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Two-step enzymatic synthesis of UDP-*N*-acetylgalactosamine

Vanessa Bourgeaux, Friedrich Piller and Véronique Piller*

Centre de Biophysique Moléculaire CNRS UPR4301 affiliée à l'Université d'Orléans et à l'INSERM, rue Charles Sadron, F-45071 Orléans Cedex 02, France

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Abstract—UDP-GalNAc has been synthesised with high yield from GalNAc, UTP and ATP using recombinant human GalNAc kinase GK2 and UDP-GalNAc pyrophosphorylase AGX1. Both enzymes have been prepared in one step from 1 L cultures of transformed *Escherichia coli* and the UDP-GalNAc produced has been purified by a simple procedure. The method described is a rapid and efficient means to produce UDP-GalNAc as well as analogues like UDP-*N*-azidoacetylgalactosamine (UDP-GalNAz).

UDP-GalNAc is the donor substrate of many *N*-acetylgalactosaminyltransferases, enzymes which transfer GalNAc from the nucleotide sugar to a saccharide or peptide acceptor. Glycoconjugates synthesised by those enzymes are of particular interest in therapeutic approaches which require the production of blood group or tumour antigens or of glycosaminoglycans.[1] Understanding the precise mechanism of action of *N*-acetylgalactosaminyltransferases would facilitate the production of glycans with GalNAc residues crucial for their biological activity. However, the main limitation to most studies is the limited availability of labelled sugar nucleotides and of sugar nucleotide analogues and derivatives due to the difficulty of their synthesis.

UDP-GalNAc can be prepared in several ways. The pure chemical approach[2,3] is long, fastidious and yields are low. As an alternative, enzymatic biosynthesis avoids the use of protection and deprotection steps required for chemical synthesis and circumvents the difficulties inherent to the formation of a pyrophosphate bond. One of the enzymatic routes used starts from UDP-GlcNAc which is converted into UDP-GalNAc by a mammalian Gal-4 epimerase.[4] The main drawback of this method is the rather low yield (30% at the equilibrium) and the difficulty to separate UDP-GalNAc from the excess UDP-GlcNAc. Another biosynthetic route starts from galactosamine and uses yeast moult galactokinase to form galactosamine-1-phosphate (Gal-NH_2-1-P) although with low yield.[5] Then the GalNH_2-1-P is purified and can be coupled to UDP chemically[2] or enzymatically[6] using a yeast UDP-Glc uridyltransferase. In both cases, the UDP-GalNH_2 formed has still to be chemically N-acetylated and moderate overall yields, not exceeding 20% of the starting product, are obtained after purification. Another chemoenzymatic synthesis was reported from UMP, sucrose, and GalNH_2-1-P through the help of seven enzymes and two cofactors and a final step of N-acetylation.[7] Besides the requirement of many enzymes, the overall yield was only 34%. Finally, an enzymatic method using a partially purified GalNAc kinase (GK2) from pig kidney allowed GalNAc-1-P formation with good yield.[8] But the subsequent use of a bacterial GlcNAc pyrophosphorylase (GlmU) with a poor specificity for GalNAc-1-P gave only 10% UDP-GalNAc.

In order to obtain substantial amounts of UDP-GalNAc, we developed a new low-cost strategy easier and more efficient than the previous ones. Starting from GalNAc, ATP and UTP we combined the activities of three enzymes. The mammalian GK2 catalyses the phosphorylation of GalNAc using ATP as phosphate donor. Then, the mammalian UDP-GalNAc pyrophosphorylase (AGX1) uses UTP to convert GalNAc-1-P into UDP-GalNAc. Yeast inorganic pyrophosphatase (PPA) drives UDP-GalNAc production forward (Scheme 1) by preventing the reverse reaction.

