Peptidoglycan-degrading Enzymes in Ether-treated Cells of *Neisseria gonorrhoeae*

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The incubation of peptidoglycan fragments with ether-treated cells of *Neisseria gonorrhoeae* resulted in breakdown products that showed the presence of previously undescribed lytic enzymes. The properties of an endopeptidase able to hydrolyse peptide-linked bis-disaccharide peptide dimer to monomer units were examined. An *exo-N*-acetyl-glucosaminidase was also shown to release free *N*-acetylglucosamine. The breakdown pattern of glycosidically-linked dimer indicated the existence of an *endo-N*-acetylglucosaminidase. The activities of the latter enzyme and of the endopeptidase were both sensitive to β -lactam antibiotics.

INTRODUCTION

The chemical structure of *Neisseria gonorrhoeae* peptidoglycan falls into chemotype I (Schleifer & Kandler, 1972) and is very similar to that in *Escherichia coli* (Hebeler & Young, 1976*a*; Rosenthal *et al.*, 1980). However, recent work in this laboratory has shown that the peptidoglycan contains *O*-acetyl groups equivalent to about 50% of the muramic acid residues (Blundell *et al.*, 1980; Blundell & Perkins, 1981).

The turnover of peptidoglycan in *N. gonorrhoeae*, mediated by autolytic enzymes, may play a significant role in the pathogenesis of this organism. A high rate of turnover has been reported in several strains (Hebeler & Young, 1976*a*; Goodell *et al.*, 1978; Rosenthal, 1979) and it has been suggested that peptidoglycan fragments released from growing gonococci may be involved in host-cell interactions (Rosenthal, 1979). Peptidoglycan degrading enzymes may also play a role in the rearrangement of cell wall during cell growth and division and could possibly be important as sites of action of β -lactam antibiotics (Blumberg & Strominger, 1974).

Several peptidoglycan degrading enzymes from *N. gonorrhoeae* have already been described. Davis & Salton (1975) characterized a D-alanine carboxypeptidase from envelope fractions. Hebeler & Young (1976*b*) extracted an *N*-acetylmuramyl-L-alanine amidase and considered it to be the major autolysin. Rosenthal (1979) deduced the presence of amidase and 'hexaminidase' (i.e. hexosaminidase) activities from the nature of released fragments and Sinha & Rosenthal (1980) showed the 'hexaminidase' to be a transglycosylase. They also show evidence of an L,D-carboxypeptidase earlier suggested by Rosenthal *et al.* (1980).

The present study describes the activities of other enzymes found in ether-treated *N. gonorrhoeae* that are able to degrade peptidoglycan fragments.

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METHODS

Strains and ether-treatment. Two strains of *Neisseria gonorrhoeae* were used. Strain 1L260 and the procedure for ether-treatment have been described by Brown & Perkins (1979). Strain FA102 has been described by Blundell & Perkins (1981).

Preparation of substrates. Peptidoglycan fragments labelled with (DL + meso)-2,6-diamino[1,7- ^{14}C]pimelic acid dihydrochloride ($[^{14}\text{C}]\text{A}_2\text{pm}$) were obtained from *Escherichia coli* UB2851 (A_2pm^- , lys $^-$). The cells were grown to $A_{578} = 1.1$ by shaking at 37 °C in brain-heart infusion broth supplemented with 20 mg $\text{A}_2\text{pm l}^{-1}$ and 14 $\mu\text{Ci } [^{14}\text{C}]\text{A}_2\text{pm l}^{-1}$ (0.52 MBq l^{-1}). The cells were harvested, washed with saline citrate buffer, (trisodium citrate 15 mM, NaCl 150 mM, brought to pH 7 with HCl) treated with 4% (w/v) SDS (Sigma) for 1 h at 100 °C and stirred at room temperature overnight. Crude peptidoglycan was sedimented at 48000 g for 2 h and washed four times with citrate buffer to remove SDS. The pellet was resuspended in buffer with α -amylase (Sigma) (1 mg ml^{-1}) for 3 h at 37 °C followed by trypsin (Sigma) (1 mg ml^{-1}) overnight at 37 °C and re-centrifuged. The pellet was washed with buffer and treated with lysozyme (Sigma) (1 mg ml^{-1}) in the same buffer and shaken overnight at 37 °C. Undigested material was sedimented and the supernatant fractionated on a P6 (Biogel, 200–400 mesh) column (16 $\times$ 1800 mm) eluted with the same buffer. Three peaks were obtained corresponding to monomer, i.e. *N*-acetylglucosaminyl-*N*-acetylmuramylpeptide, dimer and oligomeric material. The monomer contained both disaccharide tetrapeptide (GlcNAc-MurAc-L-Ala-D-isoGlu-meso- A_2pm -D-Ala) and disaccharide tripeptide (GlcNAc-MurAc-L-Ala-D-isoGlu-meso- A_2pm) while the dimer consisted of a mixture of peptide-linked dimer, glycosidically-linked dimer and cyclic dimer (i.e. having both peptide and glycosidic links (Pelzer, 1963). For some experiments the monomer and dimer were separated into their individual components by chromatography on Whatman 3MM paper in butan-1-ol/acetic acid/water (4:1:5, upper phase) (Pelzer, 1963). Peptidoglycan fragments, with or without *O*-acetyl groups and radioactively labelled with $[^{14}\text{C}]\text{glucosamine}$ or $[^3\text{H}]\text{glucosamine}$ were prepared by J. K. Blundell from *N. gonorrhoeae* 1L260 (Blundell & Perkins, 1981). *O*-acetylated monomer and dimers were separated by TLC in isobutyric acid/1 M-ammonia (5:3, v/v) (Martin & Gmeiner, 1979).

