BBA 65575

NUCLEOSIDE DIPHOSPHATE GLUCOSE PYROPHOSPHORYLASES IN MAST CELL TUMORS

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(Received December 19th, 1966)

SUMMARY

1. Mast cell tumors were shown to contain uridine diphosphate glucose pyrophosphorylase (UTP:α-D-glucose-1-phosphate uridylyltransferase, EC 2.7.7.9) and guanosine diphosphate glucose pyrophosphorylase (GTP:α-D-glucose-1-phosphate guanylyltransferase). The two activities were separated from each other by ammonium sulfate fractionation and are thus distinct enzymes.

2. The GDP-glucose pyrophosphorylase fraction was purified further on DEAE-cellulose and some of its properties were studied. The enzyme was shown to catalyze the synthesis of GDP-glucose from D-glucose-1-phosphate and GTP. Other nucleoside triphosphates did not yield nucleoside diphosphate glucose when incubated with this enzyme preparation.

3. The enzyme also catalyzed, to a lesser degree, the formation of guanosine diphosphate mannose from GTP and D-mannose 1-phosphate. Mannose 1-phosphate also had an inhibitory effect on the synthesis of GDP-glucose.

4. Fibrosarcomas and umbilical cord do not contain any detectable amount of GDP-glucose pyrophosphorylase although they do have UDP-glucose pyrophosphorylase activity.

INTRODUCTION

The important role of nucleoside diphosphate sugars in the biosynthesis of polysaccharides is well known and the work in this field has been reviewed recently[1]. These substances and the enzymes involved in their synthesis, *i.e.*, the nucleoside diphosphate sugar pyrophosphorylases, have been found to be present in numerous animal tissues and microorganisms[2]. In the formation of glycogen and various mucopolysaccharides, the nucleoside component has been shown to be uridine. Other nucleoside diphosphate sugars have also been found to be involved in biosynthetic reactions as in the case of starch or certain bacterial polysaccharides[1].

Previous reports have demonstrated that glucose can serve as a precursor in the biosynthesis of heparin[3–5]. The present investigations were initiated to elucidate some

of the intermediary reactions in the synthetic pathway. The tissue used for these studies was a transplantable mast cell tumor which contains comparatively high levels of heparin[6]. One of the primary reactions expected in the biosynthetic sequence from glucose to heparin is the formation of a nucleoside diphosphate glucose from D-glucose 1-phosphate and nucleoside triphosphate. Studies were therefore carried out to characterize the enzyme which catalyzes this reaction, *i.e.*, nucleoside diphosphate glucose pyrophosphorylase. Preliminary investigations showed that mast cell tumors contain two pyrophosphorylases. One of them is specific for uridine diphosphate glucose (UTP:α-D-glucose-1-phosphate uridylyltransferase, EC 2.7.7.9) and the other for guanosine diphosphate glucose (GTP:α-D-glucose-1-phosphate guanylyltransferase)[7]. This paper describes the separation and purification of the enzymes together with some of their characteristics.

EXPERIMENTAL PROCEDURES

Materials

The substrates, coenzymes, phosphoglucomutase (α-D-1,6-diphosphate:α-D-glucose-1-phosphate phosphotransferase, EC 2.7.5.1) and glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate:NADP oxidoreductase, EC 1.1.1.49) were purchased from Sigma Biochemical Co., and Calbiochem. Co. Glucose oxidase (β-D-glucose:O_2 oxidoreductase, EC 1.1.3.4) and hyaluronidase (hyaluronate glycanohydrolase, EC 3.2.1.35) were the products of Worthington Corp.

The tissue used in the major part of this study was the Furth mast cell tumor growing subcutaneously in LAF mice[6]. The tumors were transplanted every 10–14 days. The fibrosarcomas employed for comparative purposes were obtained from rats as described in previous report[8].

Analytical methods

Protein was assayed by the method of LOWRY *et al.*[9]. Hexose was determined by the procedure of PARK AND JOHNSON[10] and pentose according to BROWN[11]. Phosphate was analyzed by the FISKE AND SUBBAROW method[12].

