Glycoconjugate Journal (1992) **9**:180–190

Control of glycoprotein synthesis: substrate specificity of rat liver UDP-GlcNAc:Manα3R β2-*N*-acetylglucosaminyltransferase I using synthetic substrate analogues

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Received 20 January 1992

UDP-GlcNAc: Manα3R β2-*N*-acetylglucosaminyltransferase I (GlcNAc-T I; EC 2.4.1.101) is the key enzyme in the synthesis of complex and hybrid N-glycans. Rat liver GlcNAc-T I has been purified more than 25,000-fold (M_r 42,000). The V_{max} for the pure enzyme with [Manα6(Manα3)Manα6](Manα3)Manβ4GlcNAcβ4GlcNAcβ-Asn as substrate was 4.6 μmol min^{-1} mg^{-1}. Structural analysis of the enzyme product by proton nuclear magnetic resonance spectroscopy proved that the enzyme adds an *N*-acetylglucosamine (GlcNAc) residue in β1-2 linkage to the Manα3Manβ-terminus of the substrate. Several derivatives of Manα6(Manα3)Manβ-R, a substrate for the enzyme, were synthesized and tested as substrates and inhibitors. An unsubstituted equatorial 4-hydroxyl and an axial 2-hydroxyl on the β-linked mannose of Manα6(Manα3)Manβ-R are essential for GlcNAc-T I activity. Elimination of the 4-hydroxyl of the α3-linked mannose (Man) of the substrate increases the K_M 20-fold. Modifications on the α6-linked mannose or on the core structure affect mainly the K_M and to a lesser degree the V_{max}, e.g., substitutions of the Manα6 residue at the 2-position by GlcNAc or at the 3- and 6-positions by mannose lower the K_M, whereas various other substitutions at the 3-position increase the K_M slightly. Manα6(Manα3)4-*O*-methyl-Manβ4GlcNAc was found to be a weak inhibitor of GlcNAc-T I.

Keywords: GlcNAc-transferase I, substrate specificity, glycoprotein biosynthesis, N-linked glycans

Abbreviations: BSA, Bovine serum albumin; Bn, benzyl; Fuc, F, L-fucose; Gal, G, D-galactose; GalNAc, GA, *N*-acetyl-D-galactosamine; Glc, D-glucose; GlcNAc, Gn, *N*-acetyl-D-glucosamine; HPLC, high performance liquid chromatography; Man, M, D-mannose; mco, 8-methoxycarbonyl-octyl, $(CH_2)_8COOCH_3$; Me, methyl; MES, 2-(*N*-morpholino)ethanesulfonate; NMR, nuclear magnetic resonance; PMSF, phenylmethylsulfonylfluoride; pnp, *p*-nitrophenyl; SDS, sodium dodecyl sulfate; T, transferase; Tal, D-talose; Xyl, D-xylose; {0, 2+F}, Manα6 (GlcNAcβ2Manα3) Manβ4GlcNAcβ4 (Fucα6) GlcNAc; {2, 2}, GlcNAcβ2Manα6 (GlcNAcβ2Manα3) Manβ4GlcNAcβ4GlcNAc; M_5-glycopeptide, Manα6 (Manα3) Manα6 (Manα3) Manβ4 GlcNAcβ4GlcNAcβ-Asn.

Enzymes: GlcNAc-transferase I, EC 2.4.1.101; GlcNAc-transferase II, EC 2.4.1.143; GlcNAc-transferase III, EC 2.4.1.144; GlcNAc-transferase IV, EC 2.4.1.145; GlcNAc-transferase V, UDP-GlcNAc: GlcNAcβ2 Manα6-R (GlcNAc to Man) β6-GlcNAc-transferase; GlcNAc-transferase VI, UDP-GlcNAc: GlcNAcβ6(GlcNAcβ2) Manα6-R (GlcNAc to Man) β4-GlcNAc-transferase; Core 1 β3-Gal-transferase, EC 2.4.1.122; β4-Gal-transferase, EC 2.4.1.38; β3-Gal-transferase, UDP-Gal: GlcNAcβ-R β3-Gal-transferase; blood group i β3-GlcNAc-transferase, EC 2.4.1.149; blood group I β6-GlcNAc-transferase, UDP-GlcNAc: GlcNAcβ3Galβ-R (GlcNAc to Gal) β6-GlcNAc-transferase.

Complex N-glycans are thought to be involved in biological recognition phenomena and in certain diseases such as metastatic cancer. UDP-GlcNAc: Manα3R β2-*N*-acetylglucosaminyltransferase I (GlcNAc-T I; EC 2.4.1.101) is the key enzyme controlling the synthesis of complex and hybrid N-glycans. GlcNAc-T I initiates branching and is required for the subsequent action of all branching GlcNAc-transferases [1]. A specific inhibitor for GlcNAc-T

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I is not available; such a compound could be useful in the treatment of diseases involving N-glycan branching. GlcNAc-T I has been partially purified from bovine colostrum [2], and from pig liver and trachea [3], and to homogeneity from rabbit liver [4, 5]. Rabbit cDNA [6] and human cDNA and genomic DNA [7, 8] for GlcNAc-T I have recently been isolated.

Vella *et al.* [9], using synthetic substrate derivatives and a partially purified enzyme preparation from bovine colostrum, showed that the Manα6 arm of GlcNAc-T I substrate was not essential for activity but that the Manβ residue played an important role. It was later shown for rabbit liver GlcNAc-T I that an equatorial hydroxyl group at the carbon-4 position of the Manβ-residue of the substrate Manα6(Manα3)Manβ-R was essential for GlcNAc-T I activity since an axial hydroxyl at this position, or removal of the oxygen, or substitution of the hydroxyl group with a bisecting GlcNAc residue all resulted in an inactive substrate [5].

Rat liver has previously been reported to contain three of the six GlcNAc-transferases that assemble the branches of complex N-linked oligosaccharides, i.e., GlcNAc-transferases I, II and IV [1, 10–12]. In the present study, we have purified rat liver GlcNAc-T I and have used the pure enzyme to test a series of synthetic analogues of the Manα6(Manα3)Manβ-R substrate as substrates and as potential inhibitors and active site reagents. Most of these synthetic compounds have not previously been available for testing.

