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UDP-*N*-Acetyl-α-D-glucosamine as acceptor substrate of β-1,4-galactosyltransferase. Enzymatic synthesis of UDP-*N*-acetyllactosamine

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The capacity of UDP-*N*-acetyl-α-D-glucosamine (UDP-GlcNAc) as an *in vitro* acceptor substrate for β-1,4-galactosyltransferase (β4GalT1, EC 2.4.1.38) from human and bovine milk and for recombinant human β4GalT1, expressed in *Saccharomyces cerevisiae,* was evaluated. It turned out that each of the enzymes is capable to transfer Gal from UDP-α-D-galactose (UDP-Gal) to UDP-GlcNAc, affording Gal(β1-4)GlcNAc(α1-UDP (UDP-LacNAc). Using β4GalT1 from human milk, a preparative enzymatic synthesis of UDP-LacNAc was carried out, and the product was characterized by fast-atom bombardment mass spectrometry and ^{1}H and ^{13}C NMR spectroscopy. Studies with all three β4GalTs in the presence of α-lactalbumin showed that the UDP-LacNAc synthesis is inhibited and that UDP-α-D-glucose is not an acceptor substrate. This is the first reported synthesis of a nucleotide-activated disaccharide, employing a Leloir glycosyltransferase with a nucleotide-activated monosaccharide as acceptor substrate. Interestingly, in these studies β4GalT1 accepts an α-glycosidated GlcNAc derivative. The results imply that β4GalT1 may be responsible for the biosynthesis of UDP-LacNAc, previously isolated from human milk.

Keywords: **glycosyltransferase/β-1,4-galactosyltransferase/nucleotide-activated disaccharide/UDP-LacNAc**

Abbreviations: **BSA, bovine serum albumin; FAB-MS, Fast Atom Bombardment Mass Spectrometry; 2D HMBC, two-dimensional Heteronuclear Multiple-Bond Coherence; 2D ROESY, two-dimensional Rotating Frame Nuclear Overhauser Enhancement Spectroscopy; 2D TOCSY, two-dimensional Total Correlation Spectroscopy; β4GalT, β-1,4-galactosyltransferase; α-LA, α-lactalbumin; LacNAc, *N*-acetyllactosamine; NMR, Nuclear Magnetic Resonance; UDP-GlcNAc, uridine 5′-diphospho-*N*-acetyl-α-D-glucosamine; UDP-Gal, uridine 5′-diphospho-α-D-galactose; UDP-GalNAc, uridine 5′-diphospho-*N*-acetyl-α-D-galactosamine; UDP-Glc, uridine 5′-diphospho-α-D-glucose; UDP-LacNAc, uridine 5′-diphospho-*N*-acetyllactosamine; UDP-Xyl, uridine 5′-diphospho-α-D-xylose.**

Introduction

β-1,4-Galactosyltransferase (β4GalT, EC 2.4.1.38) is the best characterized Leloir-glycosyltransferase with respect to *in vitro* oligosaccharide synthesis. It is commercially available and easily purified from bovine [1] or human milk [2,3]. When bound to the milk protein α-lactalbumin (α-LA) β4GalT forms the lactose synthase complex (EC 2.4.1.22) which has a high affinity for free glucose, thus synthesizing lactose [Gal(β1-4)Glc] in lactating mammary glands [4–6]. A β-1,4-galactosyltransferase gene family has been reported recently [7] the members of which show different properties. The β-1,4-galactosyltransferase studied in this paper is the β4GalT1 according to the nomenclature in reference [7].

The broad synthetic potential of β4GalT1 has been widely exploited for the chemoenzymatic synthesis of oligosaccharides and (neo)glycoconjugates. Thus it was demonstrated that β4GalT1 transfers Gal from UDP-α-D-galactose (UDP-Gal) to GlcNAc and its β-glycosides, whereas lactose synthase is involved *in vitro* in the galactosylation of Glc and its α- and β-glycosides [8–14]. In a reaction cycle with three enzymes we have utilized β4GalT1 from bovine and human milk for the synthesis of *N*-acetyllactosamine [Gal(β1-4)GlcNAc; LacNAc] on a gram scale [15], and in combination with recombinant α1,3-galactosyltransferase for the

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synthesis of the Galili-epitope Gal(α1-3)Gal(β1-4)GlcNAc (β1-O$(CH_2)_8COOCH_3$ [16].

Our current investigations on nucleotide-activated oligosaccharides were strongly inspired by Kobata [17], who in 1963 reported the isolation and identification of a nucleotide-activated disaccharide, Gal(β1-4)GlcNAc(α1-UDP (UDP-LacNAc), and a nucleotide-activated trisaccharide, Fuc(1-2/4)Gal(β1-4)GlcNAc(α1-UDP, from human milk and colostrum. In the same period, nucleotide-activated di- and trisaccharides were also identified in goat colostrum [Neu5Ac-Gal(1-6)GlcNAc(α1-UDP and Neu5Ac-Gal(1-4)GlcNAc(α1-UDP] [18], and hen oviduct [Gal(β1-*P*-6)GlcNAc(α1-UDP and Fuc(1-4)GlcNAc(α1-UDP] [19,20]. The finding of such compounds in hen oviduct demonstrated that nucleotide-activated oligosaccharides are not unique to mammals. The biosynthesis and the physiological function of these nucleotide-activated di- and trisaccharides have not yet been elucidated. In contrast, at the end of the eighties, UDP- and GDP-activated oligosaccharides were found in archaebacteria and identified as precursors in the biosynthesis of cell wall components such as pseudomurein and the S-layer [21–25].

In the present paper we demonstrate that Gal(β1-4) GlcNAc(α1-UDP (UDP-*N*-acetyllactosamine; UDP-LacNAc) can be synthesized by human and bovine β4GalT1 *in vitro*. This implies that the nucleotide sugar UDP-*N*-acetyl-α-D-glucosamine (UDP-GlcNAc) serves as an acceptor substrate of a mammalian Leloir glycosyltransferase, β4GalT1, whereby Gal is transferred from UDP-Gal to α-glycosidated GlcNAc (Fig. 1). The synthesis of UDP-LacNAc by a Leloir glycosyltransferase promises a wide synthetic and biochemical application but also raises questions about the cellular occurrence and physiological function of this compound as will be discussed in this paper.

