β-LACTAM ANTIBIOTICS AS CARBONYL DONORS OF THE ACTIVE-SITE SERINE β-LACTAMASES, DD-PEPTIDASES AND LL-PEPTIDASES

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1. INTRODUCTION

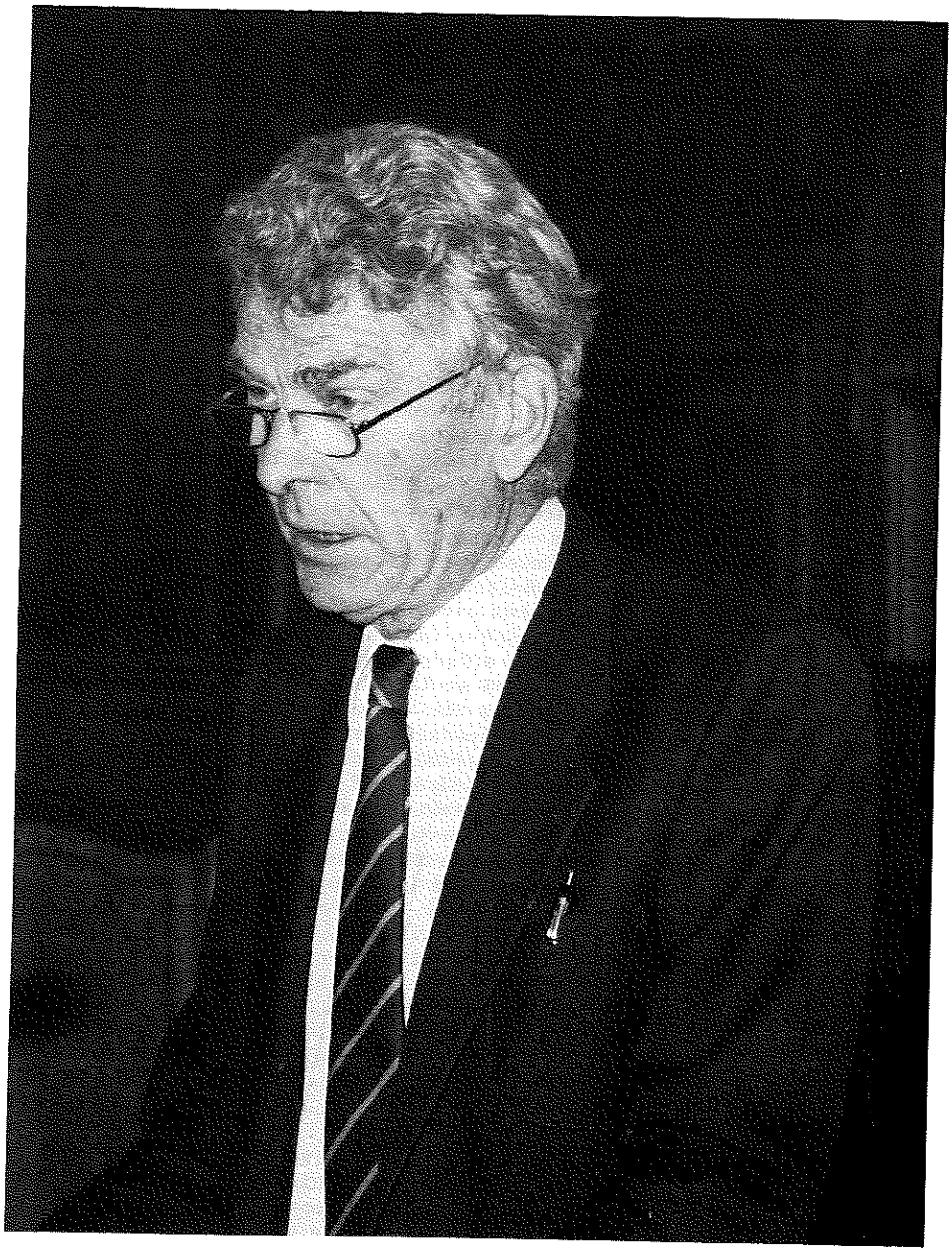
The concept of antibacterial chemotherapy goes back to the discovery of the sulfa drugs in the 1930's. This concept, however, was definitely established only when the first cures were achieved by penicillin in 1941. Penicillin opened the antibiotic era in which we are living now.

Penicillin has given rise to a flourishing family of drugs (penicillins, cephalosporins, monobactams) designated collectively as β-lactam antibiotics (Fig. 1). All of them originally came from microorganisms of the soil but many of them were rendered more effective by chemical modifications. The multiplicity of the β-lactam molecules - more than 100,000 - that the pharmaceutical industry has produced essentially by trial and error, has been dictated primarily by the various countermoves that the bacteria have made to resist our most effective armory of antibacterial chemotherapy. One of countermoves - and so far the most impressive one - has been the rapid spread of genes coding for various types of β-lactamases.

The β-lactamases degrade benzylpenicillin, and other β-lactam antibiotics discovered subsequently, into biologically inactive metabolites by opening the β-lactam ring through hydrolysis of the endocyclic amide bond. β-Lactamase genes are readily transferable elements and bacteria can create new β-lactamases by recombination of existing genes. As a result of the selective pressure exerted by the massive use of the β-lactam antibiotics, almost every bacterial species has now members that have acquired a β-lactamase gene and about 100 different types of β-lactamases have been detected.

While there is still plenty of evidence for the continuing development of resistance by β-lactamase production, other lines of defense have appeared. Tolerance to β-lactam antibiotics, though poorly understood, can be defined as diminished and/or delayed killing by bacterial growth inhibitory concentrations of the drug. Intrinsic resistance may be due to a decreased accessibility of the drug to its targets by modifications of the bacterial cell envelope and/or modifications of the targets themselves resulting in a decreased susceptibility to the drug.

In this war between man and bacteria, Professor Piet De Somer has been one of the great pioneers. As a result of his and C. De Duve's studies on penicillin production, a plant (RIT, now Smith Kline-RIT) was built in Genval (Belgium) where, in spite of the many technical problems still unsolved at that time, penicillin was manufactured commercially as early as 1947. Subsequently, P. De Somer's contributions and insights have been decisive in opening up several other areas of major importance. After he founded the Rega Institute in 1954, scientific achievements increased ever since, not only in the field of antibiotics but also in several other fields of microbiology. As to the antibiotic field, suffice it to mention



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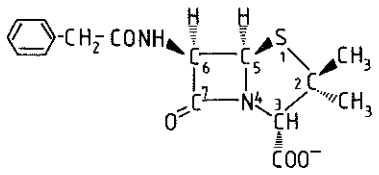
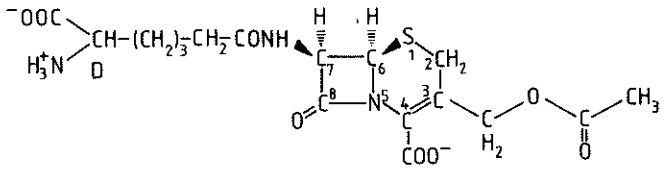
Benzylpenicillin



Cephalosporin C



Azthreonam

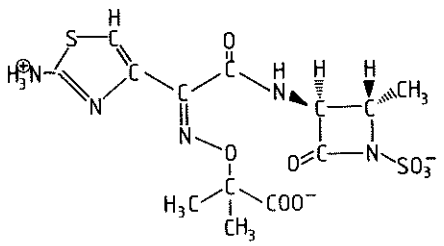


FIGURE 1. β-Lactam inactivators of the bacterial active-site serin DD-peptidases.

the discovery of griseomycin and virginiamycin and the design of one of the first semi-synthetic penicillins, clometocillin.

The aim of this article is to discuss advances made in the understanding of the catalytic mechanisms of two groups of enzymes whose common property is to recognize the β-lactam antibiotics : the DD-peptidases which are the target of penicillin action and the β-lactamases.

