Vol. 82, No. 2, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 30, 1978 Pages 568-574

Amphomycin Inhibits the Incorporation of Mannose and GlcNAc into Lipid-Linked Saccharides by Aorta Extracts

M.S. Kang, James P. Spencer and Alan D. Elbein
The University of Texas Health Science Center at San Antonio
Department of Biochemistry
7703 Floyd Curl Drive
San Antonio, Texas 78284

Received April 10,1978

Abstract

Amphomycin inhibits the incorporation of mannose from GDP-[^{14}C]mannose and GlcNAc from UDP-[^{3}H]GlcNAc into lipid-linked saccharides by either a particulate or a solubilized enzyme fraction from pig aorta. The solubilized enzyme was much more sensitive to the antibiotic than was the particulate fraction with 50% inhibition being observed at 8-15 µg of amphomycin. Although the antibiotic inhibited mannose transfer from GDP-[^{14}C]mannose into mannosyl-phosphoryl-dolichol, lipid-linked oligosaccharides and glycoprotein, the synthesis of mannosyl-phosphoryl-dolichol was much more sensitive to amphomycin. Amphomycin also inhibited the incorporation of mannose from GDP-[^{14}C]mannose into mannosyl-phosphoryl-decaprenol in particulate extracts of Mycobacterium smegmatis.

Introduction

The assembly of the $(man)_n\alpha$-Manβ-GlcNAcβ-1,4-GlcNAc core oligosaccharide that is common to many asparaginyl-linked glycoproteins involves the participation of lipid-linked saccharides (1). Two lipid-linked monosaccharides, mannosyl-phosphoryl-dolichol and GlcNAc-pyrophosphoryl-dolichol, are synthesized from their respective sugar nucleotide derivatives and these lipids then participate in the formation of lipid-linked oligosaccharides. Because of the similarity of this assembly process to that of peptidoglycan synthesis (2), it was of interest to examine the effects of cell wall inhibitors on the lipid-linked pathway in aorta. One such inhibitor is amphomycin, a polypeptide antibiotic (3,4) which has recently been shown to inhibit peptidoglycan synthesis in Bacillus cereus (5,6). In this report, we show that amphomycin inhibits the transfer of mannose and GlcNAc from sugar nucleotide to lipid-linked saccharides in extracts of aorta. The antibiotic also blocked the formation of mannosyl-phosphoryl-decaprenol in a particulate enzyme preparation of Mycobacterium smegmatis.

0006-291X/78/0822-0568$01.00/0

Materials and Methods

GDP-[^{14}C]mannose (278 μCi/μmol) and UDP-[^{3}H]GlcNAc (6.6 Ci/mmol) were from New England Nuclear Co., Boston, Massachusetts and dolichol-phosphate was from Calbiochem, La Jolla, California. Amphomycin was kindly supplied by Dr. M. Bodansky, Western Reserve University and Mr. W. Minor, Bristol Labs, Syracuse, New York. Radioactivity was measured in a Packard liquid scintillation spectrometer using a triton-toluene cocktail. Lipid samples, usually dissolved in $CHCl_3$ or $CHCl_3$:CH_3OH, were added to scintillation vials and the solvent was removed under a heat lamp. Ten ml of triton-toluene counting fluid was then added to each vial for counting. Trichloroacetic acid insoluble material (protein) was dissolved in 1 ml of Protosol prior to counting in triton-toluene fluid.

The particulate enzyme from pig aorta was prepared from the intima-media layer as previously reported (7). The enzyme preparation was solubilized by stirring the above particulate fraction with 0.5% Nonidet P-40 (NP-40) for 15 min at 5° as described (8). The mixture was centrifuged at 100,000 x g for 45 min and the supernatant liquid was used as the enzyme source. When soluble enzyme was used, dolichol-phosphate (usually 6 μg) was added first to incubation tubes and the NP-40 was added to suspend the lipid and other reaction components were added as follows to give a final reaction volume of 0.4 ml: Tris buffer, pH 7.5, 25 μmoles; $MnCl_2$, 2 μmoles; GDP-[^{14}C]mannose or UDP-[^{3}H]GlcNAc (60,000 CPM) and 100 μl of enzyme (usually 500 μg of protein). Amphomycin was generally added, in the amounts indicated in the figures, before enzyme. However, in the time course experiments, amphomycin and enzyme were preincubated for 2 min before the addition of substrates. Following incubation, usually for 20 min at 37°, reactions were stopped by the addition of 2 ml of $CHCl_3$:CH_3OH (1:1) followed by 0.5 ml of H_2O. After thorough mixing, the layers were separated by centrifugation and the bottom layer was removed and saved. The upper layer and interface were extracted with an additional 1 ml of $CHCl_3$ and the bottom layer from this extraction was combined with the first bottom layer. The combined $CHCl_3$ layer was washed with $CHCl_3$:CH_3OH:H_2O (3:48:47) and was then placed in scintillation vials for counting. This layer contained mannosyl-phosphoryl-dolichol, GlcNAc-pyrophosphoryl-dolichol and N,N'-diacetyl chitobiosyl-pyrophosphoryl-dolichol. The lipid-linked oligosaccharides and glycoprotein remained associated with the particulate material at the interface. Thus, CH_3OH was added to the aqueous layer and interface to give a single phase and the particulate material was isolated by centrifugation. The aqueous layer was discarded and the pellet was washed several times with 50% CH_3OH, before being extracted with $CHCl_3$:CH_3OH:H_2O (10:10:3). This solvent extracted the lipid-linked oligosaccharides which were dried in scintillation vials for counting. The pellet remaining from this extraction contained the glycoprotein. This material was dissolved in Protosol for counting.

