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An enzyme catalyzing the liberation of *N*-acetylglucosamine from *N*-acetylglucosaminyl pyrophosphorylpolyprenol in *Bacillus polymyxa* membranes

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A novel enzyme which specifically hydrolyzes *N*-acetylglucosaminyl pyrophosphorylpolyprenol to liberate *N*-acetylglucosamine was found in membranes of *Bacillus polymyxa* AHU 1385. The enzyme seems to be inactive toward α-*N*-acetylglucosaminyl phosphorylundecaprenol, β-*N*-acetylglucosaminyl phosphorylundecaprenol, *N*-acetylglucosamine 1-phosphate, *N*-acetylglucosamine 1-pyrophosphate, or UDP-*N*-acetylglucosamine. Much lower activities of the same enzyme were also found in membranes of several other strains of Bacilli.

N-Acetylglucosaminyl pyrophosphorylpolyprenol hydrolase; *N*-Acetylglucosamine liberation; Glycolipid acetylglucosaminidase; *(Bacillus polymyxa)*

1. INTRODUCTION

N-Acetylglucosaminyl pyrophosphorylundecaprenol (GlcNAc-PP-undecaprenol) has been shown to be an essential intermediate in the syntheses of cell wall teichoic acids and polysaccharides in Gram-positive bacteria [1–3]. In the course of studies on the biosynthesis of wall polymer, we found that incubation of membranes of *Bacillus polymyxa* AHU 1385 with GlcNAc-PP-undecaprenol led to liberation of *N*-acetylglucosamine from this glycolipid. This paper reports the characterization of the enzyme responsible for the liberation of *N*-acetylglucosamine from GlcNAc-PP-polyprenol.

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Abbreviations: GlcNAc-1-PP, *N*-acetylglucosaminyl 1-pyrophosphate; GlcNAc-1-P, *N*-acetylglucosaminyl 1-phosphate; MurAc-pentapeptide, *N*-acetylmuramyl-L-alanyl-D-γ-glutamyl-*meso*-diaminopimelyl-D-alanyl-D-alanine; ManNAc, *N*-acetylmannosamine; AMPCPP, adenosine 5′-(α,β-methylene)triphosphate

2. MATERIALS AND METHODS

2.1. *Materials*

UDP-[*acetyl*-^{14}C]GlcNAc (1.85 GBq/mmol) was enzymatically prepared from [^{14}C]acetate [4,5]. UDP-MurAc[^{14}C]pentapeptide (0.15 GBq/mmol) was prepared enzymatically [6]. [^{14}C]-GlcNAc-PP-undecaprenol, α- and β-[^{14}C]GlcNAc-P-undecaprenols were prepared from UDP-[^{14}C]-GlcNAc as described previously [3,7]. [^{14}C]GlcNAc-PP-dolichol and [^{14}C]GlcNAc(β1⟶4)[^{14}C]-GlcNAc -PP-dolichol were prepared by incubation of UDP-[^{14}C]GlcNAc with microsomes of *Saccharomyces cerevisiae* [8]. [^{14}C]GlcNAc-1-P was obtained from UDP-[^{14}C]GlcNAc by enzymatic hydrolysis. [^{14}C]GlcNAc-1- PP was prepared from [^{14}C]GlcNAc-PP-undecaprenol by phenol treatment as described in [7]. ManNAc(β1⟶4)[^{14}C]-GlcNAc-PP-undecaprenol was prepared as de-

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scribed [2]. MurAc[^{14}C]pentapeptide-PP-undecaprenol was prepared by the method of Bettinger and Young [9]. AMPCPP was purchased from Sigma. Other materials were the same as in previous papers [2,3].

2.2. Bacteria and preparation of membranes

Bacillus polymyxa AHU 1385 and other bacterial strains were given by Dr S. Takao, Hokkaido University. The methods used for culturing bacteria and for preparation of membranes were the same as those described [10].