While PPA is commercially available, the two other enzymes have been produced by bioengineering. GK2 of human or porcine origin had been previously extracted

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* Corresponding author. Tel.: +33 238 257 643; fax: +33 238 257 807; e-mail: pillere@cnrs-orleans.fr

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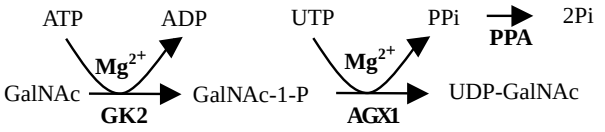

Scheme 1. Two-step enzymatic synthesis of UDP-GalNAc.

from tissues or expressed in *Saccharomyces cerevisiae*.[9–11] In this study, we expressed the GK2 of human origin in *Escherichia coli* as a tagged protein which allows its rapid and efficient purification. For AGX1, previous studies had established that addition of a tag to the recombinant form of the enzyme reduced its activity and expression without tag imposed the use of several protein purification steps.[12–14] Here, we describe the expression of AGX1 in *E. coli* as a tagged protein which is as active as the enzyme isolated from tissues and which was easily purified through its tag.

Both cDNAs encoding the enzymes GK2 and AGX1 were cloned into a high expression plasmid, the proteins were expressed in *E. coli* and purified by Ni-NTA affinity chromatography.[15] SDS-PAGE (Fig. 1) and Western blot analysis (not shown) confirmed that the main proteins obtained after purification were the expected ones with an apparent molecular weight of 57 kDa for GK2 and of 61 kDa for AGX1. From 1 L cultures we obtained 2.2 mg GK2 (82% purity) and 3.4 mg AGX1 (70% purity).

Enzymatic assays[16] indicated that the specific activities of the recombinant enzymes and the K_m values for substrates were close to those described in the literature[10,12,13] for the enzymes purified from tissues (Table 1). Both enzymes were kept at 0.5 mg/mL in a buffered (pH 7.5) solution containing 30% glycerol and showed no loss of activity after one year at −20 °C.

The purified GK2 and AGX1 enzymes were used to synthesise in one-pot UDP-GalNAc from GalNAc, ATP and UTP. Small-scale assays were first performed to optimise the reaction.[17] The reaction catalysed by AGX1 being reversible, addition of PPA was necessary to drive the synthesis in the forward direction and to reach maximum yield (68 ± 10%) after ion exchange chromatography. The optimal quantity of PPA was determined to be 2 U/mL. Another adjustment was necessary due to the difference in K_m values (Table 1). Experiments presented in Figure 2 indicate that the optimal concentration of GalNAc engaged in the mixture is 3 mM. In addition, we determined optimal quantities of enzymes for maximal formation of UDP-GalNAc in 6 h: 0.8 μg/mL AGX1 and 6.0 μg/mL GK2.

Table 1. K_m of the recombinant enzymes

	K_m (mM)	Specific activity (nmol/μg/min)
GK2	0.22 (0.14)[10]	1.80 (1.40)[10]
AGX1	0.67 (1.1)[12]	29.0 (14.9)[12,13]

Values from the literature are in parentheses.

Finally, we evaluated optimal concentrations of other substrates and found out that 3 mM GalNAc, 3 mM ATP and 3 mM UTP were the lowest concentrations required to obtain after purification maximal amounts of UDP-GalNAc. Apparition kinetics of UDP-GalNAc under those conditions are presented in Figure 3. A 2 mL production of UDP-GalNAc was performed starting from 1.33 mg GalNAc.[18] Both purified enzymes were incubated with substrates, necessary cofactors and PPA for 6 h. After a two-step purification by ion exchange and gel filtration, the pure desired product, UDP-GalNAc ammonium salt, was characterised by mass spectrometry and NMR spectroscopy.[19]

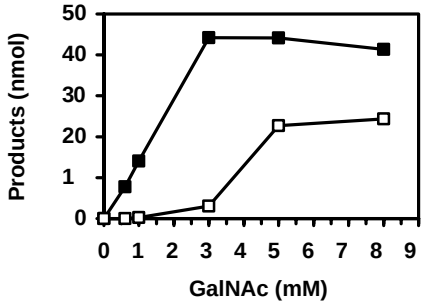

Figure 2. Kinetics of GalNAc-1-P (open squares) and UDP-GalNAc (filled squares) production as a function of GalNAc concentration.

