Identification of essential histidine residues in UDP-*N*-acetyl-D-galactosamine:polypeptide *N*-acetylgalactosaminyltransferase-T1

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UDP-*N*-acetyl-D-galactosamine:polypeptide *N*-acetylgalactosaminyltransferases (ppGaNTases) catalyse the initial step of mucin-type *O*-glycosylation. The activity of bovine ppGaNTase-T1 isoenzyme was inhibited by diethyl pyrocarbonate (DEPC) modification. Activity was partially restored by hydroxylamine treatment, indicating that one of the reactive residues was a histidine. The transferase was protected against DEPC inactivation when UDP-GalNAc and EPO-G, a peptide pseudo-substrate PPDAAGAAPLR, were simultaneously present, while presence of EPO-G alone did not alter DEPC inactivation. However, inclusion of UDP-GalNAc alone potentiated DEPC-inhibition of the enzyme, suggesting that UDP-GalNAc binding changes the accessibility or reactivity of an essential histidine residue. Deletion of the first 56 amino acids (including one hisitidine residue) yielded a fully active secreted form of the bovine ppGaNTase-T1 enzyme. Each of the 14 remaining histidines in the enzyme were mutated to alanine, and the recombinant mutants were recovered from COS7 cells. The mutation of histidine residues $His^{211} \rightarrow Ala$ and $His^{344} \rightarrow Ala$ resulted in recombinant proteins with no detectable enzymic activity. A significant decrease in the initial rate of GalNAc transfer to the substrate was observed with mutants $His^{125} \rightarrow Ala$ and $His^{341} \rightarrow Ala$ (1 % and 6 % of wild-type activity respectively). Mutation of the remaining ten histidine residues yielded mutants that were indistinguishable from the wild-type enzyme. Mutagenesis and SDS/PAGE analysis of all *N*-glycosylation sequons revealed that positions N-95 and N-552 are occupied by *N*-linked sugars in COS7 cells. Ablation of either site did not perturb enzyme biosynthesis or enzyme activity.

INTRODUCTION

The acquisition of *O*-linked *N*-acetylgalactosamine (GalNAc) is catalysed by a family of UDP-*N*-acetylgalactosamine:polypeptide *N*-acetylgalactosaminyltransferases (EC 2.4.1.41) (ppGaNTases) which transfers GalNAc from the sugar donor UDP-GalNAc to the hydroxy group of specific threonine and serine residues of proteins. In common with other glycosyltransferases [1], the predicted structure of ppGaNTase-T1 [2,3] corresponds to a type II membrane protein. The conserved features include: (1) a relatively short *N*-terminal cytoplasmic tail, (2) a hydrophobic transmembrane domain, (3) a protease-sensitive stem region which links the transmembrane anchor to the catalytic domain, and (4) the C-terminal, globular catalytic region. Using recombinant bovine ppGaNTase-T1, expressed as secreted protein from COS7 cells, we have shown previously that the order of UDP-GalNAc and peptide substrate binding is random [4]. However, the detailed mechanism of this reaction is not yet understood.

In the present study we have assessed the importance of histidine residues in ppGaNTase activity using chemical modification by diethyl pyrocarbonate (DEPC), deletion analysis and site-directed mutagenesis. We have also determined that two of three potential *N*-glycosylation sites are occupied when ppGaNTase-T1 is expressed in COS7 cells. However, ablation of either oligosaccharide-attachment site does not compromise ppGaNTase activity.

EXPERIMENTAL

Determination of polypeptide transferase activity and kinetic parameters

ppGaNTase was assayed in a final volume of 25 μl containing 40 mM sodium cacodylate, 0.1 % Triton X-100, 4 mM β-mercaptoethanol, 10 mM $MnCl_2$, pH 6.5, in the presence of 0.04–2 mM of an acceptor peptide, PPDAATAAPLR (EPO-T) and 1–80 μM UDP-[^{14}C]GalNAc (DuPont–NEN). Duplicate samples were incubated at 37 °C, the time of the assay was varied to ensure that less than 10 % of the substrate UDP-[^{14}C]GalNAc was transformed. The reaction was terminated by the addition of 150 mM EDTA (10 μl) and product formation was measured by anion-exchange chromatography (Dowex AG 1-X8, formate form; Bio-Rad) or by HPLC (reverse-phase C_{18} 0.46 × 25 cm column; Vydak). Glycosylated peptides were eluted from the column with a gradient of 5–35 % acetonitrile/0.1 % (v/v) trifluoroacetic acid at a flow rate of 1.5 ml/min [5].