Materials and methods

Materials

AG1-X8 (100–200 mesh, Cl^- form) and Bio-Gel P-4 (-400 mesh) was purchased from Bio-Rad. Bovine serum albumin, Galβ1-4GlcNAc, GalNAcβ1-3Galβ-methyl, Triton X-100, GlcNAc and GlcNAcα-benzyl were purchased from Sigma. Acetonitrile (190 nm UV cutoff) was from Fisher Scientific Co. or Caledon Laboratories. UDP-*N*-[1-^{14}C] acetylglucosamine was synthesized as described previously [13] and diluted with UDP-GlcNAc from Sigma. UDP-[U-^{14}C] galactose was purchased from Amersham and diluted with UDP-galactose from Sigma. All other reagents were purchased and prepared as described [5, 10, 11]. Oligosaccharides $\{0, 2+F\}$ and $\{2, 2\}$ were prepared as previously described [14]. Manα6 (Manα3) Manβ-$(CH_2)_8COOCH_3$, Manα6(Manα3)Manβ-octyl, Manα6-(Manα3)Glcβ-octyl and Manα6(Manα3)(2-deoxy-Manβ)-octyl were synthesized by the methods described previously [15] and were kindly donated by Dr O. Hindsgaul, Univ. of Alberta, Edmonton, Alberta, Canada. GlcNAcβ6(GlcNAcβ2)Manα-methyl was kindly provided by Dr. R. Shah, University of Toronto, Toronto, Canada. [Manα6(Manα3)-Manα6](Manα3)Manβ4GlcNAcβ4GlcNAc-Asn (M_5-glycopeptide) was prepared as described previously [5].

The following compounds were prepared by chemical synthesis: Manα3Manβ4GlcNAc [16]; Manα6(Manα3)-Manβ4GlcNAc [17]; Manα6(Manα3)Manβ4GlcNAcβ4-GlcNAc [18]; Manα6(GlcNAcβ2 Manα3)Manβ4GlcNAc and GlcNAcβ2Manα6(Manα3)Manβ4GlcNAc [19]; Manβ-(5,6-epoxy-)hexyl, Manα6(Manα3)Manβ(5,6-epoxy-)hexyl, Manα6(Manα3)Manβ-hexyl, Manα6(Manα3)Manβ-octyl, [3-*O*-(4,5-epoxy)pentyl]Manα6(Manα3)Manβ-octyl, [3-*O*-(4,4-azo)pentyl]Manα6(Manα3)Manβ-octyl, [3-*O*-(5-iodoacetamido)pentyl]Manα6-(Manα3)Manβ-octyl, Manα6(2-deoxyManα3)Manβ-octyl and Manα6(2-*O*-methyl-Manα3)-Manβ-octyl [20]; Manα6(4-deoxyManα3)Manβ-octyl (M. Springer, Diplomarbeit, University of Hamburg, 1990); Manα6(Manα3)(4-deoxy)Manβ4GlcNAc and Manα6-(Manα3)Talβ4GlcNAc [21]; Manα6(Manα3)-[GlcNAcβ4]-Manβ4GlcNAc [22]. Manα3Manβ4GlcNAc-OH was prepared from Manα3Manβ4GlcNAc by reduction as described [9]. Manα6(Manα3)(4-*O*-methyl)Manβ4GlcNAc was synthesized (R. Wilkens, Diplomarbeit, University of Hamburg, 1988) and purified by HPLC on a PAC column (Mandel Scientific) with acetonitrile–water 82:18, by vol, at 1 ml min^{-1} flow rate. The structures of all synthetic compounds were confirmed by NMR spectroscopy.

Nuclear magnetic resonance spectroscopy

Samples were prepared by exchanging twice with 99.8% ^2H_2O (Aldrich) and twice with 99.96% ^2H_2O (Merck, Sharpe and Dohme). Samples were dissolved in 99.96% ^2H_2O with acetone as the internal standard. Proton NMR spectra were accumulated at the Toronto Carbohydrate Research Centre with a Bruker 500 MHz spectrometer. The acetone signal was set at 2.225 ppm.

High performance liquid chromatography

HPLC separations were carried out with an LKB or a Waters system [1, 23]. Acetonitrile–water mixtures were used as the mobile phase for all columns at a flow rate of 1 ml min^{-1}. To separate enzyme products with hydrophobic aglycone groups, a reversed phase C_{18} column and acetonitrile–water (10:90 by vol) were used. Reducing sugars and methyl glycosides were separated on a propylamine (NH_2) column (carbohydrate analysis column from Waters) at acetonitrile–water ratios from 70:30 to 86:14 by vol. Elutions of compounds were monitored by measuring the absorbance at 195 nm, and counting the radioactivity of collected fractions [1].

Purification of rat liver GlcNAc-T I

GlcNAc-T I was purified from frozen rat liver (Pel-Freez) by the procedure previously used for rabbit liver [5] with the following modifications. (i) A smaller ratio of extraction buffer to tissue was used to reduce the extraction volume. (ii) The Triton X-100 extraction was performed using buffer containing 0.1 M (instead of 0.4 M) NaCl. (iii) Buffers did not contain PMSF or 6-aminocaproic acid. (iv)

CM-Sephadex chromatography was carried out using elution buffer containing 0.8 M (instead of 1 M) NaCl. (v) It was necessary to use 50 mM UDP (instead of 10 mM) to elute enzyme from the last 5-Hg-UDP-GlcNAc-Sepharose column.

The purified enzyme preparation was stored at 0–4 °C in 10 mM $MnCl_2$, 25 mM MES, pH 6.5, 20% glycerol, 0.1% Triton X-100, and 0.02% NaN_3, and was stable for at least one year.

Protein determination

Protein was determined by the Bio-Rad method using bovine serum albumin as a standard. Protein in highly purified enzyme preparations was determined by the bicinchoninic acid micro assay method of Smith *et al.* [24] as modified by Bendiak and Schachter [10].

