and the fraction of enzyme that remains functional under these conditions is given by $[E]_{ss}/E_0 = 1/(1 + ([I]/K) + k_3[I]/K\mathrm{k_4})$.

Initial Binding and Activation

Table 1 gives the K, k_3 and k_4 values for the interactions between the three exocellular model enzymes and several penicillins and Δ^3-cephalosporins. (Because of technical difficulties, only the k_3/K ratios could be determined in some cases.) In no case is breakdown of the EI^* complex a rapid process.

The analysis of the K and k_3 values that govern the formation of complex EI^*, shows that:

(*a*) Whether the β-lactam belongs to the penam or 3-cephem series, and irrespective of the nature of the substituents on N_{14} or of the enzyme used, the K term is never low. The relative dimensions and conformations of the various groupings influence the strength of the initial binding.

(*b*) The side chains on N_{14} exert great influences on the k_3 term. Thus, for example, k_3 for the interaction between the R61 enzyme and benzylpenicillin is 180 sec^{-1} whereas the corresponding k_3 for 6-aminopenicillanic acid is 2×10^{-4} sec^{-1}; hence, removal of the N_{14} substituent results in a 10^6-fold decreased k_3 value.

(*c*) The effects of a given side chain vary greatly depending upon the enzyme.

The best interpretation of the above observations is that: (*a*) the enzymes appear to have a binding surface that is neither very efficient nor very

Table 1 Values of the constants involved in the interaction between the exocellular model PSEs and β-lactam antibiotics (at 37°C unless otherwise stated)

		Formation of complex EI^*			Breakdown of complex EI^*	
Enzyme	Antibiotic[a]	k_3/K (M^{-1} sec^{-1})	K (mM)	k_3 (sec^{-1})	k_4 (sec^{-1})	Half-life (min)
G (45)	phenoxymethylpenicillin	0.005	150	0.0008	9×10^{-5}	130
	cephalothin	0.06	9.5	0.0005	3.3×10^{-5}	350
	cephalosporin C	0.06	1.6	0.0001	8×10^{-5}	145
R61 (44)	6-aminopenicillanic acid[b]	0.2	1	0.0002		
	cephaloglycine	22	0.4	0.009	3×10^{-6}	3,800
	ampicillin	107	7.2	0.77	1.4×10^{-4}	82
	carbenicillin	820	0.11	0.09	1.4×10^{-4}	82
	cephalosporin C	1,150	>1	>1	1×10^{-6}	10,000
	phenoxymethylpenicillin	1,500	>1	>1	2.8×10^{-4}	40
	benzylpenicillin	13,700 (25°C)	13 (25°C)	180 (25°C)	1.4×10^{-4}	82
R39 (25)	cephalosporin C	67,000 (20°C)	0.19 (20°C)	12.5 (20°C)	0.3×10^{-6}	38,000
	carbenicillin	2,920 (20°C)			5.4×10^{-6}	2,125
	cephalexin	3,000 (20°C)			2.4×10^{-6}	4,800
	ampicillin	74,000 (20°C)			4.4×10^{-6}	2,600
	cephaloglycine	74,000 (20°C)			0.8×10^{-6}	14,000
	benzylpenicillin	>90,000 (20°C)			2.8×10^{-6}	4,100
	cephalosporin 87-312	3,000,000 (10°C)			1.5×10^{-6}	7,700

[a] For the structure of the antibiotics, see Figure 1. Cephalosporin 87-312 is [3(3, 4 dinitrostyryl]-(6R-7R)-7-(2-thienylacetamido)-cephem-3-em-4-carboxylic acid, E-isomer (47).

[b] Unpublished data.

selective; (*b*) the penam or 3-cephem nuclei are the main portion of the β-lactam antibiotics concerned with the initial binding; and (*c*) both efficiency and selectivity of β-lactam action are, primarily, the consequence of efficiency and selectivity in the k_3 term.

The sequence of events may be visualized as that suggested by Rando (48). Once the β-lactam is bound to the enzyme, the substituent on a correctly positioned N_{14} (so that ϕ_2 falls inside region A) functions as the handle through which the enzyme distorts the antibiotic from its already strained ground state structure, greatly increasing the chemical reactivity of the β-lactam amide bond. As the β-lactam ring rapidly opens, the strain is relieved and acylation of a nearby enzyme side chain is completed. The free COOH grouping of the fused thiazolidine or dihydrothiazine rings may also be part of the handle or may function as an electron attracting group or both. According to this model, the k_3 term expresses the efficiency with which the β-lactam is converted by the enzyme target into a very reactive form which in turn rapidly acylates the enzyme.

Enzyme Acylation

Serine is one of the enzyme residues with which benzylpenicillin collides as a result of its binding to and activation by the R61 enzyme (49). (This enzyme possesses 29 serine residues.) Nucleophilic attack occurs on C_7 of the bound antibiotic molecule and a benzylpenicilloyl-serine ester linkage is formed (Figure 3). The process (i.e. formation of complex *EI**) causes conformational changes to the enzyme as shown by a decrease of its fluorescence and by an extensive alteration of its CD spectrum in the near ultra violet (23). Phenylmethanesulfonyl fluoride has no effect on the ability of the R61 enzyme to bind penicillin (49) but diisopropylphosphofluoridate prevents benzylpenicillin binding; (P. Charlier and J. M. Frere, unpublished). Moreover, on the basis of the effects of 2,4-dinitrofluorobenzene, O-methylisourea, and the fused ring trimer of 2,3-butanedione, benzylpenicillin binding also requires one free ε-amino group of lysine, (P. Charlier and J.-M. Frère, unpublished). (The R61 enzyme possesses 6 lysine residues.)

When bound to the R61 or R39 enzymes in the form of their *EI** complexes, cephalexin, cephalosporin C, and cephaloglycine have their molar extinction coefficients at 260 nm decreased to an extent identical to that obtained after β-lactamase action [(50) and J.-M. Frère, unpublished]. When bound to the R39 enzyme, the chromogenic cephalosporin 87–312 has a $\epsilon_{482}/\epsilon_{386}$ ratio of 2.40 which is identical to that obtained after β-lactamase action (51). With the R61 enzyme, however, the $\epsilon_{482}/\epsilon_{386}$ ratio value is only 1.20 (50) (it becomes 2.40 through breakdown of complex *EI**). This blue shift may indicate differences between the two *EI** com-

plexes with regard to the electrostatic and/or the conformational changes involved.