Modification of the recombinant polypeptide GalNAc transferase with DEPC

Modification of either native or recombinant polypeptide GalNAc transferase was carried out at room temperature using bovine ppGaNTase-T1 homogeneous enzyme preparations, described previously [2,4]. The purified native or recombinant enzyme was diluted in 50 mM Mes, pH 6.5 before use. In-

Abbreviations used: DEPC, diethyl pyrocarbonate; EPO-G, peptide pseudo-substrate, PPDAAGAAPLR; EPO-T, acceptor peptide substrate, PPDAATAAPLR; ppGaNTases, UDP-*N*-acetyl-D-galactosamine:polypeptide *N*-acetylgalactosaminyltransferases-T1.

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activation was started by the addition of one volume of DEPC (Sigma), which was diluted in ice-cold 5% (v/v) ethanol just before use. DEPC modification was terminated by the addition of L-histidine (Sigma) to a final concentration of 20 mM. Reactivation of the DEPC-modified enzyme was attempted in the presence of 40 mM hydroxylamine (final concentration), pH 7.3, at room temperature for 17 h. Hydroxylamine or 5% (v/v) ethanol had no measurable effect on the activity of the unmodified enzyme and this activity (A_0) was used for the residual activity calculation, A/A_0.

The pH dependence of DEPC modification was determined in 50 mM Mes (pH 6.06–6.65) and 30 mM Mops (pH 6.87–8.57). For each pH value, the pseudo-first-order rate constant of inactivation (k_{obs}) was calculated according to eqn. (1) [6,7]:

$$\ln(A/A_o) = -k_{obs}*t \quad (1)$$

where A/A_o is the residual enzyme activity after time t of DEPC modification, and where k_{obs} is the pseudo-first-order rate constant of inactivation for the polypeptide GalNAc transferase at a given DEPC concentration. The k_{obs} was plotted against the pH value, and the experimental results were replotted according to eqn. (2) [7]:

$$k_{obs}[H^+] = K_a(k_{obs})_{max} - K_a k_{obs} \quad (2)$$

where K_a is the dissociation constant of a reacting group and $(k_{obs})_{max}$ is the pseudo-first-order rate constant when the reacting group is unprotonated. This plot gave a straight line (obtained by linear regression) the slope of which gave the apparent pK_a.

To determine if DEPC-inactivation resulted from modification of specific residues, ppGaNTase-T1 was incubated with DEPC (as described above) in the presence of 2.5 mM EPO-G (a peptide pseudo-substrate, PPDAAGAAPLR) or 160 μM UDP-GalNAc. To prevent the transformation and cleavage of the nucleotide sugar UDP-GalNAc by the enzyme during the reaction, the peptide substrate EPO-T was replaced with the pseudo-substrate EPO-G, which acts as a dead-end inhibitor [4].

Oligonucleotide-directed site-specific mutagenesis of ppGaNTase-T1

The plasmid pSVLinGNT1 [2] directs expression of a secreted form of the bovine ppGaNTase-T1 fusion protein with a metal binding site. This construct was modified by the introduction of the unique restriction site *Dra*III between the metal binding site and the 5′ end of the coding region to produce the construct pIMD-bT1 (I, insulin signal sequence; M, metal binding site; D, *Dra*III cloning site; b, bovine; T1, ppGaNTase-T1). This new restriction site alters the N-terminus of the recombinant protein so that the N-terminal amino acids of the secreted protein are: NH_2-FVHMHHWHHHGV before the bovine ppGaNTase-T1 coding region. The region encoding the recombinant fusion protein was excised as a *Xho*I-*Bam*HI fragment from plasmid pIMD-bT1 and cloned into the *Xho*I-*Bam*HI sites of pBluescript SK + (Stratagene) to produce pBS-bT1.