2. THE DD-PEPTIDASES/PENICILLIN BINDING PROTEINS

The biochemistry of the penicillin targets started some twenty years ago. In the late 60's, the primary structure of the bacterial wall peptidoglycan - which for many years had been known to be the site of the lethal lesion caused by penicillin - had been elucidated (1) (Fig. 2) and the pathway of wall biosynthesis had been unravelled (2) (Fig. 3). Killing of bacteria by penicillin was attributed to the selective inactivation of a membrane-bound transpeptidase, whose normal function is to incorporate the lipid-transported disaccharide peptide peptidoglycan precursors, as

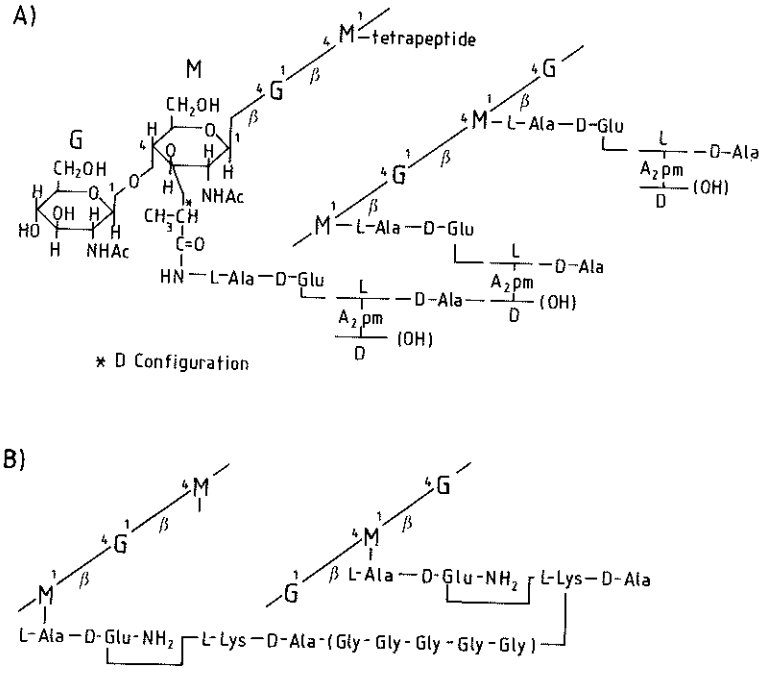


FIGURE 2. Primary structure of the wall peptidoglycans in Escherichia coli (A) and Staphylococcus aureus (B).

they emerge from the plasma membrane, to the preexisting wall peptidoglycan. Inactivation of the transpeptidase by penicillin was interpreted as a suicide process. The enzyme, mistaking the penicillin molecule for a D-alanyl-D-alanine-terminated peptidoglycan precursor (Fig. 3) undergoes derivatization in the form of a highly stable adduct. As a corollary, the transpeptidase behaves as a "penicillin binding protein".

As the research was progressing, the complexity of the reactions involved in wall peptidoglycan crosslinking became more and more evident and the unitary transpeptidase model had to be revised (3). All the bacteria possess, bound to the plasma membrane, multiple penicillin binding proteins (PBSs) of a molecular mass ranging from 25,000 to 100,000 or more (they are currently numbered in the order of decreasing size). Each bacterial species has its own assortment of PBPs. The several PBPs present in a single cell show varying susceptibility to β-lactam antibiotics so that, depending on the antibiotic concentration and the time of exposure used, the extent of inactivation of each of the PBPs may vary widely. The PBPs fulfil distinct cellular functions (in Escherichia coli, PBPs 1A/1B, 2 and 3 are related to cell elongation, shape maintenance and cell septation, respectively), do not show the same degree of essentiality for bacterial growth and may play compensatory roles.

The high-Mr PBPs (1A/1B, 2 and 3) have been assigned a role of prime importance in the killing of E.coli by the β-lactam antibiotics (3). But this is a rather exceptional situation. Often, comparison between the an-

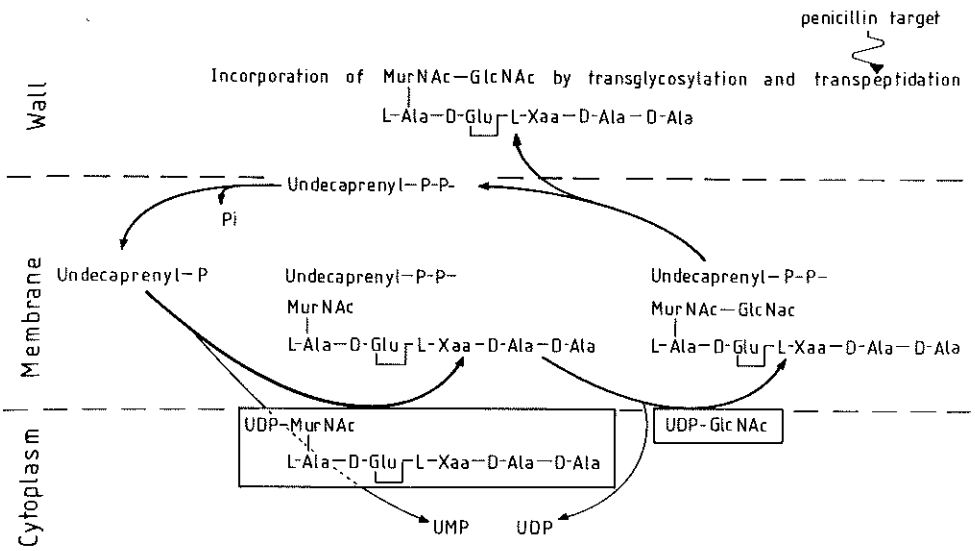


FIGURE 3. Biosynthesis of the bacterial wall peptidoglycans. Xaa = meso-diaminopimelic acid (as it occurs in E.coli) or L-lysine (as it occurs in S.aureus). Addition of the five glycine residues to the ε-amino group of the L-lysine residue (see Fig. 2) takes place at the level of the lipid intermediate (not shown).

tibacterial effectiveness of a β-lactam antibiotic and the concentration needed to derivatize some percentage (i.e. 50 %, 90 %, etc.) of one or more of the PBPs does not produce reasonable correlations and defining the killing target of a β-lactam antibiotic is difficult. There are cases where the first symptom of growth inhibition by penicillin has been related to a characteristic degree of saturation of each of the PBPs present, suggesting that the antibacterial effect arises from a summation of the extent of inactivation of several PBPs or from the abnormal relative concentrations of the PBPs left in a free form in the antibiotic-treated cells (4).

The PBPs to which catalytic activity could be assigned, catalyze various acyl transfer reactions. By transpeptidation (reaction A in Fig. 4), they perform closure of the bridges through which peptide units substituting adjacent glycan chains become linked together. This reaction is essential for the polymerization and expansion of the wall peptidoglycan in the form of an insoluble network. By carboxypeptidation, PBPs limit the amounts of donors available for transpeptidation (reaction B in Fig. 4) and/or produce new amino acceptor sites in the preformed peptidoglycan network (reaction C in Fig. 4). The significance of these hydrolytic activities is not clear. They probably control the level of cross-linking in the completed peptidoglycan and serve a role in wall remodelling throughout the bacterial life cycle.

Depending on whether the acceptor is the ω-amino group of a peptide or water, the transfer reaction leads to an increased or a decreased ex-

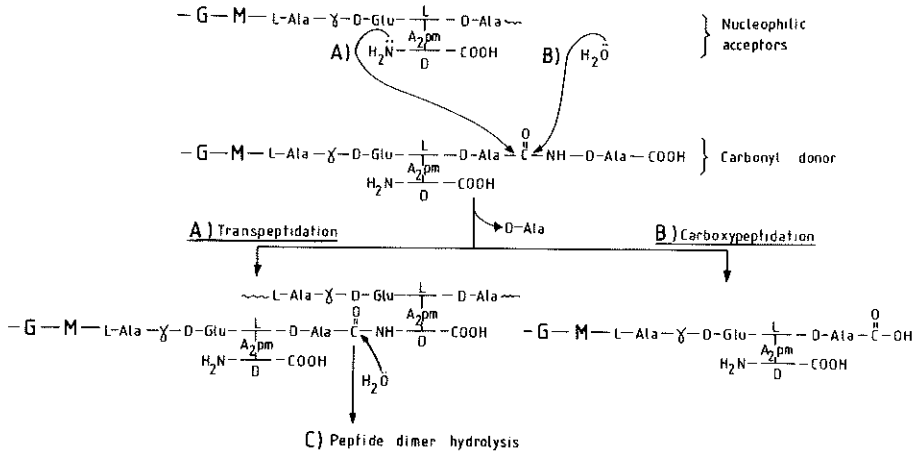


FIGURE 4. DD-peptidases/penicillin-binding proteins-catalyzed acyl transfer reactions with natural peptide donor and acceptor substrates.

tent of cross-linking. Yet, in all cases, the scissile peptide bond in the carbonyl donor is located in the α-position with respect to a free carboxylate and extends between two carbon atoms having the D configuration. This unique optical specificity justifies the name "DD-peptidase" given to the PBPs. Their selective inactivation by β-lactam derivatization explains the remarkable selective toxicity of these antibiotics.