Assay for UDP-GlcNAc: Manα3R β2-GlcNAc-T I

The standard assay for measuring activity after each purification step [5] contained, in a total volume of 25 µl, 0.26 mM M_5-glycopeptide, 0.6 mM UDP-[^{14}C]GlcNAc (5108 dpm nmol^{-1}), 1 mg ml^{-1} BSA, 0.1% Triton X-100, 10 mM $MnCl_2$, 0.1 M NaCl, 50 mM MES buffer, pH 6.1, and enzyme preparation. For the first four purification steps, 5 mM AMP and 0.1 M GlcNAc were added to the incubation mixture to inhibit pyrophosphatase and hexosaminidase, respectively. Incubations were carried out for 30–60 min at 37 °C and stopped by the addition of 0.4 ml 20 mM sodium tetraborate and 1 mM EDTA. The mixtures were passed through Pasteur pipettes filled with AG1-X8, 100–200 mesh, Cl^- form, equilibrated in water. The columns were washed with 2.6 ml water and the eluates were counted in 17 ml scintillation fluid. For HPLC analysis, eluates were lyophilized, taken up in 0.2 ml water and stored at −20 °C. Aliquots of 0.1 ml were analyzed by HPLC as described in the Tables. Enzyme activities were calculated by correcting for incubations lacking the acceptor.

Characterization studies of GlcNAc-T I

For characterization and specificity studies, 5 µl purified GlcNAc-T I containing 15 ng protein were assayed as described above with 2.1 mM UDP-[^{14}C]GlcNAc, 2678 dpm nmol^{-1}, 20 mM $MnCl_2$ and 1 h incubation time, omitting NaCl. Potential inhibitors were assayed at several concentrations in the absence and in the presence of several concentrations of added substrate Manα6(Manα3)Manβ-hexyl, Manα6(Manα3)Manβ-octyl or Manα6(Manα3)Manβ-mco. Potential inhibitors were also preincubated for 10 minutes at room temperature with the enzyme before the addition of substrate. The diazirino derivative, [3-*O*-(4, 4-azo)pentyl]Manα6(Manα3)Manβ-octyl, was irradiated at room temperature with UV light for 10 min at a distance of 3–5 cm in the presence of enzyme before the incubation.

Assays for GlcNAc-transferases II, III, IV, V and VI, for blood group i UDP-GlcNAc: Galβ-R β3-GlcNAc-transferase (i β3-GlcNAc-T, EC2.4.1.149), and blood group I UDP-GlcNAc: GlcNAcβ3Galβ-R (GlcNAc to Gal) β6-GlcNAc-transferase (I β6-GlcNAc-T)

Assays were carried out under the conditions used to characterize GlcNAc-T I (see above), or with variations as described in the Tables. GlcNAc-T II [10, 11] was assayed with 0.32 mM {0, 2 + F} or 3 mM Manα6(GlcNAcβ2Manα3)-Manβ4 GlcNAc as the substrate. The acceptor for GlcNAc-transferases III, IV and V [1] was 1 mM {2, 2}. GlcNAc-T VI [25] was assayed with 1 mM GlcNAcβ6-(GlcNAcβ2)Manα-Me; i β3-GlcNAc-T with 2 mM Galβ4-GlcNAc; and I β6-GlcNAc-T with 2 mM GlcNAcβ3Galβ-Me [23]. GlcNAc-T V and I β6-GlcNAc-T were assayed in the absence of exogenous $MnCl_2$.

Assays for UDP-Gal: GlcNAc β4-Gal-transferase (β4-Gal-T, EC 2.4.1.38), UDP-Gal: GlcNAc β3-Gal-transferase (β3-Gal-T) and core 1 UDP-Gal: GalNAcα-R β3-Gal-transferase (core 1 β3-Gal-T, EC 2.4.1.122)

Gal-transferase assays were carried out under the conditions used to characterize GlcNAc-T I (see above) except that 1.4 mM UDP-[U-^{14}C]Gal (2220 dpm nmol^{-1}), 125 mM MES, pH 7, and 20 mM $MnCl_2$ were used while UDP-GlcNAc and GlcNAc were omitted from the assays. The acceptors were 2 mM GlcNAc for β4- and β3-Gal-T, and 5 mM GalNAcα-Bn for core 1 β3-Gal-T.

Large scale GlcNAc-T I product

Large scale GlcNAc-T I product was produced from Manα6(Manα3)Manβ4-GlcNAc substrate by scaling up the standard assay 30-fold at 2-mM UDP-GlcNAc (450 dpm nmol^{-1}), 105 mM MES, pH 5.5, and 20 mM $MnCl_2$, with 5-h incubation time. After the incubation, 8 ml EDTA–borate were added and the enzyme product was applied to a column (1.6 cm × 15 cm) of AG1-X8, 100–200 mesh, Cl^- form, followed by washing with 80 ml water. The eluate was applied to a Bio-Gel P-4 (-400 mesh) column (1.6 cm × 86 cm), equilibrated in water. The radioactive fractions were purified on HPLC using a Waters carbohydrate (amine) column at 1 ml min^{-1} at an acetonitrile–water ratio of 73:27 by vol. The structure of the product (product A) was confirmed by comparison of its HPLC elution time and 500 MHz NMR spectrum with those of synthetic Manα6(GlcNAcβ2Manα3)-Manβ4-GlcNAc.

Large scale enzyme product (product B) from Manα6(Manα3)Manβ-octyl substrate was prepared as described above and isolated by HPLC using a C_{18} column with acetonitrile–water, 12:88 by vol, as the mobile phase.

Table 1. Purification scheme for rat liver GlcNAc-transferase I.

Purification step	*Total protein (mg)*	*Total activity*[a] *(mU)*	*Specific activity*[a] *(mU per mg protein)*	*Yield %*	*Purification factor*
1. Homogenate	405 000	72 000	0.18	100	1
2. Triton extract	144 000	56 000	0.39	78	2
3. CM Sephadex	6 300	5 500	0.88	8	5
4. UDP-hexanolamine-Sepharose (NaCl elution)	56	1 900	34	2.6	190
5. Affi-Gel Blue	22	770	35	1	200
6. UDP-hexanolamine-Sepharose (EDTA elution)	1.6	430	280	0.6	1 560
7. UDP-hexanolamine-Sepharose (UDP elution)	0.5	480	1 000	0.7	5 600
8. 5-Hg-UDP-GlcNAc-Sepharose (EDTA elution)	0.2	230	1 100	0.3	6 300
9. 5-Hg-UDP-GlcNAc-Sepharose (UDP elution)	0.03	150	4 600	0.2	26 000

[a] 1 mU = 1 nmol min^{-1}.

