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Polypeptide *N*-acetylgalactosaminyltransferase activity in tracheal epithelial microsomes

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Pig tracheal epithelium, a site of extensive mucin biosynthesis, contained polypeptide *N*-acetylgalactosaminyltransferase activity directed towards L-threonine residues. The enzyme preparation was broadly similar in properties to preparations from other tissues, e.g. pig and bovine submaxillary glands, bovine colostrum, BW5147 mouse lymphoma and baby-hamster kidney cells. Enzyme was membrane-bound and was released from microsomal preparations by extraction with Triton X-100. Extracted enzyme had a pH optimum of 7.5, had a requirement for Mn^{2+} (10 mM) and was inhibited by Na_2EDTA. The K_m for UDP-*N*-acetylgalactosamine was 110 μM and that for an octapeptide acceptor (VTPRTPPP) was 3.0 mM at 37 °C. Using a range of synthetic peptides of known structure related to TPPP it was established that L-threonine residues were specifically *O*-glycosylated probably in the α-configuration. Synthetic peptides containing the TPPP sequence required a peptide length of five or more for significant acceptor activity. In VTPRTPPP the two threonine residues were similarly glycosylated, as revealed by tryptic cleavage of the glycosylated product and separation of the ^{3}H-labelled fragments. The enzyme preparation also specifically catalysed the transfer of *N*-acetylgalactosaminyl residues from UDP-*N*-acetyl[1-^{3}H]galactosamine to bovine submaxillary mucin core protein and to myelin basic protein.

INTRODUCTION

The primary step in the production of mucin-type *O*-linked oligosaccharide groups in glycoproteins is the addition of a single sugar, *N*-acetylgalactosamine, at serine or threonine residues in the polypeptide chain. The enzymes that perform this first step, polypeptide *N*-acetylgalactosaminyltransferases (EC 2.4.1.41), have a key role in the overall control of *O*-glycosylation. Factors that influence initiation of glycosylation, and the subsequent addition of sugar residues, have been reviewed by Carraway & Hull [1].

Partially purified enzyme preparations from several sources have been described [2–13]. Characterization of the products and kinetics of the reaction *in vitro* has been limited. Peptide substrate-specificity also requires further clarification. Hill *et al.* [5] concluded that a minimum peptide length of 16–18 amino acid residues was needed for glycosylation by enzyme from pig submaxillary glands, but a tetrapeptide (TPPP) was later shown [9] to be slowly glycosylated by enzyme from the same source.

In this paper we have demonstrated polypeptide *N*-acetylgalactosaminyltransferase activity in pig tracheal epithelium; we have characterized enzyme specificity using proteins and a series of structurally related peptides as substrates; and we have partially characterized the product obtained using the octapeptide VTPRTPPP as substrate.

MATERIALS AND METHODS

Materials

Materials used were obtained from the sources indicated: UDP-*N*-acetyl[1-^{3}H]galactosamine (8.7 Ci/mmol) and *N*-acetyl[1-^{14}C]galactosamine (50 mCi/mmol) were from New England Nuclear Chemicals (Dupont) Ltd. Trypsin (type XIII, treated with *N*-tosyl-L-phenylalanylchloromethyl ketone; EC 3.4.21.4), α-*N*-acetylgalactosaminidase (EC 3.2.1.49) from *Charonia lampas* (specificity was checked independently by assay with *p*-nitrophenyl α-*N*-acetylgalactosaminide and *p*-nitrophenyl β-*N*-acetylgalactosaminide), bovine submaxillary mucin (BSM; type 1-S), bovine brain myelin basic protein (MBP), UDP-*N*-acetylgalactosamine, *p*-nitrophenyl α-*N*-acetylgalactosaminide and *p*-nitrophenyl β-*N*-acetylgalactosaminide were from Sigma Chemical Co. Ltd. BSA was from Miles Ltd. Aprotinin (Trasylol) was from Bayer A.G., Leverkusen, Germany. Sephadex G-25 and Optiphase 'Safe' scintillant were from LKB Pharmacia Ltd. AG1-X8 anion-exchange resin (50–100 mesh; Cl^- form) and Econocolumns for ion exchange were from Bio-Rad Laboratories Ltd. Sep-Pak C_{18} reverse-phase cartridges were from Waters Millipore (U.K.) Ltd. Trifluoromethanesulphonic acid and 2,3-dimercaptopropan-1-ol were from Aldrich Chemical Co. Ltd. All solvents for h.p.l.c. were from Rathburn Chemicals Ltd. Octapeptide, VTPRTPPP, from Boots Custom Synthesis (Boots Co., Nottingham, U.K.) was homogeneous by h.p.l.c. and m.s. RTPPP and acetyl-TPPP-amide were from Dr. J. Young, University of California, Los Angeles, CA, U.S.A. PRTPPP and TPPP were synthesized by Dr. G. Benz, Bayer A.G., Wuppertal, Germany.

Preparation of pig tracheal microsomes

Pig trachea were obtained within 1 h of death from a local abattoir. They were rinsed in several washes of 0.9% (w/v) NaCl at 0 °C containing 100 i.u. of penicillin/ml and 100 μg of streptomycin/ml. The epithelium was dissected and finely chopped with sharp scissors. The tissue was then homogenized, by using a Polytron homogenizer fitted with a PTA10S 12.5 mm (diameter) head at speed setting 4, in ice-cold 0.1 M-Tris/HCl buffer, pH 7.4 (5 ml/g of tissue), containing 0.25 M-sucrose and aprotinin (0.2 trypsin inhibitor unit/ml) for approx. 4 min. The homogenate was sedimented at 1200 $g_{av.}$ for 15 min at 4 °C. The supernatant was recovered. The pellet was re-extracted by suspension in the above buffer (0.5 × volume), homogenization and sedimentation as before. The supernatant was again recovered and pooled with the previous supernatant. This was

Abbreviations used: MBP, myelin basic protein; BSM, bovine submaxillary mucin.

