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The active centres in penicillin-sensitive enzymes

J.-M. Ghuysen,† J.-M. Frère,† M. Leyh-Bouille,†
H. R. Perkins‡ and M. Nieto§
† *Service de Microbiologie, Faculté de Médecine, Institut de Botanique, Université de Liège, Sart Tilman, B-4000 Liège, Belgium*
‡ *The University of Liverpool, Department of Microbiology, Life Sciences Building, Liverpool L69 3BX, U.K.*
§ *C.S.I.C., Centro de Investigaciones Biologicas, Instituto de Biologia Celular, Madrid 6, Spain*

The interaction between β-lactam antibiotics and the penicillin-sensitive enzymes is a multiple-step process. Binding of the β-lactam ring of the penam (or 3-cephem) nucleus occurs at binding site no. 1. Interaction between the N-14 substituent of the bound molecule and binding site no. 2 induces changes in binding site no. 1. In turn, the catalytic site thus created increases the chemical reactivity of the β-lactam amide bond. As the β-lactam ring opens and acylates an enzyme serine residue, the interaction between the thiazolidine (or dihydrothiazine) ring and binding site no. 3 stabilizes the acyl-enzyme complex. Enzyme regeneration slowly proceeds either by direct elimination of the penicilloyl moiety or via C-5–C-6 splitting of the bound metabolite. The fragment arising from thiazolidine yields free *N*-formyl-D-penicillamine while the enzyme-linked *N*-acylglycyl fragment is immediately attacked by an exogenous nucleophile correctly positioned on the acceptor site. Similarly, the enzyme action on L-X-D-Ala-D-Ala terminated peptides is mediated via a binding site no. 1 that combines with D-Ala-D-Ala, a binding site no. 2 that interacts with the side chain of the preceding L-residue, an inducible catalytic site and an acceptor site. Enzymes are known that form a transitory L-X-D-Ala-enzyme complex where the acyl group is ester-linked to the same serine residue as that involved in the formation of the penicilloyl-enzyme complex (Waxman *et al.*, this symposium). Other enzymes, however, may function as catalyst templates. Depending on the enzymes, the independence of the β-lactam and L-X-D-Ala-D-Ala active centres is more or less pronounced.

Introduction

Penicillin-sensitive enzymes (PSEs) are involved in complex processes through which (i) the nascent peptidoglycan strands as they emerge from the plasma membrane undergo attachment to the pre-existing wall peptidoglycan by transpeptidase activity, and (ii) the final extent of cross-linkages in the completed polymer is controlled and modulated throughout the bacterial life cycle by hydrolase activity (Ghuysen 1977*b*). PSEs can be detached from the bacterial cell envelope, disconnected from the other enzymes that catalyse the preceding series of steps leading to the synthesis of the nascent peptidoglycan and studied with the help of well defined substrates (Ghuysen 1977*a*).

The reactions catalysed by the PSEs

The main reactions catalysed by the PSEs during wall peptidoglycan synthesis involve L-X-D-Ala-D-Ala terminated peptides. Enzyme activity is inhibited by β-lactam antibiotics; depending on the enzyme and the structure of the antibiotic, the inhibition is more or less pronounced.

The reactions between enzyme and L-X-D-Ala-D-Ala terminated peptides or β-lactam antibiotics are analogous in that they consist of the transfer of the carbonyl carbons of the penultimate D-Ala or the β-lactam ring to an exogenous nucleophile (figure 1). Depending on the nature of the nucleophile that serves as acceptor for the L-X-D-Ala moiety, PSEs fall into several classes (figure 2). In all cases, free D-Ala is one of the reaction products.

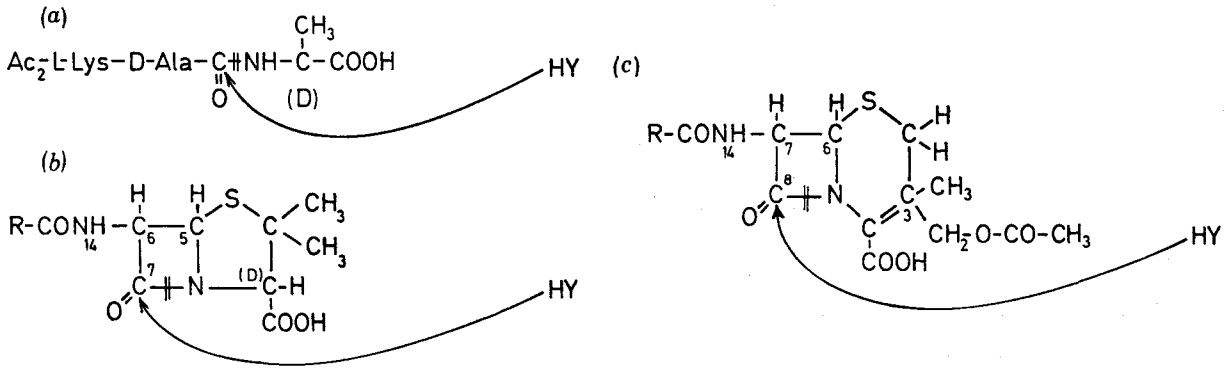


FIGURE 1. Ac_2-L-Lys-D-Ala-D-Ala (*a*), penicillins (*b*) and Δ^3-cephalosporins (*c*). Opening of the (C=O)–NH amide linkages and transfer of the relevant carbonyl carbons to an exogenous nucleophile HY by enzyme action. The β-lactam ring is fused to thiazolidine in the penam nucleus of penicillins (*b*) and to dihydrothiazine in the 3-cephem nucleus of Δ^3-cephalosporins (*c*).

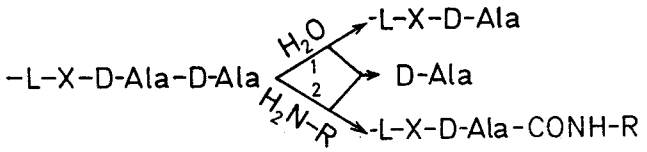


FIGURE 2. Reactions catalysed by the PSEs on L-X-D-Ala-D-Ala terminated peptides. 1, DD-carboxypeptidase activity; 2, transpeptidase activity. H_2N–R = Gly, D-Ala or a more complex amino compound.

(i) *Enzymes of class I.* Water is the only nucleophile used. The enzymes are strict DD-carboxypeptidases and function solely as hydrolases.

(ii) *Enzymes of class II.* The nucleophile may be water or a simple amino compound such as Gly or D-Ala. The enzymes catalyse simultaneously both the hydrolysis of the donor peptide and a transfer reaction through which the *C*-terminal D-Ala of the donor peptide is replaced by the exogenous amino acid. The two pathways compete with each other.