With those Δ^3-cephalosporins that have a 3'-acetoxy side chain (Figure 1), opening of the β-lactam ring may cause further alterations of the bound molecule. Migration of the electron density to the ester oxygen of the CH_2OAc on C_3 may result in the release of acetate and formation of an exocyclic methylene group. The tendency of the 3 substituents to attract or accept electrons from the 3-cephem nucleus should make the β-lactam carbonyl a stronger acylating agent (52). Cephalothin or cephaloglycine which have a 3'-acetoxy side chain might function this way. However, cephalexin (Figure 1) with its acaudal 3-methyl side chain cannot. Hence, both the chemical reactivity of the β-lactam ring in its ground state conformation, and the increased reactivity gained by the β-lactam as a result of

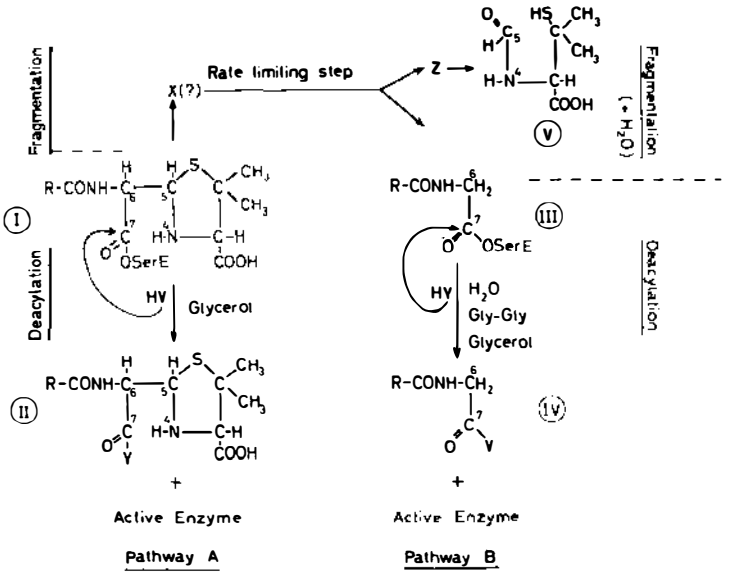


Figure 3 Breakdown of complex *EI** formed between penicillin and the R61 enzyme showing direct nucleophilic attack of the penicilloyl-ester linkage (*Pathway A*) and fragmentation of the penicilloyl moiety and attack of the acylglycyl ester linkage (*Pathway B*). (*I*) penicilloyl-enzyme (or *EI**) complex with the serine ester linkage on C_7 (R = C_6H_5-CH_2 or C_6H_5-O-CH_2); (*II*) the reaction product resulting from the direct nucleophilic attack of *I*. With the R61 enzyme, glycerol is the only functional reagent; hence *II* is the penicilloyl ester of glycerol; (*III*) acylglycyl-enzyme complex; (*IV*) the reaction product resulting from the nucleophilic attack of *III*, i.e. acylglycine if HY is H_2O; acyl-Gly-Gly-Gly if HY is Gly-Gly, and the acylglycyl ester of glycerol if HY is glycerol; (*X*) a hypothetical modified complex *EI** (see text); (*Z*) the primary product that arises from thiazolidine (see text and Figure 5); (*V*) *N*-formyl-D-penicillamine.

its binding to the enzyme, dictate the effectiveness of the acylation step. The virtual inactivity of the Δ^2-cephalosporins, which have less ring strain than the Δ^3-cephalosporins, probably results from the height of the barrier to reaction with the enzyme (53).

Nucleophilic Attack

Deacylation of the benzylpenicilloyl and phenoxymethylpenicilloyl-R61 enzyme complexes has received much attention. To reject the bound penicilloyl moiety and consequently to recover its initial conformation and activities, the R61 enzyme has developed two possible mechanisms (Figure 3). Neither of them is a rapid process (low k_4).

In Figure 3, *Pathway A* is a direct nucleophilic attack of the serine ester linkage resulting in the transfer of the penicilloyl moiety to the nucleophile HY and the regeneration of a free active enzyme. In *Pathway B,* the penicilloyl moiety is first transformed by C_5–C_6 cleavage into an N-acylglycyl residue (54–56) which remains linked to the enzyme, and a compound Z which in turn gives rise to free *N*-formyl-D-penicillamine. Z has a half-life of 10–15 min at 37°C and a neutral pH. As shown by isotopic studies with D_2O (56), the fragmentation reaction involves a rate-limiting step that is immediately followed by C_5–C_6 cleavage, protonation (or deuteration) of C_6, transfer of the acylglycyl moiety to the nucleophile, and regeneration of an active enzyme. Since the formation of free *N*-formyl-D-penicillamine is delayed when compared with the release of acylglycine, it follows that the processing of Z to *N*-formyl-D-penicillamine is irrelevant to the regeneration of the enzyme activity.

The various sites that in their ground state conformations form the β-lactam active center provide the R61 enzyme with a rather loose binding surface (Figure 4, site I). Sites II–VI in Figure 4, fulfill more selective functions. A revealing feature of *Pathway B* in Figure 3 is that C_5–C_6 cleavage is immediately followed by the release of the acylglycyl moiety. Thus, the stability of the ester bond between the penicilloyl moiety and the enzyme fixation site III (Figure 4) can be attributed to the monocyclic thiazolidine part of the molecule which by interacting with some stabilizing site IV probably confers to the penicilloyl-enzyme complex a conformation that is not favorable to nucleophilic attack. As the fragmentation step catalyzed by site V is completed, the catalytic groups of the releasing site VI undergo the correct alignment, and catalyze the immediate attack of the serine ester bond.

Figure 5 shows a hypothetical mechanism for the fragmentation step. The delayed release of *N*-formyl-D-penicillamine is tentatively attributed to the interaction of the primary fragmentation product Z with an enzyme amino group.

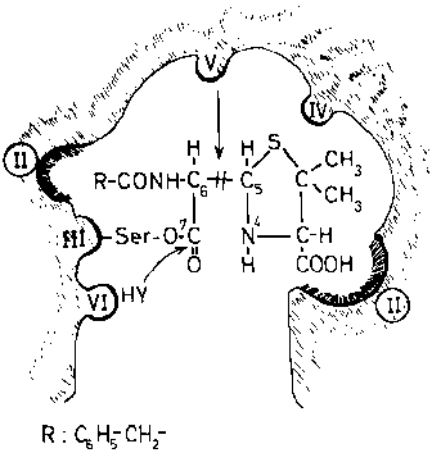


Figure 4 The penicillin-active center of the R61 enzyme. The enzyme binding surface (site *I*; not shown in the figure) is supposed to be a mosaic work of sites *II–VI*. Whether binding involves the α or β phases of the β-lactam fused ring system is not known. (*II*): activation sites : interaction with the substituents on N_{14} and the carboxyl grouping; (*III*): fixation or acylation site : nucleophilic attack of C_7 by a serine residue; (*IV*): stabilization site : interaction with the monocyclic thiazolidine part of the molecule; (*V*): fragmentation site catalyzing C_5–C_6 cleavage; (*VI*): releasing site catalyzing the attack of the serine ester linkage by a suitable nucleophile HY. Depending upon the PSEs and the nucleophile HY, *Pathways A* and/or *B* may occur (Figure 3). Although *A* has fewer steps than *B*, the overall rate of the former, where fragmentation does not occur, need not be faster.