The site-directed mutagenesis of recombinant ppGaNTase in pBS-bT1 was performed according to Kunkel [8] using *Escherichia coli* strains CJ 236 (dut$^-$, ung$^-$) and DH5αF′, and phage M13KO7. Primers were synthesized (Gibco-BRL) to direct a 56 amino acid N-terminal deletion, single histidine to alanine mutations, or asparagine to glutamine changes as follows:
Δ56, d(CACGGAGTGGGTCCTGGAGAAATG);
H125A, d(TGGTGATTGTTTTCGCAAATGAGGCT);
H137A, d(CTGCGAACTGTCGCTAGCGTCATT);
H146A, d(CGCTCACCAAGGGCCATGCTAGAA);
H179A, d(AAAAGTACCCGTTGCCGTCATTC);
H211A, d(TTTTTAGACGCGGCTTGTGAGTG);
H228A, d(GCCAGGATCAAAGCAGACAGGAAGA);
H341A, d(TTGTTACTTGCTCAGCCGTTGGACAT);
H344A, d(GCTCACATGTTGGAGCCGTGTTTCG);
H404A, d(CTTGGTCTAAGGGCTAAACTCCAAT);
H427A, d(CAGATTCCACGTGCGTATTTCTCTTT);
H460A,
d(TGGAATTTTTAACTGTGCCGGTATGGGAGGT);
H498A, d(ATGCTCAAATGCGCACACCTAAAAG);
H499A, d(GCTCAAATGCCACGCACTAAAAG);
H517A, d(TGACCCTCCAGGCCGTGAACAGTAA)- (loss of *Pst*I site);
N95Q, d(GATGATTGCACTCCAAAGATCTCTAC);
N141Q, d(CCATAGCGTCATTCAACGATCACCA);
N552Q, d(TGGCTTCTTCGGCAGGTCACCCTTC).

The mutants were identified by DNA sequencing and subcloned into pIMD-bT1. DNA encoding mutants losing any functional property were prepared independently at least twice and were fully sequenced to verify that only a single codon mutation was incorporated into the coding region.

Transient expression of mutant ppGaNTase-T1 in COS7 cells

COS7 cells were grown to 80–90% confluence at 37 °C in Dulbecco's modified Eagle's medium (Life Technologies Inc.) with 10% (v/v) fetal-calf serum, in an atmosphere of 6% CO_2 in 60-mm culture dishes. Cells were transfected with 3 μg of mutant construct DNA using 24 μl of LipofectAMINE (Life Technologies Inc.). The medium was replaced at 24 h post-transfection and cells were harvested 48–72 h post-transfection. In cases where little or no activity was detected, transfections were repeated and the cells were grown at 30 °C to increase the quantity of recombinant enzyme produced [9,10].

For metabolic labelling of recombinant enzyme, the cells were transfected as above. At 24 h post-transfection, the cells were incubated for 1 h in methionine-deficient media with 2.5% (v/v) fetal bovine serum. The medium was replaced with methionine-deficient medium containing 2.5% (v/v) fetal bovine serum supplemented with 50 μCi/ml [^{35}S]methionine (New England Nuclear) for a 2 h pulse. The medium was then replaced with Dulbecco's modified Eagle's medium containing 2.5% (v/v) fetal bovine serum and cells were incubated for an additional 2 h. The media were collected and cleared of cellular debris by low speed centrifugation.

