Scand. J. Infectious diseases 1984 in press

Bacterial cell walls, DD-peptidases and β-lactam antibiotics

Jean-Marie Ghuysen, Jean-Marie Frère, Mélina Leyh-Bouille, Martine Nguyen-Distèche, Jacques Coyette, Jean Dusart, Bernard Joris, Colette Duez, Otto Dideberg, Paulette Charlier, Georges Dive and Josette Lamotte-Brasseur

Université de Liège, Service de Microbiologie, Institut de Chimie, B6, Service de Cristallographie, Institut de Physique, B5, B-4000 Sart Tilman (Liège), Belgium, and Service de Chimie Pharmaceutique, Institut de Pharmacie, Rue Fusch, 3-5, B-4000 Liège, Belgium.

Abstract

Wall peptidoglycan expansion in bacteria rests upon i) a cytoplasmic D-Ala : D-Ala ligase (ADP) which catalyses synthesis of a D-Ala-D-Ala dipeptide (with accompanying hydrolysis of one molecule of ATP) and ii) a set of DD-peptidases which utilize this D-Ala-D-Ala dipeptide — once it has been translocated at the outer face of the plasma membrane as the C-terminal portion of a disaccharide peptide unit — as carbonyl donor for trans-peptidation and carboxypeptidation reactions (without additional energy expenditure). Four DD-peptidases have been selected which drastically differ from each other with respect to the effects that amino acceptors exert on the fate and rate of consumption of a D-Ala-D-Ala terminated amide carbonyl donor analogue. They serve as models to understand the different mechanisms by which the DD-peptidases perform catalysis and show widely varying responses to the action of β-lactams, from extreme sensitivity to very high resistance.

Correspondence should be sent to :

Professor Jean-Marie Ghuysen, Service de Microbiologie, Université de Liège, Institut de Chimie, B6, B-4000 Sart Tilman (Liège), Belgium.

1. Introduction

The bacterial wall peptidoglycan is a network structure which consists of linear strands of alternate, β,1-4 linked N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) pyranoside residues. Every D-lactyl group of N-acetylmuramic acid is substituted by a tetrapeptide L-Ala-γ-D-Glu-L-R_3-D-Ala where, most often, L-R_3 is a diamino acid such as L-Lys or meso-A_2pm . Peptide units substituting adjacent glycan chains are covalently linked together by means of "bridges". These bridges extend between the C-terminal D-Ala of a peptide and the ω amino group of the L-R_3 residue of another peptide. They either consist of a direct N^{ω}-(D-alanyl)-L-R_3 peptide bond [for example D-Ala-(L)-meso-A_2pm in the gram-negative bacteria (Fig. 1A)] or are mediated via a single additional amino acid residue or an intervening short peptide [for example, N^{ϵ}-(D-alanyl-pentaglycyl)-L-Lys in Staphylococcus aureus (Fig. 1B)].

Wall peptidoglycan biosynthesis is a three-stage process. Formation of each peptide (amide) bond is made at the expense of one molecule of ATP but different mechanisms are involved depending on the bonds to be synthesized. In particular, peptide cross-linking in wall peptidoglycan expansion rests upon the active involvement of a set of DD-peptidases. These peptidases are the targets specifically attacked by the β-lactam antibiotics. In this review, the current state of our knowledge on the functioning of the active sites of the DD-peptidases is presented. For other general reviews and more details, the reader is referred to (1-4).

2. Peptide bond formation during bacterial wall peptidoglycan biosynthesis

2.1. The ligases (ADP)

Reactions of stage 1 in peptidoglycan biosynthesis occur in the cytoplasm and leads to the manufacture of the nucleotide precursors

UDP-GlcNAc and UDP-MurNAc-L-Ala-γ-D-Glu-L-R_3-D-Ala-D-Ala (1). Addition of L-Ala to UDP-MurNAc, of D-Glu to UDP-MurNAc-L-Ala, of L-R_3 to UDP-MurNAc-L-Ala-γ-D-Glu, of D-Ala-D-Ala to UDP-MurNAc-L-Ala-γ-D-Glu-L-R_3, and synthesis of the dipeptide D-Ala-D-Ala are catalysed by specific ligases (ADP).

The D-Ala : D-Ala ligase (ADP) is of special interest to the present discussion (5). This enzyme is competitively inhibited by D-cycloserine. In the presence of D-cycloserine and ATP, a ternary complex [ligase.ATP.D-cycloserine] is formed which can be isolated by Sephadex filtration. In the reaction with the two D-Ala cosubstrates, it is assumed that an ordered sequence of substrate binding takes place [i.e. (1) donor; (2) acceptor] and that an ester-linked D-alanyl intermediate, formed at the donor site at the expense of ATP, undergoes nucleophilic attack by the amino group of the D-Ala residue bound at the acceptor site (Fig. 2). Hence, in this case, ATP-assisted conversion of one D-Ala cosubstrate into an "activated" carbonyl donor and subsequent peptide bond formation is catalysed by a single enzyme.

2.2. The aminoacyl-tRNA synthetases and peptidyl transferases

Reactions of stage 2 in peptidoglycan synthesis ensure the assembly of β,1-4 linked GlcNAc-MurNAc-pentapeptide units (from the precursors UDP-GlcNAc and UDP-MurNAc-pentapeptide) on a C55 polyisoprenoid alcohol phosphate and their transfer through the plasma membrane (1). No peptide bond is formed at this stage.

In those bacteria where the interpeptide bridges consist of one or several additional amino acid residues (Gly or L-amino acid), extension of the side chain at the L-R_3 position occurs — most often at the level of the lipid intermediate, sometimes at the level of the UDP-MurNAc-pentapeptide — by transfer of each amino acid residue from the corresponding aminoacyl-tRNA (1). The reaction requires two

distinct enzymes (Fig. 3). The first one is a synthetase which catalyses, at the expense of ATP, conversion of each glycine or L-amino acid residue destined to form or be part of a peptide bridge, to an L-aminoacyl-tRNA where the aminoacyl moiety is ester-linked to the 3'-OH ribosyl moiety of the 3' terminal adenosyl group of the tRNA. The second enzyme is a peptidase (peptidyl transferase). It catalyses attack of the carbonyl carbon of the aminoacyl-tRNA ester bond by the ω amino group of the L-R_3 side chain of the pentapeptide. Transfer results in peptide bond formation. Four species of $tRNA^{Gly}$ exist in S. aureus and support the stepwise addition of five glycine residues to the ε amino group of the L-lysine residue of the pentapeptide unit.

A different, but basically similar mechanism occurs in Streptococcus faecium and Lactobacillus casei (1). In these organisms, the peptidoglycan interpeptide bridge is a single iso-asparaginyl residue that has the D configuration. In this case, tDNA does not participate. Instead, D-Asp is converted into β-D-asparty1-Ⓟ at the expense of ATP and the β-D-aspartyl moiety is transferred to the ε amino group of the L-Lys residue at the level of the lipid intermediate.

It is interesting to note that DNA template-directed protein synthesis also implies prior conversion of each of the amino acid residues destined to form the polypeptide chain, to aminoacyl-tRNA by a specific aminoacyl-tRNA synthetase and subsequent action of a peptidyl transferase (Fig. 4). Assembly, however, is a complex process. Initiation involves binding of the specific initiator, N-formylmethionyl-tRNA (in bacteria) to the donor site of a ribosome, and binding of the next aminoacyl-tRNA to the acceptor site of the same ribosome. Binding is ensured by interaction between the anticodon region of the aminoacyl-tRNA and the corresponding codon of the mRNA on the ribosome. With the two cosubstrates thus positioned, the

peptidyl transferase catalyses transfer of the electrophilic group fMet-$\overset{O}{\overset{\|}{C}}$ from the donor site to the $H_2\ddot{N}$-aminoacyl-tRNA at the acceptor site. The donor site is then vacated by the deacylated $tRNA^{fMet}$ and reoccupied by the dipeptidyl-tRNA which is translocated from the acceptor site with the codon-anticodon interaction remaining intact. In turn, relative movement of the ribosome towards the 3' end of the mRNA exposes the third codon at the vacant acceptor site thus permitting binding of the next aminoacyl-tRNA at this site and further action of the peptidyl transferase. As a result of a repetition of this process for each consecutive amino acid residue, the nascent polypeptide chain grows by extension at its C-terminus until one of the three terminator or "nonsense" codons (UAU, UAG or UGA) appears at the acceptor site. When this happens, the peptidyl transferase now uses H_2O as nucleophilic cosubstrate for the transfer of the electrophilic peptidyl group, and causes detachment of the complete polypeptide chain at the C-terminal amino acid. Note that, in itself, the peptidyl transferase-catalysed peptide bond formation does not require any input of energy. (However, the underlying mechanical work which supports protein synthesis on ribosome is made with accompanying hydrolysis of several molecules of GTP and the involvement of several cofactors.)

2.3. The DD-peptidases

Reactions of stage 3 in peptidoglycan biosynthesis occur on the outer face of the plasma membrane and lead to the incorporation of the newly synthesized disaccharide peptide units in the pre-existing wall peptidoglycan both by transglycosylation at the level of the glycan strands and transpeptidation at the level of the peptide units (1). Transpeptidation is a reaction through which the electrophilic group ~~L-Ala-γ-D-Glu-L-R-D-Ala-$\overset{O}{\overset{\|}{C}}$ of a ~~L-Ala-γ-D-Glu-L-R-D-Ala-D-Ala pentapeptide unit (acting as carbonyl donor)

is transferred to the ω amino group at the L-R_3 position of another peptide (acting as nucleophilic acceptor). The reaction products are D-Ala, the leaving group of the carbonyl donor, and a peptide dimer (Fig. 5a).

Transpeptidation, however, is not the only reaction responsible for the assembly of the wall peptidoglycan. In most bacteria, the completed wall peptidoglycan possesses varying amounts of tetrapeptide units ~~L-Ala-γ-D-Glu-L-R_3-D-Ala. These peptide units lack the D-alanyl-D-alanine donor group required for transpeptidation and necessarily occur at the C-termini of the peptidoglycan polymer. They are the products of a carboxypeptidation reaction on pentapeptide units (Fig. 5c) or they originate by cleavage of N^{ω}-D-alanyl-L-R_3 interpeptide bonds previously made by transpeptidation (Fig. 5c). In some bacteria, hydrolysis of peptide dimers (oligomers) into tetrapeptides is achieved by true endopeptidases. However, in E. coli and other gram-negative bacteria, cross-linked peptides may be cleaved at a peptide bond between a D-Ala residue and another D-residue bearing a free carboxylate. In this case, hydrolysis is also made by carboxypeptidation.

The peptidases which catalyse the reactions shown in Fig. 5 have an unique optical specificity : the scissile amide bond extends between two D centres. For this reason, they are called D-alanyl-D-alanine-cleaving peptidases or, in short, DD-peptidases. DD-peptidases also exist which accommodate D-centers other than D-Ala at the C-terminal position of the carbonyl donor substrate and hence, function as efficient peptidoglycan hydrolases (Fig. 5c).

The transpeptidation and carboxypeptidation reactions of Fig. 5a and b depend on a D-alanyl-D-alanine sequence acting as carbonyl donor. As shown above, this dipeptide has been manufactured in the cytoplasm under the action of a D-Ala : D-Ala ligase (ADP),

with accompanying hydrolysis of one molecule of ATP (Fig. 2) and then translocated at the outer face of the plasma membrane at the C-terminal position of a disaccharide peptide unit. The D-Ala : D-Ala ligase (ADP) and the DD-peptidases in peptidoglycan synthesis play similar roles as the aminoacyl-tRNA synthetases and the peptidyl transferase, respectively, in DNA-template, ribosome-assisted protein synthesis. One may note that, during peptide bond formation, the carbonyl carbon which undergoes nucleophilic attack is an ester in the case of the ribosomal peptidyl transferase (Fig. 4) and an amide in the case of the DD-peptidases (Fig. 5). This difference between the carbonyl donor substrates is not a basic one. DD-peptidases also function as esterases (see below).

2.4. Wall peptidoglycan expansion

The process of wall peptidoglycan expansion is not uniform among bacteria. The immediate corollary is that each bacterial species has its own assortment of transglycosylases and DD-peptidases. In gram-positive bacilli, linear, uncrosslinked glycan chains, consisting of multiple disaccharide peptide units, grow by transglycosylation of the newly synthesized disaccharide peptide units at the reducing terminus of the lengthening chain (1). This nascent peptidoglycan then undergoes insolubilization by peptide crosslinking between new chains and between new chains and "old" ones. In contrast (and in spite of conflicting data), peptidoglycan extension in *E. coli* seems to proceed by transglycosylation and peptide crosslinking of the newly synthesized disaccharide peptide units directly to the "old" peptidoglycan without prior formation of nascent peptidoglycan (1,6,7).

E. coli possesses, bound to its plasma membrane, at least seven proteins (1A, 1B, 2, 3, 4, 5 and 6 in the order of decreasing molecular

weight). These proteins have been isolated and shown to have DD-peptidase activity on well-defined substrate systems (8-10).

