A convenient method for enzymatic synthesis of radiolabelled glucose-1,6-bisphosphate.

Tom Hamborg Nielsen*, Bente Wischmann, and Birger Lindberg Møller

Plant Biochemistry Laboratory, Department of Plant Biology, Royal Veterinary and Agricultural University, 40 Thorvaldsensvej, DK-1871 Frederiksberg C, Copenhagen, Denmark.

Summary

A convenient method to synthesize radiolabelled glucose-1,6-bisphosphate (Glc-1,6-P_2) of high specific activity and in a high yield is reported. The method is based on enzymatic formation of glucose-6-P from glucose and ATP using hexokinase. The labelled glucose-6-P is then converted to Glc-1,6-P_2 through an equilibrium reaction catalyzed by phosphoglucomutase. The two enzymatic steps and TLC separations necessary to afford radiochemically pure Glc-1,6-P_2 can be completed in one day. The outlined experimental procedure details the synthesis of [^{33}P]Glc-1,6-P_2 evenly labelled at the carbon 1 and the carbon 6 position. However the commercial availability of γ-^{33}P and γ-^{32}P labelled ATP and of glucose labelled with ^{3}H, ^{14}C and ^{13}C provides the option to label glucose-1,6-bisphosphate with any of these isotopes.

Key words: Glucose-1,6-bisphosphate, Glucose-6-phosphate, enzymatic synthesis.

*To whom correspondence should be addressed

Introduction.

Glucose-1,6-bisphosphate (Glc-1,6-P_2) is an important regulator of the carbohydrate metabolism (1,2) being an effector for several glycolytic enzymes, among which is phosphoglucomutase (PGM). Activation of PGM is dependent on the transfer of a phosphate group from Glc-1,6-P_2 to the catalytic site. This occurs when the enzyme has lost the phosphate group, which is essential for its catalytic mechanism. Glc-1,6-P_2 is a known metabolite in plants (3,4) where it is required to activate several isoforms of PGM (5). The availability of radiolabelled Glc-1,6-P_2 would facilitate studies of its metabolism. In our research, another motive was to synthesize radiolabelled Glc-1,6-P_2

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to test this molecule as a putative precursor for those glucose residues of potato starch which are phosphorylated at the C-6 position . In potato starch one out of every 200-300 glucose residues are phosphorylated (6,7). This phosphorylation is of major significance for the functional properties of the starch (8). Potentially Glc-1,6-P_2 could be accepted instead of Glc-1-P as precursor for the biosynthesis in the tubers and thereby introduce the phosphorylated residues.

Accordingly, we developed a method to prepare [U-^{33}P]Glc-1,6-P_2. The method is simple and based on an enzymatic approach since this permits the generation of a product with a very high specific activity and ensures the α–D conformation at the anomeric carbon. The procedure is based on the exclusive use of commercially available materials, and is equally applicable for labelling with the isotopes ^{3}H, ^{14}C, ^{13}C, ^{33}P, and ^{32}P.

Results & Discussion

The use of enzymes in the synthesis of radiolabelled biomolecules is widely applied. Bassols et al. (9) reported a method for the formation of radiolabelled [U-^{14}C]Glc-1,6-P_2 using an enzymatic system based on phosphofructokinase. We have designed an alternative method which is simpler, faster, yields several orders of magnitude higher specific activities, and may be applied to more isotopes since it is not dependent on the availability of radiolabelled Glc-1-P.

The phosphate groups of Glc-1,6-P_2 were radiolabelled using a two step enzymatic reaction. Separation of the reaction into two steps allows easy purification of the products by TLC. In the first reaction a radioactive phosphate group was introduced into Glc-6-P by hexokinase using 100 nmol glucose and 10 nmol [γ–^{33}P]ATP as substrates.

$$[\gamma\text{-}^{33}\text{P}]\text{ATP} + \alpha\text{–D-glucose} \xrightarrow{\text{hexokinase}} \text{ADP} + [^{33}\text{P}]\alpha\text{–D-Glc-6-P}$$

The purity and specificity of the enzyme ensures that the only product of the reaction is Glc-6-P. When a stoichiometric excess of glucose is used the exergonic reaction leads to efficient formation of Glc-6-P from the radioabelled ATP.

