Antibiotic Action on Enzymes Involved in Peptidoglycan Synthesis

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Peptidoglycan is a ubiquitous component of the envelopes of all bacteria that have rigid cell walls. It consists of unbranched glycan chains made up of alternating units of N-acetylglucosamine and N-acetyl (or rarely N-glycolyl) muramic acid, linked β-1,4 as in the simpler chitin. Muramic acid itself is glucosamine substituted on the 3-position by a residue of D-lactic acid in ether linkage. The resultant carboxyl groups are substituted by short specific peptide side chains consisting of alternating L and D amino acid residues, the first being L-alanine or rarely glycine or L-serine and the second D-glutamic acid. In the completed cell wall the substituted glycan chains are cross-linked by means of tri-functional amino acid residues in the primary peptide side-chain, occasionally *via* the D-glutamic acid residue but more often through a dibasic amino acid (generally diaminopimelic acid or lysine) in the third position, and sometimes with the intervention of an intercalating peptide chain (6,19). The mechanism by which this cross-linking occurs, and its inhibition, are the main topics of this paper.

In the biosynthesis of the glycan, the primary peptide side-chains are first attached to UDP-N-acetylmuramic acid by sequential addition of the appropriate amino acid at the carboxyl terminus. After the first three residues (L,D,L) a dipeptide of D-alanine is added to complete the pentapeptide side-chain of the glycan precursor. This precursor [e.g. UDP-N-acetylmuramyl-L-alanyl-D-isoglutamyl-(L)-meso-diaminopimelyl-D-alanyl-D-alanine (UDP-MurNAc-pentapeptide) in Gram negatives, Bacilli and *Streptomyces* strain R39 (see below)] is then linked to undecaprenyl phosphate with elimination of UMP and formation of a pyrophosphate linkage. The next step in glycan synthesis consists of the substitution at the muramic acid C(4) position of β-N-acetylglucosaminine from UDPGlc-NAc, UDP

being eliminated. At this stage, where appropriate, extra amino acids that will ultimately form the cross-bridges are added. The disaccharide-peptide units still linked at their reducing ends to undecaprenyl pyrophosphate are then polymerized to yield the primary glycan chains by a mechanism in which growth occurs at the reducing end (22). The newly-formed glycan chain is then linked to the pre-existing peptidoglycan network (13,23) by a transpeptidation reaction in which the sub-terminal D-alanine residue on one side-chain becomes attached to an amino group on another, either directly or *via* cross-bridging amino acids, and the terminal D-alanine is eliminated (Fig. 1). On side-chains where the *D*-ala-*D*-Ala OH dipeptide is not used to form a cross-link, the terminal D-alanine residue is often removed by a *D*,*D*-carboxypeptidase, and thereafter the next *D*-alanine residue is removed by an *L*,*D*-carboxypeptidase.

It was proposed in 1965 (20,24) and is now generally accepted, that it is the formation of the inter-chain cross-links by the transpeptidation reaction that is sensitive to β-lactam antibiotics. Further, just as the *D*,*D*-transpeptidases are often sensitive to penicillin, so too are the analogous *D*,*D*-carboxypeptidases (9). Many strains of *Streptomyces* excrete soluble *D*,*D*-carboxypeptidases and in 1972 we showed that some of these purified enzymes could perform *in vitro* transpeptidation reactions that were penicillin-sensitive (18). These transpeptidation reactions were related to those that occur in the cross-linking of peptidoglycan, and later work showed that the enzymes would join peptide fragments together *in vitro* to make products identical with the cross-link occurring in the cell walls of the parent organism (8). Much of what follows describes the

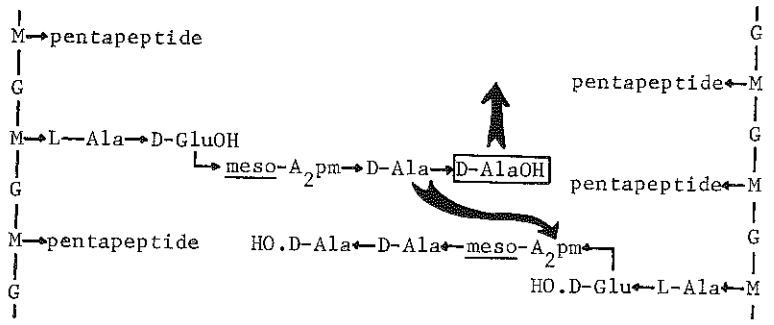

Fig. 1. Formation of a peptidoglycan cross-linkage

interaction of D,D-transpeptidases with their substrates (the carboxyl-donor peptides and the acceptors with free amino groups) and with β-lactam antibiotics.

