Bacitracin Inhibits the Synthesis of Lipid-linked Saccharides and Glycoproteins in Plants

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ABSTRACT

The particulate enzyme fraction from mung bean (Phaseolus aureus) seedlings catalyzes the incorporation of mannose from GDP-[14C]mannose into mannolysosphoryl-dolichol and of N-acetylglucosamine from UDP-[3H]N-acetylglucosamine into N-acetylglucosamine-pyrophosphoryl-polysaccharide. Bacitracin inhibits the transfer of both of these sugars into the lipid-linked saccharides with 50% inhibition being observed at 5 mM bacitracin. This antibiotic did not inhibit the transfer of glucose from UDP-[14C]glucose into steryl glycosides or the incorporation of glucose into a cell wall glucan. Bacitracin also inhibited the in vivo incorporation of [14C]mannose into mannolysosphoryl-dolichol and into glycoprotein by carrot (Daucus carota) slices. While bacitracin also inhibited the incorporation of lysine into proteins by these slices, protein synthesis was less sensitive than glycosylation. Thus at 2 mM bacitracin glycosylation was inhibited 92%, while protein synthesis was inhibited only 50%.

Bacitracin is a polypeptide antibiotic produced by Bacillus licheniformis, which has been shown to act on bacteria by interfering with the formation of lipid-linked sugars which are involved in peptidoglycan synthesis (10, 12). There is evidence that this interference is the result of the formation of a complex between bacitracin, the undecaprenyl-pyrophosphate, and a divalent cation (11). The undecaprenyl-pyrophosphate, thus bound, cannot be dephosphorylated to regenerate the undecaprenyl-monophosphate which is necessary to form lipid-sugar intermediates. This binding of phospholipids may also explain the observation that bacitracin disrupts the cell membrane (13).

That lipid-linked sugars act as intermediates in the biosynthesis of polysaccharides and glycoproteins has been well established in plants and animals as well as bacteria (4, 6, 9,14). Figure 1 is a diagram of a postulated series of reactions to explain the biosynthesis of glycoproteins in eucaryotic cells. Since the lipids involved in the above reactions are polyenyl-phosphates and are probably derived from polyenyl-pyrophosphates, it is reasonable to expect plant and animal cells to be sensitive to bacitracin. Recent work done on calf microsomes (8) indicated that bacitracin did interfere with the formation of the GlcNAc-pyrophosphoryl-dolichol intermediate but had little effect on the formation of mannolysosphoryl-dolichol.

We demonstrate here that in higher plants, bacitracin interferes with the incorporation of both GlcNAc and mannose into the lipid-linked intermediates in vitro. Furthermore, at concentrations of bacitracin which only partially inhibit protein synthesis in vivo, there is almost complete inhibition of mannose incorporation into glycoprotein.

MATERIALS AND METHODS

Reagents. Radioactive chemicals were obtained from New England Nuclear as follows: 6.6 Ci/mmol UDP-[3H]GlcNAc, 200 mCi/mmol UDP-[14C]glucose, 272 mCi/mmol GDP-[14C]mannose, 225 mCi/mmol [14C]mannose, and 275 mCi/mmol l-[14C]lysine. Pronase (grade B) was purchased from Calbiochem, and bacitracin was obtained from Sigma. Bacitracin was dissolved in water at a concentration of 100 mM and diluted to the required concentration. Silica Gel F-254 plates for TLC were from Brinkmann Instruments. All solvents were reagent grade unless otherwise indicated.

Analytical Methods. Radioactivity was measured in a Packard liquid scintillation spectrometer using scintillation fluid containing 500 ml of Triton X-100, 500 ml of toluene, 5 g of PPO and 0.2 g of POPOP.

Plant Material. Mung bean seeds and carrot roots were purchased in local supermarkets and stored at 4 C. Seeds of Phaseolus aureus (mung beans) were sterilized for 5 min in commercial bleach diluted 1:10 (about 0.5% hypochlorite) and rinsed with distilled H2O. They were then planted in moist Vermiculite and allowed to grow in the dark for 40 hr. The in vivo experiments were performed using thin discs (1 mm in thickness and 9 mm in diameter) of carrot phloem parenchyma preincubated for 16 hr in water at 27 C.

Preparation of Particulate Enzyme. Hypocotyls from mung bean sprouts were ground in cold buffer (5 ml/g containing 50 mM Tris [pH 7.5], 2 mM β-mercaptoethanol, and PVP [5 g/l]). The homogenate was filtered through cheesecloth and the particulate material was collected by centrifugation at 30,000 g for 15 min. The pellet was suspended in 50 mM Tris (1 ml/g) (pH 7.5), containing 2 mM β-mercaptoethanol.