The TLC system used to purify Glc-6-P is shown in Fig 1. After separation of the substrates and products in the first dimension, the TLC plate was developed twice in the second dimension to concentrate the band of Glc-6-P into a single spot. The Glc-6-P was then eluted from the silica gel with water and dried under vacuum. The yield of Glc-6-P as determined from the recovery of radioactivity was 80 % which corresponds to 8 nmol (Fig.2, lane 1).

Subsequent transfer of the radiolabelled phosphate group of Glc-1-P into Glc-1,6-P_2 was achieved by equilibrating the radiolabelled Glc-6-P with an approximately 4 fold stoichiometric amount of Glc-1,6-P_2 using PGM to catalyze the reaction. PGM catalyzes the equilibrium between Glc-6-P and Glc-1-P with Glc-1,6-P_2 as a catalytic intermediate:

$$\alpha\text{–D-Glc-6-P} + \text{E-P} \longleftrightarrow \alpha\text{–D-Glc-1,6-P}_2 + \text{E} \longleftrightarrow \alpha\text{–D-Glc-1-P} + \text{E-P}$$

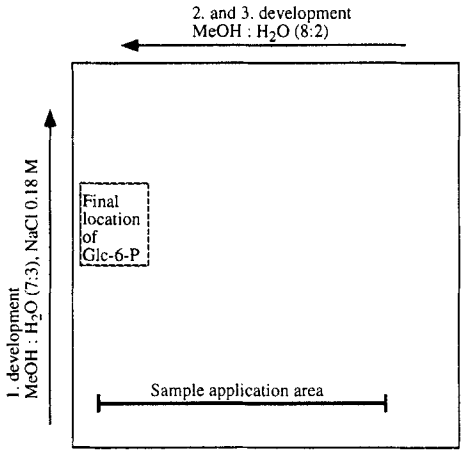

Fig. 1. TLC system for isolation of radiolabelled Glc-6-P produced by hexokinase. The silica gel within the area indicated by stipulated lines was eluted with water to recover the Glc-6-P.

where E-P and E represents the phosphorylated and unphosphorylated forms of the enzyme PGM, respectively. Since the reactions are reversible the radiolabelled P will eventually be distributed evenly between the carbon 1 and carbon 6 position.

The intermediate Glc-1,6-P_2 is tightly bound to the catalytic site of PGM (10). Consequently the exchange between enzymatically formed and exogenously added Glc-1,6-P_2 is slow. For the same reason, high concentrations of Glc-1,6-P_2 inhibit the enzyme activity. The transfer of the radiolabelled phosphate group of Glc-6-P to the pool of Glc-1,6-P_2 was therefore rather slow considering the included PGM activity. However, to achieve a high degree of transfer of radiolabelled phosphate from Glc-6-P to Glc-1,6-P_2 this latter metabolite must be present in stoichiometric excess compared to the

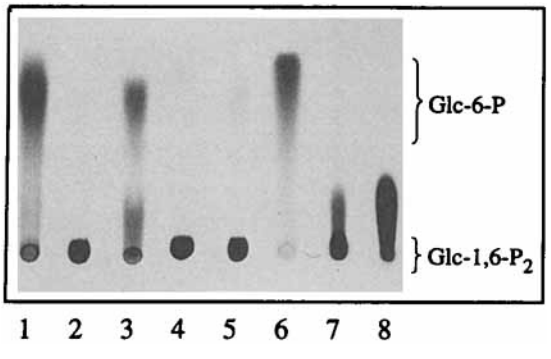

Fig 2. Autoradiography of labelled products separated by TLC. Lane 1: Product of hexokinase reaction; Lane 2: Purified Glc-1,6-P_2 radiolabelled by transfer of a ^{33}P group from Glc-6-P catalysed by PGM; Lane 3: Products obtained after acid hydrolysis of [^{33}P]Glc-1,6-P_2 ; Lane 4: Purified [^{33}P]Glc-1,6-P_2 after incubation with Glc-1-P; Lane 5: Purified [^{33}P]Glc-1,6-P_2 after incubation with PGM; Lane 6: Purified [^{33}P]Glc-1,6-P_2 after incubation with Glc-1-P and phosphoglucomutase; Lane 7: ATP; Lane 8: Pi.

