Microbial Synthesis of GDP-glucose

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Received June 17, 1972

GDP-glucose pyrophosphorylase activity was found to be widely distributed in *Streptomyces*. *Streptomyces olivaceus* IFO 3409 showed the highest activity among the strains tested. When air-dried cells of *Streptomyces olivaceus* (100 mg/ml) were incubated with GTP·(NH₄)₃ (20 μmoles/ml), D-glucose-1-phosphate (100 μmoles/ml), potassium phosphate buffer, pH 7.0 (270 μmoles/ml) and MgSO₄ (20 μmoles/ml), GDP-glucose was synthesized in 25% yield against GTP. GDP-glucose was isolated and identified. The strains capable of synthesizing GDP-glucose in higher yield were searched. *Streptomyces* sp. AKU 2801 seems to be the most suitable strain for the preparation of GDP-glucose.

The presence of GDPG was first recognized in the cells of a mold, *Eremothecium ashbyii*, by Pontis *et al.*¹⁾ This sugar nucleotide has also been found in lactating bovine mammary gland,²⁾ pig milk³⁾ and brown algae.⁴⁾ GDPG pyrophosphorylase which catalyzes the synthesis of GDPG was found in *Eremothecium ashbyii*,⁵⁾ *Gleditsia maracantha* seed,⁶⁾ fresh peas,⁷⁾ rat mammary gland and calf liver.⁸⁾ It is a glucosyl donor for the synthesis of glucomannan⁹⁾ and cellulose¹⁰⁾ in mung bean seedling, and for that of trehalose-6-phosphate in *Streptomyces hygroscopicus*¹¹⁾ and mycobacteria.¹²⁾ GDPG glucohydrolase which specifically catalyzes the hydrolysis of GDPG to GDP and D-glucose was found in baker's yeast and purified 135 fold,¹³⁾ although the presence of GDPG in yeasts has not been reported. The presence of GDPG in microorganisms is not so popular as that of GDPM.

The authors have previously reported the fermentative production of GDPM from GMP and D-glucose in the presence of potassium phosphate buffer and MgSO₄ by air-dried cells of baker's yeast^{14,15)} and *Hansenula jadinii*.¹⁶⁾ GDPG has been prepared by chemical synthesis according to the method of

Roseman *et al.*,¹⁷⁾ or a modification of the morpholidata procedure of Roseman *et al.*,¹⁸⁾ but its microbial preparation has not been reported, yet. In the further investigation on the microbial metabolism of guanosine sugar nucleotides, it was found that GDPG pyrophosphorylase activity was present in the crude extract of *Streptomyces*. Therefore, an attempt has been made for the microbial preparation of GDPG from GTP and D-glucose-1-phosphate by air-dried cells of *Streptomyces*.

This paper describes the results of microbial synthesis of GDPG.

MATERIALS AND METHODS

Materials. GTP·(NH₄)₃ was prepared from GMP as reported previously.¹⁴⁾ Phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, GDPG, NADH and 3-phosphoglyceric acid were purchased from Boehringer Mannheim Japan K. K.

Microorganisms. The microorganisms used were actinomycetes, which have been stocked in the Laboratory of Applied Microbiology, Department of Agricultural Chemistry, Kyoto University.

Medium and cultural conditions. The medium for actinomycetes was composed of 5.0 g of peptone, 2.5 g of meat extract, 5.0 g of D-glucose, 2.5 g of glycerol and 1.5 g of NaCl in 500 ml of tap water, pH 7.0. Actinomycetes were cultivated on a reciprocal shaker with a 2000 ml of Sakaguchi flask containing 500

Abbreviations. GMP, GDP, GTP: guanosine 5'-mono-, di-, triphosphates; GDPG: guanosine 5'-diphosphate D-glucose; GDPM: guanosine 5'-diphosphate D-mannose; Gu: guanine; Gu-R: guanosine.

ml of the medium for 3 days at 28°C.

