Peptidoglycan Biosynthesis by Preparations from *Bacillus licheniformis*: Cross-Linking of Newly Synthesized Chains to Preformed Cell Wall

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A wall-plus-membrane preparation from a *Bacillus licheniformis* mutant incorporated radioactivity from a peptidoglycan precursor in which the free amino group of diamino-pimelic acid was blocked by ^{14}C -labelled acetyl group. This incorporation was penicillin-sensitive. The enzymically degraded product contained cross-linked dimers, showing that newly synthesized peptidoglycan chains had been cross-linked to the pre-existing cell wall.

Incorporation of radioactive precursors into peptidoglycan by preparations of wall plus membrane from Gram-positive bacteria has been shown to be penicillin-sensitive (Mirelman & Sharon, 1972; Mirelman *et al.*, 1972; Ward, 1974). The product, identified as peptidoglycan, was a cell-wall fraction that had been extracted with 2% (w/v) sodium dodecyl sulphate and the conclusion was drawn that incorporation must be occurring by the action of a transpeptidase causing the formation of peptide cross-links. These cross-links would be formed by transfer of the subterminal D-alanine residue of a peptide side chain (L-Ala-D-isoGlu-meso-A₂pm*-D-Ala-D-Ala) to the amino group at the D centre of meso-diaminopimelic acid in another side chain, and concomitant loss of the terminal D-alanine residue. Simple transpeptidation of non-polymerized nucleotide-peptide precursors did not occur, since the process was dependent on the presence of UDP-N-acetylglucosamine. The results did not show, however, whether the cross-links were formed between separate strands of newly synthesized peptidoglycan or between new chains on the one hand and old chains on the other. It is not possible to answer this question by the simple use of radioactive precursors, since one cannot distinguish between a mixed population of cross-linked dimers some of which are doubly labelled and some unlabelled and another population with some singly labelled and some unlabelled. In the present experiments the precursor used was UDP-N-acetylmuramyl-L-alanyl-D-isoglutamyl-(D)-[^{14}C -acetyl]acetyl-meso-diaminopimelyl-D-alanyl-D-alanine (acetylated precursor), in which the possible acceptor site for cross-linking, namely the D centre of the meso-diaminopimelic acid, was blocked by an acetyl group. In this way cross-links could not be formed between separate strands of newly synthesized peptidoglycan and incorporation of radioactivity into cell-wall material would be indicative of the trans-

peptidation of new chains to acceptor sites on pre-existing peptidoglycan.

Experimental

The organism used was a penicillinase-negative mutant (Ward, 1974) obtained from one of the poorly lytic mutants, Lyt-3, previously derived from *Bacillus licheniformis* 6346 (Forsberg & Rogers, 1971). From this organism fractions consisting of membrane or wall plus membrane were prepared and used for the biosynthesis of peptidoglycan as described earlier (Ward & Perkins, 1973; also, Ward, 1974).

The radioactively labelled acetylated precursor was prepared as follows. To UDP-MurAc-L-Ala-D-isoGlu-meso-A₂pm-D-Ala-D-Ala (2 μmol in 125 μl of water), purified by column and paper chromatography (Ward, 1974), were added dioxan (450 μl), triethylamine (10 μl) and a solution in benzene of [^{14}C]acetic anhydride (The Radiochemical Centre, Amersham, Bucks., U.K.) (50 μCi , 2.5 μmol in 25 μl). The mixture was kept on ice, with intermittent shaking, for 2 h and then left at 4°C overnight. The pH value remained about 9. The sample was dried on a rotary evaporator and then 0.1 M-acetic acid (0.3 ml) was added and the sample dried (three times in all). The whole acetylation procedure was then repeated and the product was purified by chromatography on a column of Sephadex G-25. To ensure that all the amino groups of meso-diaminopimelic acid had been blocked the final material was again acetylated, this time with an excess (20 μmol) of labelled acetic anhydride. The final radioactive product (sp. radioactivity 6.8 $\mu\text{Ci}/\mu\text{mol}$) ran as a single anionic band on high-voltage electrophoresis at pH 3.5, its mobility being 1.04 times that of the untreated precursor. It gave no reaction with ninhydrin.

The absence of free amino groups was checked as follows. A sample of the final product and an equimolar sample of unacetylated precursor were dinitro-

*Abbreviations: A₂pm, diaminopimelic acid; MurAc, N-acetylmuramic acid.

phenylated with 1-fluoro-2,4-dinitro[3,5-³H]benzene (The Radiochemical Centre) at 20 μ Ci/ μ mol, in the presence of ethanol-1% NaHCO₃ (3:1, v/v). After 4h at room temperature excess of the reagent was extracted with ether, first from alkaline and then from acidified solution. The product was then subjected to high-voltage electrophoresis at pH 3.5 as described. The band from the acetylated precursor corresponding to the tritiated dinitrophenyl derivative of the parent precursor was cut out, eluted and its radioactivity measured. It contained hardly any ³H radioactivity (0.6% of that in the control), so that in the acetylated precursor certainly less than 1% of the diaminopimelic acid residues had free amino groups.