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then sedimented at 10000 $g_{av.}$ for 15 min at 4 °C. The resultant supernatant was centrifuged at 100000 $g_{av.}$ for 1 h at 4 °C. The pellet so obtained was resuspended in buffer (0.1 × volume) and homogenized by six passes of a hand-held glass/Teflon homogenizer. The suspension was again centrifuged at 100000 $g_{av.}$ for 1 h at 4 °C. The resultant microsomal pellet was resuspended in buffer at a protein concentration of 6.25 mg/ml (determined by the Pierce Coomassie Blue protein assay with BSA as the reference standard).

Chemical deglycosylation of BSM

Deglycosylation of BSM to produce BSM core protein was achieved by using trifluoromethanesulphonic acid [14]. From time-course experiments, optimum conditions for greater than 90% deglycosylation were found to be 3 h at 23 °C.

Assay for polypeptide *N*-acetylgalactosaminyltransferase activity

Assay mixtures contained the following components in a total volume of 50 μl: 0–10 mM-UDP-*N*-acetylgalactosamine; 0–12.5 mM-VTPRTPPP acceptor (or related peptides); 0.1 M-Tris/HCl buffer, pH 7.5, containing (final concentrations) 0.1% (w/v) Triton X-100, 10 mM-$MnCl_2$ and 0.05–0.25 μCi of UDP-*N*-acetyl[1-^{3}H]galactosamine; 5 mM-2,3-dimercaptopropan-1-ol and 0–100 μg of microsomal protein. All samples were prepared in duplicate at 0 °C. In certain control samples, VTPRTPPP was omitted. After the addition of the enzyme preparation, samples were incubated at 37 °C for periods from 5 min to 5 h. The reaction was stopped by the addition of 150 μl of 20 mM-Na_2EDTA at 0 °C. Products were separated in a 2 ml AG1-X8 anion-exchange resin column [9] and eluted with 0.8 ml (× 1) and 1.0 ml (× 2) of deionized water. Each pooled eluate was collected in a plastic scintillation vial, Optiphase 'Safe' scintillant (10 ml) was added and samples were counted for radioactivity. In other experiments, eluate was retained for product analysis.

When protein acceptors were used, the reaction was stopped by addition of 950 μl of 3 mM-Na_2EDTA. Proteins were co-precipitated by addition of 25 μl of 10% (w/v) BSA followed by 100 μl of 100% (w/v) trichloroacetic acid/5% (w/v) phosphotungstic acid. Tubes were placed at 4 °C overnight and then the contents of each were filtered on GFA glass-fibre filters. Each precipitate was washed under vacuum with 2 ml of 10% (w/v) trichloroacetic acid/0.5% (w/v) phosphotungstic acid (× 6) and then 2 ml of water (× 4). Filters were placed in plastic scintillation vials and 500 μl of 1 M-NaOH was added. After 2 h at room temperature to allow the precipitate to solubilize, the NaOH was accurately neutralized by the addition of 29 μl of acetic acid. Samples were made up to 3 ml in water before the addition of scintillant and counting for tritium.

Isolation of ^{3}H-labelled products by reverse-phase separation

Sep-Pak C_{18} reverse-phase cartridges were activated by passage of 10 ml of methanol followed by 15 ml of deionized water. Aqueous eluates from anion-exchange separations were applied and polar materials were washed through with 5 ml of water (× 2). Less-ionic materials were displaced with 10 ml of methanol. Combined aqueous eluates containing polar products were pooled and freeze-dried. Methanolic eluates were dried on a rotary evaporator under high vacuum.

Reverse-phase h.p.l.c.

H.p.l.c. used a Waters C_{18} μBondapak reverse-phase column (30 cm × 3.9 mm internal diameter) fitted with a 3 cm Pye–Unicam Partisil ODS guard column. Solvents were applied using a Perkin–Elmer series 3B pump at a flow rate of 1 ml/min and samples were collected at 1 min intervals. The following solvent elution programme was used: isocratic elution for 5 min in aq. 5% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid, followed by a linear gradient of 5–80% (v/v) acetonitrile in aq. 0.1% (v/v) trifluoroacetic acid over 50 min. The column was then re-equilibrated for 15 min in 5% (v/v) acetonitrile in aq. 0.1% (v/v) trifluoroacetic acid before loading the next sample. Absorbance at 214 nm was monitored. When radioactive samples were run, 1 ml fractions were collected directly into scintillation vials, 10 ml of scintillant was added and the samples were counted for radioactivity. Recoveries of radioactivity from the column were greater than 95%.

RESULTS

Optimization of reaction conditions for polypeptide *N*-acetylgalactosaminyltransferase activity

For 25 μg of pig tracheal microsomal protein, maximum reaction rate was achieved with concentrations greater than 6 mM-UDP-*N*-acetylgalactosamine and not less than 10 mM-VTPRTPPP. At these substrate concentrations, the reaction rate was linear for up to 4 h. The influence of Mn^{2+}, Triton X-100 and pH on enzyme activity was studied in samples containing 6 mM-UDP-*N*-acetylgalactosamine, 12 mM-VTPRTPPP and 25 μg of microsomal protein incubated for 1 h at 37 °C. For optimal activity, 10 mM-$MnCl_2$ and 0.1% (w/v) Triton X-100 at pH values between 7.5 and 8.5 (0.05 M- or 0.1 M-Tris buffer) were required. Na_2EDTA at 7.5 mM totally inhibited the enzyme activity under the above conditions. In later experiments addition of 5 mM-2,3-dimercaptopropan-1-ol (an inhibitor of phosphatases and pyrophosphatases [15]) prevented the breakdown of the UDP-*N*-acetylgalactosamine substrate and was included in all subsequent incubation media for the reaction.