Paper chromatography

Paper chromatography on Whatman No. 1 filter paper was carried out with the following solvent: (A) ethanol–1 M ammonium acetate (pH 7.5) (7.5:3, v/v), (B) ethanol–1 M ammonium acetate (pH 3.8) (7.5:3, v/v)[13], (C) isobutyric acid–ammonium hydroxide–water (pH 4.3) (57:4:39, v/v)[14], (D) ethyl acetate–pyridine–acetic acid–water (5:5:1:3, v/v), (E) butanol–acetic acid–water (4:1:5, v/v, upper phase).

Nucleotides and their derivatives were located with an ultraviolet lamp. For detection of sugars the papers were stained with an alkaline silver reagent[15]. D-Glucose was also identified on paper chromatograms with glucose oxidase[16]. Phosphate derivatives were located with the spray reagent of HANES AND ISHERWOOD[17].

Enzyme assays

Quantitative determinations of nucleoside diphosphate sugar pyrophosphorylase activity were carried out in the direction of pyrophosphorolysis by a modification

of the procedure of MUNCH-PETERSON[18]. The standard assay mixture contained 0.40 μmole nucleoside diphosphate sugar (UDP-, GDP-, or ADP-Glc), 8.0 μmole $MgCl_2$, 1.0 μmole cysteine, 1.0 μmole NADP, 1.25 units glucose-6-phosphate dehydrogenase, and 0.02 units phosphoglucomutase in 0.94 ml of 0.05 M Tris buffer pH 7.4. To this were added 0.05 ml of enzyme extract and 0.01 ml of 0.01 M sodium pyrophosphate. The reaction was followed by measuring the change in absorbance at 340 mμ per min for at least 10 min. Controls which were run simultaneously were made up of the same mixture except that either pyrophosphate or enzyme extract was deleted. A unit of enzyme activity was defined as the amount catalyzing the formation of 0.1 μmole of D-Glc-1-*P* per min, which corresponds to an increase in absorption at 340 mμ of 0.630 per min[19].

In certain cases where the experimental conditions for measuring pyrophosphorylase activity was expected to be suboptimal for phosphoglucomutase and glucose-6-phosphate dehydrogenase, *e.g.*, in the determination of pH optimum, the assay was carried out in two steps. After incubating the nucleoside diphosphate sugar and enzyme extract for 5 min in the desired medium, the reactions were stopped by boiling and an aliquot was then assayed for Glc-1-*P* under appropriate conditions.

Qualitative assays were also performed in the direction of nucleoside diphosphate sugar synthesis. The reaction mixture containing 3 μmole hexose 1-phosphate, 1 μmole nucleoside triphosphate, 2 μmole $MgCl_2$ and 2 mg enzyme protein in 1 ml of 0.05 M Tris buffer pH 7.4 was incubated for 1 h at 37°. The reaction was stopped by heating the mixture for 1 min in a boiling-water bath and the resultant precipitate was removed by centrifugation. The solution was passed through activated charcoal which then was washed with water. The nucleosides and their derivatives were eluted from the charcoal with 50% ethanol and chromatographed in solvents A, B, and C together with the appropriate standards.

Enzyme extract

The mastocytoma tissue was homogenized with 5 volumes of acetone at 0–5° and the mixture was filtered. The precipitate was homogenized a second time and the powder obtained was dried and kept at −20° for the next step. When kept in this form, there was no loss in pyrophosphorylase activity for at least 2 weeks.

Crude extracts for preliminary assays were prepared by extracting the acetone powder with a solution (10 ml/g) containing 0.1 M Tris, 0.01 M $MgCl_2$, 0.001 M EDTA and 0.001 M mercaptoethanol, adjusted to pH 7.5. The activity in the supernatant solution obtained after centrifugation at 18 000 × *g* was then determined.

RESULTS

Nucleoside diphosphate glucose pyrophosphorylase activity

Preliminary investigations on the tissue extract by spectrophotometric assay showed the presence of pyrophosphorylase activity for UDP-D-glucose and GDP-D-glucose. No reaction took place with ADP-D-glucose. Similarly, qualitative determination for activity in the direction of synthesis, as revealed by the paper chromatographic analysis, demonstrated that the respective nucleoside diphosphate sugars were formed only when D-glucose-1-*P* was incubated with UTP or GTP. When ATP, CTP or TTP were used as substrates together with Glc-1-*P*, no nucleoside

diphosphate glucose could be detected. Subsequent studies were therefore carried out to determine whether the UDP-Glc pyrophosphorylase could be separated from the GDP-Glc pyrophosphorylase.

