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Point of VIEW

Cutting and Stitching: The Cross-Linking of Peptidoglycan in the Assembly of the Bacterial Cell Wall

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ABSTRACT The machinery responsible for bacterial cell wall synthesis has proven to be an invaluable antibiotic target. Nearly 80 years after the discovery of penicillin, some of the mysteries surrounding this process are finally being unraveled.

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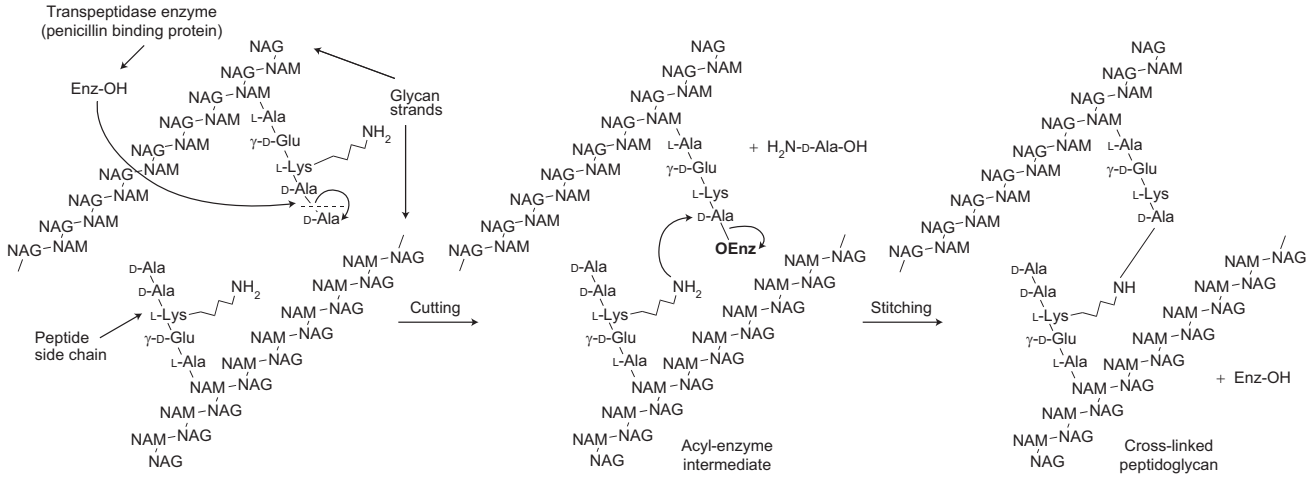
Several important classes of antibiotics, particularly the β-lactams and the glycopeptides, function by interfering with the machinery responsible for bacterial cell wall assembly. Targeting this machinery has the advantage of its being unique to bacteria and more readily accessible than the bacterial ribosome, another important antibiotic target. Acquiring a detailed understanding of the bacterial enzymes responsible for assembling the cell wall components should lead to the design of more highly effective antibiotics. One key group of enzymes is the D-Ala-D-Ala (DD)-transpeptidases, which catalyze the cross-linking of the peptide side chains of adjacent glycan strands. These enzymes represent some of the proteins collectively known as penicillin-binding proteins (PBPs), because they are the key target of penicillin, which becomes covalently attached to (and thereby inactivates) them. The most important of these are the high-molecular-weight (HMW), membrane-bound PBPs. Because they are membrane-bound, these enzymes are notoriously difficult to study, and only in the past decade have they finally yielded (as solubilized analogues) to structural analysis by X-ray crystallography (*1, 2*). But many mysteries remain, and having a precise picture of how the biological substrates bind the PBP would facilitate the process of antibiotic design. However, it has been very difficult to get solubilized derivatives of the membrane-bound PBPs to function properly, including getting them to recognize the structural features of their putative substrates. In a series of articles, the latest of which is on page xxx of this journal, Pratt *et al*. evaluate the biochemical characteristics of these enzymes, particularly their ability to interact with substrates and substrate analogues, such as β-lactams structurally decorated with peptides mimicking the stem peptides of peptidoglycan. They demonstrate a surprising dichotomy whereby some of the PBPs are able to recognize substrates and substrate-like analogues, but other PBPs, and most importantly, the HMW PBPs, do not.

