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COMMENTARY

MECHANISM OF ACTION OF β-LACTAM ANTIBIOTICS AT THE MOLECULAR LEVEL

JEAN-MARIE FRÈRE

Service de Microbiologie, Faculté de Médecine, Institut de Botanique, Université de Liège, 4000 Sart Tilman par Liège 1, Belgium

Half a century after the discovery of penicillin, the elucidation of the mechanism of action of β-lactam antibiotics has reached the stage where the reaction between antibiotics and purified, sensitive enzymes can be studied as a simple, bimolecular reaction. The main object of this paper is to discuss the recent results obtained in this respect.

These investigations were initiated and carried out in the frame of a program whose final goal is the complete characterization of the bacterial enzyme target to which penicillin binding causes cessation of cell growth. More complete reviews on this topic can be found in [1] and [2]. It should be emphasized that important questions remain unanswered at the physiological level and that the exact relationship between several sensitive enzymes and the killing target(s) of β-lactams in bacteria has not yet been clearly established.

Antibiotics of the β-lactam family (penicillins and cephalosporins) interfere with the final steps of the biosynthesis of the peptidoglycan, a major component of the bacterial cell wall. This net-like polymer completely surrounds the cell and constitutes the only protection of the Gram-positive bacteria against their internal osmotic pressure, allowing them to survive in a hypotonic environment. In Gram-negative bacteria, the strength and shape of the cell wall probably depend upon a more subtle interrelationship between the peptidoglycan and the components of the outer, membrane-like layer which is absent in Gram-positive species. The chemical structure of peptidoglycan has been discussed in detail [3] as has the three-dimensional arrangement of the polymer [4, 5].

Linear glycan chains, consisting of alternating residues of *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc), are crosslinked by short peptides (see legend of Fig. 1 for more information). These peptides, and the formation of the interpeptide linkages are directly relevant to the antibacterial action of penicillins. The biosynthesis of the polymer (for recent reviews, see Refs. [6–8] can be divided into three main stages: (1) The first stage involves the intracellular synthesis of activated precursors UDP-GlcNAc and UDP-MurNAc-pentapeptide*; (2) In a second stage, these precursors are transferred to a membrane-bound C_{55}-isoprenoid alcohol phosphate. A lipid soluble intermediate [isoprenoyl-pyrophosphate-MurNAc(pentapeptide)-GlcNAc] is formed and, if present in the final structure, the cross-bridging amino acids are added to this intermediate at the ω-amino end of the third residue of the peptide; (3) The third and final stage consists in the polymerization of the net-like polymer outside the cytoplasmic membrane by enzymes that are themselves attached to the plasma membrane. The energy of the phosphodiester bond between pyrophosphate and muramic acid is utilized for glycan chain elongation whereas peptide crosslinks are closed by a transpeptidation reaction. During this latter reaction, the carboxyl group of the penultimate D-Ala residue of one penta-