When incubated with the isolated lipid-linked disaccharide pentapeptide precursor, proteins 1A, 1B, 2 and 3, catalyse the formation of crosslinked peptidoglycan strands (unfortunately with a very low turnover number of about 0.01 s^{-1} or less). No hydrolysis of the pentapeptide moiety is observed. These proteins might be bi-headed enzymes possessing two active sites performing a transglycosylase activity and a strict transpeptidase activity, respectively. Specific inactivation of the transpeptidase site (by β-lactams) does not prevent the transglycosylase site from functioning (under which conditions, a nascent uncrosslinked peptidoglycan is synthesized). Conversely, transpeptidation requires either prior or concomitant transglycosylation.

Proteins 1A, 1B, 2 and 3 might be the products of gene fusions since in less evolved gram-positive bacteria these two activities are disconnected and performed by distinct enzymes (thus explaining that wall peptidoglycan expansion in these organisms proceeds via formation of a nascent peptidoglycan; see above). Proteins 1A, 1B, 2 and 3 are probably involved in the initial incorporation of the newly synthesized peptidoglycan material. Each of them, however, fulfil distinct cellular functions related to cell elongation (proteins 1A and 1B), cell shape maintenance (protein 2) and cell septation (protein 3).

In contrast to the high molecular weight proteins just mentioned, the E. coli low molecular weight proteins 4, 5 and 6 are effective DD-carboxypeptidases. However, protein 4 is also an efficient DD-transpeptidase (using L-Ala-γ-D-Glu-meso-A_2pm-D-Ala-D-Ala as substrate) and an efficient DD-peptidoglycan hydrolase (using the bisdisaccharide peptide dimer as substrate). Protein 4 is regarded as a "secondary" transpeptidase. Protein 4 together with proteins 5 and 6 are thought

to be involved in the maturation and remodelling of the wall peptidoglycan.

3. Model DD-peptidases

In this laboratory, we are concerned with defining the geometry and mechanistic properties of the DD-peptidases to atomic resolution. Despite progress in different areas of protein structure research, this final goal can still be approached only by crystallography and requires large amounts of ideally water-soluble enzymes.

DD-peptidases are, normally, membrane-bound and occur in a small number of copies per cell. DNA recombinant techniques have permitted amplification and may lead, by alterations of the signal peptides, to excretion in the periplasmic space or accumulation in the cytoplasm of some of the DD-peptidases of E. coli. We have undertaken another approach (2,3).

On the basis that the C-terminal sequence H_2N—L-R-D-Ala-D-Ala is the part of the natural carbonyl donors utilized by the DD-peptidases, the tripeptide Ac_2-L-Lys-D-Ala-D-Ala (which contains a potential carbonyl donor site but no free amino group) and mixtures containing the same tripeptide and a suitable amino compound NH_2-R appeared to be the simplest systems that may serve as substrate analogues. Using this procedure, DD-peptidases have been found to be excreted during growth by Streptomyces albus G (G DD-peptidase; MW : 22,000), by Streptomyces R61 (R61 DD-peptidase; MW : 38,000) and by Actinomadura R39 (R39 DD-peptidase; MW : 53,000), respectively. They have been purified to protein homogeneity and two of them, the G and R61 enzymes, have been crystallized. To complete the picture, a fourth DD-peptidase has been extracted from the membranes of Streptomyces K15 (K15 DD-peptidase) with the cationic detergent cetavlon and purified to 95 % purity in

the presence of this detergent (yield : 1.5 mg from the mycelium mass contained in 100 litres of culture medium) (11).

The G, R61, R39 and K15 DD-peptidases have one thing in common (2,3 and unpublished results). Catalytic efficiency (defined as turnover number/Km) on Ac_2-L-Lys-D-Ala-D-Ala (i) strictly requires a D-Ala at position 2; (ii) is decreased but not abolished when Gly or a D-amino acid other than D-Ala is at position 3. L-Ala, at this position, however, abolishes substrate activity; and (iii) is drastically decreased as the neutral side chain of the L-residue at position 1 is progressively shortened. Catalytic efficiency, however, is influenced differently in the different enzymes by the presence of charged groups at the end of the long side chain at position 1. Hence, whatever the DD-peptidase, initial recognition of the carbonyl donor substrate, binding energy and proper alignment of the scissile bond with regard to the enzyme catalytically active functional groups rely on the complementation of at least three enzyme binding subsites (Fig. 6). Subsites S_1' and S_1 accommodate the two methyl groups in the D configuration that occur on both sides of the scissile bond. In addition, there is, most likely located in the vicinity of subsite S_1', a cationic group involved in charge pairing with the C-terminal carboxylate of the substrate. Finally, subsite S_2 accommodates the long side chain that protrudes from the L center. Complementation of S_2 may not be essential for initial binding, but, as already mentioned, is crucial for subsequent catalysis.

In spite of these close similarities regarding the design of the enzyme donor sites, the G, R61, R39 and K15 DD-peptidases drastically differ from each other in many respects (2-3). One of them relates to the effects that the presence of amino acceptors exert on the fate and rate of consumption of the tripeptide Ac_2-L-Lys-D-Ala-D-Ala. The G DD-peptidase, on the one hand, is unable to utilize

any amino compound for the transfer of the Ac_2-L-Lys-D-alanyl moiety from the tripeptide donor substrate (Fig. 7a). It is a strict DD-carboxypeptidase. The R61 and R39 DD-peptidases, on the other, catalyse concomitant carboxypeptidation and transpeptidation reactions (Fig. 7b) and possess an acceptor site of considerable specificity. Their profiles for amino acceptors are different and the observed differences reflect specific structural features of the wall peptidoglycans of the corresponding bacteria. (The peptidoglycan crosslink is a C-terminal D-alanyl-(D)-meso-diaminopimelic acid in Actinomadura R39 but is a D-alanyl-glycyl-LL-diaminopimelic acid in Streptomyces R61.) Finally, the K15 DD-peptidase functions, almost exclusively, as a strict DD-transpeptidase (Fig. 7c). Its catalytic efficiency on the tripeptide is negligible unless the reaction mixture contains a suitable amino compound.

Central to the problem of the DD-peptidases is the elucidation of their mechanistic properties. The G, R61, R39 and K15 DD-peptidases (as well as any other peptidase, including the ribosomal peptidyl transferase discussed above) must obey the same rule. Peptidase-catalysed cleavage of peptide (ester) $\overset{O}{\overset{\|}{C}}$-N(O) bonds requires the concerted action in the enzyme active site of i) an electrophile which polarizes the bond C=O ; ii) a nucleophile which performs attack of the carbon atom; and iii) a proton donor which achieves proton donation to the nitrogen (oxygen) atom. Depending on the peptidases, these events can be carried out by different mechanisms.

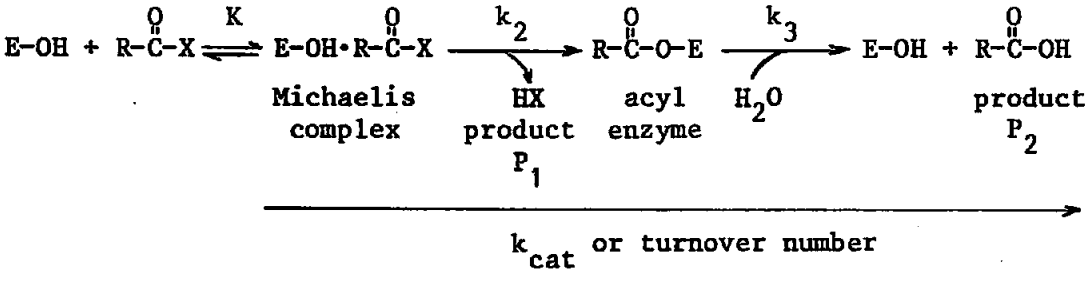
4. Covalent catalysis by DD-peptidases

4.1. The α-chymotrypsin model

Several mammalian proteases possess the catalytically active triad $Ser^{195}...His^{57}...Asp^{102}$ (using the numbering of α-chymotrypsin)

and catalyse cleavage of the scissile peptide (ester) bond via formation of a serine^{195}-linked acyl-enzyme intermediate (covalent catalysis). It is generally accepted that His^{57} fulfils two functions. The first is to abstract a proton from Ser^{195} thus "activating" a nucleophile for attack on the carbonyl carbon and the second is to donate a proton to the nitrogen (oxygen) atom. In turn, a pair of hydrogen bonds from the backbone NH of Ser^{195} and Gly^{193} acts as electrophile.

Giving E-OH = the enzyme active serine residue, X = NH-R' for an amide carbonyl donor substrate or O-R' for an ester carbonyl donor substrate; K = dissociation constant of the Michaelis complex; k_2 and k_3 = first order rate constants; and P_1 = the leaving group of the enzyme acylation step, hydrolysis proceeds through a Ping-Pong mechanism and can be written :

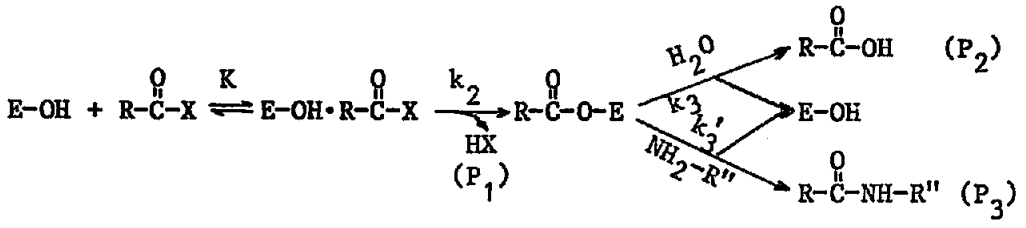


In such a model and assuming that formation of the Michaelis complex is a rapid equilibrium process, the catalytic efficiency (turnover number/Km) simplifies to k_2/K , i.e. is a valid measure of the bimolecular rate constant of enzyme acylation.

If acyl enzyme formation (k_2) is rate determining (as generally observed with amide substrates R-C(=O)-NH-R'), the acyl enzyme does not accumulate detectably at the steady state and cannot be trapped. If acyl enzyme breakdown (k_3) is rate determining (as generally observed with ester substrates R-C-O-R'), the acyl enzyme accumulates and can be trapped and isolated.

Assume the presence of an alternate amino nucleophile NH_2-R" which does not bind to the free enzyme nor to the Michaelis complex but

does react only after the leaving group P_1 has diffused away. Then, partitioning of the enzyme activity between the two nucleophiles, H_2O and NH_2-R", occurs at the level of the acyl enzyme.



If acyl enzyme formation (k_2) is rate determining, increasing concentrations of NH_2-R" cannot increase the maximal rate of carbonyl donor consumption (turnover number) but aminolysis occurs at the expense of hydrolysis on a competitive basis (Fig. 8a). If acyl enzyme breakdown (k_3) is rate determining, increasing concentrations of NH_2-R" may increase the rate of acyl enzyme breakdown and hence turnover number (Fig. 8b).

How does the above model apply to the R61, R39 and K15 DD-peptidases ? It seems well established that these three DD-peptidases catalyses a three-step reaction where the reaction flux proceeds through the transitory formation of an acyl enzyme. At least the R61 and K15 DD-peptidases have been shown to form with the ester carbonyl substrate Ac_2-L-Lys-D-Ala-D-lactate, an intermediate that accumulates at the steady state and can be trapped.

4.2. The R61 and R39 DD-peptidases (2,3)

The R61 DD-peptidase hydrolyses the amide carbonyl donor Ac_2-L-Lys-D-Ala-D-Ala (release of the C-terminal D-Ala residue) with a turnover number of 55 s^{-1}. On the basis of a Km value of 12 mM , the catalytic efficiency or bimolecular rate constant for enzyme acylation (k_2/K) is 4,600 $M^{-1}s^{-1}$. With the R39 DD-peptidase, the corresponding values are turnover number = 17.5 s^{-1}; Km = 0.8 mM ; k_2/K = 21,900 $M^{-1}s^{-1}$. With this amide substrate, no acyl enzyme accumulates detectably at the steady state.

In the presence of a suitable amino nucleophile, both enzymes catalyse concomitant hydrolysis and transpeptidation of the amide donor. Under any circumstance, the amino acceptor does not increase the maximal rate of carbonyl donor consumption (i.e. turnover) (Fig. 7b). This behavior shows that, essentially, the amino acceptor behaves as an alternate nucleophile that exerts its effect only at the level of the acyl enzyme, a situation which is reminiscent of that found with α-chymotrypsin (Fig. 8a). One should note, however, that, when complex amino acceptors (related to wall peptidoglycan) are used, the observed increase of the rate of transpeptidation is less than can be accounted for by the decrease of the rate of hydrolysis. Moreover, at high amino acceptor concentrations, both hydrolysis and transpeptidation can be inhibited so that, eventually, the enzyme is frozen in a catalytically inactive state. This and other observations show that the carboxypeptidase and transpeptidase activities of the R61 and R39 DD-peptidases are subject to precise modulation.

At variance with the model, however, kinetic studies suggest that transpeptidation is an ordered pathway in which the amino acceptor binds first to the enzyme. But, the significance of this observation is not clear since attempts to show direct binding of an effective amino acceptor to the free enzyme have failed.