Results

Enzyme purification

Rat liver GlcNAc-T I was bound by UDP-hexanolamine-Sepharose and by 5-Hg-UDP-GlcNAc-Sepharose, as previously described for the rabbit liver enzyme. The final preparation was purified more than 25 000-fold with a 0.19% yield and a specific activity of 4.6 μmol min^{-1} mg^{-1} (Table 1). SDS-PAGE analysis showed a single band at M_r 42 000 (Fig. 1). Assays for contaminating enzyme activities (Table 2) showed that the final enzyme preparation was free from core 1 β3-Gal T (GalNAcα-Bn substrate), UDP-Gal: GlcNAc β3- and β4-Gal-transferases (GlcNAc substrate), GlcNAc-T V ({2, 2} substrate in the absence of Mn^{2+}), GlcNAc-T VI (GlcNAcβ6[GlcNAcβ2]Manα-methyl substrate), blood group i β3-GlcNAc-T (Galβ4GlcNAc substrate) and blood group I β6-GlcNAc-T (GlcNAcβ3Galβ-methyl substrate). Since no free radioactive GlcNAc could be detected in HPLC assays (Fig. 2) or during purification of large scale enzyme product (see below), the enzyme

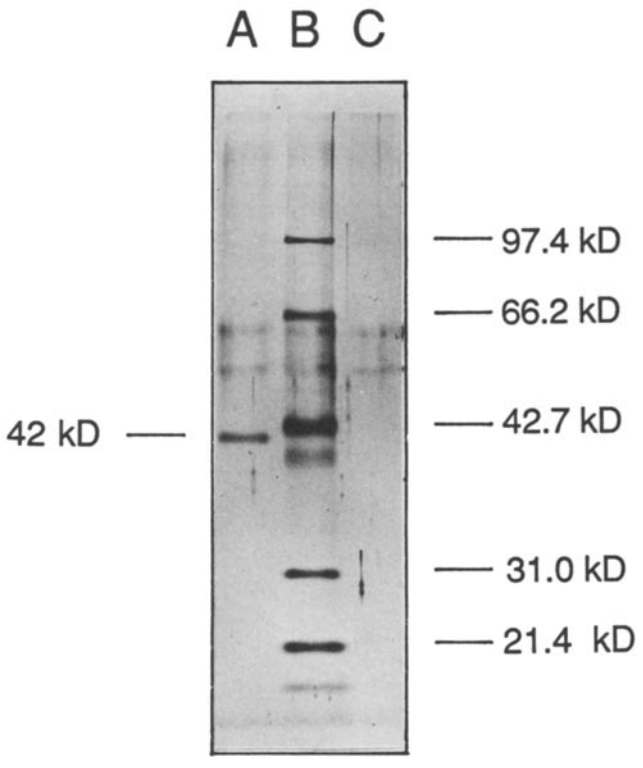


Figure 1. SDS PAGE of rat liver GlcNAc-transferase I. A, 50 ng GlcNAc-transferase I (0.2 nmol min^{-1}). B, Molecular weight standards. C, Buffer alone. The bands common to the lanes (molecular weights between 50 and 65 kD) originate from a contamination in mercaptoethanol.

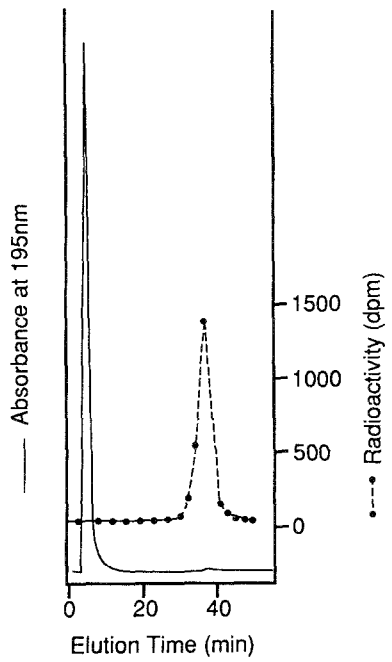


Figure 2. HPLC elution pattern of GlcNAc-T I product using Manα6(Manα3)Manβ-hexyl as the substrate and purified GlcNAc-T I. HPLC was carried out on a C_{18} column, using acetonitrile–water 12:88 by vol at 1 ml min^{-1}.

Table 2. Purity of rat liver GlcNAc-transferase I.

Acceptor	*Assay concn* (mM)	*Enzyme activity*[a] (μmol min^{-1} mg^{-1})	*Enzyme*
1. Mα6 \ Mβ-hexyl / Mα3	1	4.5	GlcNAc-T I
2. Mα6 \ Mβ4Gn / Gnβ2Mα3	3 mM	0.15	GlcNAc-T II
3. {0, 2+F}	0.32 mM	0.21	GlcNAc-T II
4. {2, 2} with Mn^{2+}	1 mM	0.06	GlcNAc-T III, IV or V
{2, 2} without Mn^{2+}	1 mM	0	GlcNAc-T V
5. Gnβ3Gβ-me	5 mM	0	I β6-GlcNAc-T
6. Gnβ6(Gnβ2)Mα-me	1 mM	0	GlcNAc-T VI
7. Gβ4Gn	5 mM	0	i β3-GlcNAc-T
8. GAα-Bn	5 mM	0	core 1 β3-Gal-T
9. Gn	5 mM	0	β3-Gal-T and β4-Gal-T

[a] GlcNAc-transferase assays were carried out as described in the Materials and methods section with 0.125 M MES pH 7, 2 mM UDP-GlcNAc (4434 dpm $nmol^{-1}$) (acceptors 1–7). The incubation time was 30 min. Acceptors 8 and 9 were assayed for Gal-T as described in the Materials and methods section, with 0.125 M MES, pH7, and 1.4 mM UDP-Gal (2220 dpm $nmol^{-1}$). The incubation time was 45 min.

preparation was also free of pyrophosphatase and hexosaminidase activities that could degrade UDP-GlcNAc or enzyme product. A low level of GlcNAc-T activity was detected with 3 mM Manα6 (GlcNAcβ2 Manα3)Manβ4-GlcNAc (0.15 μmol min^{-1} mg^{-1}) and with 0.32 mM {0, 2+F} (0.21 μmol min^{-1} mg^{-1}), possibly due to GlcNAc-T II activity. Incorporation of GlcNAc into acceptor {2, 2} in the presence of Mn^{2+} was 0.06 μmol min^{-1} mg^{-1} possibly due to GlcNAc-T III, IV or V. {0, 2+F} and {2, 2} may also contain small amounts of acceptors for GlcNAc-T I.