Materials and methods

Materials

Uridine 5′-diphospho-α-D-galactose (UDP-Gal), uridine 5′-diphospho-α-D-glucose (UDP-Glc), uridine 5′-diphospho-*N*-acetyl-α-D-glucosamine (UDP-GlcNAc), uridine 5′-diphospho- α-D-xylose (UDP-Xyl), bovine serum albumin (BSA), α-lactalbumin (α-LA), and β-1,4-galactosyltransferase (β4GalT1) from bovine milk (EC 2.4.1.38) were purchased from Sigma (Deisenhofen, Germany). Uridine 5′-diphospho-*N*-acetyl-α-D-galactosamine (UDP-GalNAc) was synthesized as reported earlier [26]. Human milk and recombinant human β4GalT1 were obtained as previously described [3,27]. Calf intestinal alkaline phosphatase (EC 3.1.3.1) was from Boehringer Mannheim (Mannheim, Germany). HEPES (*N*-[2-(hydroxyethyl) piperazine]*N*′-(2-ethanesulphonic acid)) was supplied by Roth (Karlsruhe, Germany). D_2O (99.9 and 99.96 atom % D) was purchased from Isotec, Veendaal, The Netherlands. All other chemicals were from Merck (Darmstadt, Germany).

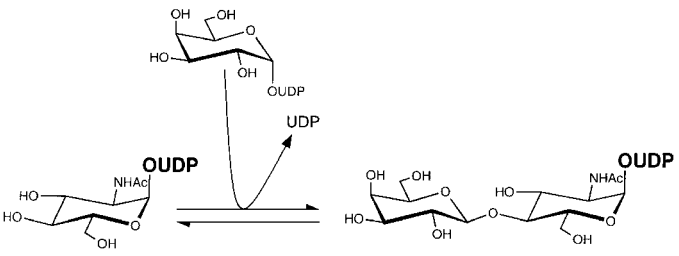

Figure 1. UDP-*N*-acetyl-α-D-glucosamine (UDP-GlcNAc) as acceptor substrate of β-1,4-galactosyltransferase (EC 2.4.1.38) with UDP-α-D-galactose as donor substrate: Enzymatic synthesis of Gal(β1-4)GlcNAc(α1-UDP (UDP-LacNAc).

Synthesis of UDP-*N*-acetyllactosamine with β-1,4-galactosyltransferase

In a standard assay, a buffer solution of 200 mM HEPES-NaOH pH 7.0, 1 mM $MnCl_2$, 1 mg ml^{-1} BSA, 0.01% NaN_3 contained 10 mM UDP-Gal and 10 mM UDP-GlcNAc. The reaction was started by the addition of 0.4 U ml^{-1} β4GalT1. The assay volume was 100 µl and the incubation temperature 30 °C. To study the effect of α-LA, variable amounts of α-LA were added to the assay as indicated below. Reactions were stopped by heating at 95 °C for 5 min as indicated in the Results and discussion section. Reaction mixtures were diluted with distilled water (1:5) and analyzed by HPLC on a Hypersil ODS colum (250 × 4.6 mm, 5 µm particle size) (CS Chromatografie Service, Langerwehe, Germany); elutions were performed with 100 mM potassium acetate pH 5.6, containing 0.013% (v/v) *n*-octylamine and 5% (v/v) methanol at a flow rate of 1 ml min^{-1}, monitoring the effluent at 260 nm [28]. Yields were determined by the relation between the peak area for uridine 5′-diphospho-*N*-acetyllactosamine (UDP-LacNAc) and the sum of the peak areas for UDP-LacNAc and the corresponding substrate (acceptor or donor).

Preparative Synthesis of UDP-*N*-acetyllactosamine with β-1,4-galactosyltransferase from human milk

To a solution of UDP-Gal (61 mg Na_2-salt, 100 µmol), UDP-GlcNAc (65.2 mg Na_2-salt, 100 µmol), and BSA (10 mg) in 200 mM HEPES-NaOH, pH 7.0 (10 ml), containing 1 mM $MnCl_2$, was added 4 U β4GalT1 from human milk, and the mixture was incubated for 7 days at 30 °C under sterile conditions. The reaction was stopped by denaturation of the enzyme at 95 °C (5 min), and an aliquot of the solution was diluted (1:5) with distilled water for analysis by HPLC [28]. The yield of the enzymatic synthesis was 14.6% (with reference to the acceptor substrate).

For the isolation of the product, proteins were removed in a 10 ml stirred ultrafiltration cell from Amicon (Witten, Germany) using a YM 10 membrane (cut-off 10 kDa). The protein-free solution was adjusted to pH 6.2, and loaded on

a column (1.2 × 8 cm) of Sepharose Q FF (Cl^--form; Pharmacia, Freiburg, Germany) equilibrated with distilled water. After washing with distilled water (30 ml), UDP-LacNAc was eluted with a linear salt-gradient (75 ml distilled water, 75 ml 1 M LiCl) at a flow rate of 1 ml min^{-1}. The fractions containing UDP-LacNAc (UV detection at 260 nm) were pooled and adjusted to pH 7.0. The solution was concentrated at 35 °C *in vacuo* and finally desalted by gel filtration on a column (2.6 × 74.5 cm) of Bio-Gel P-2 extra fine (Bio-Rad, Deisenhofen, Germany) using distilled water at a flow rate of 0.25 ml min^{-1} and at 4 °C. After lyophilisation, the product was analyzed by ^1H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy and fast-atom bombardment mass spectrometry (FAB-MS). The overall yield was 5.2% UDP-LacNAc Na_2-salt (based on the acceptor substrate; 5.2 μmol, 4.2 mg).

Mass spectrometry

Negative-ion mode Fast Atom Bombardment Mass Spectrometric (FAB-MS) analysis of the reaction product was performed on a JEOL JMS-SX/SX 102A four sector instrument operated at an acceleration voltage of 6 kV. The JEOL MS-FAB 10D FAB gun was operated at an emission current of 10 mA, producing a beam of 4 keV Xe atoms. 1 μl sample (10 μg in 0.1 ml 5% acetic acid) was mixed with 1 μl thioglycerol matrix, and linear mass scans over 1000 dalton were recorded. Recorded data were processed using JEOL complement software (Bijvoet Center, Department of Mass Spectrometry).