4.3. The K15 DD-peptidase (11 and unpublished data)

The K15 DD-peptidase drastically differs from the R61 and R39 DD-peptidases in that, when acting on the amide donor Ac_2-L-Lys-D-Ala-D-Ala, it causes only a small burst of released D-Ala. After that, the rate of hydrolysis becomes negligible (Fig. 7c). The enzyme, however, is not dead. What happens is that when D-Ala has accumulated to a certain level, it effectively competes with water as an acceptor of the transfer reaction. As a consequence, the DD-peptidase then

transpeptidates, i.e. exchanges the C-terminal D-Ala residue of the substrate for another D-Ala residue so that the substrate concentration remains, apparently, unaffected (or more precisely is consumed exceedingly slowly).

Increased consumption of Ac_2-L-Lys-D-Ala-D-Ala by the K15 DD-peptidase can be obtained in two ways. One way is to supplement the reaction mixture with D-amino acid oxidase so that the released D-Ala is eliminated by conversion to pyruvate. Under these conditions, hydrolysis proceeds at a constant velocity until an appreciable amount of substrate is consumed. However, the enzyme has still a low turnover number of 0.02 s^{-1}. On the basis of a Km value of 1.6 mM , the bimolecular rate constant of enzyme acylation is 12.5 $M^{-1}s^{-1}$.

A better way to increase the efficacy of the K15 DD-peptidase is to supplement the reaction mixture with a suitable amino compound such as Gly-Gly (Fig. 7c). This dipeptide is a much better acceptor than H_2O or D-Ala. The reaction product is, almost exclusively, the transpeptidated tetrapeptide Ac_2-L-Lys-D-Ala-Gly-Gly. Turnover number is 0.61 s^{-1} and on the basis of a Km value of 5.6 mM (for the carbonyl donor), the bimolecular rate constant of enzyme acylation is 110 $M^{-1}s^{-1}$, i.e. a value which is almost 10 times that obtained in the absence of Gly-Gly (and with added D-amino acid oxidase).

Fig. 7a for the K15 DD-peptidase is reminiscent to Fig. 8b for α-chymotrypsin. However, the increased turnover number of α-chymotrypsin caused by the amino compound is obtained with an ester carbonyl donor, under conditions where, in the absence of added amino acceptor, the acyl enzyme largely accumulates. In marked contrast, the increased turnover number of the K15 DD-peptidase caused by Gly-Gly is observed with an amide carbonyl donor, under conditions where, in the absence of added Gly-Gly, 10 %, at the most, of the DD-peptidase occurs in the form of a detectable intermediate, i.e. the rate of acyl enzyme formation is much smaller than the rate of acyl enzyme breakdown.

The effect of Gly-Gly (or other suitable amino compounds such as Gly-L-Ala) on the attack of the amide carbonyl donor Ac_2-L-Lys-D-Ala-D-Ala by the K15 DD-peptidase is not that of a simple alternate nucleophile acting at the level of the acyl enzyme. The effect is much more complex and results in a dramatic increase of the efficacy with which this DD-peptidase undergoes acylation by the carbonyl donor substrate. The K15 DD-peptidase might serve as model of the high molecular weight proteins 1A, 1B, 2 and 3 of E. coli. Remember that these proteins are, apparently, deprived of carboxypeptidase activity and catalyse transpeptidation only under conditions where transglycosylation is effective. Transglycosylation may be necessary to confer on the carbonyl donor and amino acceptor groups that relative specific disposition required for transpeptidation.

5. β-Lactams as suicide substrates of the DD-peptidases

β-Lactam antibiotics interfere with the enzyme machinery responsible for crosslinking the wall peptidoglycan and cause inhibition of cellular growth. Usually, this primary bacteriostatic effect is accompanied by cellular lysis and death due to the unleashing or triggering of the autolytic system of the sensitive bacteria. These antibacterial effects have been related to the ability of the β-lactams to form adducts with various membrane-bound proteins, adducts that are sufficiently stable to be analysed by gel electrophoresis in the presence of sodium dodecylsulphate (8). The penicillin binding proteins which — at present — have been identified as enzymes are DD-peptidases.

5.1. Mechanism of enzyme inactivation (2,3)

The β-lactam antibiotics immobilize the R61 and R39 DD-peptidases in the form of stable acyl enzyme intermediates and thus confer on these enzymes the property to behave as penicillin binding proteins.

The R61 and R39 DD-peptidases have a serine residue that is specifically involved in penicillin binding. The amino acid sequence around this active serine is Val-Gly-Ser* in the R61 enzyme and Leu-Pro-Ala-Ser*-Asn-Gly-Val in the R39 enzyme. Partial degradation by different proteases of each of the [^{14}C]-labelled intermediates obtained by reaction of the R61 DD-peptidase with Ac_2-L-Lys-D-Ala-D-lactate (in which case the acyl enzyme accumulates) and benzylpenicillin, respectively, gives rise to very similar "proteolytic maps" (12). Hence, at least with the R61 DD-peptidase, carbonyl donor substrate and penicillin bind either to residues which are located close to each other in the polypeptide backbone or to the same serine residue. This latter alternative seems to be the most probable one. Whether or not the same situation applies to the R39 DD-peptidase is under current investigation.

Inactivation of the R61 and R39 DD-peptidases by β-lactams is a suicide commitment. β-Lactams are recognized and taken up by the DD-peptidases as carbonyl donor substrates but the reaction flux does not proceed to reaction products. Instead, it stops at the level of the acyl enzyme. Because of the endocyclic nature of the scissile amide bond of the β-lactam ring, what should be regarded as the leaving group during acyl enzyme formation cannot leave the enzyme site which therefore remains occupied (Fig. 9).

The trapping effect of the β-lactams can be overcome by rupture of the C_5-C_6 linkage in the enzyme-bound benzylpenicilloyl moiety (Fig. 9). This reaction, which occurs spontaneously and causes the release of the leaving group with concomitant formation of phenylacetylglycyl-enzyme, is immediately followed by deacylation of the enzyme and recovery of the activity. Depending on the cases, enzyme deacylation may also occur without prior fragmentation of the bound acyl moiety. When this occurs, the DD-peptidase functions, essentially, as a classical β-lactamase. Whatever the case, enzyme recovery is always a slow or very slow process.

Whether they possess a bicyclic (penicillins, cephalosporins) or monocyclic (monobactams) framework and whatever the structure of the substituents, the β-lactams interact with the R61 and R39 DD-peptidases according to a three-step reaction similar as that observed with carbonyl donor substrates

$$E + I \overset{K}{\rightleftharpoons} E{\cdot}I \xrightarrow{k_2} \text{acyl enzyme} \xrightarrow{k_3} E + \text{product(s)}$$

The higher the bimolecular rate constant k_2/K of enzyme acylation and the smaller the rate constant k_3 of enzyme deacylation, the more potent the β-lactam as enzyme inactivator. If the β-lactam (I) is able to inactivate the enzyme at $[I] \ll K$, then the level of acyl enzyme $[E\text{-}I^*]_{ss}$ at the steady state is

$$[E\text{-}I^*]_{ss} = E_o \frac{1}{1 + \frac{k_3 K}{k_2 [I]}}$$

and the time ($t_{0.95}$) necessary for the concentration of E-I* to reach 95 % of its steady state level is

$$t_{0.95} = \frac{3}{k_3 + \frac{k_2 [I]}{K}}$$

If k_3 is negligible, then

$$t_{0.95} = \frac{3K}{k_2 [I]} .$$

Thus, for example, at a 10 μM concentration (which is lower than the dissociation constant K of the Michaelis complex E·I), a β-lactam that exhibits for a given enzyme, a k_2/K value of 1000 $M^{-1}s^{-1}$ and a k_3 value of $10^{-4}s^{-1}$, immobilizes 99 % of the enzyme as acyl enzyme at the steady state and the time required for the acyl enzyme to reach 95 % of the steady state level is about 5 min .

Tables 1 and 2 give the k_2/K and k_3 values and in some cases the individual values of K and k_2 for the interactions between the two DD-peptidases under consideration and various bicyclic and monocyclic β-lactam compounds [for details, see (13-18)].

5.2. Structure-activity relationship (2,3)

For a long time, the bicyclic framework has been held responsible for the unique properties of the penicillins and cephalosporins. Since in all these compounds, the substituents attached to the amide linkage of the β-lactam ring are prevented from being coplanar, much emphasis has been given to the "suppressed amide resonance" or "increased intrinsic reactivity" of the β-lactam ring as the basis of the enzyme inactivating and antibacterial potency. We, in this laboratory, never shared this view and the recent discovery, at the Squibb Institute for Medical Research, of active monobactams where all of the atoms attached to the β-lactam linkage are essentially coplanar, much contributed to the demonstration that non-planarity of the amide bond is, at least, not the essential factor.

Similarly, it is true that an electron withdrawing substituent at the 4 position of the penicillin or cephalosporin β-lactam ring (i.e. the thiazolidine or dihydrothiazine sulfur atom) decreases the basicity of the β-lactam nitrogen atom and makes it a better leaving group. The same effect is obtained if an electron withdrawing group is directly attached to the β-lactam nitrogen atom (as the $SO_3^{\ominus}$ group in the monobactams). It remains, however, that the goodness of fit of the β-lactam molecule in the enzyme active site is the primary parameter that dictates the efficacy of enzyme inactivation (17). The data of Table 1 are, in this respect, convincing and show the proeminent effect exerted, in most cases, by the structure of the acyl substituent of the β-lactam ring.

Assuming that all DD-peptidases commit suicide by binding the β-lactams to the carbonyl donor substrate active site, then the D-Ala-D-Ala peptide bond in the substrate and the amide bond in the β-lactam are functionally equivalent in that they are predisposed to attack by the same enzyme active site functional groups. In these two types of compounds the scissile $\overset{\overset{O}{\|}}{C}-N$ bond is flanked on one side by the C-terminal carboxylate (or SO_3^- group in the monobactams) and, on the other, by another $\overset{\overset{O}{\|}}{C}-N$ amide bond. These three functional groups create, around the common backbone $\overset{\overset{O}{\|}}{C}-N-C-\overset{\overset{O}{\|}}{C}-N-C-\overset{\overset{O}{\|}}{C}-OH$ (or $\overset{\overset{O}{\|}}{C}-N-C-\overset{\overset{O}{\|}}{C}-N-SO_3^{\ominus}$) zones of positive and negative electrostatic potentials whose relative spatial disposition and strength must be important for the reactivity and orientation of the whole molecule within the enzyme active site. Examination of a set of active peptide and β-lactam conformers show very important variations in the electrostatic environments of the molecules, suggesting enzyme-ligand associations of widely varying complementarity and productiveness (4,19).

In addition, the part of a D-Ala-D-Ala terminated peptide and the part of the β-lactam molecule (Tables 1 and 2) which largely dictates the effectiveness of the acylation step is the lateral chain on the L residue which precedes the D-Ala-D-Ala sequence and (in most cases) the acyl substituent on the β-lactam ring. These substituents show complete lack of isosterism.

Finally, cases exist where reagents do not affect to the same extent, the activities of the DD-peptidases as peptidyl transferases and as penicillin binding proteins. Thus the K15 DD-peptidase (whether it is membrane-bound or purified to 95 % homogeneity) can be completely inactivated by β-lactams. With the purified enzyme, the bimolecular rate constant (k_2/K) of inactivation by benzylpenicillin is 150 $M^{-1}s^{-1}$ and the rate constant (k_3) of enzyme recovery is $1 \times 10^{-4} s^{-1}$. Remarkably, the presence of Gly-Gly — which, as

shown above, greatly increases the rate of enzyme acylation by the carbonyl donor Ac_2-L-Lys-D-Ala-D-Ala — has no effect at all on the rate of enzyme acylation by benzylpenicillin. In addition, the transpeptidase activity of the K15 DD-peptidase can be totally suppressed by low concentrations of p-chloromercuribenzoate while substantial binding of benzylpenicillin remains intact.

The above observations reinforce the idea, put forward ten years ago (20), that the independence between carbonyl donor substrate and β-lactam sites in the DD-peptidases may be more or less pronounced. More recently (3), we have proposed that by interacting with specific binding sites [$S_{2(peptide)}$ and $S_{2(\beta\text{-lactam})}$], the peptide side chain and the β-lactam substituent would confer on the catalytic site a conformation specifically devised to operate on the peptide link of D-Ala-D-Ala and the amide bond of the β-lactam ring, respectively. Following this view, the β-lactams should be regarded as weak isosteric inhibitors of the DD-peptidases (high K values) that can be converted into powerful inactivators (high k_2 value and low k_3 value) if a correct interaction is achieved between the acyl substituent and an "allosteric" binding subsite $S_{2(\beta\text{-lactam})}$.

Substantial progress in the determination of the three-dimensional structure of the R61 DD-peptidase is being made (21,22). The general folding of the polypeptide backbone has been established as well as the presence of a serine residue within the β-lactam binding site. Before long, the catalytically important enzyme side chains of at least this serine DD-peptidase and a precise picture of how it works and is inactivated by β-lactams will be known at the atomic level.