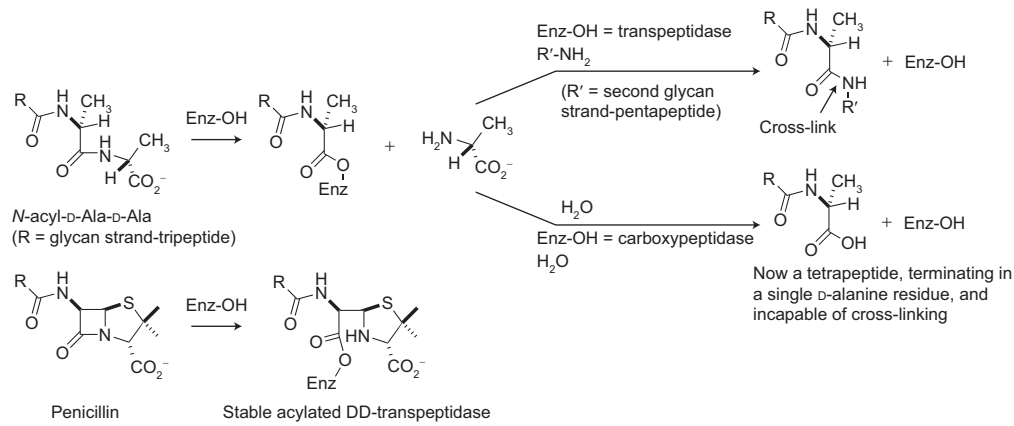
Penicillin, the first broadly effective antibacterial agent, is arguably the most important medical discovery of the 20th century, still accounting for approximately half of all antibiotic prescriptions. It began in 1928 when bacteriologist Alexander Fleming made a chance discovery of a bactericidal fragment of *Penicillium notatum* (now called *Penicillium chrysogenum)* mold growing in a discarded Petri dish. He was aware that one organism may secrete substances that are toxic to other organisms. Driven by his desire to discover new antibacterial substances, he isolated and grew the mold and demonstrated its ability to produce a substance with high potency against common human pathogens. Ten years later, his discoveries were further developed into a viable antimicrobial product by pathologist and pharmacologist Howard Florey and



Scheme 1. The cross-linking of peptidoglycan strands.

chemist/biochemist Ernst Chain at Oxford University, U.K. The three shared the Nobel Prize in Physiology or Medicine for these discoveries in 1945.

Mechanistically, it had been appreciated that bacteria treated with penicillin displayed unusual morphology, thus implicating interference with the bacterial cell wall formation. The bacterial cell wall, a strong, rigid, highly cross-linked polymer composed of polysaccharide chains with peptide cross-links, hence known as peptidoglycan or murein, is essential for bacteria to maintain their shape and to enable them to withstand large changes in osmotic pressure. However, nearly 20 years later, as the details of bacterial cell wall assembly were elucidated, a hypothesis was advanced for precisely how penicillin interfered with the process. Scheme 1 illustrates the final steps of the peptidoglycan synthesis, involving a cross-linking reaction of the pentapeptide side chains (known as stem peptides) protruding from the glycan strands, themselves consisting of the two alternating amino sugars *N*-acetylmuramic acid and *N*-acetylglucosamine. A transpeptidase enzyme first cleaves the terminal D-Ala-D-Ala linkage, with the formation of a Ser-D-Ala ester bond, then the resultant acyl-enzyme reacts with the side chain amino group of the third residue, which can be the ε-amino group of lysine, as shown, in the case of *Streptococcus pneumoniae*, to cleave the ester linkage of the acyl-enzyme and form a cross-linking peptide amide bond. The amine donor (called the "acceptor") can also be the ε-amino group of diaminopimelic acid, in the case of *Escherichia coli*, or also the terminal amino group of a pentaglycine unit, further appended to the lysine side chain, in the case of *Staphylococcus aureus*, a more flexible arrangement that allows *S. aureus* to achieve a high level of cross-linking (*3*).



Scheme 2. The enzymatic mechanism of transpeptidation.

