STRUCTURE AND FUNCTION OF PEPTIDOGLYCANs

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ABSTRACT. Detailed knowledge of bacterial cell wall peptidoglycan structures has expanded rapidly in the past few years, and very definite patterns have emerged which can be attributed to common biosynthetic pathways, and, more speculatively, to a common structural function. The glycan is β-1,4 linked and is therefore a substituted form of chitin, probably retaining its linear conformation. The peptide may be more flexible, and varied in its cross-linking, but all contain a sequence derived from a probable common precursor, UDP-N-acetyl muramyl-(A)-D-glu-(B)-D-alal-D-alal, when (A) and (B) are amino acids with the carboxyl and α-amino groups of a center with L-configuration in the main chain. The conformation of this sequence is discussed.

As my title suggests, I shall discuss the common factors in known cell wall peptidoglycan structures, and try to rationalise these factors in terms of the function of these polymers. The first objective has become much easier of late thanks to the rapid expansion of knowledge in this field, for which we are particularly indebted to Dr. Ghuysen and his co-workers and, among the more exotic varieties, to Dr. Perkins and Dr. Kandler and his co-workers. My task is made much simpler by the excellent review recently published by Jean-Marie Ghuysen (1968).

Any common facet of cell wall peptidoglycan structures that has persisted throughout the millenia of fierce competition for survival must be essential for that survival, and be best suited to ensure it, and therefore should have a clearly discernible role in the function of this polymer. This function is, of course, the preservation of the integrity of the bacterial cell during growth and division within its relatively fragile plasmic membrane, which, in many bacteria, contains very large osmotic pressures. It is worth comparing this polymer with those that serve similar structural functions in fungi and plants. Most contain considerable amounts of β-1,4 linked polysaccharides, stable linear molecules which are ideally suited as backbone material for the weaving of structural meshworks. These polymers are, of course, cellulose and chitin, while the glycan of peptidoglycan is itself a substituted form of chitin, as I will demonstrate.

Pure chitin packs into a semi-crystalline material that is apparently unsuitable for cell wall function, so that chitin is always found in a fibrillar complex, embedded in a ground substance, containing large amounts of protein where flexibility is required (Rudall 1967). This relationship
with protein is formalized in peptidoglycan by the ether linkage to D-lactyl peptide. Figure 1 is a representation of peptidoglycan in the preferred conformation of α-cellulose and chitin (c.f. Carlström 1957). In both, hydrogen bonds between C2-OH and the C'5 ring oxygen of the adjacent sugar residue stabilizes a flat linear chain as shown. In chitin, planar acetamido groups lie in a plane perpendicular to the plane of the diagram, and hydrogen bond with each other to form a sheet of chains, one on top of another. Note that the presence of 3-O-D-lactyl groups, on alternating residues in the glycan of cell wall peptidoglycan, would halve the number of C3-OH to C',-O interchain hydrogen bonds. However, they may provide the opportunity for others, for example between the lactyl carbonyl group and the adjacent GlcNAc C'4 primary hydroxyl groups, as illustrated. This hydrogen bond may only be possible with a lactyl moiety having the D-configuration. The conformation of the glycan backbone may well be similar to that shown here, and if so, the exigencies of spatial packing between N-acetylmuramyl acetamido groups and N-acetylglucosaminyl primary hydroxyl groups would tend to line up the lactylpeptide side chains in a precise fashion. In this conformation, the C6 hydroxyls of N-acetylmuramic acid residues are relatively unhindered, and would be available for acetylation (as in Staphylococcus aureus, Tipper et al. 1965) or phosphodiester linkage to other polymers, as has been demonstrated in Lactobacillus casei (Knox and Hall 1965; Knox and Holmwood 1968), Streptococcus pyogenes (Munoz et al. 1967) and Staphylococcus lactis (Button et al. 1966; Archibald et al. 1968).