6. The G DD-peptidase

As we have seen, the R61, R39 and K15 DD-peptidases perform covalent catalysis (through an essential serine residue in the two former enzymes), achieve both carboxypeptidation and transpeptidation reactions and are inactivated by β-lactams. In marked contrast, the G DD-peptidase performs liganding catalysis through an essential Zn^{++} cofactor, achieves only carboxypeptidation reactions and shows very high resistance to β-lactams.

The inability of the G DD-peptidase to perform transpeptidation reactions is a property shared by the mammalian Zn^{++} peptidases such as thermolysine and carboxypeptidases A and B. The current view of thermolysin-catalysed hydrolysis of peptide and ester bonds is that i) Glu^{143} acts as a proton abstractor heightening the nucleophilicity of the zinc-bound water; ii) Zn^{++} acts as an electrophile and iii) His^{231} facilitates proton donation to the nitrogen (oxygen) atom of the scissile bond at the level of the tetrahedral intermediate. Collapse of the intermediate with re-entry of a water molecule causes the release of the reaction products. No covalent intermediate is formed during the process.

The G DD-peptidase (212 amino acid residues; sequence known) (23) consists of two globular domains connected by a single link and the Zn^{++}-containing active site is an open cleft in the larger C-terminal domain (24). The G DD-peptidase, thermolysin and carboxypeptidases A and B show complete lack of amino acid sequence homology and overall structural relatedness.

Although refinement of the three-dimensional structure is still in progress (at 0.18 nm), a provisional picture of how the G DD-peptidase works can be proposed (4). Models show that (using the numbering of amino acid residues derived from X-ray data) accommodation of the tripeptide Ac_2-L-Lys-D-Ala-D-Ala in the active site leads to

a close interaction between the C-terminal carboxylate and the guanidinium side chain of Arg^{136} (most likely involved in charge pairing) and to a positioning of the scissile $\overset{O}{\overset{\|}{C}}$-N bond such that the oxygen atom is oriented toward the Zn^{++} ion and the nitrogen atom is oriented towards the imidazole ring of His^{190} (apparently ready to donate a proton). In turn, Ser^{151} may play the role of an orienter (and/or activator) of the water-bound Zn^{++} ion. Hence, the G DD-peptidase would also possess a catalytically important serine residue. Its function, however, would be entirely different from that of the active serine residue in the R61 and R39 DD-peptidases. Finally, the Zn^{++} protein ligands are His^{152}, His^{193} and His^{196} (24).

When the tripeptide Ac_2-L-Lys-D-Ala-D-Ala is aligned in the enzyme active site (Fig. 6), then i) the methyl group of the C-terminal D-Ala points to a large subsite S_1' (which, in fact, is able to accommodate a much larger side chain, a property which is compatible with the peptidoglycan hydrolase activity of the G DD-peptidase); ii) the methyl group of the penultimate D-Ala finds its place in a subsite S_1 of small size (larger side chains cannot be accommodated); and iii) the side chain at the L-centre complements subsite S_2 which appears as a large cavity. Note that each side chain of the substrate can be accommodated in the corresponding subsite of the enzyme only if it has the proper configuration, L or D, as indicated. Following binding, catalysis proceeds with a turnover number of 2.5 s^{-1}. The Km value is 0.33 mM and catalytic efficiency is 7,600 $M^{-1}s^{-1}$.

Kinetically, the interaction between the G DD-peptidase and β-lactams is a three-step reaction :

$$E + I \overset{K}{\rightleftharpoons} E \cdot I \xrightarrow{k_2} E\text{-}I^* \xrightarrow{k_3} E + Ps$$

β-Lactams may acylate a residue in the G DD-peptidase to form an adduct E-I* of high stability (low k_3 value) but the efficacy of the process is very low and the nature of E-I* is unknown. With

penicillins, the dissociation constant K is very high (150 mM with phenoxymethylpenicillin). With cephalosporins, it is similar (1 - 10 mM) as that measured with the R61 and R39 serine DD-peptidases. But, in all cases, k_2 is negligible ($1 - 8 \times 10^{-4} s^{-1}$) so that the β-lactams behave essentially as weak reversible inhibitors of the G DD-peptidase.

Depending on the β-lactams, inhibition of the G DD-peptidase is competitive or non-competitive. The competitive inhibitor p-iodo-7-β-phenylacetamido cephalosporanate binds to the active site and gives rise to an isomorphous crystal enzyme derivative (24). The noncompetitive inhibitors cephalosporin C and cephalothin cause disruption of the protein crystal lattice and induces large conformational changes (and most likely aggregates) of the enzyme molecules in solution (25). Models show that with both penicillins and cephalosporins, it is not possible to align simultaneously the β-lactam amide bond with the Zn^{++} ion and the C-terminal carboxylate with Arg^{136} (4).

6-β-Iodopenicillanate, at high concentrations, inactivates the G DD-peptidase with a first order rate constant of $7 \times 10^{-4} s^{-1}$ (limit value). It binds to the active site just in front of the Zn^{++} ion and superimposes the proton donor His^{190} (which probably undergoes alkylation with concomitant loss of the iodine from the inactivator) (26). Bifunctional compounds possessing both a C-terminal carboxylate susceptible to interact with Arg^{136} and either a thiol, hydroxamate or carboxylate susceptible to bind the Zn^{++} ion inhibit the G DD-peptidase competitively. β-Mercaptopropionate is very effective ($K_i = 5 \times 10^{-9}$ M) (26).

7. Conclusions

At least two classes of DD-peptidases are well characterized which perform catalysis through entirely different mechanisms. The G Zn^{++} DD-carboxypeptidase has no amino acid sequence homology nor structural relatedness with its mammalian counterparts, the Zn^{++}-containing thermolysin and carboxypeptidase A. Among all the enzymes known to consist of two distinct domains, the G DD-peptidase is unique in that the active site is not located at the junction between the two domains but is an open cleft in the large, C-terminal domain. The catalytic mechanism of the G DD-peptidase is similar as that of thermolysin or carboxypeptidase A but there are distinct differences in the relevant active sites. The β-lactams are neither good substrates nor good inactivators and, essentially, behave as weak reversible inhibitors. This lack of activity is caused by the active site geometry which permits enzyme ligand association of high productiveness only with carbonyl donor peptides (or esters).

The R61 and R39 serine DD-carboxypeptidases/transpeptidases may be mechanistically related to α-chymotrypsin and other mammalian serine peptidases, at least with respect to the effect that amino acceptors exert on the channelling of the enzyme activity through hydrolysis and aminolysis (transpeptidation). Establishment of the amino acid sequence and three-dimensional structure of the R61 DD-peptidase is well in progress and will permit in a near future, identification of the catalytically important side chains (in addition to the active serine). It is clear, however, that the R61 DD-peptidase shows no three-dimensional relatedness with any known serine peptidase.

The K15 DD-peptidase also performs covalent catalysis. Its active site has developed an unique feature which prevents it from hydrolysing D-Ala-D-Ala terminated amide carbonyl donor substrates and gives it the capacity of performing transpeptidation with high efficacy.

Covalent catalysis, which confers on the R61, R39 and K15 DD-peptidases, the capacity of performing transpeptidation, also make them vulnerable to β-lactams. With β-lactams, part of the normal catalytic mechanism occurs but stops at the abortive acyl enzyme level. β-Lactams are mechanism-based or k_{cat} inactivators of this group of DD-peptidases.

At the present stage of our knowledge, enzyme acylation by D-Ala-D-Ala terminated carbonyl donor substrates and β-lactam inactivators seem to rely on an induced fit mechanism (2,3,20). Following initial binding, the enzyme would be made catalytically active as a result of a specific interaction between the lateral chain of the L residue which precedes the D-Ala-D-Ala sequence of the bound peptide or the substituents of the β-lactam ring, and some specific amino acid(s) (4) of the enzyme cavity. Depending on the case, the independence between the carbonyl donor substrate and β-lactam sites may be more or less pronounced.

Acknowledgements

Part of the work has been supported by the Fonds de la Recherche Scientifique Médicale (FRSM), Brussels (convention n°3.4507.83), and an Action concertée with the Belgian Government (convention n°79/84-I1).

References

1. Ghuysen JM. Biosynthesis and assembly of bacterial cell walls. In : Poste G, and Nicolson GL, eds. The synthesis, assembly and turnover of cell surface components. North-Holland Publishing Co, 463-595, 1977.

2. Ghuysen JM, Frère JM, Leyh-Bouille M, Coyette J, Dusart J, Nguyen-Distèche M. Use of model enzymes in the determination of the mode of action of penicillins and Δ^3-cephalosporins. Ann. Rev. Biochem. 48: 73-101, 1979.

3. Ghuysen JM, Frère JM, Leyh-Bouille M, Dideberg O, Lamotte-Brasseur J, Perkins HR, De Coen JL. Penicillins and Δ^3-cephalosporins as inhibitors and mechanism-based inactivators of D-alanyl-D-Ala peptidases. In : Burgen ASV, and Roberts GCK, eds. Topics in molecular pharmacology. Elsevier/North-Holland Biomedical Press, 63-97, 1981.

4. Charlier P, Dideberg O, Dive G, Dusart J, Frère JM, Ghuysen JM, Joris B, Lamotte-Brasseur J, Leyh-Bouille M, Nguyen-Distèche M. The active sites of the D-alanyl-D-alanine-cleaving peptidases. In : Hakenbeck R, Höltje JV, Labischinski H, eds. The target of penicillin. The murein sacculus of bacterial cell walls. Architecture and growth. International FEMS Symposium, March 1983, Berlin (West). Berlin : W de Gruyter and Co, 369-386, 1983.

5. Neuhaus FC, Hammes WP. Inhibition of cell wall biosynthesis by analogues of alanine. Pharmac. Ther. 14:265-319, 1981.

6. Mirelman D. Assembly of wall peptidoglycan polymers. In : Salton MRJ, Shockman GD, eds. β-Lactam antibiotics. Mode of action, new developments and future prospects. Academic Press, 67-87, 1981.

7. Burman LG, Park JT. Change in the composition of Escherichia coli murein as it ages during exponential growth. J. Bact. 155:447-453, 1983.

8. Spratt BG. Penicillin binding proteins and the future of β-lactam antibiotics. J. Gen. Microbiol. 129:1247-1260, 1983

9. Tomioka S, Ishino F, Tamaki S, Sudo A, Nakagawa JL, Matsuhashi M. Enzymatic synthesis of peptidoglycan-crossbridges in E. coli. In : Hakenbeck R, Höltje JV, Labischinski H, eds. The target of penicillin. The murein sacculus of bacterial cell walls. Architecture and growth. Berlin : W de Gruyter, 505-510, 1983.

10. Waxman DJ, Strominger JL. Penicillin binding proteins and the mechanism of action of β-lactam antibiotics. Ann. Rev. Biochem. 52:825-869, 1983.

11. Nguyen-Distèche M, Leyh-Bouille M, Ghuysen JM. Isolation of the membrane-bound 26,000-Mr penicillin-binding protein of Streptomyces strain K15 in the form of a penicillin-sensitive D-alanyl-D-alanine-cleaving transpeptidase. Biochem. J. 207:109-115, 1982.

12. Yocum RR, Amanuma H, O'Brien TA, Waksman DJ, Strominger JL. Penicillin is an active site inhibitor for four genera of bacteria. J. Bact. 149:1150-1153, 1982.

13. Dusart J, Marquet A, Ghuysen JM, Frère JM, Moreno R, Leyh-Bouille M, Johnson K, Lucchi C, Perkins HR, Nieto M. DD-carboxypeptidase-transpeptidase and killing site of β-lactam antibiotics in Streptomyces strains R39, R61 and K11. Antimicrob. Ag. Chemother. 3:181-187, 1973.

14. Frère JM, Ghuysen JM, Iwatsubo M. Kinetics of interaction between the exocellular DD-carboxypeptidase-transpeptidase from Streptomyces R61 and β-lactam antibiotics. A choice of models. Eur. J. Biochem. 57:343-351, 1975.

15. Frère JM, Ghuysen JM, Reynolds PE, Moreno R, Perkins HR. Binding of β-lactam antibiotics to the exocellular DD-carboxypeptidase-transpeptidase of Streptomyces R39. Biochem. J. 143:241-249, 1974.

16. Kelly JA, Frère JM, Klein D, Ghuysen JM. Interaction between non-classical β-lactam compounds and the Zn^{++} G and serine R61 and R39 D-alanyl-D-alanine peptidases. Biochem. J. 199:129-136, 1981.

17. Frère JM, Kelly JA, Klein D, Ghuysen JM, Claes P, Vanderhaeghe H. Δ^2- and Δ^3-Cephalosporins, penicillanate and 6-unsubstituted penems. Intrinsic reactivity and interaction with β-lactamases and D-alanyl-D-alanine-cleaving serine peptidases. Biochem. J. 203:223-234, 1982.

18. Laurent G, Durant F, Frère JM, Klein D, Ghuysen JM. Des-, syn- and anti-oxyimino Δ^3-cephalosporins. Intrinsic reactivity and reaction with RTEM-2 serine β-lactamase and D-alanyl-D-alanine-cleaving serine and Zn-containing peptidases. Biochem. J., in press.

19. Lamotte-Brasseur J, Dive G, Ghuysen JM. On the structural analogy between D-alanyl-D-alanine terminated peptides and β-lactam antibiotics. Eur. J. Med. Chem., in press.