Isolated DD-peptidases/PBPs may have high activity on well-defined peptides. These peptides may be of natural origin (as those shown in Fig. 4) or especially designed to function as donor and acceptor analogues (as those shown in Fig. 5). Other PBPs (of high molecular mass) catalyze, in concert, peptide cross-linking by transpeptidation and glycan synthesis by transglycosylation. Their bifunctionality is revealed by the products that they generate upon incubation with the lipid-linked precursor N-acetylglucosaminyl-N-acetylmuramyl(pentapeptide)-diphosphoryl-undecaprenol (Fig. 6). These reactions proceed with a low turnover number of 0.01 s^{-1} or less, suggesting that the in vitro conditions poorly mimick the in vivo situation. As shown with PBP3 of E.coli, removal by gene fusion of the amino terminal 240 amino acid region which, presumably, contains the penicillin-insensitive transglycosylate site, results in a truncated, 348 amino acid polypeptide that binds penicilin (5).

The DD-peptidase/PBPs are not easily accessible to biochemical studies. They occur in a small number of copies per cell, from perhaps 100 to 2000, and their membraneous nature poses obvious problems (though some of them can be detached from the plasma membrane by careful proteolytic action without apparent alteration of the catalytic properties). DNA recombinant techniques as means to prepare large quantities of water-soluble DD-peptidase/PBPs are actively investigated. In addition, such enzymes are excreted spontaneously during the growth of bacteria (i.e. actinomycetes strains).

The gene encoding the extracellular DD-peptidase/PBP of Streptomyces

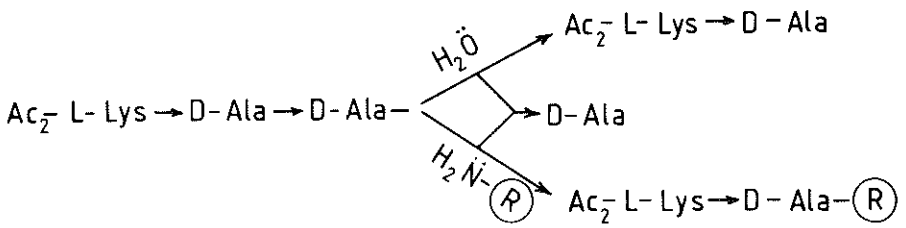


FIGURE 5. DD-peptidases/penicillin-binding proteins-catalyzed acyl transfer reactions with peptide donor and acceptor analogues.

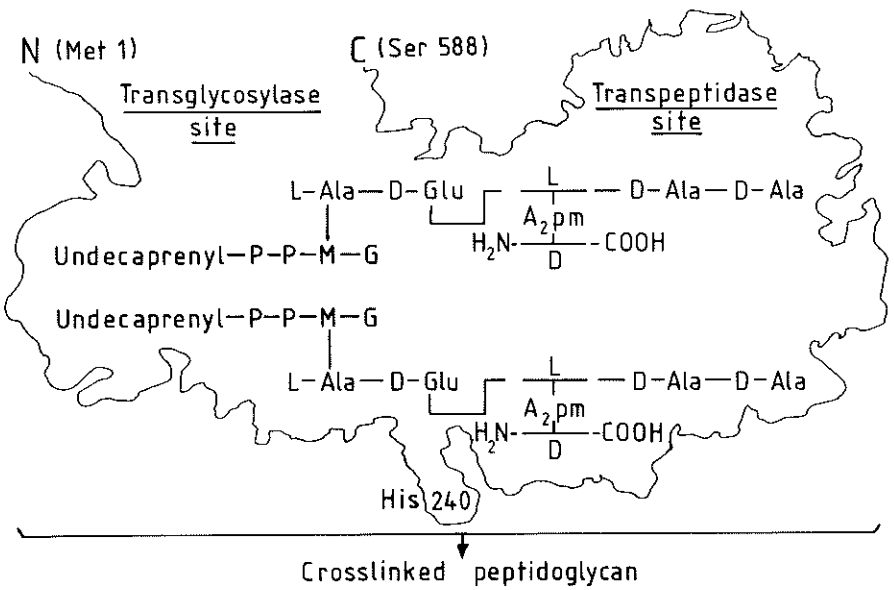


FIGURE 6. Lipid-linked disaccharide (pentapeptide)-diphosphoryl-undecaprenol as substrate of the bifunctional penicillin-binding proteins. The transglycosylase site catalyzes glycan elongation (with release of the undecaprenyl-P-P). The transpeptidase site catalyzes cross-linking between peptide units.

R61 has been cloned with amplified expression of the excreted protein (6). The gene codes for a 406 amino acid precursor which possesses both a cleavable N-terminal signal peptide and a cleavable C-terminal extension. This C-terminal segment has no runs of hydrophobicity typical of a membrane anchor. But, by analogy with E.coli PBP no 5, it may be hypothesized that, should it not be cleaved during maturation, this segment would function as a halting signal through which the enzymes would become membrane-bound by

a mechanism not previously described. Whatever the mechanism and physiological significance of excretion, this water-soluble 349 amino acid enzyme has served its role in the unravelling of the enzymatic, biochemical and structural properties of the DD-peptidases/PBPs.

3. DD-PEPTIDASES AND β-LACTAMASES-CATALYSED ACYL TRANSFER REACTIONS

In recent years we have witnessed one of those remarkable convergences which not unfrequently lead to episodes of high drama in science. The convergence is that, following initial studies made in 1976 (7) and 1979 (8), all the DD-peptidases/PBPs and the majority of the β-lactamases which have so far been characterized are active-site serine enzymes which can be regarded as counterparts of the LL-peptidases of the trypsin family. They all operate by an acyl enzyme mechanism.

3.1. General reaction and basic equations (9)

The DD-peptidases and β-lactamases-catalysed reactions proceed via the formation of a covalent intermediate in which the electrophilic carbon of the carbonyl donor substrate becomes ester-linked to the hydroxyl group of the enzyme's serine residue (Fig. 7). Enzyme acylation produces a leaving group or product P_1. In turn, transfer of the acyl moiety to an exogenous nucleophilic acceptor HY causes release of $R\text{-}\overset{O}{\overset{\|}{C}}\text{-}Y$, or product P_2, and enzyme regeneration.

This three-step reaction can be presented by

$$E + D \underset{}{\overset{K}{\rightleftharpoons}} E.D \underset{P_1}{\overset{k_{+2}}{\rightleftharpoons}} E\text{-}D^{*} \xrightarrow[HY]{k_{+3}} E + P_2 \qquad 1$$

where E = enzyme; D = carbonyl donor; HY = exogenous nucleophile; E.D = Michaelis complex; $E\text{-}D^{*}$ = serine ester-liked acyl enzyme; K = dissociation constant; k_{+2} and k_{+3} = first-order rate constants; P_1 = leaving group and P_2 = second reaction product. It is assumed that [D] >> [E], E.D is in rapid equilibrium with free enzyme and free donor, and HY (i.e. water) is at a saturating concentration.

The values of k_{cat} and Km are related to K, k_{+2} and k_{+3} by

$$k_{cat} = \frac{k_{+2}k_{+3}}{k_{+2} + k_{+3}} \qquad Km = \frac{Kk_{+3}}{k_{+2} + k_{+3}}$$

so that k_{+2}/K (i.e. the second-order rate constant of enzyme acylation by the carbonyl donor) is equal to k_{cat}/Km.