Preparation of enzyme source. After 3 days-cultivation, cells were harvested by filtration and washed two times with 0.01 M potassium phosphate buffer, pH 7.0. The washed cells were suspended in suitable amount of ice-cold 0.01 M potassium phosphate buffer, pH 7.0, containing 0.005 M 2-mercaptoethanol and then treated with ultrasonic oscillator (20 kHz) for 20 min below 10°C. The disrupted cells were centrifuged off and the supernatant was used as a cell-free extract. Air-dried cells of *Streptomyces* were prepared as described previously.¹⁴⁾

Analyses. GDPG, GDP, GTP, guanosine and guanine were determined as reported previously.¹⁵⁾ The reaction products were separated by column chromatography with Dowex 1×2 (Cl⁻ form, 200~400 mesh) according to the method of Cohn and Carter.¹⁹⁾ Glucostat method²⁰⁾ was used to identify the sugar moiety of GDPG. Inorganic phosphate was determined by the method of Fiske-Subbarow.²¹⁾

Assay of GDPG pyrophosphorylase. GDPG pyrophosphorylase activity was determined by following the formation of GTP from GDPG and pyrophosphate according to the method of Verachtert *et al.*²²⁾ The assay mixture contained 1.0 μmole of GDPG, 5.0 μmoles of sodium pyrophosphate, 3.0 μmoles of 3-phosphoglyceric acid, 0.63 μmole of NADH, 1.25 μmoles of neutralized hydrazine sulfate, 5.0 μmoles of MgSO₄, 20 μg of phosphoglycerate kinase, 60 μg of glyceraldehyde-3-phosphate dehydrogenase, 50 μmoles of triethanolamine buffer, pH 7.8, and a suitable amount of cell-free extract of actinomycetes, in a total volume of 2.56 ml. The reaction was carried out in a quartz cuvette at 30°C, and the oxidation of NADH was followed in the Hitachi model 124 double beam type spectrophotometer at the wavelength of 340 mμ. A unit was defined as the amount of enzyme which catalyzes a decrease of 0.01 absorbance at 340 mμ per 1 min under the conditions described above.

Reaction system for GDPG synthesis. The reaction system contained 20 μmoles of GTP·(NH₄)₃, 100 μmoles of D-glucose-1-phosphate, 270 μmoles of potassium phosphate buffer, pH 7.0, 20 μmoles of MgSO₄ and 100 mg of air-dried cells of *Streptomyces* in a total volume of 1.0 ml. One hundred mg of the air-dried cells in a test tube were further dried with P₂O₅ *in vacuo* for 12 hr before use and 1.0 ml of the reaction system except the cells was pipetted into the test tube. The reaction was carried out at 28°C with shaking and terminated by immersing the tube in boiling water for 3 min. The cells were centrifuged off and the supernatant was employed for the determination of the reaction products.

RESULTS AND DISCUSSION

Distribution of GDPG pyrophosphorylase activity in actinomycetes

Although the presence of GDPG glucosylhydrolase in baker's yeast has been reported¹³⁾ and GDPG was found to be the glucosyl donor for the synthesis of trehalose-6-phosphate in *Streptomyces*¹¹⁾ and mycobacteria,¹²⁾ the presence of GDPG pyrophosphorylase activity in microorganisms has not been recognized except for that in *Eremothecium ashbyii*.¹⁾ Therefore, the authors have surveyed yeasts and actinomycetes having GDPG pyrophosphorylase activity, but have not succeeded to find out such a strain in yeasts. The results with actinomycetes are shown in Table I. GDPG pyrophosphorylase activity was widely distributed in *Streptomyces*, and *Streptomyces olivaceus* IFO 3409 showed the highest activity among the strains tested.

Isolation and identification of GDPG

When air-dried cells of *Streptomyces olivaceus* (100 mg/ml) were incubated in the reaction system with GTP·(NH₄)₃ (20 μmoles/ml), D-glucose-1-phosphate (100 μmoles/ml), potassium phosphate buffer (270 μmoles/ml, pH 7.0) and MgSO₄ (20 μmoles/ml), four UV-absorbing spots were detected on the paper chromatogram of the reaction system. Three of them were identified as guanosine, guanine and GDP plus GTP by their chromatographic behaviours. The *R_f* value of the fourth derivative was in good agreement with authentic GDPG or GDPM. The derivative was not synthesized, when any one of GTP, D-glucose-1-phosphate and the air-dried cells was omitted from the reaction system. D-Glucose could not be substituted for D-glucose-1-phosphate.

