BioMetals **17:** 35–43, 2004.

Effect of the manganese ion on human α3/4 fucosyltransferase III activity

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Received 25 January 2003; accepted 3 March 2003; Published online: June 2003

Key words: α3/4 fucosyltransferase III, electrophilic catalyst; Lewis determinants; manganese ion, mass spectrometry

Abstract

The effect of manganese and other divalent cations on the activity of a soluble recombinant form of human α3/4 fucosyltransferase III (SFT3) expressed in *Spodoptera frugiperda* (Sf9) insect cells was studied. SFT3 was active in the absence of divalent cations with an optimum pH of 4.5. In the absence of Mn^{2+} increasing the pH from 4.5 to 7.0 caused a decrease in the affinity of SFT3 for the acceptor Galβ3GlcNAcO$(CH_2)_3$NHCO$(CH_2)_5$NH-biotin, as monitored by the 4-fold increase in the apparent K_M value (0.9 to 3.3 mM). At pH 7.0, the addition of Mn^{2+} activated the enzyme and caused an increase in the affinity of SFT3 for the acceptor, as monitored by the 5-fold decrease of the apparent K_M value (3.3 to 0.7 mM). In solution, a complex between GDP-Fuc donor and the divalent cation Mn^{2+} was observed by electrospray ionization mass spectrometry, in a 1:1 stoichiometry. These results indicated that Mn^{2+} bound the enzyme and increased its affinity for the acceptor; one possible functional role of manganese in catalysis could be as an electrophilic catalyst, co-ordinating the negative charges of the phosphate groups of the GDP-Fuc donor and promoting Fuc transfer. At low pH values such role would be played by the proton.

Abbreviations: ESI – electrospray ionization; FT – fucosyltransferase; FT3 – human α3/4 fucosyltransferase III; Galβ3GlcNAc-sp-biotin – Galβ3GlcNAcO$(CH_2)_3$NHCO$(CH_2)_5$NH-biotin; GDP-Fuc – guanosine diphosphate-β-L-fucose; Le^a – Lewis^a; Le^b – Lewis^b; Le^x – Lewis^x; Le^y – Lewis^y; Mops – 4-Morpholinepropanesulfonic acid; SFT3 – soluble FT3; sLe^a – sialyl Lewis^a; sLe^x – sialyl Lewis^x.

Introduction

Cell surfaces express several fucosylated glycoconjugates, including Lewis^a (Le^a), Lewis^b (Le^b), sialyl Lewis^a (sLe^a), Lewis^x (Le^x), Lewis^y (Le^y) and sialyl Lewis^x (sLe^x). These determinants have been identified as ligands for selectins that mediate cell recognition events occurring in inflammation (Huang *et al.* 2000) and metastases formation (Takada *et al.* 1991). Cell adhesion occurring in the initial stages of certain pathogenic events such as gastric cell infection by *Helicobacter pylori* have also been shown to be partially associated to the Le^b determinant (Ilver *et al.* 1998).

The biosynthesis of the Lewis determinants is directed by α3 and α3/4 fucosyltransferases (FTs), which catalyse the transfer of the fucose residue, from the donor sugar substrate, guanosine diphosphate-β-L-fucose (GDP-Fuc), to a type I (Galβ3GlcNAc-R) or type II (Galβ4GlcNAc-R) based acceptor oligosaccharide substrate. The human α3/4 fucosyltransferase III (FT3, EC 2.4.1.65) has predominant α4 fucosyltransferase activity, acting preferentially on type I based acceptors (Kukowska-Latallo *et al.* 1990; Costa *et al.* 1997; Sousa *et al.* 2001). Therefore, it is involved in the final step of the biosynthesis of Le^a, Le^b and sLe^a.

Only few subfamilies of glycosyltransferases share significant primary structure homology (Breton *et al.*

1998). For FT3, FT5 and FT6 subfamily, DNA sequence analyses revealed homology greater than 85% (Weston *et al*. 1992); the main differences among them are located in the stem amino-terminal region (hypervariable region) (Dupuy *et al*. 1999).

Little is known about the mechanism of FT3 catalysis. The FT3 enzyme catalyses the Fuc transfer with inversion of configuration at the anomeric carbon of L-Fuc (Breton *et al*. 1998). By analogy with glycosidase reactions (Sinnott 1991) the acceptor hydroxyl group possibly attacks the C1 of Fuc in an SN_2-like mechanism leading to the inversion of configuration.