Enzyme fractionation and purification

The following procedures were carried out at 0–5°. Acetone powder obtained from 90 g of mastocytoma was stirred for 30 min with 250 ml of the extracting medium described in the preceding section and the mixture was centrifuged at 18 000 × *g*. The precipitate was then extracted with another 200 ml and the two supernatants were combined (crude extract).

20 ml of 0.7 M $MnCl_2$ were added to the crude extract and the resultant precipitate was discarded. To 400 ml of the supernatant were added 80 g of ammonium sulfate and the precipitate was collected by centrifugation (P-I). Another 80 g of ammonium sulfate were then added to the supernatant .This gave a second precipitate (P-II) which was collected by centrifugation.

The two precipitates were redissolved in a solution of the same composition as that used in the extraction of the acetone powder and dialyzed overnight against 30 volumes of the same solution. The small amount of solid which appeared after dialysis was separated and discarded.

The results of this fractionation are shown in Table I. It is seen that Fraction

TABLE I

FRACTIONATION OF NUCLEOSIDE DIPHOSPHATE GLUCOSE PYROPHOSPHORYLASE ACTIVITIES

The P-I fraction precipitated from 20% ammonium sulfate; the P-II fraction precipitated between 20–40% ammonium sulfate.

Fraction	*Volume (ml)*	*Protein (mg)*	*UDP-Glucose**		*GDP-Glucose**	
			Total units	*Specific activity***	*Total units*	*Specific activity***
Crude extract	410	3497	102	0.029	63.6	0.018
P-I	32	253	2.6	0.08	31.0	0.122
P-II	48	1070	27.9	0.026	0.423	0.005

* Each of these were used as substrates. ADP-Glc showed no activity. The units are defined in the text.

** Activity per mg of protein.

P-I contains the GDP-Glc pyrophosphorylase and only trace amounts of UDP-Glc pyrophosphorylase. Furthermore, this step effected an appreciable increase in specific activity of the GDP-Glc enzyme. Fraction P-II, on the other hand, had UDP-Glc pyrophosphorylase activity with only negligible amounts of the GDP-Glc enzyme. There was no purification of the UDP-Glc enzyme by this procedure and the recovery was only 27%. Subsequent observations revealed that there was an appreciable amount of UDP-Glc pyrophosphorylase activity still left in the supernatant after precipitation from 40% ammonium sulfate.

The separate fractions were incubated with nucleoside triphosphates and Glc-1-*P* and the products were identified by paper chromatography in solvents A, B and C.

The results further substantiated the separation of the pyrophosphorylases between the two fractions.

GDP-glucose pyrophosphorylase

Purification with DEAE-cellulose—10 ml of Fraction P-I were dialyzed overnight against a liter of 0.2 M Tris buffer pH 8. The dialyzed solution was passed through a column of DEAE-cellulose, 1.5 cm × 12 cm, previously equilibrated with 0.2 M Tris at pH 8. After washing the column with 50 ml of the same buffer, the protein was eluted with 30 ml portions of buffer–NaCl solutions. The concentrations of the NaCl in the eluting mixtures were from 0.05–0.40 M in successive increments of 0.05. Fractions of 3 ml were collected and aliquots from each tube were assayed for protein and pyrophosphorylase activity. The GDP-Glc pyrophosphorylase was found in fractions eluted with 0.25 M NaCl (Fraction D-I). The fractions eluted with 0.40 M NaCl contained minute amounts of UDP-Glc pyrophosphorylase activity. Table II shows the data on Fraction D-I together with related information on Fraction P-I. This fraction did not contain any detectable UDP- or ADP-Glc pyrophosphorylase activity when determined spectrophotometrically in the direction of pyrophosphorolysis. Similarly, when assayed for activity in catalyzing synthesis of nucleoside diphosphate glucose from Glc-1-*P* and different nucleoside triphosphates, positive results were obtained only when GTP was the nucleotide component.