Channelling of the enzyme-bound penicilloyl moiety through *Pathways A* and/or *B* (Figure 3) depends upon the exogenously available nucleophile. With the R61 enzyme and in water, *B* only is operational (54–56): phenoxymethyl- and benzylpenicillin are quantitatively fragmented into acylglycine and *N*-formyl-D-penicillamine. The effects of nucleophiles other than water are necessarily studied in aqueous media, i.e. under competition conditions (59). With Gly-Gly and other NH_2-R nucleophiles, the reaction also proceeds exclusively through *Pathway B:* acylglycine (from H_2O), acyl-Gly-Gly-Gly (or the corresponding acyl-Gly-Gly-CONH-R derivatives) and *N*-formyl-D-penicillamine are the end products (59). In a 20% solution of glycerol both *A* and *B* (Figure 3) occur. The reaction products are the penicilloyl ester of glycerol from *A*, and acylglycine, the acylglycyl ester of glycerol, and *N*-formyl-D-penicillamine from *B*. Attack on the penicilloyl moiety by glycerol through *Pathway A* is rather rapid but ceases after a short time (10–15 min). This observation suggests that the rate-limiting step of penicillin fragmentation is preceded by a faster reaction through which an intermediate (*X* in Figure 3) is formed which escapes

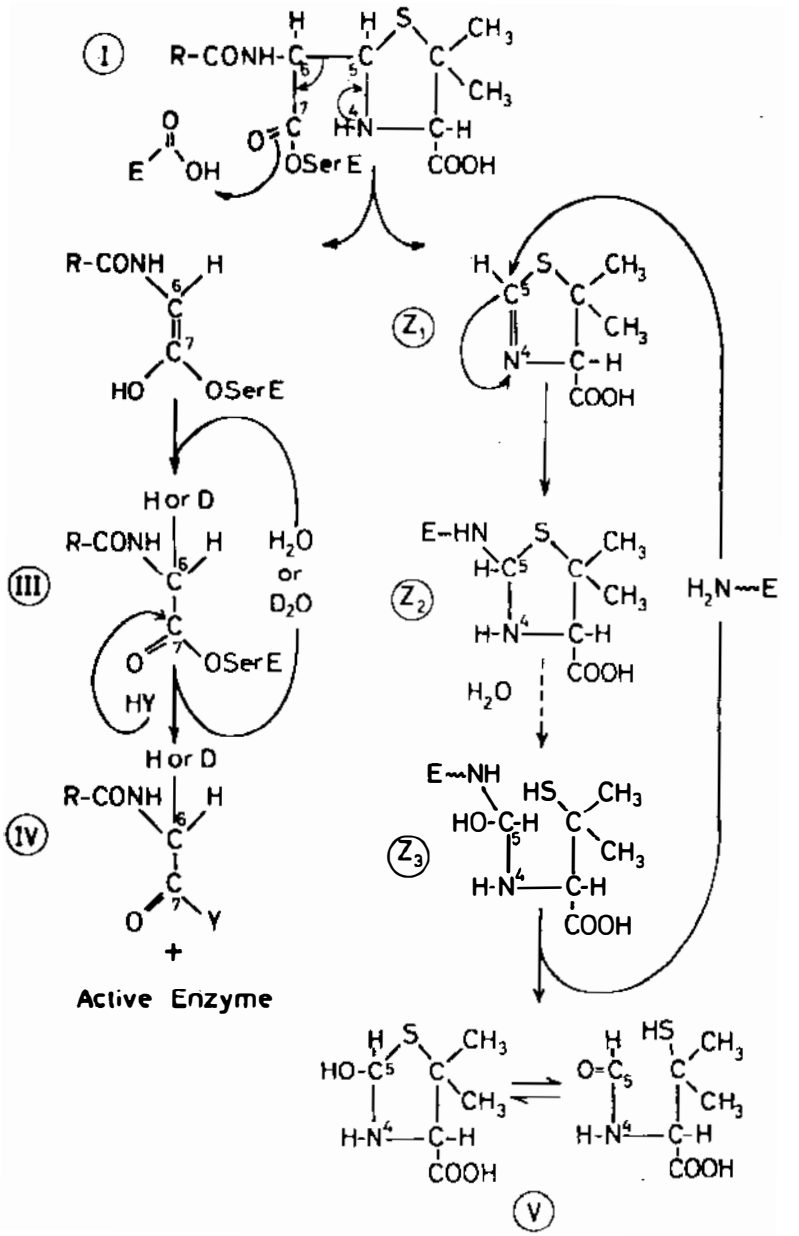


Figure 5 Hypothetical mechanism for the fragmentation of the penicilloyl-R61 enzyme complex *EI** (*Pathway B* in Figure 3). (*I*), (*III*), (*IV*), and (*V*) see Figure 3 except that here N-formyl-D-penicillamine, i.e. *V*, is represented in equilibrium with hydroxythiazolidine.

nucleophilic attack by glycerol. When fragmentation is achieved, attack of the acylglycyl moiety by both H_2O and glycerol occurs in a competitive manner and the partitioning between the two nucleophiles present does not alter the total rate of transfer.

The attacks on the enzyme-bound penicilloyl or enzyme-bound acylglycyl moieties by the exogenous nucleophile HY are directed against the same C_7 of the original penicillin molecule (Figures 3 and 4). The two processes exhibit great differences in their specificity profiles, which suggests that they are catalyzed by distinct enzyme sites. However, these sites may be different conformations containing the same grouping of amino acid residues, the change in conformation being induced by the fragmentation process (59). Finally, denatured complex *EI** also breaks down but an active enzyme is not regenerated, and in water, penicilloate is very slowly released (59).