In order to isolate the derivative, the reaction was carried out on a large scale. The reaction mixture contained 2.6 mmoles of GTP·(NH₄)₃, 13 mmoles of D-glucose-1-phosphate, 19.5 ml of 1.8 M potassium phosphate buffer, pH 7.0, 2.6 mmoles of MgSO₄ and 13 g of

TABLE I. DISTRIBUTION OF GDPG
PYROPHOSPHORYLASE ACTIVITY IN CELL-FREE
EXTRACTS OF ACTINOMYCETES

The activities were assayed as described in Materials and Methods.

Strain	GDPG pyrophosphorylase activity (units/mg of protein)
<i>Mycobacterium avium</i> IFO 3154	0
" " IFO 3082	0
" " IFO 3153	0
" <i>phlei</i> IFO 3158	0
<i>Nocardia gardneri</i> IFO 3385	0
" <i>asteroides</i> IFO 3424	0
" " IFO 3384	0
" " IFO 3423	0
" <i>corallina</i> IFO 3338	0
<i>Streptomyces albus</i> AKU 2201	0
" <i>coelicolor</i> AKU 2202	0
" <i>rimosus</i> IFO 3326	0
" " IFO 3441	0.278
" <i>antibioticus</i> IFO 3117	0
" <i>aureus</i> IFO 3175	0
" <i>lavendulae</i> IFO 3145	0.147
" <i>flavus</i> IFO 3359	0
" <i>griseus</i> IFO 3122	1.03
" " IFO 3430	0.726
" " IFO 3356	0.159
" " IFO 3355	0
" <i>ruber</i> IFO 3310	0.563
" <i>olivaceus</i> IFO 3409	2.96
" <i>fradiae</i> IFO 3439	1.05
" " IFO 3123	0.471
" <i>purpurascens</i> IFO 3389	0.142
" <i>scabies</i> IFO 3111	0.505

the air-dried cells in a total volume of 130 ml. This reaction mixture was divided into 13 portions and each portion in a test tube (24 mm of diameter) was incubated with shaking at 28°C for 2.5 hr. The reaction was terminated by immersing the tubes in boiling water for 3 min. The cells were centrifuged off. The resultant supernatant solution was acidified with 1 N HCl to pH 3.8, and active charcoal was added. The adsorbed nucleotides on the charcoal were eluted with ethanol-ammonia-water solution (50:5:45). The eluate was concentrated at pH 8.0 below 30°C, and the concentrate was charged on a Dowex 1×2 (Cl⁻ form) column. The elution was carried out according to the method of Cohn and

Carter.¹⁹⁾ The derivative was eluted with a mixture of 0.01 N HCl and 0.15 M NaCl as shown in Fig. 1. Active charcoal was added to the fraction, and the adsorbed derivative was eluted with the ethanol-ammonia-water solution. The eluate was concentrated to a small volume below 30°C and lyophilized. About 200 mg of the derivative were obtained as the ammonium salt.

The paper chromatograms of the derivative and its acid-hydrolysate are shown in Fig. 2. The derivative exhibited the same mobility as authentic GDPG or GDPM in the two solvent systems. The hydrolysis of the derivative with 0.1 N HCl for 1 min gave GDP.

The UV absorption spectra of the derivative indicate that it is a guanosine nucleotide, as shown in Fig. 3.

The derivative was treated in 0.01 N H₂SO₄ at 100°C for 20 min, the resultant solution was neutralized with 0.05 N Ba(OH)₂ and the precipitate was centrifuged off. The supernatant was subjected to paper chromatography. D-Glucose was detected in the acid-hydrolysate. This acid-lability indicates that there is a glycosidic bond between D-glucose and GDP. The presence of D-glucose in the acid-hydrolysate was also confirmed by the glucostat method as shown in Table II.

The NMR spectrum of the derivative is shown in Fig. 4. The signals in the spectrum were assigned in the usual manner as follows; δ 8.08 s. (H-8, one proton), δ 5.93 d. ($J_{1',2'} = 5.0$ Hz) (H-1', one proton), δ 5.69 q. ($J_{1'',2''} = 3.5$ Hz, $J_{1'',P} = 6.5$ Hz) (H-1'', one proton). The spectrum was in good agreement with that of authentic GDPG.

