STUDIES ON BACTERIAL CELL WALL INHIBITORS

X. PROPERTIES OF PHOSPHO-*N*-ACETYLMURAMOYL-PENTAPEPTIDE-TRANSFERASE IN PEPTIDOGLYCAN SYNTHESIS OF *BACILLUS MEGATERIUM* AND ITS INHIBITION BY AMPHOMYCIN*

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The phospho-*N*-acetylmuramoyl-pentapeptide-transferase from *Bacillus megaterium* KM was characterized by the transfer reaction. The particulate enzyme preparation had the activity to transfer phospho-*N*-acetylmuramoyl-pentapeptide from UDP-*N*-acetylmuramoyl-pentapeptide to undecaprenoid-1-ol-phosphate. The optimum pH for activity was about 8.5. The reaction required the presence of Mg^{2+} and an SH-protector. With 25 mM Mg^{2+} the maximum activity was observed. The reaction was reversible and so the addition of UMP decreased the formation of undecaprenoid-1-ol-diphospho-*N*-acetylmuramoyl-pentapeptide.

Amphomycin inhibited non-competitively the transferase for the substrate UDP-*N*-acetylmuramoyl-pentapeptide.

In the previous papers[1~3], we have described that amphomycin[4,5] is a specific inhibitor of bacterial cell wall peptidoglycan synthesis[1,2] and that it inhibits phospho-MurNAc-pentapeptide-transferase (UDP-*N*-acetylmuramoyl-L-alanyl-D-γ-glutamyl-γ-lysyl (or *meso*-diaminopimelyl)-D-alanyl-D-alanine: undecaprenoid-1-ol-phosphate phospho-*N*-acetylmuramoyl-pentapeptide-transferase, EC 2.7.8.13), the first step of a lipid cycle in peptidoglycan synthesis in *Bacillus*[3]: the step is of interest because it catalyzes the transfer of a hydrophilic substrate into the lipophilic environment of the membrane.

The properties of the enzyme have been characterized only with particulate and solubilized preparations from *Staphylococcus aureus*[6] and with a solubilized enzyme from *Escherichia coli*[7]. However, the enzymes from Gram-positive rods have never been reported. So, the authors examined the properties of the transferase from the Gram-positive rod *B. megaterium*. It was found that the enzyme could be differentiated from that of *S. aureus* although they both catalyze the following reaction.

$$\text{UDP-MurNAc-pentapeptide} + \text{undecaprenoid-1-ol-phosphate (lipid-P)} \xrightleftharpoons{Mg^{2+}} \text{lipid-P-P-MurNAc-pentapeptide} + \text{UMP}$$

For example, the transferase from *S. aureus* is activated by monovalent cations such as K^+ and NH_4^+[8], but the enzyme from *B. megaterium* KM is not stimulated by these cations.

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Abbreviations: MurNAc-pentapeptide or MurNAc-L-Ala-D-γ-Glu-*meso*-Dpm-D-Ala-D-Ala for *N*-acetylmuramoyl-L-alanyl-D-γ-glutamyl-*meso*-diaminopimelyl-D-alanyl-D-alanine; lipid-P for undecaprenoid-l-ol phosphate.

The present paper deals with the properties of the enzyme of *B. megaterium* KM and with the mode of its inhibition by amphomycin.

Materials and Methods

Bacterial Strain

B. megaterium KM was obtained from Dr. P. E. REYNOLDS, Department of Biochemistry, University of Cambridge, England.

Chemicals

Amphomycin (Na salt) was given by Dr. L. DELCAMBE, International Center of Information of Antibiotics, Belgium. Other chemicals were obtained commercially.

