DEOXYCHOLATE ENHANCEMENT OF AN INTERMEDIATE OF PEPTIDOGLYCAN SYNTHESIS IN *MICROCOCCUS LUTEUS*

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1. Introduction

The synthesis of peptidoglycan by wall plus membrane preparations [1] is known to yield polymer soluble in cold aqueous sodium dodecylsulphate (SDS) and also a residue, presumably of material crosslinked to the pre-existing wall, that cannot be extracted even by hot aqueous SDS [2]. The cold SDS-soluble fraction is thought to consist of uncrosslinked peptidoglycan chains that have not been attached to the preformed wall by transpeptidation, probably because of partial disruption of the wall/membrane interface [2]. In the presence of penicillin, synthesis of crosslinked material decreased and a concomitant increase in the SDS-soluble fraction occurred [2,3]. In an attempt to dissociate some of the reactions involved in peptidoglycan synthesis deoxycholate (DOC) was added to a wall plus membrane preparation synthesising peptidoglycan, and the reaction mixture was treated sequentially with cold and hot SDS [2] to yield 3 separate fractions: a cold 5% SDS-soluble fraction; a hot (100°C for 15 min) 1% SDS-soluble fraction; and a residual hot SDS-insoluble fraction. In the absence of DOC, the majority of incorporation was into the hot SDS-insoluble and cold SDS-soluble fractions of peptidoglycan with a very small amount into the hot SDS-soluble fraction. However, in the presence of 0.25% DOC, incorporation into the hot SDS-soluble fraction increased $\geqslant$ 15-fold and synthesis of the other fractions was almost totally inhibited. The majority of the material in the hot SDS-soluble fraction was polymeric. Pulse-chase experiments showed it to be an intermediate in peptidoglycan synthesis. It had muramic acid at its reducing end, which was linked by a mild acid-labile bond, probably to undecaprenol.

2. Materials and methods

Micrococcus luteus NCTC 2665 was grown, harvested and the wall plus membrane preparations isolated as in [1–3]. The complete reaction mixture for peptidoglycan synthesis contained (in 200 μl final vol.) 50 mM Tris/HCl (pH 7.8), 20 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 100 mM NH_4Cl, 15 mM ATP, UDP-*N*-acetyl-[^{14}C]glucosamine (UDP-[^{14}C]GlcNAc, Radiochemical Centre, Amersham, 10 nmol, 12 dpm/pmol), UDP-*N*-acetylmuramyl–L-alanyl–D-isoglutamyl–L-lysyl–D-alanyl–D-alanine (UDP-MurNAc pentapeptide, prepared as in [2,4]) (100 nmol), glycine (500 nmol) and wall plus membrane preparation (100 μl, 3–7 mg protein/ml). The suspensions were incubated at 28°C for 30 min, treated with 0.2 ml 10% (w/v) SDS and centrifuged. The SDS was precipitated from the supernatant by cooling to 0°C and removed by centrifuging. To remove unchanged precursor, the resulting supernatant was applied to a paper chromatogram and developed for 2–3 days in solvent A, isobutyric acid : 0.5 M ammonia (5:3, v/v). The material remaining on the origin, corrected for losses, was taken as the cold SDS-soluble fraction. The wall/membrane pellet from the original suspension was washed once with 0.5 ml 50 mM Tris/HCl buffer (pH 7.8) containing 20 mM $MgCl_2$ and 1 mM 2-mercaptoethanol (TMM buffer), to which 1% SDS had been added, and 4 times with TMM buffer. It was then resuspended in 0.5 ml TMM buffer containing

1% SDS, heated for 15 min at 100°C, cooled and centrifuged. The supernatant was the hot SDS-soluble fraction and the final pellet washed once in TMM buffer and twice in water (0.5 ml), the hot SDS-insoluble.

