Vol. 86, No. 3, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 14, 1979 Pages 902-908

Amphomycin Inhibits Phospho-N-acetylmuramyl-pentapeptide Translocase in Peptidoglycan Synthesis of Bacillus*

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Received January 3, 1979

Summary

Amphomycin, a selective inhibitor of peptidoglycan synthesis of bacteria, inhibited the lipid intermediates accumulation and the peptidoglycan synthesis from UDP-N-acetylmuramyl-L-Ala-D-Glu-[^{3}H]-meso-Dpm-D-Ala-D-Ala (UDP-MurNAc-pentapeptide) and UDP-N-acetylglucosamine (UDP-GlcNAc) with a particulate fraction from Bacillus megaterium KM, and also inhibited the formation of MurNAc-(-pentapeptide)-P-P-lipid in the absence of UDP-GlcNAc. But it did not inhibit the formation of peptidoglycan from MurNAc(-pentapeptide)-P-P-lipid and UDP-GlcNAc with the same system of the organism.

Thus, it is concluded that the site of action of amphomycin is phospho-MurNAc-pentapeptide translocase in peptidoglycan synthesis.

Introduction

In the previous papers (1, 2), the present authors reported that amphomycin (3, 4) and tsushimycin (5), antibiotics consisting of a fatty acid and a straight peptide chain and possessing inhibitory activities against Gram-positive bacteria, selectively inhibit cell wall peptidoglycan synthesis in Bacillus cereus T. The antibiotics also give rise to accumulation of nucleotide precursors in the organism (1) and Staphylococcus aureus (2). The nucleotide precursors accumulated in the cells of S. aureus incubated in the presence of amphomycin were identified as UDP-MurNAc-L-Ala-D-Glu-L-

Abbreviation used: MurNAc, N-acetylmuramyl; GlcNAc, N-acetylglucosamine; Dpm, diaminopimelic acid.
*Studies on Bacterial cell wall inhibitors. Part IV. Part III of this title appeared in Biochim. Biophys. Acta, 498, 223-228 (1977).
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0006-291X/79/030902-07$01.00/0

Lys-D-Ala-D-Ala, UDP-MurNAc-L-Ala and UDP-N-acetylmuramic acid (2). In the experiment using a particulate enzyme system of B. megaterium KM, it has been shown that amphomycin inhibits the synthesis of peptidoglycan from UDP-MurNAc-L-Ala-D-Glu-meso-Dpm-D-Ala-D-Ala (UDP-MurNAc-pentapeptide) and UDP-GlcNAc and does not induce the accumulation of lipid intermediates.

In this paper, we now present evidence indicating directly that the primary site of action of amphomycin is phospho-MurNAc-pentapeptide translocase, the first step of a lipid cycle of peptidoglycan synthesis in bacteria.

Materials and Methods

Bacterial strain. B. megaterium KM was obtained from Dr. P. E. Reynolds, Department of Biochemistry, University of Cambridge, U. K.

Chemicals. Amphomycin (sodium salt) and ristocetin were given by Dr. L. Delcambe, International Center of Information of Antibiotics, Belgium. Vancomycin is a gift from Dr. R. J. Hosley of Eli Lilly and Company, USA. UDP-MurNAc-L-Ala-D-Glu-[^{3}H]-meso-Dpm-D-Ala-D-Ala and cold UDP-MurNAc-pentapeptide are generous gifts from Dr. T. Oka, Kyowa Hakko Kogyo Co., Ltd., Japan.

Syntheses of lipid intermediates and peptidoglycan with a particulate fraction from B. megaterium KM. The assay was performed using a particulate fraction prepared from B. megaterium KM according to the method reported by Oka (6). A reaction mixture (30 µl) containing 3.3 x 10^{-4}M UDP-MurNAc-L-Ala-D-Glu-[^{3}H]-meso-Dpm-D-Ala-D-Ala (11.6 µCi/µmole), 3.3 x 10^{-4}M UDP-GlcNAc, 2.5 x 10^{-2}M $MgCl_2$, 1.0 x 10^{-3}M dithiothreitol, 0.25M Tris-HCl buffer (pH 8.5) and 10 µl of particulate fraction (200 µg protein) of the organism was incubated for 10-60 minutes at 25°C. After the reaction was stopped by addition of 15 µl of 6M pyridinium acetate (pH 4.2), the lipid intermediates in the mixture were extracted twice with 100 µl of n-butanol. The extracts were transferred to a scintillation vial and dried up. The radioactivity was estimated using a toluene scintillation fluid with a liquid scintillation counter (lipid intermediates accumulation, steps(s) 1 or 1 and 2).

Peptidoglycan remained in the water layer was precipitated by adding an excess of 5% trichloroacetic acid. The precipitate was collected on a Millipore filter, and washed twice with an excess of 5% trichloroacetic acid. The radioactivity of the precipitate on a filter was counted using a toluene scintillation fluid with a scintillation counter (peptidoglycan synthesis, steps 1, 2 and 3).