INTERACTION OF TRANSPEPTIDASES WITH DONORS AND ACCEPTORS

The D,D-carboxypeptidase-transpeptidases under discussion are those of Streptomyces strains R61 and R39. These enzymes have been obtained in a high state of purity (2,5), and their specificities for various peptides acting as carboxypeptidase substrates have been examined in detail (11,12). In each case the C-terminal sequence giving the highest activity is R.D-Ala-D-Ala but the preceding residue also has a considerable effect on the enzyme kinetics. Thus for the R61 enzyme, Km = 12 mM and V_{max} = 5160 μmol/mg protein/h when Ac_2-L-Lys-D-Ala-D-Ala is the substrate, whereas the values are 15 mM and 23 μmol/mg protein/h with α-Ac-L-Lys-D-Ala-D-Ala, i.e. with a free ε-amino group (11). The corresponding values for the R39 enzyme are 0.8 mM and 1200 μmol/mg protein/h with the diacetylated substrate and 0.2 mM and 2200 μmol/mg protein/h when there is a free ε-amino group (12). Although not every carboxypeptidase substrate has been tested, it seems probable that all these substrates will function as donors in the transpeptidase reaction when the mixture has been supplemented with a suitable acceptor. The range of substrates that will function as acceptors seems to be related to the type of cross-linkage that exists in the peptidoglycan of the organism producing the enzyme. Thus in strain R61 the cross-link is formed between the penultimate D-alanine of one primary side-chain and a glycine residue that is attached to the ε-amino group of the L,L-diaminopimelic acid belonging to another side-chain. Correspondingly, glycine and many peptides with an N-terminal glycine residue, will act as acceptors in transpeptidation reactions, although other amino compounds can also function (17). In strain R39, on the other hand, the peptidoglycan linkage is directly from a D-alanine residue to the amino group at the D-centre of meso-diaminopimelic acid and in this case suitable transpeptidation acceptors must have an amino group in α-position to the carboxyl group of a D-amino acid or glycine (17).

Whenever these soluble Streptomyces enzymes perform transpeptidation, concomitant hydrolysis of the substrate by carboxypeptidase action also

occurs, and the kinetics are therefore necessarily complex (1,3). Nevertheless, in the presence of saturating concentrations of donor, it is possible to determine from Lineweaver-Burk plots an apparent K_m for the acceptor. The values obtained with the R61 enzyme were about 5 mM for meso-diaminopimelic acid as acceptor and 3 mM for the peptide Gly-L-Ala. Thus the 'affinity' of this enzyme for donor and acceptor is about the same. When complex acceptors related to peptidoglycan fragments were used, it became apparent that certain features of those substances exerted a profound effect on the relative amount of transpeptidation and donor hydrolysis that occurred (7,8,17). Thus, for instance, when increasing concentrations of the tetrapeptide L-Ala-D-isoGln-(L)-meso-A_2pm-D-Ala were provided as acceptor for the R39 enzyme, with Ac_2-L-Lys-D-Ala-D-Ala as donor, then transpeptidation rose to a maximum at an acceptor concentration of about 0.8 mM and at higher concentrations both transpeptidation and hydrolysis reactions were progressively inhibited until eventually the tripeptide donor present in the reaction mixtures remained unused (7). This particular phenomenon was dependent on the α-amide group on the glutamic acid residue, since high concentrations of the same tetrapeptide, non-amidated, inhibited overall attack on the donor by limiting its hydrolysis, but did not decrease the amount of transpeptidation product formed. More extensive studies (8) showed that when the donor site of the enzyme was saturated, the rate of the total reaction (hydrolysis and transpeptidation) was the same as the rate of hydrolysis alone when no acceptor was added. Thus the enzyme had the same turnover number for D-alanine release whether or not acceptor was present. The concentration of amidated tetrapeptide required to inhibit transpeptidation as well as hydrolysis was lower when the enzyme was less saturated by donor. Thus, as indicated above, a high concentration of amidated tetrapeptide acceptor can 'freeze' the enzyme so that both substrates remain completely unattacked. These and similar results led to the conclusion that mechanisms able to change the acceptor and donor concentrations or to alter some particular structural feature of these peptides are important for control of the activity of the exocellular D,D-carboxypeptidase-transpeptidase of strain R39. Similar mechanisms could well be involved in the control of the peptide cross-linking system *in vivo* (8).