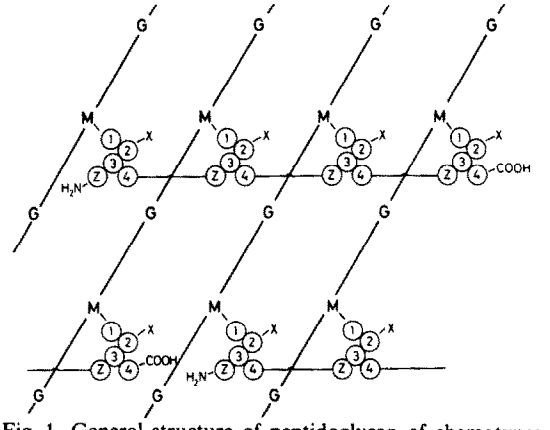


Fig. 1. General structure of peptidoglycan of chemotypes I, II and III. G: *N*-acetylglucosamine, M: *N*-acetylmuranic acid. The circles represent amino acid residues: ① is L-alanine, ② D-glutamic acid, which is bound to residue ③ via its γ-carboxyl group. The α-carboxyl is either free or amidated (X). The third residue is either a diamino acid, such as lysine or a diamino diacid such as diaminopimelic acid (A_2pm). The fourth residue is D-alanine. Z represents the amino acid residue(s) which may be present in the crossbridge between two tetrapeptides. In chemotype I, Z is absent and a direct linkage is formed between the carboxyl group of the D-Ala of one peptide and the ω-amino group of the third residue of another peptide (Gram-negative bacteria, Bacilli, *Gaffkya homari* and *Actinomadura* have a peptidoglycan of chemotype I). In chemotype II, Z is one amino acid residue (e.g. glycine in *Streptomyces* spp. and L-asparagine in *Streptococcus faecalis* ATCC 9790) or a short peptide (pentaglycine in *Staphylococcus aureus*). In chemotype III, Z is one or several tetrapeptides having a structure identical to that of the basic tetrapeptide. Chemotype IV peptidoglycans are not represented on the figure. In these cases, the Z-bridge is a diamino acid or a diamino acid-containing peptide which extends between the α-carboxyl group of glutamic acid and the carboxyl group of D-alanine. The figure also shows some peptide units which have retained either a free amino or a free carboxyl group.

* The sequence of the pentapeptide is generally: -L-Ala → D-Glu → ③ → D-Ala → D-Ala.

peptide unit is transferred onto the free amino group of another peptide unit (either on the crossbridge in chemotypes II, III, IV or on the third residue itself in chemotype I). Formation of the peptide bond is accompanied by the release of the ultimate D-Ala residue and can be represented by the simplified reaction: R-D-Ala-D-Ala + R'-NH_2 → R-D-Ala-NH-R' + D-Ala where R-D-Ala-D-Ala is the donor peptide and R'-NH_2 the acceptor peptide. In the presence of penicillin, the transpeptidation reaction is inhibited, which causes a profound disorganization of the cell envelope of the growing cells. The delicate balance between synthesis of cross-linked peptidoglycan and its degradation by autolysins is disrupted and bacteria growing in a hypotonic environment undergo lysis (peptidoglycan hydrolysing enzymes, which constitute the cellular autolytic system are normally present in all bacteria where they probably play important functions by creating new growth sites in the polymer).

In Gram-negative bacteria, the phenomenon is more complex: lysis is only obtained at high concentrations of penicillin; low concentrations of antibiotic induce morphological abnormalities (round cells, non dividing chains, formation of bulges, etc.).

In vitro assays for penicillin-sensitive transpeptidation reactions were first devised by Araki *et al.* [9] and Izaki *et al.* [10], independently. They showed that membrane fragments of *E. coli*, when incubated with the precursors UDP-GlcNAc and UDP-MurNAc-pentapeptide, catalysed a series of reactions which culminated in the synthesis of crosslinked peptidoglycan. In the presence of penicillin, the reactions did not proceed further than the stage of linear, uncrosslinked peptidoglycan. These membrane preparations also contained a penicillin-sensitive D-Ala-D-Ala carboxypeptidase (DD-Cbase), which caused the release of the C-terminal D-Ala from the UDP-MurNAc-pentapeptide. The D-alanine released by this carboxypeptidase could be distinguished from the D-alanine released by the transpeptidase by omitting UDP-GlcNAc from the incubation mixture. A DD-carboxypeptidase penicillin-sensitive activity has been shown to occur in many bacterial species [11–23]. Its exact role has not yet been clearly elucidated but this enzyme is thought to be concerned with the control of the extent of peptidoglycan crosslinking. In some bacilli, the specific inhibition of the DD-carboxypeptidase activity does not cause detectable damages to the cell but in other bacteria, such as *Gaffkya homari*, the role of the DD-carboxypeptidase is essential. In this latter bacterium, a peptide unit is utilized as an acceptor only after release of the C-terminal D-Ala residue by DD-carboxypeptidase action; in consequence, polymerization cannot proceed further than the formation of peptide dimers and a maximum degree of 50 per cent of crosslinking should then be expected. The experimental data confirm these predictions. Moreover, the DD-carboxypeptidase, and not the transpeptidase, appears to be the lethal target of penicillin [24]. Similarly, in a thermosensitive mutant of *E. coli*, the amount of nascent peptidoglycan which is incorporated in the wall by transpeptidation to the preexisting peptidoglycan is controlled by DD-carboxypeptidase action. Furthermore, an exact balance between transpeptidase activity and DD-carboxypeptidase activity may be an important feature of the mechanism concerned with cell septation [25 and D. Mirelman, personal communication].