The two mechanisms described above for the deacylation of complex *EI** formed between the R61 enzyme and benzylpenicillin are probably general but the channelling through either *Pathway A or B* (or both) appears to be the result of a complex interplay between the three reagents involved: the enzyme, the nucleophile and, most likely, the β-lactam itself. Thus in water, the R39 enzyme (54) fragments penicillin with formation of acylglycine (as observed with the R61 enzyme) but the G enzyme (46) catalyzes the hydrolysis of penicillin to penicilloate.

Membrane-Bound PSEs

Although it is not impossible that a suitable amino acid other than serine (for example, cysteine) might be involved in the initial attack of the β-lactam, membrane-bound PSEs essentially behave like the exocellular enzymes and give rise to rather stable *EI** complexes. Depending upon the β-lactam antibiotics, the k_3/K values range from 3–400 M^{-1} sec^{-1} with the

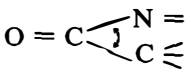
$Z_1 \rightarrow V$ represents the enzyme-catalyzed processing of 5,5-dimethyl-Δ^2-thiazoline-4-carboxylate. Pathway $Z_1 \rightarrow V$ would proceed with a half-life of about 10–15 min (at 37°C and neutral pH). The proposed mechanism rests upon the following observations: (*a*) Isotopic studies with D_2O show that the hydrogen or deuterium atom that is fixed on C_6 comes from H_2O or D_2O (56); (*b*) Benzylpenicillin methylester can be degraded nonenzymatically to methyl 5,5,-dimethyl-Δ^2-thiazoline-4-carboxylate in trifluoroacetic acid. The phenylacetylglycyl fragment was isolated by conversion to its *N*-benzyl amide (57). Enzymatically, however, free 5,5-dimethyl-Δ^2-thiazoline-4-carboxylate is not the primary product released from the thiazolidine part of penicillin (58). Evidence suggests the existence of an enzyme site that interacts with the monocyclic thiazolidine part of the bound benzylpenicilloyl molecule (see text). However, the proposed mechanism implies that the processing $Z_1 \rightarrow V$ occurs on enzyme sites unrelated to those involved in DD-carboxypeptidase-transpeptidase activity.

Streptomyces membrane-bound DD-transpeptidase (34, 36) and from 1–1200 M^{-1} sec^{-1} with the purified *S. faecalis* enzyme (38, 39). With this latter enzyme and benzylpenicillin, *EI** formation is a two-step process with $K = 0.025$ mM and $k_3 = 0.025$ sec^{-1} ($k_4 = 2.8 \times 10^{-5}$ sec^{-1}) (38). A similar mechanism has been proposed for the DD-carboxypeptidase of *B. subtilis* (60); with all the antibiotics tested, the K values are high, ranging from 0.1 mM to more than 10 mM. Finally, PBPs of high molecular weight and of unknown enzyme activity also form with [^{14}C]benzylpenicillin radioactive complexes that subsequently decay more or less rapidly (5, 61, 62).

With the *S. faecalis* enzyme (39) and the DD-carboxypeptidases from various bacilli (63, 64), breakdown of the complex *EI** formed with penicillin proceeds in water via the fragmentation pathway and acylglycine is produced. Incidentally, fixation of penicillin to the *B. subtilis* enzyme involves the formation of a penicilloyl-ester bond and the hydroxylaminolysis of the complex is catalyzed enzymatically (65). In contrast to the above enzymes, the 46,000 dalton PBP (a DD-carboxypeptidase-transpeptidase) of *S. aureus* (66) and the enzyme of the L-form of *Proteus* (67) catalyze in water the hydrolysis of penicillin to penicilloate. They are thus β-lactamases of low efficiency. PSEs of this type may have served as protoenzymes in the evolution of true β-lactamases. True β-lactamases have tremendously increased turnover numbers on β-lactam antibiotics but they have lost the ability to react with DD-carboxypeptidase-transpeptidase substrates. The *Streptomyces* DD-transpeptidase, when it is membrane-bound, is also a penicillinase of this type (34) but once it has been solubilized with the help of the cationic detergent, the same enzyme catalyzes penicillin fragmentation (36).

INTERACTION BETWEEN PSEs AND L-*R*-D-ALA-D-ALA-TERMINATED SUBSTRATE ANALOGUES

The natural substrates that undergo the nucleophilic attacks catalyzed by the PSEs do not end in an *N*-substituted β-lactam-fused ring system but in an *N*-substituted L-*R*-D-Ala-D-Ala sequence. Tipper & Strominger (68) had proposed that penicillin would be isosteric with the C-terminal D-Ala-D-Ala portion of the natural substrate and thus would function as an affinity labelling agent. However, the dihedral angles ω_2 in penicillins and Δ^3-cephalosporins (135 and 155°) in their ground state conformations are considerably smaller than that about the peptide bond (180°), and, in addition, the



bond angle of the β-lactam is 90.5°, whereas the corresponding bond angle in the dipeptide is 117° (Figure 6). Alternatively, it was proposed (68, 69) that penicillin would be isosteric with the transition state structure of the D-Ala-D-Ala portion of the natural substrate. On the basis of steric maps of D-Ala-D-Ala generated according to the ω_2 angle of penicillins and Δ^3-cephalosporins, β-lactams have conformations similar to the distorted dipeptide (12). Hence, according to this modified model, enzyme action on the natural substrate or its analogues should involve the distortion of the