Assay methods. Strain 1L260 was used as a source of enzymes throughout unless otherwise indicated. Reaction mixtures for enzyme assays typically contained 1 μl ether-treated cell suspension (approximately 30 μg protein) and 5–10 μl substrate (approximately 11–22 nmol). Unless otherwise indicated the final buffer at the stated pH value contained 0.04 M-Tris/maleate, 0.015 M- Mg^{2+} and 0.8 mM-2-mercaptoethanol (MMM buffer). After incubation at 37 °C for an appropriate time the mixture was acidified with 15 μl 2.27 M-acetic acid (to prevent β -elimination) and heated to 100 °C for 5 min. The suspension was centrifuged at 14000 g for 6 min; the supernatant was spotted on a paper chromatogram and developed as described for 24 or 72 h. After drying, 1 cm strips were cut and counted in non-aqueous scintillant (butyl-PBD). Efficiencies were 76% for ^{14}C and 7.8% for ^3H . The products were identified by comparison with markers of *E. coli* disaccharide tetrapeptide (C_6) and comparison of the R_{C_6} values (Primosigh *et al.*, 1961). Radioactively-labelled *N*-acetylglucosamine was identified by co-chromatography with authentic *N*-acetylglucosamine revealed by the method of Salton (1959). β -Elimination was performed in 4 M- NH_4OH at 37 °C for 6 h (Tipper, 1968). Released peptide, if present, was recognized by rechromatography: radioactivity disappeared from the original glycopeptide position and appeared as a fast-running lactyl-peptide.

RESULTS

Ether-treated cells of *N. gonorrhoeae* evidently contained endopeptidase activity since their incubation with *E. coli* peptide-linked dimer gave monomeric breakdown products identified as disaccharide tetrapeptide (C_6), disaccharide tripeptide (C_5) and *N*-acetylmuramyl tripeptide. Table 1 shows the R_{C_6} values, which were found to be reproducible providing fresh solvent was prepared.

Table 1. *Relative chromatographic mobilities of peptidoglycan components*

Paper chromatography in butanol/acetic acid/water (4:1:5, upper phase).

Fragment	R_{C_6}
Glycosidically-linked dimer	0.18
Peptide-linked dimer	0.28
Cyclic dimer	0.43
C_5 (disaccharide-tripeptide)	0.74
C_6 (disaccharide-tetrapeptide)	1.00
<i>N</i> -Acetylmuramyl tripeptide	1.12
<i>O</i> -Acetylated monomer	1.46
<i>N</i> -Acetylglucosamine	2.6

Fig. 1. Effect of temperature and pH on endopeptidase and glycosidase-X activities. (a) Effect of temperature. Samples were incubated in MMM buffer, pH 6, for 60 min. Endopeptidase activity (○), glycosidase-X activity (●). (b) Effect of pH. Endopeptidase activity, open symbols; glycosidase-X activity, closed symbols. MMM buffer (○, ●); 0.04 M-Tris/HCl (□, ■); 0.02 M-sodium carbonate buffer (△, ▲).

Initial assays to obtain the optimum conditions for endopeptidase activity were performed by using mixed dimer. In this case the cyclic dimer was converted to glycosidically-linked dimer by the action of the endopeptidase. Endopeptidase activity was measured as the percentage of total peptide links broken, i.e. in both peptide-linked and cyclic dimer, given by the loss of the sum of these two. Concomitantly some breakdown of glycosidic linkage was observed (glycosidase-X), indicated by a decrease in the sum of glycosidically-linked + cyclic dimer. The relationships may be summarized thus:

Measurement of endopeptidase activity at various temperatures showed an optimum at 37 °C although activity was maintained up to 52 °C (Fig. 1a). The pH optimum was at pH 8.0 with no significant difference between Tris/HCl and MMM buffer (Fig. 1b). No effect on activity in Tris/HCl buffer at pH 8.0 was observed by the addition of either magnesium ions (0–30 mM) or Triton X-100 (0–0.1%, v/v).

