TUNICAMYCIN INHIBITION OF BACTERIAL WALL POLYMER SYNTHESIS

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1. Introduction

Tunicamycin is a glucosamine containing antibiotic [1] which inhibits the biosynthesis of glycoproteins in both yeast [2,3] and mammalian cells [4,5] by preventing the formation of dolichyl-pyrophosphoryl-*N*-acetylglucosamine. This intermediate is involved in the early stages of the biosynthetic pathway leading to glycosylation of the various proteins. In bacterial systems tunicamycin was shown to inhibit the incorporation of glucosamine into macromolecular material, presumably the wall of the organisms [6]. More recently Bettinger and Young [7] reported the inhibition of peptidoglycan synthesis in *Bacillus subtilis* 168 and that the site of action of the antibiotic was the formation of the second lipid intermediate undecaprenyl-P-P-*N*-acetylmuramyl-(pentapeptide)-*N*-acetylglucosamine. In the biosynthesis of peptidoglycan this intermediate is formed by the transfer of *N*-acetylglucosamine from UDP-*N*-acetylglucosamine to undecaprenyl-P-P-*N*-acetylmuramyl-pentapeptide. In addition they also observed inhibition by tunicamycin of the formation of an additional lipid intermediate in which one or several *N*-acetylglucosamine residues are pyrophosphate-linked to undecaprenol. The function of this intermediate which is also present in other bacilli remains unclear.

In contrast to the above observations Tamura et al. [8] using particulate enzyme preparations from *Micrococcus lysodeikticus* (luteus), concluded that tunicamycin inhibited peptidoglycan synthesis by preventing the formation of the first lipid intermediate, undecaprenyl-P-P-*N*-acetylmuramyl pentapeptide.

The present study, which was initiated before the publication of Tamura et al. [8] was undertaken to determine at what site tunicamycin inhibited peptidoglycan synthesis. The results obtained both support and extend to several other bacteria, their [8] conclusion that tunicamycin acts by inhibiting phospho-*N*-acetylmuramyl pentapeptide translocase, the initial membrane-bound reaction in peptidoglycan synthesis.

2. Materials and methods

The bacteria used were *Bacillus licheniformis* 94, a β-lactamase and *N*-acetylmuramyl-L-alanine amidase deficient mutant derived from *B. licheniformis* 6346 [9], *B. subtilis* 168 trp thy, *B. subtilis* W23, *Micrococcus luteus* NCTC 2665 and *Staphylococcus aureus* H.

Organisms were grown at 35°C with shaking in a medium containing tryptone 1% (w/v), yeast extract 1% (w/v) (both from Difco) and KH_2PO_4 0.5% (w/v). Glucose, sterilized separately was added to a final concentration of 0.4% (w/v). Cultures were harvested in the exponential phase of growth, A_{600} 1.5–2.0, and the bacteria were washed once with 50 mM Tris (hydroxymethyl) amino methane–Tris–HCl buffer, pH 7.8, containing 60 mM $MgCl_2$ and 1 mM dithiothreitol.

Membranes were prepared from the washed organisms by grinding with alumina and differential centrifugation as described previously [9]. Membrane preparations to be used for the determination of phospho-*N*-acetylmuramyl pentapeptide translocase were first treated to remove endogenous lipid intermediates by prior incubation with uridine-5′-monophosphate as described by Hammes and Neuhaus [10].