Studies on FT5 revealed a catalytic residue with pKa 4.1, presumably an active site carboxylate anion that acts as a catalytic base in deprotonating the nucleophilic hydroxyl group of the acceptor (Murray *et al*. 1996). Evidences for a catalytic Asp residue were provided by previous work (Dupuy *et al*. 1999) where the substitution of Asp112 by the corresponding amide resulted in inactive forms of FT3.

It has been observed that divalent cations, namely Mn^{2+}, play an important role in glycosyltransferase catalysis. For some glycosyltransferases, such as N-acetylglucosaminyltransferase I, the ion acts as a co-factor (Ünligil *et al*. 2000), whereas for others, such as FT3 (Johnson *et al.* 1993), and other fucosyltransferases (Beyer & Hill 1980; Murray *et al*. 1996; Kaneko *et al*. 1999) the ion is an activator. The role of Mn^{2+} in fucosyltransferase catalysis is not yet clear, but it might co-ordinate the pyrophosphate group of the donor sugar substrate, stabilising the FT3 active site, similarly to that observed in the 3-D structure of N-acetylglucosaminyltransferase I (Ünligil *et al*. 2000). Mn^{2+} binding is accomplished by the residues of the conserved DxD motif, which has been identified in many glycosyltransferases families (Wiggins & Munro 1998; Breton & Imberty 1999; Ünligil *et al*. 2000).

In the present work, we have studied the effect of Mn^{2+} on the kinetic properties of a soluble form of FT3 that contained its catalytic domain (SFT3). We have shown that in the absence of Mn^{2+} SFT3 was active and had a maximal activity at pH 4.5. In the presence of Mn^{2+}, the maximum was shifted to pH 7.0 with nine-fold activation. At pH 7.0, Mn^{2+} caused a five-fold decrease of K_M for the acceptor substrate. Additionally, in solution GDP-Fuc formed a stable non-covalent complex with Mn^{2+} in a 1:1 stoichiometry. These results suggested that Mn^{2+} bound the enzyme increasing its affinity for the acceptor, and acted as an electrophilic catalyst in the Fuc transfer.

Materials and methods

Chemicals

The acceptor substrate Galβ3GlcNAcO$(CH_2)_3$NHCO $(CH_2)_5$NH-biotin (Galβ3GlcNAc-sp-biotin), linked to a hydrophobic spacer, was obtained from Syntesome; GDP-[^{14}C]-Fuc was obtained from Amersham Pharmacia Biotech.

SFT3 analysis

Soluble FT3, SFT3, (Ala^{47}-Thr^{361}) where the cytoplasmic and transmembrane domains, and part of the stem region, have been replaced by the signal sequence of human β trace protein, was produced from the baculovirus (AcMNPV)/insect cell (*Spodoptera frugiperda*) expression system, and purified as previously described (Morais *et al*. 2001). SFT3 was purified by a two-step procedure using an anion exchange chromatography, on a CM-Sepharose column, followed by an affinity chromatography on a GDP-Fractogel column (Morais *et al*. 2001). SFT3 concentration was estimated by the bicinchoninic acid (BCA) protein assay (Smith *et al*. 1985) after protein precipitation with sodium deoxycholate (DOC) and trichloroacetic acid (TCA) (Bensadoun & Wienstein 1976). Bovine serum albumin (BSA) was used as the standard. The active pool from the GDP-Fractogel was analysed by SDS-PAGE, using a discontinuous buffer system according to Laemmli (Laemmli 1970). The polyacrylamide gel composition was 12%T and 2.6%C. The gel was stained with Coomassie Blue R-250.

Enzyme assays and metal activation studies

The activity of SFT3 was detected by the assay described previously (Costa *et al*. 1997). Typically, the reaction mixtures (70 μl) contained 50 mM Mops-NaOH, pH 7.5, 100 mM NaCl, 20 mM $MnCl_2$, 0.047 mM GDP-Fuc and 0.0017 mM GDP-[^{14}C]-Fuc (21622 dpm/nmol of GDP-[^{14}C]-Fuc), and 0.54 mM Galβ3GlcNAc-sp-biotin. One unit of enzyme activity (U) was defined as the amount of enzyme that catalysed the transfer of 1 μmol of Fuc/min to the type I acceptor at 37 °C. It was observed linearity of product formation with time up to 31 min. The scintillation counter used was the Beckman LS 6500.