Assay of phospho-MurNAc-pentapeptide translocase (step 1) with a particulate fraction from B. megaterium KM. The above-mentioned reaction mixture (30 µl) without UDP-GlcNAc was incubated at 25°C. The lipid intermediate formed was extracted twice with n-butanol. The radioactivity of the extract was counted as described above. The lipid intermediate formed under this condition is MurNAc(-pentapeptide)-P-P-lipid.

Assay of other steps (2 and 3) than phospho-MurNAc-pentapeptide translocase in peptidoglycan synthesis from UDP-MurNAc-pentapeptide and UDP-GlcNAc. A reaction mixture without UDP-GlcNAc (5 µl of 2.0 x 10^{-3}M [^{3}H]-UDP-MurNAc-

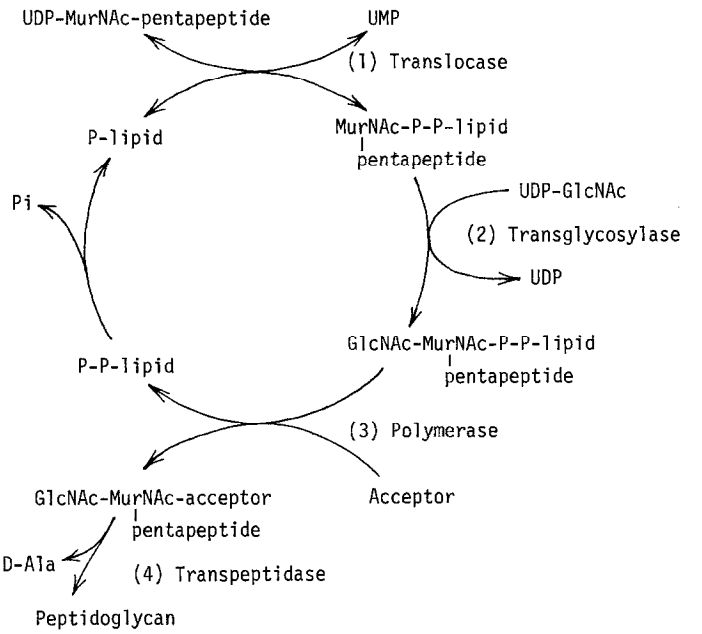

Fig. 1. Pathway of Peptidoglycan Synthesis from UDP-MurNAc-pentapeptide and UDP-GlcNAc with a Particulate Fraction of B. megaterium KM.

pentapeptide, 10 µl of 0.75M Tris-HCl buffer containing 7.5 x 10^{-2}M $MgCl_2$ and 3.3 x 10^{-3}M dithiothreitol, and 10 µl of particulate fraction) was incubated for 20 minutes at 25°C to accumulate radioactive MurNAc(-pentapeptide)-p-p-lipid, and then 5 µl of 0.2M cold UDP-MurNAc-pentapeptide (100 times the amount of the hot substrate added first), 5 µl of 1.0 x 10^{-3}M UDP-GlcNAc and 5 µl of an antibiotic solution (600 µg/ml) or water were added. After additional incubation of the reaction mixture for 120 minutes, lipid intermediates and peptidoglycan synthesis were assayed as described above.

Results and Discussion

With a particulate enzyme system of B. megaterium KM, peptidoglycan is synthesized from UDP-MurNAc-pentapeptide and UDP-GlcNAc via two lipid intermediates as shown in Fig. 1 (7, 8). Effect of amphomycin on lipid intermediates accumulation and peptidoglycan synthesis was examined with the system. The time courses of lipid intermediates accumulation and peptidoglycan synthesis from [^{3}H]-meso-Dpm-labeled UDP-MurNAc-pentapeptide and UDP-GlcNAc in the presence or the absence of amphomycin are shown in Fig. 2. Both with and without UDP-GlcNAc, lipid intermediate(s) accumulation and peptidoglycan synthesis were inhibited by amphomycin. This suggests that amphomycin inhibited

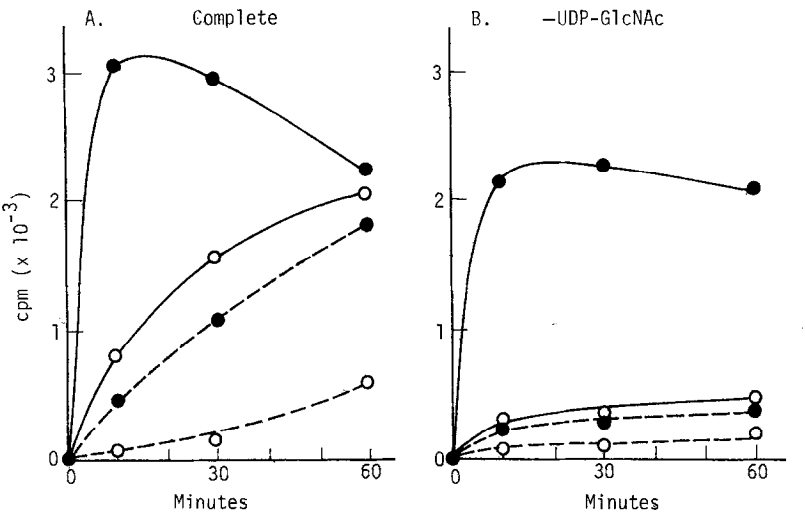

Fig. 2. Effect of Amphomycin on Lipid Intermediate Accumulation and Peptidoglycan Synthesis by a Particulate Enzyme of B. megaterium KM.
● : lipid intermediate ——: no antibiotic
○ : peptidoglycan ——: amphomycin (100 μg/ml)