INTERACTION OF TRANSPEPTIDASES WITH β-LACTAM ANTIBIOTICS

As indicated, the transpeptidation reactions are sensitive to penicillin. Tipper and Strominger (20) proposed that penicillin functioned as an analogue of a terminal acyl-D-Ala-D-Ala sequence, which having arrived at the active site of a transpeptidase, permanently inactivated the enzyme by penicilloylation of the protein. Recent work with the purified D,D-carboxypeptidase-transpeptidases has now begun to throw some new light on how these enzymes interact with β-lactam antibiotics.

When a purified preparation of the R61 enzyme was mixed with benzylpenicillin, the circular dichroism spectrum of the enzyme in the near u.v. was extensively altered (Fig. 2) (14). The peptide region in the far u.v. remained unchanged. The interaction between the enzyme and the antibiotic showed high affinity and no further change in the c.d. of the enzyme occurred after the addition of one mol of penicillin for each mol of enzyme (38,000 mol wt). Examination of the fluorescence of the enzyme proved more convenient for studying the interaction with penicillin. Excitation was at 273 nm and emission was observed at 320 nm. When one molar proportion of benzylpenicillin was added to 7.5×10^{-7} M enzyme in 10 mM sodium phosphate buffer, pH 7.0, at 25°C the fluorescent emission decreased over a period of some 10 min, the final quenching being 25-30% (Fig. 3). By making successive additions of penicillin to a sample of enzyme and allowing 10 min after each addition for completion of the interaction, the quenching of fluorescence was used to follow penicillin binding. The result once more was that the enzyme bound one molar proportion of antibiotic and further additions did not affect the fluorescence (14). The inactivation by penicillin was evidently reversible, because fully inactivated enzyme recovered all its activity during overnight dialysis against 10 mM buffer, pH 7. The binding of penicillin or

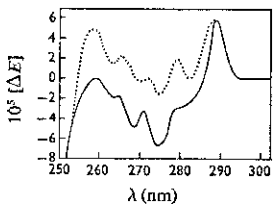

Fig. 2. Circular dichroism of the R61 enzyme with (.....) and without (———) penicillin (14).

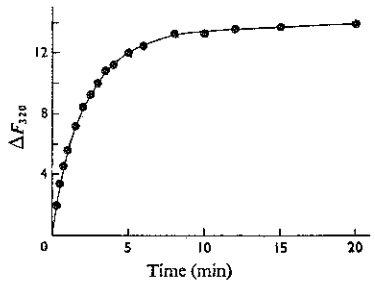

Fig. 3. Time course of the binding of sodium benzylpenicillin to the R61 enzyme. The ordinate represents quenching of fluorescence (14).

its release were unaffected by the presence of thiol compounds, which contrasts with the behaviour of an enzyme from Bacillus subtilis (10).

The forward rate constant for the association of penicillin and R61 enzyme was calculated from second-order kinetic plots during the first 3-4 min after mixing. The value measured at 25°C in 10 mM sodium phosphate buffer pH 7 was $k_f = 1.8 \times 10^4$ litre. mol^{-1}. s^{-1}, and in the presence of 57 mM Ac_2-L-Lys-D-Ala-D-Ala (a donor substrate at a concentration about five times its K_m value) $k_f = 5.7 \times 10^3$ litre. mol^{-1}. s^{-1}. This relatively small change in the reaction rate could not be ascribed to competition between antibiotic and substrate, however, because the non-substrate, non-inhibitor peptide Ac_2-L-Lys-D-Ala produced a similar effect ($k_f = 4.4 \times 10^3$ litre. mol^{-1}. s^{-1}) (14). It was also observed that penicillin binding, as measured by fluorescence quenching, could occur with enzyme that had been made enzymically inactive by either urea or guanidinium chloride.

From the titration of penicillin with the R61 enzyme, as followed by quenching of fluorescence, an association constant could be calculated. Hence, with the k_f determined as described above, the rate constant for the dissociation of the complex could be deduced - in 10 mM sodium phosphate buffer it was 0.8×10^{-4} s^{-1}, corresponding to a half-life of 2.4 h at 25°C (14). This is comparable to a value of 1.35 h observed at 37°C, in experiments in which an excess of penicillinase was present throughout and enzyme recovery was followed by measuring its carboxypeptidase activity (unpublished work).