However interesting they are for the unravelling of the reactions leading to peptidoglycan biosynthesis, the membrane + wall systems are of little value in studying the interaction between penicillin and the sensitive enzymes at the molecular level. Indeed, these systems are, *per se*, extremely complex and transpeptidation can only be observed as the last step of a series of reactions with the result that it cannot be studied directly and independently.

In order to overcome these difficulties, two approaches were used, which were (1) the isolation and purification of the penicillin binding components (PBCs) present in the plasma membranes of the bacteria and their characterization by gel electrophoresis and (2) the use of simple peptide donor and acceptor systems as direct substrates for isolated transpeptidases. In this respect, *Streptomyces* R61 and *Actinomadura* R39 were interesting organisms because of their ability to excrete during growth soluble transpeptidases-DD-carboxypeptidases which could be purified to protein homogeneity by conventional techniques [26, 27].

A. *The penicillin binding components (PBCs)*

The main result of these studies has been to demonstrate that bacterial cytoplasmic membranes possess several proteins which form relatively stable complexes with penicillins. In some cases, enzymic activities or physiological roles could be attributed to some of these PBCs (see Table 1 for a summary of the properties of the PBCs). Transpeptidase activity has been demonstrated for at least 3 of the PBCs of *Salmonella typhimurium* [33]: these proteins utilize the pentapeptide L-Ala → D-Glu ↳ (L)-*meso*-A_2pm-(L) → D-Ala → D-Ala to form crosslinked dimers. The importance of these results should be emphasized, since, in all other cases, DD-carboxypeptidase was the only enzymic activity which could be attributed to one or several PBCs. The interactions between penicillin and these DD-carboxypeptidases have been studied with some detail. The results are discussed below.

B. *Interaction of penicillins with transpeptidases and* DD-*carboxypeptidases*

1. *Soluble* DD-*carboxypeptidases-transpeptidases from Actinomycetes.* The DD-carboxypeptidases-transpeptidases excreted by both *Streptomyces* R61 and *Actinomadura* R39 have been purified to protein homogeneity [26, 27]. They catalyse transfer reactions between donor and acceptor peptides identical or very similar to the peptidoglycan peptide units in the corresponding strains [37, 38]. Simultaneously with the transpeptidation reaction, these enzymes also catalyse the hydrolysis of the donor peptide. The transpeptidase/carboxypeptidase ratio can be increased by raising either the pH or the acceptor concentration or by decreasing the water content of the incubation mixture. With the R61 enzyme, up to 80 per cent of the water can be replaced by a mixture of ethyleneglycol and glycerol, which decreases the rate of hydrolysis to a greater extent than the rate of transpeptidation, resulting in a 7-fold increase of the transpeptidase/carboxypeptidase ratio value [39].

Table 1. Properties of the penicillin binding components

Species	Number	mol. wt*	Enzymic activities	Physiological function	Ref.
Bacillus subtilis	5	1 122,000 2 96,500 3 88,000 4 78,000 5 50,000	No 5 = DD-Cbase	Nos 1, 2, 4: possible killing targets of penicillin	[28, 29, 30, 31]
Bacillus megaterium	5	1 123,000 2 94,000 3 83,000 4 70,000 5 45,000	No 5 = DD-Cbase	No. 1 possible killing target of penicillin	[32]
Bacillus cereus	3	1 ≃ 122,000 2 ≃ 96,000 3 50,000			[28]
Bacillus stearothermophilus	4	1 105,000 2 78,000 3 76,000 4 45,000	No. 4 = DD-Cbase	Nos 1, 2, 3: possible killing targets of penicillin	[a]†
Staphyloccocus aureus	2	$\frac{1}{2}$ ± 100,000			[28]
Streptococcus faecalis ATCC 9790	6		No. 6 = DD-Cbase		[b]†
Salmonella typhimurium	5	1 120,000 2 78,000 3 69,000 4 52,000 5 38,000	Nos 1, 4, 5 = DD-Cbases and Tpases	No. 4: possible killing target of penicillin	[33]
Escherichia coli	6	1 91,000 2 66,000 3 60,000 4 49,000 5 42,000 6 40,000	Nos. 5 and 6 = DD-Cbases	No. 1: cell elongation No. 2: shape No. 3: cell division	[23, 34, 35, 36]

* PBCs are numbered in order of decreasing mol. wt.