The proportion of total enzyme $[E]_o$ which occurs as acyl enzyme $[E\text{-}D^{*}]_{ss}$ at the steady state of the reaction is

$$\frac{[E]_o}{[E\text{-}D^{*}]_{ss}} = 1 + \frac{k_{+3}}{k_{+2}} + \frac{Kk_{+3}}{k_{+2}[D]} \qquad 2$$

The time necessary for the acyl enzyme to reach a certain percentage of its steady state level is

$$t = \frac{-\ln\left(1 - \frac{[E\text{-}D^{*}]}{[E\text{-}D^{*}]_{ss}}\right)}{k_{+3} + k_a} \qquad 3$$

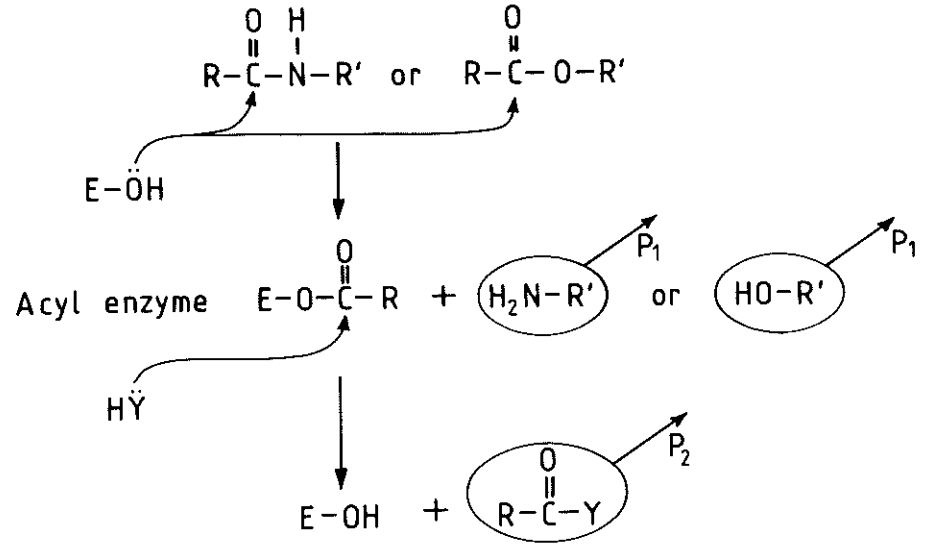


FIGURE 7. Active-site serine enzyme-catalyzed acyl transfer reaction between an amide (or ester) carbonyl donor and an exogenous nucleophilic acceptor. R-CONH-R' or R-COO-R' = amide or ester carbonyl donor; E-OH = enzyme with the hydroxyl group of the serin residue; H_2N-R' or HO-R' = leaving group; HY = exogenous acceptor (H_2O, an amino compound, etc.).

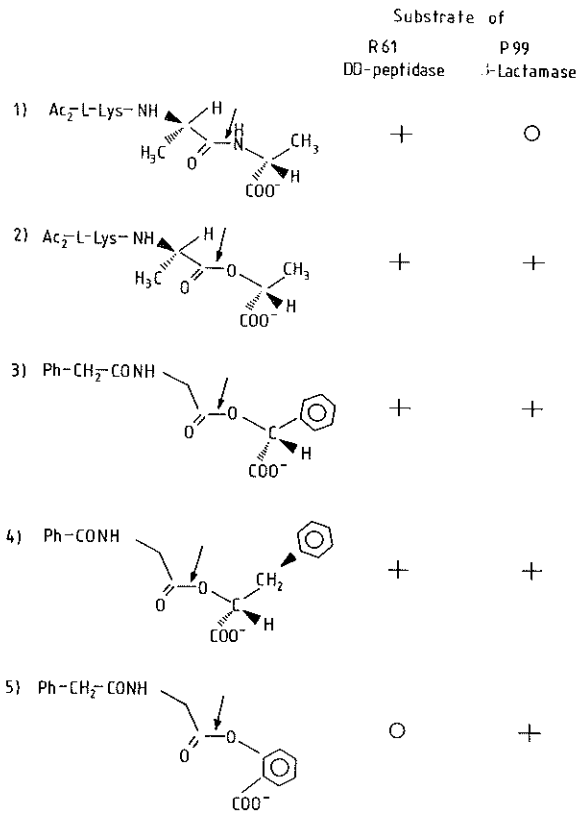
where $k_a = \frac{k_{+2}}{1 + (K/[D])}$ is the pseudo-first-order rate constant of acyl enzyme formation at a given concentration of carbonyl donor.

For $k_{+3} >> k_{+2}$, the steady-state proportion of enzyme present as acyl enzyme is low ($k_{cat} = k_{+2}$ and Km = K). For $k_{+2} >> k_{+3}$, this proportion is high ($k_{cat} = k_{+3}$ and $Km = Kk_{+3}/k_{+2}$) and the accumulated acyl enzyme can be trapped, isolated and characterized. If k_{+3} has a very small absolute value, the acyl enzyme is highly stable and the donor is a mechanism-based inactivator.

The presence of competing nucleophiles (alcohol, hydroxylamine, other amino compounds) in the aqueous reaction mixture modifies the fate of the donor since the enzyme activity undergoes partitioning at the level of the acyl enzyme between hydrolysis, alcoholysis, aminolysis or transpeptidation. An alternate nucleophile may also increase the rate of consumption of the donor (i.e. k_{cat}) if, in water, the acyl enzyme accumulates ($k_{+2} > k_{+3}$).

3.2. DD-peptidases and β-lactamases-catalysed reactions with non-cyclic peptide and ester carbonyl donors

The substrate profiles of some DD-peptidases and β-lactamases have been explored using several peptides and depsipeptides as donors (Fig. 8) and by carrying the reactions both in water and in aqueous solutions con-



(1) Ac_2-L-Lys^2-D-Ala^1-D-Ala^1; (2) Ac_2-L-Lys-D-Ala-D-Lac;
(3) Phenylacetylglycyl-D-mandelate; (4) Hippuryl (D) phenyllactate;
(5) Phenylacetylglycylsalicylate.

FIGURE 8. Amide and ester compounds as carbonyl donors of some DD-peptidases and β-lactamases. The arrows indicate the scissile bond. (1) : Ac_2-L-Lys-D-Ala-D-Ala; (2) Ac_2-L-Lys-D-Ala-D-lactate; (3) phenylacetylglycyl-D-mandelate; (4) hippuryl-(D)-phenyllactate; (5) phenylacetylglycylsalicylate.

taining alternate acceptors.

Not surprisingly, the conclusion of these studies is that a peptide or a depsipeptide of high donor activity for a DD-peptidase (such as Ac_2-L-Lys-D-Ala-D-Ala or Ac_2-L-Lys-D-Ala-D-lactate) closely resembles the C-terminal portion of a peptidoglycan precursor. With the DD-peptidases studied, the k_{+3}/k_{+2} ratio is such that enzyme acylation is usually slower or much slower than enzyme deacylation, the efficacy of binding of D-alanyl-D-alanine-terminated peptides is relatively weak ($Km \cong K = 0.1\text{-}10$ mM; a situation which favors maximization of the overall reaction rates) and the

k_{+2} values of enzyme acylation may range from $\simeq 1\ s^{-1}$ to $\simeq 50\ s^{-1}$. Replacement of the scissile peptide bond by an ester bond (as it occurs in the depsipeptides) usually results in an increased rate of enzyme acylation (k_{+2}).

Relatively little is known on the β-lactamases-catalysed acyl transfer reactions involving non-cyclic donors. But three observations have been made. 1) All the β-lactamases appear to be devoid of peptidase activity. 2) Some β-lactamases (such as that produced by Enterobacter cloacae P99) have esterase activity (10); hence β-lactamases and DD-peptidases exist which catalyse acyl transfer reactions from the same depsipeptide donors. 3) Other β-lactamases (such as that produced by Bacillus licheniformis) have no or little esterase activity; β-lactam compounds are, apparently, the only donors on which they operate.

As expected from an acyl enzyme mechanism, the presence of alternate nucleophiles causes partitioning of the acyl enzymes formed during interaction between non-cyclic (peptide and/or ester) donors and the DD-peptidases or β-lactamases. The following examples illustrate the underlying mechanisms.

The use of D-alanyl-D-alanine-terminated peptides as donors by the DD-peptidases reveals a subtle "push and pull" interplay at the level of the acyl enzyme, between the leaving group D-Ala, water and an exogenous amino compound NH_2-R structurally related to the wall peptidoglycan interpeptide bridge. Depending on the DD-peptidases, the relative acceptor activity may be D-Ala << 55.5 M H_2O << mM NH_2-R or 55.5 M H_2O << D-Ala << mM NH_2-R (Fig. 9).

The DD-peptidases of the first type hydrolyze the donor to completion without any significant accumulation of acyl enzyme and without any sign of interference by the leaving group D-Ala (which accumulates in the reaction mixture). Millimolar concentrations of an exogenous, suitable peptide acceptor channel part of the donor to the transpeptidated product at the expense of its hydrolysis (Fig. 9A). These DD-peptidases (for example the PBPs 4, 5 and 6 of E.coli and the Streptomyces R61 DD-peptidase) catalyze concomitant carboxypeptidation and transpeptidation reactions on a competitive basis.