In 1965, Donald Tipper and Jack Strominger proposed that penicillin structurally resembled the terminal D-Ala-D-Ala linkage of the stem peptide, thus enabling the antibiotic to irreversibly acylate the active site serine of the DD-transpeptidase,

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with consequent opening of the β-lactam (*4*). Strominger also coined the term "penicillin-binding proteins" to describe the large group of enzymes known to bind penicillin, which is now known to include, on the basis of the initially employed SDS-PAGE process, both HMW and low-molecular-weight (LMW) PBPs. The HMW PBPs are further subdivided into classes A and B. Class A HMW PBPs are bifunctional, having the ability to catalyze not only transpeptidation but, at a second active site, the coupling of the two sugars (glycosyltransferase (GT) activity) leading to formation of the polysaccharide chain. The GT site currently represents a prospective but unexplored antibiotic target, with the first structural data of this site recently made available (*5, 6*). Class B HMW PBPs are solely transpeptidases. Many of the LMW PBPs are carboxypeptidases, not transpeptidases, utilizing water as an acceptor, rather than a peptide amino group, thus resulting in simple hydrolysis of the D-Ala-D-Ala linkage. The purpose of these nonessential (*7*) carboxypeptidases is still not known, but one hypothesis is that, by inactivating some of the stem peptides, they regulate the extent of cross-linking (*8*). Scheme 2 illustrates the structural similarity between the penicillin and the terminal D-Ala-D-Ala, as well as the double displacement enzymatic mechanism responsible for the cross-linking process of Scheme 1, carboxypeptidation, and also inactivation of the enzyme by the β-lactam antibiotic.

Not all PBPs are membrane-bound. The study of these enzymes was accelerated by the discovery of LMW PBPs that were secreted by the cell, many of which are carboxypeptidases, but some of which also have transpeptidase activity, including the exocellular DD-carboxypeptidase–transpeptidase produced by *Streptomyces* strain R61 (*9*). This enzyme was also the first PBP for which X-ray structural data were available (*10*).

Intensive study of PBPs has demonstrated that the essential killing targets are the HMW PBPs and that mutations of these proteins can lead to low affinity for penicillin and consequently high-level antibiotic resistance (*11*). But the enzymes have been difficult to study. In the solubilized form, these HMW enzymes did not appear to catalyze the transpeptidase reactions with analogues of their putative substrates at rates sufficient to explain their *in vivo* activity (*12*). Pratt and Kumar (*13, 14*), however, were recently able to synthesize substrate analogues of the water-soluble LMW *Streptomyces* R61 transpeptidase–carboxypeptidase, which sufficiently resembled the natural substrates to obtain kinetic data and to elucidate some aspects of substrate and acceptor specificity. However, when he and his colleagues tried to extend these studies to solubilized forms of the HMW membrane PBPs, they found no evidence for the recognition of the structural features resembling natural substrates (incorporated into either peptidoglycan mimetics or β-lactams) (*15*). The present work also includes intriguing *in vivo* studies that indicate that the β-lactams that had been modified to resemble peptidoglycan are not recognized more effectively than commercial β-lactam antibiotics. This implies that no such specific recognition may exist, even when the proteins are present in their natural (membrane) environment.

The results of Pratt and colleagues, coupled with other observations, lead to intriguing hypotheses. Recently, for example, two separate crystal forms, with the transpeptidase active site closed (*16*) or, alternatively, open (*17*), of the class A PBP1b from *S. pneumoniae* have been reported, a suggestion that nature may have a mechanism for switching transpeptidation off and on. Recently, an interesting study of PBP1a from *E. coli* documented the *in vitro* synthesis of cross-linked murein and also a link between the GT and transpeptidase activities of this enzyme (*18*). Thus, one possibility is that for some of the HMW bifunctional class A PBPs, the two processes (*i.e.*, synthesis of the polysaccharide strands and cross-linking of the stem peptides) are coordinated by the single enzyme in such a way that the transpeptidase activity cannot proceed without ongoing transglycosylation. However, the *in vivo* studies of Pratt *et al.* seem to imply that the proteins may simply be nonspecific.

As the cell wall assembly process becomes further defined, it is likely that new strategies for its inactivation will emerge, potentially leading to new antibacterial products. In addition, the study of these enzymes has provided, and continues to offer, rich insight into fundamental aspects of substrate recognition and enzyme mechanism. A detailed picture of how the bacterial cell coordinates the events leading to its own reproduction is now gradually being exposed.

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