The enzymic hydrolysis at the optimum pH and temperature of peptide-linked dimer into the various products was followed with the course of time (Fig. 2). The initial major product was the monomer tetrapeptide, thus indicating that the substrate was initially in the form of the bis-disaccharide octapeptide. After 20 min, monomer tripeptide was formed at the expense of the monomer tetrapeptide indicating the loss of D-alanine through L,D carboxypeptidase activity. After 40 min an appreciable quantity of substrate was converted to a peak with an R_c corresponding to *N*-acetylmuramyltripeptide (Höltje *et al.*, 1975), thus indicating *N*-acetylglucosaminidase activity (see below). Experiments with purified monomer tetrapeptide as substrate similarly showed degradation to the monomer tripeptide and to *N*-acetylmuramyltripeptide (data not shown).

Fig. 2. Time course of breakdown of peptide-linked dimer to monomeric products. Initial dimer concentration, 1.08 mM. Buffer, 0.025 M-Tris/HCl, pH 8. Incubation at 37 °C. Total monomer (○); monomer tetrapeptide (●); monomer tripeptide (□); N-acetylmuramyltripeptide (■).

Fig. 3. Effect of various antibiotics on endopeptidase and glycosidase-X activities. Incubation was in 25 mM-Tris/HCl buffer, pH 8, at 37 °C for 60 min. Activities are given as percentage of control (67–82% of peptide links or 18–35% of glycan links broken). (a) Endopeptidase activity; substrate, peptide-linked dimer. (b) Glycosidase-X activity; substrate, glycosidically-linked dimer. Antibiotics: benzylpenicillin (△); ampicillin (▽); methicillin (□); mecillinam (■).

The K_m of the endopeptidase measured by the initial rate of the breakdown of peptide-linked dimer (0.27–2.2 mM) to monomeric products under optimum conditions was found to be 4.4 ± 2.9 mM (95% confidence limits).

The effect of four β -lactam antibiotics on endopeptidase activity was also examined (Fig. 3a). Each gave considerable inhibition but a residual activity of about 15% of the control remained. The concentrations producing 50% inhibition of endopeptidase activity were as follows: penicillin G, 3×10^{-8} M; ampicillin, 3×10^{-8} M; methicillin, 3×10^{-7} M; mecillinam, 3×10^{-6} M.

For comparison with the Liverpool isolate 1L260, strain FA102 altered at the *penA2* locus, reputedly more resistant to penicillin and with more highly cross-linked peptidoglycan (Guymon *et al.*, 1978), was also examined. Once more endopeptidase and L,D-carboxypeptidase were found with approximately similar activities (data not shown). In further experiments wherein di-*O*-acetylated dimer (Blundell & Perkins, 1981) was used as substrate, endopeptidase activity yielded *O*-acetylated monomer, suggesting either that the enzyme was unaffected by the presence of *O*-acetyl groups, or less probably that another specific enzyme was present.

Fig. 4. Effect of pH on *exo-N*-acetylglucosaminidase activity. Samples of hexosamine-labelled monomer were incubated with ether-treated cells in MMM buffer at 37 °C for 2 h. Values represent the percentage of the total radioactivity recovered as free *N*-acetylglucosamine.

Fig. 5. Possible reactions for hydrolysis of glycosidically-linked dimer.

N-Acetylglucosaminidase activity was confirmed by the release of *N*-acetyl-[^3H]glucosamine from peptidoglycan monomers labelled initially with [^3H]glucosamine, in which the label was equally distributed between the two hexosamine moieties (Blundell & Perkins, 1981). The pH optimum of this activity was around pH 7 (Fig. 4). However, some difficulty was found in accurately determining the optimum pH, as resuspending the ether-treated cells in various buffers appeared to reduce enzyme activity; at pH 6 up to 25% of the total counts could be converted to *N*-acetylglucosamine, representing 50% hydrolysis of the glycosidic link. *N*-Acetylglucosamine was released from *O*-acetylated monomer, indicating that this enzyme, too, was unaffected by the presence of the *O*-acetyl group.

The nature of the glycosidase-X referred to earlier was investigated by using isolated glycosidically-linked dimer (Fig. 5) as the substrate. Essentially the enzyme could either be a muramidase, the transglycosylase that has already been described (Sinha & Rosenthal, 1980), or an *endo-N*-acetylglucosaminidase. The muramidase would yield only monomer tetrapeptide, the transglycosylase would yield monomer tetrapeptide and the faster running anhydro monomer, and the *endo-N*-acetylglucosaminidase would yield a trisaccharide tetrapeptide and the faster

running muramyl tetrapeptide (Fig. 5). Under the experimental conditions L,D carboxypeptidase may additionally produce the corresponding tripeptides and the *exo-N*-acetylglucosaminidase just described may remove the non-reducing *N*-acetylglucosamine residues. When glycan-linked dimer was incubated with ether-treated cells two products were obtained as peaks on a paper chromatogram. To distinguish between the above three possibilities these products were then subjected to β -elimination. Of the two peaks, only the slower moving underwent β -elimination. This result, as indicated in Fig. 5, supported the conclusion that an *endo-N*-acetylglucosaminidase was responsible for cleaving the glycan links (glycosidase-X activity).