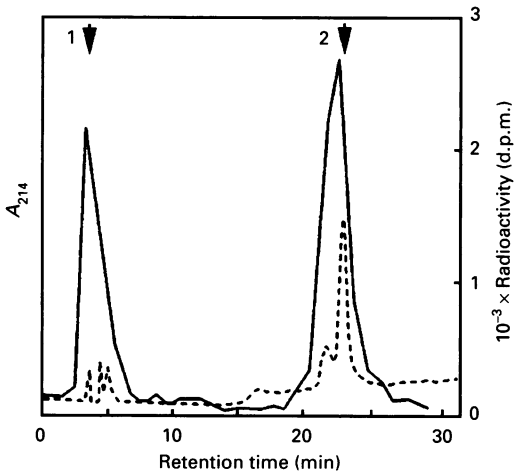

Fig. 1. Glycosylation of VTPRTPPP by *N*-acetylgalactosaminyltransferase: reverse-phase analysis of radiolabelled products and starting substrates

Incubation mixture (final volume 50 μl) contained 0.1 M-Tris/HCl buffer, pH 7.5, 0.1% Triton X-100, 10 mM-$MnCl_2$, 5 mM-2,3-dimercaptopropan-1-ol, 0.25 μCi of UDP-*N*-acetyl[1-^{3}H]-galactosamine, 12.5 mM-VTPRTPPP and 25 μg of microsomal protein. The mixture was incubated for 1 h at 37 °C and then passed over an anion-exchange resin (AG1-X8), eluted with water (final volume 3 ml). A portion (1.5 ml) of the eluate was freeze-dried and then reconstituted in 5% (v/v) acetonitrile in aq. 0.1% (v/v) trifluoroacetic acid. H.p.l.c. analysis was as described in the Materials and methods section. The elution profile of the reaction products (10212 d.p.m.) is shown. Absorbance (---) was measured at 214 nm, and radioactivity as d.p.m. (——). Arrow 1 indicates peak elution position in h.p.l.c. of *N*-acetyl[1-^{14}C]galactosamine. Arrow 2 indicates peak elution position of VTPRTPPP.

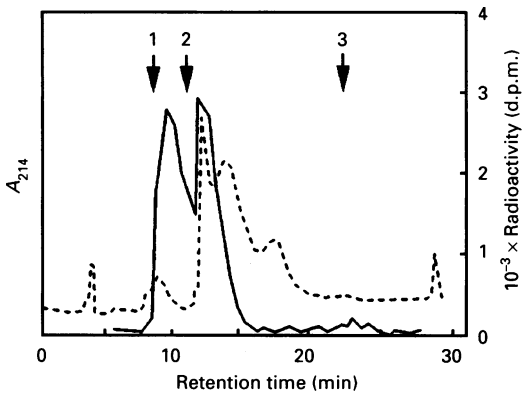

Fig. 2. Reverse-phase h.p.l.c. analysis of ^{3}H-labelled glycosylated VTPRTPPP after incubation with trypsin

^{3}H-labelled product was isolated from transferase enzyme incubations (conditions as for Fig. 1) by methanolic elution from a Sep-Pak C_{18} reverse-phase column. Samples were dried, reconstituted in 0.2 M-ammonium bicarbonate buffer, pH 8.4, and then incubated with trypsin (2 μg) in 30 μl (total volume) of the same buffer. After 30 min incubation at 20 °C, a 20 μl sample of the mixture was transferred to 480 μl of 5% (v/v) acetonitrile in aq. 0.1% (v/v) trifluoroacetic acid. The resulting solution (13570 d.p.m.) was analysed by h.p.l.c. as described in the Materials and methods section and the elution profile is shown. Absorbance (-----) was measured at 214 nm, and radioactivity as d.p.m. (——). Arrow 1 shows the peak elution position in h.p.l.c. of ^{3}H-labelled glycosylated TPPP. Arrow 2 indicates peak elution position of ^{3}H-labelled glycosylated VTPR. Arrow 3 shows the peak elution position of ^{3}H-labelled glycosylated VTPRTPPP.

Analysis of glycosylated VTPRTPPP by h.p.l.c.

Incubation of enzyme with VTPRTPPP and UDP-*N*-acetyl[1-^{3}H]galactosamine led to the formation of two radiolabelled products that were separable by means of reverse-phase h.p.l.c. One radioactive product was present closely preceding non-glycosylated VTPRTPPP substrate (peak 2, Fig. 1). The other product (peak 1, Fig. 1) was eluted during the first 5 min of isocratic elution and corresponded to *N*-acetylgalactosamine. A product eluted in the same position as peak 1 was produced when the enzyme incubation was performed in the absence of VTPRTPPP. Here no component corresponding to peak 2 was observed.

Identification of the glycosylation sites in VTPRTPPP

VTPRTPPP contained two threonine residues (Thr-2 and Thr-5), both of which were potential sites for *O*-glycosylation. Initial results gave no indication of whether the first, the second or both threonine residues became glycosylated by the enzyme reaction. To distinguish between glycosylation at Thr-2 and Thr-5, advantage was taken of the Arg–Thr (4–5) bond as a potential site for tryptic digestion. Cleavage here would result in the production of two non-identical tetrapeptides, either or both of which might be glycosylated. Glycosylated octapeptide was digested with trypsin for 30 min at 20 °C at pH 8.4. Reverse-phase h.p.l.c. (Fig. 2) showed the presence of two radiolabelled peaks that contained approximately equal amounts of radiolabel. This suggested that both Thr-2 and Thr-5 had been glycosylated to a comparable extent under the conditions used.