monophosphorylated sugars. At the experimental conditions used about 61 % of the radioactivity (80 μCi) was transferred to the Glc-1,6-P_2 pool (30 nmol) after a 5 h incubation period (Fig. 3). The fast initial incorporation of approximately 7% of the radiolabel most likely represent radiolabelling of PGM which like Glc-1,6-P_2 remains at the origin in the TLC system. However, the radiolabeling of PGM will decrease with time as the radiolabelled is transferred to the considerably larger pool of Glc-1,6-P_2. The TLC system used for separation of Glc-1,6-P_2 and Glc-6-P (and Glc-1-P) is as shown in Fig 1.

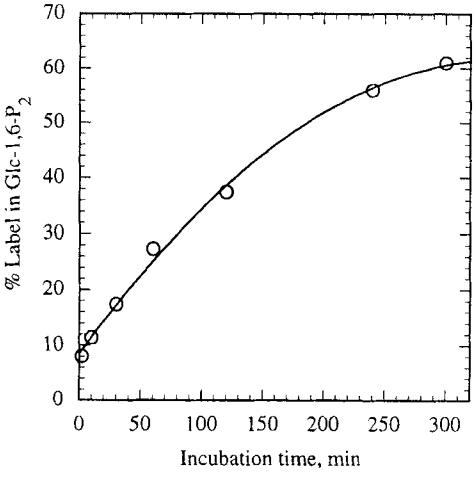

Fig 3. PGM catalysed transfer of radiolabel from Glc-6-P to compounds which do not move in the TLC system (same system as in Fig. 1). Samples of reaction mixture were separated on TLC and after localizing radioactive areas these were cut out, eluted with water and the radioactivity determined by liquid scintillation counting.

Identification of the product obtained as Glc-1,6-P_2.

Acid treatment: Glc-1,6-P_2 is labile under acidic conditions. Hydrochloric was added acid to a concentration of 0.1 N to a sample of the radiolabelled compound which was then boiled for 10 min. The solution was added 25 mM Mops and then neutralized by titration with 0.1 N NaOH. When analysed by TLC the radioactivity in the hydrolysate comigrated with Glc-6-P and Pi (Fig 2, lane 3), the two products expected to be formed. The intensity of th two radiolabelled products was similar indicating that the radiolabel was distributed evenly between the carbon 1 and carbon 6 position.

Enzymatic conversion. The radiolabelled compound (5 pmol) was added to a mixture containing PGM (2 U) and a surplus of Glc-1-P (50 nmol) and incubated for 15 min at 20 °C. The radioactive component formed comigrated with the hexose phosphates when analyzed by TLC. The transfer of the radiolabelled phosphate group was completely dependent on the presence of both Glc-1-P (or Glc-6-P) and PGM (Fig 2, lanes 4-6).

Dionex Chromatography. The radiolabelled compound comigrated with Glc-1,6-P_2 during anion exchange chromatography (Fig. 4).

On the basis of these different identification criteria it is concluded that we have achieved efficient enzyme catalyzed radiolabeling of Glc-1,6-P_2, and that no other labelled products are present in the final preparation. Although Glc-1,6-P_2 is a labile glucoside, no sign of hydrolysis was seen at the mild experimental conditions used.