The activity of the translocase was determined both by utilizing the exchange assay of Hammes

and Neuhaus [10] and also in a transfer assay which measured the formation of butan-1-ol soluble radioactivity from UDP-*N*-acetyl-muramyl-(diamino [^{3}H] pimelic acid)-pentapeptide. The reaction mixture contained in total vol. 50 μl, 50 mM Tris–HCl pH 7.8, 0.21 M KCl, 42 μM $MgCl_2$, alkaline phosphatase (Boehringer) 5 μg, 0.17 mM UDP-MurAc-[^{3}H]DAP-pentapeptide (17.4 mCi/mmol) and membrane preparation (250–350 μg protein). This was incubated for 2 min at 25°C and the reaction was then stopped by the addition of butan-1-ol 6 M–pyridinium acetate, pH 4.2 (2:1 v/v) (300 μl) and water (250 μl). After mixing and centrifuging the organic phase was removed and the aqueous phase re-extracted with butan-1-ol (water saturated) (2 × 400 μl). The combined extracts were backwashed with water (butanol-1-ol saturated) (2 × 1 ml) and air-dried in vials prior to the determination of radioactivity.

Formation of the glucosamine-containing lipid was determined in reaction mixtures containing in total vol. 50 μl, 50 mM Tris–HCl, pH 7.8, 60 mM $MgCl_2$, 1 mM dithiothreitol, 0.15 mM UDP-[^{14}C] GlcNAc (4.4 mCi/mmol) and membrane preparation (250–350 μg protein). After incubation for 10 min at 25°C the reaction was stopped and the lipid-linked [^{14}C]GlcNAc isolated either by the procedure outlined above in the transfer assay of translocase activity or as described by Bettinger and Young [7]. Identical results were obtained using either technique.

The effects of tunicamycin on the synthesis of peptidoglycan and 1,3-poly(glycerol phosphate) teichoic acid were determined using membrane + wall preparations of *B. licheniformis* 94 as previously described [11]. After incubation at 28°C for 30 min the reactions were terminated by addition of sodium dodecyl sulphate (10% w/v) and the wall (SDS-insoluble) and membrane (SDS-soluble) fractions prepared [11].

Tunicamycin, supplied by Dr G. Tamura, University of Tokyo, was the kind gift of Professor A. H. Rose, University of Bath. UDP-MurAc pentapeptide labelled with diamino [^{3}H]pimelic acid (400 mCi/mmol) and CDP-[^{3}H]glycerol (100 mCi/mmol) were prepared as described previously [11] UDP-*N*-acetyl [^{14}C]glucosamine (300 mCi/mmol) and [^{14}C]uridine-5′-monophosphate (264 mCi/mmol) were purchased from the Radiochemical Centre, Amersham, Bucks. Non-radioactive UDP-MurAc pentapeptide was isolated from Mg^{2+}-deprived cultures of *B. licheniformis* and the corresponding lysine-containing UDP-MurAc pentapeptide was the gift of Dr A. Weston, University of Liverpool. Other non-radioactive precursors were purchased from Sigma.

3. Results and discussion

With the exception of *S. aureus* H which grew in the highest concentration of antibiotic tested (50 μg/ml) tunicamycin inhibited growth of all the other bacteria used in this study, although there were marked differences in the amount of antibiotic required to effect this inhibition. Growth of both *B. subtilis* 168 and W23 was inhibited at antibiotic concentrations of 0.2 and 0.5 μg/ml whereas 10 μg/ml and 50 μg/ml were required to inhibit the growth of *B. licheniformis* 94 and *M. luteus*, respectively.

The effect of tunicamycin on the in vitro synthesis of both peptidoglycan and teichoic acid was determined using membrane + wall preparations of *B. licheniformis* 94. Peptidoglycan synthesis was inhibited by 59% at an antibiotic concentration of 10 μg/ml (fig.1). At lower concentrations (1–2 μg/ml) a stimulation of the synthesis of cross-linked (SDS-insoluble) peptidoglycan was obtained although there was no concomitant change in the synthesis of uncross-linked (SDS-soluble) peptidoglycan. Tunicamycin also inhibited the synthesis of wall-associated (SDS-insoluble) 1,3-poly(glycerol phosphate) teichoic acid. In this case the lower concentrations of antibiotic did not stimulate the formation of either SDS-insoluble or soluble polymeric material. At the higher antibiotic concentrations the amount of diamino [^{3}H]pimelate present in the SDS-soluble fraction as lipid intermediates was also decreased from 960 pmol/mg protein in the control to 375 pmol and 242 pmol in the presence of 10 μg/ml and 20 μg/ml of tunicamycin. Thus, tunicamycin appeared to inhibit the initial membrane bound step of peptidoglycan synthesis, the formation of undecaprenyl-P-P-*N*-acetylmuramyl pentapeptide.