Properties of GlcNAc-T I

Only one radioactive peak was found in the large scale enzyme product preparations. Enzyme product A and synthetic Manα6(GlcNAcβ2Manα3)Manβ4GlcNAc (compound 2, Table 2) both eluted on HPLC at 43 min. About 350 nmol product A were purified with at least a 45% conversion of substrate to product. The 500 MHz proton NMR spectrum of product A (Table 3) was identical to that of the synthetic compound Manα6(GlcNAcβ2Manα3)-Manβ4GlcNAc [19]. This proves that the purified rat liver GlcNAc-T I attaches GlcNAc in β1–2 linkage only to the Manα1–3 residue of Manα6(Manα3)Manβ4GlcNAc substrate.

Large scale enzyme product B, Manα6(GlcNAcβ2Manα3)-Manβ-octyl, prepared from Manα6(Manα3)Manβ-octyl, showed an NMR spectrum similar to that of product A differing mainly in the Manβ- protons due to the replacement of GlcNAc by the octyl group (Table 3).

The conversion of substrate to product was linear with respect to time and enzyme concentration in the standard assay (data not shown). Purified GlcNAc-T I had a K_M of 0.4 and 0.37 mM and a V_{max} of 9.3 and 8.0 μmol min^{-1} mg^{-1} for substrates M_5-glycopeptide and UDP-GlcNAc, respectively. The enzyme showed 75% of optimal activity in the absence of exogenous Triton X-100 (the incubation mixture contained 0.008% Triton-X-100 due to the detergent present in the enzyme preparation); activity was stimulated by adding Triton X-100 to the assay with a broad optimum above 0.17% (data not shown). The pH optimum was at pH 5.5 (Fig. 3). The enzyme was inactive in the presence of 20 mM EDTA, but was stimulated by $MnCl_2$ with a broad optimum above 40 mM (Fig. 4). Other divalent cations (Fig. 5) also stimulated GlcNAc-T I activity; Co^{2+} was more effective than Mn^{2+}. Addition of mercaptoethanol, dithiothreitol, UMP, AMP and UDP-hexanolamine to the assay mixture had little effect on enzyme activity, but UDP and 5-Hg-UDP-GlcNAc (as mercaptide) inhibited the activity by about 50%. However, both UDP-hexanolamine and 5-Hg-UDP-GlcNAc bound to Sepharose are capable of strongly binding the enzyme (Table 1).

Table 3. 500 MHz proton NMR data.

Proton	*Chemical shifts/ppm and coupling constants/Hz (in parentheses)*				
	Enzyme products				
	A	B	M \ M-octyl / M	M \ Glc-octyl / M	M \ 2-deoxy-M-octyl / M
GlcNAcβ2					
H-1	4.552 (8.5)	4.553 (8.5)	–	–	–
N-acetyl	2.054	2.051	–	–	–
core GlcNAc					
H-1	4.715β	–			
	5.213 (3.5)α	–	–	–	–
N-acetyl	2.054	–	–	–	–
Manβ					
H-1	4.841	4.671	4.7	–	nd
H-2	4.256	4.129	4.133	–	nd
Manα6					
H-1	4.919	4.911	4.908	4.887	4.89
H-2	nd	3.990	3.990	3.980	3.993
Manα3					
H-1	5.119	5.129	5.105	5.217	5.098
H-2	4.189	4.188	4.065	4.051	4.017
Glcβ					
H-1	–	–	–	4.469 (8.0)	–

nd, not detected.

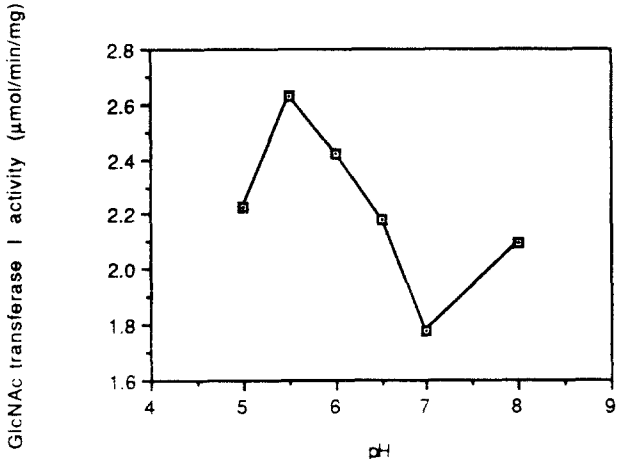


Figure 3. Dependence of GlcNAc-transferase I activity on buffer pH.

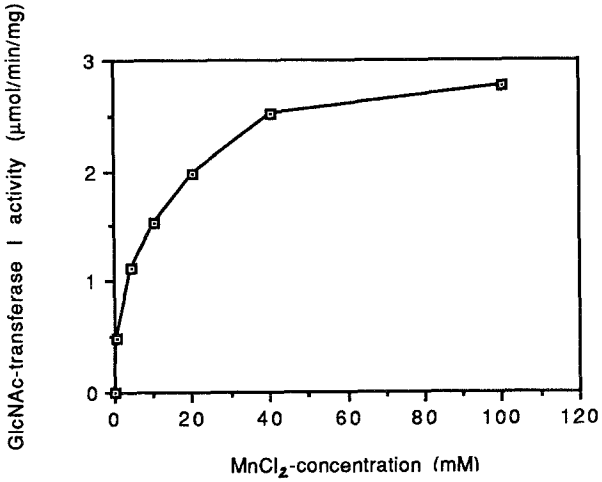


Figure 4. Stimulation of GlcNAc-transferase I activity by $MnCl_2$.