(iii) *Enzymes of class III.* The nucleophile may be water, Gly or D-Ala, or a much more complex amino compound related to wall peptidoglycan. Hydrolysis and transpeptidation also occur simultaneously and compete with each other. The enzymes of class III (DD-carboxypeptidases–transpeptidases) possess complex acceptor sites. The reaction products of enzyme action on peptide monomers acting both as carbonyl donors and amino acceptors are dimers whose structures reflect the type of cross-linkages found in the wall peptidoglycans (figure 3).

(iv) *Enzymes of class IV.* The nucleophile is preferentially, if not exclusively, an amino compound. These enzymes are strict or at least highly specialized DD-transpeptidases.

The above classification does not reveal the real complexity of the reactions catalysed. (i) A single enzyme may have different catalytic activities (e.g. carboxypeptidase and transpeptidase), but the channelling of the total enzyme activity in either pathway depends on the nature of the substrates and the physico-chemical conditions prevailing in the reaction mixture. Because

of their allotopic properties, many enzymes are potentially bifunctional. (ii) DD-Carboxypeptidases may hydrolyse those interpeptide bonds previously made by transpeptidation and which extend between D-Ala and another D-centre in the α-position to a free carboxyl group (arrow in fig. 3). Usually, the 'endopeptidase' activity is low. However, some DD-carboxypeptidases of classes I and II are efficient endopeptidases and function as powerful autolytic agents. (iii) The direction of the transpeptidation reaction between nascent and pre-existing

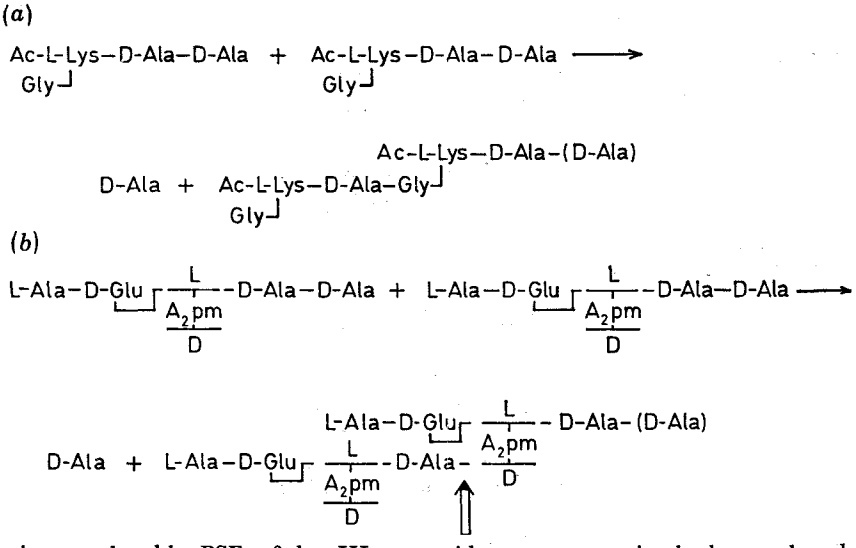


FIGURE 3. Reactions catalysed by PSEs of class III on peptide monomers acting both as carbonyl donor through the D-Ala-D-Ala sequence and as nucleophilic acceptor through the N^{ε}-glycine residue (in *a*) or the amino group located on the D carbon of *meso*-diaminopimelic acid (in *b*). (*a*) Reaction catalysed by the DD-carboxypeptidase–transpeptidase R61; (*b*) reaction catalysed by the DD-carboxypeptidase–transpeptidase R39. Arrow, linkage hydrolysed by endopeptidase activity.

peptidoglycans is not uniform among bacteria. Thus the pre-existing wall peptidoglycan provides the amino acceptor groups for the transpeptidation reaction in *Bacilli* (Ward & Perkins 1974) but is the carbonyl donor in *Gaffkya homari* (Hammes & Kandler 1976). Because of these different mechanisms of peptidoglycan cross-linking, enzymes performing the same activity may act at different stages of wall peptidoglycan metabolism. (iv) PSEs catalysing the same reactions may fulfil distinct physiological functions related to septum formation, wall elongation and cellular shape maintenance, respectively (Spratt, this symposium).

The reactions between enzyme and L-X-D-Ala-D-Ala terminated peptides or β-lactam antibiotics are also analogous in that they proceed according to a three-step mechanism (Frère *et al.* 1975*a*):

$$E + D \overset{K}{\rightleftharpoons} ED \xrightarrow{k_3} ED^* \xrightarrow{k_4} E + Ps \qquad (1)$$

or

$$E + I \overset{K}{\rightleftharpoons} EI \xrightarrow{k_3} EI^* \xrightarrow{k_4} E + Ps, \qquad (2)$$

where E is enzyme; D is L-X-D-Ala-D-Ala peptide; I is antibiotic; ED* and EI* are intermediate complexes; and Ps are reaction products. K is the dissociation constant of the stoichiometric complexes ED or EI; k_3 and k_4 are first-order rate constants. The main difference between reactions (1) and (2) is that k_4 is high in (1) (high turnover numbers) and very low in (2) (very low turnover numbers). Because of the low k_4 in reaction (2), complex EI* exhibits high stability and the enzymes can be identified as penicillin binding proteins. Some enzymes,

however, may react with β-lactam antibiotics in a seemingly reversible manner ($E + I \overset{K}{\rightleftharpoons} EI$). This situation occurs if k_3 is low. Under these conditions, much more enzyme is immobilized in the form of complex EI than in the form of complex EI*. Such enzymes may escape detection as penicillin binding proteins.

In the strict sense, the interaction between enzyme, L-X-D-Ala-D-Ala peptide and penicillin is a competition between two substrates for the same enzyme (Frère *et al.* 1975*b*). However, since penicillin immobilizes the enzyme in the form of a rather stable complex EI* (low k_4 in reaction (2)), it behaves as an inhibitor of the enzyme action on L-X-D-Ala-D-Ala terminated peptides. Antibiotics that are good inhibitors of a given enzyme have k_3/K ratios of 1000 $M^{-1}\,s^{-1}$ or more and k_4 values of $1 \times 10^{-4}\,s^{-1}$ or less. Indeed, at the steady state (s.s.),

$$\frac{[EI^*]_{s.s.}}{E_0} = \frac{k_a}{k_4 + k_a}$$

and the time at which $[EI^*]/E_0$ is 95 % of the value at the steady state, is

$$t_{0.95} = 3/(k_4 + k_a),$$

where

$$k_a = k_3 \Big/ \left(1 + \frac{K}{[I]}\right),$$

or if

$$[I] < K, \quad k_a = k_3[I]/K.$$

Thus for $k_3/K = 1000\ M^{-1}\,s^{-1}$, $k_4 = 1 \times 10^{-4}\,s^{-1}$ (which corresponds to a half-life of 80 min for complex EI*) and $[I] = 1 \times 10^{-5}$ M (a value which is always much smaller than K; see further), 99 % of the total amount of enzyme is inactive at the steady state and the time required for the reaction to reach 95 % of the steady state is about 5 min.