NMR spectroscopy

Prior to analysis the reaction products were repeatedly exchanged in D_2O (99.9 atom % D) with intermediate lyophilisation and finally dissolved in 450 μl D_2O (99.96 atom % D). Proton-decoupled 75.469-MHz ^{13}C NMR spectra were recorded on a Bruker AC-300 spectrometer at a probe temperature of 300 K. Chemical shifts (δ, ppm) are referenced to external acetone (δ 31.08). Resolution enhanced ^1H 1D and 2D NMR spectra were recorded on Bruker AMX-500 or Bruker AMX-600 instruments (Department of NMR spectroscopy, Utrecht University) at a probe temperature of 300 K. Chemical shifts (δ) were expressed in ppm relative to internal acetate (δ 1.908, acetone δ 2.225). HOD signal suppression was achieved by applying a WEFT pulse sequence in 1D ^1H experiments and by pre-saturation for 1 s in 2D experiments. 2D TOCSY (total correlation spectroscopy) spectra were recorded by using MLEV-17 mixing sequences with effective spin-lock times between 20 and 100 ms. 2D ROESY (rotating frame nuclear Overhauser enhancement spectroscopy) spectra were recorded with a mixing time of 250 ms [29]. The spin-lock field strength corresponded to a 90° pulse of about 115 μs. A proton detected ^{13}C-^1H 2D HMBC (heteronuclear multiple-bond coherence) experiment was performed at a ^1H frequency of 600.140 MHz (150.916 MHz for ^{13}C) using a pulse sequence as described by Summers *et al.* [30]. The delay time for the detection of long-range ^{13}C-^1H couplings was set to 60 ms. ^1H 1D and 2D spectra were processed on Silicon Graphics IRIS work stations (Indigo 2 and O2) using Bruker UXNMR software (Bijvoet Center, Department of NMR Spectroscopy). ^{13}C 1D spectra were elaborated on Silicon Graphics IRIS work stations (Indigo 2 and O2) using TRITON software (Bijvoet Center, Department of NMR Spectroscopy).

Effect of different parameters on UDP-LacNAc synthesis

First, the effect of the $MnCl_2$ concentration (1–20 mM) and the amount of α-LA on UDP-LacNAc synthesis with β4GalT1 from human or bovine milk was investigated using the assay as described above.

Secondly, the concentration of the acceptor substrate UDP-GlcNAc was varied at a constant concentration of 10 mM UDP-Gal. The assay was performed as described above with an incubation time of 4.5 days.

Thirdly, the synthesis of UDP-LacNAc with β4GalT1 was performed using the assay as described above with 10 mM UDP-Gal and 40 mM UDP-GlcNAc in the presence of 2 U ml^{-1} calf intestinal alkaline phosphatase at 30 °C [31].

Substrate specificity of β-1,4-galactosyltransferase

In a standard assay, a 10 mM donor nucleotide sugar/10 mM acceptor nucleotide sugar buffer solution (200 mM HEPES-NaOH, pH 7.0, 1 mM $MnCl_2$, 1 mg ml^{-1} BSA, 0.01% NaN_3; 100 μl) containing 0, 1 or 8 mg ml^{-1} α-LA, was incubated with 0.4 U ml^{-1} recombinant human β4GalT1, bovine β4GalT1, and human milk β4GalT1, respectively. After an incubation period of 6 days, the reaction was stopped by denaturation of the enzymes at 95 °C (5 min) and the mixture was analyzed by HPLC [28]. The following donor/acceptor combinations of nucleotide sugars were tested: UDP-Gal/UDP-Glc, UDP-Gal/UDP-Xyl, UDP-Gal/UDP-GalNAc, UDP-Gal/UDP-Gal, UDP-Glc/UDP-GlcNAc, UDP-Glc/UDP-Glc, UDP-Xyl/UDP-Xyl, UDP-GalNAc/UDP-GlcNAc, and UDP-GalNAc/UDP-Glc. Control experiments contained only the donor substrate and were treated in the same way as described above.

Results and discussion

UDP-*N*-Acetyl-α-D-glucosamine as acceptor substrate of β-1,4-galactosyltransferase

β-1,4-Galactosyltransferase (β4GalT1) accepts a wide spectrum of derivatives of the natural donor UDP-Gal and the natural acceptor GlcNAc [12]. In all reported cases the enzyme catalyzes the transfer of galactose or its derivative to the HO-4 position of a β-linked GlcNAc or its derivative.

It was also stated that β4GalT1 is not the suitable biocatalyst for preparative synthesis when *N*-acetyl-α-D-glucosaminides are used as acceptors [32,33].

In our initial experiments β4GalT1 from bovine and human milk were tested for their ability to accept UDP-*N*-acetyl-α-D-glucosamine (UDP-GlcNAc) as acceptor substrate (Fig. 1). After 7 days of incubation at 30 °C, HPLC profiling revealed in each case the appearance of a product eluting after UDP-Gal and UDP-GlcNAc; the yields were 15% and 14.6% (based on acceptor), respectively. The retention time of the product peak corresponded to that of Gal(β1-4)GlcNAc(α1-UDP (UDP-LacNAc) synthesized via a transgalactosylation reaction from lactose and UDP-GlcNAc in the presence of β-galactosidase from *Bacillus circulans* [Zervosen A, Nieder V, Gutiérrez Gallego R, Kamerling JP, Vliegenthart JFG, Elling L, unpublished results]. To exclude the possibility of contamination of the milk β4GalT1 preparations used in this work with other enzymes, the experiment was repeated with purified recombinant human β4GalT1 [27]. After an incubation time of 6 days the same HPLC profile was obtained with a product in a yield of 9.7% (based on the acceptor substrate) (Fig. 2). In order to characterize the product, a preparative enzymatic synthesis was performed with β4GalT1 from human milk. After 7 days of incubation at 30 °C, a product was formed in a yield of 14.6% (HPLC analysis; based on the acceptor substrate). Isolation of the product led to an overall product yield of 5.2% (5.2 μmol, 4.2 mg).