Substrate specificity of GlcNAc-T I

GlcNAc-T I acts on a number of compounds with the structure Manα6(Manα3)Manβ-R, where R can be a hydrophobic group or part of the natural N-glycan core structure (Table 4). GlcNAc is transferred to carbon-2 of the α3-Man residue. This is shown by the structure of the product of the large scale incubation, and by the lack of activity towards Manα6(2-deoxy-Manα3)Manβ-octyl (compound 17) and Manα6(2-*O*-methyl-Manα3)Manβ-octyl (compound 19). Substrates with hydrophobic R groups (compounds 1, 10, 11) have a similar V_{max} but a lower K_M than the corresponding compound with a single GlcNAc residue (compound 12). When R is (5,6-epoxy)hexyl

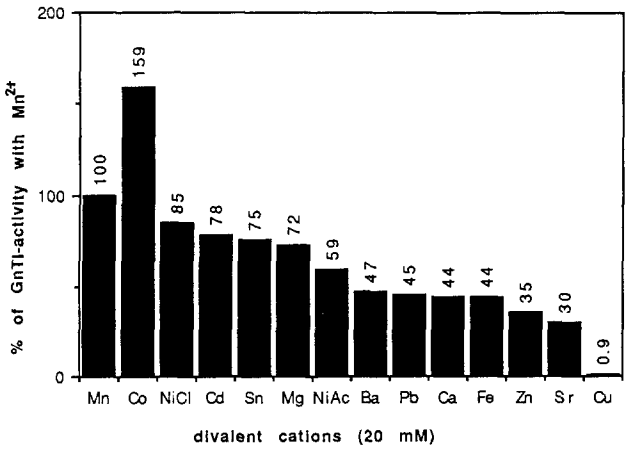


Figure 5. Stimulation of GlcNAc-transferase I activity by divalent cations. $NiAc_2$, nickel acetate.

(compound 16), the V_{max}/K_M is lowered, possibly due to decreased hydrophobicity.

Substitutions at the Manα6 residue of Manα6(Manα3)-Manβ-R substrate have variable effects on activity, i.e., substitution at the 2-hydroxyl by GlcNAc (compound 20) increases the V_{max}/K_M and substitution at the 3- and 6-hydroxyl by mannose yields the best substrate, M_5-glycopeptide (compound 14). Several reactive groups were attached to a pentyl chain linked to the 3-hydroxyl of the α6-linked Man (compounds 21–23). The iodoacetamido derivative was tested in the presence of daylight and in the dark. The diazirino derivative was activated by irradiation over a broad range of UV light. Under the given assay conditions, the epoxide (compound 21), iodoacetamido (compound 23) and diazirino derivatives (compound 22) were active substrates with the V_{max}/K_M comparable to other Manα6(Manα3)Manβ-R substrates (Table 4).

An equatorial hydroxyl group at carbon 4 of the β-linked Man residue of Manα6(Manα3)Manβ-R is essential for GlcNAc-T I activity, since removal of the oxygen (compound 25), inversion of configuration to an axial hydroxyl (compound 24), or substitution by GlcNAc (compound 26) or methyl (compound 27) all result in an almost completely inactive substrate. Inversion of configuration (compound 29) or removal (compound 28) of the 2-hydroxyl of the β-Man also result in an inactive substrate (Table 4).

When the 4-hydroxyl of the Manα3 residue of Manα6(Manα3)Manβ-R is removed (compound 18), the V_{max}/K_M is reduced 20-fold. The high K_M of 21.6 mM of this compound suggests poor binding to the enzyme.

Inhibition studies

Mixed substrate studies were carried out with substrates Manα6(Manα3)Manβ-hexyl, Manα6(Manα3)Manβ-octyl or Manα6(Manα3)Manβ-mco and substrate analogues. Compounds that inhibited enzyme activity 10% or less at a 1 to 10 mM concentration were considered poor inhibitors. Compounds 15, 17–19, 24 to 26, 28 and 29 are substrate analogues which showed little or no substrate activity (Table 4); these compounds were poor inhibitors at 1 mM concentration (Table 5). Compounds 17–19, 24, 25, 28 and 29 were also poor inhibitors at a 10-fold excess over substrate (data not shown). Manα6(Manα3)(4-*O*-methyl-Manβ4)GlcNAc (compound 27) acted as a weak uncompetitive inhibitor with a K_I of about 13 mM (data not shown). Compounds 16 and 21–23, which are good substrates for the enzyme (Table 4), behaved as expected for competing substrates (Table 5). Although these compounds are epoxide, diazirino or iodoacetamido derivatives, they did not reduce incorporation into Manα6(Manα3)Manβ-hexyl. Experiments in which potential inhibitors were preincubated with the enzyme before the addition of substrate gave similar results.

Discussion

GlcNAc-T I is the enzyme that adds the first GlcNAc-branch in the biosynthesis of complex N-glycans. This branch is the structural requirement for the next biosynthetic steps catalyzed by GlcNAc-transferases II, III and IV, α6-fucosyltransferase and mannosidase II. GlcNAc-T II product in turn is the structural requirement for GlcNAc-T V action, which is the prerequisite for GlcNAc-T VI [25, 26]. Inhibition of GlcNAc-T I would therefore be expected to prevent conversion to all complex forms of N-glycans. This inhibition may be beneficial in certain disease states, such as cancer, where increased occurrence of certain complex structures has been reported [27–30].

The purification (Table 1) and characterization of rat liver GlcNAc-T I indicated that the enzyme from rat liver is very similar to the rabbit liver enzyme. The molecular weight of M_r42, 000, the pH optimum of pH 5.5, the stimulation by Triton X-100, and the influence of nucleotides, nucleotide derivatives and reducing agents, are almost identical. Both enzymes increase in activity with increasing $MnCl_2$ concentration up to 100 mM, although the rabbit liver enzyme reaches a plateau at 40 mM $MnCl_2$. The effectiveness of rat liver GlcNAc-T I stimulation by divalent metal ions was: $Co^{2+} > Mn^{2+} > Ni^{2+} > Cd^{2+} > Sn^{2+} > Mg^{2+} > Ba^{2+} > Pb^{2+} > Ca^{2+} = Fe^{2+} > Zn^{2+} > Sr^{2+} >>> Cu^{2+}$. Thus, rat liver GlcNAc-T I was much more stimulated by Co^{2+}, Sn^{2+}, Ba^{2+}, Pb^{2+} and Ca^{2+} than the rabbit liver enzyme. This is possibly due to the presence of contaminants which differ between the two enzyme preparations, or may be due to small differences in the metal binding sites of the enzymes. Human GlcNAc-T I shows significant homology (92% amino acid sequence identity) to the rabbit liver enzyme [8]. Our studies suggest that the rat liver sequence may also be similar.