The DD-peptidases of the second type (for example the 26,000-Mr, membrane-bound DD-peptidase of Streptomyces K15) (11) behave differently. The leaving group D-Ala effectively suppresses the acceptor activity of water, performs attack of the acyl enzyme and regenerates the initial carbonyl donor. Though the DD-peptidase turns over and a non-negligible proportion of it occurs as acyl enzyme, little of the carbonyl donor is consumed. In turn, this "pace-making" mechanism can be overcome by supplementing the reaction mixture with a suitable peptide acceptor. This peptide, at millimolar concentrations, largely saturates the enzyme (and is the only acceptor HY of reaction 1). Consequently, the carbonyl donor is transpeptidated until completion, no acyl enzyme accumulates and the DD-peptidase functions as a strict transpeptidase (Fig. 9B). Experimental evidence also shows that the peptide acceptor does not behave as a simple alternate nucleophile. It influences the initial binding between the enzyme and the donor and enhances the efficacy of the ensuing enzyme acylation step.

These properties offer a possible explanation for the observations that certain PBPs have no detectable or weak enzymic activity in vitro. The high-Mr PBPs 1A/1B, 2 and 3 of E.coli, already mentioned, may lack hydrolytic potency because they possess a "pace-making" mechanism like that described above. They may have low intrinsic transpeptidase activity because in the assays so far devised, they do not find the right amino acceptor needed for an efficient acylation of the protein by the donor.

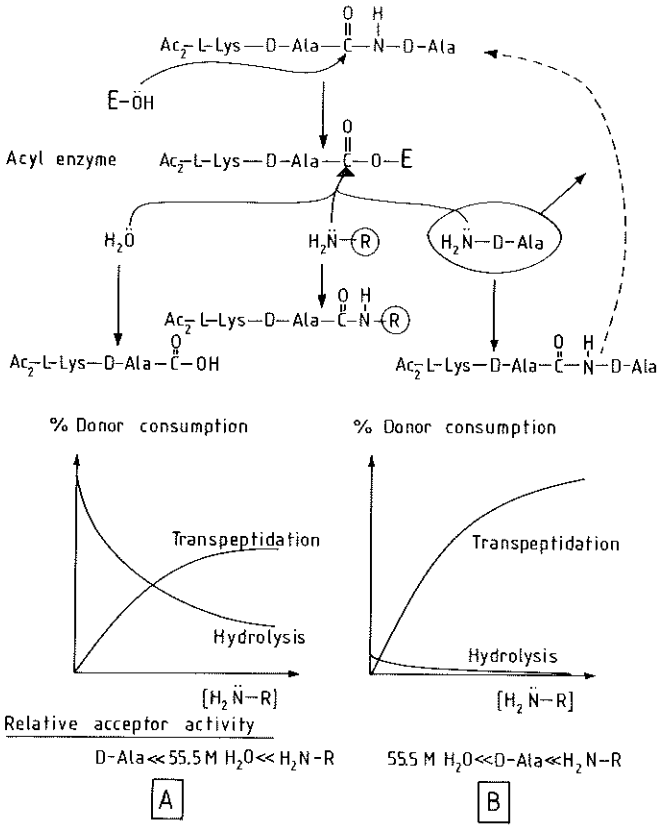


FIGURE 9. Competition between the leaving group D-Ala, water and an exogenous amino acceptor at the level of the acyl enzyme formed during DD-peptidase-catalyzed acyl transfer reaction with Ac_2-L-Lys-D-Ala-D-Ala as carbonyl donor. A) The DD-peptidase catalyzes concomitant carboxypeptidation and transpeptidation reactions. B) The DD-peptidase catalyzes, almost exclusively, a transpeptidation reaction. E-OH = enzyme with the hydroxyl group of the serine residue.

3.3. DD-peptidases and β-lactamases-catalyzed reactions with cyclic (β-lactam) carbonyl donors

β-Lactam compounds may have high donor activity for the β-lactamases and the DD-peptidases if the two following minimal requirements are fulfilled. A negative charge must occur at the 3' in the penicillins and 4' in the cephalosporins (this group is usually a carboxylate but a sulfate is found in the monobactams) and the acyl side chain borne by the β-lactam ring must be on the β face in a cis-position to the thiazolidine ring in the penicillins or dihydrothiazine ring in the cephalosporins (Fig. 1).

Enzyme acylation of β-lactam compounds produces a leaving group which remains covalently attached to the enzyme ester-linked electrophilic por-

tion of the donor. Consequently, the enzyme's active site remains occupied by a leaving group which cannot leave. In this respect, the reactions catalyzed by the β-lactamases contrast with those catalyzed by the DD-peptidases (Fig. 10).

The β-lactamases have adapted themselves to the situation created by a non-diffusable leaving group. Water is an excellent attacking nucleophile of the acyl (penicilloyl, cephalosporoyl, etc.) enzyme which, usually, is very short-lived. The β-lactamases can hydrolyze β-lactam antibiotics with very high k_{cat} values (up to 1000 s^{-1}).

In contrast, β-lactam compounds are mechanism-based inactivators of the DD-peptidases. Constant k_{+3} of reaction 1 may have a very small absolute value. However, the efficacy of the inactivation depends on the va-

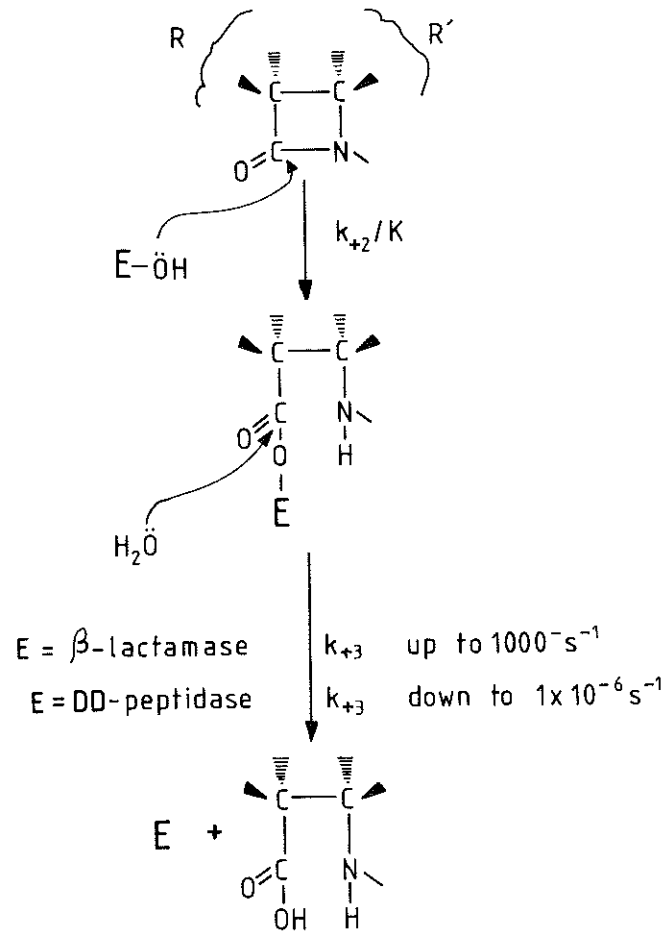


FIGURE 10. β-Lactam compounds as carbonyl donors of the β-lactamases and DD-peptidases. E-OH = enzyme with the hydroxyl group of the serine residue.

lues of all the kinetic parameters (9). The higher the second-order rate constant of enzyme acylation (k_{+1}/K; $M^{-1}s^{-1}$) and the smaller the first-order rate constant of acyl enzyme breakdown (k_{+3}; s^{-1}), the lower the β-lactam compound concentration ([D]) for which the term $Kk_{+3}/k_{+2}[D]$ of equation 2 (which for $k_{+3} << k_{+2}$ simplifies to $[E]_o/[E\text{-}d^*]_{ss} = 1 + (Kk_{+3}/k_{+2}[D])$ becomes negligible. When this term is smaller than 0.01, virtually all the DD-peptidase is immobilized as acyl enzyme at the steady state of the reaction.

The time required for the reaction to reach the steady state is another important feature of the inactivation process. Assume four β-lactam compounds acting on a given DD-peptidase. They are used at the same concentration ([D] = 1 μM), the k_{+3} value for acyl enzyme breakdown is the same ($k_{+3} = 1 \times 10^{-4} s^{-1}$; i.e. the acyl enzyme has a half-life of 125 min) but the relevant k_{2+}/K values are 10, 100, 1000 and 10,000 $M^{-1}s^{-1}$. It follows from equations 2 and 3 that 9.1 %, 58 %, 90.9 % and 99 %, respectively, of the DD-peptidase is acylated at the steady state of the reaction and that it takes 698 min, 348 min, 70 min and 7.6 min, respectively, for the reaction to reach 99 % of the steady-state.