Radiochemicals

(DL+*meso*)-2,6-Diamino-[G-^{3}H]pimelic acid hydrochloride (45 Ci/mmole) was obtained from Commissariat a L'Energie Atomique, France, UDP-MurNAc-L-Ala-D-γ-Glu-*meso*-[^{3}H]Dpm-D-Ala-D-Ala was prepared according to the method of REYNOLDS[9] with some modification. An example of the preparation is described below. A culture (1%) of *B. megaterium* KM grown overnight at 37°C in a medium (pH 5.0), containing 2.0% dehydrated Nutrient broth (Difco), 0.5% yeast extract, 200 μg/ml of L-lysine and 25 μg/ml of *meso*-diaminopimelic acid, was transferred into the same fresh medium (100 ml in a 500-ml Sakaguchi flask), and incubated with shaking at 37°C until the absorbance at 660 nm reached 0.4. The cells were harvested by centrifugation and washed twice with 0.01 M potassium phosphate buffer (pH 7.0) containing 1 mM $MgCl_2$ and 0.8% NaCl. The washed cells were suspended at the concentration of 100 mg wet cells per ml in a medium, containing 4 mg of KCl, 4 mg of $MgCl_2$, 2 mg of NH_4Cl, 0.15 mg of Na_2SO_4, 0.35 mg of $Na_2HPO_4 \cdot 12H_2O$, 10 mg of glucose, 0.04 mg of uracil, 0.125 mg of L-alanine, 0.125 mg of L-glutamic acid, 0.2 mg of L-lysine, 0.05 mg of chloramphenicol, 0.02 mg of vancomycin and 2 μCi (DL+*meso*)-2,6-diamino-[G-^{3}H]pimelic acid hydrochloride per ml, and then incubated with aeration at 37°C for 35 minutes. The incubated cells were harvested by centrifugation, extracted overnight with 5% trichloroacetic acid at 4°C, and then centrifuged at 4,000$\times g$ for 10 minutes. After the supernatant fluid had been washed with ethyl ether to remove the remaining trichloroacetic acid and adjusted to pH 7.0 with 1 N NaOH, it was submitted to column chromatography on Dowex 1$\times$2 (Cl^-) as described by STROMINGER *et al.*[10] Fractions containing UDP-MurNAc-pentapeptide were further submitted to gel filtration through Sephadex G-10 and paper chromatography using Toyo Roshi No. 51A paper and *iso*-butyric acid-acetic acid-1 N NH_4OH (10: 1: 5) as the developing solvent. The extract from the paper chromatogram (Rf 0.25) was desalted by gel filtration with Sephadex G-10 to give a solution of UDP-MurNAc-L-Ala-D-γ-Glu-*meso*-[^{3}H]Dpm-D-Ala-D-Ala (980 μl, 0.7 μmole, specific activity: 12.1 mCi/mmole).

Assay of Phospho-MurNAc-pentapeptide-transferase 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 described by OKA[11]. The assay procedures are as follows unless otherwise stated. A reaction mixture (30 μl), containing 0.11 or 0.33 mM UDP-MurNAc-[^{3}H]pentapeptide (12.1 mCi/mmole), 250 mM tris-HCl buffer (pH 8.5), 25 mM $MgCl_2$, 1.0 mM dithiothreitol and 10 μl of the particulate fraction (200~300 μg of protein), was incubated for 3 minutes at 25°C. After the reaction was stopped by addition of 20 μl of 6 M pyridinium acetate (pH 4.2), the lipid intermediate (undecaprenoid-1-ol diphosphate-MurNAc-pentapeptide) formed was extracted twice with 100 μl of *n*-butanol. The extracts were transferred to a scintillation vial and dried. The radioactivity was determined using a toluene scintillation fluid with a liquid scintillation counter (Aloka ASC-651).

Results

Properties of Phospho-MurNAc-pentapeptide-transferase from *B. megaterium* KM

A particulate enzyme preparation from *S. aureus* is known to catalyze the following two reactions[6].

(1) Transfer reaction

$$\text{UDP-MurNAc-pentapeptide} + \text{lipid-P} \overset{Mg^{2+}}{\rightleftharpoons} \text{lipid-P-P-MurNAc-pentapeptide} + \text{UMP}$$

(2) Exchange reaction

$$[^3\text{H}]\text{UMP} + \text{UDP-MurNAc-pentapeptide} \rightleftharpoons [^3\text{H}]\text{UDP-MurNAc-pentapeptide} + \text{UMP}$$

The phospho-MurNAc-pentapeptide-transferase from *B. megaterium* KM was characterized by the transfer reaction. The particulate enzyme preparation had the activity to transfer phospho-MurNAc-pentapeptide from UDP-MurNAc-pentapeptide to lipid-P as shown in Fig. 1. The reaction proceeded in a straight line for 4 minutes at 25°C at pH 8.5, and then the velocity was decreased. In the following

Fig. 1. Transfer of phospho-MurNAc-pentapeptide from UDP-MurNAc-pentapeptide to lipid-P as a function of time.