To confirm that the radioactivity was associated with pentapeptide precursor with its D-Ala-D-Ala terminus intact, the acetylated precursor (2.8 nmol) was mixed with vancomycin (0.11 μ mol) and subjected to paper electrophoresis at pH 7. All the radioactivity was associated with vancomycin complex and none ran in the position of the untreated acetylated precursor, thus showing that D-Ala-D-Ala termini were present (Perkins, 1969).

Results

The wall-plus-membrane preparation from a mutant of *B. licheniformis* was used to demonstrate the incorporation of radioactively labelled peptidoglycan precursors into cell-wall material (Table 1). Acetylated precursor was utilized less efficiently than the natural compound, only about 23% as much being incorporated under identical conditions. Nevertheless, the sensitivity to penicillin of the incorporation of each precursor was almost identical, 1 μ g of benzylpenicillin/ml causing about 80% inhibition in one experiment (Expt. 1) and greater than 95% in another (Expt. 2). Such variation was no doubt due to the proportion of membrane present in a particular preparation. The incorporation of both precursors was almost entirely dependent on the presence of UDP-*N*-acetylglucosamine. This result virtually ruled out the possibility that ¹⁴C-labelled acetyl residues were being removed and incorporated as such by some process irrelevant to peptidoglycan synthesis, but in any case an additional experiment showed that incorporation of radioactivity from ¹⁴C-acetylated precursor took place almost equally well when acetate was present in the

Table 1. *Peptidoglycan biosynthesis by cell-free preparations from Bacillus licheniformis*

Incubation mixtures contained 0.56 mM-UDP-MurAc-pentapeptide labelled with either diamino[³H]pimelic acid (34.5 mCi/mmol) or [¹⁴C]acetyldiaminopimelic acid (6.75 mCi/mmol), 0.67 mM-UDP-GlcNAc, enzyme prepared in 0.05 M-Tris-HCl buffer, pH 7.7 (100 μ l), and antibiotic to give the final concentrations indicated. Biosynthetic activity in the presence of acetate buffer was determined by using a preparation of wall plus membrane that had been twice washed with 0.05 M-Tris-acetate buffer, pH 7.7, and resuspended in the same buffer. Incorporation into cell-wall-free membrane preparations was carried out as described previously (Ward & Perkins, 1973). Mixtures were incubated at 24°C for 30 min. Incorporation of radioactivity into cell-wall material was determined after extraction of the pellet with sodium dodecyl sulphate (2%; 3 $\times$ 2.0 ml) and washing with NaCl (1 M; 2 $\times$ 2.0 ml) and water (3 $\times$ 2.0 ml) (Ward, 1974). Incorporation of UDP-MurAc-pentapeptide in the complete wall-plus-membrane system was 4.56 nmol/h per mg of protein when diamino-[³H]pimelic acid-labelled precursor was used and 1.06 nmol/h per mg of protein for the acetylated precursor. The cell-wall-free membrane preparations incorporated 8.65 nmol of acetylated precursor/h per mg of protein. Values in the Table represent the percentage of the radioactivity found in the respective complete system.

	System	Peptidoglycan biosynthesis	
		[³ H]A ₂ pm-labelled	[¹⁴ C]Acetyl-A ₂ pm-labelled
Incorporation into a cell-wall fraction	UDP-MurAc-pentapeptide* ...		
	Complete	100	100
	-UDP-GlcNAc	6.5	6.8
	+Benzylpenicillin		
	Expt. 1. 0.5 μ g/ml	20.5	23.9
	1.0 μ g/ml	17.4	23.1
	Expt. 2. 0.5 μ g/ml	—	24.9
	1.0 μ g/ml	4.5	3.5
Incorporation into a membrane fraction	+Acetate buffer	—	83.0
	Complete		100
	+Benzylpenicillin		
	1.0 μ g/ml		101.7
	5.0 μ g/ml		93.1

* UDP-MurAc-pentapeptide was either UDP-*N*-acetylmuramyl-L-Ala-D-isoGlu-*meso*-A₂pm-D-Ala-D-Ala (³H-labelled in diaminopimelic acid) or the same compound that had been fully acetylated at the D-amino group of diaminopimelic acid (¹⁴C-labelled in the acetyl group).

buffer system (Table 1). Acetylated precursor was also incorporated by a wall-free membrane preparation, but in this case as expected the reaction was insensitive to penicillin.