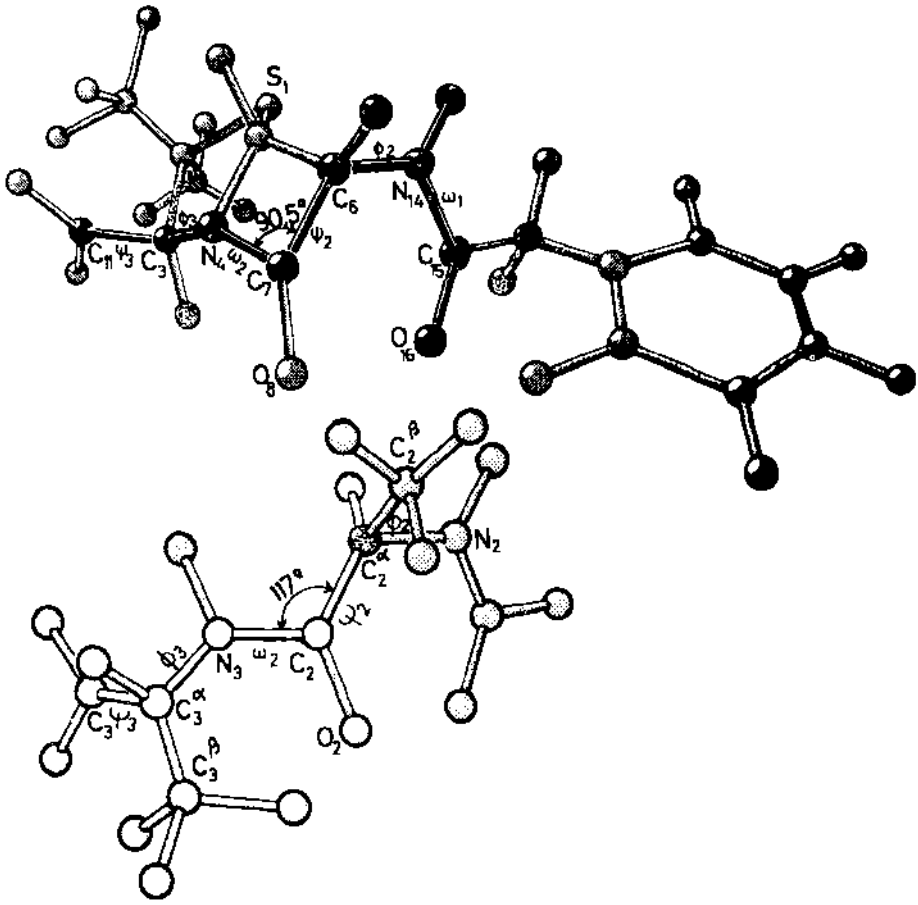


Figure 6 Ground state conformation of benzylpenicillin and its comparison with D-alanyl-D-alanine. [Adapted from (48) with the permission of Pergamon, Oxford.]

D-Ala-D-Ala peptide bond to a pyramidal shape. Such a mechanism seems to be very unlikely (48). Finally, to add confusion to the picture, it is worth mentioning that the LD-transpeptidase of *S. faecalis* (40) and the LD-carboxypeptidase of *Gaffkya homari* (70) are penicillin-sensitive.

Hydrolysis Pathway

The substrate requirements of the R39, R61, and G enzymes have been characterized by measuring the effects that each residue in the standard tripeptide Ac_2-L-Lys-D-Ala-D-Ala exerts on the K_m and V_{max} parameters, respectively, for the release of the C-terminal residue in water (hydrolysis pathways) (33, 71–73). These observations were made (Table 2). (*a*) The occurrence of L-Ala instead of D-Ala at the C-terminal or the penultimate positions and the occurrence of D-amino acids other than D-Ala at the penultimate position change the peptide into a nonsubstrate. The enzymes have a less strict specificity for a D-Ala residue at the C-terminal position where it can be replaced by other D-amino acids, although most often at the expense of substrate activity. Substitution of D-Ala by Gly at either one of the two positions under consideration also causes a more or less pronounced decrease of substrate activity; (*b*) Shortening the neutral side chain of the L-residue that precedes D-Ala-D-Ala (i.e. replacement of Ac_2-L-Lys by Ac_2-L-A_2bu, Ac-L-Hse, Ac-L-Ala, and Ac-Gly) causes a drastic decrease of substrate activity. Succinylation instead of acetylation of the L-lysine tripeptide, lack of substitution of the ϵ-amino group, or substitution with other charged groups may increase, decrease, or suppress substrate activity; (*c*) K_m values of good substrates are not much different and are often as high as those of poor substrates but good substrates have much higher V_{max} values. Hence, efficiency in substrate activity is primarily the consequence of efficiency in the V_{max} term; (*d*) Nonsubstrate peptides or peptides exhibiting very low substrate activity may act as inhibitors of the G enzyme (as measured on Ac_2-L-Lys-D-Ala-D-Ala). Ac-D-Ala-D-Ala is a weak inhibitor but Ac-Gly-D-Ala-D-Glu is a good inhibitor. Since Ac_2-L-Lys-D-Ala-D-Glu is a fair substrate, it follows that lack of the acetylated side chain of L-lysine can change a good substrate into a nonsubstrate inhibitory peptide. Ac_2-L-Lys-D-Glu-D-Ala is also a good inhibitor, whereas Ac_2-L-Lys-D-Ala-D-Ala is, of course, an excellent substrate; hence, replacement of the penultimate D-Ala whose carbonyl carbon undergoes nucleophilic attack, by another D-amino acid residue such as D-Glu can also change a substrate into an inhibitor. Inhibition of the R61 enzyme is usually smaller than that observed with the G enzyme, and none of the peptide inhibitors of the G and R61 enzymes have any effect on the R39 enzyme (in fact some of them are fair substrates of this latter enzyme). Obviously, the substrate, nonsub-

Table 2 Substrate activity of LDD terminated peptides for the exocellular model PSEs, (hydrolysis pathway)[a]

Peptide	G enzyme				R61 enzyme				R39 enzyme			
	K_m[b]	V_{max}[c]	Eff.[d]	Act.[e] (%)	K_m[b]	V_{max}[c]	Eff.[d]	Act.[e] (%)	K_m[b]	V_{max}[c]	Eff.[d]	Act.[e] (%)
1. Ac_2-L-Lys-D-Ala-D-Ala	0.33	100	300	100	12	890	72	100	0.8	330	410	100
Ac_2-L-Lys-D-Ala-D-Lys	0.80	85	106		13	90	7					
Ac_2-L-Lys-D-Ala-D-Leu	0.33	33	100		10	50	5		0.7	230	320	
Ac_2-L-Lys-D-Ala-D-Glu				100				10				
Ac_2-L-Lys-D-Ala-Gly	2.5	60	24		36	200	6		2.5	100	40	
Ac_2-L-Lys-D-Ala-L-Ala				0				0				0
2. Ac_2-L-Lys-Gly-D-Ala	15.0	107	7		15	1.7	0.1					0
Ac_2-L-Lys-D-Leu-D-Ala				0	10	10	1					0
Ac_2-L-Lys-D-Glu-D-Ala				0				0				
Ac-L-Lys-L-Ala-D-Ala				0				0				0
3. Ac-L-A_2bu-D-Ala-D-Ala	0.6	42	70					85				16
R_1-L-Hse-D-Ala-D-Ala[f]	1.0	16	16					3				7
Ac-L-Ala-D-Ala-D-Ala	3.3	0.7	0.2					1.4				0.2
Ac-Gly-D-Ala-D-Ala	1.1	1.9	2									
Ac-D-Ala-D-Ala	-			0				1				5
4. N^{α}-Ac-L-Lys-D-Ala-D-Ala	6	20	3		15	4	0.3		0.2	600	3,000	
R_2-(L)-*meso*-A_2pm-(L)-D-Ala-D-Ala[f]	0.4	10	25		11	8	0.3		0.25	400	1,600	
R_3-L-Lys-D-Ala-D-Ala[f] $(Gly)_5$	0.28	9	32		14	800	57		0.30	420	1,400	
Suc_2-L-Lys-D-Ala-D-Ala				5				16				36