Formation and Analysis of Products. Assays for the incorporation of GlcNAc, mannose, or glucose into lipid in vitro, at different bacitracin concentrations, were prepared by incubating 0.3 ml of particulate enzyme (containing about 1.2 mg of protein) with the appropriate sugar nucleotide (usually 50,000 cpm of 14C or 3H) and 1 mM MgCl2 in a final volume of 0.5 ml for various periods of time. These reactions were terminated by the addition of 2 ml of CHCl3/CH3OH (1:1). The reaction mixtures were mixed vigorously and the phases were separated by centrifugation. The lower organic phase was removed and saved. The upper aqueous phase was extracted a second time with 1 ml of CHCl3. After separation of the phases, the lower layer was removed and combined with the first organic phase. The combined organic phases were extracted with CHCl3/CH3OH/H2O (3:48:47) and the lower layer was removed and dried in scintillation vials for the measurement of radioactivity, or subjected to TLC.
for various lengths of time at several bacitracin concentrations in H2O in a final volume of 5 ml. Reactions were terminated by the addition of 10 ml of CHCl3/CH3OH (1:1). The carrots were then ground in an Omni-Mix grinder, mixed vigorously, and the phases were separated by centrifugation. The lower, organic phase was removed and saved. The upper, aqueous phase and interface was extracted a second time with 5 ml of CHCl3. After separation of the phases, the lower layer was removed and combined with the first organic phase. The combined organic phases were extracted with CHCl3/CH3OH/H2O (3:48:47) and the lower phase was removed and dried in scintillation vials for radioactive measurements. The upper, aqueous layer from the original extraction was then centrifuged to isolate the pellet which was washed extensively with 100% methanol, 50% methanol, and H2O. After washing, the pellet was treated with 5 ml of 10% trichloroacetic acid in order to be certain that any noncovalently bound 14C]lysine or 14C]mannose was removed. Trichloroacetic acid was removed from the residue by washing twice with ether followed by washes with absolute ethanol, 50% ethanol, and H2O. The pellets were then given a final wash in 0.01 M Tris (pH 8.0) containing 0.01 M CaCl2 before being subjected to pronase digestion.

**Pronase Digestion.** The above washed pellets were suspended in 0.01 M Tris (pH 8.0) containing 0.01 M CaCl2 such that the final volume of pellet plus buffer was 10 ml for each sample. The samples were incubated with 10 mg of pronase and 2 drops of toluene (to prevent bacterial growth) at 37°C for 24 hr. At the first 24 hr, an additional 10 mg of pronase and drop of toluene were added to each incubation mixture. The reactions were terminated by placing the samples in a boiling water bath for 10 min. Protein was then precipitated by treatment with 5% trichloroacetic acid and the insoluble material was removed by filtration. Release of radioactivity by pronase was determined by counting 1/10 of the filtrate.

**Chromatographic Methods.** TLC of the lipid products was done on Silica Gel F-254 (2-mm thickness) plates in two solvent systems: solvent system A, CHCl3/CH3OH/H2O (65:25:4, v/v/v) and solvent system B, CHCl3/CH3OH/concentrated NH4OH (75:25:4, v/v/v). Samples were evaporated in a stream of air, redissolved in 100 µl of CHCl3/CH3OH (2:1) and applied to the silica gel plate. The location of the radioactive lipids was determined by cutting the plates into 0.5-cm sections which were scraped into scintillation vials and counted in toluene scintillation fluid. DOL-PP was run as a standard on each plate and it was visualized by iodine vapor.

Paper chromatography of radioactive sugars released by acid hydrolysis of both lipid and glycoprotein products was done on Whatman 3MM paper in a solvent consisting of 15% formic acid-formic acid-H2O (18:3:1:4) or 1-butanol-pyridine-0.1 M HCl (5:3:2). Standard sugars were visualized with periodate-permanate spray.