20. Ghuysen JM, Leyh-Bouille M, Frère JM, Dusart J, Marquet A, Perkins HR, Nieto M. The penicillin receptor in Streptomyces. Ann. N.Y. Acad. Sci. 235:236-268, 1974.

21. Kelly JA, Moews PC, Knox JR, Frère JM, Ghuysen JM. Structure of a penicillin target enzyme and location of the antibiotic binding site. Science 218:479-481, 1982.

22. Kelly JA. X-ray structure of a penicillin target enzyme. In : Hakenbeck R, Höltje JV, Labischinski H, eds. The target of penicillin. The murein sacculus of bacterial cell walls. Architecture and growth. Berlin : W de Gruyter and Co, 387-392, 1983.

23. Joris B, Van Beeumen J, Casagrande F, Gerday C, Frère JM, Ghuysen JM. The complete amino acid sequence of the Zn^{++}-containing D-alanyl-D-alanine-cleaving carboxypeptidase of Streptomyces albus G. Eur. J. Biochem. 130:53-69, 1983.

24. Dideberg O, Charlier P, Dive G, Joris B, Frère JM, Ghuysen JM. Structure at 2.5 Å resolution of a Zn^{++}-containing D-alanyl-D-alanine-cleaving carboxypeptidase. Nature 299:469-470, 1982.

25. Labischinski H, Giesbrecht P, Fischer E, Barnickel G, Bradaczek H, Frère JM, Houssier C, Charlier P, Dideberg O, Ghuysen JM. Study of the Zn-containing DD-carboxypeptidase of Streptomyces albus G by small angle X-ray scattering in solution. Eur. J. Biochem., in press.

26. Charlier P, Dideberg O, Jamoulle JC, Frère JM, Ghuysen JM, Dive G, Lamotte-Brasseur J. Active-site directed inactivators of the Zn^{2+} D-alanyl-D-alanine-cleaving carboxypeptidase of Streptomyces albus G. Biochem. J., in press.

Legends for Figures

Fig. 1. Wall peptidoglycan in Escherichia coli (A) and Staphylococcus aureus strain Copenhagen (B).

G = N-acetylglucosamine; M = N-acetylmuramic acid.

Fig. 2. Schematic view of the active site of the D-Ala : D-Ala ligase (ADP).

Fig. 3. Extension of the L-lysine side chain of the peptidoglycan pentapeptide of Staphylococcus aureus strain Copenhagen.

Fig. 4. Peptide bond formation and polypeptide termination in DNA-template-directed protein synthesis on ribosome.

Fig. 5. Transpeptidation (A), carboxypeptidation (B) and peptide dimer hydrolysis (C) during wall peptidoglycan expansion in Escherichia coli.

Fig. 6. Accommodation of the amide carbonyl donor Ac_2-L-Lys-D-Ala-D-Ala in the active site of the G, R61, R39 and K15 DD-peptidases.

Fig. 7. Schematic representation of the effects of amino acceptors on the consumption of the amide carbonyl donor Ac_2-L-Lys-D-Ala-D-Ala by the G DD-peptidase (A), the R61 and R39 DD-peptidases (B) and the K15 DD-peptidase (C).

In all cases, the amide carbonyl donor is Ac_2-L-Lys-D-Ala-D-Ala. Gly-Gly and Gly-L-Ala are used as acceptors by the R61 and K15 DD-peptidases. To be utilized by the R39 DD-peptidase, the amino group $N\bar{H}_2$-R must be located on a D carbon, in α position to a

free carboxylate as it occurs, for example, in

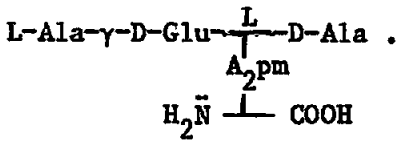


Fig. 8. α-Chymotrypsin-catalysed attack of amide and ester bonds. Effect of an alternate nucleophile NH_2-R on the channelling of an amide carbonyl donor (a) and an ester carbonyl donor (b) through hydrolysis and aminolysis.

The rate determining step is acyl enzyme formation in (A) and acyl breakdown in (B). The figures illustrate the behaviour observed with α-chymotrypsin. The amide carbonyl donor in (A) is acetyl-L-phenylalanine p-trimethylammonium anilide. The ester carbonyl donor in (B) is acetyl-L-phenylalanine methyl ester. In both cases, NH_2-R is L-alanine amide.

Fig. 9. Reaction between the R61 DD-peptidase (Z = the hydroxyl group of the active serine) and benzylpenicillin. Formation of the benzylpenicilloyl enzyme and breakdown of benzylpenicilloyl enzyme via rupture of C_5-C_6.

The benzylpenicilloyl enzyme has a half-life of 80 min . The intermediate Z, which gives rise to N-formyl-D-penicillamine, has a half-life of about 15 min .

Table 1. Interaction between β-lactams and the R61 and R39 DD-peptidases. Bimolecular rate constants (k_{+2}/K) of enzyme acylation (inactivation) and first order rate constants (k_{+3}) of enzyme deacylation (recovery). (at 37° unless otherwise stated).

β-Lactams	R61 DD-peptidase		R39 DD-peptidase	
	k_2/K ($M^{-1}s^{-1}$)	k_3 (s^{-1})	k_2/K ($M^{-1}s^{-1}$)	k_3 (s^{-1})
Penams				
Penicillanate	6×10^{-2}	$< 6 \times 10^{-5}$	14	6×10^{-3}
β-Iodopenicillanate	0.7	$< 3 \times 10^{-5}$	7,600	$< 10^{-5}$
6-Aminopenicillanate	0.25	$< 6 \times 10^{-5}$	1,200	6×10^{-3}
Benzylpenicillin	13,000 (a)	1.4×10^{-4}	300,000	3×10^{-6}
Phenoxymethylpenicillin	1,500	2.8×10^{-4}	[> 70,000]	—
Carbenicillin	830	1.4×10^{-4}	6,000	5×10^{-6}
Ampicillin	110	1.4×10^{-4}	70,000 (b)	4×10^{-6}
Methicillin	[15]	—	1,200 (b)	3×10^{-5}
Oxacillin	[130]	—	[40,000]	—
Cloxacillin	[30]	—	[15,000]	—
Quinacillin	no inactivation at 5 mM , 15 min		400	6×10^{-5}
Quinacillin sulfone	no inactivation at 5 mM , 15 min		10	6×10^{-5}
Mecillinam	0.22	$< 2 \times 10^{-4}$	32	1×10^{-4}
Δ^3-Cephems				
7-Aminocephalosporanate	33	4×10^{-3}	200	1×10^{-6}
Cephalosporin C	1,500	1×10^{-6}	65,000 (b)	0.3×10^{-6}
Cephalothin	[3,000]	—	> 70,000	—
Cephaloglycine	22	3×10^{-6}	70,000 (b)	0.8×10^{-6}
Cephalexine	[4]	—	3,000 (b)	2×10^{-6}
Nitrocefin	460 (c)	3×10^{-4}	$2.6\ 10^6$ (c)	1.5×10^{-6}
Benzyl-3-cephem (d)	80	5×10^{-6}	4,900	$<1 \times 10^{-7}$
RU 23345	24	5×10^{-6}	2,300	$<2 \times 10^{-6}$
Cefotaxime	16	$< 4 \times 10^{-6}$	2,600	$<2 \times 10^{-6}$
RU 25159	23	$< 2 \times 10^{-6}$	2,000	$<2 \times 10^{-6}$
HR 109	1.5	4×10^{-6}	200	$<2 \times 10^{-6}$
HR 979	1.5	1×10^{-6}	1,200	$<2 \times 10^{-6}$
Cefuroxime	350	4×10^{-6}	3,900	$<2 \times 10^{-6}$
RU 25238	2	$< 2 \times 10^{-6}$	2,300	$<2 \times 10^{-6}$
S 810592	1.5	$< 5 \times 10^{-6}$	450	$<5 \times 10^{-6}$
RU 25519	1.5	$< 2 \times 10^{-6}$	1,300	$<2 \times 10^{-6}$
Δ^2-Cephem				
Benzyl-2-cephem (d)	70	3×10^{-3}	52	$<2 \times 10^{-5}$
Cephamycine				
Cefoxitine	1,500	5×10^{-5}	7,000	$<3 \times 10^{-5}$
Penems				
Unsubstituted	670	0.05	1,750	8×10^{-3}
2-Methyl	800	0.01	5,400	8×10^{-3}
2-Phenyl	1,400	0.02	10,000	0.01
Monobactams				
Sulfazecin	40	2×10^{-5}	6,500	$<1 \times 10^{-5}$
SQ 26180	100	$< 1 \times 10^{-5}$	2,500	$<1 \times 10^{-5}$
SQ 26324	70	2.5×10^{-5}	8,300	$<1 \times 10^{-5}$
Azthreonam	< 0.002		15	$<0.5 \times 10^{-5}$
Others				
Clavulanate	21	5×10^{-6}	32	$< 10^{-6}$
N-formimidoylthienamycin	1,000	7×10^{-6}	10,000	2×10^{-6}

(a) = at 25°; (b) = at 20°; (c) = at 10°; (d) Respectively, Δ^3- and Δ^2-desacetoxy-7-phenylacetamido cephalosporanate. Values between square brackets were computed from ID_{50} values obtained in the absence of titration phenomena. From references 13-19 and unpublished data (β-iodopenicillanate).

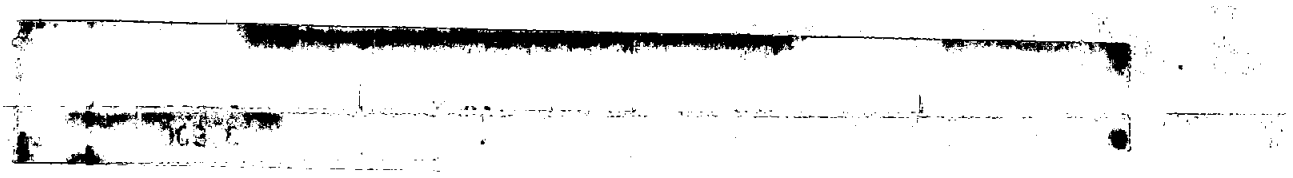


Table 2. Acylation of the R61 and R39 DD-peptidases. Individual values of K and k_2 (at 37° unless otherwise stated).

β-Lactams	R61 DD-peptidase			R39 DD-peptidase		
	K (mM)	k_2 (s^{-1})	k_2/K ($M^{-1}s^{-1}$)	K (mM)	k_2 (s^{-1})	k_2/K ($M^{-1}s^{-1}$)
Penams						
Penicillanate	36	2×10^{-3}	0.06			
β-iodopenicillanate	4	3×10^{-3}	0.7			
6-Aminopenicillanate	0.8	2×10^{-4}	0.25			
Benzylpenicillin	13 (a)	180 (a)	13,000 (a)			
Phenoxymethylpenicillin	> 1	> 1	1,500			
Carbenicillin	0.11	0.09	830			
Ampicillin	7	0.8	110			
Δ^3-Cephems						
Cephalosporin C	> 1	> 1	1,500	0.2 (b)	12.5 (b)	65,000 (b)
Cephaloglycine	0.4	9×10^{-3}	22			
Monobactams						
Sulfazecin	0.032	1.3×10^{-3}	40			
Azthreonam	12	$< 2 \times 10^{-5}$	< 0.002	0.3×10^{-3}	2.9×10^{-3}	15

(a) = at 25°; (b) = at 20°.

For references, see Table 1.