[a] From (33, also 71–73). The experiments reported here were carried out at a time when none of the enzymes had been purified to homogeneity.
[b] K_m values are expressed in mM.
[c] V_{max} values are in μmoles of peptide hydrolyzed/mg protein/h.
[d] Eff. (efficiency) = V_{max}/K_m.
[e] Act. = substrate activity, simply expressed as a percentage of that of the standard tripeptide Ac_2-L-Lys-D-Ala-D-Ala.
[f] R_1: UDP-MurNAc-L-Ala-Gly-D-Glu┘; R_2: UDP-MurNAc-D-Glu┘; R_3: β-1,4 GlcNAc-MurNAc-L-Ala-D-Glu(amide)┘.

strate, and inhibitor activities of LDD-terminated peptides result from a complex interplay between the enzyme and each of the three amino acid residues under consideration.

The most general equation for the interaction between PSEs and L-*R*-D-Ala-D-Ala-terminated substrates is

$$E + D \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ED \xrightarrow{k_3} EP \xrightarrow{k_4} E + P,$$

where D is the carbonyl donor and P is the reaction product. If k_4 is very high (a likely assumption at least with the exocellular PSEs and good substrates as shown by the high turnover numbers), then $V_{max} = k_3 \times [E_o]$; if, in addition, $k_2 \gg k_3$, then $K_m = (k_3 + k_2)/k_1$ reduces to $K_m = k_2/k_1$ and becomes equal to the dissociation constant K of the reversible complex *ED*. Hence, as a first approximation the above observations suggest that (*a*) the enzymes have a binding surface that is neither very efficient nor very selective (high K_m or K); (*b*) the C-terminal D-Ala-D-Ala dipeptide is the main portion of the molecule concerned with the initial binding; and (*c*) a side chain of very definite molecular characteristics is required in the L residue that precedes D-Ala-D-Ala to induce enzymic action on the bound peptide (high V_{max} or k_3). In other words, an L residue whose lateral chain has the appropriate size, shape, and charge may be regarded as the handle through which the peptide, once bound to the enzyme, induces a correct alignment of the catalytic groups which in turn forces the amide bond of the D-Ala-D-Ala portion to adopt a configuration intermediate between *cis* and *trans* and to undergo rapid nucleophilic attack. With poor substrates, the induced change in the enzyme would be unfavorable or incorrect, markedly decreasing the V_{max} (or k_3) value or even preventing the enzyme action (73).

The above model is analogous to that proposed for the interaction with β-lactam antibiotics; (*a*) the D-Ala-D-Ala and the penam or 3-cephem nuclei, respectively, would be mainly involved in the initial binding. However, the corresponding ground state conformations exhibit little isosterism; and (*b*) the L residue that precedes D-Ala-D-Ala and the substituents on N_{14} of the β-lactam ring, respectively, would be mainly responsible for the activation of the relevant bound molecules. These side chains, however, are not structurally related.

The membrane-bound DD-carboxypeptidase of *S. faecalis* (40) and the enzyme that in the membrane-wall system of *Gaffkya homari* (74) catalyzes the cross-linking stage of peptidoglycan synthesis (presumably the transpeptidase) also discriminate between the residues in the C-terminal position of the peptide donor with a high specificity for a D-Ala-D-Ala sequence.

Whether the exocellular model enzymes function as catalyst templates or form Ac_2-L-Lys-D-Ala-enzyme intermediates remains to be established. PSEs isolated from membranes of various bacilli, *Escherichia coli,* and *S. aureus,* are transitorily acylated by the Ac_2-L-Lys-D-Ala moiety (66, 75, 76). By using trapping procedures, acyl enzyme intermediates can be isolated by Sephadex filtration (76) and/or detected after SDS polyacrylamide gel electrophoresis and fluorography (66). Enzyme acylation is probably mediated through an ester linkage (76). On the basis of these observations, Strominger and his colleagues have proposed a Ping Pong Bi Bi mechanism. An Ac_2-L-Lys-D-Ala-enzyme intermediate (*ED**) would be formed in the first step of the reaction and the Ac_2-L-Lys-D-Ala moiety (*D**) would be transferred to a suitable nucleophile in the second step:

$$E + D \underset{}{\overset{K}{\rightleftharpoons}} ED \xrightarrow{k_3} \begin{cases} ED^* + HY \xrightarrow{k_4} D^*Y + E \\ \text{D-Ala} \end{cases}$$

The release of D-Ala precedes the nucleophilic attack. With two competing nucleophiles (such as H_2O and NH_2-R), partitioning occurs at the level of complex *ED**.

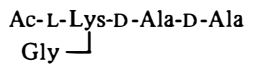
Transpeptidation Pathway

Some PSEs utilize either H_2O or amino compounds as nucleophilic reagents. They behave exclusively as DD-carboxypeptidases or DD-transpeptidases. Many PSEs, however, catalyze concomitant hydrolysis and transpeptidation reactions, the two pathways competing with each other. Their transpeptidation abilities may be limited to simple amino nucleophiles but several PSEs are known that catalyze much more complex reactions. The DD-carboxypeptidases of *E. coli* (77–79), the PBPs 4 and 5 of *Salmonella typhimurium* (61), and both R61 (26, 27, 30, 80) and R39 (26, 28, 29) enzymes perform cross-linking reactions leading to the formation of peptide dimers. Such PSEs must possess sites especially devised to operate with complex amino nucleophiles.

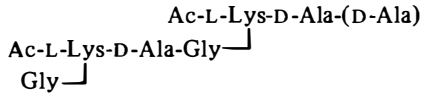
The exocellular model enzymes have revealed interesting features:

1. The profiles of the R61 and R39 enzymes for amino nucleophiles closely reflect the type of cross-linkage that exists in the peptidoglycan of the strain that produces the enzyme. In strain R39, the cross-link is from D-Ala to the amino group at the D-center of *meso*-diaminopimelic acid (28). Correspondingly, suitable amino groups must be in α-position to the carboxyl group of glycine or a D-amino acid (26). In strain R61, on the other hand, the cross-link is between D-Ala and a glycine residue that is attached to the ε-amino group of LL-diamino pimelic acid (81). In this case, glycine

and various peptides with an N-terminal glycine residue are efficient nucleophiles, although other amino compounds such as ω-amino acids, aminohexuronic acids, D-cycloserine, and 6-aminopenicillanic acid can also function (26). In addition, the R39 enzyme catalyzes dimerization reactions such as that shown in Figure 2 (29), and the R61 enzyme transforms the tetrapeptide monomer

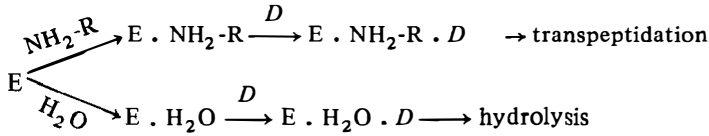


into the hexa- and heptapeptides,



(30, 80). The dimers thus made in vitro are identical or very similar to those formed in vivo during peptidoglycan synthesis in the corresponding bacteria.