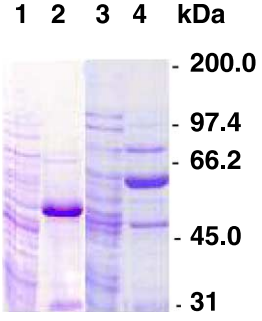

Figure 1. Coomassie-stained gel after SDS-PAGE of the soluble proteins extracted from transformed bacteria by gentle lysis (lanes 1 and 3) and purified on Ni-NTA resin (lanes 2 and 4). Lanes 1 and 2: GK2; lanes 3 and 4: AGX1.

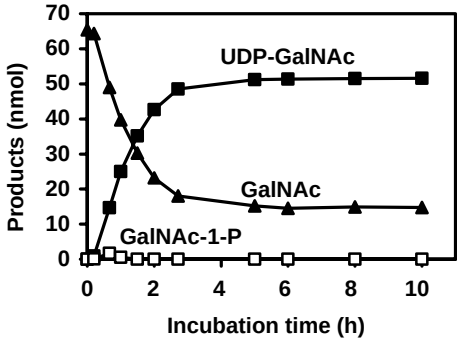

Figure 3. Time kinetics of UDP-GalNAc (filled squares) and GalNAc-1-P (open squares) formation and of GalNAc consumption (triangles).

In order to test whether this enzymatic synthesis was applicable to GalNAc derivatives, we chose *N*-azidoacetylgalactosamine (GalNAz)[20] as starting substrate since it was recently used to enable the detection in the cells of mucin type GalNAc-bearing glycoproteins.[21] First, we determined the K_m of GK2 for GalNAz to be 0.50 mM,[16] a value in the same order of magnitude as for GalNAc (0.22 mM). Under the conditions described above[18] GalNAz was very efficiently converted into UDP-GalNAz (99% yield). Previous studies had suggested[21] that GalNAz was processed in cells by the hexosamine salvage pathway enzymes.[11] Here, we provide evidence that GalNAz and GalNAz-1-P, respectively, are substrates for GK2 and AGX1 in vitro.

The paper describes an efficient method for the enzymatic synthesis of UDP-GalNAc (68 ± 10%) from the inexpensive substrates GalNAc, ATP and UTP. With the amount of enzymes isolated after 1 L *E. coli* cultures, it is possible to obtain up to 500 mg of UDP-GalNAc. The method proposed here was also successfully employed on GalNAz, showing that both GK2 and AGX1 can accept substrates with modified N-acyl groups. Surprisingly, GalNAz appeared to be an excellent substrate for the three-enzyme system. Considering the K_m value of GK2 for GalNAz and the high specificity of this enzyme for GalNAc, AGX1 must be responsible for the high conversion into UDP-GalNAz suggesting a broad acceptance for this latter enzyme. Procedures using the same enzymatic system for the synthesis of other UDP-GalNAc analogues are currently under investigation. The same efficient method was also applied to the synthesis of radioactive UDP-GalNAc, a very useful substrate for enzymatic tests of *N*-acetylgalactosaminyltransferases and which is no longer commercially available. By this procedure we have produced [^{3}H]-labelled UDP-GalNAc with the same overall yield using [^{3}H]GalNAc. It is noteworthy that the method presented here offers, for the synthesis of radioactive UDP-GalNAc, an appreciable alternative to a pure chemical approach.