For analysis of the mutants by PAGE, each [^{35}S]methionine labelled mutant was enriched using either metal-chelate affinity resin or Affi-Gel Blue (Bio-Rad). The supernatants were adjusted with SBB buffer (30 mM Mops/10 mM $MgCl_2$/100 mM NaCl/20 mM imidazole, pH 6.5) and 1 ml was mixed with 50 μl of a slurry of either $NiCl_2$-charged Chelating Fast Flow Sepharose beads (Pharmacia) or Affi-Gel Blue (Bio-Rad) by rocking overnight at 4 °C. The immobilized $NiCl_2$ beads were washed with SBB buffer and the recombinant transferase protein was eluted by the addition of 250 mM imidazole in 30 mM Mops/10 mM $MgCl_2$/100 mM NaCl, pH 7.1. To characterize the enzymic properties of these mutants, metabolically-labelled enzyme containing similar amounts of radioactivity was assayed using 0.2 and 2 mM EPO-T and [^{3}H]UDP-GalNAc.

For SDS/PAGE analysis, recombinant enzyme adsorbed to Affi-Gel Blue beads was loaded directly, or equal amounts of ^{35}S-labelled material were added to Tricine-SDS/PAGE gel loading buffer [4% (w/v) SDS, 12% (w/v) glycerol (w/v), 50 mM Tris/HCl, pH 6.8, 2% (v/v) mercaptoethanol, 0.01% (w/v) Serva Blue G]. The samples were subjected to Tricine-SDS/PAGE (10%/3% gel) [11], the gel was soaked in En^{3}Hance (DuPont-NEN), dried and radioactively-labelled proteins were exposed by autoradiography on XAR film (Kodak).

RESULTS

Effect of DEPC on ppGaNTase-T1

To favour *N*-carbethoxylation of the imidazole ring of histidine residues, pH conditions were chosen so that the DEPC modification would minimize reactivity with tyrosine, lysine, arginine and cysteine [12]. Incubation of ppGaNTase-T1 with 4.8 mM DEPC in 50 mM Mes buffer, pH 6.5, resulted in a time-dependent inactivation of the enzyme. Less than 20 % of ppGaNTase-T1 activity remained within 5 min of incubation (Figure 1). Since a histidine-rich metal-binding site was engineered into the recombinant form of the ppGaNTase-T1 [2], we first compared the time course of DEPC inactivation of native and recombinant enzyme (using 4.8 mM DEPC in 50 mM Mes buffer, pH 6.5) and found that the rate of inactivation was similar (results not shown). To ensure that subsequent measurements were being made on ppGaNTase-T1 and not a mixture of closely related isoforms, all remaining studies were conducted with recombinant ppGaNTase-T1. To test if histidine was modified by DEPC, DEPC-inactivated recombinant enzymes were treated with hydroxylamine. Hydroxylamine removes *N*-carbethoxy groups from histidine residues and *O*-carbethoxy groups from tyrosine [12], but does not readily remove derivatives of lysine, arginine and cysteine. ppGaNTase-T1 that had been inactivated to less than 20 % residual activity by treatment with DEPC (4.8 mM DEPC in 50 mM Mes buffer, pH 6.5, for 5 min) was treated with hydroxylamine (40 mM, pH 7.3, at room temperature for 17 h). This treatment led to a restoration of more than 60 % of the enzyme activity indicating that at least part of the DEPC-mediated inactivation could be attributed to the modification of histidine residues.

Kinetic parameters for ppGaNTase-T1 and the DEPC-treated transferase were estimated from Lineweaver–Burk plots and a five-fold reduction in the V_{max} of reactions catalysed by the DEPC-treated enzyme was determined. No significant difference in the apparent K_m values for either of the substrates UDP-GalNAc or EPO-T was observed (Table 1). The pH-dependence of DEPC modification is shown in Figure 2. Pseudo-first-order rate k_{obs} constants obtained from eqn. (1) were plotted against pH. Experimentally determined K_{obs} values were replotted according to eqn. (2), revealing an apparent pK_a of 7.6 for the residues modified by DEPC (see inset, Figure 2).