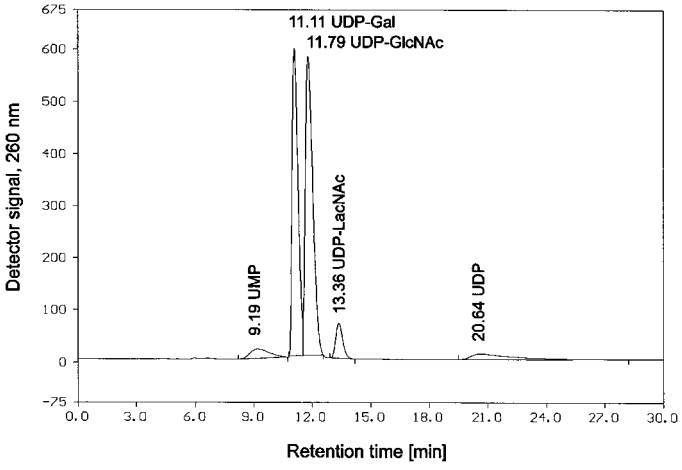

Figure 2. Synthesis of UDP-LacNAc with recombinant human β4GalT1 as biocatalyst, as analyzed by HPLC (column: Hypersil ODS colum (250 × 4.6 mm, 5 μm particle size); solvent system: 100 mM potassium acetate pH 5.6, containing 0.013% (v/v) *n*-octylamine and 5% (v/v) methanol; flow rate: 1 ml min^{-1}; UV-detection: 260 nm) [28]. A buffer solution of 200 mM HEPES-NaOH pH 7.0, 1 mM $MnCl_2$, and 0.01% NaN_3 contained 10 mM UDP-Gal and 10 mM UDP-GlcNAc. The incubation was carried out in the presence of 0.4 U ml^{-1} β4GalT1 and 1 mg ml^{-1} BSA for 6 days at 30 °C.

Structural studies of UDP-*N*-acetyllactosamine

The negative-ion mode FAB-MS spectrum of the nucleotide-activated saccharide showed one intense peak in the high-mass region at *m/z* 790.0 corresponding to the $[M-Na]^-$ pseudo-molecular ion of Hex-HexNAc-UDP ($X_{1,2}$ = Na, Fig. 3). Two fragment ions were found at *m/z* 462.0 and 321.0 belonging to Hex-HexNAc-*P* and UMP, respectively.

The 1D ^1H NMR spectrum (Fig. 4) showed four signals downfield of the HOD signal (δ 4.754). Two of these signals, at δ 8.002 and δ 5.969 (^3J 8.1 Hz), were attributed to the uracil-ring protons based on literature [34,35]. The anomeric signal at δ 5.969 ($^3J_{1,2}$ 2.6 Hz) was assigned to the ribose residue R (β configuration, furanose ring form), whereas the remaining anomeric signal at δ 5.511 ($^3J_{1,2}$ 3.2 Hz, $^3J_{1,P}$ 7.2 Hz) was assigned to the GlcNAc residue A (α configuration, pyranose ring form) linked to the phosphate group. Two chemical shifts, upfield of the HOD resonance could also be assigned; the singlet at δ 2.072 originating from the *N*-acetyl protons of GlcNAc A and the anomeric doublet at δ 4.465 ($^3J_{1,2}$ 7.5 Hz) which reflects the Gal residue B (β configuration, pyranose ring form).

The ^{13}C NMR revealed four signals in the anomeric region at δ 103.70 (B_{C-1}), 103.20 (U_{C-5}), 95.00 (A_{C-1}), and 89.80 (R_{C-1}) (Figure 4). Furthermore, four signals were observed in the downfield region at δ 175.55 ($A_{C=O}$), 166.70 (U_{C-2}), 152.30 (U_{C-4}), and 142.10 (U_{C-6}). Typical GlcNAc resonances were found at δ 22.72 ($A_{NAc-CH3}$) and δ 53.89 (A_{C-2}). The signals at δ 60.30, 61.62 and 65.13 reflected the presence of three hydroxymethyl groups, the latter of which is substituted.

By means of 2D TOCSY, ROESY and HMBC experiments all ^{13}C and almost all ^1H resonances in the 1D spectra could be assigned (Table 1). In the TOCSY spectra (not shown) the three identified anomeric ^1H signals were used to assign the complete spin systems; a mixing time of 100 ms allowed the identification of all resonances corresponding to a single residue, whereas a mixing time of 20 ms made the sequential assignment possible.

To establish the linkage type between residues B and A, 2D ROESY and HMBC experiments were performed. In the ROESY spectrum (not shown) the anomeric track of residue B revealed several cross-peaks at δ 3.998, 3.79, 3.737, 3.659 and 3.573. The cross-peak at δ 3.573 was attributed to TOCSY transfer to H-2 of the same residue. The cross-peaks at δ 3.737 and 3.659 were identified as intraresidual connectivities to H-5 and H-3, respectively. The remaining cross-peaks at δ 3.998 and 3.79 were assigned as interresidual contacts to H-5 and H-4/3 of residue A, respectively. In the ^{13}C-^1H HMBC spectrum (Fig. 4) the visualization of the interresidual three-bond connectivities over the glycosidic bond yielded the unambiguous determination of the B(β1-4)A sequence via two long-range couplings between A_{C-4} and B_{H-1} (δ 79.12 and 4.465) and between B_{C-1} and A_{H-4} (δ 103.70 and 3.767).