Model PSEs

Each bacterial species probably possesses its own assortment of PSEs. The study of the enzyme active centres in every PSE would be a formidable task. Three water-soluble enzymes (table 1) have been selected for the following reasons. (i) They are available as homogenous proteins. (ii) They utilize Ac_2-L-Lys-D-Ala-D-Ala as a carbonyl donor substrate with high efficiency. (iii) The G enzyme is a DD-carboxypeptidase–endopeptidase of class I; the R61 and R39 enzymes are DD-carboxypeptidases–transpeptidases of class III exhibiting distinct requirements for amino nucleophiles (figure 3). (iv) The enzymes have very high (the R39 enzyme), moderate (the R61 enzyme) and very low (the G enzyme) sensitivities to β-lactam antibiotics. (v) The R61 enzyme (excreted by *Streptomyces* R61), the R39 enzyme (excreted by *Actinomadura* R39) and the G enzyme (excreted by *Streptomyces albus* G) have different molecular masses and are not immunologically related to each other. (vii) The exocellular R61 enzyme catalyses the same reactions with Ac_2-L-Lys-D-Ala-D-Ala as carbonyl donor substrate as the lysozyme-releasable and membrane-bound DD-carboxypeptidases–transpeptidases of class III that can be detected in and isolated from the *Streptomyces* cell envelopes, and antibodies against the R61 enzyme inhibit the activities of these cell bound enzymes. Similarly, the R61 enzyme catalyses in the transpeptidation pathway the same reactions as the corresponding membrane-bound DD-transpeptidase of class IV (Leyh-Bouille *et al.* 1977; Nguyen-Distèche *et al.* 1977).

The *Streptomyces* membrane-bound DD-transpeptidase of class IV has also received special attention (Marquet *et al.* 1974; Dusart *et al.* 1975*a*, *b*, 1977). In many respects, it behaves as an integral membrane protein. A temperature as low as −30 °C is necessary to prevent the isolated membranes from catalysing transpeptidation (Ac_2-L-Lys-D-Ala-D-Ala + Gly-Gly → Ac_2-L-Lys-D-Ala-Gly-Gly + D-Ala). Solubilization of the enzyme requires disruption of the lipid bilayer itself by treatment with the cationic detergents *N*-cetyl or *N*-dodecyl-*N*,*N*,*N*-trimethyl ammonium bromide or chloride. In the presence of the detergent the 'solubilized' enzyme continues to function almost exclusively as a transpeptidase but is inactive in the frozen state. Removal of the detergent causes enzyme inactivation.

TABLE 1. SOME PROPERTIES OF THE R39, R61 AND G ENZYMES

			activity on Ac_2-L-Lys-D-Ala-D-Ala in water				
enzyme	class	10^{-3} M_r	K_m/mM	specific activity IU/mg	efficiency†	turnover number	sensitivity to penicillin
R39	III‡	53	0.8	20	25	1050	very high
R61	III‡	38	10	80	8	3300	moderate
G	I§	19	0.3	8	27	150	very low

† Defined as specific activity divided by K_m.

‡ In the transpeptidation pathway, the DD-carboxypeptidases–transpeptidases R61 and R39 catalyse the reactions shown in figure 3*a* and *b*, respectively.

§ The DD-carboxypeptidase G enzyme is an endopeptidase hydrolysing interpeptide bonds as that shown in figure 3*b* (arrow).

The use of the R39, R61 and G enzymes as models for the determination of the mechanisms involved in the DD-carboxypeptidase and transpeptidase reactions and the mode of action of penicillin has been discussed in a recent review (Ghuysen *et al.* 1979), where the reader can find the complete list of references.

THE ENZYME DONOR SITES

Procedure

The donor site of the enzyme active centre for L-X-D-Ala-D-Ala peptides or β-lactam antibiotics is characterized by the K and k_3 terms (reactions (1) or (2)). It is that part of the protein molecule involved in the initial binding of the compounds and where the corresponding carbonyl carbons are made susceptible to attack by the exogenous nucleophile.

The profiles of the enzyme donor sites have been studied by measuring the effects that each residue of the tripeptide Ac_2-L-Lys-D-Ala-D-Ala or each portion of the β-lactam antibiotics exerts on the K and k_3 terms of reactions (1) or (2) (with water as the nucleophilic reagent). For this purpose, the D-Ala residues at either the *C*-terminal or the penultimate positions of Ac_2-L-Lys-D-Ala-D-Ala were replaced by Gly, L-Ala and other D-amino acids, respectively; the neutral side chain of L-lysine was progressively shortened and, finally, the ε-amino group of L-lysine was exposed or substituted by other charged groups (Leyh-Bouille *et al.* 1970, 1971, 1972; Nieto *et al.* 1973). Similarly, antibiotics of the penam or 3-cephem nucleus series (figure 1) bearing various substituents on N-14 (and, for the Δ^3-cephalosporins, on C-3) have been used as probes.

Because of the high stability of the intermediates EI*, the values of the K and k_3 terms of reaction (2) or at least the k_3/K ratios can be determined directly. The intermediate EI* formed with penicillin is a penicilloyl-enzyme complex. With the R61 (Frère *et al.* 1976*b*) and the R39 (C. Duez, unpublished data) enzymes, attachment of the penicilloyl group to the enzyme is mediated via a serine ester linkage. No other alteration of the bound molecule in complex EI* has been detected (the penicilloyl group, however, is able to epimerize while still attached to the R61 enzyme; Degelaen *et al.* 1979). With Δ^3-cephalosporins possessing an acetoxy side chain on C-3 (figure 1*c*), acylation of the enzyme may lead to the release of acetate from the dihydrothiazine ring and the formation of an exocyclic methylene group.

Because of the very short half-lives of the complexes ED*, the terms of reaction (1) that can be measured directly are K_m and V_{max}. However, if $k_4 > k_3$, then $V_{max} = k_3[E_0]$; if, in addition, $k_2 \gg k_3$, then the dissociation constant K of the stoichiometric complex ED,

$$E + D \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ED, \quad \text{with} \quad k_2/k_1 = K,$$

becomes equivalent to K_m. During hydrolysis of [^{14}C]Ac_2-L-Lys-D-Ala-D-Ala by the R61 or the R39 enzymes, attempts failed to trap transitory complexes ED* with sodium dodecyl sulphate (SDS) (B. Joris, unpublished data). Traces of labelled proteins were detected by SDS polyacrylamide gel electrophoresis but the yield was as low as that obtained with bovine serum albumin, indicating that either the enzymes function as catalyst templates or the [^{14}C]-Ac_2-L-Lys-D-Ala-enzyme complex (that would be equivalent to the penicilloyl-enzyme complex EI*) has an extremely short half-life. In this latter case, formation of complex ED* is not rate-limiting ($k_4 > k_3$).