Fig 1A

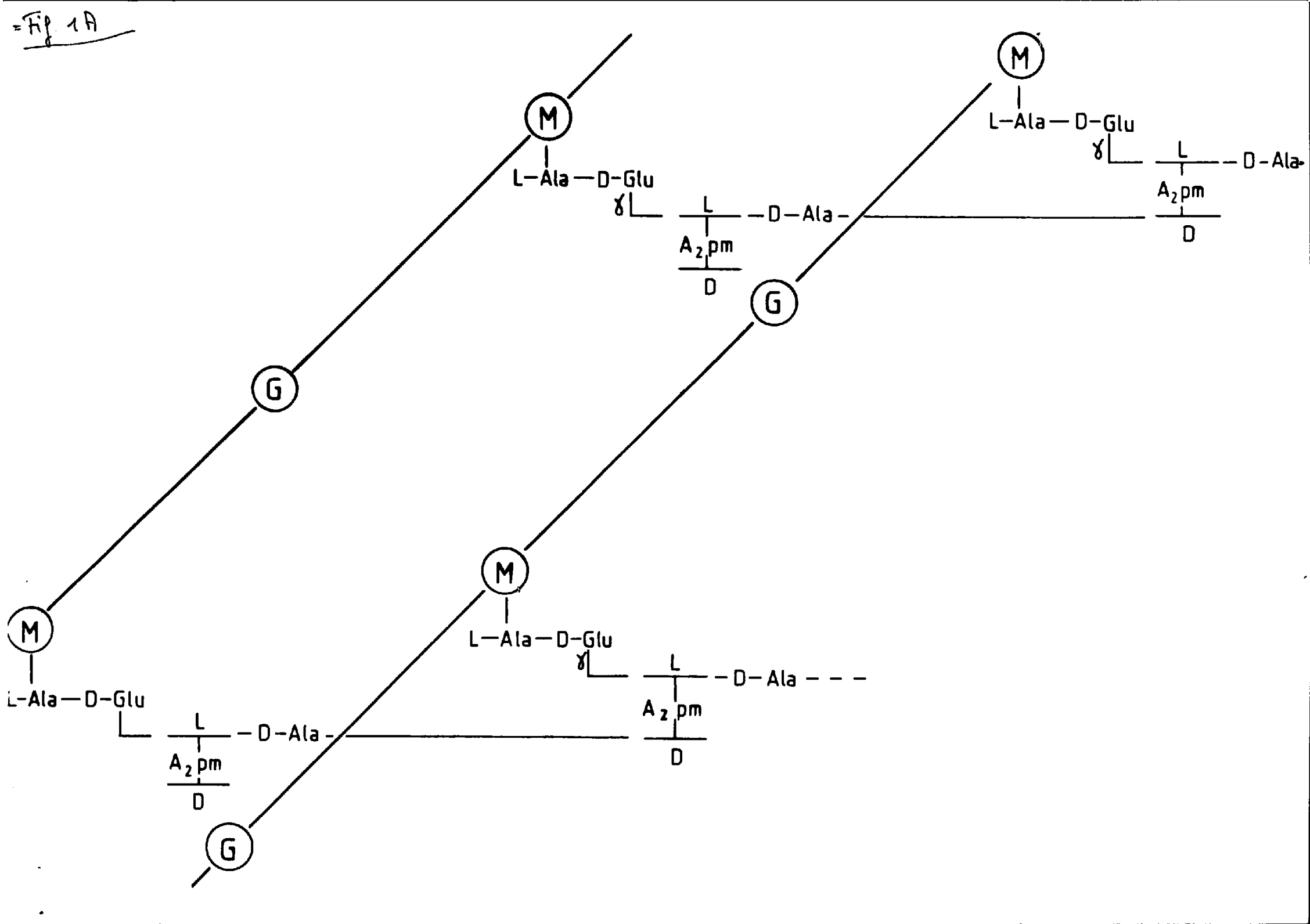


Fig 15

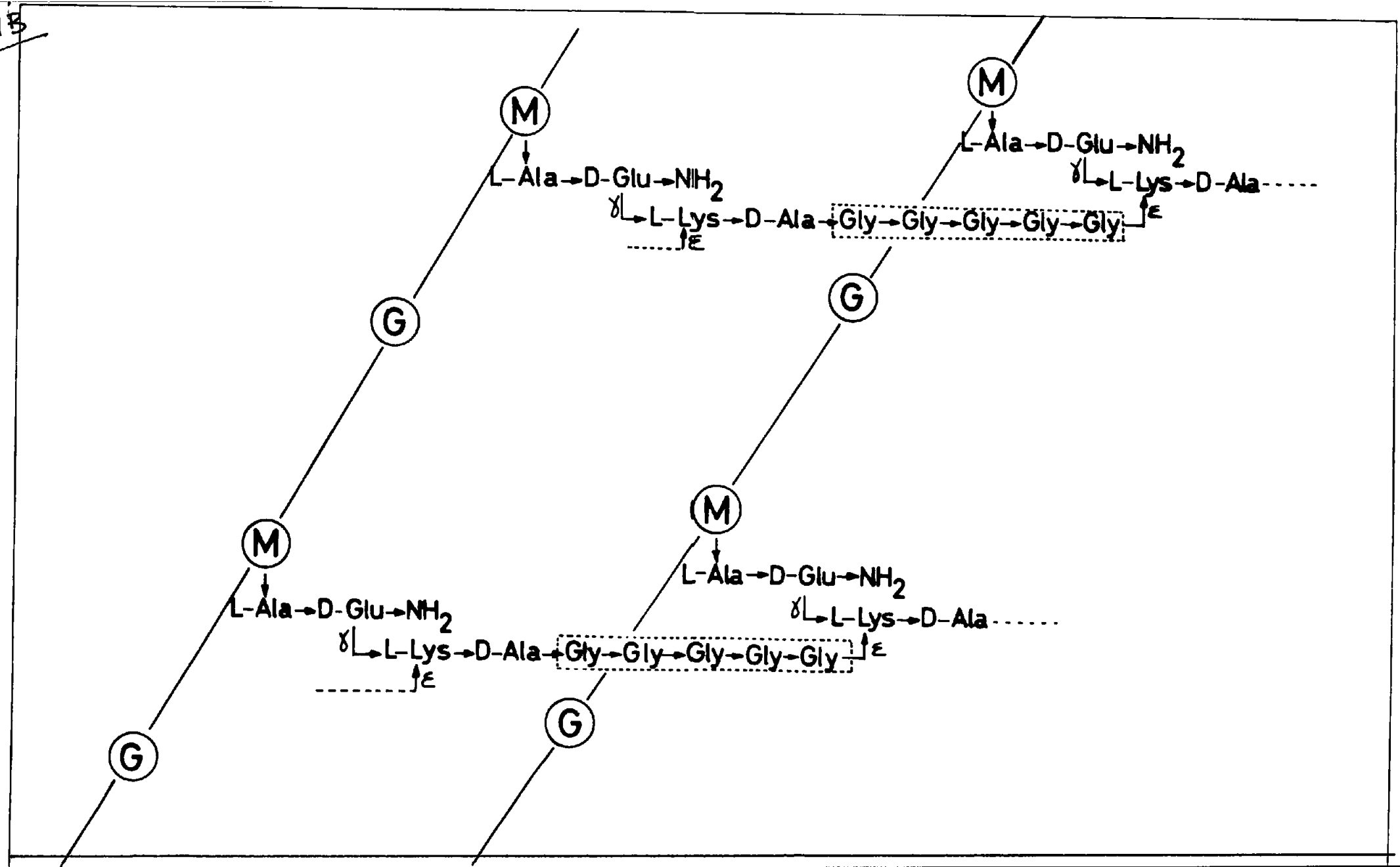
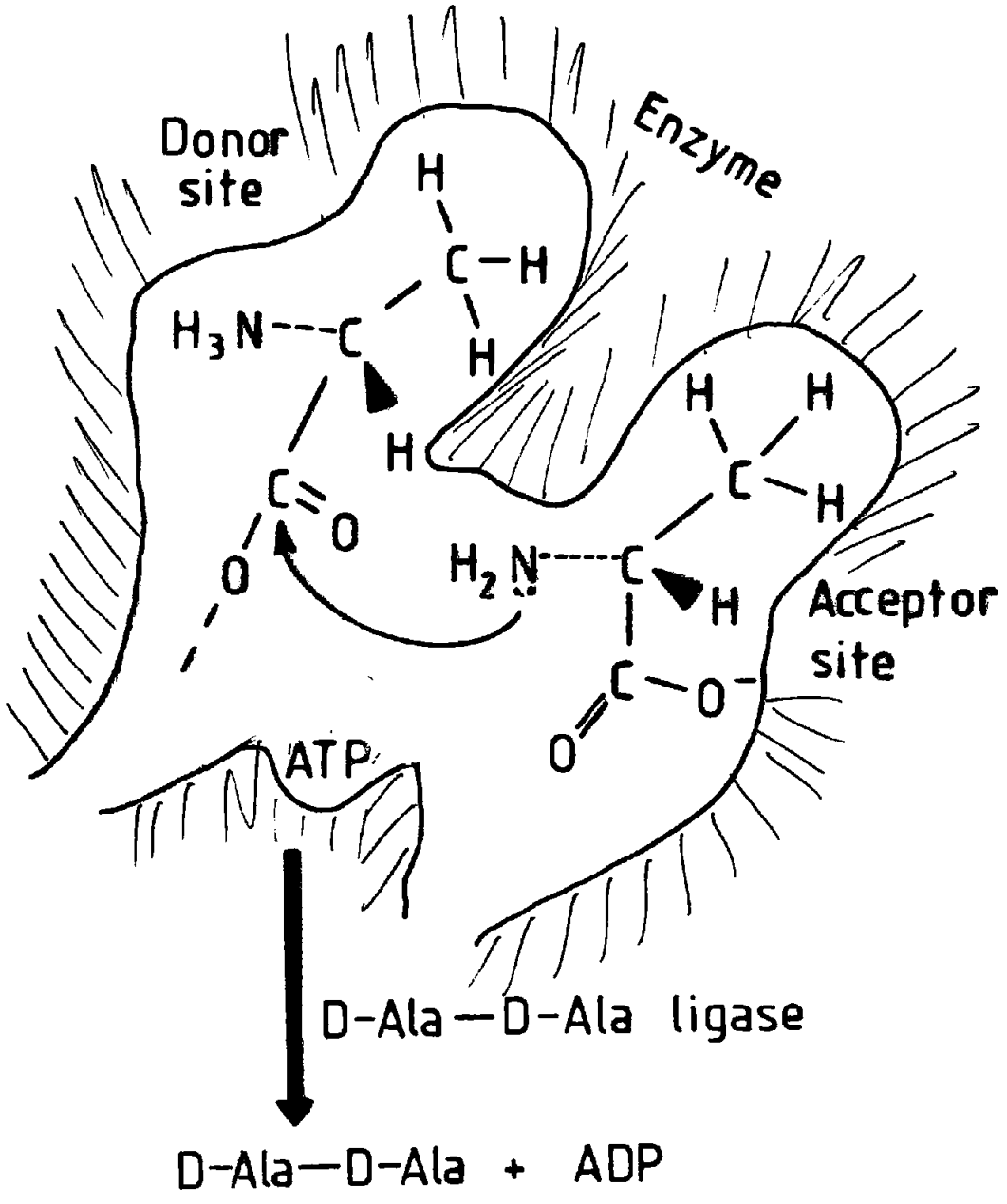

M
L-Ala→D-Glu→NH2
γL→L-Lys→D-Ala→Gly→Gly→Gly→Gly→Gly
ε
G
M
L-Ala→D-Glu→NH2
γL→L-Lys→D-Ala
ε
G
M
L-Ala→D-Glu→NH2
γL→L-Lys→D-Ala→Gly→Gly→Gly→Gly→Gly
ε
G
M
L-Ala→D-Glu→NH2
γL→L-Lys→D-Ala→Gly→Gly→Gly→Gly→Gly
ε
G

Fig 2



D-Ala—D-Ala + ADP

Fig 3

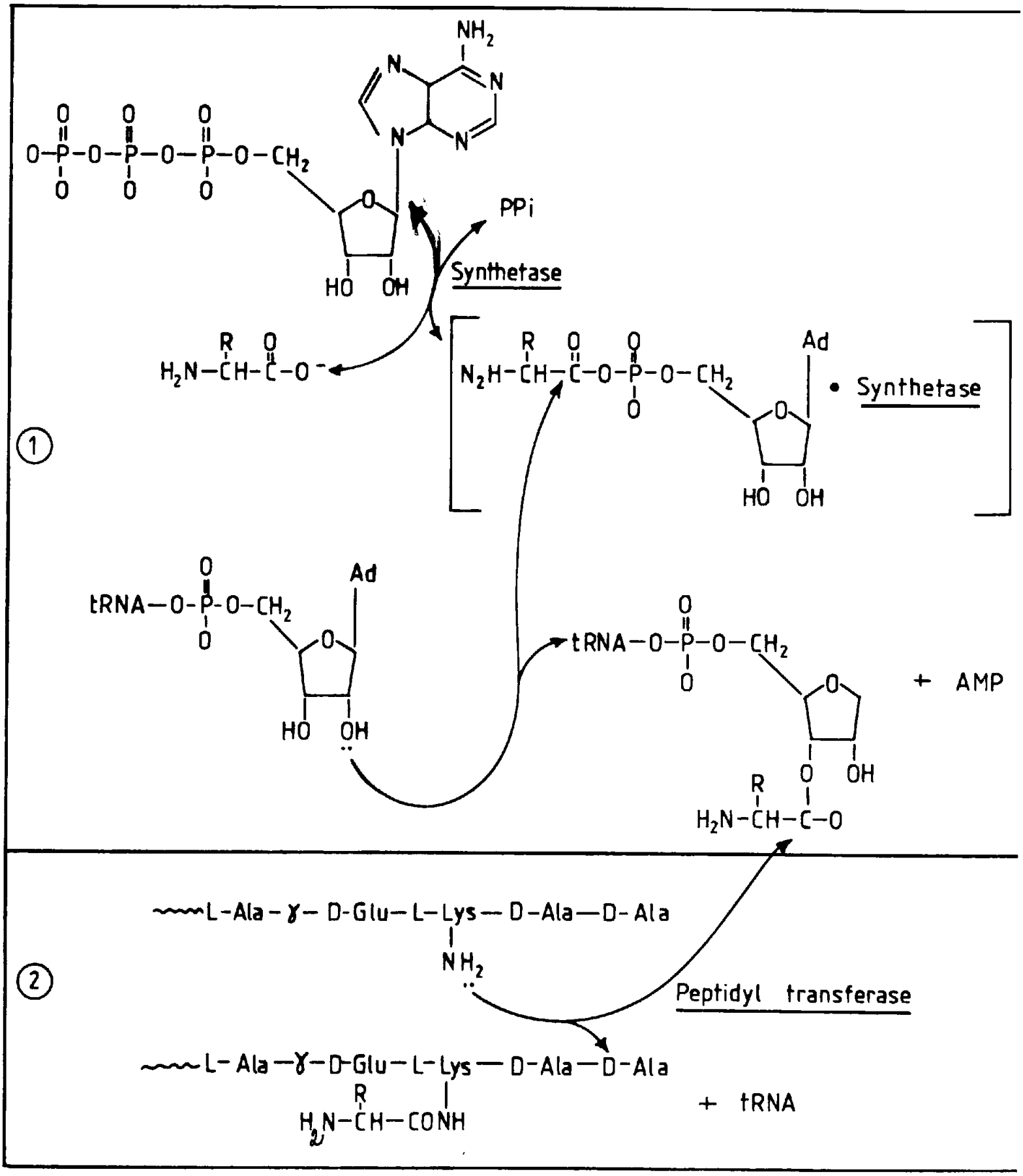

NH2
PPi
Synthetase
HO OH
Ad
• Synthetase
tRNA
+ AMP
1
2
L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ala
Peptidyl transferase
+ tRNA