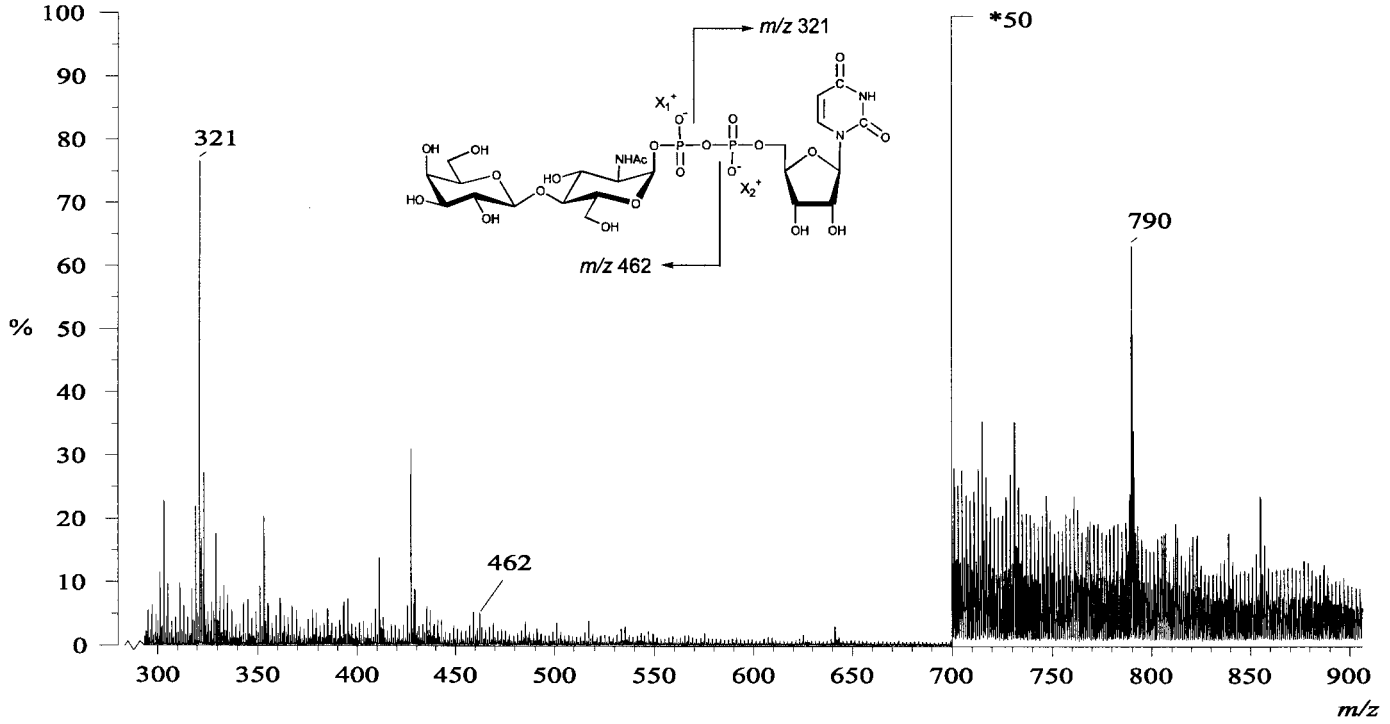

Figure 3. FAB-MS (negative-ion mode) of UDP-LacNAc, Gal(β1-4)GlcNAc(α1-UDP.

Evaluation of different conditions for UDP-*N*-acetyllactosamine synthesis

In order to gain further insight into the enzymatic synthesis of UDP-LacNAc from UDP-GlcNAc and UDP-Gal with β4GalT1 as a biocatalyst, the influence of different parameters was investigated. In earlier reports an optimal synthesis of Gal(β1-4)GlcNAc with β4GalT1 from human and bovine milk was achieved by enzyme stabilization with BSA and a concentration of Mn^{2+} between 20 and 50 mM [36,37]. In another study, an inhibitory effect of Mn^{2+} concentrations >4 mM at pH 8.0 on the activity of bovine milk β4GalT1 was found with 10 mM GlcNAc and 0.25 mM UDP-Gal [38].

In the present study, applying incubation periods of 7 days, a Mn^{2+} concentration of 1 mM at pH 7.0 was found to be optimal for UDP-LacNAc synthesis using the human or bovine β4GalT1. It was noted for the bovine enzyme that Mn^{2+}, at concentrations between 10 and 20 mM, and a pH between 7.5 and 7.9 catalyzes efficiently the decomposition of UDP-Gal (within 4 h) to UMP and Gal 1,2-cyclic phosphate, leading to a decrease in the product formation. These results are in accordance with studies on the stability of UDP-Gal [39]. However, UDP-GlcNAc was found to be stable for at least 9 h under the same conditions [39]. Based on these data, we suggest that a Mn^{2+} concentration of 1 mM reflects more the physiological conditions and may explain the biosynthesis of UDP-LacNAc, previously described to occur in human milk [17].

In additional experiments, the effect of higher acceptor concentrations on the yield of UDP-LacNAc was investigated. In Figure 5 it is shown that the yield of UDP-LacNAc can be further increased by increasing the concentration of UDP-GlcNAc. At 30 mM UDP-GlcNAc and 10 mM UDP-Gal a yield of 19.3% (based on UDP-Gal) after 4.5 days was obtained. Figure 5 also suggests that higher yields can be obtained at even higher UDP-GlcNAc concentrations. In comparison to the former conditions with 10 mM for each of the substrates the yield could be increased more than two-fold when incubated for 4.5 days.

The addition of calf intestinal alkaline phosphatase as used in other glycosyltransferase-mediated syntheses [31] has a beneficial effect on the yield of UDP-LacNAc. In Figure 6 it is shown that the yield increases up to 80% (based on UDP-Gal) within 19 days. However, at such long incubation periods decomposition of UDP-Gal has to be considered. Therefore, the yields based on the donor substrate were corrected by comparison with the yields based on the acceptor substrate. A yield of 17.4% based on acceptor substrate after 19 days corresponds to a corrected yield of 69.6% based on donor substrate indicating that 69.6% of UDP-Gal are converted to UDP-LacNAc and 10.4% of UDP-Gal are decomposed within 19 days (Fig. 6). It is obvious that decomposition of UDP-Gal is not a severe problem within 4.5 days of incubation. The yield does not increase very much after 4.5 days when compared to Figure 5 in the absence of alkaline phosphatase; this may