† [a]: H. A. Chase and P. E. Reynolds, personal communication; [b]: R. Fontana and J. Coyette, personal communication.

DD-Cbase = DD-carboxypeptidase; Tpase = transpeptidase.

(a) *Kinetic analysis.* The interaction between enzyme and β-lactam inhibitors has been studied by various kinetic methods; the following simple model best explains all the observed data:

$$E + I \overset{K}{\rightleftharpoons} EI \xrightarrow{k_3} EI^* \xrightarrow{k_4} E + X + Y$$

Model A

The free enzyme E reversibly binds the antibiotic I to form a first stoichiometric complex EI. This complex then undergoes an irreversible transformation into a second stoichiometric complex EI* which finally breaks down, regenerating the active enzyme and releasing a fragmented antibiotic molecule. The dissociation constant K and the first-order rate constants k_3 and k_4, which characterize such a system have been measured [40, 41] for the interaction between both R61 and R39 enzymes and several penicillins and cephalosporins (see Table 2). At low concentrations of antibiotic (i.e. for $I \ll K$), the rate of

Table 2. Values of the constants according to model A and effect of the antibiotic on the germination of conidia

	Streptomyces R61 Exocellular enzyme [40]					*Actinomadura* R39 Exocellular enzyme [41]				
Antibiotic	K mM	k_3 s^{-1}	k_3/K $M^{-1}\,sec^{-1}$	k_4 sec^{-1}	$1/LD_{50} \times 10^{-1}$ (M^{-1}) (30°)	K mM	k_3 sec^{-1}	k_3/K $M^{-1}\,sec^{-1}$	k_4 sec^{-1}	$1/LD_{50} \times 10^{-1}$ (M^{-1}) (30°)
Penicillin G	13 (25°)	179 (25°)	13,700 (25°)	1.4×10^{-4}	18,000	—	—	300,000	3×10^{-6}	140,000
Carbenicillin	0.11	0.09	830	1.4×10^{-4}	3,000	—	—	5,750	5×10^{-6}	12,000
Ampicillin	7.2	0.77	107	1.4×10^{-4}	18,000	—	—	74,000 (20°)	4×10^{-6}	41,000
Penicillin V	>1	>1	1,500	2.8×10^{-4}	20,000	—	—	—	—	—
Methicillin	—	—	—	—	—	—	—	1,150 (20°)	3×10^{-5}	14,000
Cephalosporin C	>1	>1	1,150	1×10^{-6}	90	0.19 (20°)	12.5 (20°)	67,000 (20°)	0.3×10^{-6}	1,100
Cephaloglycine	0.4	9×10^{-3}	22	3×10^{-6}	350	—	—	74,000 (20°)	0.8×10^{-6}	84,000
Cephalexine	—	—	—	—	—	—	—	3,000 (20°)	2.4×10^{-6}	700,000
Cephalosporin 87/312	>0.2(10°)	>0.1(10°)	460 (10°)	3×10^{-4}	—	—	—	2.6×10^{6}(10°)	1.5×10^{-6}	—

The LD_{50} values represent the concentrations of antibiotic allowing 50 per cent of cell survival [50]. On the table, the reciprocal of this value is given and the units have been chosen to allow direct comparison with the k_3/K value.

All the results were obtained at 37°, unless otherwise indicated.

For technical reasons, the individual values of K and k_3 could only be obtained for cephalosporin C in the case of the R39 enzyme [see 41].

formation of EI* is dependent on the ratio k_3/K. The steady-state concentration of free enzyme is a function of the concentration of inhibitor ([I]) and of the values of k_4 and k_3/K. A good inhibitor should have a high k_3/K value and a low k_4 value. For the soluble enzymes, however, a relationship could not be obtained between these values and the sensitivity of the organisms to various penicillins (see Table 2). Obviously, the exocellular enzymes are not the killing targets of the β-lactams. However as, until recently, these enzymes were the only transpeptidases which had been purified to protein homogeneity, they constituted models of choice for the study of the interaction between penicillins and sensitive enzymes.