**RESULTS**

**Effect of Bacitracin on Formation of Polyprenol-P-Sugars in Vitro.** When the enzyme preparation from the hypocotyls of mung beans (P. aureus) was incubated with UDP-[3H]GlcNAc or GDP-[14C]mannose, radioactiveity from both of these sugars was incorporated into lipid-linked saccharides (4, 6). The addition of bacitracin to such incubation mixtures greatly inhibited this formation of labeled glycolipids (Fig. 2). As shown in Figure 2, the incorporation of both labeled mannose and labeled GlcNAc from their respective nucleotide sugars into lipid-soluble material was inhibited approximately 50% (mannose was actually 43% inhibited and GlcNAc 50%) at a bacitracin concentration of 5 mM and about 80% at a bacitracin concentration of 10 mM. The decrease in lipid-soluble radioactive products, in the presence of bacitracin, was not due to an effect of bacitracin on the partitioning of lipid products. That is, bacitracin did not block the extraction of lipids from reaction mixtures. Thus, reaction mixtures in which the bacitracin was added at the end of the incubation showed no decrease in lipid-soluble radioactivity when compared to the controls with no bacitracin (data not shown). The inhibition by bacitracin was followed during the course of a 20-min incubation as shown in Figure 3. It can be seen that inhibition was observed...
as early as 1 min after the start of the incubation and persisted throughout the 20-min incubation. The formation of mannosyl-phosphoryl-dolichol was inhibited approximately 40% at 4 μmol of bacitracin while the synthesis of GlcNAc-pyrophosphoryl-polysoprenol was inhibited about 55% at that concentration.

In order to show that bacitracin is a specific inhibitor of polyisoprenyl-linked sugars and not simply an inhibitor of the formation of glycolipids in general, the effect of bacitracin on the production of steryl glucosides was investigated. Enzyme preparations were incubated with UDP-[14C]glucose at bacitracin concentrations up to 30 mM and steryl glucoside formation was determined as previously described (7). The incorporation of glucose into steryl glucoside was not inhibited at any bacitracin concentration, but mannone incorporation into mannosyl-phosphoryl-dolichol was inhibited as before (Fig. 4).

The labeled glycolipid formed in the above experiments from UDP-glucose in the presence of bacitracin was subjected to TLC in two solvent systems. Only one peak of radioactivity was detected on thin layer plates run in both of these solvents and the Rf values of this product were similar to those reported for steryl glucosides (7).

Bacitracin, a suspected inhibitor of glycoprotein synthesis, was also found to have no effect on the formation of glucans from UDP-glucose (Fig. 4). In this experiment, the incorporation of glucose from UDP-[14C]glucose into cell wall glucan was assayed as previously described in the presence of increasing amounts of bacitracin (1). This polymer was previously characterized as a β-1,3-glucan. Bacitracin did not affect the synthesis of this polymer except possibly at very high concentrations (Fig. 4). The formation of steryl glucosides and glucan polymers, therefore, both are insensitive to the action of bacitracin.

Effect of Bacitracin in Vivo. Aged carrot (Daucus carota) discs were incubated in water for different periods of time with either [14C]mannonse or [14C]lysine and various concentrations of bacitracin. As can be seen in Figure 5B, a labeled glycolipid was formed from [14C]mannonse in carrot slices in the absence of bacitracin. The production of this labeled glycolipid was almost completely inhibited when the bacitracin concentration of the surrounding media was 2 mM, while 1 mM bacitracin was somewhat less inhibitory. Concentrations up to 10 mM were used, although 2 mM is the highest concentration shown in the figure. The glycolipid formed in the absence of bacitracin was subjected to mild acid hydrolysis (0.01 M HCl in 50% propanol at 100 C for 15 min). All of the radioactivity was released as water-soluble material which was shown by paper chromatography to be mannone. Thus the lipid formed in carrot slices from [14C]mannonse appears to be mannosyl-phosphoryl-dolichol, and in vivo synthesis of this lipid is inhibited by bacitracin.

The effect of bacitracin on the synthesis and glycosylation of glycoprotein was also investigated. Insoluble material from the above reactions (containing both protein and polysaccharide material) was digested with pronase, and the amount of water-soluble radioactivity released by this treatment was measured. Figure 5A shows the amount of radioactivity released by pronase from the insoluble products of carrots incubated with [14C]mannonse and 0, 1 mM or 2 mM bacitracin. It can be seen that bacitracin inhibited the incorporation of mannone into insoluble material that was digestible with pronase. Complete acid hydrolysis of this water-soluble material released all of the radioactivity as mannone suggesting that this sugar was incorporated intact into glycopeptides which were rendered water-soluble by pronase digestion.

Protein synthesis by carrot slices, in the presence or absence of bacitracin, was also studied by following the incorporation of radioactive lysine into trichloroacetic acid insoluble material. The incorporation of [14C]lysine into pronase-digestible material was less sensitive to bacitracin than was the incorporation of [14C]mannonse (Table I). After 20 min, at a bacitracin concentration of 2 mM, the incorporation of mannone into insoluble material was inhibited 92%, whereas the incorporation of lysine into protein was inhibited only 50%.

