The synthesis of guanosine 5′-diphosphate D-glucose by enzyme extracts of mung beans *(Phaseolus aureus)* and other higher plants

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In response to a statement in the recent literature that the enzyme responsible for the synthesis of guanosine 5′-diphosphate D-glucose had been found only in pea seeds, enzyme extracts were prepared from a variety of higher plants. When incubated with guanosine 5′-triphosphate, α-D-glucopyranosyl phosphate and $MnCl_2$, they were all shown to catalyze the synthesis of a compound characterized as guanosine 5′phate D-glucose. The most active fractions from maize and mung bean extracts were obtained by consecutive precipitation of protein with monomethyl polyethylene glycol and ammonium sulfate. Based upon these results, it seems likely that the synthesis of GDP-D-glucose is ubiquitous in higher plants.

GDP-D-glucose Phaseolus aureus *β-1,4-Glucan*

1. INTRODUCTION

In a recent review concerned with the biosynthesis of cellulose Delmer [1], gave as an argument against the participation of guanosine 5′-diphosphate D-glucose (GDP-D-glucose) in that process that the enzyme catalyzing the synthesis of that sugar nucleotide had been found only in the pea seed. She further stated that neither she, Heiniger and Franz [2] nor other unnamed investigators had been able to find it elsewhere. In fact, the presence of the activity in several other species was reported 20 years ago [3]. Here, I wish to confirm that observation and give further evidence to suggest that it is probably ubiquitous in higher plants. The role of GDP-D-glucose, if any, in the formation of cellulose is not clarified thereby, but its widespread synthesis indicates that, at least, it should be considered in any overall theory of cell wall formation and its regulation.

2. MATERIALS AND METHODS

2.1. *Reagents*

α-D-Glucopyranosyl phosphate labeled uniformly with ^{14}C (261 μCi/μmol) was obtained from Amersham. Monomethyl polyethylene glycol (Me-PEG) (average M_r 5000) was from Polysciences, and nucleoside triphosphates and purified $(NH_4)_2SO_4$ were from Sigma.

2.2. *Paper chromatography and electrophoresis*

Paper electrophoresis was carried out on Schleicher and Schuell no.589 Orange Ribbon paper with an apparatus like that described by Crestfield and Allen [4] at 25–30 V/cm. Schleicher and Schuell no.589 White Ribbon paper was used for partition chromatography with solvent I, *n*-propanol:ethyl acetate:water (7:1:2, v/v), or solvent II, 95% ethanol:1 M ammonium acetate (7:3, v/v).

2.3. *General preparation of enzyme extracts*

Plant material was obtained from local markets when possible, green barley and mung bean leaves were grown under Gro-Lux bulbs in the laboratory, and tobacco leaves were a gift from Mr Richard Wadleigh, Department of Entomology. Ammonium sulfate fractions (25–40% saturation) were prepared from plant extracts as described [3].

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2.4. *Estimation of GDP-D-glucose formation*

A typical reaction mixture contained: 0.01 μmol α-D-glucopyranosyl phosphate (U-^{14}C, about 0.05 μCi), 0.1 μmol GTP (2Na), 0.15 μmol $MnCl_2$, 0.5 μmol Tris-HCl, pH 7.5, 0.2 μmol β-mercaptoethanol in a total volume of 28 μl. The mixture was incubated in a sealed, thin-walled capillary tube for 30 min at 37°C. It was applied to an electrophoresis paper moistened with 0.1 M ammonium formate buffer, pH 2.7, on the apparatus. At 1400 *v* (32 *v*/cm) GDP-D-glucose is separated from its ^{14}C-labeled precursor, α-D-glucopyranosyl phosphate, in about 100 min. Radioactive compounds were made visible by exposure of the dried electrophoresis papers to X-ray film (Kodak, XAR-5). For purposes of the survey, visual examination of the film sufficed to detect the synthesis of GDP-D-glucose. Its identity was confirmed by mixing the radioactive compound eluted from the electrophoresis paper with 0.1 μmol authentic GDP-D-glucose and chromatographing the mixture with solvent II. The coincidence of radioactivity and UV absorption on that chromatogram was taken as evidence of the synthesis of GDP-D-glucose. When quantitative data were required, the appropriate areas were cut from the electrophoresis or chromatography papers, shaken for 30 min in a scintillation vial with 1 ml water and, after the addition of 10 ml of a Triton scintillation cocktail [5], counted in a Beckman Model LS-230 scintillation spectrometer.