the formation of lipid intermediate (step 1) and consequently caused the inhibition of peptidoglycan synthesis.

Inhibition effect of amphomycin on phospho-MurNAc-pentapeptide translocase (step 1) was examined and compared with those of ristocetin and vancomycin. As shown in Table I, amphomycin inhibited the translocase activity by 45 and 90% at 20 and 100 μg/ml, respectively. The inhibitory activity of amphomycin was stronger than those of ristocetin and vancomycin, both of which had been known to inhibit the translocase activity of a particulate fraction of S. aureus (9) of Gaffkya homari (10).

As amphomycin lyses growing cells of the same organism at a concentration of 10-100 μg/ml (2), the concentrations of amphomycin required for inhibition of phospho-MurNAc-pentapeptide translocase seemed to be small enough to explain the bactericidal action by this mechanism.

In the next place, we tested the effect of the antibiotic on other steps (steps 2 and 3) than phospho-MurNAc-pentapeptide translocase in peptidoglycan synthesis from UDP-MurNAc-pentapeptide. After the formation of MurNAc-

Table I. Effect of Amphomycin on the Phospho-MurNAc-pentapeptide Translocase Activity of a Particulate Fraction of B. megaterium KM.

Antibiotic	Concentration (μg/ml)	Lipid-PP-Mp formation cpm (%)
None	-	2168 (100)
Amphomycin	1	2018 (93)
	10	1845 (85)
	20	1193 (55)
	50	534 (25)
	100	223 (10)
Ristocetin	10	2075 (96)
	100	1685 (78)
Vancomycin	10	1139 (53)
	100	922 (43)

Reaction time: 3 min. Other methods are described in the text.

Table II. Effect of Amphomycin on the Formation of Peptidoglycan from MurNAc(-pentapeptide)-P-P-lipid with a Particulate Fraction of B. megaterium KM.

	Lipid intermediate cpm (%)	Peptidoglycan cpm (%)
1st incubation	839	-
2nd incubation		
No antibiotic	350 (100)	488 (100)
Amphomycin (75μg/ml)	233 (67)	478 (98)
Ristocetin (75μg/ml)	532 (152)	286 (58)

1st incubation: - UDP-GlcNAc, 20 min, 25°C
2nd incubation: + UDP-GlcNAc,
+ excessive cold UDP-MurNAc-pentapeptide
120 min, 25°C

(-pentapeptide)-P-P-lipid from [^{3}H]-meso-Dpm-labeled UDP-MurNAc-pentapeptide in the absence of UDP-GlcNAc in the system for peptidoglycan synthesis, excessive cold UDP-MurNAc-pentapeptide, UDP-GlcNAc and an antibiotic were added to the reaction mixture. As shown in Table II, amphomycin did not inhibit the formation of peptidoglycan from MurNAc(-pentapeptide)-P-P-lipid and UDP-GlcNAc (steps 2 and 3), but ristocetin which was known to inhibit the peptidoglycan synthesis in B. megaterium KM (2) did inhibit the reaction.

It has already been reported that amphomycin is a selective inhibitor of cell wall peptidoglycan synthesis, induces accumulation of UDP-MurNAc-pentapeptide, and does not inhibit the cross-linking (step 4) of the organism (1, 2). From the facts and the above results, it is concluded that the primary site of action of the antibiotic is phospho-MurNAc-pentapeptide translocase (step 1), the first step of a lipid cycle in peptidoglycan synthesis in bacteria. Recently, Tamura et al. (11) reported that tunicamycin inhibits this step in Micrococcus lysodeikticus. Tunicamycin is known to inhibit the synthesis of GlcNAc-P-P-lipid that is involved in the biosynthesis of a yeast glycoprotein (12) and a mammalian cell surface glycoprotein (13). More recently, amphomycin has been reported by Kang et al. (14) to inhibit the incorporation of mannose and GlcNAc into lipid-linked saccharides by pig aorta extract. It will be of interest whether amphomycin interfers with the synthesis of lipid-linked glycoprotein in eukaryotes and of such a saccharide as teichoic acid in prokaryotes.

Acknowledgements

We are indebted to Dr. T. Oka for a gift of hot and cold UDP-MurNAc-pentapeptide and for valuable discussion and to Miss H. Ueda for her helpful assistance. This work was partially supported by a Research Grant from the Ministry of Education of Japan.

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