The proposed model explains the fact that inhibition may appear to be irreversible in some cases and reversible in others: if k_4 is low, an inhibited complex is easily isolated; if k_4 is high, regeneration of the active enzyme is observed after elimination of the excess of penicillin. The isolation of a stable complex at low temperature, and complete recovery of activity upon incubation of this isolated complex at 37° may be observed with the same enzyme [42, 43]. Moreover, this model allows a good interpretation of earlier, puzzling observations that cells which have been saturated with penicillin can start to grow and to synthesize peptidoglycan after reincubation in a fresh penicillin-free medium [44, 45].

When donor substrate is added to the enzyme together with the antibiotic, the ternary interaction may follow either a competitive or a non-competitive pathway. The non-competitive model shown below is the most general.

$$\begin{array}{ccccc} E & \overset{+I}{\rightleftharpoons} & EI & \rightarrow EI^* \rightarrow & E + X + Y \\ {\scriptstyle +S}\uparrow\downarrow & & \downarrow\uparrow{\scriptstyle +S} & & \\ ES & \overset{+I}{\rightleftharpoons} & ESI & & \\ \downarrow & & & & \\ E + P & & & & \end{array}$$

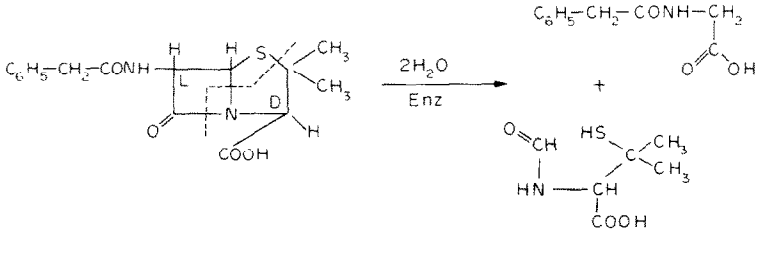
Model B

In the competitive model, the ternary complex ESI cannot be formed as binding of inhibitor and substrate are mutually exclusive. In several cases, apparently competitive kinetics have been obtained for the interaction between the three reagents [11–13, 19, 20]. According to Blumberg and Strominger [8], these results suggest that substrate and inhibitor compete for the same site on the enzyme. However, a careful analysis [46] shows that, in systems involving the formation of a rather stable intermediate, such as EI*, the concentrations of EI and ESI might be equally negligible with the result that perfectly competitive Lineweaver–Burk plots are obtained even if the interaction is non-competitive. This conclusion strictly applies to the exocellular R61 enzyme, but most likely it is also true for the DD-carboxypeptidases from *B. subtilis* and *B. stearothermophilus*, for which both 'competitive inhibition' and formation of rather stable complexes with β-lactams have been observed [12, 13].

(b) *Fragmentation of the antibiotics.* The fragments produced by the action of the R61 enzyme on benzylpenicillin (penicillin G) are phenylacetylglycine and *N*-formyl-D-penicillamine [47, 48]. The dashed line indicates that, with respect to its biosynthesis, penicillin can be regarded as the condensation product of L-cysteine and D-valine. Hence, fragmentation by the R61 enzyme follows a very different pathway. Recovery of enzyme activity and release of phenylacetylglycine are simultaneous events; consequently, if an intermediate such as phenylacetylglycyl–enzyme, is formed, its half-life is very short compared to the half-life of the original enzyme–penicillin complex. Phenoxymethylpenicillin (penicillin V) is degraded in a similar manner into phenoxyacetylglycine and *N*-formyl-D-penicillamine. The R39 enzyme also produces phenylacetylglycine from benzylpenicillin, but much more slowly (compare the values of k_4 on Table 2). For this reason, the fate of the thiazolidine ring during the interaction of benzylpenicillin with this latter enzyme has not yet been elucidated.