Several experiments were done with extracts of *Mycobacterium smegmatis* to determine the effects of amphomycin on the formation of mannosyl-phosphoryl-decaprenol (9). *M. smegmatis* was grown in Trypticase soy broth and a particulate enzyme preparation was made as previously described (10). Reaction mixtures with GDP-[^{14}C]mannose, $MgCl_2$ and particulate enzyme were prepared as reported before, except that various amounts of amphomycin were added to the incubations. Mannosyl-phosphoryl-decaprenol was isolated by extraction with $CHCl_3$:CH_3OH (10).

Results and Discussion

The pig aorta enzyme preparation that synthesizes the lipid-linked saccharides is sensitive to amphomycin. This inhibition could be demonstrated

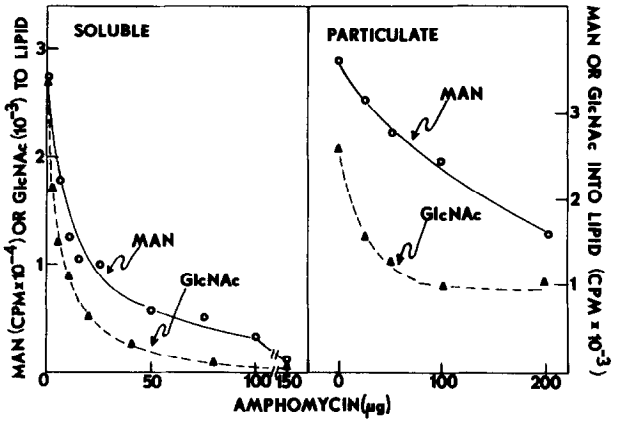

Figure 1

Effect of amphomycin concentration on the formation of Dol-P-Man from GDP-[^{14}C]mannose (o—o—o) and Dol-PP-GlcNAc from UDP-[^{3}H]GlcNAc (Δ—Δ—Δ) in the soluble and particulate enzyme fraction. 100 µl of the enzyme preparations were used and the reaction was terminated after 20' of incubation at 37°C. Incubations and extractions were as described in the text.

in both the particulate and the solubilized enzyme preparations. Figure 1 shows that the incorporation of mannose from GDP-[^{14}C]mannose to mannosyl-phosphoryl-dolichol (Dol-P-Man), as well as the incorporation of GlcNAc from UDP-[^{3}H]-GlcNAc to GlcNAc-pyorophosphoryl-dolichol (Dol-PP-GlcNAc), is inhibited by amphomycin. It can be seen that the solubilized enzyme system was much more sensitive to this antibiotic than was the particulate enzyme with 50% inhibition being observed at 7-15 µg of amphomycin. It should also be noted that the transfer of GlcNAc was inhibited at lower amphomycin concentrations than the transfer of mannose. Figure 2 shows a time course of incorporation of mannose and GlcNAc by the solubilized enzyme in the presence and absence of amphomycin. It can be seen that amphomycin inhibition was detectable as early as 1 min and persisted throughout a 20 min incubation. Again 50% inhibition was observed at about 7-15 µg of antibiotic. Both Figures 1 and 2 show that GlcNAc transferase was more sensitive to amphomycin than the mannose transferase.