Table 4. Specificity of purified rat liver β2-GlcNAc-transferase I[a].

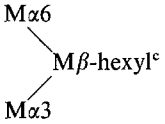
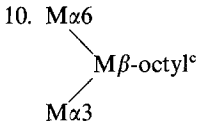
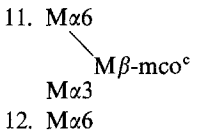
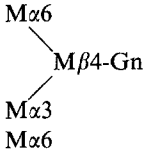
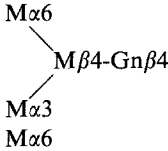
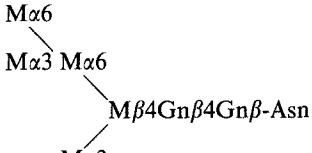
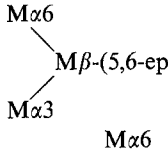
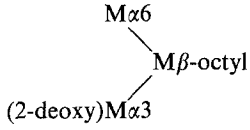
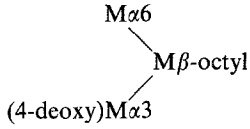
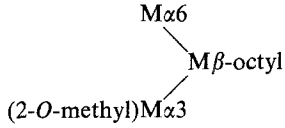
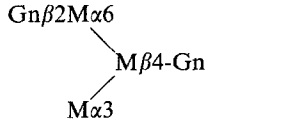
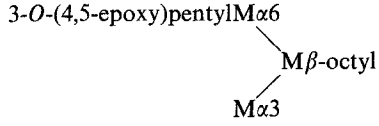
Acceptor	*Assay concn (mM)*	*Enzyme activity (μmol min^{-1} mg^{-1})*	K_M[b] *(mM)*	V_{max}[b] *(μmol min^{-1} mg^{-1})*	$\frac{V_{max}}{K_M}$
1. 	1	4.5	1.7	10.7	6.3
10. 	1	4.4	1.1	8.9	8.1
11. 	1	4.0	1.5	9.8	6.5
12. 	1	3.2	2.7	11.8	4.4
13. 	1	4.3	1.8	11.4	6.3
14. 	0.2	3.7	0.4	9.3	23.3
15. Manβ-(5,6-epoxy)hexyl	6	<0.1			
16. 	1	2.3	2.6	8.3	3.2
17. 	1	<0.1			
18. 	1	0.3	21.6	7.5	0.4
19. 	1	<0.1			
20. 	1	3.9	1.9	11.4	6.0
21. 	1	2.8	1.8	8.2	4.6

Table 4 (*continued*).

Acceptor	*Assay concn* (*mM*)	*Enzyme activity* (*μmol min*$^{-1}$ *mg*$^{-1}$)	K_M[b] (*mM*)	V_{max}[b] (*μmol min*$^{-1}$ *mg*$^{-1}$)	$\frac{V_{max}}{K_M}$
22. 3-*O*-(4,4-azo)pentylMα6 Mβ-octyl Mα3	1	2.0	1.2	5.1	4.3
23. 3-*O*-(5-iodoacetamido)pentyl Mα6 Mβ-octyl Mα3	1	3.7	1.4	6.7	4.8
24. Mα6 Talβ-Gn Mα3	1	<0.1			
25. Mα6 (4-deoxy)Mβ4-Gn Mα3	1	<0.1			
26. Mα6 Gnβ4Mβ4-Gn Mα3	1	<0.1			
27. Mα6 (4-*O*-methyl)Mβ4-Gn Mα3	1	<0.1			
28. Mα6 (2-deoxy)Manβ-octyl Mα3	1	<0.1			
29. Mα6 Glcβ-octyl Mα3	1	<0.1			
30. Mα3Mβ-Gn-OH	0.73	0.16			

[a] GlcNAc-transferase I assays were carried out as described in the Materials and methods section using 0.105 M MES, pH 6.1, 20 mM $MnCl_2$, and 2.09 mM UDP-GlcNAc (2678 dpm nmol^{-1}) and 1 h incubation time.
[b] K_M and V_{max} were determined from at least four acceptor concentrations by linear double reciprocal Lineweaver–Burk plots.
[c] Acceptors 1, 10 and 11 were also assayed by HPLC separation, using a C_{18} column at 1 ml min^{-1} flow rate, with acetonitrile–water 12:88 or 16:84 by vol.

Rabbit liver GlcNAc-T I requires an equatorial hydroxyl at carbon 4 of the Manβ-residue, since compounds 24–26 (Table 4) were inactive as substrates [5]. The possibility could not be excluded that the bisected compound (26, Table 4) was inactive due to steric hindrance or to a change in conformation. However, the 4-*O*-methyl-Manβ-derivative of Manα6(Manα3)-Manβ-GlcNAc (27, Table 4) probably has a conformation similar to Manα6(Manα3)Manβ-GlcNAc; this compound has not been studied previously and was shown to be inactive as a substrate. This suggests that the enzyme needs both the oxygen and the hydrogen of the equatorial hydroxyl group at carbon 4 of the β-linked Man, probably to form an essential hydrogen bond with the enzyme. The 2-hydroxyl of the β-linked Man is also essential for enzyme binding to the substrate, since the 2-deoxy compound (28, Table 4) is inactive as a substrate and does not inhibit. The requirement for this 2-hydroxyl to be in the axial position is demonstrated by the lack of activity of the Glc analogue (29, Table 4). The 4-hydroxyl of the Manα3 residue is also required for optimal substrate binding but residual activity is possible in its absence (18, Table 4).