(c) *Presence of serine at the penicillin binding site.* Pronase or thermolysin digestion of the denatured R61 enzyme–[^{14}C]benzylpenicillin complex released a [^{14}C]labelled Val Gly Ser tripeptide. Further action of pronase or treatment with leucine aminopeptidase yielded valine and a radioactive Gly–Ser dipeptide. Hence, the only group to which penicillin could have been bound was the hydroxyl group of the serine residue [49].

2. *The membrane-bound transpeptidases.* Isolated membranes of various *Streptomyces* species [50] catalyse transpeptidation reactions with donor and acceptor peptides which are very similar to the natural peptides occurring in the *Streptomyces* wall peptidoglycan. In *Streptomyces* R61, a parallelism exists between the effects of several penicillins and cephalosporins on the membrane-bound transpeptidase and on the germination of conidia suggesting that this enzyme is the killing site of β-lactam antibiotics. Model A applies to the interaction between the enzyme and various penicillins, although, for technical reasons, it has not been possible to demonstrate that formation of complex EI* is also a two-step process. The important point, however, is that, with all β-lactams tested, EI* complexes spontaneously break down to release active enzyme. The half-lives of these complexes at



37° vary from 3 min to several hours depending upon the antibiotic. The structure of the degradation product(s) of penicillin is still under current investigation. The enzyme can be solubilized with a cationic detergent *N*-acetyl-*N*,*N*,*N*-trimethylammonium bromide and partially purified by chromatography on Sephadex G-100. Model A remains valid for the partially purified enzyme; in this case, phenylacetylglycine is released upon breakdown of the EI* complex previously formed with benzylpenicillin. In other *Streptomyces* species (K15, *rimosus*), a spontaneous degradation of the EI* complexes was found in all cases, either with membrane-bound or solubilized transpeptidases (Dusart, J. and Leyh-Bouille, M., personal communication). Recently, two of the membrane-bound DD-carboxypeptidases-transpeptidases (PBCs 4 and 5) from *Salmonella typhimurium* have been isolated and purified. The complexes formed between these enzymes and benzylpenicillin decay with half-lives of 90 min (PBC 4) and 5 min (PBC 5) at 37° [33].

C. *The carboxypeptidases*

Streptomyces albus G secretes a soluble DD-carboxypeptidase, which is only inhibited by extremely high (10–20 mM) concentrations of benzylpenicillin [51]. This enzyme appears to be more resistant to penicillin, by at least two order of magnitude, than all the other DD-carboxypeptidases or transpeptidases which have been described so far. The understanding of the reasons for this relative absence of sensitivity should supply new insights into the phenomenon of resistance.

With the exception of this latter enzyme, all the DD-carboxypeptidases described below originate from cytoplasmic membranes. Some of them were solubilized with detergents and partially purified in the presence of the solubilizing agent. In some instances [12, 23, 30], homogeneity was claimed on the basis of the presence of one single protein band after gel electrophoresis in the presence of sodium dodecylsulfate. However, such a test is insufficient; indeed, penicillin titration of the DD-carboxypeptidases thus 'purified' from *B. subtilis* and *B. stearothermophilus* indicated that only 0.28 and 0.04 moles of penicillin were bound per mole of enzyme, respectively [43].

Some of the enzymes listed as carboxypeptidases in this paragraph also catalyse simple transpeptidation reactions, using amino acids (glycine, D-alanine), a simple dipeptide (glycyl–glycine) or hydroxylamine as acceptors (carboxypeptidases from *B. stearothermophilus*, *B. megaterium*, *S. faecalis* and *E. coli*). The data which are presently available do not justify the classification of these enzymes as transpeptidases, since none of them is able to utilize more complex acceptors, which would be similar to the natural cell wall acceptors, nor to catalyse the formation of dimers from donor–acceptor substrates [12, 17, 23, 54]. However, with these enzymes as well as with other DD-carboxypeptidases, suitable experimental conditions might still be found in which they would catalyse transpeptidations similar to the physiological reactions.