The results of the chemical analysis of the derivative are shown in Table II. These data indicate that it contains one mole of GDP for each mole of D-glucose.

Based on the results described above, the derivative was identified as GDPG.

Synthesis of GDPG by Streptomyces sp. AKU 2801

Under the conditions described above, air-dried cells of *Streptomyces olivaceus* rapidly

FIG. 1. Separation of the Products by Dowex 1×2 Column Chromatography.

The products were eluted with mixtures of 0.01 N HCl and NaCl solution whose concentration was varied as shown in the figure. Resin: Dowex 1×2 (Cl^- form), 200~400 mesh, $12.5 \text{ cm}^2 \times 37 \text{ cm}$. One fraction: 20 ml/3 min.

FIG. 2. Paper Chromatograms of the Isolated Derivative and Its Acid-hydrolysate.

A: the isolated derivative, B: its acid-hydrolysate, C: authentic samples. Solvent system I: 95% ethanol: ammonium acetate=7.5: 3, pH 7.5, solvent system II: isobutyric acid: 0.5 N NH_4OH =5: 3. The paper chromatograms were detected under ultraviolet light (2536Å). The acid-hydrolysate was prepared as follows; a solution containing the derivative was acidified to pH 1 and allowed to stand at 100°C for 1 min.

hydrolyzed GTP to GDP, and consequently, the yield of GDPG was low, being only 25% of the GTP added to the reaction mixture (Table III). In order to prepare GDPG on a large scale, we have searched for micro-organisms capable of synthesizing GDPG from GTP in higher yields. As shown in Table IV, *Streptomyces* sp. AKU 2801 was found to be

FIG. 3. UV Absorption Spectra of the Isolated Derivative.

the most suitable strain for the synthesis of GDPG among the strains tested. The yield of GDPG was 60~70% against GTP. A typical time course of GDPG synthesis by *Streptomyces* sp. AKU 2801 is shown in Fig. 5. Intact cells of this strain were not able to synthesize GDPG under the conditions. The effect of various surfactants on the synthesis of GDPG by the intact cells was investigated. As shown in Table V, anionic surfactants were effective for the synthesis of GDPG. When 0.5% of sarcosinate-LT was

FIG. 4. NMR Spectrum of the Isolated Derivative.

The spectrum was recorded on a Varian A-60 spectrometer at 60 MHz in D_2O with DSS as the internal standard.

TABLE II. CHEMICAL ANALYSIS OF THE ISOLATED DERIVATIVE

The results were expressed as μ moles per one mole of guanosine.

Test	Results (μ moles)
Acid-labile phosphate	1.08
Total phosphate	2.01
D-Glucose ^{a)}	0.89
GDP ^{b)}	0.80

^{a)} The hydrolysis was carried out for 20 min at 100°C in 0.01 N H_2SO_4 .

^{b)} The hydrolysis was carried out for 1 min at 100°C in 0.1 N HCl. The amount of GDP was paper-chromatographically determined as reported previously.¹⁵⁾

TABLE III. SYNTHESIS OF GDPG BY AIR-DRIED CELLS OF *Streptomyces olivaceus*

The reaction mixture contained 20 μ moles of $GTP \cdot (NH_4)_3$, 100 μ moles of D-glucose-1-phosphate, 270 μ moles of potassium phosphate buffer (pH 7.0), 20 μ moles of $MgSO_4$ and 100 mg of the air-dried cells in a total volume of 1.0 ml. The reaction was carried out with shaking at 28°C.

Incubation time (hr)	Substrate and products found (μ moles/ml)					
	GTP	GDP	GDPG	GMP	Gu-R	Gu
1	8.1	10.6	1.4	0	0	0
2	2.4	13.6	3.6	0	0.2	0.3
3	0	10.7	5.0	1.5	1.3	1.0
4	0	8.1	4.4	2.5	2.5	2.0

FIG. 5. A Typical Time Course of GDPG Synthesis by Air-dried Cells of *Streptomyces* sp. AKU 2801.