TABLE II

PURIFICATION OF GDP-GLUCOSE PYROPHOSPHORYLASE

Purification and *Recovery* were calculated with respect to activity in the crude extract (Table I).

Fraction	*Volume (ml)*	*Protein (mg)*	*Total units*	*Specific activity*	*Purification*	*Recovery (%)*
P-I	10	79	9.70	0.12	7	48
D-I	6	14	6.49	0.46	27	32

Enzymatic synthesis of GDP-glucose—In order to establish unequivocally the reaction catalyzed by the enzyme, a larger scale experiment was carried out wherein the nucleoside diphosphate sugar could be isolated and characterized. 6 ml of a solution containing 3 mM Glc-1-*P*, 2 mM GTP, 1 mM EDTA, 1 mM mercaptoethanol, 10 mg enzyme (Fraction D-I), and 0.1 M Tris pH 7.4 were incubated for 2 h at 38°. The solution was boiled for 1 min and the coagulated protein was removed by centrifugation. The clear supernatant solution was shaken with a sufficient amount of charcoal to absorb the material showing ultraviolet absorption between 250 to 260 mμ. The charcoal was then eluted with 50% ethanol until the absorbance of the eluate at 260 mμ became minimal. Paper chromatography of this material with solvents, A, B and C showed that the major ultraviolet absorbing substance had the same mobility as GDP-Glc. Specifically, the mobilities (R_{GMP}) in solvents A, B and C were 1.47, 0.43 and 0.63, respectively. The component which corresponded with GDP-Glc was isolated by preparative paper chromatography in solvent B. When the isolated product was chromatographed again in the above mentioned three solvents, only one spot corresponding with GDP-Glc was detected.

The enzymatically synthesized product had an ultraviolet absorption spectrum identical with guanosine nucleotides. It showed the characteristic spectral shifts with variation in pH and the reported ratios at 250, 260 and 280 mμ (ref. 14). Perchloric acid hydrolysis followed by chromatography[20] further substantiated the conclusion that the base component was guanine.

Analysis of the enzymic reaction product showed it to be composed of guanine, ribose, acid-labile phosphate, total phosphate and hexose in the ratio 1.0:1.0:1.1:1.9:1.1. The hexose was identified as glucose by hydrolysis with 0.1 N HCl for 30 min and paper chromatography of the hydrolyzate in solvents D and E. Staining with alkaline silver nitrate revealed the presence of a component having the same R_F as glucose. This component also gave a pronounced reaction with glucose oxidase.

Hydrolysis of the product with 0.01 M HCl for 15 min and 3 h gave rise to GDP and GMP, respectively. Quantitative analyses of reducing sugar during the course of the reaction gave hydrolysis rates similar to those previously reported for GDP-Glc[21].

When the enzymatically synthesized GDP-Glc was incubated with mast cell tumor enzyme and the complete pyrophosphorylase assay mixture, it reacted like authentic GDP-Glc. Since the Glc-1-*P* released is acted upon by phosphoglucomutase, it must be in the α form. It may therefore be concluded that glucose to phosphate linkage in the nucleotide sugar is in the α configuration.

pH optimum—The effect of pH on the activity of GDP-Glc pyrophosphorylase from mastocytoma is shown in Fig. 1. Tris buffer was employed for pH 7.3 and above, while phosphate buffer was used for pH 7.3 and below. At pH 7.3 both buffers gave the same activity. It is seen that maximal activity is obtained at pH 7.2–7.7.

Magnesium requirement—No activity was detectable in the absence of $MgCl_2$. As shown in Fig. 2 optimum activity under conditions of the assay was when the Mg^{2+} concentration was $8–10 \cdot 10^{-3}$ M.

Effect of substrate concentration—The effect of GDP-Glc concentration on the rate of reaction is shown in Fig. 3. The apparent K_m calculated from the reciprocal plot[22] is about $1 \cdot 10^{-4}$ M at 30°. The dependence of reaction rate on pyrophosphate concentration is shown in Fig. 4. The apparent K_m at 30° is $8 \cdot 10^{-4}$.