3. RESULTS

Enzyme extracts of the following plant parts were examined for their ability to catalyze the synthesis of GDP-D-glucose from α-D-glucopyranosyl phosphate and GTP: tobacco leaf, spinach leaf, green barley sprouts, broccoli flower buds, broccoli stem, carrot root, celery stem, mung bean primary leaves, mung bean seedlings, ground dry mung bean seeds, fresh maize kernels and maize seedlings. All of those brought about the synthesis of GDP-D-glucose by the criteria given above. In many cases, much of the α-D-glucopyranosyl phosphate in the reaction mixtures was diverted to maltodextrins, presumably by starch phosphorylase, and, as usual with crude plant enzyme extracts, there was considerable hydrolysis of GTP by endogenous phosphatases.

Extracts of fresh maize kernels and, later, mung bean seedlings were chosen for more detailed examination since they provided a considerably more concentrated source of protein. For experimental purposes, newly germinated mung bean seedlings proved to be a better source of the enzyme since the bean extracts were less active in the synthesis of ADP-D-glucose and CDP-D glucose than were those from maize. A modification of the usual procedures prevented the loss of activity that frequently occurred in preparations from the maize and mung bean seedlings. It was apparently due to the adsorption of enzyme by extraneous protein and its irreversible precipitation by $(NH_4)_2SO_4$. After treatment with $MnCl_2$ and before the precipitation of protein with salt, the extract was fractionated by the addition of a 50% aqueous solution of Me-PEG [6]. The precipitate obtained between 3 and 8% Me-PEG was dissolved in buffer and fractionated with $(NH_4)_2SO_4$ as usual.

Enzyme extracts of seedlings germinated overnight in an aerosol cabinet at 37°C catalyzed the synthesis of GDP-D-glucose and UDP-D-glucose and, to a lesser extent, CDP-D-glucose and ADP-D-glucose when incubated with α-D-glucopyranosyl phosphate, $MnCl_2$, and the appropriate nucleotide triphosphate. Seedlings germinated under water at room temperature overnight were less advanced in growth than the others, and it was found by the above criteria that they produced UDP-D-glucose and GDP-D-glucose but little CDP-D-glucose or ADP-D-glucose. Those extracts were chosen for further purification.

A column containing 1 ml of GTP-agarose gel (Sigma) was washed extensively with 0.05 M Tris-HCl, 0.02 M mercaptoethanol buffer, pH 7.5. One ml (15 mg protein) of the mung bean 25–40% $(NH_4)_2SO_4$ fraction of the 3–8% Me-PEG precipitate was applied to the column, allowed to flow through and then returned to the column. That was repeated 3 times. The agarose on the column was then washed with 30 ml of the buffer. Enzyme was eluted with 3 ml of 0.025 M GTP. The enzyme in this eluate appeared to be quite unstable although the addition of 10 mg bovine serum albumin improved the stability somewhat. The enzyme was concentrated to that volume originally applied to the column by passage of the eluate through an ultrafilter (Amicon, Y-10 membrane). If GTP was to be removed, the concentra-

tion process was repeated several times following dilution with 10 ml Tris buffer before the next concentration. Considerable activity was lost in this purification even with the addition of serum albumin. Freezing destroyed the activity completely, and most was lost after storage for 2 or 3 days at refrigerator temperatures.

3.1. *Characterization of the product*

At pH 2.7 GDP-monosaccharides migrate upon electrophoresis more slowly than hexose monophosphates because, in addition to the two negative charges on the phosphate groups, there is also a partial positive charge on one of the ring nitrogens in guanine (position 7, pK 1.9) [7]. The relative movements of the D-glucosyl nucleotide sugars and hexose monophosphates are given in table 1. The radioactive compound formed in these experiments moved at pH 2.7, as predicted, and was coincident with authentic GDP-D-glucose. Upon paper chromatography in solvent I, it was also indistinguishable from authentic GDP-D-glucose. When hydrolyzed (pH 2, 15 min, 100°C), the ^{14}C-labeled compound moved with authentic D-glucose upon electrophoresis in 0.5 M sodium borate buffer, pH 9, and upon chromatography in solvent I. Those two systems separate D-glucose from any of the other hexoses ordinarily found in nature.