The reaction mixture contained 20 μ moles of $GTP \cdot (NH_4)_3$, 100 μ moles of D-glucose-1-phosphate, 270 μ moles of potassium phosphate buffer, pH 7.0, 20 μ moles of $MgSO_4$ and 100 mg of the air-dried cells in a total volume of 1.0 ml. The reaction was carried out with shaking at 28°C.

added to the reaction system, GDPG was synthesized from GTP in 70% yield by the intact cells.

From the data described above, *Streptomyces* sp. AKU 2801 seems to be suitable for the preparation of GDPG.

TABLE IV. SYNTHESIS OF GTPG BY AIR-DRIED CELLS OF *Streptomyces*

The reaction mixture contained 20 μ moles of GTP $\cdot$ (NH₄)₃, 100 μ moles of D-glucose-1-phosphate, 270 μ moles of potassium phosphate buffer, pH 7.0, 20 μ moles of MgSO₄ and 100 mg of air-dried cells of *Streptomyces* in a total volume of 1.0 ml. The reactions were carried out with shaking at 28°C for 4 hr.

Strain	GTPG produced (μ moles/ml)
<i>Streptomyces albus</i> AKU 2201	3.05
" <i>coelicolor</i> AKU 2202	1.78
" <i>rimosus</i> IFO 3341	2.12
" <i>olivochromogenes</i> IFO 3173	2.21
" <i>antibioticus</i> IFO 3117	2.12
" <i>aureus</i> IFO 3175	2.41
" <i>lavendulae</i> IFO 3145	0
" <i>flavus</i> IFO 3359	4.40
" <i>ruber</i> IFO 3110	0
" <i>griseus</i> IFO 3122	2.20
" " IFO 3430	3.60
" " IFO 3356	0
" <i>fradiae</i> IFO 3360	0
" " IFO 3123	5.92
" <i>purpurascens</i> IFO 3389	2.45
" <i>hygroscopicus</i> IFO 3192	2.20
" <i>griseolus</i> IFO 3403	5.50
" <i>scabies</i> IFO 3111	4.02
" sp. AKU 2801	12.7
" sp. AKU 2802	7.61
" sp. AKU 2811	1.14
" sp. AKU 2823	1.78
" sp. AKU 2831	0
" sp. AKU 2841	4.91

Acknowledgements. The authors wish to thank Professor T. Tochikura, Department of Food Science and Technology, Kyoto University and Professor H. Yamada, Research Institute for Food Science, Kyoto University for their helpful discussion and advice during the course of this work. The authors are also indebted to Dr. T. Shingu, Faculty of Pharmacology, Kyoto University for measuring the NMR spectrum and Dr. N. Kashimura, Department of Agricultural Chemistry, Kyoto University for his kind interpretation of the NMR spectrum.

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TABLE V. EFFECT OF SURFACTANTS ON GTPG SYNTHESIS BY THE INTACT CELLS

The reaction mixture contained 20 μ moles of GTP $\cdot$ (NH₄)₃, 100 μ moles of D-glucose-1-phosphate, 270 μ moles of potassium phosphate buffer (pH 7.0), 20 μ moles of MgSO₄, 30 mg of intact cells and 5.0 mg of surfactant in a total volume of 1.0 ml. The reactions were carried out with shaking at 28°C.

Surfactant ^{a)}	GTPG found (μ moles/ml) 2 hr	4 hr
LPC	0	0
Diamitol	0	0
Tween 80	0	0
BWA-20	0	0
PMS-IG	0	0
KLS	6.6	8.7
SLP	0.7	1.8
OTP	9.6	—
SBL-4T	10.6	11.5
Sarcosinate-LT	11.9	14.9

^{a)} LPC: laurylpyridinium chloride; Diamitol: benzalkonium chloride; Tween 80: polyoxyethylene sorbitan monooleate; BWA-20: lanolinalcohol derivative; PMS-IG: propyleneglycol monostearate; KLS: potassium lauryl sulfate; SLP: sodium lauryl phosphate; OTP: sodium di-2-ethylhexyl sulfosuccinate; SBL-4T: triethanolamine lauryl polyethenoxy sulfate; Sarcosinate-LT: triethanolamine lauryl sarcosinate.

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