Acknowledgments

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15. DNAs encoding human GK2 and AGX1 were obtained from Open Biosystems. The complete coding sequences were amplified by PCR in order to create *Bam*HI restriction sites in 5′ and 3′ ends of both inserts. For GK2 the primers used were 5′GGATCCAATGGCTAC AGAGAGCCCTG3′, sense and 5′GGATCCGGCCTC AAGCAAAACC3′, antisense. For AGX1 the primers used were 5′CGGGATCCAATGAACATTAATGACC TCAAACTCCG3′, sense and 5′GCGGATCCTT CAAATACCATTTTTCACCAGCTCATG3′, antisense. The PCR products were digested by *Bam*HI, purified and ligated into a similarly digested pTrcHisB vector (Invitrogen). The resulting plasmids were used to transform *E. coli* ER 2566 (NEB) for GK2 and Rosetta™ (Novagen) for AGX1. Selected clones were grown in SOB medium at 37 °C for GK2 and 15 °C for AGX1 until the OD 600 nm reached 0.5–0.8. Then, recombinant protein syntheses were induced by addition of 0.5 mM (for AGX1) or 1 mM (for GK2) IPTG and cells were allowed to grow for an additional 5 h for GK2 and 23 h for AGX1. The pellets obtained after centrifugation were lysed in 2.5 mL/g pellet Y-PER plus™ (Pierce) containing 1 mM PMSF, 5 μg/mL DNAse, 10 μg/mL RNAse, and 0.5 mM DTT. After shaking at room temperature for 20 min and centrifugation, a second extraction was performed on the pellets. 6His-tagged recombinant proteins recovered in the lysis supernatants were isolated by affinity chromatography on Ni-NTA superflow beads (Qiagen) after a 3-fold dilution in 50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 10 mM imidazole, and 0.5 mM DTT (buffer A). After incubating for 2 h with the beads at 4 °C and three washes with buffer A, the proteins were eluted with 250 mM imidazole, pH 7.0, 0.5 mM DTT.
16. GK2 assays: unless otherwise stated, all products were purchased from Sigma–Aldrich. Reaction mixtures (25 μL) contained [^{3}H]GalNAc (American Radiolabeled Chemicals) adjusted to 1 mM and 1300 cpm/nmol, 5 mM ATP, 5 mM $MgCl_2$, 0.1 g/100 mL (0.1%) BSA and various amounts of GK2 in 75 mM Tris–HCl, pH 8.8. After 15 min at 37 °C, samples were diluted with 700 μL water/methanol 50:50 and applied to a 0.5 mL DOWEX 1 × 8

anion exchange column. The column was washed with 25 mM NH_4HCO_3 (3 × 1.5 mL) to remove unbound material, and then GalNAc-1-P was eluted with 250 mM NH_4HCO_3 (3 × 1.5 mL). All fractions were analysed by scintillation counting. For GalNAc-1-P preparation, the same protocol as above was scaled up to 23 mL with nonradioactive GalNAc; after DOWEX, fractions were pooled, concentrated and applied to a gel filtration column on G10 Sephadex (Amersham) gel (60 mL, flow rate 30 mL/h) before NMR analysis: GalNAc-1-P (53%, 3.59 mg). ^{1}H NMR (500 MHz, D_2O) δ: 5.46 (dd; 1H; $J_{H\text{-}1,P}$ = 7.5; $J_{1,2}$ = 3.5; H-1); 4.205 (ddd; 1H; $J_{2,3}$ = 11.0; $J_{2,P}$ = 2.3; H-2); 4.155 (ddd; 1H; $J_{5,6a}$ = 7.5; $J_{5,6b}$ = 5.1; H-5); 4.04 (d; 1H; $J_{4,3}$ = 3.0; H-4); 3.96 (dd; 1H; $J_{3,2}$ = 11.0; $J_{3,4}$ = 3.0; H-3); 3.785 (dd; 1H; $J_{6a,6b}$ = 11.9; $J_{6a,5}$ = 7.5; H-6a); 3.745 (dd; 1H; $J_{6b,6a}$ = 11.8; $J_{6b,5}$ = 5.0; H-6b); 2.09 (s; 3H; CH3). For K_m calculation, 70 ng of GK2 was used with 5 mM ATP and various amounts of GalNAc from 25 μM to 1400 μM in 25 μL total volume. The same method was used with GalNAz but with [^{32}P]ATP (Amersham). All assays were performed in triplicate. AGX1 assays: reaction mixtures (25 μL) contained [^{3}H]UTP (ARC) adjusted to 4 mM and 250 cpm/nmol, 5 mM GalNAc-1-P, 5 mM $MgCl_2$, 0.1% BSA and various amounts of AGX1 in 75 mM Tris–HCl pH 8.8. After 30 min at 37 °C, the samples were diluted with water/methanol 50:50 and applied to DOWEX 1 × 8 as described for GK2, except that UDP-GalNAc was eluted with 300 mM NH_4HCO_3 (10 × 1.5 mL) and non-reacted UTP with 1 M NH_4HCO_3. All fractions were analysed by scintillation counting. For Km calculations, 1.6 μg of enzyme was used with 4 mM UTP and various amounts of GalNAc-1-P from 0.275 mM to 6.6 mM in 25 μL total volume. All assays were performed in triplicate.