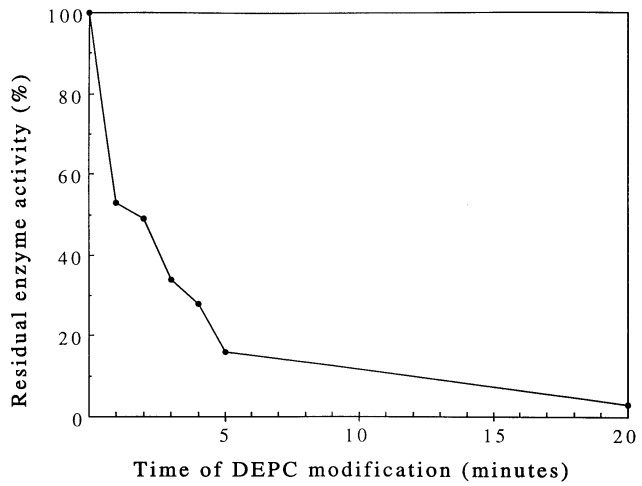

Figure 1 Time dependence of DEPC inactivation of native bovine ppGaNTase

The purified enzyme was modified with 4.8 mM DEPC as described in the Experimental section and then used in the enzyme assay. The enzyme activity was plotted as a percentage relative to the enzyme activity determined in 5% (v/v) ethanol at the same time point.

Table 1 Estimated kinetic parameters of unmodified and DEPC-modified purified recombinant ppGaNTase

The parameters are representative of two separate experiments. The kinetic values were obtained as described in the Experimental section. The concentrations used for the sugar donor and acceptor were 1–80 μM UDP-GalNAC and 0.04–2 mM EPO-T respectively.

	UDP-GalNAc		EPO-T	
Enzyme	K_m(app) (μM)	V_{max}(app) (pmol/h)	K_m(app) (mM)	V_{max}(app) (pmol/h)
Unmodified	22.9	1095	0.6	782
DEPC-modified	18.8	147	0.5	163

The simultaneous presence of the sugar donor and pseudo-acceptor substrates, UDP-GalNAc and EPO-G, provided the greatest protection against DEPC-inactivation, increasing the time required for enzyme inactivation from 2 to 6 min (Figure 3). The presence of EPO-G alone did not provide any significant protection against DEPC inactivation. In contrast, the binding of UDP-GalNAc alone accelerated the DEPC inactivation rate 4-fold, from 2 min to 0.5 min. This site is protected when both the donor and acceptor substrates are present, suggesting that the peptide acceptor substrate may be in close proximity to the reactive amino acid residue(s).

Identification of histidine residues which are essential for ppGaNTase-T1 activity

There are 15 histidine residues in ppGaNTase-T1, distributed throughout the coding region. Replacement of the first 56 residues of ppGaNTase with a His-rich metal-binding site resulted in deletion of His^{55} and its flanking sequence, producing a truncated enzyme that was fully active (results not shown). The role of the remaining 14 histidine residues was evaluated by mutagenesis of each individual histidine to an alanine residue. Each construct, together with the wild-type enzyme, was expressed in COS7 cells using a mammalian secretion vector, described previously [2,4]. The mutant proteins were metabolically labelled with [^{35}S]methionine and equal quantities of radioactively-labelled culture media were applied to Affi-Gel Blue resin and were subsequently analysed by SDS/PAGE (Figure 4). With the exception of one of these mutants (H341A), all were synthesized and secreted to roughly the same extent as the wild-type enzyme.

When similar quantities of the mutant proteins were assayed (verified by autoradiography of proteins separated by SDS/PAGE), the initial rates of sugar transfer for four of the mutant bovine enzymes, $His^{125} \rightarrow Ala$, $His^{211} \rightarrow Ala$, $His^{341} \rightarrow Ala$ and $His^{344} \rightarrow Ala$, were $\leqslant 6\,\%$ of the wild-type level. The small amount of recombinant protein produced precluded determination of kinetic parameters for these four mutant enzymes. In contrast, the remaining ten mutants catalysed the transfer of GalNAc to acceptor substrates at a similar initial rate to the wild-type enzyme (Table 2). Similarly, the apparent K_m values of these ten mutants (determined using Lineweaver–Burk plots) for donor and acceptor substrates were similar to that of the wild-type enzyme.