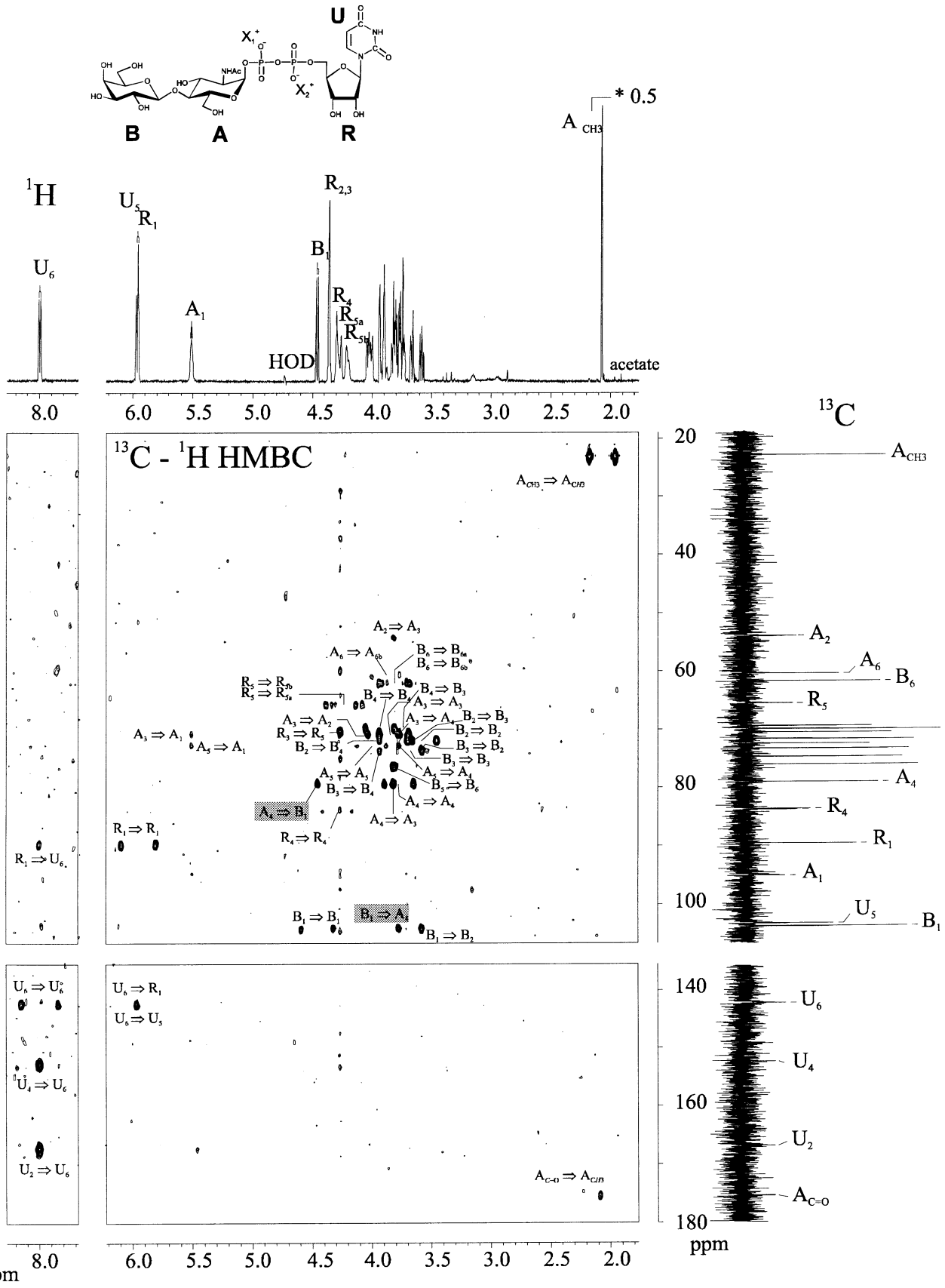

Figure 4. 75-MHz 1D ^{13}C, 500-MHz 1D ^{1}H, and 600-MHz 2D ^{13}C-^{1}H HMBC NMR spectra (at 300 K, D_2O) of UDP-LacNAc, Gal(β1-4)GlcNAc(α1-UDP. A = GlcNAc; B = Gal; R = Rib; and U = uracil. In the HMBC spectrum B_1. . . . A_4 means a cross-peak between $B_{C\text{-}1}$ and $A_{H\text{-}4}$, etc.

Table 1. ^{1}H and ^{13}C NMR chemical shifts of Gal(β1-4)GlcNAc (α1-UDP, prepared with β-1,4-galactosyltransferase from human milk, recorded at 300 K in D_2O. Coupling constants (Hz) are given between brackets.

Residue	*H or C*	^{1}H[a]	^{13}C[b]
GlcNAc	A_1	5.511 (3.2, 7.2)	95.00
	A_2	4.032	53.89
	A_3	3.806	70.29
	A_4	3.767	79.12
	A_5	3.998	72.37
	$A_{6(a)}$	n.d.[c]	60.30
	A_{6b}	3.885[c]	—
	A_{CH3}	2.072	22.72
	$A_{C=O}$	—	175.55
Gal	B_1	4.465 (7.5)	103.70
	B_2	3.573	71.66
	B_3	3.659	73.11
	B_4	3.933	68.97
	B_5	3.737	75.98
	$B_{6(a)}$	3.872[c]	61.62
	B_{6b}	3.792[c]	—
Rib	R_1	5.969 (2.6)	89.80
	R_2	4.364	74.76
	R_3	4.364	69.69
	R_4	4.289	83.93
	$R_{5(a)}$	4.265	65.13
	R_{5b}	4.212	—
Uracil	U_2	—	166.70
	U_4	—	152.30
	U_5	5.969 (8.1)	103.20
	U_6	8.002 (8.1)	142.10

[a]In ppm relative to the signal of internal acetate (δ 1.908).
[b]In ppm relative to the signal of external acetone (δ 31.08).
[c]The assignment of H-6a and H-6b may have to be interchanged within one residue; n.d. = not determined.

be due to differences in commercial preparations of the used bovine enzyme. However, alkaline phosphatase should be added in order to obtain a maximum yield.

Effect of α-lactalbumin and substrate specificity

As reported previously, the modification of the β4GalT1 activity by α-lactalbumin (α-LA) depends on the type and the concentration of the acceptor. α-LA acts as an inhibitor when the GlcNAc concentration is varied near its K_m value of 5.8 mM [6,38,40,41] and as an activator when the GlcNAc concentration is well below the K_m value (<2mM) [37,40,41]. The effect of α-LA on the UDP-LacNAc formation when using bovine β4GalT1 is shown in Figure 7. The yield of UDP-LacNAc decreases up to an α-LA concentration of 1 mg ml^{-1} (70 μM), and the synthesis is completely inhibited at 8 mg ml^{-1} (560 μM). In fact, our results can be compared to the classical studies by Brew *et al.* [40] which indicated 85% inhibition of the LacNAc synthesis at 2 mg

