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Note

High yielding one-pot enzyme-catalyzed synthesis of UDP-glucose in gram scales☆

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Abstract

Uridine diphosphoglucose is an important cofactor of glucosylating enzymes. A simple and high yielding one-pot enzymatic synthesis of UDPG on a gram scale from glucose via hexokinase, phosphoglucomutase and UDPG pyrophosphorylase (UGPase) is described. Repetitive addition of substrate was used to avoid inhibition of UGPase. The approach allows recovery of active enzymes and their re-use. The synthesis of UDP-[4-^{13}C]-glucose on a 0.5 g scale resulted in a final yield of 70% and a purity of $>95\%$ after chromatographic purification.

Keywords: Uridine diphosphoglucose; Enzymatic synthesis; Hexokinase; Phosphoglucomutase; UDPG pyrophosphorylase; ^{13}C NMR

Uridine diphosphoglucose (UDPG) is one of the most significant glucosyl donors in a variety of enzymatic reactions including the biosynthesis of simple or complex glucosides, oligo- and polysaccharides, glycoproteins and related macromolecules.[1,2] It also serves as a biogenetic precursor for a range of nucleotide sugars such as UDP-galactose, UDP-glucuronic acid and UDP-Rha.[3] Both, chemical[4–6] and enzymatic[7–10] syntheses of UDPG have been reported. Several chemical methods have been applied for the synthesis of this cofactor, but the overall yields attainable were, in general, exceedingly low. Therefore, enzymatic syntheses have been designed in the past by using the enzymes pyrophosphorylase[7–9] or sucrose synthase.[10] We have tried to use previously described methods for the synthesis of ^{13}C-labeled UDPG that use pyrophosphorylases; however, in our hands only low yields of ca. 10% were obtained and the second approach, based on sucrose synthase, resulted in an overall yield not higher than 21%.[10] Such a yield is too low in the case where specially labeled, and therefore rare and expensive, glucose is the starting material. In contrast to these strategies, we report here on an economic and simple method to synthesize UDPG enzymatically on a gram scale in much higher yields, from glucose via hexokinase, phosphoglucomutase and uridine-5′-diphosphoglucose pyrophosphorylase. As an example, pure UDP-[4-^{13}C]-glucose ($>95\%$) has been synthesized with a final yield of 70%

☆ Abbreviations described in the text.

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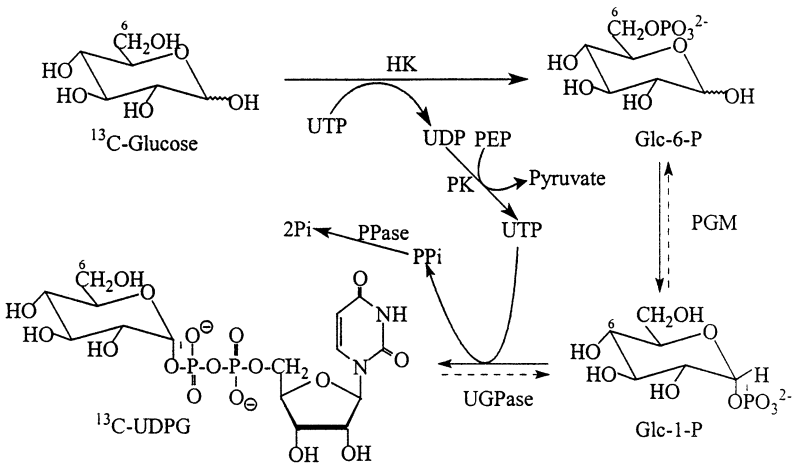
(Scheme 1). ^{13}C-glucose was phosphorylated by UTP in the presence of HK, and the Glc-6-P formed was converted to Glc-1-P by PGM. For sufficient UTP concentrations a UTP regeneration system was applied based on UDP and PEP with catalysis by PK. Because PK is not expensive it was usually added in an excess in order to maintain the concentration of UTP. Glc-1-P reacted with UTP in the presence of UGPase to form UDPG and PPi. The limiting step of these reactions is the Glc-1-P formation, in which the equilibrium is distinctly unfavorable for the formation of Glc-1-P.[11] However, inorganic pyrophosphatase allowed the overall reaction to pull efficiently in the direction of UDPG by cleavage of the PPi formed.