The results so far implied that incorporation was occurring by the penicillin-sensitive transpeptidation of newly synthesized peptidoglycan chains to pre-existing cell wall, since the only possible acceptor site on the modified precursor was blocked by acetylation. To confirm this idea, the radioactive cell-wall preparation was degraded with a muramidase from *Streptomyces griseus* (Ward & Perkins, 1968) to yield a mixture of bis-disaccharide heptapeptide (dimer), disaccharide tripeptide and disaccharide tetrapeptide (monomers), with and without amidation (Ward & Perkins, 1973; Ward, 1974). The degradation product was applied to washed paper (Whatman no. 3) and chromatographed in freshly prepared isobutyric acid–0.5M-NH₃ (5:3, v/v). The radioactivity incorporated from ¹⁴C-labelled acetyl precursor was found in two distinct bands, 21.4% of the total being recovered from the region occupied

by dimer and 73.2% from that occupied by monomers. The samples eluted from the paper were then chromatographed separately on a column (70cm×2cm) of Sephadex G-25 (Hughes, 1968). The radioactive dimer was eluted in exactly the same position as the peak of ninhydrin-positive material that represented the unlabelled dimer derived from the peptidoglycan of the preparation itself, whereas as expected the radioactive monomer was eluted later. The radioactive monomer was further characterized by comparison with synthetic samples. For this purpose monomer samples prepared from unlabelled walls were acetylated with [¹⁴C]acetic anhydride as described for the acetylated precursor. They were then subjected to paper electrophoresis at pH 6.5 with the result shown in Fig. 1. The monomer fraction from the biosynthesized material separated into two bands. One, moving further towards the anode and representing 81.7% of the total radioactivity present as monomer, corresponded exactly to authentic acetylated disaccharide tetrapeptide and the other (the remaining 18.3%) corresponded exactly to disaccharide tetrapeptide amide. Evidently the enzyme preparation used was capable of catalysing the amidation, although no special attempt was made to provide the wherewithal to procure maximum substitution of the carboxyl groups, namely NH₄⁺ ions and ATP (Siewert & Strominger, 1968). The monomer products may or may not have retained the C-terminal alanine residues; the electrophoretic system used would have been unlikely to produce any great separation of these molecules. For a peptide of comparable molecular weight the loss of an alanine residue should increase the mobility by only about 5% (Offord, 1966).

Discussion

The results described above provide for the first time clear evidence that in the biosynthesis of peptidoglycan by a cell-free system newly synthesized glycan chains can be cross-linked by transpeptidation to older chains already in the wall. In these particular experiments, of those peptide chains incorporated about one in every five was so cross-linked, whereas in the cell walls of *B. licheniformis* grown under laboratory conditions the value is greater than one chain in two (Ward, 1974). However, the present precursor was modified by acetylation, which may have influenced the degree of cross-linking achieved. For instance, with the natural precursor many cross-links might well have been formed between the new chains themselves, linkages precluded by the use of acetylated precursor. The same could be true in growing bacteria. The biosynthesis of peptidoglycan from natural precursor by a similar preparation resulted in 39% of the labelled side chains being found in cross-linked dimers (Ward,

Fig. 1. Separation of acetylated monomers by electrophoresis

The acetylated-monomer peak obtained by chromatography on Sephadex G-25 as described in the text was subjected to paper electrophoresis at pH 6.5 (pyridine-acetic acid–water, 25:1:474, by vol.; 70V/cm, 30min). Bands (1cm) were cut from the paper and their radioactivities were measured in a scintillation counter (a). Authentic [¹⁴C]acetylated disaccharide tetrapeptide amide (A) and [¹⁴C]acetylated disaccharide tetrapeptide (B) were prepared as described and subjected to paper electrophoresis at the same time and their positions located by scanning the paper in a Packard Radiochromatogram Scanner (b). The parent disaccharide peptides gave the expected analysis on an automatic amino acid analyser for amino acids and amino sugars after hydrolysis in 4M-HCl at 100°C for 4h.

1974). This value would correspond to cross-linking of one in five of the new peptide chains if links were formed solely between new glycan chains, or more than one in three if all links were between new and old peptidoglycan. For the reasons described above these two possibilities cannot be differentiated.

It could be argued that the cross-linking of new chains to old was entirely dependent on the use of the acetylated analogue of the natural precursor, but it seems much more likely that the same enzyme system would function as far as possible in the same way in both cases.

The cross-linking of newly synthesized peptidoglycan chains to pre-existing wall would be of considerable importance during bacterial growth. Necessary changes in the shape of the peptidoglycan network could occur through the action of lytic enzymes, followed by 'invisible mending' with newly synthesized chains cross-linked to the old wall at appropriate points. By such means the retaining

network around the cell could be changed during growth without at any time having to lose its strength or overall continuity.

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