Confirmation of this was obtained when membrane preparations of both *B. licheniformis* 94 and the other organisms listed in table 1 were assayed for phospho-*N*-acetylmuramyl pentapeptide translocase activity. In each organism the translocase was inhibited by tunicamycin measured in either the exchange or

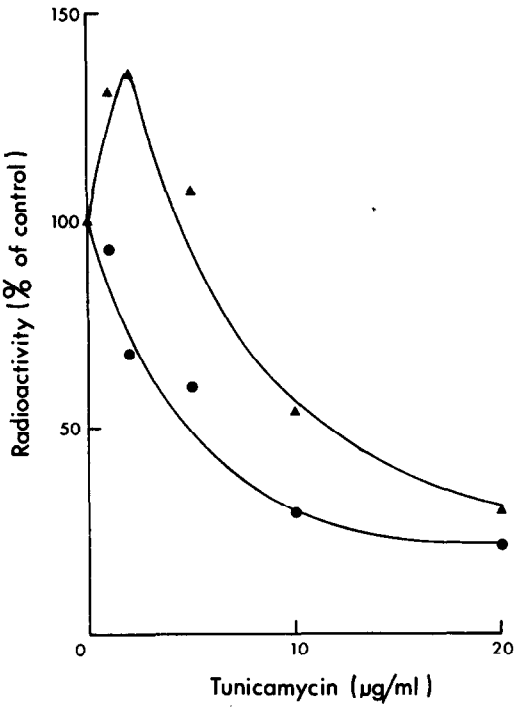

Fig.1. Effect of tunicamycin on the incorporation of *N*-acetylmuramyl-[^{3}H]A_2pm pentapeptide into peptidoglycan and [^{3}H]glycerol phosphate into teichoic acid by a membrane + wall preparation of *B. licheniformis* 94. Radioactivity incorporated into SDS-insoluble peptidoglycan (▲) and teichoic acid (●) was determined. In the absence of antibiotic the membrane + wall preparation synthesized 1.44 nmol peptidoglycan and 1.93 nmol teichoic acid.

transfer assays (table 1). Using membrane preparations isolated from the two strains of *B. subtilis* examined, 50% inhibition of enzyme activity was obtained at antibiotic concentrations somewhat higher than those required to inhibit growth whereas the reverse situation was found in *B. licheniformis* 94, *M. luteus* and *S. aureus* H. The differences observed in these three organisms between the amount of antibiotic required to inhibit growth and translocase activity may simply reflect a difference in the ability of the antibiotic to reach and interact with the membrane-bound enzyme. Alternatively, the relative resistance of the two cocci and *B. licheniformis* 94 to autolysis may allow these organisms to survive the inhibiting effects of tunicamycin. The role of autolysins in the bactericidal effects of other antibiotics known to inhibit various stages of peptidoglycan synthesis has already been well documented [12].

Formation of the second lipid intermediate involved in peptidoglycan synthesis requires the transfer of *N*-acetylglucosamine from UDP-*N*-acetyl glucosamine to undecaprenyl-P-P-*N*-acetylmuramyl pentapeptide. The following experiment was carried out to determine whether this stage of peptidoglycan synthesis is inhibited by tunicamycin. Membranes (652 μg protein) from *B. licheniformis* 94 were incubated with UDP-MurAc-[^{3}H]DAP-pentapeptide (22.4 nmol), for 10 min at 28°C to allow the formation of undecaprenyl-P-P-*N*-acetylmuramyl pentapeptide. At this time UDP-*N*-acetyl [^{14}C]glucosamine