The reaction mixture contained 0.11 mM UDP-Mur-NAc-[^{3}H]pentapeptide.

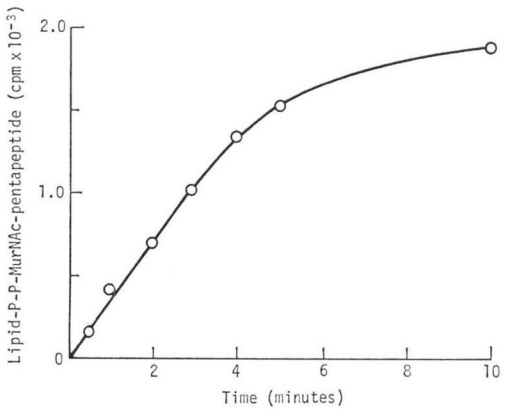


Fig. 2. Effect of pH on the phospho-MurNAc-pentapeptide-transferase activity.

The reaction mixture contained 0.11 mM UDP-MurNAc-[^{3}H]pentapeptide. Incubation time: 5 minutes; ○, 0.1 M tris-HCl buffer; △, 0.1 M sodium citrate buffer.

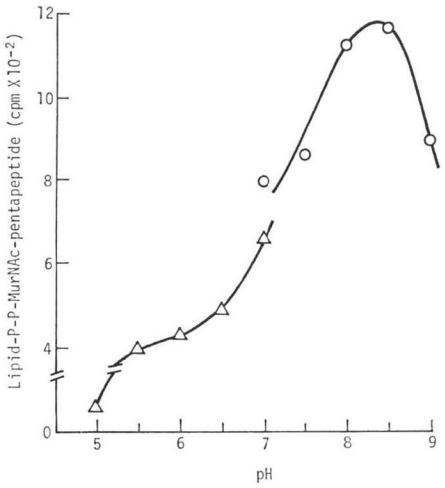


Table 1. Requirements for the phospho-MurNAc-pentapeptide-transferase activity.

Complete reaction mixture: 0.11 mM UDP-MurNAc-[^{3}H]pentapeptide (12.1 mCi/mmole), 0.1 M tris-HCl buffer (pH 8.5), 25 mM $MgCl_2$, 10 mM dithiothreitol and 10 μl of particulate fraction (153 μg of protein). Total volume: 35 μl.

Reaction mixture		Lipid-P-P-MurNAc-pentapeptide cpm (%)
Complete		1,003 (100)
Omission	Mg^{2+}	179 (18)
	Dithiothreitol	329 (33)
	Particulate fraction	13 (1)
Addition	2.5 mM UMP	191 (19)
	25 mM UMP	37 (4)
	0.2 mM ATP	931 (93)

Table 2. Effect of monovalent ions on the phospho-MurNAc-pentapeptide-transferase activity.

Basal reaction mixture: 0.33 mM UDP-MurNAc-[^{3}H]pentapeptide (12.1 mCi/mmole), 10 mM dithiothreitol, 0.1 M tris-HCl buffer (pH 8.5) and 10 μl of particulate fraction (153 μg of protein). Total volume: 35 μl.

Addition*	Lipid-P-P-MurNAc-pentapeptide cpm (%)
Mg^{2+}	1,158 (100)
$Mg^{2+} + NH_4^+$	712 (62)
$Mg^{2+} + Li^+$	791 (68)
$Mg^{2+} + Na^+$	855 (74)
$Mg^{2+} + K^+$	926 (80)
$Mg^{2+} + Rb^+$	931 (80)
$Mg^{2+} + Cs^+$	694 (60)

* Concentrations of $MgCl_2$ and monovalent cations (chloride) were 25 mM and 170 mM, respectively.

experiments, the reactions were stopped after 3 minutes unless otherwise stated, and the amounts of lipid-P-P-MurNAc-pentapeptide formed were determined.