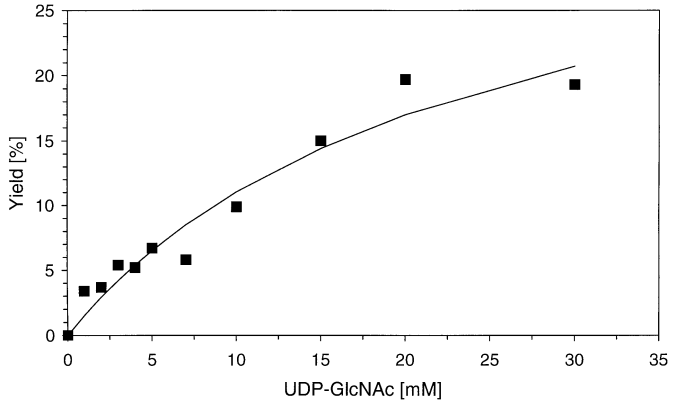

Figure 5. Effect of increasing UDP-GlcNAc concentrations on the yield of UDP-LacNAc. The yield is based on the initial UDP-Gal concentration. A buffer solution of 200 mM HEPES-NaOH pH 7.0, 1 mM $MnCl_2$, and 0.01% NaN_3 contained different concentrations of UDP-GlcNAc and 10 mM UDP-Gal. Incubations were carried out in the presence of 0.4 U ml^{-1} β4GalT1 from bovine milk and 1 mg ml^{-1} BSA for 4.5 days at 30 °C.

ml^{-1} (140 μM) α-LA. On the other hand, Berliner *et al.* [42] demonstrated a 100% activation of bovine β4GalT1 in the presence of 0.4 mg ml^{-1} (30 μM) α-LA and 2 mM GlcNAc. In this study it was also stated that the enzymatic conversion of glucosamine acceptors carrying longer *N*-acyl chains (e.g., *N*-octanoyl-) is inhibited by α-LA concluding that α-LA binds to a hydrophobic region of β4GalT1 and that both binding sites are juxtaposed. The effect of α-LA on the conversion of UDP-GlcNAc may also be similarly interpreted. Although being an α-glycosidated acceptor,

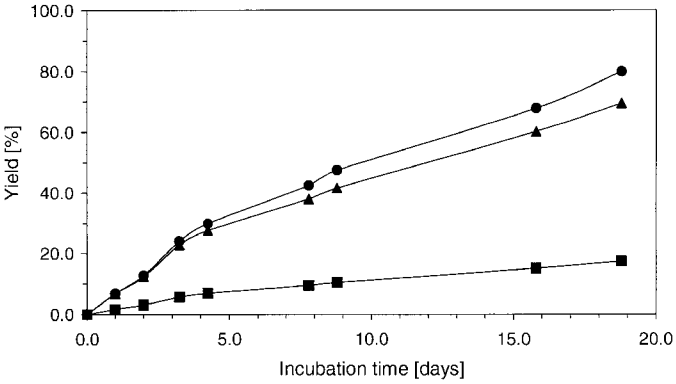

Figure 6. Effect of alkaline phosphatase (2 U ml^{-1}) on the yield of UDP-LacNAc. A buffer solution of 200 mM HEPES-NaOH pH 7.0, 1 mM $MnCl_2$, and 0.01% NaN_3 contained 40 mM UDP-GlcNAc and 10 mM UDP-Gal. Incubations were carried out in the presence of 0.4 U ml^{-1} β4GalT1 from bovine milk and 1 mg ml^{-1} BSA for several days at 30 °C. ●, Yield based on donor substrate; ■, yield based on acceptor substrate; ▲, corrected yield indicating the conversion of UDP-Gal into the product UDP-LacNAc. The difference between the yield based on donor substrate and the corrected yield is due to partial decomposition of UDP-Gal.

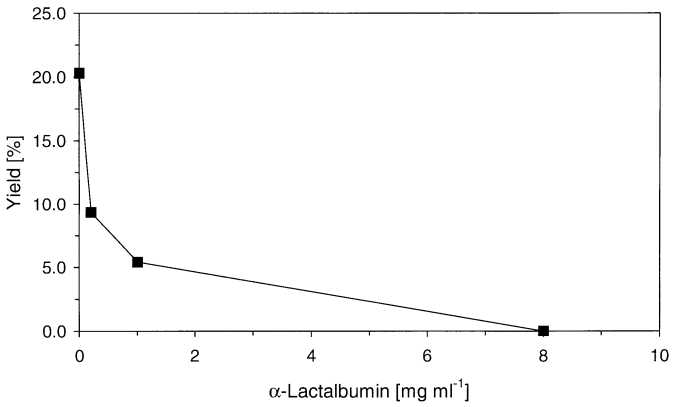

Figure 7. Effect of α-lactalbumin on the formation of UDP-LacNAc by β4GalT1 from bovine milk. A buffer solution of 200 mM HEPES-NaOH pH 7.0, 1 mM $MnCl_2$, and 0.01% NaN_3 contained 10 mM UDP-Gal and 10 mM UDP-GlcNAc. Incubations were carried out at 30 °C in the presence of 0.4 U ml^{-1} β4GalT1, 1 mg ml^{-1} BSA and varying concentrations of α-lactalbumin.

the nucleotide moiety of the activated sugar may occupy a part of the α-LA binding site, thereby contributing to the binding of the acceptor substrate. Elucidation of the exact mechanism must await three-dimensional structural analysis of β4GalT1.

In order to investigate further the substrate specificity of β4GalT1 other nucleotide sugars in different donor/acceptor combinations in the absence and presence of α-lactalbumin were tested. The results are summarized in Table 2 for recombinant human β4GalT1. The effect of α-LA on the formation of UDP-LacNAc (Fig. 7) supports the well-known role of α-LA as a modulator of β4GalT1 decreasing the enzyme's affinity for *N*-acetyl-D-glucosamine [6]. However, although it has been shown previously [6] that α-LA increases the affinity of β4GalT1 for glucose, UDP-Glc was not found to be an acceptor substrate for the enzyme in the presence of α-LA (UDP-lactose could not be detected by HPLC). This result was not surprising because β4GalT1 accepts α- and β-glucosides in the presence of α-LA provided that the aglycon is small [8,32,42]. In earlier studies it was demonstrated that Glcα1-*P* is not an acceptor in the presence of α-LA [42,43] implying that a negative charge hinders the recognition by the enzyme. However, in the donor/acceptor combination UDP-Gal/UDP-Glc UDP is formed in the presence and absence of α-LA indicating that UDP-Gal is enzymatically hydrolyzed. A control experiment with UDP-Gal as acceptor (20 mM UDP-Gal in the reaction) also exhibited hydrolysis. In addition, Table 2 clearly indicates that no other combination of nucleotide sugars with or without α-LA gives rise to the formation of a nucleotide-activated oligosaccharide. In some combinations, UDP is produced by enzymatic hydrolysis. With UDP-Xyl and UDP-GalNAc as acceptors UDP is only formed in the presence of α-LA. In earlier studies, xylose