β-Lactam compounds have been extensively used to probe the active sites of the DD-peptidases/PBPs (12). The values of k_{+3} and k_{+2}/K (and sometimes the individual values of K and k_{+2}) have been determined accurately in some cases. An interesting conclusion of these studies is that a cyclic β-lactam compound is not necessarily a better acylating agent of the active-site serine residue than a non-cyclic peptide (or ester) donor. Moreover, as observed with non-cyclic donors, the efficacy of binding of the β-lactam compounds may be relatively weak (K = 0.1-10 mM). However, because of high k_{+2} values, the DD-peptidases/PBPs can be (almost) completely immobilized as acyl enzymes in a few seconds or minutes by β-lactam compound concentrations much smaller than the dissociation constant K.

Plots of extents of protein acylation versus β-lactam compound concentrations give access to the β-lactam compound concentration ($[D]_{0.5}$) which is necessary to achieve 50 % saturation of a given DD-peptidase/PBP after a given time of incubation ($t_{0.5}$) and at a given temperature. Under certain conditions (9), these $[D]_{0.5}$ and $t_{0.5}$ values are related to the second-order rate constant of protein acylation by

$$([D]t)_{0.5} = 0.69 \frac{K}{k_{+2}} \qquad 4$$

Equation 4 is valid if i) the incubation time is much smaller than k_{+3} (or $k_a >> k_{+3}$); ii) [D] << K; and iii) the β-lactam compound concentrations used are such that a 100 % level of protein acylation would be achieved at the steady-state of the reaction. That these conditions are fulfilled can be checked graphically; a 23-fold (or a 7.2-fold etc.) increase in the β-lactam concentration should be sufficient to cause a change in the extent of enzyme acylation from 10 % to 90 % (or from 20 % to 80 %, etc.). Hundreds of $[D]_{0.5}$ values concerning the interactions between β-lactam compounds and PBPs have been published. These data may give at least rough estimates of the values of the second-order rate constant of protein acylation (although in many instances, the experimental conditions are not described in sufficient detail to be sure that equation 4 applies).

3.4. Properties of the acyl enzymes formed by reaction between β-lactam carbonyl donors, the DD-peptidases and the β-lactamases

The above discussion is an oversimplification. The acyl enzymes formed by reaction between some DD-peptidases and β-lactam compounds may be relatively short-lived. Thus the benzylpenicilloyl PBP4 of Staphylococcus aureus has a half-life of about 1.5 min. Conversely, the acyl enzymes formed by reaction between β-lactamases and β-lactam compounds may be long-lived. This is the case for the pairs cefoxitin-RTEM β-lactamase (k_{+3} = $5 \times 10^{-3} s^{-1}$) and cloxacillin-E.cloacae β-lactamase ($k_{+3} = 1.5 \times 10^{-4} s^{-1}$). Finally, long-lived acyl enzymes (whether they are formed with a DD-peptidase or a β-lactamase) may undergo intramolecular rearrangements. Enzyme regeneration is then a complex pathway.

The first acyl enzyme rearrangement reported in the literature describes the rupture of the C_5-C_6 bond in an acyl (benzylpenicilloyl) DD-peptidase (13) (Fig. 11). This reaction, in which water donates the proton on C_6, has two consequences. The leaving group of the enzyme acylation step can now leave the enzyme's active site (to give rise to N-formyl-penicillamine) and the new phenylacetylglycyl enzyme (which is identical to that formed by reaction with phenylacetylglycyl-D-mandelate) is susceptible to concomitant and immediate attack by water and a structured amino compound. Hydrolysis of the benzylpenicilloyl enzyme with release of benzylpenicilloate and rupture of C_5-C_6 proceed at approximately the same low rates ($= 0.5 \times 10^{-4} s^{-1}$) so that C_5-C_6 rupture is not an effective means for the enzyme to get relieved of the bound inactivator. Release of the phenylacetylglycyl moiety is pH-independent. Release of the benzylpenicilloyl moiety increases at high pHs. The second-order rate constant computed for the attack of the benzylpenicilloyl enzyme by OH^- ions is about 4 $M^{-1} s^{-1}$, a value which is not much higher than that observed for the hydrolysis of α-methylpenicilloate.

A second type of acyl enzyme rearrangement is observed with those cephalosporins which have a good 3' leaving group, for example an acetoxy substituent (14). Such groups are eliminated from the acyl enzyme before deacylation occurs and an exocyclic methylene is formed. This elimination may have important consequences with respect ot cephalosporin turnover by the β-lactamases and inactivation of the DD-peptidases since the acyl enzymes which have undergone elimination at position 3 are more hydrolytically inert than their parents. As shown with a DD-peptidase, the 3' substituent influences enzyme acylation in a way not predictable solely on the basis of the electronic effects and, conversely, the DD-peptidase may strongly influence the rates of the elimination reaction.

A third type of acyl enzyme rearrangement, which is an integral part of the β-lactamase inactivation mechanisms, involves ring opening between S_1 and C_5 in the penam sulfones and 6-β-bromo(iodo)penicillanate or between O_1 and C_5 in clavulanate (15). Ring opening launches a series of rearrangements which proceed ultimately to enzyme inactivation. The established common feature is that of a branched pathway where β-elimination of the acyl enzyme leads to a hydrolytically inert acyl enzyme E-d^1 and/or further modification of some residues of the enzyme's active site.

$$E + D \underset{}{\overset{K}{\rightleftharpoons}} E.D \xrightarrow{k_{+2}} E\text{-}D^{*} \xrightarrow{k_{+3}} E + P$$

$$E\text{-}D^{*} \xrightarrow{k_{+4}} E\text{-}D^{1}$$

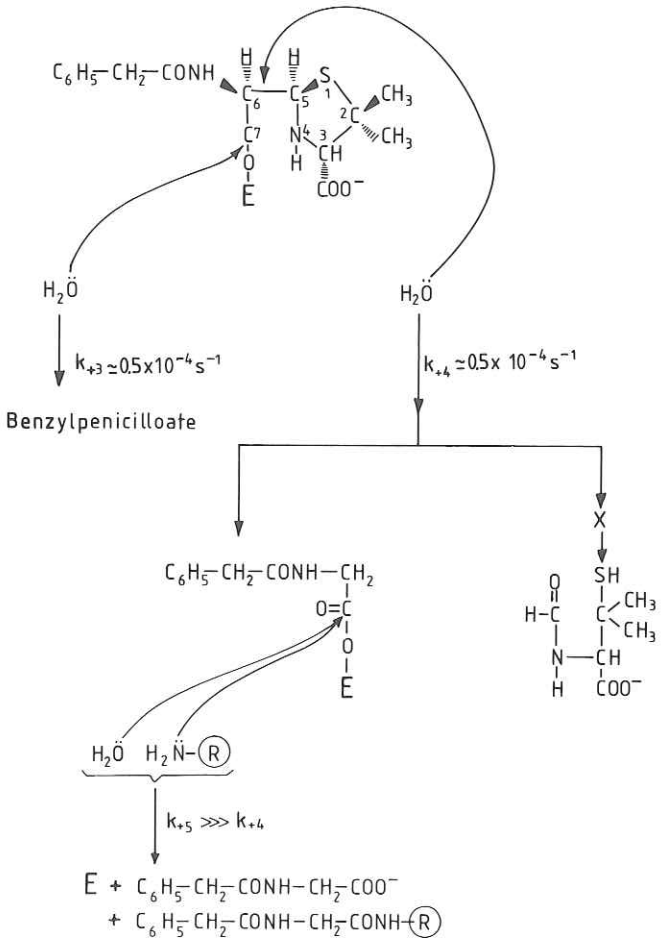


FIGURE 11. Rearrangement of the acyl enzyme formed by reaction between benzylpenicillin and a DD-peptidase. Rupture of the C_5-C_6 bond (k_{+4}) causes release of the leaving group of the enzyme acylation step.

For $k_{+3} << k_{+4}$, the fate of the β-lactamase is inactivation. Under other conditions, the β-lactamase can be totally inactivated only if $[D]/[E]_o \geq (k_{+3} + k_{+4})/k_{+4}$. The final composition of the reaction mixture depends on both the concentration of the penam and the $[D]/[E]_o$ ratio value. For $[D]/[E]_o < (k_{+3} + k_{+4})/k_{+4}$, the final mixture contains free enzyme, $E\text{-}D^i$ and hydrolyzed penam. For $[D]/[E]_o > (k_{+3} + k_{+4})/k_{+4}$, the final mixture contains $E\text{-}d^i$ and both hydrolyzed and intact penam.