The effect of metal ion in SFT3 activity was investigated by using the divalent cations Mn^{2+}, Mg^{2+},

Ca^{2+}, Co^{2+}, Zn^{2+} and Cu^{2+} at 20 mM final concentration. The counterions for all of the metals were chloride ions. The activity calculated was expressed in percentage and was relative to that found in the absence of added metal. For manganese concentrations of 0.005, 0.05, 0.5, 5, 10, 20 and 50 mM $MnCl_2$, a reaction mixture consisting of 100 mM Mops-NaOH, pH 7.0 with 3 or 1.5 μg ml SFT3 was used.

pH rate studies and kinetic measurements

The effect of pH value on SFT3 activity (7.9 μg/ml) was determined in the presence or absence of 20 mM $MnCl_2$. The pH value in the assay mixtures was varied between pH 3.0 to pH 8.7, using 100 mM Mops-NaOH or 100 mM citrate in the presence or absence of Mn^{2+}, respectively. Citrate could not be used with Mn^{2+} due to its chelating activity. For example, the specific activity values at pH 6.0 were, in the presence of Mn^{2+}, 54.9 and 20.9 mU/mg, in Mops or citrate buffers, respectively.

The kinetic parameters of SFT3 with type I acceptor substrate, Galβ3GlcNAc-sp-biotin, were determined at pH 4.5 and pH 7.0, using the 100 mM Mops-NaOH buffer, in the presence or absence of 5 mM $MnCl_2$. The concentration of Galβ3GlcNAc-sp-biotin typically varied between 0.056 and 5.5 mM, at saturating concentrations of 0.05 mM of GDP-Fuc. SFT3 concentrations in the assays were 12.5 and 8.3 μg/ml, at pH 4.5 in absence or presence of $MnCl_2$, respectively, or 11.8 and 5.9 μg/ml, at pH 7.0 in absence or presence of $MnCl_2$, respectively. The reaction was monitored for 180 s, product being quantified at 10, 30, 60, 90, 120 and 180 s. The enzymatic reaction was initiated with Galβ3GlcNAc-sp-biotin in the absence of $MnCl_2$. To overcome the initial lag phase observed in the presence of $MnCl_2$, the enzymatic reaction in these assays were initiated with the enzyme pre-incubated with $MnCl_2$ at 37 °C.

Initial rates were obtained from the slope of the line corresponding to the linear least square fit of experimental data, using the LINEST function from Excel. For kinetic calculations, initial rates were determined from the slope of the tangent at right angles at the origin to the curves of product vs. time (Cornish-Bowden 1995). Kinetic constants were obtained from the Hanes-Woolf linearization of the Michaelis-Menten equation (Cornish-Bowden 1995).

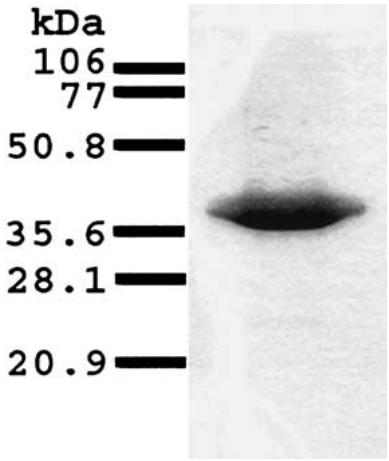

Figure 1. SDS-PAGE analysis of SFT3. The polyacrylamide gel was constituted of 12% T and 2.6% C. The gel was stained with Coomassie Blue R-250.

Mass spectrometry

An Esquire 3000^{Plus} mass spectrometer (Bruker Daltonics, Germany), equipped with an electrospray (ESI) or nano-electrospray offline (nano-ESI offline) ionization sources and an ion-trap analyser, was used. The analysis of the GDP-Fuc donor substrate (50 μM) was done in the negative ion mode, using 50:50 aqueous acetonitrile. The sample was injected into the ESI source with a 50 μl glass syringe at a constant flow of 150 μl/h. The Mn^{2+}.GDP-Fuc complex was observed at pH 7.0 using the nano-ESI (offline) source in the positive ion mode. Five μl of GDP-Fuc in 2 mM triethylamine-CO_2 at pH 7.0, were pre-incubated for 3 min with 0.005, 0.05, 0.5 or 5 mM $MnCl_2$ at 37 °C. In the negative or positive ion modes, currents at the electrospray capillary were 17.09 or 38 nA, respectively, and mass spectra were acquired in the standard mass range m/z 50 to 3000 with scan resolution of 13.000 m/z sec. In this operation mode the mass accuracy obtained was $\pm$ 0.2 m/z. Representative mass spectra were the average of 20 or 30 scans.