In further experiments the synthetic tetrapeptides VTPR and TPPP were found to be poor glycosyl acceptors compared with VTPRTPPP. Glycosylation of the threonine residue in VTPR was at a rate of 8 nmol/h per mg of protein at 10 mM. The rate for TPPP was also low, 60 nmol/h per mg of protein at 12.5 mM. By contrast, the rate of glycosylation of VTPRTPPP was 1 μmol/h per mg of protein at 12.5 mM. To increase the yields of glycosylated tetrapeptides, the enzyme incubation period was extended to 6 h and the products were then analysed by h.p.l.c. Despite some variability in column behaviour, the ^{3}H-labelled glycosylated tetrapeptide peaks (arrows 1 and 2, Fig. 2) appeared in positions similar to those of the two peaks from tryptic digestion of glycosylated VTPRTPPP.

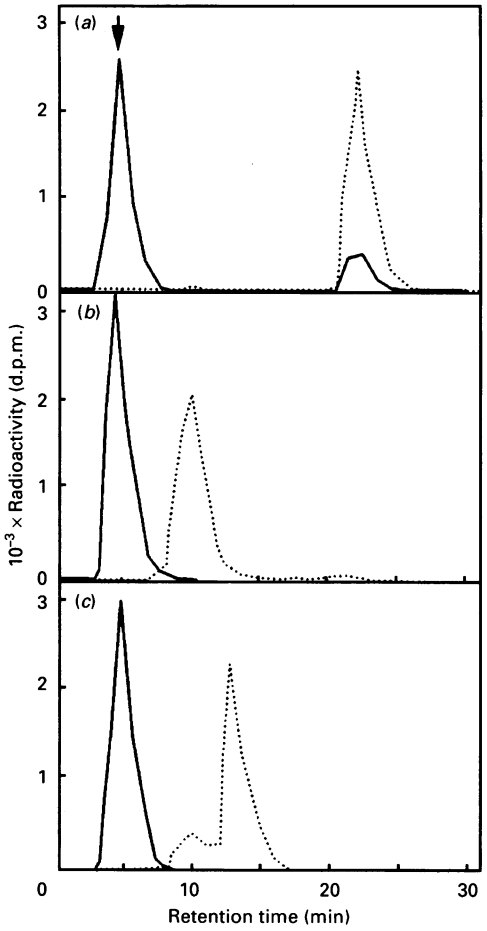

Fig. 3. Reverse-phase h.p.l.c. analysis of ^{3}H-glycosylated VTPRTPPP and tryptic glycopeptides after incubation with α-*N*-acetylgalactosaminidase

(*a*) ^{3}H-glycosylated VTPRTPPP was isolated from transferase enzyme incubations (conditions as Fig. 1) by methanolic elution from a Sep-Pak C_{18} reverse-phase column after passage over an anion-exchanger (AG1-X8) as described in the Materials and methods section. The ^{3}H-labelled product, dried, reconstituted in 0.01 M-sodium acetate buffer, pH 3.7, to give 6000 d.p.m. in 45 μl, was incubated with α-*N*-acetylgalactosaminidase (3 munits in 3 μl of the same buffer) for 66 h at 37 °C. The mixture was diluted with 450 μl of 5% (v/v) acetonitrile in aq. 0.1% (v/v) trifluoroacetic acid. H.p.l.c. was as described in Materials and methods section, and gave the elution profile shown; radioactivity is d.p.m.: ·····, without α-*N*-acetylgalactosaminidase incubation; ——, after α-*N*-acetylgalactosaminidase incubation. Arrow indicates peak elution position in h.p.l.c. of *N*-acetyl[1-^{14}C]galactosamine. (*b*) Peak 1 tryptic-digest glycopeptide from Fig. 2, and (*c*) peak 2 tryptic-digest glycopeptide from Fig. 2 were dried, reconstituted in 0.01 M-sodium acetate buffer, pH 3.7, and incubated with α-*N*-acetylgalactosaminidase as in (*a*). Radioactivity (d.p.m.) elution profiles from h.p.l.c. are shown: ·····, without α-*N*-acetylgalactosaminidase incubation; ——, after α-*N*-acetylgalactosaminidase incubation.