The enzyme donor site for LDD *peptides*

Although quantitative differences are observed, the donor sites of the three enzymes studied have similar profiles (table 2). The following rules apply to each of them.

(i) The enzymes have a strict specificity for a D-Ala at the penultimate position (which is the residue to be eventually transferred to water) and have a less strict specificity for D-Ala at the *C*-terminal position where it can be replaced by other D-amino acids, although often at the expense of substrate activity. (ii) The observed K_m values range between 0.3 and 3 mM for the R39 and G enzymes, and between 10 and 30 mM for the R61 enzyme. Remarkably, the K_m values for good substrates are not much different and are often as high as those for poor substrates (not shown in table 2). (iii) Variations in substrate activity are mainly reflected in variations in the V_{max} term, whose values greatly depend on the structure of the side chain of the L-residue that precedes D-Ala-D-Ala. Shortening the neutral side chain of the L-residue in the standard tripeptide (replacement of Ac_2-L-Lys by Ac_2-L-A_2bu, Ac-L-Hse, Ac-L-Ala or Ac-Gly) causes a drastic decrease in V_{max} in all cases. In contrast, the occurrence of charged groups at the terminal position of the L-X side chain exerts very specific effects on the activity of each of the enzymes under consideration.

Within the limits of the approximations, $K_m = K$ and $V_{max} = k_3[E_0]$; the best and simplest interpretation of the above observations is as follows (Nieto *et al.* 1973). The *C*-terminal D-Ala-D-Ala dipeptide is the portion of the molecule mainly concerned with the initial binding to the enzyme binding site no. 1. The process is not very efficient (high K_m or K). However, once

the substrate is bound, the L-residue whose lateral chain has the appropriate size, shape and charge interacts with some specific enzyme grouping (binding site no. 2) and serves as the handle through which binding site no. 1 is made catalytically active. In turn, the catalytic site thus created forces the amide bond of D-Ala-D-Ala to adopt a configuration between *cis* and *trans* and causes the immediate transfer of the L-X-D-Ala moiety to the exogenous nucleophile, either directly (if the enzyme works as catalyst template) or via the transitory formation of an extremely unstable L-X-D-Ala-enzyme complex. In this model, the conformational change in the enzyme only occurs in the presence of bound substrate, and distortion of the amide bond in the substrate only occurs as a result of the induced change in the enzyme (which correctly positions the catalytic site). Thus, the free enzyme need not have any affinity for the transition state of the substrate.

TABLE 2. SUBSTRATE ACTIVITY OF LDD TERMINATED PEPTIDES FOR THE MODEL ENZYMES (HYDROLYSIS PATHWAY)

(The activity is expressed as a percentage of that of Ac_2-L-Lys-D-Ala-D-Ala.)

	enzyme		
peptide	G	R61	R39
Ac_2-L-Lys-D-Ala-D-Ala	100†	100†	100†
Ac_2-L-Lys-D-Ala-D-Leu	35	7	80
Ac_2-L-Lys-D-Ala-Gly	8	9	10
Ac_2-L-Lys-D-Ala-L-Ala	0	0	0
Ac_2-L-Lys-D-Leu-D-Ala	0	1.4	0
Ac_2-L-Lys-Gly-D-Ala	2	0	0
Ac_2-L-Lys-L-Ala-D-Ala	0	0	0
Ac_2-L-A_2bu-D-Ala-D-Ala	23	85	16
Ac-L-Ala-D-Ala-D-Ala	0.1	1.4	0.2
Ac-D-Ala-D-Ala	0‡	1‡	5
N^{α}-Ac-L-Lys-D-Ala-D-Ala	1	0.4	750
Suc_2-L-Lys-D-Ala-D-Ala	5	16	36
§R–L-Lys-D-Ala-D-Ala $(Gly)_5$–┘	10	72	340

† For K_m and V_{max} values, see table 1.
‡ Ac-D-Ala-D-Ala is an inhibitor of the activity of the G and R61 enzymes on Ac_2-L-Lys-D-Ala-D-Ala.
§ R = β-1,4-GlcNAc-MurNAc-L-Ala-D-Glu(amide).

According to the above model, an unfavourable or incorrect side chain on the L-residue would cause an induced change in binding site no. 1 that would be unfavourable or incorrect for catalytic activity, decreasing k_3 or preventing enzyme action. Each enzyme probably differs with respect to the substrate requirements of binding site no. 2. Ac-D-Ala-D-Ala has no L-residue; it binds to enzyme binding site no. 1 but is a non-substrate or a weak inhibitor (of the G and R61 enzymes). Ac-DD-cyclodiaminoadipic acid, an analogue of D-Ala-D-Ala where the two methyl side chains are linked covalently and where ring formation makes it obligatory for the peptide bond to be *cis*, binds more strongly to the G and R61 enzymes than Ac-D-Ala-D-Ala, as judged by the inhibitory activities of the two compounds (Nieto *et al.* 1973). These observations suggest that these enzymes might combine only to the small proportion of L-X-D-Ala-D-Ala molecules that have a *cis*-configuration in the *C*-terminal amide linkage, thus displacing the configuration equilibrium.

The enzyme donor site for penicillins and Δ^3-cephalosporins

In no case is breakdown of the complexes EI* a rapid process (see later). The least stable complex, which is that formed between phenoxymethylpenicillin and the R61 enzyme, still has a half-life of 40 min ($k_4 = 2.8 \times 10^{-4}$ s^{-1}). Hence, K and k_3 (or at least the ratios k_3/K) may be determined directly. The following observations were made (Frère *et al.* 1975*a*, *b*, 1978*b*; Fuad *et al.* 1976).