The purified enzyme from strain R39 was also inactivated by β-lactam antibiotics (12). The binding of penicillin to this enzyme could not be studied by the use of fluorescence or c.d. because neither parameter was affected. Instead, binding was examined by measuring the loss of carboxypeptidase activity as more penicillin was added. Again it was found that one mol of penicillin was needed to inactivate one mol of enzyme (53,300 mol wt) (4). A sample of the enzyme was also inactivated by addition of an excess of ^{14}C-benzylpenicillin and filtered on a Sephadex column to remove unfixed antibiotic. At 37°C and in pH 7.7 buffer (0.1 M Tris, 0.2 M NaCl, 0.05 M $MgCl_2$) in which the untreated enzyme was completely stable, the radioactive penicillin was released and enzymic activity recovered at almost exactly the same rate (half-lives 73 and 65 h, respectively). The kinetic characteristics of the recovered enzyme, both as carboxypeptidase and as transpeptidase, were unchanged and furthermore the regenerated enzyme reacted with fresh penicillin exactly as before.

The nature of the radioactive compounds released from the ^{14}C-benzylpenicillin-R39 enzyme complex was also examined (4). It was found that the main compound released in Tris-NaCl-$MgCl_2$ buffer was not benzylpenicillin or benzylpenicilloic acid, nor was it the same as the compound that arose when penicillin alone was incubated under the same conditions, in which case the main product was probably a benzylpenicilloyl-Tris compound. When the ^{14}C-penicillin-enzyme was dissociated in phosphate buffer (10 mM, pH 7) release of radioactivity was more rapid (half-life at 37°C 11-14 h) but enzymic activity did not recover; the enzyme is, in any case, unstable in this buffer. Under these conditions the main product of penicillin release seems to be benzylpenicilloic acid, as judged by both chromatography and electrophoresis, but the identification was not certain.

It did not appear that SH groups were involved in either enzyme activity or penicillin binding, since neither iodoacetate nor 5,5'-dithio-bis-(2-nitrobenzoic acid) had any effect.

The reaction of the R39 enzyme with β-lactam antibiotics was also studied with two cephalosporins (4). The chromogenic cephalosporin 87-312 (3-[2,4-dinitrostyryl]-[6R-7R]-7-[2-thienylacetamido]-ceph-3-em-4-carboxylic acid, E-isomer) (15) immediately changed colour when added to

the enzyme, even at 0°C. The new absorption spectrum was found to be almost identical with that of cephalosporin 87-312 that had been hydrolysed by β-lactamase (i.e. the absorption maximum had shifted from 386 to 482 nm). Thus the reaction between the enzyme and the antibiotic could be followed by the loss of enzymic activity, on the one hand, and the change in absorption maximum on the other. As the activity disappeared there was a parallel change in the absorption spectrum of the antibiotic (Fig. 4). According to the published extinction coefficient for cephalosporin 87-312, the end point of the enzyme-antibiotic reaction occurred when 1.27 molar proportions of cephalosporin had been added, compared with a value of 0.92 for benzylpenicillin and the same 90% pure enzyme preparation. It seems most likely that the true stoicheiometry is in fact 1:1. This view was supported by observations with cephaloridine, another cephalosporin that gives a change of absorption spectrum when its β-lactam ring is opened (16). A titration with R39 enzyme similar to that described for cephalosporin 87-312 produced a molar ratio of antibiotic:enzyme of 0.92. The rate constants for the dissociation of the cephalosporin-enzyme complexes, based on recovery of enzyme activity, were also calculated for the decomposition in Tris-NaCl-$MgCl_2$ buffer at 37°C. The half-lives were 130 h for cephalosporin 87-312 and 300 h for cephaloridine, compared with 65 h for benzylpenicillin (4).

Parallel experiments performed with the R61 enzyme showed that the spectrum of cephalosporin 87-312 bound to this enzyme was not the same as that of the β-lactamase product (Frère, Leyh-Bouille, Ghuysen & Perkins, unpublished work). In this case the latter spectrum only appeared after the breakdown of the complex accompanied by restoration of enzymic activity. As with the R39 enzyme, the complex with R61 enzyme appeared to require 1.25 mol of cephalosporin 87-312.