17. Combined GK2-AGX1 assays: optimisation tests were performed at 37 °C. Reaction mixtures (25 μL) contained [^{3}H]GalNAc (3 mM, 50 cpm/nmol), 5 mM ATP, 4 mM UTP, 5 mM $MgCl_2$, 0.1% BSA, yeast PPA (0.05 U) and various amounts of GK2 and AGX1 in 75 mM Tris–HCl, pH 8.8. After 2 h, samples were diluted with water/methanol 50:50 and applied to DOWEX 1 × 8, the column washed with 25 mM NH_4HCO_3 (3 × 1.5 mL) to remove unbound material, non-reacted GalNAc-1-P was eluted with 100 mM NH_4HCO_3 and UDP-GalNAc with 300 mM NH_4HCO_3.
18. For UDP-GalNAc synthesis a 2 mL reaction mixture contained 3 mM GalNAc, 3 mM UTP, 3 mM ATP, 5 mM $MgCl_2$, 0.1% BSA, GK2 (12 μg), AGX1 (1.6 μg) and PPA (4 U) in 75 mM Tris–HCl, pH 8.8. The reaction was left at 37 °C for 6 h, stopped by addition of methanol/water 50:50 (13 mL) and applied to DOWEX 1 × 8 (4 mL). After extensive washing first with 25 mM then with 100 mM NH_4HCO_3, UDP-GalNAc was eluted with 300 mM NH_4HCO_3. After evaporation, the sample was applied to a gel filtration column as described in Ref. 16. Detection was performed by absorbance reading at 260 nm.
19. UDP-GalNAc ammonium salt (68%, 2.62 mg). ^{1}H NMR (500 MHz, D_2O) δ: 7.96 (d; 1H; $J_{6'',5''}$ = 8.1; H-6″); 5.98 (d; 1H; $J_{1',2'}$ = 4.8; H-1′); 5.97 (d; 1H; $J_{5'',6''}$ = 8.1; H-5″); 5.55 (dd; 1H; $J_{H\text{-}1,P}$ = 7.3; $J_{1,2}$ = 3.3; H-1); 4.45–4.3 (m; H-2′; H-3′); 4.3–4.16 (m; H4′; H5′a; H5′b; H2; H5); 4.05 (br d; 1H; J = 2.1; H-4); 3.97 (dd; 1H; $J_{3,2}$ = 10.9; $J_{3,4}$ = 3.0; H-3); 3.78 (dd; 1H; $J_{6a,6b}$ = 11.8; $J_{6a,5}$ = 7.3; H-6a); 3.73 (dd; 1H; $J_{6a,6b}$ = 11.8; $J_{6b,5}$ = 5.2; H-6b); 2.09 (s; 3H; CH_3); MS (ESI): $(M+H)^+$, 608.13; $(M+NH_4)^+$, 625.25; $(M+Na)^+$, 630.5; $(M+K)^+$, 646.00.
20. GalNAz was synthesised according to Ref. 21. For UDP-GalNAz formation, the same conditions were used as for UDP-GalNAc.[18] UDP-GalNAz was characterised directly from the 25 μL reaction mixture after protein precipitation in MeOH/H_2O 50:50 by mass spectroscopy. MS (ESI): $(M-H)^-$ 647.08.
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