TABLE 3. THE K AND k_3 VALUES FOR THE INTERACTION IN WATER BETWEEN THE MODEL ENZYMES AND β-LACTAM ANTIBIOTICS

(Experiments carried out at 37 °C unless otherwise indicated.)

enzyme	antibiotic	K/mM	k_3/s^{-1}
G	cephalosporin C	1.6	0.0001
	cephalothin	9.5	0.0005
	phenoxymethylpenicillin	150	0.0008
R61	6-aminopenicillanic acid	1	0.0002
	cephaloglycine	0.4	0.009
	carbenicillin	0.11	0.09
	ampicillin	7.2	0.77
	cephalosporin C	> 1	> 1
	phenoxymethylpenicillin	> 1	> 1
	benzylpenicillin	13 (25 °C)	180 (25 °C)
R39	cephalosporin C	0.19 (20 °C)	12.5 (20 °C)

(1) Irrespective of the enzyme, whether the antibiotic belongs to the penam or 3-cephem nucleus series and irrespective of the nature of the substituents, the values of K range between 0.1 and 13 mM (with the exception of the interaction between phenoxymethylpenicillin and the G enzyme, for which K is 150 mM) (table 3). Remarkably, the K values for good inhibitors are not much different from, and are often as high as, those for poor inhibitors. Hence, although the relative dimensions and conformations of the various groupings of the antibiotic molecules may have some influence on the strength of the initial binding, the recognition of the antibiotic by its enzyme target appears to be a rather inefficient and non-selective process (high K). Moreover, the β-lactam ring of the penam or 3-cephem nuclei appears to be the main portion of the molecule concerned with the initial binding.

(2) As shown with the R61 enzyme (table 3), the k_3 term exhibits very great variations depending on the side chains on N-14 of the antibiotic molecule. The k_3 value with 6-aminopenicillanic acid (which has no side chain) is 2×10^{-4} s^{-1} (J.-M. Frère, unpublished data); it is about 1 s^{-1} with ampicillin and 180 s^{-1} with benzylpenicillin. With the penicillin-resistant G enzyme, k_3 is always very low; in fact it is as low as the k_3 observed for the interaction between the R61 enzyme and the unsubstituted 6-aminopenicillanic acid. For technical reasons, the individual K and k_3 values could not be determined in all cases. Since, as shown above, the K values do not vary to a great extent, variations in the measured k_3/K ratios are assumed to reflect mainly the k_3 variations. For each of the antibiotics tested (table 4), the k_3/K ratios observed with the highly penicillin-sensitive R39 enzyme are higher or much higher than those observed with the less penicillin-sensitive R61 enzyme. For each enzyme, the k_3/K values vary greatly depending on the structure of the β-lactam antibiotics. These values range from 900 to 3 000 000 M^{-1} s^{-1} with the R39 enzyme and from 0.2 to 14 000 M^{-1} s^{-1} with the R61

enzyme. With the penicillin-resistant G enzyme and irrespective of the antibiotic, k_3/K is extremely low (6×10^{-2} M^{-1} s^{-1} or less). The detection of the G enzyme as a penicillin binding protein (with [^{14}C]benzylpenicillin for which k_3/K is less than 2×10^{-3} M^{-1} s^{-1}) thus requires high antibiotic concentrations and prolonged incubation times. In fact, penicilloylation of the G enzyme occurs under conditions where bovine serum albumin is also at least partly penicilloylated (B. Joris, unpublished data).

TABLE 4. THE RATIOS $(k_3/K)/(\text{M}^{-1}\ \text{s}^{-1})$ FOR THE INTERACTION IN WATER BETWEEN THE MODEL ENZYMES AND β-LACTAM ANTIBIOTICS

	enzyme		
antibiotic	G	R61	R39
6-aminopenicillanic acid	—	0.2	900
benzylpenicillin	< 0.002	14000†	> 90000†
phenoxymethylpenicillin	0.005†	1500†	> 70000
oxacillin	—	130	40000
cloxacillin	< 0.002	30	15000
methicillin	< 0.002	15	1000
carbenicillin	< 0.002	820†	2900†
ampicillin	< 0.002	107†	74000†
cephalexin	< 0.002	4	3000†
cephaloglycine	—	22†	74000†
cephalosporin C	0.06†	1150†	67000†
cephalothin	0.06†	3000	> 70000
nitrocefin	—	460	3000000

† These values were measured directly as described in Frère *et al.* (1975*a*, 1978*b*) and Fuad *et al.* (1976). The other values were estimated as indicated in Frère *et al.* (1979).

The best and simplest interpretation of the above observations is as follows (Ghuysen *et al.* 1979). Binding of the β-lactam ring to the enzyme binding site no. 1 is neither very efficient (high K) nor very selective. However, once the β-lactam antibiotic is bound to the enzyme, a suitable substituent on N-14 interacts with some specific enzyme grouping (binding site no. 2) and serves as the handle through which the binding site no. 1 is made catalytically active. In turn, the catalytic site thus created distorts the antibiotic molecule from its already strained ground state structure, greatly increasing the chemical reactivity of the β-lactam amide bond. Thus, as proposed above for the mechanism of enzyme action on L-X-D-Ala-D-Ala peptides, (i) each enzyme differs with respect to the substrate requirements of binding site no. 2; (ii) the change in the enzyme only occurs in the presence of bound antibiotic, and (iii) the increase in the reactivity of the β-lactam amide bond only occurs as a result of the induced change in the enzyme. This latter process leads to the acylation of a serine residue of, presumably, the catalytic site (formation of complex EI*) but not to the immediate transfer of the bound metabolite. The high stability exhibited by complex EI* is due to the interaction between the monocyclic thiazolidine or dihydrothiazine rings and a third enzyme grouping (binding site no. 3), which confers on complex EI* a conformation that is not favourable for nucleophilic attack (Ghuysen *et al.* 1979).

According to the proposed model, an unfavourable or incorrect substituent on N-14 of the antibiotic molecule would cause an incorrect alignment of the catalytic site, thus decreasing k_3. 6-Aminopenicillanic acid has no N-14 substituent; consequently, it binds to the enzymes but binding does not lead to any great increased reactivity. The G enzyme has no binding site

no. 2; consequently, the G enzyme combines with the antibiotics but the β-lactam ring of the bound molecules remains at a low level of reactivity. Depending on the enzyme, however, a β-lactam molecule may be converted, at least to some extent, to a reactive form without the involvement of binding site no. 2. Thus, for example, the ability of the R39 enzyme to form complex EI* with 6-aminopenicillanic acid (table 4) is much higher (k_3/K = 900 M^{-1} s^{-1}) than that of the R61 enzyme (k_3/K = 0.2 M^{-1} s^{-1}). It may be that with the R39 and other enzymes, binding of the antibiotic molecule to binding site no. 1 together with the interaction of the fused thiazolidine or dihydrothiazine rings (or other fused substituents as those found in the novel β-lactam antibiotics recently discovered) induce in the β-lactam ring an increased reactivity sufficient to confer on the molecule a substantial inhibitory activity.