Table 1

Relative electrophoretic movement of various compounds on paper at pH 2.7

Compound	Distance migrated (cm)	$R_{picrate}$
Reference compound (picrate)	16	1.00
UDP-D-glucose	17	1.06
Hexose monophosphate	14	0.87
GDP-D-glucose	11.7	0.73
ADP-D-glucose	9	0.56
CDP-D-glucose	9	0.56

3.2. *Characteristics of the reaction*

The reaction rate was proportional to time. In a typical reaction mixture the $(NH_4)_2SO_4$ fraction from mung bean seedlings (0.3 mg protein) catalyzed the synthesis of 7 nmol GDP-D-glucose in 30 min at 37°C. When enzyme preparations at that stage of purification were used, the extent of the reaction was increased markedly by $CoCl_2$ and less so by several other divalent cations (table 2).

Table 2

The effect of several divalent cations on the enzyme-catalyzed synthesis of GDP-D-glucose

Enzyme preparation	Percent of total ^{14}C in GDP-D-glucose upon addition of metal chloride				
	Addition:				
	None	$MnCl_2$	$MgCl_2$	$CoCl_2$	$NiCl_2$
3–8% Me-PEG/ 25–40% $(NH_4)_2SO_4$	2	23	12	47	20
Eluate from GTP affinity column	2	10.6	7	12.4	7.5

Reaction mixtures contained: enzyme (derived from 1–2 g mung bean seedlings), 0.5 μmol Tris-HCl, pH 7.5, 0.2 μmol β-mercaptoethanol, 0.01 μmol (0.05 μCi) α-D-[^{14}C]glucopyranosyl phosphate, 0.15 μmol metal chloride in a total volume of 28 μl. Reaction mixtures were incubated in thin-walled capillaries for 30 min at 37°C. The extent of synthesis of GDP-D-glucose was estimated as described in the text

Table 3

Evidence that GDP-D-glucose and UDP-D-glucose are not synthesized by the same enzyme

Enzyme	Percent of total ^{14}C	
	GDP-D-glucose	UDP-D-glucose
3–8% Me-PEG/ 25–40% $(NH_4)_2SO_4$	23	76
GTP-agarose eluate	13.7	2.2

Enzyme was obtained from mung bean seeds germinated overnight under water at room temperature. Enzyme was prepared by precipitation with monomethyl polyethylene glycol and ammonium sulfate and subsequently by adsorption and elution from a GTP-agarose column. Reaction mixtures contained: enzyme (derived from 1–2 g mung bean seedlings), 0.5 μmol Tris-HCl, pH 7.5, 0.2 μmol β-mercaptoethanol, 0.01 μmol (0.05 μCi) α-D-[^{14}C]glucopyranosyl phosphate, 0.15 μmol $MnCl_2$, 0.1 μmol GTP or UTP in a total volume of 28 μl. Mixtures were incubated in thin-walled capillaries and nucleotide sugars were isolated and the extent of their synthesis estimated as described in the text

However, when the enzyme was further purified on the GTP-agarose affinity column, the effects of Co^{2+} and Mn^{2+} were about the same (table 2). Synthesis of GDP-D-glucose seems to be brought about by a specific enzyme, not UDP-D-glucose pyrophosphorylase, since purification of the extract on an affinity column considerably enhanced the synthesis of GDP-D-glucose over UDP-D-glucose (table 3). There was nothing to indicate that the mung bean enzyme or the reaction it catalyzes is significantly different from that in peas [3,8] or mammary gland [9].

4. DISCUSSION

The purpose of this investigation was to demonstrate that despite contrary statements the specific synthesis of GDP-D-glucose, or of the enzyme that catalyzes that synthesis, is widespread in higher plants. The compound itself has already been isolated from several plant species [10–14] although no systematic study of its distribution has been made. For example, in extracts of *Mycobacterium smegmatis* GDP-D-glucose can serve as a donor of D-glucose in the synthesis of trehalose phosphate, but in that system ADP-D-glucose is equally effective [15]. Sucrose synthetase will also use GDP-D-glucose, but that enzyme is quite unspecific for its glucosyl nucleotide [16]. As far as I know, there is no biochemical reaction in which it has been found to function as the specific donor of D-glucose units except in the much disputed synthesis of a β-1,4-D-glucan and glucomannan in particulate fractions from mung bean roots and other plant parts [5,17].

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