The four mutant enzymes ($His^{125} \rightarrow Ala$, $His^{211} \rightarrow Ala$, $His^{341} \rightarrow Ala$ and $His^{344} \rightarrow Ala$) were subsequently expressed under conditions which yield higher levels of recombinant enzyme (see the Experimental section and [10]). This allowed us to detect

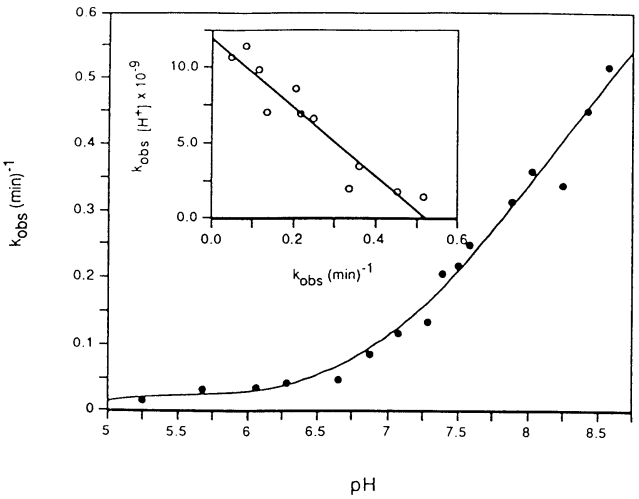

Figure 2 pH dependence of DEPC modification

Purified recombinant bovine ppGaNTase-T1 was modified at various pH values before analysis using the standard enzyme assay. The methodology and calculations are described in the Experimental section. Pseudo-first-order rate constants (k_{obs}) obtained from eqn. (1) were plotted against pH. Inset: the experimental k_{obs} values were replotted according to eqn. (2). The slope and the *x*-intercept reveal a p$K_a = 7.6$ and a $k_{obs(max)} = 0.55$ min^{-1}.

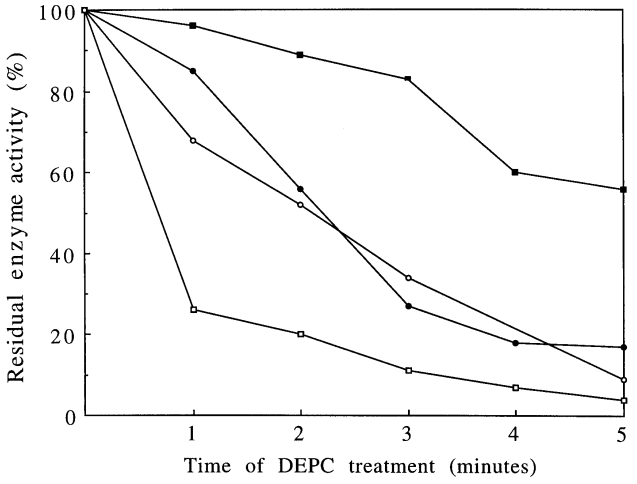

Figure 3 Influence of peptide and nucleotide sugar substrate on the rate of DEPC inactivation of ppGaNTase-T1

Recombinant enzyme was treated with DEPC in the absence (●) or presence of peptide pseudo-substrate and nucleotide sugar: 2.5 mM EPO-G (○), 160 μM UDP-GalNAc (□), or both 2.5 mM EPO-G and 160 μM UDP-GalNAc (■).

some residual activity in mutants $His^{125} \rightarrow Ala$ and $His^{341} \rightarrow Ala$ (equal to 1 and 6 % of the wild-type levels respectively); however, no activity above that of the mock-transfected controls was observed for $His^{211} \rightarrow Ala$ and $His^{344} \rightarrow Ala$.