Samples for reduction were treated with KB^3H_4 (10 mCi/mmol; 0.1 M) in 12.5 mM NaOH at 4°C for 6 h [5]. After destruction of excess reagent by acidification, the reduced material was hydrolysed in 4 M HCl for 4 h at 100°C and the HCl then removed in vacuo. The hydrolysate was fractionated on a column (0.8 X 20 cm) of Dowex 50 X 2 (100–400 mesh) [5].

Radioactivity was measured in a scintillation counter (Intertechnique), areas of paper chromatograms being placed in scintillation fluid containing 2(4′-*t*-butylphenyl)-5(4″-biphenyl)-1,3,4-oxadiazole (butyl-PBD) 4 g in 1 l of toluene and aqueous samples in aqueous scintillant (butyl-PBD 6 g, toluene 1 l, Triton X-100 0.5 l, water 150 ml).

3. Results and discussion

Peptidoglycan was synthesised by a wall plus membrane preparation of *M. luteus* in the presence of concentrations of DOC up to 0.5% (w/v). Increasing concentrations of DOC inhibited incorporation into the hot SDS-insoluble and the cold SDS-soluble fractions giving complete inhibition at 0.3%, but in the hot SDS-soluble fraction incorporation rose to a maximum at 0.25% DOC and thereafter declined (fig.1a). Thus DOC appeared to disorganize the wall synthesising apparatus and to prevent either of the final products observed [2,3] from appearing. The hot SDS-soluble fraction evidently represents a distinct population of peptidoglycan that can only be freed from the wall plus membrane preparation by hot, rather than cold, SDS. Why heat is necessary for its release is not known. Perhaps there is associated protein, which requires heat for complete denaturation in SDS before the peptidoglycan can be released.

In the absence of DOC, benzylpenicillin inhibited formation of the hot SDS-insoluble peptidoglycan and increased the cold SDS-soluble material as reported [1–3] but the hot SDS-soluble fraction remained very small (fig.1b). The effects of increasing concentrations of DOC on incorporation into all 3 fractions of peptidoglycan were largely unaffected by

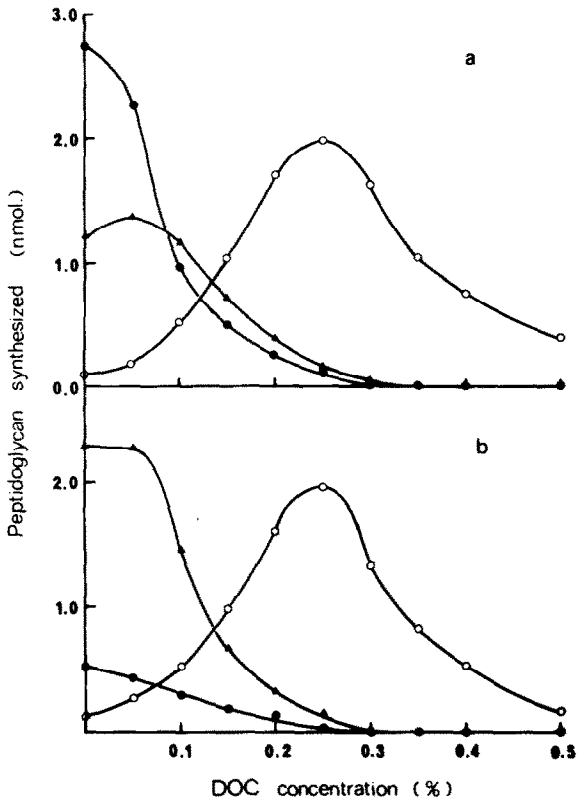

Fig.1. Effect of DOC on peptidoglycan synthesis by a wall plus membrane preparation: (a) in the absence and (b) in the presence of benzylpenicillin (10 μg/ml). The peptidoglycan was fractionated with SDS as described. The amount of [^{14}C]-GlcNAc incorporated in each assay into hot SDS-insoluble (•), cold SDS-soluble (▲) and hot SDS-soluble (○) peptidoglycan was determined.

the simultaneous presence of benzylpenicillin (10 μg/ml) (fig.1b). Penicillin is thought to act by inhibiting the transpeptidation of peptidoglycan [1] and since the drug did not affect synthesis of the hot SDS-soluble fraction, formation of this fraction is unlikely to involve transpeptidation.