2. The amino nucleophiles act as modulators of the enzyme activities. The effects of Ac_2-L-Lys-D-Ala-D-Ala and amino nucleophile concentrations on the rates of hydrolysis and transpeptidation suggest, for the transpeptidation reaction, an ordered pathway mechanism in which the amino nucleophile NH_2-R binds first to the R61 enzyme (27, 82)



In such a mechanism, hydrolysis is decreased (by competition) whereas the overall reaction (hydrolysis + transpeptidation) may be unchanged, increased, or decreased. With the R61 enzyme and simple amino nucleophiles (such as *meso*-diaminopimelic acid), the overall reaction is unchanged and the transpeptidation/hydrolysis ratio is proportional to the nucleophile concentration. However, at high concentrations of complex nucleophiles (related to peptidoglycan structure), transpeptidation is also inhibited and the transpeptidation/hydrolysis ratio is smaller than expected, which suggests that a second molecule of the amino nucleophile is fixed on the enzyme, yielding a nonproductive quaternary complex E. D. $(NH_2\text{-R})_2$. Features of complex amino nucleophiles also exert profound effects on the relative amounts of transpeptidation and hydrolysis catalyzed by the R39 enzyme (28, 29). Thus, when increasing concentrations of the amidated tetrapeptide L-Ala-D-Glu (amide)—(L)-*meso*-A_2pm-(L)-D-Ala are provided as nucleophile to the R39 enzyme with Ac_2-L-Lys-D-Ala-D-Ala,

hydrolysis is progressively inhibited and transpeptidation rises to a maximum at a definite tetrapeptide concentration; at higher concentrations, both transpeptidation and hydrolysis are progressively inhibited until eventually the tripeptide donor Ac_2-L-Lys-D-Ala-D-Ala remains unused (Figure 7). Excess of amidated tetrapeptide can thus freeze the enzyme. The phenomenon is dependent on the α-amide group of the D-Glu residue, since high concentrations of the same nonamidated L-Ala-D-Glu┌(L)-*meso*-A_2pm-

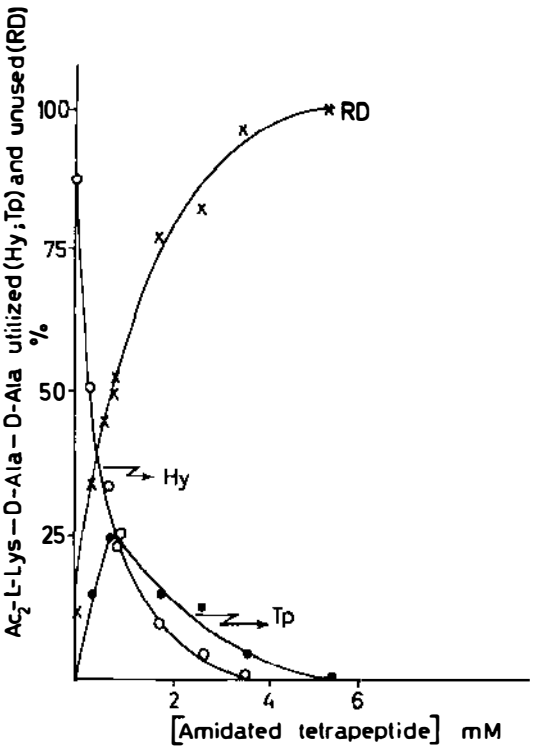
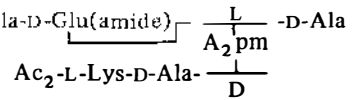


Figure 7 Competition between H_2O and increasing concentrations of the tetrapeptide L-Ala-D-Glu(amide)┌(L)-A_2pm-(L)-D-Ala for the nucleophilic attack of Ac_2-L-Lys-D-Ala-D-Ala (0.27 m*M*). Hy : hydrolysis (formation of Ac_2-L-Lys-D-Ala); Tp : transpeptidation, i.e. formation of



RD : residual Ac_2-L-Lys-D-Ala-D-Ala. Note that at 5.4 mM of amidated tetrapeptide, the enzyme is completely inactive. [Adapted from (29)].

(L)-D-Ala tetrapeptide limits the hydrolysis of Ac_2-L-Lys-DAla-D-Ala but does not decrease the amount of transpeptidation product formed. The phenomenon is also dependent on the degree of saturation of the enzyme by the carbonyl donor peptide; the less saturated the enzyme, the lower is the concentration of amidated tetrapeptide required to inhibit transpeptidation as well as hydrolysis. The complex interplay between enzyme, D-Ala-D-Ala-terminated substrates and nucleophilic acceptor that the above experiments reveal is not accounted for by a simple acylation and deacylation model.

3. Whether the substrate is a β-lactam or an L-*R*-D-Ala-D-Ala terminated peptide, PSEs exhibit great differences in their specificities for the nucleophilic reagents. Thus, glycerol (a 20% solution) is an excellent nucleophilic reagent for the attack of the penicilloyl- and acylglycyl- R61 enzyme complexes, but it is a very poor acceptor for the R61 enzyme-catalyzed transfer of Ac_2-L-Lys-D-Ala from Ac_2-L-Lys-D-Ala-D-Ala (59). The tetrapeptide

Ac-L-Lys-D-Ala-D-Ala
Gly—┘

is not utilized as a nucleophile by the R61 enzyme for the attack of the penicilloyl or acylglycyl ester linkages. The *Streptomyces* DD-transpeptidase utilizes water to degrade penicillin to penicilloate or to fragment it with formation of free acylglycine but the same enzyme utilizes preferentially, if not exclusively, amino compounds for the attack of Ac_2-L-Lys-D-Ala-D-Ala.