Table 3 summarizes the properties of some DD-carboxypeptidases. In general, the interactions between these enzymes and β-lactams appear to proceed according to model A, although results never allow a clear distinction between a direct or a two-step formation of complexes EI*. Nevertheless, in all case, complexes EI* break down, releasing the active enzyme and an inactive, degraded antibiotic. Carboxypeptidase IB from *E. coli* might be the only exception [23]: although this enzyme is strongly inhibited by penicillin, a stable complex could not be isolated. This result implies that the complex is extremely unstable or, alternatively, that the inhibition is a truly reversible phenomenon ($E + I \rightleftharpoons EI$).

In some cases, the structure of the released compound(s) is far from being firmly established: (1) With carboxypeptidase IA from *E. coli* [23], the possible contamination of the preparation by a small amount of β-lactamase cannot be excluded since the EI* complex was not isolated prior to the release of the inactivated antibiotic; (2) *N*-formyl-D-penicillamine was found by Chase and Reynolds (personal communication) while the intact thiazoline (D-5,5-dimeth-

Table 3. Properties of some DD-carboxypeptidases

Bacterial species	Physical state	Half-life of complex formed with benzylpenicillin	Released compound(s) upon decay of complex	Ref.
Bacillus subtilis	M.B solubilized (Triton X-100)	200 min (37°)	Probably phenylacetylglycine	[43]
Bacillus stearothermophilus	M.B. solubilized (Triton X-100)	10 min (55°) 10 min (55°)	Phenylacetylglycine + *N*-formyl-D-penicillamine or phenylacetylglycine + 5,5 Dimethyl-Δ^2 thiazoline-4-carboxylate	[a]* [43] [52] [53]
Bacillus megaterium	M.B.	180 min (25°)		[17]
Streptococcus faecalis ATCC 9790	M.B. or solubilized (Genapol)	260 min (37°)	Phenylacetylglycine + *N*-formyl-D-penicillamine	[54] [b]*
Escherichia coli H2143				
IA	solubilized	5 min (37°)	Penicilloic acid?	[23]
IB	solubilized	no stable complex isolated	Penicilloic acid?	
Proteus mirabilis unstable L-form	solubilized (Genapol)	5.5 min (30°) 3.5 min (37°)	Penicilloic acid and other, minor compounds	[55] [56]

* [a] H. A. Chase and P. E. Reynolds, personal communication; [b] J. Coyette, personal communication.
M.B. = membrane-bound.

yl-Δ^2-thiazoline-4-carboxylate) was reported by Hammarström and Strominger [53] as the reaction products arising from the thiazolidine moiety of benzylpenicillin after interaction with the *Bacillus stearothermophilus* membranes. However, the identification of the intact thiazoline appears rather dubious, since this compound spontaneously decays (half-life: 130 sec; J. M. Frère, unpublished results) under the conditions in which it was supposedly isolated (50 min at 55°).

Finally, steady state studies of the hydrolysis of the substrate in the presence of penicillin by the DD-carboxypeptidase from the *Proteus* unstable L-form indicated a non-competitive inhibition [55, 56]. These results demonstrate a non-competitive model might be necessary for a general description of the interaction between penicillin and sensitive enzymes.

GENERAL COMMENTS

The most striking and novel feature of the interaction between β-lactams and sensitive enzymes has been the instability of the inhibited EI* complexes. Degradation of these complexes and enzyme reactivation appear to be general phenomena. Depending upon the enzyme, the antibiotic and physical factors, such as pH and temperature, the half-lives of the inhibited EI* complexes vary from 3 min to more than 10 days (which corresponds to first-order rate constants ranging from 10^{-3} to less than 10^{-6} sec^{-1}). In general, EI* complexes are more stable with cephalosporins than with penicillins. The discovery of this degradation step suggests a new mode of bacterial resistance to β-lactams which is the degradation of the antibiotic by the target enzyme itself. Indeed, assuming all other factors to be identical, the faster the decay of the EI* complex, the higher the concentration of antibiotic which is needed to cause the same enzyme inhibition. In these studies, the purified DD-carboxypeptidases-transpeptidases from actinomycetes were extremely useful tools.