Formation of complex EI* requires possibilities for electron delocalization outside the β-lactam ring. The occurrence of active hydrogen(s) on the α-carbon of the acyl side chain of Δ^3-cephalosporins probably facilitates acylation of the enzyme (Morita *et al.*, this symposium). Cefuroxine, however, lacks such active hydrogen and has a potent antibacterial activity. The free –COOH grouping of thiazolidine or dihydrothiazine, on the other hand, probably functions as an electron-attracting grouping and, similarly, the tendency of the C-3 substituents of Δ^3-cephalosporins to attract or accept electrons from the 3-cephem nucleus should make the β-lactam carbonyl a stronger acylating agent. The Δ^3-cephalosporins that have a 3′-acetoxy side chain might function this way, but cephalexin, with its acaudal 3-methyl side chain, cannot. Hence, if the intrinsic acylating activity of a β-lactam compound is an important parameter, the increased reactivity gained through specific interaction with the enzyme binding sites 1, 2 and 3 is the main event that dictates the effectiveness with which complex EI* is formed.

Uniqueness or duality of the enzyme donor sites

At this stage, the question arises whether the enzyme sites involved in the processing of L-X-D-Ala-D-Ala peptides are distinct or not from those involved in the processing of penicillins and Δ^3-cephalosporins (table 5).

1. *Binding site(s) no.* 1. To establish whether the initial binding of the two compounds under consideration occurs at one single or two distinct binding sites no. 1, the inhibition by β-lactam antibiotics of enzyme action on Ac_2-L-Lys-D-Ala-D-Ala has been studied under conditions where inhibition is exclusively due to the first stoichiometric complex EI (in the absence of complex EI*). Such conditions can be fulfilled in particular when the R61 enzyme is inhibited by 6-aminopenicillanic acid (k_3 = 2×10^{-4} s^{-1}) or when the G enzyme is inhibited by cephalosporin C (k_3 = 10^{-4} s^{-1}). Kinetically, the R61 enzyme is competitively inhibited, indicating that the ternary complex EID cannot be formed (J.-M. Frère, unpublished data). The G enzyme, on the other hand, is non-competitively inhibited, indicating that the ternary complex EID is formed. The dissociation constants of the various complexes ($E + D \rightleftharpoons ED$; $E + I \rightleftharpoons EI$; $EI + D \rightleftharpoons EID$; and $ED + I \rightleftharpoons EID$) have similar values of about 0.5 mM (Frère *et al.* 1978*b*).

With other antibiotic-L-X-D-Ala-D-Ala-enzyme systems (and in particular with the R39 enzyme) where a very rapid formation of a stable complex EI* cannot be avoided (high k_3), kinetically the observed inhibitions are most often competitive. Under these conditions, however, the concentrations of EI and EID may be so small that a seemingly competitive inhibition may be observed even if the inhibition is truly non-competitive and therefore, the experiments are not conclusive. Nevertheless, even under these unfavourable conditions, the activity of the

TABLE 5. THE ENZYME ACTIVE CENTRES

(HY is an exogenous nucleophile.)

step	enzyme site involved	reactions on L-X-D-Ala-D-Ala peptides		reactions on penicillin	
1	binding site no. 1	interaction with D-Ala-D-Ala	E + D	interaction with the β-lactam ring	E + I
			$\Leftrightarrows K$		$\Leftrightarrows K$
		initial binding	ED	initial binding	EI
2	binding site no. 2	interaction with the L-X residue		interaction with the N-14 substituent	
		change of binding site no. 1 in catalytic site	k_2	change of binding site no. 1 in catalytic site	k_3
3	catalytic site†	opening of D-Ala-D-Ala amide bond		opening of β-lactam amide bond	
		formation of complex ED* §	ED*	formation of complex EI* §	EI*
4	binding site no. 3			interaction with the thiazolidine ring	
5	fragmentation site‡			stabilization of complex EI*	
				splitting of C-5–C-6 of the penicilloyl moiety	
			k_4	release of *N*-formyl-D-penicillamine	
4 or 6	acceptor site	transfer of L-X-D-Ala to HY correctly positioned on the enzyme acceptor site		transfer of penicilloyl (from step 4) or *N*-acylglycyl (from step 5) to HY correctly positioned on the enzyme acceptor site.	k_4
		release of L-X-D-Ala-Y		release of penicilloyl-Y or *N*-acylglycyl-Y	
		enzyme regeneration	E + Ps	enzyme regeneration	E + Ps

† Catalytic site is a modified binding site no. 1.

‡ Fragmentation site is a combined catalytic site and binding sites no. 2 and 3.

§ With some enzymes, ED* has been shown to be an L-X-D-Ala-enzyme complex equivalent to the penicilloyl-enzyme complex EI* (Waxman *et al.*, this symposium). Other enzymes may function as catalyst templates.

43000 molecular mass DD-carboxypeptidase for the stable L-form of *Proteus* is inhibited by penicillin in a non-competitive manner (Schilf *et al.* 1978). With PSEs isolated from *Bacilli*, however, both the penicilloyl and L-X-D-Ala groups have been shown to be covalently linked to the same enzyme serine residue (Waxman *et al.*, this symposium).

The above studies suggest that some PSEs might be able to accommodate L-X-D-Ala-D-Ala peptides and β-lactam antibiotics at the same time. With other PSEs, binding of the two compounds is mutually exclusive and the enzyme surface probably possesses one single binding site no. 1 or two overlapping binding sites no. 1. These conclusions probably apply to the catalytic and, when present and functional, the acylation sites since these sites are probably different conformations of binding site no. 1.

2. *Binding sites no.* 2. Binding sites no. 2 are made operational as a result of their interaction with the lateral chain of the L-residue that precedes D-Ala-D-Ala or the β-lactam N-14 substituent, respectively. The R39, R61 and G enzymes have equivalent efficiencies on Ac_2-L-Lys-D-Ala-D-Ala (table 1) but, very remarkably, the efficiency of the same enzymes on β-lactam antibiotics is very high, moderate and negligible, respectively (tables 3 and 4). In this respect, the G enzyme is an extreme case of dissociation between the two processes under consideration. Moreover, the structural features of the lateral chain of the L-residue and the β-lactam N-14 substituent required to induce enzyme action are completely unrelated. All of these observations strongly suggest that the R61 and R39 enzymes have one single binding site no. 1 but two distinct binding sites no. 2 devised to combine with the L-residue of the bound peptide or the N-14 substituent of the bound antibiotic. By interacting with their corresponding binding sites no. 2, these side chains change binding site no. 1 into a catalytic site whose conformation is specifically devised to operate on the amide linkages of D-Ala-D-Ala or the β-lactam ring, respectively. The G enzyme, on the other hand, probably possesses one single binding site no. 2 that works exclusively with the L-X-D-Ala-D-Ala binding site no. 1. Thus equipped, the G enzyme performs high DD-carboxypeptidase activity but has a very low capacity for activating the bound antibiotic molecule.