Identification of *N*-linked glycosylation sites

SDS/PAGE analysis and *N*-glycanase digestions of the purified enzyme suggest that at least one of these sites is modified [13,14]. Amino-acid-sequence analysis revealed three potential *N*-glycosylation sites in the bovine ppGaNTase-T1 enzyme (Asn^{95}, Asn^{141}, Asn^{552}). To determine which of the three potential *N*-glycosylation sites was modified, each asparagine was mutated to glutamine. Each construct, together with the wild-type enzyme, was expressed in COS7 cells as described above. Figure 5 shows the SDS/PAGE separation of the metabolically-labelled enzymes secreted from the COS7 cells. The apparent decrease in size of the mutant enzymes $Asn^{95} \rightarrow Gln$ and $Asn^{552} \rightarrow Gln$ indicated that residues Asn^{95} and Asn^{552} are *N*-glycosylated in the wild-type enzyme. The $Asn^{141} \rightarrow Gln$ mutation did not alter the electrophoretic mobility of the enzyme. Kinetic parameters for each mutant protein were determined and the apparent K_m values for both UDP-GalNAc and peptide acceptor EPO-T were similar to

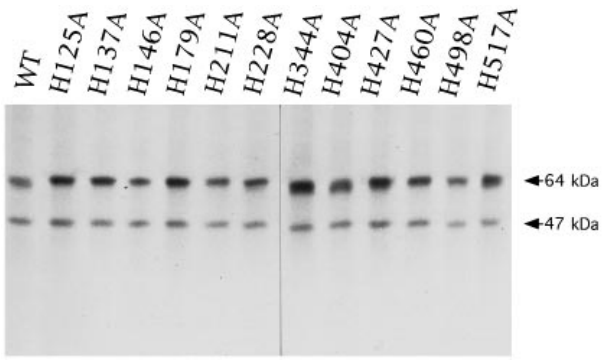

Figure 4 Tricine-SDS/PAGE analysis of histidine mutants

Constructs containing wild-type (WT) and twelve His → Ala point mutants of bovine ppGaNTase-T1 were transfected into COS7 cells and radioactively labelled metabolically with [^{35}S]methionine. Recombinant proteins were purified with Affi-Gel Blue and subjected to Tricine-SDS/PAGE, as described in the Experimental section. The band at 45 kDa is present in mock-transfected cells. The 64 kDa protein corresponds to the bovine ppGaNTase-T1. Molecular masses are indicated on the right (arrow-heads).

Table 2 Kinetics of the wild-type and mutant ppGaNTase-bT1

For determination of the K_m(app) of UDP-GalNAc, the concentration range of UDP-GalNAc was 1–40 mM and the EPO-T concentration was 0.5 mM. For determination of the K_m(app) of EPO-T, the concentration range of EPO-T was 0.07–2 mM and the UDP-GalNAc concentration was 40 mM. The K_m(app) shown are the means of two experiments carried out in duplicate. To determine the initial rate, analysis of SDS/polyacrylamide gels of mutants (see Figure 4) was used to normalize recombinant enzyme concentrations and the enzyme activities for H125A, H211A, H341A, H344A and H499A were determined by using the modified COS7 transfection protocol [10]. n.d., not determined.

	K_m(app)		
Construct	UDP-GalNAc (μM)	EPO-T (mM)	Initial rate (%)
Wild-type	17.4	0.37	100
H125A	n.d.	n.d.	1.1
H137A	17.6	0.32	86
H146A	12.8	0.32	89
H179A	17.9	0.29	92
H211A	–	–	< 1 %
H228A	13.4	0.33	136
H341A	–	–	6
H344A	–	–	< 1 %
H404A	16.9	0.33	120
H427A	16.9	0.30	152
H460A	19.0	0.31	110
H498A	14.3	0.33	130
H499A	–	–	138
H517A	17	0.33	91
N95Q	14.4	0.53	83
N141Q	13.2	0.41	94
N552Q	12.6	0.50	86