I would like briefly to review the evidence for the β-1, 4 linkages in the glycan, since some fairly recent data has yet to be published. This evidence is only complete for one organism, S. aureus. Appropriate enzymatic hydrolysis of the cell walls of this organism gives the two possible disaccharides, 4-O-β-N-acetylg glucosaminyl-N-acetylmuramic acid (GM) and 4-O-β-N-acetylmuramyl-N-acetylglucosamine (MG), both in high yield (Tipper et al. 1965; Tipper and Strominger 1966). The non-identity of GM from Micrococcus lysodeikticus with synthetic 1, 6 linked disaccharide was demonstrated by Jeanloz et al. (1963), and 1, 4 linkage in both disaccharides from S. aureus was deduced from periodate oxidation data (Tipper et al. 1965; Tipper and Strominger 1966), and from their reactions in alkali, as exemplified by their slow conversion to Morgan-Elson chromogen, dependent on hydrolysis of their glycosidic linkage (Tipper 1968). β-linkage in GM is demonstrated by susceptibility to the specific β-N-acetyl-D-glucosaminidase of hog epididymis (Ghuyes and Strominger 1963), and β-linkage in MG is demonstrated by NMR spectroscopy as illustrated in Figure 2 (Tipper, Tomoeda and Strominger, unpublished observations), which is a 100 megacycles spectrum of MG in D2O. The doublet at 8.75 T (J = 6.5 cps) is derived from the three protons on the lactate methyl group, the peak at 8.01 T is due to the six protons of the two acetamido carboxymethyl groups, and the broader peak at 5.37 T is due to HDO with its two side bands at 5.07 and 5.67 T. The single proton doublet at 5.83 T (J = 6.5 cps) is due to the C'5 proton on the lactate group, since, as shown in the upper trace, irradiation at the frequency of the lactate methyl group (8.75 T) decouples this doublet. Apart from the ring protons in the region from 6.0 T to 6.75 T, this leaves only the doublets at 6.88 and 5.48 T to be assigned. It has been shown that C1 protons in α-D-glucosides resonate in the region near 4.8 T with a J of 3 cps; while in β-D-glucosides they resonate at higher field,
A POSSIBLE CONFORMATION FOR TWO PEPTIDOGLYCAN SUBUNITS

\[ M = N\text{-ACETYL MURAMYL} \]
\[ G = N\text{-ACETYL GLUCOSAMINYL} \]

\[ \text{CARBON} \]
\[ \text{OXYGEN} \]
\[ \text{ACETAMIDO} \]

FIGURE 1.

near 5.5 \( \tau \) with a \( J \) of 7 cps (van der Veen 1963). \( N\text{-acetyl-D-glucosamine} \) exists in aqueous solution predominantly as the \( \alpha \)-anomer (van der Veen 1963), and was found to give a doublet at 4.8 \( \tau \) (\( J = 3 \) cps), which was not present in \( N\text{-acetyl-D-glucosaminitol} \). Similarly, the doublet at 4.88 \( \tau \) (\( J = 3 \) cps) in MG disappeared on reduction, and is therefore due to the \( C_1 \) proton of its reducing \( N\text{-acetyl-\( \alpha \)-D glucosamine moiety} \). On the other hand, the single proton doublet at 5.48 \( \tau \) (\( J = 7 \) cps) is present in both reduced and unreduced MG, and is therefore due to the \( C_1 \) proton of its \( \beta \) glycosidic linkage. Similar properties in the disaccharides isolated from \textit{M. lysodeikticus} (Leyh-Bouille et al. 1966) and several other Gram-positive organisms indicate that all have the same glycan, as does common susceptibility to a variety of \( N\text{-acetylmuramidases} \) such as lysosyme and the endo-\( N\text{-acetylglucosaminidase} \) of lysostaphin (Browder et al. 1963; Tipper and Strominger 1966; Hungerer et al. 1969).

The biosynthetic scheme for cell wall peptidoglycan that has been elaborated from work in a few bacteria, notably \textit{S. aureus}, \textit{M. lysodeikticus}
Figure 2.
The process can be divided into several stages: stage (1), synthesis of the UDP-MurNac-pentapeptide appropriate to the final peptidoglycan product. These reactions involve soluble substrates, enzymes and products in the cell cytoplasm; stage (2), transfer of P-MurNac-pentapeptide to a phosphorylated C_{55}-polyisoprenoid alcohol (Higashi et al. 1967) and conversion of it to the completed subunit of the peptidoglycan. This
involves addition of N-acetylglucosamine (completing the subunit of the glycan), of cross-bridge and side chain amino acids where these occur, of amide ammonia and O-acetyl groups where these occur, and other modifications such as hydroxylation of glut, lys or DAP where these occur (c.f. Perkins 1965; Schleifer et al. 1967). These events involve soluble substrates, some of which are fairly large, like aminoacyl tRNA's, but membrane-bound enzymes and products, and presumably occur at the inner surface of the membrane. The products are then transported through this membrane and polymerised in stage (3), in which the acceptor is most probably the non-reducing N-acetylglucosaminyl end group of the growing glycan, which is topologically outside the plasma membrane. Pre-synthesis of lipid-bound GM disaccharides ensures the alternating sequence of sugars in the glycan. Finally, peptide cross-linking is introduced by transpeptidation (stage (4)) and the product is subjected to controlled hydrolysis by autolytic enzymes (stage (5)), converting it into the state in which it is isolated. The transpeptidation mechanism of peptide cross-link formation (Wise and Park 1965; Tipper and Strominger 1965) is probably common to all types of peptidoglycan, since all known cross-links have, at their C-terminus, a single D-alanine residue on a tetrapeptide linked to N-acetylmuramic acid. This cross-link is produced by attack by the amino group of the acceptor amino acid on the D-alanyl-D-alanine peptide linkage, with elimination of the terminal D-alanine residue and formation of a new peptide linkage, and this is the reaction inhibited by penicillins and cephalosporins (Wise and Park 1965; Tipper and Strominger 1965; Izaki et al. 1966; Araki et al. 1966). The presence of D-alanyl-D-alanine groups (whose synthesis is specifically inhibited by D-cycloserine) in the precursor of all peptidoglycans would be required by the common mechanism of cross-link formation, and accounts for the common susceptibility to D-cycloserine.