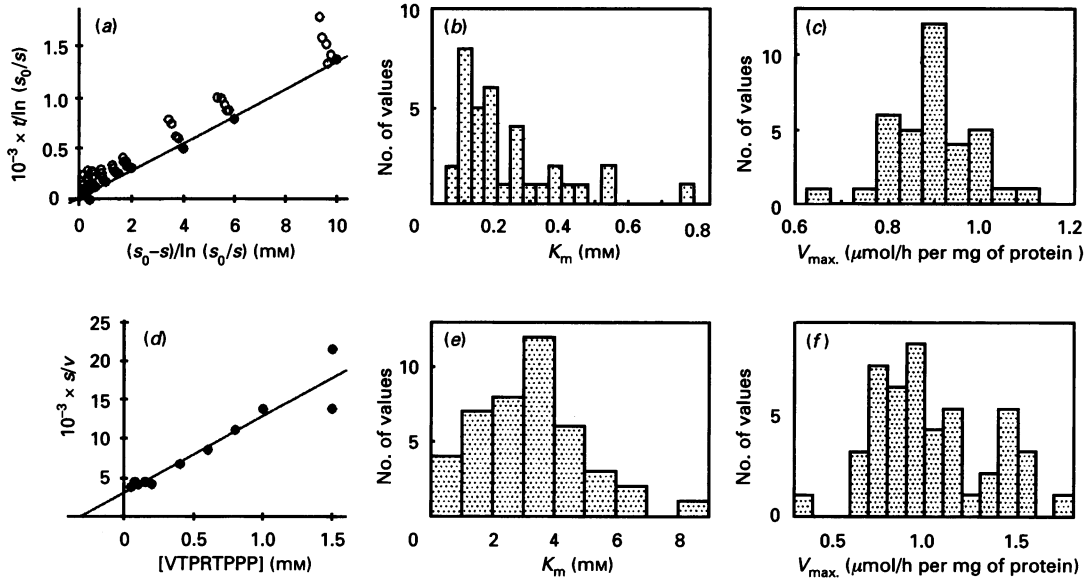

Fig. 4. Kinetic parameters for *N*-acetylgalactosaminyltransferase reaction with respect to UDP-*N*-acetylgalactosamine and VTPRTPPP

(*a*) Integrated Michaelis–Menten plot [17] of $t/\ln(s_0/s)$ against $(s_0-s)/\ln(s_0/s)$. Incubation mixtures (total volume 50 μl) contained 0.1 M-Tris/HCl buffer, pH 7.5, 0.1% Triton X-100, 10 mM-$MnCl_2$, 0.1 μCi of UDP-*N*-acetyl[1-^{3}H]galactosamine, 0.2–10 mM-UDP-*N*-acetylgalactosamine, 12.5 mM-VTPRTPPP, 5 mM-2,3-dimercaptopropan-1-ol and 25 μg of enzyme protein, and were incubated for between 15 min and 240 min at 37 °C. *s* is UDP-*N*-acetylgalactosamine concentration (mM) in incubation mixture at time *t* (min). *s* values at t_0 for each substrate concentration were obtained by extrapolation of non-linear curves fitted to data points (○) covering the range of incubation times. For this experiment linear extrapolation of the t_0 values (●) gave values for K_m of 0.109 mM and $V_{max.}$ of 0.9 μmol/h per mg of protein for UDP-*N*-acetylgalactosamine. Distribution of K_m (*b*) and $V_{max.}$ (*c*) values obtained by direct linear analysis [16] of the same data. (*d*) Hanes plot of data from enzyme incubations carried out as in (*a*) except that 6 mM-UDP-*N*-acetylgalactosamine and 0.5–15 mM-VTPRTPPP were used. For the data shown, K_m was 3.1 mM and $V_{max.}$ was 1.01 μmol/h per mg of protein for VTPRTPPP. Distribution of K_m (*e*) and $V_{max.}$ (*f*) values obtained by direct linear analysis of the data in (*d*).

Glycosidic linkage of *N*-acetylgalactosaminyl substituents

^{3}H-glycosylated VTPRTPPP produced by enzyme incubation was separated from *N*-acetyl[1-^{3}H]galactosamine by passage through a C_{18} Sep-Pak cartridge. By h.p.l.c. analysis free *N*-acetyl[1-^{3}H]galactosamine was found solely in the aqueous eluate. The glycosylated peptide in the methanolic eluate was evaporated to dryness by rotary evaporation, then redissolved in 0.01 M-sodium acetate buffer, pH 3.7. This glycosylated product was treated with α-*N*-acetyl-D-galactosaminidase, and resulted in the partial release of 85% of the radiolabel as *N*-acetyl[1-^{3}H]galactosamine (Fig. 3*a*) consistent with the presence of an α-glycosidic linkage.

The two radiolabelled peaks from tryptic digestion of glycosylated VTPRTPPP were isolated by h.p.l.c., neutralized, evaporated to dryness and reconstituted in 0.01 M-sodium acetate buffer, pH 3.7. Each was then treated with α-*N*-acetyl-D-galactosaminidase, and then re-analysed by h.p.l.c. (Figs. 3*b* and 3*c*). In both cases enzymic hydrolysis was complete and resulted in the appearance of a peak corresponding to *N*-acetyl-[1-^{3}H]galactosamine consistent with both Thr-2 and Thr-5 having been *O*-glycosylated each with a single α-linked *N*-acetylgalactosamine residue.

Kinetic parameters

K_m and $V_{max.}$ were determined for both VTPRTPPP and UDP-*N*-acetylgalactosamine substrates. The formation of radiolabelled product was studied over a range of substrate concentrations and over a range of incubation times to determine initial rates. Results were analysed by both direct linear plot [16] and either integrated Michaelis–Menten method [17] (UDP-*N*-acetylgalactosamine) or Hanes plot (VTPRTPPP). Values for K_m and $V_{max.}$ from integrated Michaelis–Menten analysis for UDP-*N*-acetylgalactosamine (Fig. 4*a*) were in close agreement with the modal values from direct linear analysis (Figs. 4*b* and 4*c*). K_m and $V_{max.}$ from a Hanes plot (Fig. 4*d*) for VTPRTPPP were also in good agreement with direct linear analysis (Figs. 4*e* and 4*f*) modal values. The K_m for UDP-*N*-acetylgalactosamine was 110 ± 24 μM (mean ± S.D.; $n = 3$), and the K_m for VTPRTPPP was 3.08 ± 0.74 mM (mean ± S.D.; $n = 3$). $V_{max.}$ for UDP-*N*-acetylgalactosamine was in the range 0.48–1.14 μmol/h per mg of protein. $V_{max.}$ for VTPRTPPP was in the range 0.84–1.05 μmol/h per mg of protein.