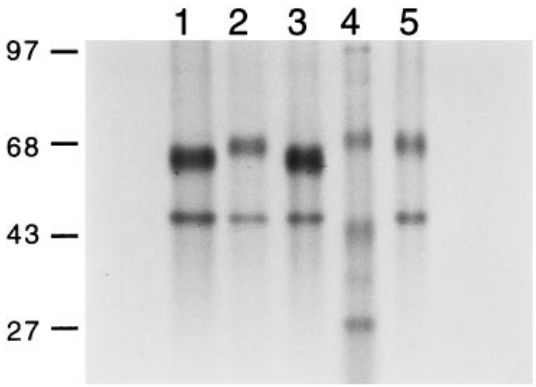

Figure 5 Tricine-SDS/PAGE analysis of *N*-glycosylation mutants

Constructs containing wild-type and three asparagine point mutants of ppGaNTase-T1 were transfected into COS7 cells and radioactively labelled metabolically with [^{35}S]methionine. Recombinant proteins were subjected to Tricine-SDS/PAGE. The gel shows: lane 1, Asn^{95} → Gln mutant; lane 2, Asn^{141} → Gln mutant; lane 3, Asn^{552} → Gln mutant; lane 4, molecular-mass markers (kDa, indicated on the left); and lane 5, wild-type ppGaNTase-T1. The band at 45 kDa is present in mock-transfected cells.

that of the wild-type enzyme. The enzyme activities of all of the single *N*-glycosylation-site mutants were also similar to that of the wild-type enzyme (Table 2).

DISCUSSION

Using a combination of chemical modification and site-directed mutagenesis, we have identified two histidine residues which appear to be essential for the catalytic function of a recombinant bovine ppGaNTase-T1. Under conditions which favour modification of histidine residues, DEPC treatment caused both a time- and concentration-dependent inactivation of the enzyme. The rate of inactivation was pH-dependent, suggesting that modification of titratable residues with a pK_a of 7.6 was responsible for the inactivation. Essential histidine residues with such pK_a values have been identified in thermolysin [15] and acid phosphatase [16].

DEPC modification in the presence of a pseudo-substrate peptide EPO-G alone did not provide protection to the enzyme, but the presence of UDP-GalNAc alone led to an accelerated inactivation by DEPC, suggesting that binding of the nucleotide sugar introduced a conformational change in the enzyme leading to either the exposure of, or increased reactivity of residues sensitive to DEPC. Substrate protection against DEPC modification was only observed when UDP-GalNAc and EPO-G were both present. Similar findings of enhanced inactivation and protection were observed for a human UDP-glucuronyltransferase. UDP-glucuronic acid enhanced enzyme inactivation by DEPC while co-incubation of both substrates UDP-glucuronic acid and 4-methylumbelliferone provided protection [6].

Single amino acid substitutions of histidine to alanine were prepared for each histidine residue of the enzyme by site-directed mutagenesis. Of the fourteen mutants, ten displayed glycosylation activities similar to the wild-type enzyme and exhibited K_m values for substrates UDP-GalNAc and EPO-T within 0.7–1.0-fold of that of the wild-type enzyme. Mutant His^{341} → Ala retained 6% of the wild-type level of activity. The role of His^{341} is unclear; this residue is not conserved among the ppGaNTases. However, the proximal position of His^{341} relative to the conserved His^{344} may influence the properties of this essential residue (His^{344}) in the His^{341} → Ala mutant. In contrast, three of the mutants, His^{125} → Ala, His^{211} → Ala and His^{344} → Ala, which are highly conserved in this gene family had a very low to undetectable rate of sugar transfer ($\leqslant 1\%$ of the wild-type). Although these findings are consistent with the results of DEPC modification experiments, the small levels of recombinant protein produced by the COS7 transient transfection system precluded identification of the DEPC-modified residues. It is noteworthy that, of the four histidine residues identified in this study to be required for efficient enzymic activity, three (His^{125}, His^{211} and His^{344}) are conserved among all forms of ppGaNTase described to date [2,9,10,17–20]. It is not known if any of these histidines is part of the enzyme active site. It is plausible that some (or all) of these residues are engaged in some common structural feature. One possibility is the co-ordination of manganese which is known to be an essential co-factor of ppGaNTase [13,14,21,22]. Another potential role would involve binding of either the nucleotide sugar donor or the protein substrate, although binding to the nucleotide sugar site seems less likely, since all of the histidine mutants were bound to Affi-Gel Blue suggesting that the dye-binding site on ppGaNTase-T1 does not require these residues. We are presently attempting to over-express the histidine mutants in quantities which will allow us to distinguish among these possibilities.

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