Transpeptidation does not involve large changes in free energy, and is a reversible process that never goes to completion. My own investigations of peptide chain lengths indicate cross-link formations to be a random process, about 75% efficient in staphylococci (Tipper and Berman 1969), while it is only 55-65% efficient in several other organisms such as B. subtilis (Warth 1968), B. sphaericus (Hungerer and Tipper 1969), L. casei (Hungerer et al. 1969), and A. crystallopoietes (Kulwich et al. 1967b). Efficiency is about 80% in Proteus mirabilis (Martin 1967), while in E. coli it is only 35% efficient, and only dimers are formed (cf. Weidel and Pelzer 1964). The residual noncross-linked C-termini retain D-alanyl-D-alanine groups in S. aureus (Tipper and Strominger 1965), but many organisms contain one or several D-alanine carboxypeptidases which convert these C-terminal groups to single D-alanine residues, or even expose the next amino acid (cf. Izaki and Strominger 1968). The latter is especially true of bacilli, in which an enzyme that removes D-alanyl-D-alanine as a unit may exist (Egan 1969).

Peptide of the Peptidoglycan

The variety of amino acid compositions in cell wall peptidoglycans has been exemplified by the pioneering work of Cummins and Harris (1956) and Elizabeth Work (e.g. Hoare and Work 1957), and has been reviewed by Martin (1967). Figure 4 shows the most common type of cell wall peptidoglycan peptide. It contains the components of the peptide L-alanyl-D-isoglutamyl-meso-diaminopimelyl-D-alanine. The exact structure, as
Peptidoglycan of E. coli, B. megaterium and B. subtilis Vegetative Cell Walls

<table>
<thead>
<tr>
<th>Organism</th>
<th>R₁</th>
<th>R₂</th>
<th>C-Terminus</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>OH</td>
<td>OH</td>
<td>D-Ala</td>
</tr>
<tr>
<td>B. subtilis veg. cell wall</td>
<td>OH</td>
<td>NH₂</td>
<td>DAP</td>
</tr>
<tr>
<td>B. subtilis spore cortex</td>
<td>OH</td>
<td>OH</td>
<td>D-Ala</td>
</tr>
<tr>
<td>C. diphtheriae</td>
<td>NH₂</td>
<td>NH₂</td>
<td>D-Ala</td>
</tr>
</tbody>
</table>

Illustrated here, is known in only two organisms, E. coli (van Heijenoort 1969) and B. subtilis (Warth 1968), but many parts of it are known in a variety of other organisms. Its constituents are common to all Gram-negative organisms, most Gram-positive spore-forming bacilli and clostridia, and is found in several other common Gram-positive species, and this precise structure may be common to all of them. Note that both the α-NH₂ and COOH groups at the end of meso-DAP with the L-configuration are involved in the main peptide chain. Cross-linking is to the amino group at the D-end, and direct cross-linking as shown here is the only presently known type. The cross-links in the LL-DAP-containing peptidoglycan of Streptomyces albus have recently been shown to be D-alanyl-glycyl-LL-DAP (M. Leyh-Bouille and R. Bonaly, personal communication). Amidation of the D-glu-α-COOH and meso-DAP-D-COOH is variable (Warth 1968; Kato and Strominger 1968) and species variation in D-alanyl carboxypeptidases must account for the variable nature of the C-terminal amino acid (Figure 4).