Previous reported results[8] suggested that a high concentration of magnesium was the inhibitor of phosphoglucomutase and the yield of UDPG in a one-step incubation was low when the UTP/Mg^{2+} ratio was 1:1 or 1:2. However, in our experiment, 4 mM Mg^{2+} with an UTP/Mg^{2+} ratio of 1:2 was used in a one-pot reaction and yields of $>85\%$ were usually obtained. This finding indicated that the concentration of Mg^{2+} was not an important factor that determines the yield in a one-step incubation. If UTP in a concentration of 10 mM was applied in our experiments, only a very low yield of $\sim 10\%$ of UDPG was obtained (data not shown). This result was based on the fact that UTP at higher concentration than 2 mM strongly inhibits UGPase.[12] Therefore, the concentration of UTP is the crucial factor that determines the final yield of enzymatically formed UDPG. The described methods[7–9] are designed to synthesize ^{14}C-labeled UDPG on a rather small scale (<10 μmol). Since only low substrate concentrations are applied in these cases no substrate inhibition occurs. When these conditions are applied for a larger scale (more than 500 μmol) the volume of the reaction system must, however, be drastically increased which makes very large amounts of enzymes necessary. Because of all these disadvantages, we devised a one-pot method in which UTP and glucose were both used at a concentration below 2 mM.

Every enzyme used in the described synthesis is very stable and therefore the reactions can be performed over several days. When more than 80% of UTP and glucose was consumed, a new substrate solution was added. This procedure was repeated until the conversion of substrate was below 80%. Five cycles (60 mL batch) could usually be performed with incubation periods between 4 and 8 h. No product inhibition occurs using this procedure so it was not necessary to add more enzymes during the whole experimental procedure. Finally, a satisfactory yield of ca. 85% for the total five cycles was measured, indicating that these conditions are excellent for the preparative synthesis of UDPG. Moreover,



Scheme 1. Enzymatic synthesis of ^{13}C-labeled UDPG from ^{13}C-glucose. Abbreviations: HK, hexokinase; PGM, phosphoglucomutase; PK, pyruvate kinase; PPase, inorganic pyrophosphatase; UGPase, uridine-5′-diphosphoglucose pyrophosphorylase; UTP, uridine 5′-triphosphate; UDP, uridine 5′-diphosphate; PEP, phospho(enol)pyruvate; PPi, inorganic pyrophosphate; Pi, phosphoric acid; UDPG, uridine 5′-diphosphoglucose.

Table 1
Yields of UDP-[4-^{13}C]-glucose at each cycle by repetitive addition of substrates in a 60 mL reaction system

Cycle	UDP-[4-^{13}C]-glucose	
	μmol	Yield (%)
1 (4 h)	98.4	90
2 (4 h)	95.8	87
3 (6 h)	95.8	87
4 (8 h)	91.2	83
5 (8 h)	85.8	78

Table 2
R_f values of UDPG and related compounds in two TLC solvent systems

Compounds	R_f	
	S1	S2
Glucose	0.52 ± 0.03	0.61 ± 0.02
G-1-P	0.13–0.14	0.23 ± 0.01
G-6-P	0.11 ± 0.01	0.16 ± 0.01
UDPG	0.36 ± 0.02	0.54 ± 0.01
UTP	0–0.03	0.05 ± 0.04
UDP	0.07 ± 0.02	0.22 ± 0.04
UMP	0.35 ± 0.01	0.49 ± 0.02
Uridine	0.64 ± 0.02	0.69 ± 0.02

the enzymes can be recovered and can be re-used. After recovery, the concentrated enzymes can be applied to another batch and re-used for the next cycles. Table 1 shows the amounts of UDPG synthesized and the yield of each cycle in a single 60 mL incubation. The method we developed here can also be used for the synthesis of other isotope-labeled UDP-glucose on a preparative scale from commercially available isotope-labeled substrates such as glucose, glucose-1-P, glucose-6-P and UTP. It is an efficient method and the costs can be greatly reduced by the described repetitive addition technique.

As an example, 434 mg UDP-[4-^{13}C]-glucose (0.77 mmol) was obtained from 200 mg (1.10 mmol) [4-^{13}C]-glucose after purification. The total yield was 70% based on [4-^{13}C]-glucose. The ratio of phosphorous:glucose:uridine in the product was 1.95:0.96:1.00, which is close to the theoretical value of 2:1:1 indicating a high purity of the synthesized UDP-[4-^{13}C]-glucose. It was also pure based on chromatography by two TLC systems. The R_f values of UDPG and related compounds are shown in Table 2. The synthesized UDP-[4-^{13}C]-glucose was accepted as cofactor in two enzyme systems, UGPase system and UDPG-DH. Both can be used to assay UDPG quantitatively and qualitatively. On the basis of its UV absorption, 98% UDP-[4-^{13}C]-glucose was active in the UGPase system and 96% in UDPG dehydrogenase system. From all these data we conclude, that the formed product had a purity > 95%.