6-β-Iodopenicillinate has been used as a means to probe the active

sites of ß-lactamases. In all cases, rearrangement of the acyl enzyme gives rise to a dihydrothiazine chromophore (16). But depending on the ß-lactamases, the value of second-order rate constant of enzyme acylation varies from 1 x 10^6 $M^{-1}s^{-1}$ to 100 $M^{-1}s^{-1}$. Moreover, k_{+3} may not be negligible in which case complete enzyme inactivation requires high molar ratios of 6-ß-iodopenicillinate to enzyme.

6-ß-Iodopenicillanate has also been used as a DD-peptidase labelling reagent. Rearrangement of the acyl enzyme also gives rise to the dihydrothiazine chromophore. Though a weak acylating donor (k_{+2}/K = 0.75 $M^{-1}s^{-1}$), 6-ß-iodopenicillinate has the advantage to form an adduct which is much more stable than that formed by reaction with benzylpenicillin.

4. STRUCTURE OF, AND EVOLUTIONARY RELATIONSHIP BETWEEN DD-PEPTIDASES AND ß-LACTAMASES

Advances have been made in three areas.

Gene cloning and sequencing (and sometimes direct analysis of the expressed proteins) has given access to the primary structure of ten ß-lactamases (from plasmid pBR322, E.coli, Citrobacter freundii, Enterobacter P99, Salmonella typhimurium, Bacillus licheniformis, Bacillus cereus, Staphylococcus aureus, Streptomyces albus G and Streptomyces cacaoi) (17), three DD-peptidase/PBPs of low molecular mass (from E.coli, B.subtilis and Streptomyces R61) (6,18) and four bifunctional PBPs of high molecular mass, (PBPs 1A, 1B, 2 and 3 of E.coli) (19). The proteins or the penicillin-binding (transpeptidase) domains of the bifunctional PBPs are 250- to 350-amino acid polypeptide chains. Using a program which compares protein sequences on the basis of the relative amino acid substitution frequencies found among families of homologous proteins, some of these proteins (or domains) form pairs or groups which match very well with respect to the amino acid sequences. However, when the pairs or groups thus defined are compared to each other, the overall similarity becomes marginally significant or vanishes.

Enzymic degradation of long-lived acyl enzymes formed with ß-lactam compounds and analysis of the ß-lactam-labeled peptide fragments has led to the identification of the active-site serine residue. This serine residue is always close to the amino terminus of the protein (at position 62 in the Streptomyces R61 DD-peptidase) or the penicillin-binding (transpeptidase) domain of the bifunctional PBPs. Moreover, in all cases, a lysine residue occurs at the third position on the carboxyl side of the active-site serine ($Ser^{\ddagger}$-Xaa-Xaa-Lys).

The exocellular DD-peptidase of Streptomyces R61 and several ß-lactamases have been crystallized. The tertiary structure and active site of the DD-peptidase are known at 2 Å resolution (20). X-ray analysis at 3.5 Å of the ß-lactamases of Bacillus licheniformis (20) and B.cereus (21) (these two ß-lactamases have highly homologous amino acid sequences) has provided clear images of the polypeptide folding. Though the Streptomyces DD-peptidase and the baccilus ß-lactamases lack structural relatedness in the primary structure, they have very similar tertiary structures. They contain eight helices and a ß-sheet of five strands protected on the front face by two helices and on the back face by another helix (Fig. 12). Matching of the helices and sheet strands between the three structures is so great that it is impossible to argue against an evolutionary relationship.

Alignment of the primary structures (with appropriate deletions or insertions) of the seventeen penicillin-recognizing enzymes for which the data are available, generates boxes which contain identical and/or functionally homologous residues (Fig. 13). These boxes occur at similar posi-

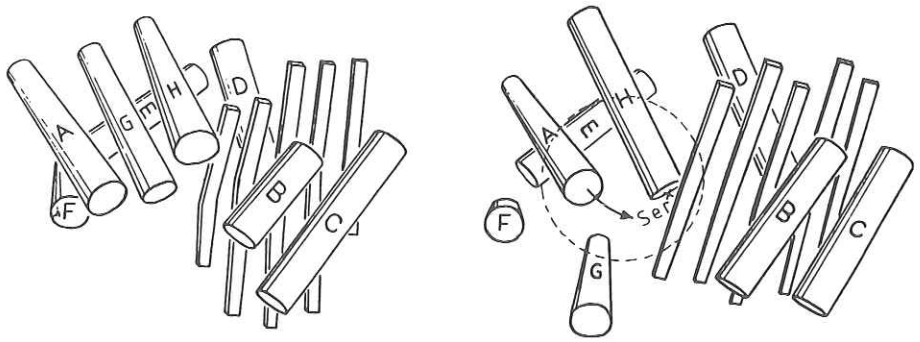


FIGURE 12. Spatial disposition of secondary structures in the Bacillus licheniformis β-lactamase and the Streptomyces R61 DD-peptidase. The eight α helices (cylinders) are marked A through H. The β sheet (five strands) is protected by helices B and C on one face and by helix D on the other. The active-site serine (star) is at the N-terminus of helix H. The active site area is circled.

Enzymes	Box I	Box II	Box III	Box IV	Box V	Box VI	Box VII	
a	G_{42} D_{45}	R_{57} F R V G S* V T K	Y_{90}	T_{101} V R	D_{225}	W_{271}	G_{297} H T G	Streptomyces R61 DD-peptidase
b	G_{44} D_{47}	L_{59} F E L G S* V S K I	Y_{92}	T_{97} A K G	D_{217}	W_{260}	H_{314} K T G	
c	G_{45} D_{50}	R_{65} F A F A S* T S K P Y M I M Y	Y_{105}	T_{109} E K L	E_{166}	W_{210} L	D_{233} K S G T	
d	G_{24} D_{29}	L_{43} F P M C S* V F K	Y_{74}(?)	T_{90} G K	E_{152}	W_{194}	D_{217} K T G	β-lactamases
e	G_{23} D_{28}	R_{42} F A Y G S* T F K	Y_{83}(?)	T_{95} G K	E_{260}	W_{200}	D_{223} K S G	
f	G_{42} D_{48}	R_{67} Y S P A S* T F K	H_{107}	D_{112} L R	D_{157}	L_{196}	A_{209} K T G	
g	G_{20} D_{29}	R_{31} L P I A S* M T K	Y_{61or68}	L_{81} R K	D_{490}	W_{207}	L_{226} K T G	Low MW
h	G_{28} D_{37}	R_{39} R D P A S* L T K	N_{80}	F_{90} L K	D_{175}	W_{203}	I_{212} K T G H	PBPs
i	G_{444} D_{447}	L_{460} R Q V G S* N I K	Y_{511}	α_{523} S K	D_{588}	W_{697} 702	G_{715} K T G	
j	G_{489} E_{492}	R_{505} R S I G S* L A K	Y_{557}	L_{569} T R	D_{647}	W_{672}	G_{697} K T G	High MW
k	G_{300} D_{305}	V_{325} Y P P A S* T V K	Y_{367}	H_{375} G R	D_{490}	W_{512}	A_{543} K S G	PBPs
l	G_{288} E_{292}	V_{302} F E P G S* T V K	Y_{334}	V_{344} A R	E_{456}	L_{476}	I_{493} K T G T	

FIGURE 13. Conserved boxes along the primary structures of penicillin-recognizing enzymes. a) Streptomyces R61 DD-peptidase; b) enterobacterial β-lactamases; c) B.licheniformis, B.cereus, pBR322 and S.aureus β-lactamases; d) Streptomyces albus G β-lactamase; e) Streptomyces cacaoi β-lactamase; f) Salmonella typhimurium Oxa-2 β-lactamase; g) B.subtilis PBP5; h) E.coli PBP5; i) E.coli PBP1A; j) E.coli PBP1B; k) E.coli PBP2; l) E.coli PBP3. The boxes in i to l refer only to the penicillin-binding (transpeptidase) domains of the PBPs.

tions along the amino acid sequences (22) and some of them are known to occupy critical positions in the tertiary structure of the Streptomyces DD-peptidase (23). It has been proposed (22) that this conserved pattern probably expresses a common polypeptide scaffolding.

Assuming that hundreds, perhaps thousands, of different DD-peptidases and β-lactamases occur in the bacterial world, the accumulated information is still very limited. Yet, it has led to significant conclusions and allows predictions to be made.