Figure 5 shows the second most common type of peptide, containing the sequence Nα-(L-alanyl-D-isoglutamyl)-L-lysyl-D-alanine. This sequence has been characterised in all the organisms listed here, and also in M. lysodeikticus (cf. Ghuysen 1968). In all of these organisms, cross-linking is between D-alanine and the ε-amino group of L-lysine. This
linkage is direct in M. lysodeikticus (see below), but in all other organisms involves at least one intervening amino acid in a cross-bridge (cf. Ghuysen 1968). The cross-bridge amino acids, usually activated as their tRNA derivatives, are added sequentially to the ε-amino group of lysine in the lipid intermediate (Matsuhashi et al. 1967). An exception is D-aspartic acid, which is activated as D-aspartyl-β-phosphate (Staudenbauer 1968) and is β-linked to the lysine residue in Streptococcus faecium (Ghuysen et al. 1967), L. casei (Hungerer et al. 1969) and Bacillus sphaericus (Hungerer and Tipper 1969). Note that all three of these diverse Gram-positive organisms have exactly the same D-isoasparaginyl cross-link and differ only in glutamic amidation and in their C-termini, which are lysine in B. sphaericus.

Only in these two most common types of peptides, in which either L-lysine or LL- or meso-DAP occupies the third position from the muramic acid-linked N-terminus, is cross-linking at this position known to be normal. In all other cases (with the exception of N°-D-alanyl-diglycyl-L-ornithine cross-links in Micrococcus radiodurans, M. Lache, personal communication), cross-linking probably involves the α-COOH of D-glutamic acid. In all, however, as previously mentioned, cross-linking appears to involve a D-alanine residue in position 4 at the other end.

Figure 6 illustrates the structure of M. lysodeikticus peptidoglycan.
Micrococcus lysodeikticus (S. lutea; M. flavus; M. citreus)

FIGURE 6.
In this and related organisms (Campbell et al. 1969), transpeptidation and autolysis are integrated, and N-terminal alanine resulting from N-acetylmuramyl-L-alanine amidase action is probably an acceptor for peptidyl D-alanine, as suggested by Schleifer and Kandler (1967), resulting in the production of "head to tail" linked tetrapeptide subunits, as demonstrated by the structural work of Ghuysen et al. (1968). This structure is also unique in containing direct $N^\alpha$-(D-$\alpha$-alanyl)-lysine cross-links and in being substituted on its D-glutamic acid groups by glycine residues (Tipper and Strominger 1965; Tipper et al. 1967b). These residues are added, as in the synthesis of the nucleotide pentapeptide, in a reaction directly coupled to ATP hydrolysis (Katz et al. 1967). Amidase action also results in the formation of extensive segments of glycan (up to 60%) unsubstituted by peptide, though the original 1:1 muramic to glutamic ratio remains unchanged.

Figure 7 illustrates the structure of Clostridium poinsettiae (Perkins 1967) and Butyribacterium rettgeri (Miller et al. 1966, 1968; Guinand et al. 1968), two of the less common types of peptidoglycan, which have chiefly been investigated by Drs. Perkins and Kandler. In both, the third residue is an $L^-\alpha$-amino acid, and cross-links are formed by di-basic amino acids of the D-configuration between D-alanine and the $\gamma$-COOH of glutamic acid, with their $\alpha$-amino group linked to D-alanine. Most probably these cross-links are derived synthetically, as is the glycine in M. lysodeikticus (Katz et al. 1967), by addition to the $\alpha$-COOH of glutamic acid in the lipid intermediate in a reaction directly linked to ATP hydrolysis. The residual free amino group would subsequently be the acceptor for transpeptidation in the usual fashion. Two variants in the first amino acid, and several in the third are known, and Figure 8 shows the structure of peptidoglycan which Clostridium insidiosum would have if it followed the same pattern, as indicated by recently published results of Dr. Perkins (1968).

Figure 9 illustrates the structure which is common to all known peptidoglycans, and is compatible with all known data on others: Note that there is a strict DLDLD sequence, starting with D-lactate, and that the glutamyl linkage in this chain is always $\gamma$, giving a flexibility that might allow the chain to double back in itself. All the other peptide links in the chain, particularly those linking the third amino acid, are $\alpha$. In the $L^-L$-DAP-containing cell walls of Streptomyces and Clostridium welchii, one $L^-L$-DAP amino group is acylated by a $\gamma$-$D$-isoglutaminyl residue and the carboxyl group at the same end of the DAP molecule acylates D-alanine (J. M. Ghuysen, personal communication). This kind of repeating structure is reminiscent of some of the peptide antibiotics like the Enniatins and the Gramicidins (cf. Lardy et al. 1967), which suggests that they may have some properties in common. The most striking property of these antibiotics is the stability of their folded conformations, that in some cases forms an octahedral array of ligands that are ideally situated to form a hexadentate coordination shell around an unhydrated alkali metal ion (cf. Lardy et al. 1967). It seems possible that appropriate folding of the common peptidoglycan peptide sequence could result in a preferred conformation, perhaps stabilised by metal chelation, which could be important in several ways:

(a) The conformation could be that required for binding to the active sites of enzymes such as peptidoglycan synthetase, transpeptidase, carboxypeptidases and enzymes that add ammonia and cross-bridge amino acids.
In particular, some such fixed conformation might greatly facilitate the interaction of two large substrate peptides in transpeptidation, when these peptides are already linked to a relatively inflexible polymeric network.

(b) Chelation might increase the hydrophobic nature of the peptide, aiding its membrane transport.

(c) Finally, such a fixed peptide conformation, in concert with that of the glycan, would conceivably be important for the structural function of the peptidoglycan, since the combined effects could fix the network in an expanded yet fairly rigid configuration that might be economical in material and leave gaps for insertion of other cell wall polymers and diffusion of nutrients, etc.

I would therefore like to suggest that this sequence of glycan and peptide is present in all cell wall peptidoglycans because they determine a particular conformation that is functionally important. One might expect such a conformation to result in a sufficient uniformity of spacing to yield an X-ray small angle scattering pattern, but this does not seem to be the case. Possibly this is because the semi-crystalline domains are very small, because of average peptide chain lengths of 3-4 units and glycan
chains of 6-12 disaccharides (Tipper et al. 1967a; Krulwich et al. 1967a; Hungerer et al. 1969; Tipper 1969; Warth 1968). Fragments of *S. aureus* peptidoglycan are presently being tested for ability to interfere with ion transport in mitochondria in the laboratory of Dr. Henry Lardy. Note that if this proposition is correct, all D-amino acids other than the single common residues of D-glu and D-alal must be involved in cross-bridges or side chains and this would, for example, apply to DD-DAP which has been detected in several organisms (Hoare and Work 1957).

Addendum

Studies in the laboratory of Dr. H. Lardy showed that disaccharide decapéptide monomer and dimer, isolated from cell walls of *S. aureus* Copenhagen after hydrolysis with Chalaropsis B enzyme, at $5 \times 10^{-5}$ M, inhibited by 50 to 85% the respiration of mitochondria induced by $2 \times 10^{-7}$ M valinomycin. This respiration is linked to transport of potassium ions into the mitochondria, but this influx was not markedly affected by the cell wall fractions. Peptide-free disaccharide was without effect on either parameter. The mechanism of the inhibitory action of these fractions is unknown, but these studies do not indicate that they specifically chelate alkali metal ions. A more promising proposal for the conformation of *S. aureus* peptidoglycan has been made recently by H. J. Rogers and coworkers (personal communication), who reported that it can assume a $\beta$-pleated sheet conformation with maximal hydrogen bonding, but only by virtue of the $\gamma$-D-glutamyl linkage. If such a sheet conformation can be generalised, its stabilisation by hydrogen bonding would still have the potential benefits listed under (a) and (c) above.

Several previously undescribed peptide sequences have recently been described by O. Kandler and coworkers (see this issue, also personal communication), but they all fit the pattern given in Figure 9. They include structures with N$^\gamma$(γ-D or γ-L-glutamyl) lysine linkages, presumably synthesised via the $\gamma$ phosphate derivatives, as in the case of the $\beta$-D-aspartyl residues already described. *Mycobacteria* (Petit et al. 1969, Biochem. Biophys. Res. Commun. 35:478) and Nocardia (M. Guinand, personal communication) have been found to contain N-glycolyl muramic acid rather than the N-acetyl derivatives, and spore cortex peptidoglycans have been shown to contain about 50% muramic lactam,
FIGURE 9.
nonrandomly distributed in long glycan chains (A. D. Warth, 1969, Fed. Proc. 28:657). The lactyl moieties in muramic lactam can no longer be involved in the type of hydrogen bonding shown in Figure 1, and so the presence of muramic lactam may well result in a much more flexible glycan, more suited to the possible contractile function of cortex. It has also been found that S. albus produces a D, D-carboxypeptidase which, like the parent organism, is insensitive to penicillins. This suggests that the conformation of acyl-D-alanyl-D-alanine accepted by this carboxypeptidase and the transpeptidase in this organism is not that mimicked by penicillins (M. Leyh-Bouille et al., 1970, Biochemistry, in press).

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