**DISCUSSION**

In bacterial systems, bacitracin has been shown to inhibit the formation of undecaprenyl-pyrophosphoryl-sugars which are intermediates in peptidoglycan synthesis (10, 12). Apparently bacitracin binds the undecaprenyl-phosphosphate and prevents its dephosphorylation which is necessary for recycling of this lipid carrier (12, 13). We have found that bacitracin also interferes with the formation of the lipid-linked saccharides which are intermediates in plant glycoprotein synthesis. Thus, this antibiotic inhibits the transfer of mannone from GDP-[14C]mannonse to mannosyl-phosphoryl-dolichol as well as the transfer of GlcNAc from UDP-

**Fig. 4.** A: steryl glucoside synthesis in presence of bacitracin. Production of steryl glucosides from UDP-[14C]glucose (○—○) is compared with production of mannosyl-phosphoryl-dolichol (□—□) at concentrations of bacitracin up to 30 mM. B: production of glucan polymer from UDP-[14C]glucose in presence of bacitracin. Insoluble products, from incubations of mung bean particulate enzyme with UDP-[14C]glucose, containing both glycoprotein and polysaccharide, were subjected to pronase digestion. The insoluble radioactive product which was not pronase-digestible was presumed to be glucan.

**Fig. 5.** In vivo incorporation of [14C]mannonse into (A) pronase-digestible material and (B) mannosyl-phosphoryl polysoprenol in the presence of 2 mM (△—△), 1 mM (□—□) bacitracin and in the absence (○—○) of bacitracin. Incubations and pronase digestion were as described in the text.

**Table I.** Incorporation of [14C] Lysine Into Protein in the Presence of Bacitracin

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<tr>
<th>Concentration of Bacitracin</th>
<th>Radioactivity Released by Pronase</th>
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<tr>
<td>0 mM</td>
<td>100%</td>
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<tr>
<td>1 mM</td>
<td>80%</td>
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<tr>
<td>2 mM</td>
<td>60%</td>
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[\textsuperscript{14}H]GlcNAc to GlcNAc-pyrophosphoryl-polysisoprenol.

These results are in contrast to those of Herscovic et al. (8) who reported that bacitracin blocked the formation of GlcNAc-pyrophosphoryl-dolichol by microsomal preparations of calf pancreas, but had no effect on the synthesis of mannosyl-phosphoryl-dolichol. In mammalian systems, the lipid carrier for both mannose and GlcNAc has been reported to be a dolichol but in plants these two lipid carriers may be different. Thus the mannose-containing lipid from Phaseolus vulgaris has been characterized by mass spectrometry as mannosyl-phosphoryl-dolichol (3). Although the lipid portion of the GlcNAc-containing lipid has not been chemically characterized in plants, studies from our laboratory suggest that this lipid is different from the dolichol which carries mannose (5). In spite of the differences, it seems likely that the lipid to which the GlcNAc is attached is of the polysisoprenol type. It appears unlikely that these differences between the plant and animal lipids can explain the differences reported in bacitracin action, especially since this antibiotic also inhibits bacterial systems which involve lipids of the undecaprenol type. Further work will be necessary to explain the mode of action of this antibiotic.

In oviduct tissue extracts, bacitracin was shown to cause the accumulation of the trisaccharide-lipid, mannose-\(\beta\)-GlcNAc-GlcNAc-pyrophosphoryl-dolichol (2).

Although bacitracin clearly inhibited the formation of mannosyl-phosphoryl-dolichol and GlcNAc-pyrophosphoryl-polysisoprenol, it has no effect on the transfer of glucose from UDP-[\textsuperscript{14}C]glucose to steryl glucosides. This was an interesting observation since it showed that bacitracin did not inhibit all \textit{in vitro} reactions and more importantly that the antibiotic did not prevent the formation of neutral glycolipids such as steryl glucosides. Thus the antibiotic appears to have some specificity for phosphorylated lipids of the polysisoprenol type. Bacitracin also inhibited the \textit{in vivo} incorporation of [\textsuperscript{14}C]mannose into mannosyl-phosphoryl-dolichol and into glycoprotein in carrot slices. \textit{In vitro} 80\% inhibition of mannose incorporation was seen at 10 mm bacitracin, whereas \textit{in vivo} 70\% inhibition was observed at 2 mm. This may indicate that the bacitracin concentration of the surrounding medium is different than that inside the cell. Bacitracin may accumulate in the cell or it could be that \textit{in vivo} systems are more sensitive. Bacitracin also inhibited the incorporation of mannose into glycoprotein but did not block the transfer of glucose from UDP-glucose into \(\beta,1\to5\) glucan. Again, this showed that the antibiotic did not inhibit all glycosyl transferase reactions and also provided additional evidence for the role of mannosyl-phosphoryl-dolichol in the mannosylation of proteins.

**Literature Cited**