3. *Binding site no.* 3. This site is operational only for the processing of the β-lactam antibiotics. In complex EI* formed with penicillin, the penicilloyl moiety is covalently linked at C-7 to the catalytic site whereas the N-14 substituent and the monocyclic thiazolidine are in close interaction with binding sites 2 and 3, respectively. Elimination of the penicilloyl moiety, and enzyme regeneration, is a difficult task.

The G enzyme (as shown with phenoxymethylpenicillin; J.-M. Frère, unpublished data) and various PSEs (Kozarich 1978; Schilf *et al.* 1978) slowly eliminate an intact penicilloyl moiety in the form of penicilloate (figure 4, VIII) and thus behave as β-lactamases of low efficiency. The R61 and R39 enzymes (Frère *et al.* 1975*c*, 1976*a*, 1978*a*; Adriaens *et al.* 1978), and several DD-carboxypeptidases from *Bacilli* (Hammarström & Strominger 1975; Georgopapadakou *et al.* 1977) and *Streptococcus faecalis* (Coyette *et al.* 1977) fragment the penicilloyl grouping. As shown with the R61 enzyme, the fragmentation consists of C-5–C-6 cleavage and protonation of C-6. It involves one molecule of water and yields an *N*-acylglycyl residue (figure 4, III) and a compound Z which in turn gives rise to free *N*-formyl-D-penicillamine (figure 4, IV). On the basis of kinetics data, the fragmentation step is a slow process and is rate-limiting. However, once it is achieved, thus suppressing the stabilization effect due to the interaction between the thiazolidine ring and binding site no. 3, the *N*-acylglycyl moiety is immediately detached from the serine ester bond and released as *N*-acylglycine (figure 4, V).

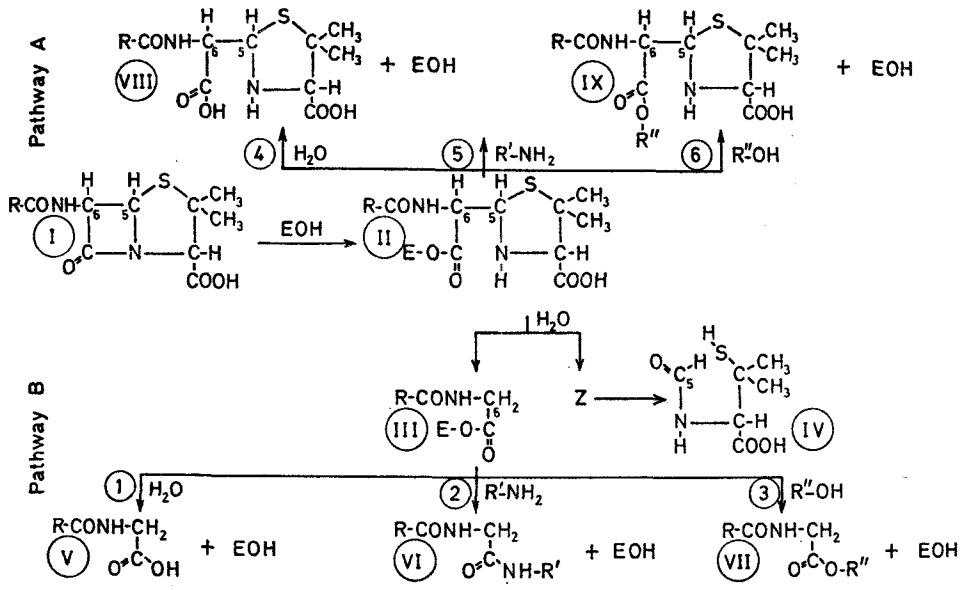


FIGURE 4. Degradation of penicilloyl-enzyme complex EI* via pathways A or B. Pathway A, direct transfer of the penicilloyl moiety to the exogenous nucleophile. Pathway B, fragmentation of the penicilloyl moiety and transfer of the *N*-acylglycyl moiety to the exogenous nucleophile. EOH, free enzyme; I, penicillin; II, complex EI*; III, *N*-acylglycyl-enzyme complex; IV, *N*-formyl-D-penicillamine; V, *N*-acylglycine; VIII, penicilloic acid; R'-NH_2, amino acid or peptide; R''-OH, glycerol; VI, acyl-Gly-CONH-R'; VII, acylglycyl ester of glycerol; IX, penicilloyl ester of glycerol. Note: R'-NH_2 is not an acceptor for the penicilloyl moiety (reaction 5).

TABLE 6. THE k_4 VALUES FOR THE INTERACTION IN WATER BETWEEN THE MODEL ENZYMES AND β-LACTAM ANTIBIOTICS

enzyme	antibiotic	$k_4/10^{-6}$ s^{-1}	half-life of complex EI*/min
G	phenoxymethylpenicillin†	90	130
	cephalosporin C	50	145
	cephalothin	33	350
R61	phenoxymethylpenicillin‡	280	40
	ampicillin, carbenicillin and benzylpenicillin‡	140	80
	nitrocefin†	300	40
	cephaloglycine	3	3800
	cephalosporin C	1	10000
R39	carbenicillin	5.4	2125
	ampicillin	4.4	2600
	benzylpenicillin‡	2.8	4100
	cephalexin	2.4	4800
	nitrocefin	1.5	7700
	cephaloglycine	0.8	14000
	cephalosporin C	0.3	38000

† Breakdown of complex EI* does not involve fragmentation of the bound metabolite.
‡ Breakdown of complex EI* involves fragmentation of the penicilloyl moiety.

Channelling through pathways A and/or B of the penicilloyl moiety depends on the enzyme and various other factors. These factors include the following. (i) the structure of the antibiotic. The R61 enzyme fragments benzylpenicillin and phenoxymethylpenicillin but it behaves as a cephalosporinase for nitrocefin (J. M. Frère, unpublished data). (ii) The microenvironmental conditions. The *Streptomyces* membrane-bound transpeptidase of class IV behaves as a penicillinase (Marquet *et al.* 1974) but, once it is extracted with cationic detergent, it fragments penicillin (Dusart *et al.* 1977). (iii) The nature of the exogenous nucleophile (see further).

The k_4 values for various complexes are known (table 6), but in most cases it is not known whether breakdown of complex EI* involves the fragmentation or the simple release of the bound metabolite. From the limited data available, it appears that although penicillin fragmentation (pathway B in figure 4) implies a much more sophisticated mechanism than the release of penicilloate (pathway A in figure 4), the overall rate of pathway B need not be faster and does not confer any advantage on the enzymes where it is operational. Channelling through pathways A or B is probably governed by the relative disposition of the enzyme binding sites 1, 2 and 3, and the distortion that they confer on the bound penicilloyl moiety. The fate of complex EI* thus depends at least in part on the conformation of complex EI* which in turn appears to be influenced by both the structure of the antibiotic molecule involved in the reaction and the environmental conditions.