2.3. *Assay of enzymatic hydrolysis of GlcNAc-PP-polyprenol*

Membranes (40 μg protein, determined according to Lowry et al. [11]) were incubated with 2.5 μM [^{14}C]GlcNAc-PP-undecaprenol (7.63 cpm/pmol) for 10 min at 25°C in 30 μl of 50 mM Tris-HCl, pH 8.2, containing 0.4% Nonidet P-40. The reaction was terminated by the addition of 5 μl of isobutyric acid/0.5 M NH_4OH (5:3, v/v), and the mixture was chromatographed on Toyo no.50 filter paper in the same solvent. The radioactivity of the area of *N*-acetylglucosamine was counted in a liquid scintillation counter.

3. RESULTS AND DISCUSSION

When [^{14}C]GlcNAc-PP-undecaprenol was incubated with membranes of *Bacillus polymyxa* AHU 1385 in the presence of Nonidet P-40, a radioactive substance with the mobility of *N*-acetylglucosamine (R_F = 0.45) on paper chromatograms in isobutyric acid/0.5 M NH_4OH (5:3) was produced (fig.1). The radioactive product was coincident with *N*-acetylglucosamine on paper chromatography in butan-1-ol/pyridine/acetic acid/water (6:4:0.3:3, by vol.) and paper electrophoresis in 50 mM sodium tetraborate, pH 10.0. No intermediary formation of phosphorylated sugar such as GlcNAc-1-P and GlcNAc-1-PP was detected. The hydrolytic activity seems to be located in membranes. Thus, the activity in the soluble fraction resulting from centrifugation of the cell extract at 100000 × *g* was one tenth of the activity in the membrane fraction. The rate of the hydrolytic reaction was maximum at temperatures between 25 and 30°C. The optimal pH of the reaction was between 8.0 and 8.8. Nonidet P-40 was required for the hydrolysis, giving the maximum activity at a concentration of 0.4%. 0.27% Triton X-100 added together with 0.4% Nonidet P-40 inhibited the reaction (table 1). A stronger inhibition was caused by the addition of sodium cholate or sodium deoxycholate. The hydrolase was activated by $MgCl_2$ and $MnCl_2$ and inhibited by EDTA and AMPCPP. NaCl, KCl, a tunicamycin-like antibiotic, 24010 (50 μg/ml), which inhibits the formation of GlcNAc-PP-polyprenol [3], or *N*-acetylglucosamine did not affect the activity.

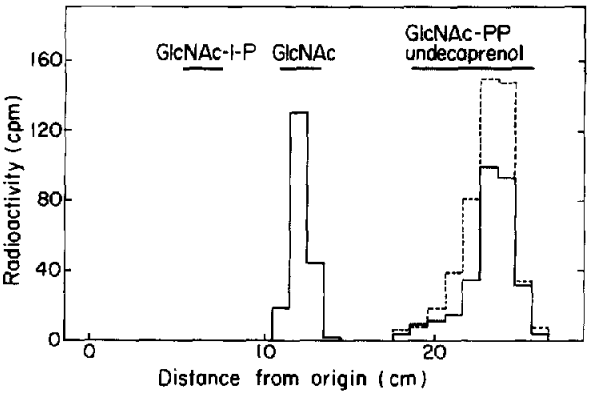

Fig.1. Paper chromatography of the reaction product. 2.5 μM [^{14}C]GlcNAc-PP-undecaprenol was incubated with native enzyme (solid line) or enzyme boiled for 2 min in a water bath (dashed line), and the reaction mixtures were chromatographed on paper as described in section 2. The radioactivity of 1-cm segments from the chromatograms was measured. The positions of standards are shown above by horizontal bars.