Comparisons between MBP, BSM core protein and VTPRTPPP as substrates for glycosylation

The enzyme preparation was tested for its ability to glycosylate MBP (Fig. 5*a*), a 180-amino-acid-residue protein that contains the VTPRTPPP sequence at positions 96–103. The threonine residues at positions 97 and 100 are potential sites for *O*-glycosylation within this protein. Glycosylation of BSM core protein, which contains multiple potential sites for glycosylation, was also studied (Fig. 5*b*). The microsomal enzyme preparation glycosylated VTPRTPPP and both proteins. VTPRTPPP was a more efficient substrate than was BSM core protein on a weight yield basis. The reaction rate for MBP was also lower than that for the VTPRTPPP (Fig. 5*a*).

Competitive studies with VTPRTPPP and BSM core protein in the enzyme assay

In control experiments, samples were incubated in the absence of both acceptor and microsomal protein. One radioactive degradation product (approx. 1% of total radioactivity added) was consistently produced from UDP-*N*-acetyl[1-^{3}H]-

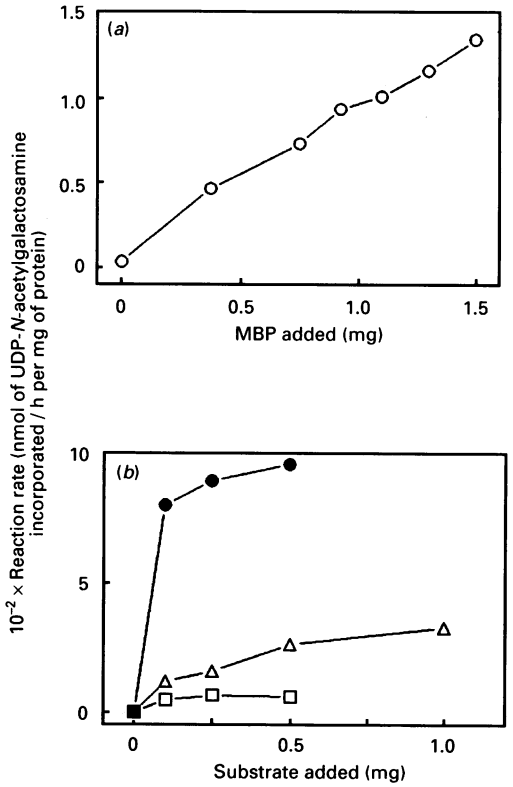

Fig. 5. Comparative rates of glycosylation of VTPRTPPP, BSM core protein and bovine brain MBP by *N*-acetylgalactosaminyltransferase

(*a*) Incubation mixtures contained (50 μl total volume) 0.1 M-Tris/HCl buffer, pH 7.5, 0.1% (w/v) Triton X-100, 10 mM-$MnCl_2$, 0.25 μCi of UDP-*N*-acetyl[1-^{3}H]galactosamine, 6 mM-UDP-*N*-acetylgalactosamine, 25 μg of enzyme protein and MBP (○) (0.5–1.75 mM). Incubations were for 2 h at 37 °C. The labelled product was isolated by precipitation as described in the Materials and methods section. Values throughout are corrected for substrate and enzyme controls. (*b*) The foregoing conditions were employed except that 5 mM-2,3-dimercaptopropan-1-ol, 0.10 μCi of UDP-*N*-acetyl[1-^{3}H]galactosamine, VTPRTPPP (●) (0.1–0.5 mg) or BSM core protein (△) (0.1–1 mg) or native BSM (□) (0.1–0.5 mg) were included. Incubations were for 1 h at 37 °C. Products were separated by anion-exchange chromatography as described in the Materials and methods section.

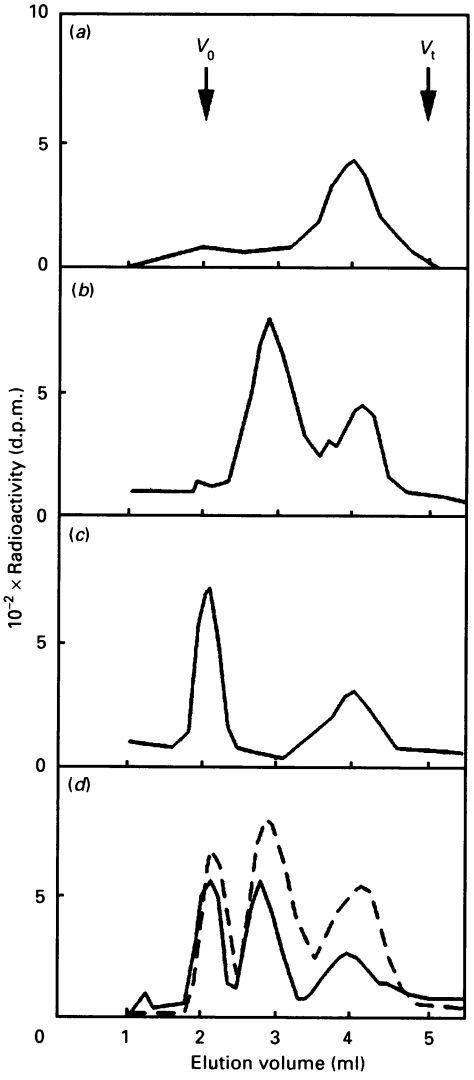

Fig. 6. VTPRTPPP and BSM core protein as competitive acceptors for *N*-acetylgalactosaminyltransferase