(1) A model has been proposed for the interaction between penicillins and sensitive enzymes (model A). From an integration of the data obtained with numerous enzymes, the model appears to be general. In particular, it explains how reversibility or irreversibility of the inactivation can be observed, sometimes with the same enzyme, depending upon the experimental method used. Moreover, a careful analysis of this type of situation provides an explanation for the apparent paradox in which some enzymes, while exhibiting apparently reversible, competitive kinetics of inhibition by penicillin, nevertheless form stable complexes with the inhibitor. Once the enzyme, the substrate and the inhibitor have been mixed together, a non steady-state situation prevails during the major part of the incubation time. A theoretical estimation of the amount of product formed at any given time indicates that, in such cases, Lineweaver–Burk plots would yield curves which, within the limits of experimental errors, may be easily mistaken for straight lines and would converge on the ordinate, as in a classical competitive system ([46] and J. M. Frère, unpublished results).

(2) The structure of the degradation products of penicillin has been established. Although it is by no means certain that the degradation of all penicillin–enzyme complexes yields *N*-acyl-glycine and *N*-formyl-D-penicillamine (directly or via the intact thiazoline), this fragmentation represents a new and unexpected enzymatic reaction. Although slow, the reaction is genuinely enzymatic since no fragmentation of penicillin is observed after denaturation of the complexes [43, 49, 57]. In fact, this type of degradation might well occur in some resistant bacterial strains but would have escaped detection for the simple reason that degradation of penicillin to penicilloic acid by classical penicillinases is the mechanism to which resistance is usually attributed.

(3) The original hypothesis of Tipper and Strominger [58] has been examined under a new light: firstly, the suggestion by Rando [59] that penicillins might be 'k_{cat} inhibitors' is supported by the fact that the main cause of inhibition is more likely to be rapid irreversible inactivation step (high (k_3) rather than an especially good recognition of the antibiotics by the binding site (low K); secondly, competitive kinetics of inhibition on which the structural analogy hypothesis was initially proposed, are compatible with the general, non-competitive model B. Furthermore, the DD-carboxypeptidase from the unstable L-form of *P. mirabilis* is non-competitively inhibited by penicillins, an observation which can only be explained by this latter model; thirdly, the proposed formation of a thioester bond between an active –SH group on the enzyme and the carboxyl group of the β-lactam ring is at variance with the finding that penicillin is bound to a serine residue in the exocellular enzyme from *Streptomyces* R61. However, this situation may not be general.

Important information concerning the mechanism of action of penicillins has also been obtained by the detailed studies of the penicillin binding components. Firstly, they indicate that multiple PBCs are present in the cytoplasmic membrane of all the bacterial strains examined. Secondly, they demonstrate that some of these PBCs fulfil physiological functions concerned with cell septation or cell elongation. Surprisingly, the major penicillin binding component is often a DD-carboxypeptidase, an enzyme to which no physiological role could be attributed in several organisms.

It should be understood, however, that penicillin-sensitive, physiologically important components may escape detection, if they form truly reversible complexes with penicillins or if denaturation of the complex formed does not prevent the release of a degraded β-lactam.

Finally, it should be emphasized that the detailed pathway of the enzymatic catalysis of the transpeptidation reactions remains rather mysterious. The ping-pong mechanism proposed by Tipper and Strominger [58] never received any serious experimental support. In fact, our data obtained with the exocellular transpeptidase-DD-carboxypeptidase from *Streptomyces* R61 indicate that the acceptor binds first to the enzyme in the transpeptidation pathway, a conclusion which is not in agreement with the proposed ping-pong mechanism [39].

The exocellular enzymes of *Streptomyces* are not the killing targets of penicillins; yet, until recently, they were the only enzymes purified to protein hom-

ogeneity which were able to catalyse *in vitro* reactions similar, sometimes identical to the physiological transpeptidations and with which the interaction with β-lactam antibiotics could be directly studied as simple cases of bimolecular reactions. They still remain the models of choice for the study of the mode of action of this class of inhibitors at the molecular level. It is hoped that other transpeptidases of membrane origin, such as the newly purified PBCs from *Salmonella typhimurium* will soon extend and confirm our present concept of a multistep interaction between target enzymes and penicillins, resulting in a steady-state system, in which the level of active enzyme is a function of the velocities of formation and degradation of the EI* complex.

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