GlcNAc-PP-dolichol was also hydrolyzed by the enzyme. However, neither α-GlcNAc-P-undecaprenol nor β-GlcNAc-P-undecaprenol was hydrolyzed by membranes. Furthermore, it was shown that MurAc-pentapeptide-PP-undecaprenol, ManNAc(β-1⟶4)GlcNAc-PP-undecaprenol, or GlcNAc(β-1⟶4)GlcNAc-PP-dolichol was not decomposed by this enzyme. The enzyme did not act on GlcNAc-1-P, GlcNAc-1-PP or UDP-GlcNAc at all. This result also excludes the possibility that *N*-acetylglucosamine was released from GlcNAc-PP-polyprenol through the intermediate formation of GlcNAc-1-P or GlcNAc-1-PP. Thus, it is most likely that this hydrolase recognizes the *N*-acetylglucosamine moiety, the pyrophosphate bridge and the lipid moiety and

Table 1

Effects of detergents, metals and other compounds on GlcNAc-PP-polyprenol hydrolase activity, and substrate specificity of the enzyme

Substrate	Other additions	*N*-Acetylglucosamine liberated (pmol)
GlcNAc-PP-undecaprenol	none	26
	Triton X-100 (0.27%)	14
	cholate (0.10%)	9
	deoxycholate (0.10%)	5
	$MgCl_2$ (10 mM)	57
	$MnCl_2$ (10 mM)	64
	EDTA (1.8 mM)	12
	AMPCPP (1.1 mM)	14
	N-acetylglucosamine (5.4 mM)	25
GlcNAc-PP-dolichol	none	26
	$MgCl_2$ (10 mM)	45
α-GlcNAc-P-undecaprenol	none	0
β-GlcNAc-P-undecaprenol	none	0
GlcNAc-1-P	none	0
GlcNAc-1-PP	none	0
UDP-GlcNAc	none	0

Incubations were carried out as described in section 2 with 2–5 μM *N*-[^{14}C]acetylglucosamine-labeled substrates and other additions, as indicated, in the presence of 0.4% Nonidet P-40. The radioactivity of the area of *N*-acetylglucosamine from the chromatogram was measured

directly hydrolyzes the bond between the *N*-acetylglucosamine and polyprenyl pyrophosphate moieties. However, the formation of polyprenyl pyrophosphate was not ascertained.

The same hydrolytic activity was also found in several other Bacilli, as shown in table 2, but the values of the relative activity were much lower than the value found in *B. polymyxa* AHU 1385. Some bacteria also have an activity which liberates GlcNAc-1-P from GlcNAc-PP-undecaprenol. In the case of *B. cereus* membranes, GlcNAc-1-P was not decomposed to *N*-acetylglucosamine. Thus,

Table 2

Distribution of GlcNAc-PP-polyprenol-hydrolyzing enzymes among Bacilli

Microorganism	*N*-Acetylglucosamine liberated	GlcNAc-1-P liberated
B. polymyxa AHU 1385	1180	0
B. polymyxa AHU 1226	0	87
B. circulans AHU 1365	84	108
B. cereus AHU 1355	42	44
B. subtilis AHU 1035	46	0
B. licheniformis AHU 1371	23	0
B. coagulans AHU 1366	0	280

Membranes (40–70 μg protein) prepared from various bacteria were incubated with 2.5 μM [^{14}C]GlcNAc-PP-undecaprenol in the presence of 10 mM $MgCl_2$ as described in section 2. Radioactivities in *N*-acetylglucosamine and GlcNAc-1-P separated by paper chromatography were determined (results expressed as pmol/10 min per mg protein)

some of these Bacilli may have two kinds of GlcNAc-PP-polyprenol hydrolases; the one liberates *N*-acetylglucosamine and the other liberates GlcNAc-1-P.

The physiological role of the *N*-acetylglucosamine-liberating enzyme in this strain is unknown at present. The cell walls of this strain contain an acidic polysaccharide composed of *N*-acetylglucosamine, *N*-acetylmannosamine and pyruvate (unpublished). It is probable that GlcNAc-PP-undecaprenol participates in the synthesis of this polymer. Therefore, the hydrolase may regulate the amount of undecaprenyl phosphate and, as a result, the synthesis of peptidoglycan and acidic polysaccharide. Further studies on the properties and functions of the hydrolase and on solubilization and partial purification of the enzyme are now in progress.

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