To find out whether the hot SDS-soluble material was an intermediate in peptidoglycan synthesis, pulse-chase experiments were carried out. Peptidoglycan synthesised as before was pulse-labelled from UDP-[^{14}C]GlcNAc for 30 min in the presence of 0.25% DOC. 85–90% of the incorporation was into the hot SDS-soluble fraction. After centrifugation the supernatant was removed and the pellets were washed once

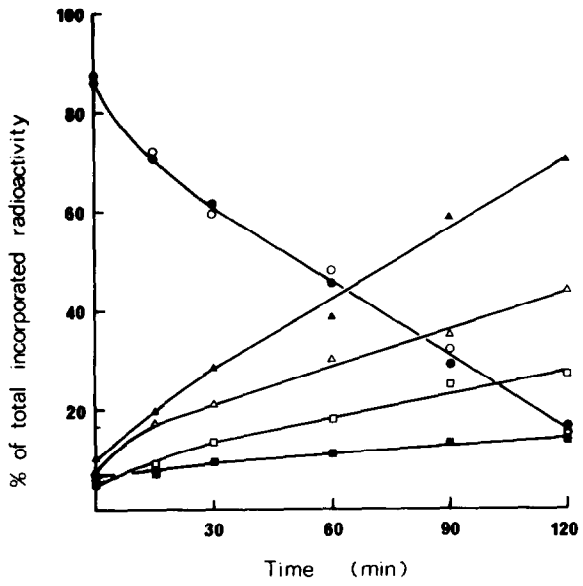

Fig.2. Pulse-chase of hot SDS-soluble peptidoglycan into cold SDS-soluble and SDS-insoluble fractions. Wall membrane preparations labelled from UDP-[^{14}C]GlcNAc in the presence of 0.25% DOC were washed as described and re-incubated in the presence of excess unlabelled nucleotide precursors with (closed symbols) and without (open symbols) benzylpenicillin (10 μg/ml). At intervals samples were fractionated with SDS and radioactivities were measured. Hot SDS-soluble fraction (○, ●). Cold SDS-soluble fraction (△, ▲). Hot SDS-insoluble fraction (□, ■).

with 1 ml TMM buffer. Each pellet was resuspended in 0.2 ml TMM buffer containing 100 mM NH_4Cl, 15 mM ATP, UDP-MurNAc-pentapeptide (200 nmol), unlabelled UDP-GlcNAc (1 μmol) and glycine (500 nmol). Incubation was continued for ⩽ 2 h, reactions being stopped by addition of 0.2 ml 10% SDS. Label originally in the hot SDS-soluble fraction appeared in both the cold SDS-soluble and the hot SDS-insoluble fractions (fig.2). In a control preparation without the initial labelling, peptidoglycan was synthesised from labelled precursors after removal of the 0.25% DOC. The ratio of incorporation into the hot SDS-insoluble and cold SDS-soluble fractions was the same in the control as in the pulse-chased preparation. Thus even after removal of the DOC, the ratio of incorporation into cold SDS-soluble relative to hot SDS-insoluble material was higher than in preparations never treated with bile-salt (fig.1). This suggests that the effect of DOC upon the orientation of the enzyme system that normally transfers the polymeric material from the membrane to the wall is only partially reversible by simple washing. The effects of benzylpenicillin added after the removal of DOC was also examined. Chase preparations (fig.2) and controls in which synthesis from new precursors was measured were affected similarly, incorporation into the cold SDS-soluble fraction being increased at the expense of the hot SDS-insoluble fraction.