Table 1
The inhibition of growth and of the phospho-*N*-acetylmuramyl pentapeptide translocase activity of membrane preparations by tunicamycin

Organism	Growth inhibitory concentration[a] (μg/ml)	Phospho-*N*-acetylmuramyl pentapeptide translocase	
		Rate of exchange[b]	Concentration giving 50% inhibition of activity (μg/ml)
B. licheniformis 94	10.0	5.75	1.2
B. subtilis 168	0.2	4.92	1.5
B. subtilis W23	0.5	4.54	1.4
M. luteus	50.0	8.98	3.8
S. aureus H	> 50.0	7.10	5.2

[a] The concentration of tunicamycin required to inhibit overnight growth of the organism in tryptone–yeast extract–glucose medium from an inoculum of 10^4 bacteria/ml

[b] The rate of exchange calculated as described [10] given in mol/litre/min × 10^6

(16.3 nmol) was added in the presence or absence of tunicamycin (25 μg/ml). After a further 5 min incubation the reaction was stopped by the addition of chloroform–methanol. Lipid intermediates containing ^{14}C but only negligible amounts of ^{3}H were extracted with the organic phase. After removal of the chloroform the aqueous phases were dried in vacuo, resuspended in water (0.5 ml) and peptidoglycan lipid intermediates extracted with butan-1-ol–pyridinium acetate. This procedure solubilised 936.4 pmol and 776.1 pmol of ^{3}H and 57.4 pmol and 51.3 pmol of ^{14}C from the membranes incubated in the absence and presence of tunicamycin.

Hence, tunicamycin reduced the amount of ^{14}C present by 12% and that of ^{3}H by 17%. Finally the aqueous phases were concentrated and the residues subject to chromatography. The peptidoglycan synthesized contained 90.8 pmol and 67.9 pmol of ^{3}H, with essentially equimolar amounts of ^{14}C in each case. Thus, the formation of the second lipid intermediate, undecaprenyl-P-P-*N*-acetylmuramyl-(pentapeptide)-*N*-acetylglucosamine and the polymerization of disaccharide-pentapeptide units are not markedly inhibited by tunicamycin. Clearly, the site of inhibition of peptidoglycan synthesis by this antibiotic is the phospho-*N*-acetylmuramyl pentapeptide translocase. Recently Lehle and Tanner [3] have shown that tunicamycin inhibits the synthesis of dolichyl-P-P-*N*-acetylglucosamine by membrane preparations of *Saccharomyces cerevisiae* but not the subsequent transfer of a second residue of *N*-acetylglucosamine to this precursor. Thus in both systems tunicamycin inhibits the phospho-translocase reaction but not the *N*-acetylglucosaminyl transferase.

The inhibition by tunicamycin of the synthesis of *N*-acetylglucosamine-containing lipid intermediates in bacilli may reflect a similar situation [7]. Low concentrations of tunicamycin (2 μg/ml) inhibited the formation by membranes of *B. licheniformis* 94 of chloroform–methanol extractable *N*-acetyl [^{14}C] glucosamine by 60.4%. Membranes incubated in the absence of antibiotic incorporated 829 pmol/mg protein. At higher antibiotic concentrations (10 μg/ml and 20 μg/ml) inhibition increased only to 77%. The transfer of further *N*-acetylglucosaminyl residues to endogenous undecaprenyl-P-P-*N*-acetylglucosamine and its higher analogues may explain the residual 23% incorporation [7]. Although the role of such lipid intermediates in biosynthesis remains unclear the involvement of such precursors has been described in the synthesis of membrane glycoproteins by *B. licheniformis* 9945A [13]. In addition the tunicamycin-sensitivity of teichoic acid biosynthesis in *S. aureus* [14,15] *B. subtilis* W23 [15,16] and *B. licheniformis* 94 suggests the involvement of *N*-acetylglucosamine-containing lipid intermediates in this process.

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