Table 2. Substrate specificity of recombinant human β4GalT1 with different donor/acceptor combinations of nucleotide monosaccharides. In a standard assay, a 10 mM donor nucleotide sugar/10 mM acceptor nucleotide sugar buffer solution (200 mM HEPES-NaOH, pH 7.0, 1 mM $MnCl_2$, 1 mg ml^{-1} BSA, 0.01% NaN_3; 100 μl) containing 0, 1 or 8 mg ml^{-1} α-LA, was incubated with 0.4 U ml^{-1} recombinant human β4GalT1. After an incubation period of 6 days, the reaction was stopped by denaturation of the enzymes at 95 °C (5 min) and the mixture was analyzed by HPLC [28]. Control experiments contained only the donor nucleotide sugar.

		Formation[a] of NAO[b]/UDP		
		Addition of α-lactalbumin (mg ml^{-1})		
Donor substrate	*Acceptor substrate*	*None*	*1*	*8*
UDP-Gal	UDP-Glc	−/+	−/+	−/+
UDP-Gal	UDP-Xyl	−/−	−/+	−/+
UDP-Gal	UDP-GalNAc	−/−	−/+	−/+
UDP-GalNAc	UDP-Glc	−/−	−/−	−/−
UDP-GalNAc	UDP-GlcNAc	−/−	−/−	−/−
UDP-Glc	UDP-GlcNAc	−/−	−/−	−/−
UDP-Glc	UDP-Glc	−/−	−/−	−/−
UDP-Xyl	UDP-Xyl	−/−	−/−	−/−
UDP-Gal	UDP-Gal	−/−	−/+	−/+

[a]− no formation, + formation, of NAO and UDP, respectively.
[b]NAO: nucleotide activated oligosaccharide.

was reported to be a good acceptor in the presence of α-LA [36,42] and has been recently identified as the first acceptor of bovine milk β4GalT1 giving rise to two disaccharide products (Gal(β1-4)Xyl and Gal(β1-β1)Xyl) [14]. UDP-GalNAc was reported to serve as a good donor substrate of β4GalT1 from bovine milk in the presence of a high α-LA concentration (8 mg ml^{-1}) and GlcNAc as acceptor yielding GalNAc(β1-4)GlcNAc [44]. In our experiments the donor/acceptor combinations UDP-GalNAc/UDP-GlcNAc and UDP-GalNAc/UDP-Glc gave neither a nucleotide disaccharide nor UDP, respectively, in the presence or absence of α-LA (Table 2). UDP-Glc was also identified to be a donor substrate of bovine β4GalT1 with GlcNAc as acceptor [45,46]. A transfer reaction to UDP-GlcNAc or hydrolysis of UDP-Glc could not be detected in our experiments. Similar results were also obtained for β4GalT1 from human and bovine milk (data not shown).

The findings of UDP-GlcNAc as an acceptor substrate may also be interpreted with respect to previous findings concerning the donor specificity of β4GalT1. It has been shown that in addition to UDP-Gal also other naturally occurring UDP-sugars can serve *in vitro* as donor sub-

strates of β4GalT [47]. These were interpreted as side activities which can be exploited for synthesis. UDP-Glc and UDP-GalNAc were utilized as donor substrates of bovine β4GalT1 [46,48] for the synthesis of Glc(β1-4)GlcNAc(β1-OR and, in the presence of α-LA, for the synthesis of GalNAc(β1-4)GlcNAc [44].

More experimental evidence concerning the ability to synthesize UDP-LacNAc may come from a new human β4GalT family [7]. The new members, β4GalT2, β4GalT3 [7], β4GalT4 [49], β4GalT5 [50,51], and β4GalT6 [52] show a different response of activity towards the modulation by α-LA. β4GalT3 and 5 are insensitive to α-LA and transfer little if any Gal from UDP-Gal to Glc. They do not show any inhibition of LacNAc synthesis, however, β4GalT2 reacts like the bovine β4GalT. On the other hand, β4GalT4 activity is increased by α-LA for Glc and GlcNAc as acceptor substrates [49] and is required together with β-1,3-*N*-acetylglucosaminyltransferase for the efficient biosynthesis of poly-*N*-acetyllactosamine in core 2 branched O-glycans [53]. β4GalT6 is involved in the biosynthesis of lactosylceramide [52]. Whether one of these new β4GalT family members has a higher affinity and activity towards UDP-GlcNAc as an acceptor substrate remains to be investigated. In this context it may also be elucidated whether UDP-LacNAc plays a donor substrate role in the biosynthesis of poly-LacNAc glycans in glycoproteins and glycolipids.

General remarks

The observation that UDP-GlcNAc is *in vitro* an acceptor substrate of β4GalT1 from human milk is of biochemical importance and gives a plausible explanation for the biosynthesis of UDP-LacNAc identified in human milk [17]. The biochemical and physiological role of UDP-LacNAc is still unclear, but can now be addressed since we have established the preparative access to this compound by using β4GalT1 (present paper) as well as by using β-galactosidase from *Bacillus circulans* in a transgalactosylation reaction (Zervosen A, Nieder V, Gutiérrez Gallego R, Kamerling JP, Vliegenthart JFG, Elling L, unpublished results). The enzymatic synthesis of UDP-LacNAc as a nucleotide-activated building bloc may promise novel applications of prokaryotic and eukaryotic *N*-acetylglucosaminyltransferases (GlcNAcTs) in oligosaccharide synthesis, e.g., synthesis of poly-*N*-acetyllactosamine (poly-LacNAc) or branched N- and O-glycan structures. Work is in progress in our laboratory to check different *N*-acetylglucosaminyltransferases for their ability to transfer LacNAc *en bloc* from UDP-LacNAc onto specified acceptor structures. In addition, the cellular appearance and the physiological function of UDP-LacNAc as well as inhibition studies of GlcNAcTs and nucleotide sugar transporters can be addressed.

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