Fig 4

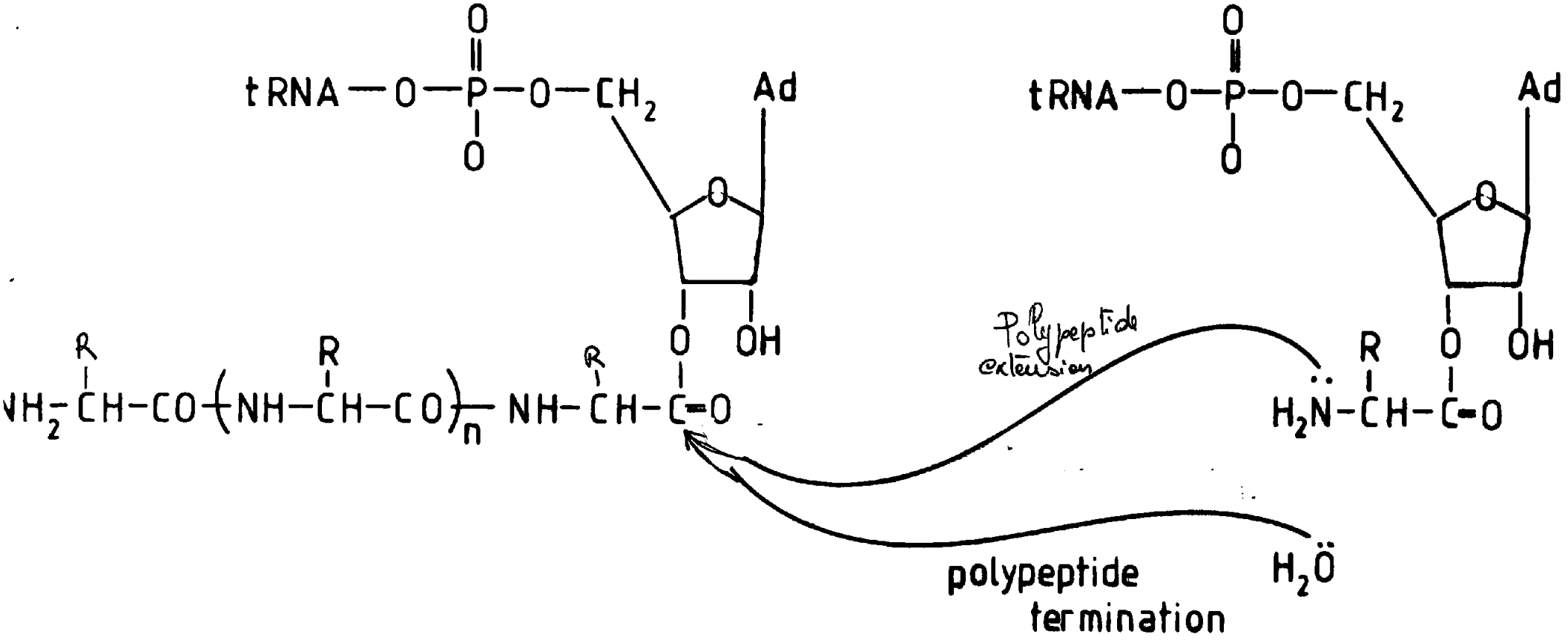

tRNA
Ad
OH
Polypeptide extension
polypeptide termination
H_2O

Fig 5

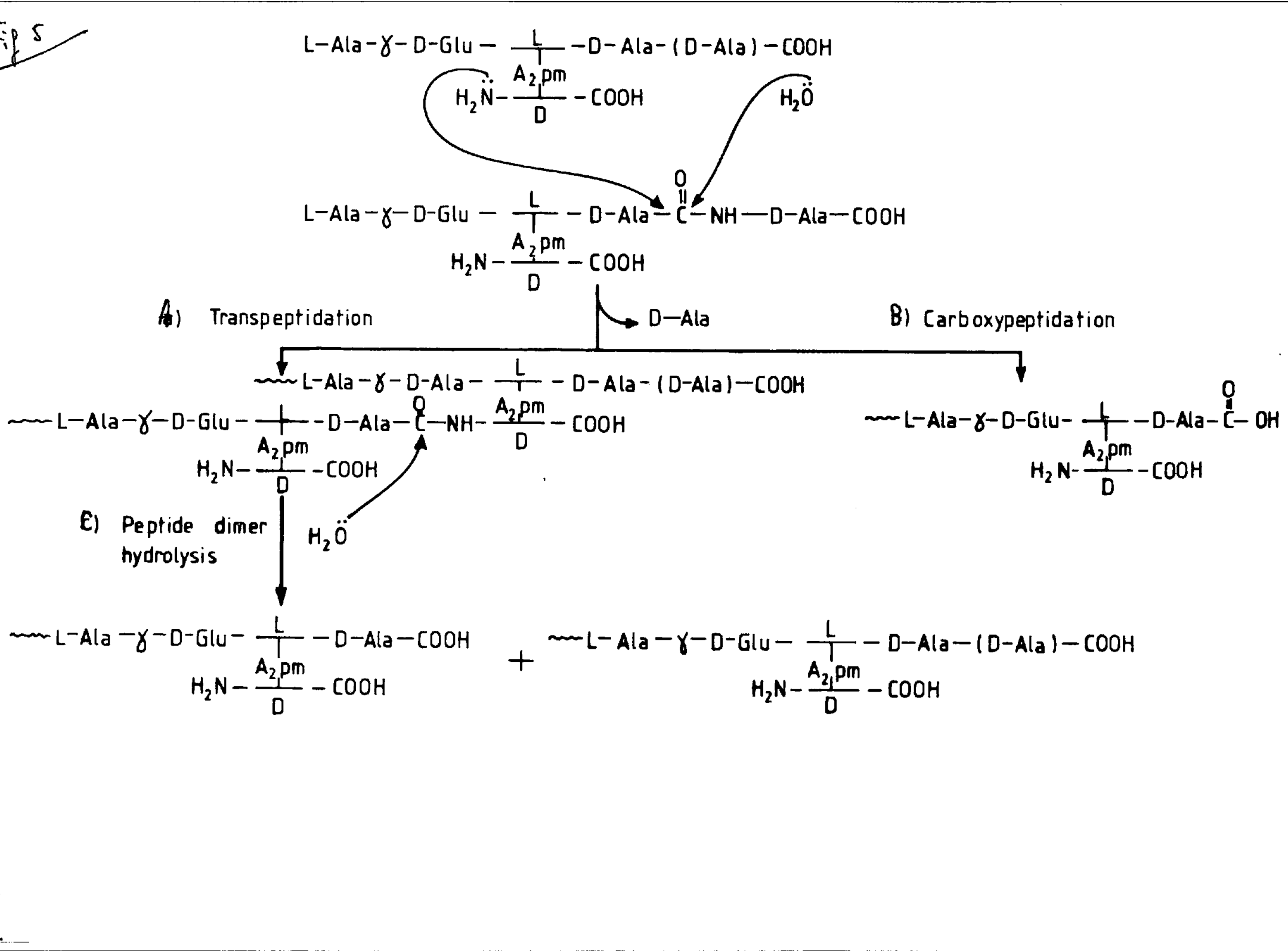

L-Ala-γ-D-Glu-L-D-Ala-(D-Ala)-COOH
A2pm
H2N-D-COOH
H2O
L-Ala-γ-D-Glu-L-D-Ala-C(=O)-NH-D-Ala-COOH
A2pm
H2N-D-COOH
D-Ala
A) Transpeptidation
B) Carboxypeptidation
L-Ala-γ-D-Ala-L-D-Ala-(D-Ala)-COOH
L-Ala-γ-D-Glu-L-D-Ala-C(=O)-NH-A2pm-D-COOH
A2pm
H2N-D-COOH
L-Ala-γ-D-Glu-L-D-Ala-C(=O)-OH
A2pm
H2N-D-COOH
C) Peptide dimer hydrolysis
H2O
L-Ala-γ-D-Glu-L-D-Ala-COOH
A2pm
H2N-D-COOH
+
L-Ala-γ-D-Glu-L-D-Ala-(D-Ala)-COOH
A2pm
H2N-D-COOH

Fig 6

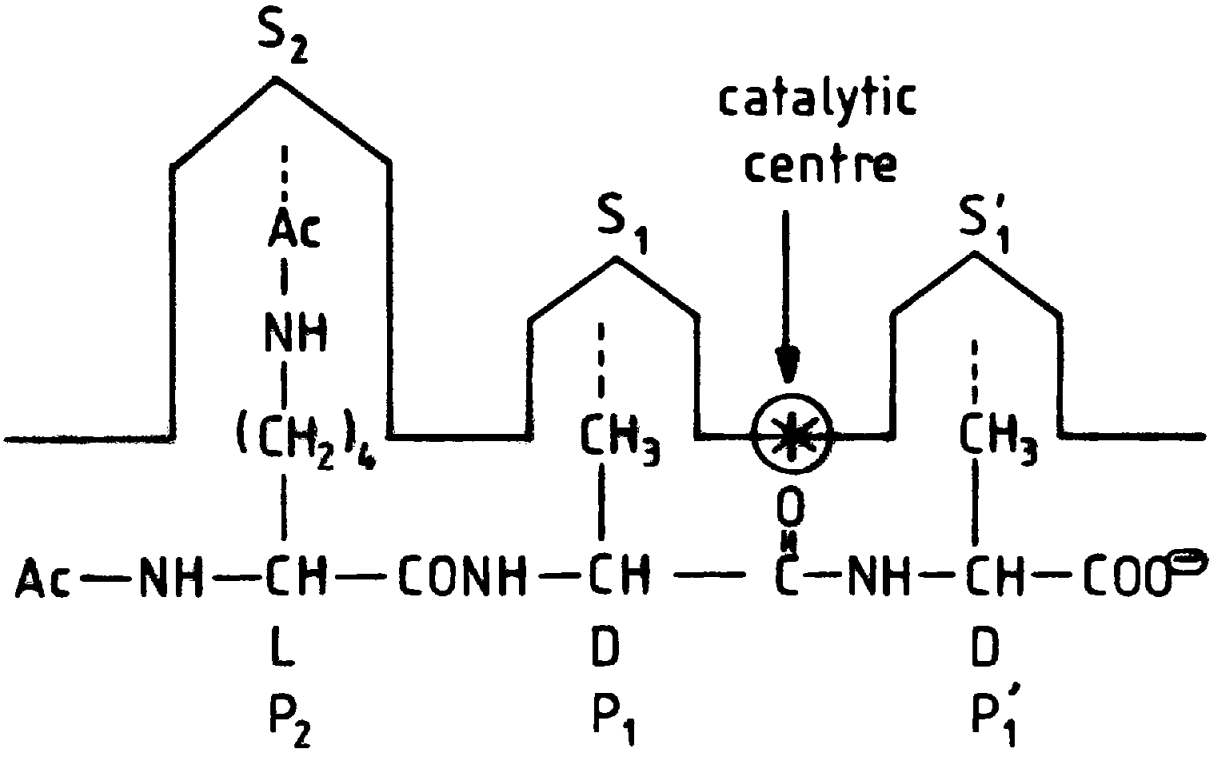

S2
catalytic
centre
S1
S'1
Ac
NH
(CH2)4
CH3
CH3
O
Ac—NH—CH—CONH—CH — C—NH—CH—COO⊖
L
D
D
P2
P1
P'1