There seemed no doubt that the cephalosporin 87-312 and benzylpenicillin were competing for the same site on both enzymes, because experiments showed that their reactions with the protein were mutually exclusive.

The above results suggest that although both enzymes caused the ultimate destruction of penicillin, they did not do so by a simple opening of the β-lactam ring, since benzylpenicilloic acid was not the product, at least in Tris-NaCl-$MgCl_2$ buffer. Since the regenerated enzyme had also recovered

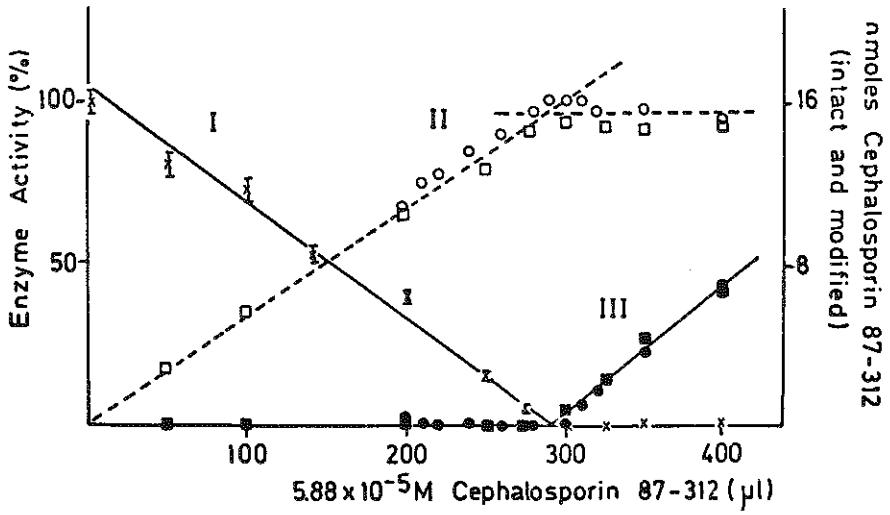

Fig. 4. Titration of R39 enzyme with cephalosporin 87-312. I: Residual enzyme activity, II: modified antibiotic concentration deduced from E_{482}, III: unchanged antibiotic concentration deduced from E_{386}(4).

its sensitivity to penicillin, it was evident that the released penicillin had not carried with it some labile part of the protein. What bond is formed between β-lactam antibiotics and the soluble D,D-carboxypeptidase-transpeptidases of Streptomyces species remains unknown at present, except that no sulphydryl group seems to be involved. This situation is different from that with the D,D-carboxypeptidase of B. subtilis, where benzylpenicillin apparently formed a thioester bond (21). The main difference between the enzymes from strains R61 and R39 appears to be that the penicillin complex of the former is much more unstable, with a half-life of 1.35 h compared with 65 h for the latter. As with the R39 enzyme, the 'penicillin' released from its complex with the R61 enzyme was neither benzylpenicillin nor benzylpenicilloic acid (unpublished work). It is now thought (P.M. Blumberg, personal communication) that the DD-carboxypeptidases of B. subtilis and B. stearothermophilus also convert penicillin to a product that is not penicilloic acid.

At present it is not possible to say what site on the enzymes is occupied by β-lactam antibiotics. Penicillins prevent enzyme action, either by

stopping the initial binding of the donor peptide or by preventing the ensuing chemical reaction that leads either to hydrolysis or to transpeptidation. Until there is an independent method of measuring donor binding, these possibilities cannot be clearly distinguished. With antibiotics like cephaloridine that are firmly bound, it should prove possible to isolate fragments of enzyme retaining the modified antibiotic, thus eventually defining the nature of the binding site.

Acknowledgements

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DISCUSSION

Sund: It seems to me that the experimental data of your fluorescence titration follow a sigmoidal shaped curve rather than a normal hyperbolic curve.

Perkins: You may be right, but I think one would need to carry out experiments in the light of more recent results

using a more sensitive method of following the association. The change in fluorescence at low molar proportions of penicillin was rather small to measure accurately.

Veeger: Have you compared enzyme activities in buffers of equal ionic strength, in order to elucidate specific effects of polyvalent ions on your enzyme?

Perkins: We did not use phosphate buffer at high ionic strength because the enzyme is particularly sensitive to that anion, and rapidly loses its activity.