β-Lactams as Inhibitors of the PSEs

L-*R*-D-Ala-D-Ala-terminated substrates (*D*) inhibit the formation of complex *EI**; reciprocally, β-lactams (*I*) inhibit the processing of *D* both in the carboxypeptidase and transpeptidase pathways. Mathematical treatments giving the concentration of complex *EI** at time *t* in the presence of *D*, and the amount of *D* utilized at time *t* in the presence of *I*, can be found in (83). For very large values of *t*, the phenomenon looks like a competition between two substrates for the same enzyme. The question therefore arises whether *D* and *I* are processed on the same enzyme center (in which case the inhibition is competitive and the ternary complex *EID* is not formed) or on two distinct enzyme centers (in which case the inhibition is noncompetitive and the ternary complex *EID* is formed). In many cases, kinetics suggest a competitive inhibition. However, because of the very rapid formation of a stable complex *EI**, the concentration of *EI*, and of *EID*, may be so small that a seemingly competitive inhibition may be obtained even if the interaction is truly noncompetitive (83). However, with the enzyme

of the L-form of *Proteus,* in spite of a very rapid formation of complex EI^* (k_3/K = 2–8 × $10^4 M^{-1}sec^{-1}$) benzylpenicillin inhibits the hydrolysis of UDP-*N*-acetylmuramyl-pentapeptide in a noncompetitive manner, with a dissociation constant K'

$$EI + D \overset{K'}{\rightleftharpoons} EID$$

lower than 3 μM (67). The interactions between the R61 enzyme and 6-aminopenicillanic acid or between the G enzyme and cephalosporin C are characterized by a very low k_3 value (10^{-4} sec $^{-1}$; Table 1) so that the inhibition of the enzyme activity by the β-lactams can be studied under conditions where it is due almost exclusively to the formation of complex *EI,* since complex EI^* is virtually not formed. With the R61 enzyme, the inhibition is competitive (the K value is about 1 mM; J. M. Frere, unpublished); with the G enzyme, the inhibition is noncompetitive (49) and the dissociation constants of the various complexes formed ($E + D \rightleftharpoons ED$; $E + I \rightleftharpoons EI$; $EI + D \rightleftharpoons EID$; $ED + I \rightleftharpoons EID$) exhibit similar values of about 0.5 mM. The ternary complexes *EID* which occur with the G and *Proteus* enzymes are dead ends at least with regard to substrate *D.*

To be a good inhibitor of the enzyme activity on L-*R*-D-Ala-D-Ala-terminated substrates, a β-lactam must be able to immobilize a large proportion of the enzyme in the form of complex *EI* (very low K) or of complex EI^* (high k_3 and low k_4). Although the first alternative may occur, with all those systems where inhibition is observed at low [*I*] values, in fact [*I*] is much smaller than K and therefore inhibition is mainly due to the immobilization of the enzyme in the form of complex EI^*. Under these conditions, the concentration of free enzyme at the steady state is a function of [*I*], k_3/K, and k_4. The higher the k_3/K and the lower the k_4, the better is the β-lactam as an inhibitor. For a given k_3/K, the higher the k_4, the better is the antibiotic as a substrate and the poorer it is as an inhibitor. At equal values of K, intrinsic resistance to β-lactams may be caused by different mechanisms. The main cause of resistance of the G enzyme is a very low k_3 value (Table 1) whereas the resistance of the *Proteus* enzyme to benzylpenicillin (44, 67) is due to a relatively high k_4 value of 3.3 × $10^{-3}sec^{-1}$ (half life of complex EI^*: 3.5 min).

CONCLUDING REMARKS

The mechanisms of the PSEs-catalyzed reactions on β-lactams and L-*R*-D-Ala-D-Ala-terminated substrates, respectively, differ in many respects:

1. The stabilizing site *IV* and the fragmentation site *V* in Figure 4 which play important roles in the processing of the β-lactams, are not involved in the processing of the L-*R*-D-Ala-D-Ala substrates.

2. The penicillin sensitivity of the PSEs and their ability to degrade the β-lactams according to either one of the two pathways described above are not related to the ability of the same enzymes to function as carboxypeptidases, transpeptidases, or endopeptidases.

3. The DD-carboxypeptidase-transpeptidase activities of the PSEs can be regulated by changing the carbonyl donor and amino acceptor concentrations or by altering some particular structural features of these compounds. Conversely, the β-lactam-degrading activity of the same PSEs is not subject to such precise regulations.

4. The corresponding functional portions of the β-lactam and L-*R*-D-Ala-D-Ala molecules exhibit very little isosterism or no isosterism at all.

5. The binding surface of at least some PSEs seems to be able to accomodate β-lactam antibiotics and L-*R*-D-Ala-D-Ala substrates at the same time.

All these observations lead to the two following proposals:

1. The β-lactam and the L-*R*-D-Ala-D-Ala active centers are essentially distinct entities although, depending upon the PSEs, the independence between them may be more or less pronounced (84), in which case the two centers may share one or more active sites.

2. The normal physiological function of the β-lactam center may be regulatory rather than catalytic. Such a model has the following additional implications: (*a*) Activation of one center by its relevant substrate freezes the other center as a result of the conformational changes; (*b*) mutations affecting one center may exist that have no effect on the functioning of the other center; and (*c*) loss of the L-*R*-D-Ala-D-Ala center and further alterations of the β-lactam center might have been important steps in the evolution of the PSEs to true β-lactamases. The R61 enzyme (now available in large quantities) (85) which is a highly penicillin-sensitive DD-carboxypeptidase-transpeptidase, and the G enzyme, which is a highly penicillin-resistant DD-carboxypeptidase-endopeptidase have been crystallized (86) (O. Dideberg, J. M. Frère, and J. M. Ghuysen, in preparation). X-ray crystallographic and sequencing studies should provide more information on the exact geometries of the relevant active centers.

The complex series of reactions catalyzed by the peptidoglycan cross-linking enzyme system during bacterial wall synthesis have to be regulated. Mechanisms similar to the modulating effects that the carbonyl donor and the nucleophilic acceptor exert on the DD-carboxypeptidase-transpeptidase activities of some model PSEs could well be involved in vivo. As suggested above, the β-lactam center of the PSEs probably fulfills a regulatory function; however, the natural effector thus postulated is unknown. By interacting with this regulator center, penicillins and Δ^3-cephalosporins impart to the catalytic center a conformation that is incorrect for enzyme action. Inactivation of the wall peptidoglycan cross-linking system as a whole or

of some of its essential components, causes inhibition of wall synthesis and cessation of cell growth. With the continuous action of the autolytic system (or perhaps its triggering) (8), the process eventually leads to cell death and cell lysis.

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