Fig 7

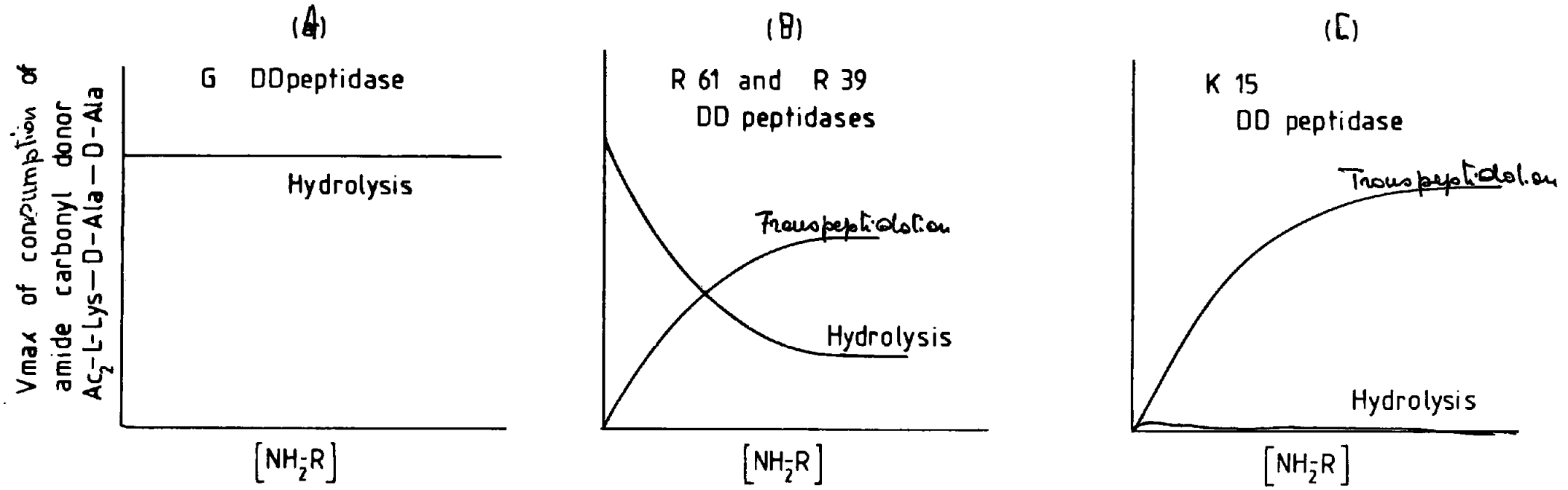
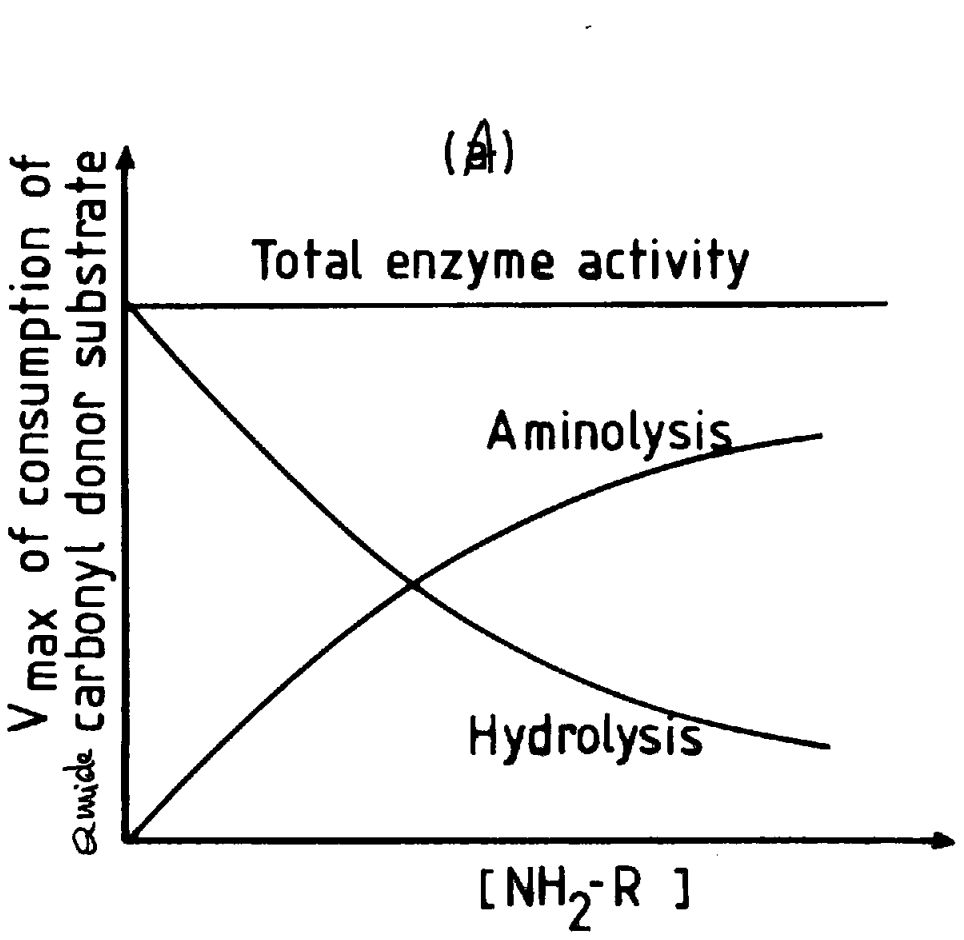

(A)
G DDpeptidase
Hydrolysis
Vmax of consumption of amide carbonyl donor Ac2-L-Lys-D-Ala-D-Ala
[NH2R]
(B)
R 61 and R 39
DD peptidases
Transpeptidation
Hydrolysis
[NH2R]
(C)
K 15
DD peptidase
Transpeptidation
Hydrolysis
[NH2R]

Fig 8


(A)
Total enzyme activity
Aminolysis
Hydrolysis
Vmax of consumption of amide carbonyl donor substrate
[NH2-R]

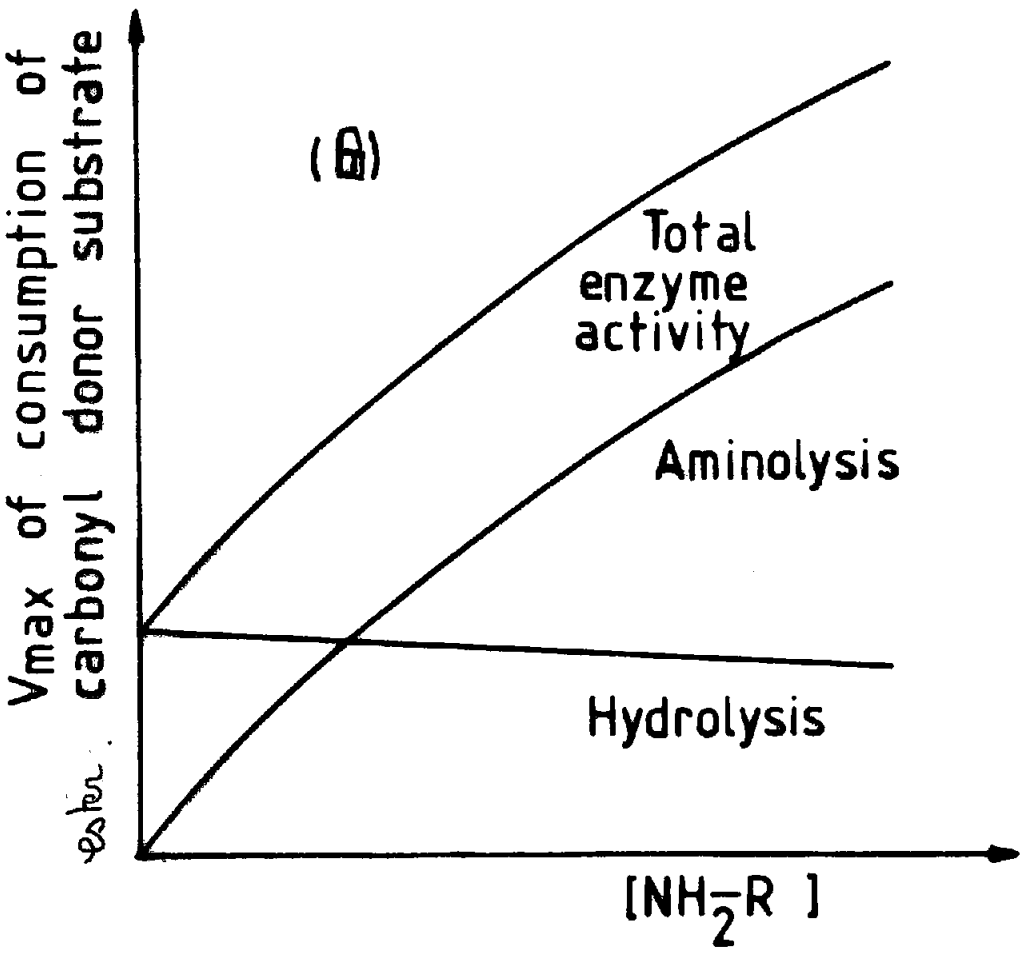
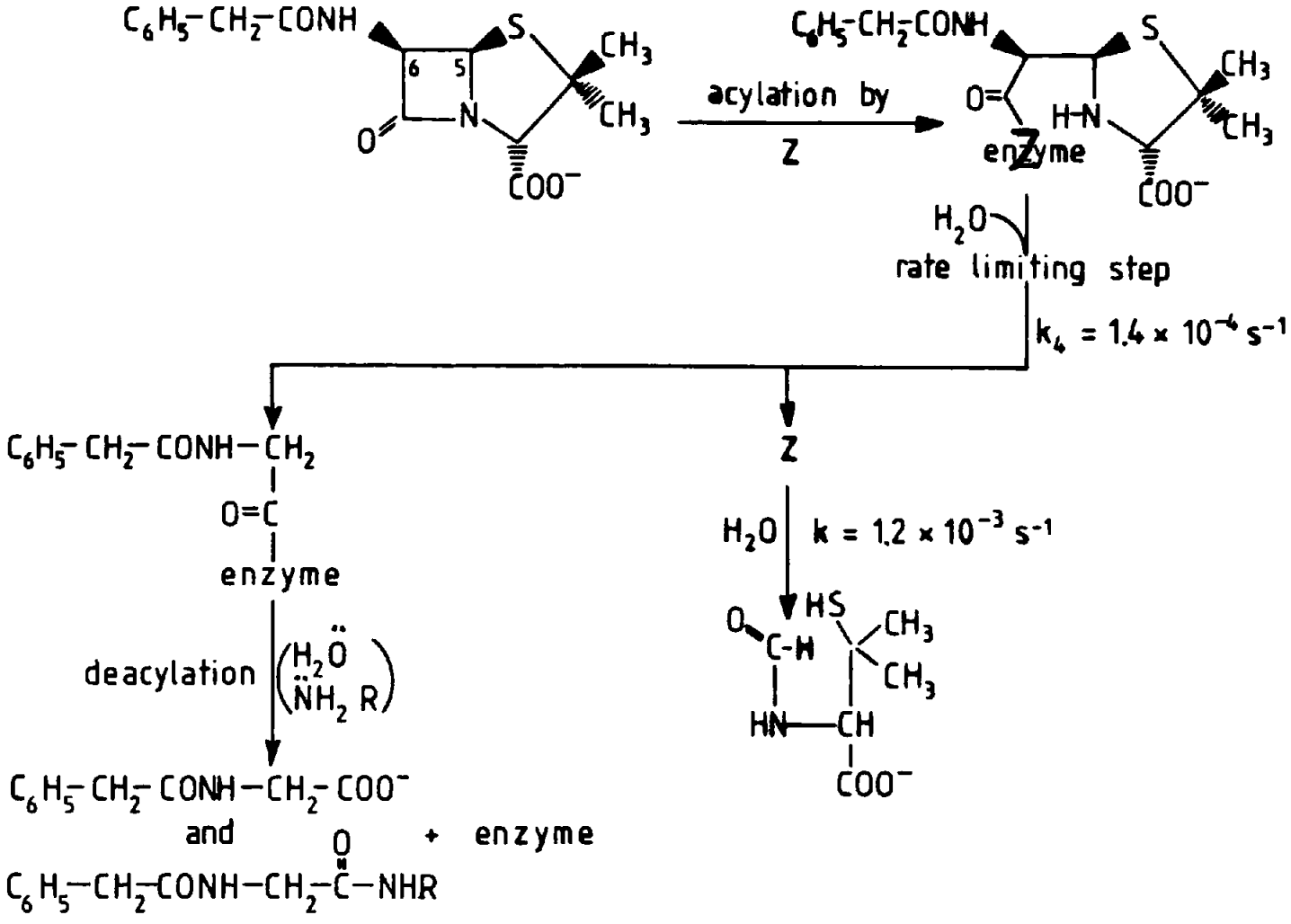

(B)
Total enzyme activity
Aminolysis
Hydrolysis
Vmax of consumption of ester carbonyl donor substrate
[NH2-R]

Fig 9


C6H5-CH2-CONH
6
5
S
CH3
CH3
O
N
COO⁻
acylation by
Z
C6H5-CH2-CONH
S
CH3
O=
HN
CH3
enzyme
COO⁻
H2O
rate limiting step
k4 = 1.4 × 10⁻⁴ s⁻¹
C6H5-CH2-CONH-CH2
O=C
enzyme
deacylation
H2O
NH2 R
C6H5-CH2-CONH-CH2-COO⁻
and
O
+ enzyme
C6H5-CH2-CONH-CH2-C-NHR
Z
H2O
k = 1.2 × 10⁻³ s⁻¹
HS
O
C-H
CH3
CH3
HN
CH
COO⁻