If, in the absence of DOC, the small amount of hot SDS-soluble fraction was pulsed with radioactivity then this too diminished during a chase incubation. Hence we conclude that the hot SDS-soluble fraction contains an intermediate in peptidoglycan synthesis which can be linked to the wall and that the linkage process is subject, in part, to inhibition by benzylpenicillin. Experiments with *Pseudomonas aeruginosa* [6] also described a hot SDS-soluble peptidoglycan fraction that could be chased completely into an SDS-insoluble fraction, thus probably being similar to the intermediate we describe.

When the hot SDS-soluble fraction labelled in the presence of 0.25% DOC was chromatographed in solvent A, 90% remained at the origin and the remaining 10% had R_F 0.26. Similarly, 86% was excluded from a Sephadex G-50 column (3 × 30 cm) eluted with water and 14%, which was completely included, also chromatographed with R_F 0.26 in solvent A. When the material excluded by Sephadex G-50 was applied to a Sephadex G-100 column (2.2 × 55 cm) and eluted with 0.1 M NaCl, a broad peak of included material was obtained. Digestion of this material by lysozyme (100 μg/ml, 37°C, 48 h in ammonium acetate buffer 0.1 M (pH 6.5) yielded only 2 peaks of radioactivity after paper chromatography in solvent A. The major peak (R_F 0.51) corresponded in mobility to disaccharide pentapeptide GP2 [2,7], and a minor peak (R_F 0.85) to lipid intermediate, i.e., disaccharide (pentapeptide)-pyrophosphate-undecaprenol. In two experiments the ratios of radioactivity found in the lipid intermediate to total radioactivity gave values of 46 and 63 disaccharides for the length of the undegraded polymer. The average biosynthetic chainlength of hot SDS-insoluble peptidoglycan synthesised in the absence of penicillin was shown to be 66 disaccharides [2], a value which agrees well with a role for the hot SDS-soluble fraction as an intermediate in the formation of crosslinked peptidoglycan. A lower chain-

length (26–28 disaccharides) was observed for cold SDS-soluble material [2] but since this fraction is probably an artefact of the wall/membrane preparation (see section 1) differences are likely to occur. Additional factors in the present experiments include the use of DOC and the possible loss of a proportion of the undecaprenol during the fractionation procedure.

Material excluded from Sephadex G-50 as described above was reduced with KB^3H_4 and hydrolysed. A small amount of muramitol was detected on subsequent fractionation on Dowex 50. However, when the reduction step was preceded by heating with 0.1 M HCl for 2 h at 60°C [8], the amount of muramitol detected increased 9-fold. These findings imply that the muramic acid at the reducing end of the intermediate has its reducing group blocked by a mild acid-labile linkage, probably to undecaprenol.

An oligomeric intermediate in peptidoglycan synthesis was first described in *Bacillus megaterium* [9]. It was extractable with SDS at 32°C, could be chased into SDS-insoluble material and consisted mainly of an oligomer, 12 disaccharides in length. The intermediate described here is similar in many ways, but we have not as yet obtained a definitive value for the chainlength.

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References

[1] Mirelman, D., Bracha, R. and Sharon, N. (1972) Proc. Natl. Acad. Sci. USA 69, 3355–3359.
[2] Weston, A., Ward, J. B. and Perkins, H. R. (1977) J. Gen. Microbiol. 99, 171–181.
[3] Mirelman, D., Bracha, R. and Sharon, N. (1974) Ann. NY Acad. Sci. 235, 326–347.
[4] Ward, J. B. (1974) Biochem. J. 141, 227–241.
[5] Ward, J. B. (1973) Biochem. J. 133, 395–398.
[6] Mirelman, D. and Nuchamowitz, Y. (1979) Eur. J. Biochem. 94, 541–548.
[7] Mirelman, D. and Sharon, N. (1967) J. Biol. Chem. 242, 3414–3427.
[8] Ward, J. B. and Perkins, H. R. (1973) Biochem. J. 135, 721–728.
[9] Fuchs-Cleveland, E. and Gilvarg, C. (1976) Proc. Natl. Acad. Sci. USA 73, 4200–4204.