The β-lactamases, DD-peptidases and penicillin-binding/transpeptidase domains of bifunctional PBPs have derived by divergent evolution from a common ancestor gene that must have coded for an essential DD-transpeptidase. These enzymes offer an example of persistence of similarities between three-dimensional structures while i) the similarity between primary structures has progressively disappeared as a function of the evolutionary distance; and ii) the active sites have been modified to give rise to multiple penicillin-binding DD-peptidase of varying functionality and essentiality and multiple penicillin-hydrolysing β-lactamases of varying anti-β-lactam antibiotics spectra.

The tertiary arrangement of packed α-helices and β-strands shown in Fig. 12 is unprecedented. Though structural changes have occurred as the sequences have diverged, the active-site serine DD-peptidase and β-lactamases form a very large family of enzymes which are characterized by a similar fold of the polypeptide chains.

Differences in the catalyzed reactions can, at least in part, be viewed as the expression of the variations seen in the amino acid composition of some of the conserved boxes. As a corollary, these amino acids are targets of choice to be studied by site-directed mutagenesis.

5. CATALYTIC MECHANISM OF THE β-LACTAMASES, DD-PEPTIDASES AND LL-PEPTIDASES OF THE TRYPSIN FAMILY, AN UNIFIED VIEW

Understanding the established catalytic properties of an enzyme at atomic resolution can be approached only by cross-fertilization between various disciplines and technologies : tertiary structure determination, gene analysis and site-directed mutagenesis, molecular modelling and theoretical chemistry. As described above, recent advances in the field of the DD-peptidases and β-lactamases have brought the goal closer.

The DD-peptidases and β-lactamases as well as the LL-peptidases of the trypsin family catalyze rupture of a peptide (amide, ester) bond in a carbonyl donor. Such a bond has a permanent dipole which confers a partial positive charge on the carbonyl carbon atom. Bond rupture requires polarization of the C=O by an electrophile, attack of the carbonyl carbon atom by a nucleophile and proton donation on the nitrogen (oxygen) atom. The reaction as it is catalyzed by the active-site serine LL-peptidases (and using the numbering of chymotrypsin) is currently attributed to the concerted action between the conserved triad Ser 195 ... Hist 57 ... Asp 102 acting as proton abstractor and donator and a pair of hydrogen bonds from the backbone NH of Gly 193 and Ser 195 acting as electrophile.

The tertiairy structure of the penicillin-recognizing enzymes is not known to a resolution that permits identification of the amino acid side chains possibly involved in catalysis in concert with the active-site serine. But histidine is not a conserved residue and one histidine-free β-lactamase (from Streptomyces albus G) is known.

Since histidine is not always involved in enzyme-catalyzed rupture of a peptide (amide, ester) bond, the role of the diad His 57 ... Asp 102 in chymotrypsin has been addressed by theoretical studies (24). Ab initio

calculations have led to an unified concept according to which the essential components of the catalytic machinery of the β-lactamases, DD-peptidases and LL-peptidases are a serine residue, a polarizing agent and a water molecule. This water molecule acts as the proton carrier while the diad His 57 ... Asp 102, characteristic of the LL-peptidases, simply orients the interacting system and is dispensable. The functioning of the active-site serine β-lactamases, DD-peptidases and LL-peptidases thus depends only on the initial geometry of the interacting system that is generated by the carbonyl donor bound to the enzyme active site. Depending on the R and R' groups which flank the scissile bond (Fig. 14), the dihedral

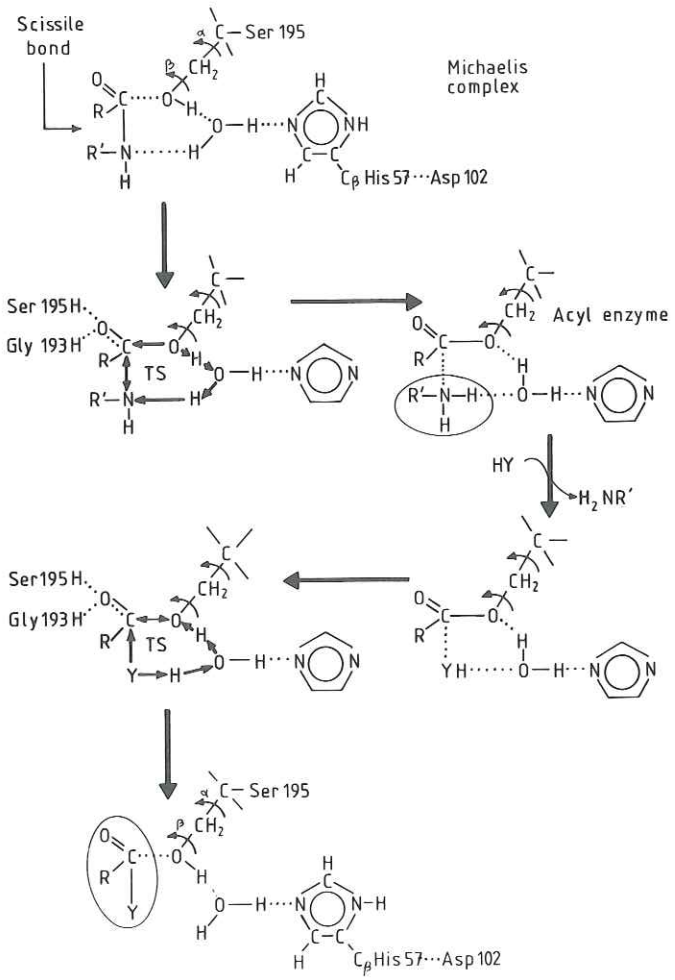


FIGURE 14. Proton shuttle during chymotrypsin-catalysed rupture of the scissile bond of a susceptible amide carbonyl donor. TS = transition state. A water molecule serves a protein carrier. The diad His 57 ... Asp 102 simply serves in orienting the interacting system.

angles α and β of the side chain of the active-site serine adopt varying orientations which greatly influence the polarization of the C=O bond and, consequently, the sense and ease of the proton shuttle. Under optimal geometrical conditions, the whole interacting system rearranges itself in such a way that enzyme acylation (and enzyme deacylation) occur at high rates without any significant energy barrier.

The above concept has a corollary. β-Lactam compounds may have donor activity for the mammalian LL-peptidases (trypsin, chymotrypsin, elastase, plasmin, thrombin) if their structures match the active sites. Assuming that the LL-peptidases differ from the DD-peptidases in having endopeptidase versus carboxypeptidase activity and in preferring L versus D configuration, cephalosporins have been modified accordingly and converted into effective mechanism-based inactivators of the LL-peptidases (25). These cephalosporins are 4' esters or amides and bear the acyl substituent in the 7α position so that they better mimick the mammalian substrates. Inactivation occurs by generation of a stable acyl enzyme. Reaction 1 and equations 2, 3 and 4 apply.

In the case of porcine pancreatic elastase and as shown by refined X-ray crystallography studies (25), the acyl enzyme formed with the long-lasting inactivator 3-acetoxymethyl-7-α-chloro-3-cephem-4-carboxylate-1,1-dioxide tert-butyl ester undergoes elimination of both the 7' chlorine and the 3' substituent. In this case, His 57 rotates away from its normal position in the enzyme's active site and becomes exposed to the exocyclic methylene. Slow alkylation of His 57 by Michael addition (t1/2 = 2 hours) provides the acyl enzyme with a second covalent bond which is not subject ot hydrolysis (or reversal by hydroxylamine). This particular cephalosporin is a potent inactivator of the human leukocyte elastase. The value of the second-order rate constant of enzyme acylation is 161,000 $M^{-1}s^{-1}$ (with $K = 1.8 \times 10^{-7}$ M and $k_{+2} = 0.029\ s^{-1}$).

Hence, 45 years after the first use of penicillin as a drug, β-lactam compounds - which so far have served their therapeutical role as antibacterial agents by inactivating the DD-peptidases - can now be remodelled to attack other enzyme targets of great medical importance.

ACKNOWLEDGEMENTS

It gives me great pleasure to thank my colleagues who contributed to the work described here : P. Charlier, J. Coyette, O. Dideberg, G. Dive, C. Duez, J. Dusart, J.M. Frère, B. Joris, J. Lamotte-Brasseur, M. Leyh-Bouille, M. Nguyen-Distèche from the University of Liège, Belgium and J. Kelly and J. Knox from the University of Connecticut, Storrs, CN, USA.

The work in Liège has been supported by an Action concertée from the Belgian Government (convention 79/84-11), the Fonds de la Recherche Scientifique Médicale, Brussels (contract no 3.4507.83) and a Convention tripartite between the Région wallonne, Continental Pharma and the University of Liège.

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