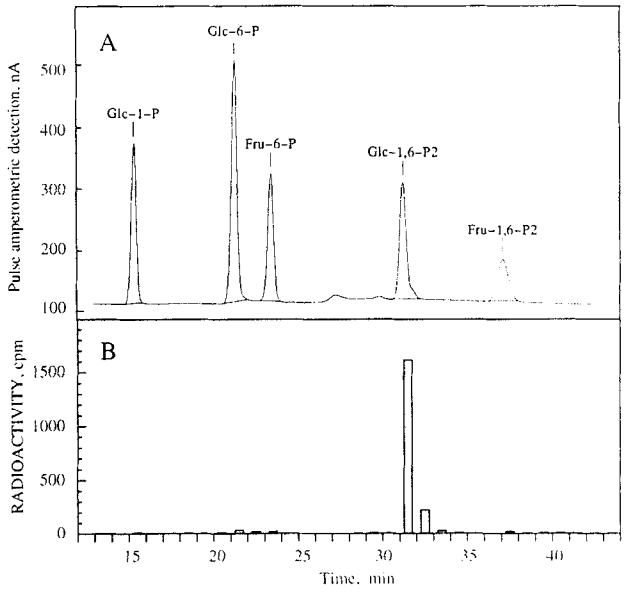

Fig 4. Separation of authentic standards and analysis of the radiolabelled sample by anion exchange HPLC. A, pulse amperometric detection of standard compounds. B, radioactivity in fractions.

Experimental

Chemicals and enzymes: α–D-glucose, disodium salt of α–D-Glc-1,6-P_2, trisodium salt of ATP, $MgCl_2$, NaCl, Mops, hexokinase (bakers yeast) and phosphoglucomutase (rabbit muscle) were all purchased from Sigma Chemical Company, St. Louis, USA. [γ-^{33}P]ATP with a specific activity of 1000 Ci mmol^{-1} was purchased from Amersham International plc, Buckinghamshire, England.

[^{33}P]Glucose-6-phosphate (Glc-6-P) was obtained by mixing 200 μCi [γ-^{33}P]ATP (0.2 nmol), 100 nmol α–D-glucose, and 1U hexokinase in a final volume of 29 μL 20 mM Mops-KOH (pH 7.5) and 2 mM $MgCl_2$. The incubation period was 25 min with portions of 5 nmol unlabelled ATP being added after 5 min and 15 min.

The total reaction mixture was streaked onto a 7×10 cm TLC plate (Silica gel 60, Merck, Darmstadt, Germany). The TLC plate was developed once in the first dimension using a solvent containing 35 mL methanol, 15 mL H_2O, and 0.5 g NaCl. To concentrate the stripe of Glc-6-P into a single spot the plate was developed twice in the second dimension with a solvent composed of methanol:H_2O (8:2). The TLC was placed against an X-ray film (Hyperfilm-MP, Amersham International plc, Buckinghamshire, England) for 1 min to localize the position of the Glc-6-P formed. The silica gel area (4 cm^2) was eluted with 2×1 mL H_2O. The combined eluates were concentrated to app. 200 μL in a rotovapor at 50 °C and diluted to 400 μL with buffer to reach a final concentration of 10 mM Mops-KOH (pH 7.5), 1 mM imidazol and 1 mM $MgCl_2$. The final yield of Glc-6-P was 80 % (160 μCi, 8 nmol).

The preparation of Glc-6-P was incubated with 7 U PGM and 30 nmol Glc-1,6-P_2 for 5 h at 20 °C. The total reaction mixture was purified by TLC using the same solvent system as in the previous step. The distribution of radiolabel was visualized by autoradiography. The region of the silica gel near the baseline and containing Glc-1,6-P_2 was cut out and eluted with 15 mL H_2O. Silica gel was removed by centrifugation (1000 g, 1 min) and the supernatant was frozen and lyophilized over the night. The dry powder was dissolved in 200 µL H_2O. Insoluble material was removed by centrifugation (15,000 g, 1 min) and the supernatant containing Glc-1,6-P_2 was stored at -80 °C. Final yield (80 µCi, 30 nmoles)

The radiochemical purity of the final preparation of Glc-1,6-P_2 was analyzed by anion exchange HPLC using a Dionex 4500i (Sunnyvale, CA, USA) chromatographic system. Samples and authentic reference compounds were separated on a CarboPac PA1 (Dionex) column (4X250 mm) eluted (flow rate 1 mL min^{-1}) isocratically with 2 mL solvent A: (54 mM NaOH, 100 mM sodium acetate) then eluted with a linear gradient (28 mL) from solvent A to solvent B: (75 mM NaOH, 500 mM sodium acetate), and finally eluted isocratically with solvent B. Fractions of 1 mL were collected and their radioactivity determined by liquid scintillation counting in a 1214 Rackbeta (LKB Wallac, Turku, Finland) after addition of 10 mL Ecoscint A (National Diagnostics, Manville, NJ, USA).

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