The optimum pH for activity was determined to be about 8.5 as shown in Fig. 2. The activity was significantly weaker at pH values below 7.5 or over 9.0.

The reaction required the presence of Mg^{2+} and an SH-protector and was unaffected by the addition of ATP (Table 1). The maximum activity was observed with 25 mM Mg^{2+}, but the activity decreased rather with over 25 mM of the ion as shown in Fig. 3, while the activity of the enzyme from *S. aureus* has been reported not to decrease even in the presence of over 25 mM of Mg^{2+} [12]. Since HEYDANEK *et al.*[8] have reported that the transferase from *S. aureus* is activated with monovalent cations in addition to Mg^{2+}, the effect of monovalent ions on the enzyme activity of *B. megaterium* KM was examined. As Table 2 shows, the cations tested inhibited the transferase from *B. megaterium* KM, distinguishing this enzyme from that of *S. aureus*. NH_4^+ and Cs^+ (each 25 mM) inhibited the enzyme by 38 and 40%, respectively.

As shown in Table 1, the addition of UMP, another product of the reaction, suppressed the transfer reaction. The addition of increasing concentrations of UMP after 4 minutes of the initiation of the reaction resulted in the progressively increasing effect on the concentration of lipid-P-P-MurNAc-pentapeptide present (Fig. 4). Furthermore, when UMP was added at zero time, the amount of lipid-P-P-MurNAc-pentapeptide formed was equivalent to that which remained when the same amount of UMP was added after 4 minutes. Thus, equilibrium conditions were reached by either route, indicating that the reaction is reversible.

Fig. 3. Effect of Mg^{2+} on the phospho-MurNAc-pentapeptide-transferase activity.

The reaction mixture contained 0.11 mM UDP-MurNAc-[^{3}H]pentapeptide.

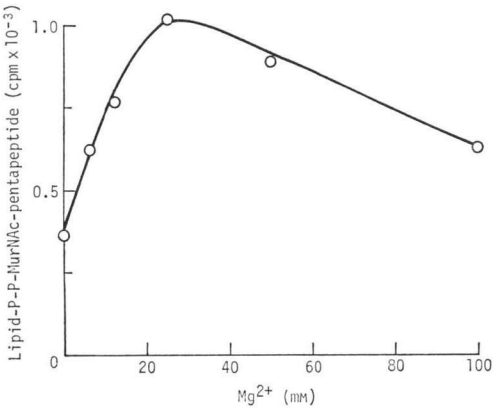
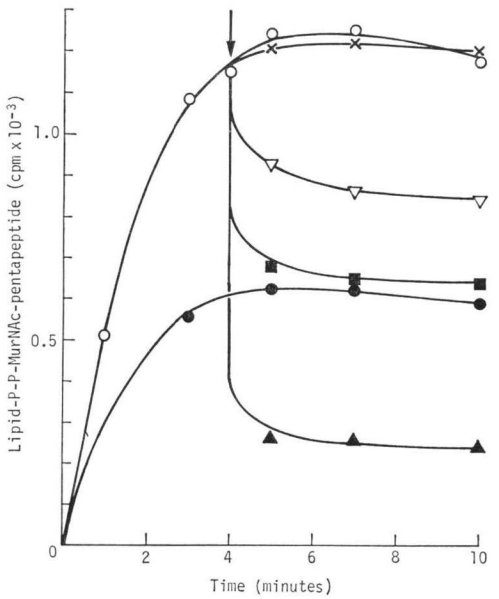


Fig. 4. Effect of UMP on lipid-P-P-MurNAc-pentapeptide formation.

The concentration of UDP-MurNAc-[^{3}H]pentapeptide was 0.11 mM. The arrow shows the addition time for UMP or UDP. ○, control (no UMP or UDP added); ▽, 1 μM UMP; ■, 10 μM UMP; ▲, 100 μM UMP; ×, 100 μM UDP; ●, 10 μM UMP was added at zero time.