In the particulate enzyme preparation, amphomycin not only inhibited the transfer of mannose from GDP-[^{14}C]mannose to Dol-P-Man but it also inhibited

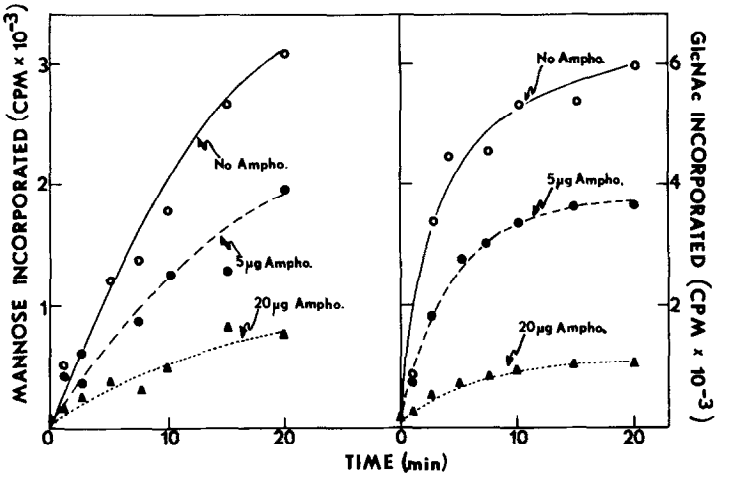

Figure 2

Time course of incorporation of mannose from GDP-[^{14}C]mannose and GlcNAc from UDP-[^{3}H]GlcNAc into Dol-P-Man and Dol-PP-GlcNAc with the soluble fraction in the absence and presence of two concentrations of amphomycin. The enzyme was preincubated at 37°C for 2 min with either 5 µg or 20 µg of antibiotic per 100 µl of the enzyme before starting the incubation. Controls were also run in which enzyme alone was preincubated.

mannose transfer to lipid-linked oligosaccharides and to protein (Figure 3). However, Dol-P-Man formation was much more sensitive to inhibition than was the formation of lipid-linked oligosaccharide or glycoprotein. If mannose incorporation into lipid-linked oligosaccharide and protein requires the participation of Dol-P-Man, then one would expect an inhibition in these products when Dol-P-Man formation is blocked. However, it can be seen from Figure 3 that mannose is still incorporated into lipid-linked oligosaccharides at concentrations of amphomycin which completely inhibit Dol-P-Man synthesis. These results suggest that some of the mannose residues in the lipid-linked oligosaccharides come directly from GDP-[^{14}C]mannose. Several other studies have also implied a direct transfer of mannose from GDP-[^{14}C]mannose to the lipid-linked oligosaccharides (11,12).

In order to determine whether amphomycin inhibited polyprenol-linked sugar formation in other systems, the formation of mannosyl-phosphoryl-decaprenol was examined in extracts of Mycobacterium smegmatis. Figure 4 shows that

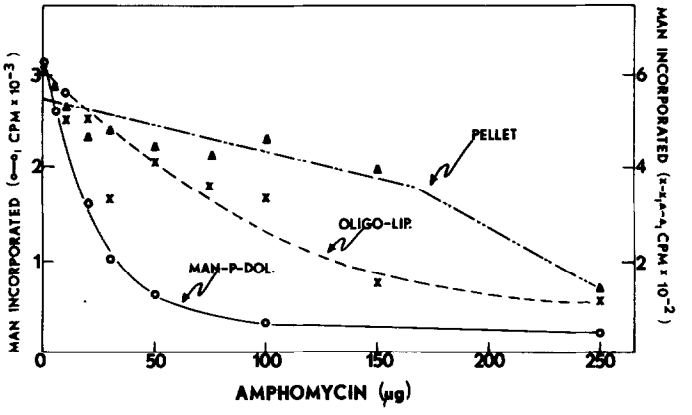

Figure 3

Formation of Dol-P-Man, lipid-linked oligosaccharide and acid-insoluble material (glycoprotein) from GDP-[^{14}C]mannose by the particulate fraction in the presence of increasing amounts of amphomycin. Incubations were for 30 min at 37°C. Enzyme assays and extractions were as described in the text.