The enzyme acceptor site

Procedure

The enzyme acceptor site is that part of the protein molecule where the nucleophilic reagent (not shown in reactions (1) and (2)) is correctly positioned and serves as the final acceptor of the reactions (k_4). The profiles of the enzyme acceptor sites have been studied by investigating the enzyme requirements for various nucleophiles (HO–R; H_2N–R) other than water, for the attack of Ac_2-L-Lys-D-Ala-D-Ala or penicillin. These nucleophiles were necessarily tested in an aqueous medium, i.e. in competition with water.

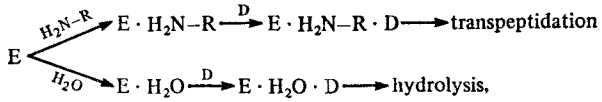
The enzyme acceptor site involved in the attack of Ac_2-L-*Lys*-D-*Ala*-D-*Ala*

The G enzyme, a DD-carboxypeptidase performing high endopeptidase activity, utilizes only H_2O as nucleophilic reagent (class I) (Pollock *et al.* 1972). This enzyme lacks a structured acceptor site able to operate with amino compounds.

The R61 and R39 enzymes have specific requirements for amino nucleophiles that closely reflect the type of cross-linkage that exists in the wall peptidoglycans of the corresponding bacteria. In *Actinomadura* R39, the cross-linkage is from D-Ala to the amino group at the D-centre of *meso*-diaminopimelic acid. Correspondingly, suitable amino nucleophiles for the R39 enzyme must be in the α-position to the carboxyl group of a D-centre (Ghuysen *et al.* 1973, 1974). In *Streptomyces* R61, on the other hand, the cross-linkage is from D-Ala to N^{ε}-(glycyl)-LL-diaminopimelic acid. Correspondingly, various peptides with an *N*-terminal glycine residue are efficient nucleophiles for the R61 enzyme (Perkins *et al.* 1973). By using peptide monomers where both the *C*- and *N*-terminal groups fit the donor and acceptor sites, respectively, of each of the two enzymes under consideration, peptide dimers can be synthesized *in vitro* (figure 3) that are identical or very similar to those synthesized *in vivo* during wall peptidoglycan metabolism in the corresponding bacteria (Ghuysen *et al.* 1974; Zeiger *et al.* 1975).

The amino nucleophiles may act as modulators of the enzyme activities. On the basis of

steady-state kinetics (Frère *et al.* 1973), (i) the transpeptidation reaction appears to follow an ordered pathway in which the amino compound H_2N–R binds first to the enzyme and (ii) the simultaneously catalysed hydrolysis and transpeptidation reactions probably proceed according to the following scheme:



where D (for carbonyl donor) is Ac_2-L-Lys-D-Ala-D-Ala. In such a mechanism the hydrolysis pathway is decreased by competition and the overall reaction (hydrolysis plus transpeptidation) may be unchanged, increased or decreased. With the R61 and R39 enzymes and simple amino compounds (Gly or D-amino acids), the overall reaction is unchanged. Moreover, at a given concentration of Ac_2-L-Lys-D-Ala-D-Ala, the ratio V_t/V_{hy} (where V_t is the rate of transpeptidation and V_{hy} is the rate of hydrolysis) is directly proportional to the amino acid concentration, a property which is expected if the enzyme does not fix more than one molecule of amino acceptor or one molecule of peptide donor at the same time. However, with H_2N–R compounds related to wall peptidoglycan structures, the observed V_t/V_{hy} ratio is a more complex function of the amino acceptor concentration, suggesting the occurrence of an additional acceptor binding site on the enzyme leading to the formation of an [enzyme-$(H_2N$–R$)_2$-peptide donor] complex, which is non-productive for transpeptidation. With increasing concentrations of such amino compounds, hydrolysis is progressively inhibited and transpeptidation, after rising to a maximum, is in turn progressively inhibited. Eventually, the enzyme may be frozen in a non-operational state (Ghuysen *et al.* 1974).

The enzyme acceptor site involved in the attack of penicillin

The effects of various nucleophiles on breakdown of the penicilloyl-R61 enzyme complex EI* has been studied in some detail (Marquet *et al.* 1979). In water, breakdown of complex EI* proceeds only through pathway B (figure 4). Similarly, in a water solution containing a suitable H_2N–R′ compound, pathway B only is operational. The acylglycyl moiety is simultaneously transferred to both H_2O and the amino compound, the two processes competing with each other (figure 4, reactions 1 and 2). In a 20% solution of glycerol, however, both pathways A and B occur (figure 4, reactions 1, 3 and 6). Hence, H_2O (figure 4, reaction 1), various suitable amino compounds (figure 4, reaction 2) and glycerol (figure 4, reaction 3) can function as acceptors of the *N*-acylglycyl moiety. However, (i) glycerol is a very poor acceptor for the R61 enzyme-catalysed transfer of Ac_2-L-Lys-D-Ala from Ac_2-L-Lys-D-Ala-D-Ala; (ii) the transfer reactions of the Ac_2-L-Lys-D-Ala or *N*-acylglycyl groups exhibit quantitative differences with respect to their specificity profiles for the amino nucleophiles (in addition, the tetrapeptide N^{α}-Ac,N^{ε}-Gly-L-Lys-D-Ala-D-Ala (figure 3*a*) is efficiently utilized by the R61 enzyme both as carbonyl donor through its D-Ala-D-Ala sequence and as nucleophilic acceptor through its *N*-glycine residue and gives rise to a peptide dimer; the same tetrapeptide is not utilized as acceptor for the transfer of the *N*-acylglycyl moiety); (iii) the amino acceptors have no regulating effects on the processing of the β-lactam antibiotics. These observations strongly suggest that the R61 enzyme acceptor sites for β-lactam antibiotics and L-X-D-Ala-D-Ala terminated peptides are distinct entities. However, the possibility cannot be excluded that these sites might be different conformations containing the same grouping of amino acid residues, the change in conformation being produced by the different structures of the two metabolites.

Conclusion

The biochemical studies so far accumulated have shed some light on the structural and functional complexity of the active centres of the PSEs. They suggest that the β-lactam and L-X-D-Ala-D-Ala active centres are at least partly distinct entities. Recently, the R61 and G enzymes have been crystallized (Knox *et al.* 1979; Dideberg *et al.* 1979). X-ray crystallographic and sequencing studies should provide more information on the exact geometries of the enzyme active centres.

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