Mode of Inhibition of the Phospho-MurNAc-pentapeptide-transferase by Amphomycin

The authors[3] reported that amphomycin is a specific inhibitor of the transferase: it does not inhibit

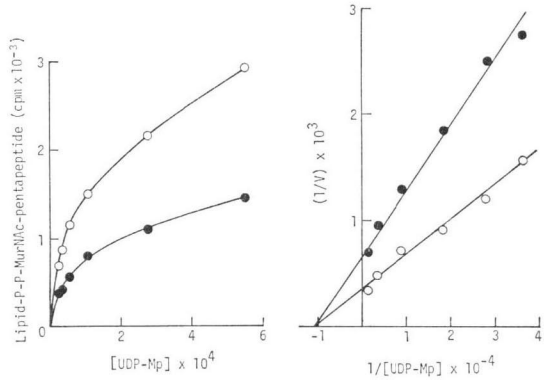
Table 3. Effect of amphomycin on the phospho-MurNAc-pentapeptide-transferase activity.

The concentration of UDP-MurNAc-[^{3}H]pentapeptide was 0.33 mM. Other conditions are described in the text.

Amphomycin (μg/ml)	Lipid-P-P-MurNAc-pentapeptide cpm (%)
0	1,945 (100)
1	1,795 (92)
10	1,622 (83)
20	970 (50)
50	311 (16)
100	0 (0)

Fig. 5. Inhibition of the transfer of phospho-MurNAc-pentapeptide from UDP-MurNAc-pentapeptide (UDP-Mp) to lipid-P by amphomycin.

○, no additions; ●, with amphomycin (30 μg/ml).



the formation of peptidoglycan from lipid-P-P-MurNAc-pentapeptide and UDP-GlcNAc. Table 3 shows the inhibition of the transferase activity by various concentrations of amphomycin. The mode of inhibition by amphomycin was then examined by measuring the formation of lipid-P-P-MurNAc-pentapeptide from various concentrations of UDP-MurNAc-pentapeptide with or without amphomycin (30 μg/ml). As shown in Fig. 5, the LINEWEAVER-BURK plot revealed that the antibiotic inhibits non-competitively the reaction for the substrate UDP-MurNAc-pentapeptide. The *Km* value of the enzyme was tentatively calculated from Fig. 5 to be 93 μM for UDP-MurNAc-pentapeptide but it must be noted that the enzyme was not purified.

Discussion

The transferase of *B. megaterium* KM has the properties similar to those of *S. aureus*[12] with respect to optimum pH and Mg^{2+} requirement. However, it is differentiated from the enzyme of *S. aureus* by the finding that the *B. megaterium* enzyme is somewhat inhibited by monovalent cations and requires an SH-protector (the *S. aureus* enzyme is stimulated by monovalent cations and does not require any SH-protector).

Amphomycin is a specific inhibitor of cell wall peptidoglycan synthesis[1,2], inhibiting non-competitively the phospho-MurNAc-pentapeptide-transferase of *B. megaterium* for the substrate UDP-MurNAc-pentapeptide. After the authors[2] suggested that amphomycin-group antibiotics inhibit the formation of lipid intermediate in peptidoglycan synthesis, several reports concerning its mode of action have appeared. ELBEIN and coworkers[13~15] reported that amphomycin inhibits the transfer of mannose from GDP-mannose and of GlcNAc from UDP-GlcNAc by particulate enzyme preparations from pig aorta[13,14], *Mycobacterium smegmatis*[13], and mung beans[15], and that tsushimycin, an amphomycin-group antibiotic, also inhibits the formation of dolichyl-P-mannose, dolichyl-P-Glc and dolichyl-P-P-GlcNAc with a particulate fraction from pig aorta[16].

On the other hand, tunicamycin[17,18] inhibits selectively the formation of polyisoprenol-P-P-GlcNAc in the biosynthesis of glycoproteins[19,20] and teichoic acid[21], and the phospho-MurNAc-pentapeptide-transferase of *Micrococcus lysodeikticus*[22], but does not inhibit the transfer of mannose and glucose.

Thus, the mechanism of inhibition by amphomycin of the formation of lipid intermediate seems to be different from that by tunicamycin. A determination of mode of inhibition of the phospho-MurNAc-pentapeptide-transferase of *B. megaterium* by tunicamycin would be of interest.

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