The ^{1}H NMR spectrum of the synthesized UDPG was superimposable with that of standard UDPG. In the ^{13}C NMR spectrum, ^{13}C UDPG showed only one signal at 70.2 ppm (Fig. 1(A)). The spectrum of the mixture of UDP-[4-^{13}C]-glucose synthesized and 20 mg authentic unlabelled UDPG is illustrated in Fig. 1(B). The spectrum of UDP-[4-^{13}C]-glucose coincided with the signal at 70.2 ppm. This result is different to previous observations,[10] in which the C-4 of the glucose residue

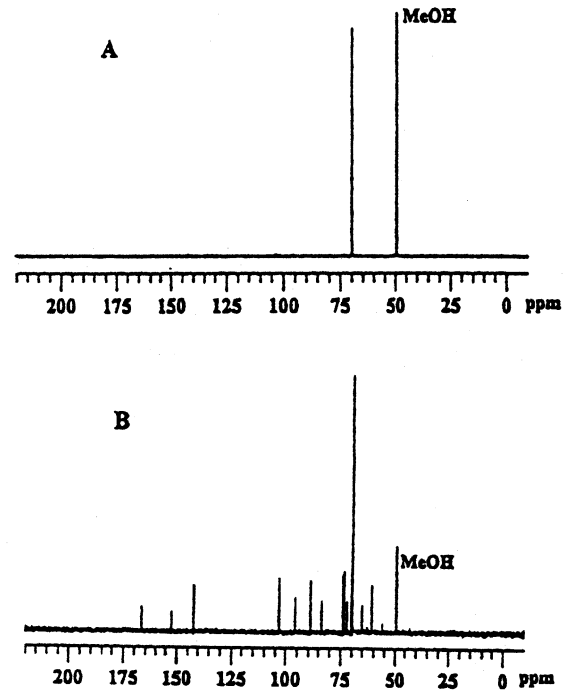


Fig. 1. ^{13}C NMR spectra of UDP-[4-^{13}C]-glucose. All the data were recorded at 100.6 MHz with a Brucker AM 400 spectrometer. Methanol (δ 49.8) was used as internal reference and D_2O was used as solvent. (A) 2.3 mg UDP-[4-^{13}C]-glucose in 0.65 mL D_2O and 0.05 mL methanol, measuring time: 15 min; (B) 0.7 mg UDP-[4-^{13}C]-glucose + 20 mg unlabelled UDPG in 0.7 mL D_2O and 5 μL methanol, measuring time: 3 h.

of UDPG was attributed to the signal at 75.7 ppm (corresponding to 73.8 ppm in Fig. 1(B)) and C-3 was assigned to the signal at 72.0 ppm (corresponding to 70.2 ppm in Fig. 1(B)). From this point of view, the previous assignment must therefore be revised.

In conclusion, the here described method allows a simple and efficient synthesis of appropriate ^{13}C labeled UDP-glucose. Such highly enriched ^{13}C UDPG, in principal, can be monitored with great sensitivity by ^{13}C NMR. A very low concentration of UDP-[4-^{13}C]-glucose (2.3 mg in 0.7 mL D_2O) gave a high signal/noise ratio (>50) when it was measured for only 15 min at 100.6 MHz (Fig. 1(A)). UDPG can act as a glucosyl donor in a broad variety of enzymatic reactions for the biosynthesis of various glucosides, but also for elucidation of the biosynthesis of oligo- and polysaccharides. The good ^{13}C NMR sensitivity, which can still be much enhanced by using higher fields, would be of a great advantage for the search of any novel UDPG-dependent enzymes and the use of ^{13}C labeled UDPG would facilitate the structural elucidation of in vivo or cell-free formed glucosides, especially as to their glycosidic $1\rightarrow4$-linkages.

1. Experimental

Materials.—[4-^{13}C]-Glucose (99% enriched) was obtained from Promochem GmbH (Wesel, Germany). Hexokinase (HK, EC 2.7.1.1) from yeast, phosphoglucomutase (PGM, EC 5.4.2.2) from chicken muscle, yeast uridine-5′-diphosphoglucose pyrophosphorylase (UGPase, EC 2.7.7.9), pyruvate kinase (PK, EC 2.7.1.40) from chicken muscle, yeast inorganic pyrophosphatase (PPase, EC 3.6.1.1), glucose-6-phosphate dehydrogenase (G-6-P-DH, EC 1.1.1.49) from *Leuconostoc mesenteroides*, α-D-glucose 1,6-diphosphate (G-1,6-PP, cyclohexylammonium salt) and phosphoenolpyruvate (PEP) were obtained from Sigma (Deisenhofen, Germany). Uridine-5′-diphosphoglucose dehydrogenase (UDPG-DH, EC 1.1.1.22) from beef liver, glucose-1-phosphate (Na salt, G-1-P), glucose-6-phosphate (Na salt, G-6-P) and NAD^+ were purchased from Boehringer (Mannheim, Germany). Uridine-5′-triphosphate-Na_3 (UTP) was from Serva (Heidelberg, Germany). Centriprep YM-10 MW Centrifugal Filter Devices were from Amicon Inc. (Witten, Germany).