Incubation mixtures (50 μl total volume) were as in Fig. 5(*b*) except that VTPRTPPP (0.1 mg) or BSM core protein (0.5 mg) was included as acceptor. Incubations were for 1 h at 37 °C. Reactions were terminated by addition of 20 mM-Na_2EDTA (150 μl) and the products were separated by anion exchange as described in the Materials and methods section. Eluted products were freeze-dried, then reconstituted in 200 μl of 10 mM-sodium phosphate buffer, pH 7.2, and applied to a Sephadex G-25 (fine grade) column (5 ml bed volume). Fractions (0.1 ml) were collected and counted for radioactivity. Duplicate experiments were performed throughout. (*a*) Incubation mixture without enzyme or acceptor, showing *N*-acetyl[^{3}H]galactosamine as minor degradation product (present also in subsequent experiments). (*b*) Incubation mixture including enzyme and VTPRTPPP as above. (*c*) Incubation mixture with enzyme and BSM core protein as above. (*d*) Incubation mixture with enzyme and both VTPRTPPP and BSM core protein as above (——). Chromatographic separation of a mixture of products from (*b*) and (*c*) is also shown (– – – –) in (*d*).

galactosamine. It was identified as *N*-acetyl[1-^{3}H]-galactosamine when eluted from a column of Sephadex G-25 (Fig. 6*a*). All other incubations produced a comparable peak in a similar position (Figs. 6*b*–6*d*). Samples incubated with VTPRTPPP additionally produced the expected glycosylated product (Fig. 6*b*). Samples incubated with BSM core protein produced a distinctive radioactive peak of glycosylated BSM (Fig. 6*c*). When the enzyme was incubated with both acceptors simultaneously, the radioactivity in both corresponding products decreased relative to the separate glycosylation of the substrates (Fig. 6*d*). To ensure that this decrease in radioactivity was not due to adsorption on the column, a portion containing a mixture of the two products prepared in separate incubations was run on the column (Fig. 6*d*). The two peaks separated in consistent positions with full recoveries of radioactivity. Competitive assays were performed in which either increasing amounts of BSM were incubated with a fixed amount (0.1 mg) of VTPRTPPP (Fig. 7*a*) or increasing amounts of VTPRTPPP were incubated with a fixed amount (1 mg) of BSM (Fig. 7*b*). In both assays the total rate of transfer of *N*-acetylgalactosamine was not additive. Attachment of 50% of the radiolabel to each of the acceptors would be achieved by incubation of 0.1 mg of VTPRTPPP with 0.54 mg of BSM (Fig. 7*a*) or 0.022 mg of VTPRTPPP with 1 mg of BSM (Fig. 7*b*).

Glycosylation of peptides related in sequence to TPPP

A range of synthetic peptides of sequence related to TPPP was assessed as potential substrates for the enzyme (Fig. 8). The

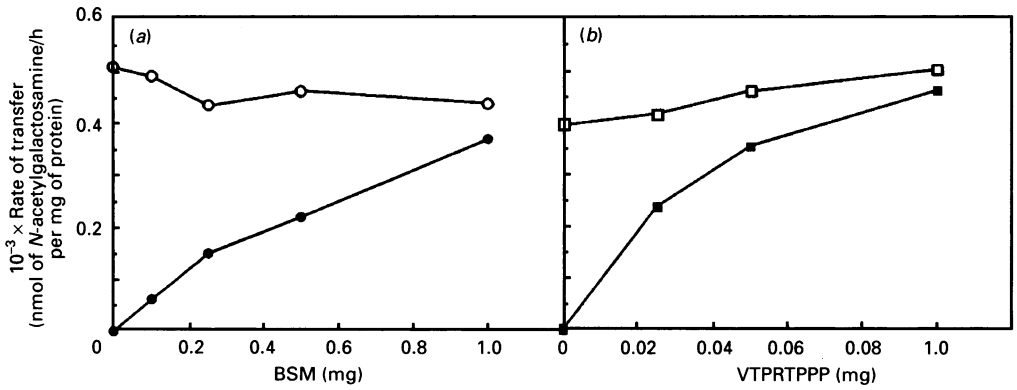

Fig. 7. Reaction rate of *N*-acetylgalactosaminyltransferase for mixtures of VTPRTPPP and BSM core protein as acceptors over a range of concentrations

Incubation mixtures (50 μl total volume) were as in Fig. 5(*a*) except that in (*a*) BSM core protein (0–1 mg) (●) or VTPRTPPP (0.1 mg) plus BSM core protein (0–1 mg) (○) were included as acceptors, and in (*b*) VTPRTPPP (0–0.1 mg) (■) or BSM core protein (1 mg) and VTPRTPPP (0–0.1 mg) (□) were included as acceptors. Incubations were for 1 h at 37 °C. Reactions were terminated by addition of 20 mM-Na_2EDTA (150 μl) and the products were separated by anion exchange as described in the Materials and methods section.

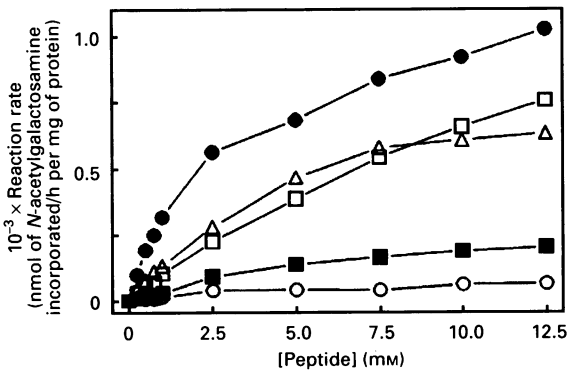

Fig. 8. Rates of glycosylation of related peptides containing the TPPP sequence

Incubation mixtures (50 μl total volume) and conditions were as described in Fig. 5(*b*) except that potential peptide acceptors were included in the range 0–12.5 mM. Labelled products were separated as in Fig. 5(*b*). Values are of duplicate experiments, and are corrected throughout for enzyme and substrate controls. ●, VTPRTPPP; ○, TPPP; □, acetyl-TPPP-amide; ■, RTPPP; △, PRTPPP.