The high V_{max}/K_M for the best substrate, M_5-glycopeptide (14, Table 4), indicates the importance of the Manα6-arm in substrate binding. However,the Manα6-arm is not essential

Table 5. Mixed substrate assays with purified rat liver β2-GlcNAc-transferase I[a].

Substrate[b]	*Assay concn*	*Enzyme activity*[c] *(substrate alone)*	*Enzyme activity*[c] *(mixed with 0.4 mM substrate 1) (dpm)*		*Enzyme activity*[d] *(mixed with 1 mM substrate 10 with preincubation)*
			Calculated[f]		
	(mM)	*(dpm)*	*NComp.*	*Comp.*	*(dpm)*
1.	0.4 mM	5086	–	–	
15.	1 mM	0	5402	5086	–
16.	0.4 mM	2684	6059	7770	6752
	1 mM	5531	8440	10617	8606
18.	0.4 mM	328	5857	5414	5275
	1 mM	803	5856	5889	5559
21.	0.4 mM	3690	7913	8776	7406
	1 mM	6857	10342	11943	9464
24.	1 mM	32	6066	5118	–
25.	1 mM	126	5915	5212	–
26.	1 mM	101	6096	5187	–
27.	1 mM	75	4651	5161	–
10.	1 mM	13117		–	–
17.	1 mM	0		13117	–
19.	1 mM	29		13146	–
22.[e]	1 mM	10396		23513	16087
23.	1 mM	8484		21601	15090
28.	1 mM	460		13577	–
29.	1 mM	9		13126	–

[a] GlcNAc-transferase I assays were carried out as described in the Materials and methods section for characterization studies.
[b] The structures of the compounds are listed in Table 4.
[c] Enzyme was incubated with the designated substrate alone, or with additional 0.4 mM Manα6(Manα3)Manβ-hexyl (substrate 1) for 1 h.
[d] Substrates were preincubated with the enzyme for 10 min at room temperature before the addition of 1 mM Manα6(Manα3)Manβ-octyl (substrate 10).
[e] Substrate was irradiated in the presence of enzyme with UV light for 10 min before the assay or during the preincubation.
[f] The dpm enzyme product expected for the presence of a competing substrate (Comp.) was calculated with the formula:

$$v_t = \frac{v_1 S_1/K_1 + v_2 S_2/K_2}{1 + S_1/K_1 + S_2/K_2}$$

where v^t = total activity; v_1, v_2 = enzyme rates using substrates 1 and 2, respectively; S_1, S_2 = concentrations of substrates 1 and 2, respectively; K_1, K_2 = K_M for substrates 1 and 2, respectively. The dpm enzyme product expected for two non-competing substrates (NComp.) is the sum of the dpm for the individual products. Inactive acceptors (compounds 15, 17–19, 24–29) did not significantly reduce incorporation into substrate.

since Manα1-3Manβ1-4GlcNAc is a reasonably good substrate from bovine colostrum [9], and even Manα1-3Manβ1-4GlcNAcOH (30, Table 4) shows activity. Substitution of the 2-, 3-, and 6-hydroxyl of the Manα6 residue does not interfere with activity.

Since the 6-arm of the trimannosyl substrate is not essential for substrate binding, we synthesized compounds containing epoxide, diazirino and iodoacetamido groups attached to the Manα6-arm (Table 4) as possible irreversible inhibitors. The epoxides were expected to react with nucleophilic groups in the enzyme; the diazirino group is activated in UV light and may react with any group; the iodoacetamido group may react with sulfhydryl and other groups. None of these reactions apparently took place, since these compounds were very good substrates and apparently not irreversible inhibitors. This may be due to the absence of the required reactive groups in the enzyme.

Replacement of the core GlcNAc by hydrophobic groups 1, 10, 11, Table 4) yields more effective substrates. However, two GlcNAc residues in the core also increase activity slightly. The presence of modified pentyl groups attached to the 3-position of the Manα6 residue of the substrate 21–23, Table 4) has a minor influence on activity as it increases or decreases the V_{max}/K_M slightly. This suggests that there may be hydrophobic binding sites in the enzyme to bind the core but not the Manα6 arm of the substrate.

The results of our specificity studies are summarized in Fig. 6. The shaded areas, i.e., the 2- and 4-hydroxyls of both the Manα3 and the Manβ residue, are requirements for optimal GlcNAc-T I activity. The 2-, 3- and 6-hydroxyls of Manα6, and the core GlcNAc residue do not appear to be essential. The roles of the 4-hydroxyl of the Manα6 residue and the 3- and 6-hydroxyl of the Manα3 residue have yet to be established.

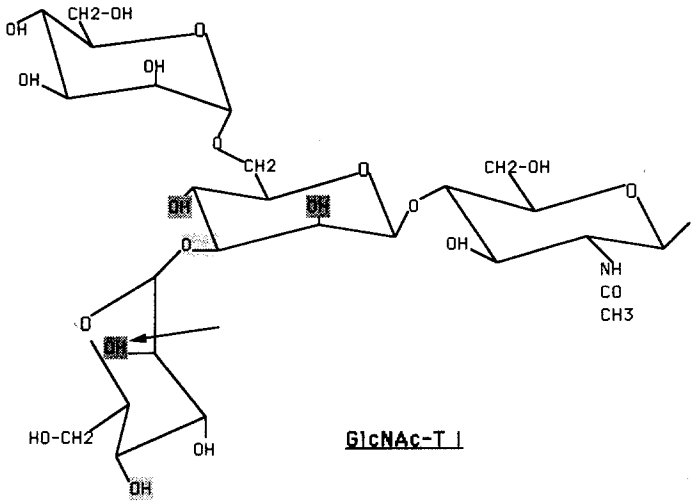


Figure 6. GlcNAc-transferase I specificity towards Manα6-(Manα3)Manβ4GlcNAc substrate. The hydroxyls found to be essential for activity are marked by shaded areas.

Acknowledgements

This research was supported by grants from the Medical Research Council of Canada and the Canadian Cystic Fibrosis Foundation to Harry Schachter and to Inka Brockhausen; from the Deutsche Forschungsgemeinschaft to Hans Paulsen, and by a NATO grant for international collaboration in research.

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