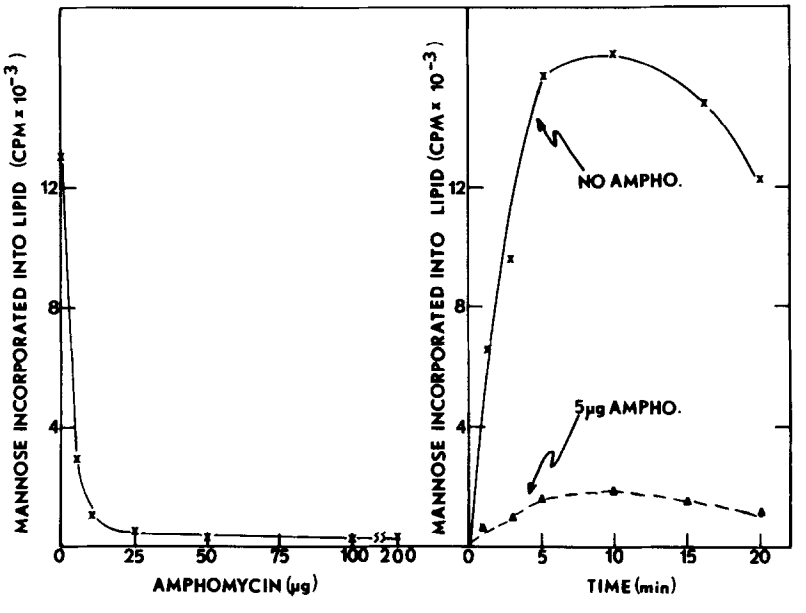

Figure 4

Inhibition of mannosyl transfer of Mycobacterium smegmatis by amphomycin. The curve on the left shows the effect of amphomycin concentration on the transfer of mannose from GDP-[^{14}C]mannose into mannosyl-phosphoryl-decaprenol while the curve on the right shows a time course in the absence or presence of amphomycin (5 µg). Incubation mixutres were as described in the text and mannosyl-phosphoryl-decaprenol was isolated by extraction with $CHCl_3$:CH_3OH (10).

amphomycin was a potent inhibitor of the mannose transferase in this organism with 70 or 80% inhibition being observed at 5 μg of antibiotic. The curve on the right of Figure 4 is a time course experiment showing that mannose incorporation into lipid was linear for about 5 min and that the effect of amphomycin was detectable as early as 1 min and persisted throughout the 20 min incubation. Thus amphomycin also inhibits mannosyl-phosphoryl-polyisoprenol synthesis in bacteria.

Antibiotics which inhibit the synthesis of the oligosaccharide chain of glycoproteins should be useful in studies of the function of the carbohydrate portion of the molecule as well as in studies on the mechanism of assembly of glycoproteins. Several antibiotics have been described which interfere with this assembly process. One of these is tunicamycin (13) which inhibits for formation of Dol-PP-GlcNAc preventing buildup of the lipid-linked oligosaccharides (14). Thus, tunicamycin inhibits glycosylation of proteins, in vivo. Another antibiotic which has been reported to inhibit the transfer of GlcNAc from UDP-GlcNAc into lipid-linked saccharides is bacitracin. However, this antibiotic had no effect on the incorporation of mannose into lipid (15). In this paper we show that amphomycin, which is a polypeptide antibiotic like bacitracin but contains a fatty acid linked to one of the amino acids, inhibits the incorporation of both mannose and GlcNAc into lipid-linked saccharides. The amount of amphomycin required for inhibition appears to be considerably lower than the amount of bacitracin. It will be of interest to examine the in vivo effect of amphomycin on glycoprotein synthesis and function.

Acknowledgment

This research was supported by grants from the Robert A. Welch Foundation, the National Institutes of Health (HL 17783) and the National Science Foundation (PCM 77-08801).

References

1. Waechter, C.J. and Lennarz, W.J. (1976) Annu. Rev. Biochem. 45, 95-112.
2. Lennarz, W.J. and Scher, M.G. (1972) Biochim. Biophys. Acta 265, 417-441.
3. Heinemann, B., Kaplan, M.A., Muir, R.D. and Hooper, I.R. (1953) Antibiotics and Chemotherapy 3, 1239-1242.
4. Bodansky, M., Sigler, G.B. and Bodansky, A. (1973) J. Am. Chem. Soc. 95, 2352-2357.

5. Omura, S., Janaka, H., Shinokara, M., Oiwa, R. and Hata, T. (1975) Chemotherapy, Proc. 9th Inter. Cong. Chemo., 5, 355-361.
6. Tanaka, H., Iwai, Y., Oiwa, R., Shinohara, S., Shimizer, S., Oka, T. and Bmura, S. (1977) Biochim. Biophys. Acta 497, 633-640.
7. Chambers, J. and Elbein, A.D. (1975) J. Biol. Chem. 250, 6904-6915.
8. Heifetz, A. and Elbein, A.D. (1977) J. Biol. Chem. 252, 3057-3063.
9. Takayama, K. and Goldman, D.S. (1970) J. Biol. Chem. 245, 6251-6257.
10. Schultz, J. and Elbein, A.D. (1974) Arch. Biochem. Biophys. 160, 311-322.
11. Chambers, J., Forsee, W.T. and Elbein, A.D. (1977) J. Biol. Chem. 252, 2498-2506.
12. Forsee, W.T., Griffin, J.A. and Schutzbach, J. (1977) Biochem. Biophys. Res. Commun. 75, 799-805.
13. Takatsuki, A., Aruna, K. and Tamura, G. (1971) J. Antibiotics (Tokyo) Ser. A 24, 215-273.
14. Tkacz, J.S. and Lampen, J.O. (1975) Biochem. Biophys. Res. Commun. 65, 248-2571.
15. Hercovics, A., Bugge, B. and Jeanloz, R.W. (1977) FEBS Lett. 82, 215-218.