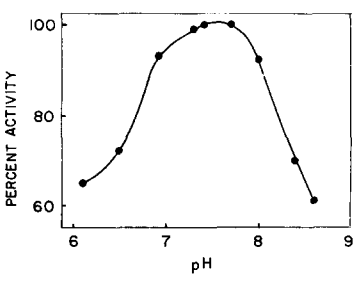


Fig. 1. Effect of pH on the activity of GDP-glucose pyrophosphorylase. The procedures and conditions are described in the text.

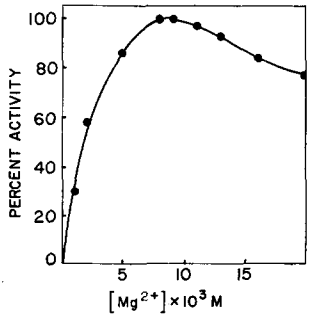
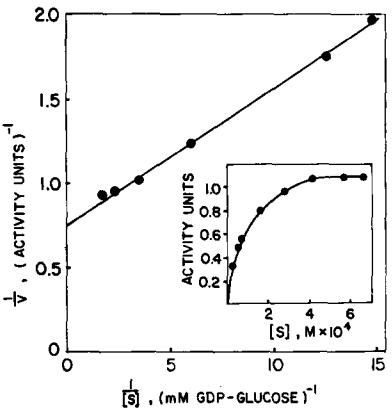


Fig. 2. Effect of magnesium concentration on GDP-glucose pyrophosphorylase activity. The standard concentrations were employed except for that of $MgCl_2$. The rates at different concentrations were calculated as percentage of that shown with 0.008 M $MgCl_2$ where maximum activity was obtained.



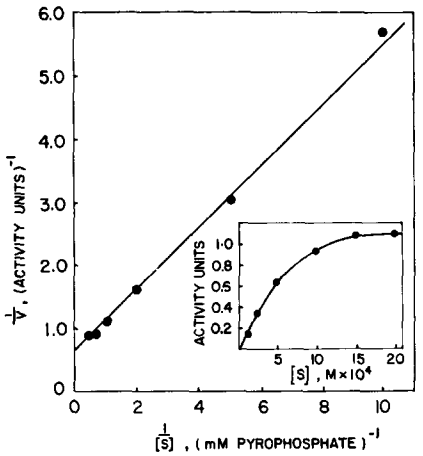


Fig. 3. Effect of GDP-Glc on reaction rate. The conditions were the same as those given in the text except that GDP-Glc concentration was varied. Velocity is expressed as units per ml of enzyme preparation containing 2.4 mg protein per ml, specific activity 0.46.

Fig. 4. Dependence of reaction rate on concentration of pyrophosphate. The conditions were the same as those described in the text for the assay except that the concentration of pyrophosphate was varied. Velocity is expressed as units per ml of enzyme solution containing 2.4 mg protein per ml, specific activity 0.46.

Effect of D-*mannose 1-phosphate*—Man-1-*P* ($7 \cdot 10^{-2}$ mM) did not inhibit the rate of pyrophosphorolysis of GDP-Glc when the concentration of the latter was 7 mM or above. However, at GDP-Glc concentrations of 3.5 and 0.7 mM, there was an inhibition of 18 and 40%, respectively. Similar inhibition also ocurred when GDP-Man was added.

When assayed qualitatively in the direction of synthesis by paper chromatographic identification of the product (see EXPERIMENTAL PROCEDURES), it was found that the enzyme preparation also catalyzes the synthesis of GDP-Man from Man-1-*P* and GTP. This reaction occurred to a lesser degree than that observed in the synthesis of GDP-Glc under the same conditions. When equivalent amounts of Glc-1-*P* and Man-1-*P* were incubated with GTP and limiting amounts of enzyme, the only detectable product was GDP-glucose.