octapeptide VTPRTPPP was the best substrate. The hexapeptide PRTPPP was glycosylated at a rate 50–70% of that for VTPRTPPP. But considerably less effective were the pentapeptide RTPPP and tetrapeptide TPPP in which the *N*-terminal Pro and Pro-Arg residues respectively were absent. These were glycosylated at 10–20% of the rate for VTPRTPPP. Capping of the end groups of TPPP with both an *N*-acetyl and an amide group (acetyl-TPPP-amide) resulted in a glycosylation rate similar to that of PRTPPP. Enzyme specificity for substitution at L-threonine residues was indicated, since acetyl-L-allothreonyl-PPP-amide and acetyl-SPPP-amide were less than 10% as effective as VTPRTPPP as substrates.

DISCUSSION

Pig tracheal epithelial microsomes contain polypeptide *N*-acetylgalactosaminyltransferase activity with specificity directed towards L-threonine residues in TPPP-containing sequences. Conditions for optimal activity were similar to those for similar enzyme preparations from other sources [4,5,10–13,18,19]. The K_m for UDP-*N*-acetylgalactosamine was also in the same range described [4,5,20] for other sources.

The synthetic peptide substrates chosen for study were based on the octapeptide VTPRTPPP, which was a good substrate for polypeptide *N*-acetylgalactosaminyltransferase from pig submaxillary gland [9]. The sequence occurs in a range of species-derived MBPs [21]. The TPPP sequence also occurs in a variety of proteins, though not necessarily glycosylated, including the *C*-terminal sequence of simian virus 40 antigen [22], and in the hinge region of human IgG_3 [23].

As well as the synthetic substrates, the pig tracheal enzyme preparation also glycosylated BSM core protein and MBP. BSM core protein contains many potential *O*-glycosylation sites (64 serine residues and 101 threonine residues) and three *N*-glycosylation sites [24]. Bovine MBP contains only potential *O*-glycosylation sites (17 serine residues and seven threonine residues) [21]. Mixtures of VTPRTPPP and BSM core protein competed (at least in part) for glycosylation by the same enzyme.

Pig submaxillary enzyme [9] glycosylated VTPRTPPP at a rate twice that for RTPPP or PRTPPP. A similar relationship was observed for the pig tracheal enzyme preparation, and the tetrapeptides VTPR and TPPP gave the lowest reaction rates. The higher rate of glycosylation of VTPRTPPP compared with other peptides may reflect glycosylation of both Thr-2 and Thr-5. Alternatively extension of the pentapeptide beyond its *N*-terminus may favour increased glycosylation of the second threonine (Thr-5 in VTPRTPPP). A recent survey [25] concluded that there was little evidence of peptide sequence-specificity around *O*-glycosylation sites in glycoproteins. There was increased frequency of proline, alanine, serine and threonine in the vicinity, but no absolute requirement. This contrasts with the high degree of specificity around asparagine residues at *N*-glycosylation sites. Surveying a wide range of *O*-glycosylation sites may, however, mask structural specificity around the very first site of substitution in a protein, or at the initiating site in a region of multiple glycosylation. It is possible that initiation of glycosylation requires binding of the enzyme to a target protein at a specific recognition site, and that this is a rate-limiting step. Once addition at the first site has been completed, then glycosylation may continue by translocation of the enzyme to an adjacent site on the protein, followed by further substitution. Second and subsequent modifications may be energetically more favourable than was the initial glycosylation. Such a sequence of events envisages a decrease in K_m and an increase in $V_{max.}$ for glycosylations after that at the initial site. After initiation, further substitution would proceed with progressive ease, rather like a 'zipper effect'.

Glycosylation of VTPRTPPP could give rise to three possible products: two monoglycosylated peptides and a diglycosylated peptide. We were only able to resolve a single radiolabelled glycosylated VTPRTPPP product peak by fractionation by anion-exchange chromatography, by reverse-phase h.p.l.c. or by Sephadex G-15 chromatography. Trypsin hydrolysis of the glycosylated octapeptide resulted in two radiolabelled tryptic glycopeptides, which were separated by reverse-phase h.p.l.c. They each contained similar amounts of radioactivity, suggesting that both Thr-2 and Thr-5 in VTPRTPPP were glycosylated to a similar level. But we could not discriminate between the possibilities that either a diglycosylated product had been produced or that two monoglycosylated peptides had been formed in equal amounts. The two tryptic-digest glycopeptides exhibited elution profiles on h.p.l.c. similar to those of the products of glycosylation of VTPR and TPPP. That the elution profiles did not overlap exactly probably reflected partial degradation of peptides, especially the tetrapeptides, during incubation with the microsomal preparation.

The microsomal enzyme preparation that we used had not been purified. It is therefore possible that more than one *N*-acetylgalactosaminyltransferase was involved in the glycosylation of the two threonine residues in VTPRTPPP. Furthermore, the possibility must also be considered that in pig tracheal epithelial cells there are separate enzymes for glycosylation at serine and threonine residues. We found that Ac-TPPP-NH_2 was a considerably better acceptor substrate for polypeptide *N*-acetylgalactosaminyltransferase in the pig tracheal microsomal preparation than was Ac-SPPP-NH_2. This result would tend to favour separate enzymes, but the possibility cannot be excluded that conformational differences between the acceptors had an important influence on the rate of reaction of a single enzyme.

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