Chromatography.—Thin-layer chromatography (TLC) was carried out with the following solvent systems: solvent 1 (S1), 85:15:0.1 EtOH–water–diethylamine (v/v), solvent 2 (S2), 80:20:0.1 EtOH–water–85% H_3PO_4 (v/v). Analytical chromatography was carried out on 2×10 cm Silica Gel 60 F_{254} TLC aluminum plates (E. Merck, Darmstadt). Preparative chromatography was performed on 20×20 cm Silica Gel 60 F_{254} 0.5 mm TLC glass plates obtained from the same company. UV absorbing compounds on chromatograms were visually detected at 254 nm and glucose-containing compounds were detected by spraying the plates with a thymol soln (0.5 g thymol in 95 mL 95% EtOH and 5 mL 98% H_2SO_4) followed by heating at 110 °C for 10 min.

Preparative synthesis of UDP-[4-^{13}C]-glucose.—A total volume of 60 mL reaction mixture contained 0.1 M triethanolamine (TEA) buffer (pH 8.0), 4 mM $MgCl_2$, 10 mM β-mercaptoethanol, 1.8 mM ^{13}C-glucose, 1.9 mM UTP, 33 μM G-1,6-PP, 2.0 mM PEP, 60 U HK, 80 U PGM, 200 U PK, 20 U UGPase, 40 U PPase. The reaction mixture was incubated at 25 °C. The synthesis was monitored by TLC (S1 and S2) and the UDPG formed was assayed with UDPG-DH. When more than 80% of ^{13}C-glucose had been converted to UDPG, 1 mL of fresh substrate soln containing 110 μmol glucose, 112 μmol UTP, 116 μmol PEP and 2 μmol G-1,6-PP was added to the reaction mixture. This substrate soln was added four times. After the reaction, the enzymes were separated from the reagents by centrifugation with Amicon Centrip YM-10. The concd enzymes were re-used in an additional 60 mL reaction soln.

The combined solns were pooled (total 120 mL) and concd by lyophilization. The remaining powder was dissolved in 35 mL 70% EtOH and in five equal batches purified by column chromatography on Silica Gel G (2.0×70 cm, 230–400 mesh, 1 mL/min) with 80% EtOH. Fractions containing UDPG were combined, concd under reduced pressure (12

kPa) at 35 °C and lyophilized. Final purification was carried out on preparative TLC plates with solvent system S1. UDPG was eluted with 60% MeOH and centrifuged (8000*g*) for 15 min. The UDPG soln was evaporated under reduced pressure (22 kPa) at 35 °C. Another preparative TLC with solvent system 2 was carried out if necessary. Quantitative estimation of UDPG was performed by UV at 262 nm and the amounts calculated using its molar extinction coefficient.[13] UDPG was chemically analyzed by measuring the glucose, uridine and phosphate released after acid hydrolysis as described earlier.[14]

Enzymatic assays of UDP-[4-^{13}C]-glucose.—UGPase and UDPG-DH, respectively, were used to determine the content of UDPG both qualitatively and quantitatively.

UGPase system[15].—UDPG was determined by Glc-1-P formed in the presence of the enzyme UGPase. Glc-1-P was transformed to Glc-6-P by an excess of PGM. Glc-6-P concentrations were determined by G-6-P-DH at 340 nm. The assay mixture (1 mL) contained 80 mM tricine buffer (pH 7.6), 4.0 mM $MgCl_2$, 1.4 mM NAD, 0.01 mM G-1,6-PP, 2.0 mM sodium pyrophosphate (PPi), 0.13 U PGM, 0.44 U G-6-P DH, 0.1 U UGPase and 0.02–0.10 μmol UDPG. The reaction was started by the addition of PPi. NADH formation (25 °C) was continuously monitored at 340 nm until constant absorption. The assay mixture, but without UDPG, was used as a blank and standard UDPG (between 0.02 and 0.10 mM) were used as positive references. Absorption at 340 nm was linear with the concentration of UDPG in the range 0.02–0.10 mM.

UDPG-DH system[16].—UDPG is oxidized to UDP-glucuronic acid in the presence of NAD^+ and UDPG-DH. NAD^+ (2 mol) is reduced per mol of UDPG oxidized. The reaction mixture (1 mL) contained 0.1 M Tris–HCl buffer (pH 8.5), 1 mM NAD^+, 0.05 U UDPG-DH and 0.01–0.1 mM UDPG. The reaction was started by the addition of the enzyme. The absorption at 340 nm was monitored until no further reaction was detected.

^{13}C NMR spectra.—^{13}C NMR spectra were measured at 100.6 MHz with a Bruker AM 400 spectrometer, protons were de-coupled. Chemical shifts were measured by using MeOH (δ 49.8 ppm) as internal reference when D_2O was used as solvent.

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