UDP-glucose pyrophosphorylase

Extended studies were not carried out on the UDP-Glc pyrophosphorylase since this activity has already been shown to be present in mast cell tumors[23]. The UDP-Glc pyrophosphorylase fraction obtained in the present experiments (Fraction P-II, Table I) was completely separated from GDP-Glc pyrophosphorylase by a second ammonium sulfate fractionation. Incubation of the UDP-Glc pyrophosphorylase with Glc-1-*P* and UTP followed by paper chromatography revealed comparatively small amounts of a component having the R_G of UDP-Glc. Instead the paper chromatogram showed large spots with mobilities similar to those of UMP and uridine. When the enzyme fraction (P-II) was incubated with UDP-Glc these same spots, in addition to Glc-1-*P* were detected. It thus appears that the tissue extract contains a UDP-Glc pyrophosphatase which during the 2 h incubation

period destroys most of the UDP-Glc which is formed. This activity is not detected during the short period (6–10 min) required for the spectrophotometric assay. The decomposition of UDP-Glc to Glc-1-P and UMP followed by the hydrolysis of the latter to uridine and inorganic phosphate has previously been shown to be catalyzed by microsomal enzymes from skin[24].

Nucleoside diphosphate glucose pyrophosphorylases from other sources

It was of interest to investigate the pyrophosphorylase activity of other tissues containing high concentrations of mucopolysaccharides. Fibrosarcomas which have been shown to contain chondroitin sulfate and hyaluronic acid[8] were extracted and fractionated in the same manner as mast cell tumors. Umbilical cord, which is rich in hyaluronic acid and chondroitin sulfate C and B[25], was first digested for 15 min with hyaluronidase and then extracted and fractionated like the other tissues. These tissues were found to contain comparatively high levels of UDP-Glc pyrophosphorylase but no detectable amount of GDP-Glc pyrophosphorylase.

DISCUSSION

The results of the present study demonstrate that mast cell tumor contains an enzyme which catalyzes the following reaction:

$$\text{GTP} + \text{D-glucose 1-phosphate} \rightleftarrows \text{GDP-glucose} + \text{pyrophosphate}$$

This enzyme, GDP-glucose pyrophosphorylase (GTP:D-glucose-1-phosphate guanylyltransferase), can be separated from UDP-glucose pyrophosphorylase (UTP:α-D-glucose-1-phosphate uridylyltransferase, EC 2.7.7.9) which is also present in the same tissue, by fractional precipitation with ammonium sulfate. The GDP-glucose pyrophosphorylase is specific with respect to the nucleotide in that it shows no activity when Glc-1-*P* is incubated with ATP, CTP, UTP or TTP.

GDP-glucose pyrophosphorylase has been shown to be present in mammary gland, liver, muscle and kidney of various animals[21,26], in milk[27], and in the microorganism *Eremothecium ashbyii*[28]. Subsequently it was demonstrated in plants[29] where it is involved in the synthesis of cellulose[30].

The present finding that GDP-glucose pyrophosphorylase is not detected in rat fibrosarcomas which contain hyaluronic acid and chondroitin sulfate[8] or in umbilical cord which contains chondroitin sulfate B and C in addition to hyaluronic acid[25] indicates that this enzyme is not necessary for the biosynthesis of these mucopolysaccharides. The function of this enzyme or of GDP-Glc in the sequence of metabolic intraconversions in mast cell tissue or possibly in the biosynthesis of heparin is yet to be determined. Previous reports have described the synthesis of UDP-Glc and UDP-glucosamine[23] as well as the oxidation of UDP-Glc to UDP-glucuronic acid by cell-free preparations from the DUNN AND POTTER mastocytoma[31]. The formation of a non-sulfated polymer of glucosamine and glucuronic acid after incubation of UDP-*N*-acetylglucosamine and UDP-glucuronic acid with a preparation from this tissue has also been reported[32]. These findings suggest that uridine nucleotide monomers may be involved in the biosynthesis of heparin. However, this does not preclude the possibility that GDP-Glc, formed by the mast cell tissue, is converted to the requisite monosaccharide derivative which in turn may act as a glycosyl

donor in the formation of heparin. Further studies in this laboratory are concerned with investigating this problem.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. JACOB FURTH for providing them with animals bearing the mastocytomas and for helpful instructions with regard to carrying the tumors.

This work was supported by Research Grant HE-07514 from the National Institutes of Health, U.S. Public Health Service.

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