The effect of temperature on this activity is shown in Fig. 1(a). The optimum was at 37 °C but temperature sensitivity was greater than with the endopeptidase. The pH optimum was about pH 8.5 (Fig. 1b), which is higher than that found for the *exo-N*-acetylglucosaminidase (Fig. 4). The antibiotic sensitivity of the *endo-N*-acetylglucosaminidase activity closely paralleled that of the endopeptidase except that all activity disappeared at high antibiotic concentrations (Fig. 3b).

DISCUSSION

Endopeptidase activity in *N. gonorrhoeae* has been described for the first time. However, an inspection of the properties, particularly the pH optimum and the antibiotic sensitivities, suggests that it may be identical with the D-alanine-carboxypeptidase already described (Davis & Salton, 1975; Davis *et al.*, 1978). A penicillin-sensitive D,D-endopeptidase identical with a D-alanine-carboxypeptidase has been found in *E. coli* (Tamura *et al.*, 1976). Our results have also shown residual endopeptidase activity in the presence of high concentrations of β -lactam antibiotics, which may indicate that there is a second endopeptidase, insensitive to these drugs, similar to that described in *E. coli* (Tomioka & Matsushashi, 1978; Keck & Schwarz, 1979).

The role of the endopeptidase is not clear but it may be involved in controlling cross-linking in the wall, being able to split bridges already formed by a transpeptidase. Endopeptidase activity may be involved in causing the large reduction in cross-linking in released soluble peptidoglycan fragments compared to native insoluble peptidoglycan in *N. gonorrhoeae* (Sinha & Rosenthal, 1980) and may explain why released peptides greater than tetra- or pentapeptide are not found (Rosenthal, 1979).

The role of the *exo-N*-acetylglucosaminidase is also unclear. Combined with the action of *endo-N*-acetylglucosaminidase it would have the effect of producing glycan chains terminated at the reducing end with an *N*-acetylglucosamine residue, as reported for bacilli and *Staphylococcus aureus* (Ward, 1973), and at the non-reducing end with an *N*-acetylmuramylpeptide unit. It is not possible to state whether the *exo-N*-acetylglucosaminidase and the *endo-N*-acetylglucosaminidase are the same enzymes; the difference in pH optima suggests that they may be different. The inhibition of the apparent *endo-N*-acetylglucosaminidase activity by β -lactam antibiotics was unexpected, since these drugs typically inhibit peptidases specific for D,D linkages. The transglycosylase from *E. coli* that yields 1,6-anhydromuramic acid termini (Höltje *et al.*, 1975) was also reported to be weakly inhibited by penicillin (20% inhibition by 4.2×10^{-4} M-penicillin) but that is some three orders of magnitude less sensitive than the *endo-N*-acetylglucosaminidase activity in the present experiments. A possible explanation would be that the latter enzyme has a binding site for the peptide side-chain on the proximal *N*-acetylmuramic acid residue, and that β -lactams can interact with this site.

The present study confirms the presence of an L,D-carboxypeptidase in *N. gonorrhoeae* indicated by earlier studies (Rosenthal *et al.*, 1980; Sinha & Rosenthal, 1980). It seems likely that there is no single major autolysin in *N. gonorrhoeae* (Hebeler & Young, 1976b) but that the enzymes described here, together with those already found to hydrolyse peptidoglycan are jointly responsible for any turnover of peptidoglycan in *N. gonorrhoeae* and the release of fragments into the growth medium.

Since the completion of this work Gubish *et al.* (1982) have described the purification of a gonococcal *endo- β -N*-acetylglucosaminidase that cleaves peptidoglycan. They examined the effect of the enzyme in cleaving peptidoglycan at internal *N*-acetylglucosamine residues to yield

free reducing groups and shorter chains of peptidoglycan, which was still insoluble. Their purified enzyme also cleaved *p*-nitrophenyl- β -*N*-acetylglucosaminide, and therefore had *exo*-enzyme activity. The main difference from our work was that Gubish *et al.* (1982) studied activities at pH 4.5, whereas we found an optimum of pH 8.5 for the *endo*-enzyme (Fig. 1*b*) and pH 6.5 to 7.5 for the *exo*-enzyme (Fig. 4). We did not examine pH values as low as pH 4.5 in either assay.

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