Results and discussion

Divalent cation effect on SFT3 activity

In the present work, we have studied the role played by manganese and other divalent cations in the reaction catalysed by the human FT3. We used a catalytic active soluble form of the enzyme, SFT3 (Ala^{47} to Thr^{361}), containing the catalytic domain and part of

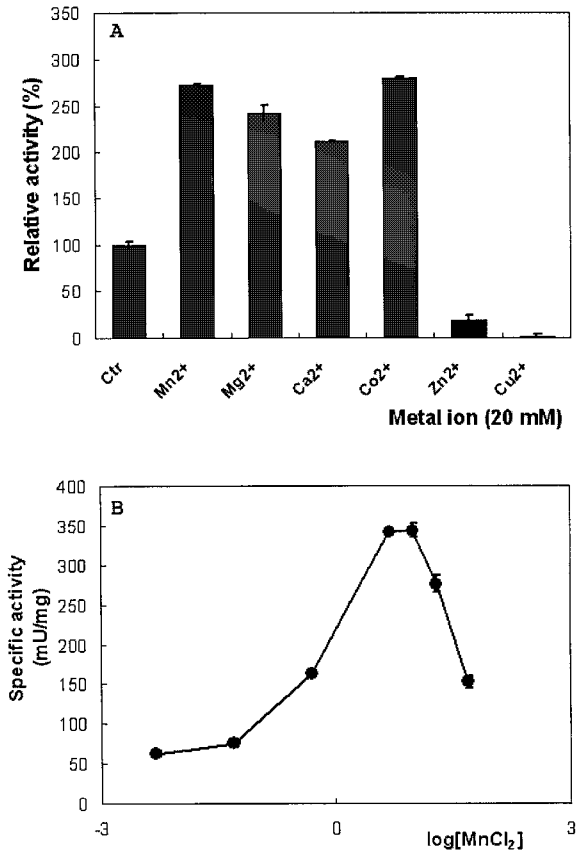

Figure 2. A) Effect of metal ions on SFT3 activity. The divalent cations Mn^{2+}, Mg^{2+}, Ca^{2+}, Co^{2+}, Zn^{2+} and Cu^{2+}, in the chloride salt form were tested at 20 mM concentration. The α4 FT activity was monitored using the type I acceptor, Galβ3GlcNAc-sp-biotin, under standard assay conditions. The activity, expressed in percentage, was relative to that found in the absence of added metal (21.6 mU ml). B) Specific activity of SFT3 measured as function of Mn^{2+} concentration (0.005–50 mM). The type I acceptor and GDP-Fuc were used at 1.7 and 0.05 mM concentration, respectively.

the stem region, expressed in *Spodoptera frugiperda* (Sf9) insect cells (Morais *et al.* 2001). The purified enzyme migrated as a single band on SDS-PAGE (Figure 1).

The activity pattern of SFT3 at pH 7.5, with a panel of divalent cations, indicated that it could be modulated by these metals (Figure 2A). The cations were used at 20 mM similarly to that described by other authors (Johnson *et al.* 1992, 1993; De Vries *et al.*, 1995). The most effective activators were Mn^{2+} and Co^{2+}, leading to 2.7-and 2.8-fold activation of SFT3, respectively. The SFT3 reaction was enhanced to a lower extent in the presence of Mg^{2+} and Ca^{2+} (2.5- and 2.1-fold activation, respectively). SFT3 was approximately 80% inhibited with Zn^{2+}, and in the presence of Cu^{2+} no significant activity was detected. Similarly to the wild-type FT3 (Johnson *et al.* 1993), and other fucosyltransferases (Beyer & Hill 1980; Murray *et al.* 1996; Kaneko *et al.* 1999), SFT3 was activated by metal ions preferentially co-ordinated by oxygen ligands in an octahedral geometry (Mn^{2+}, Co^{2+}, Mg^{2+}, and Ca^{2+}) (Fraústo da Silva & Williams 1997). The divalent cations Zn^{2+} and Cu^{2+}, which show preferences for other geometries and are co-ordinated by ligand groups containing nitrogen and sulphur atoms (Fraústo da Silva & Williams 1997), inhibited the enzyme, possibly due to the involvement of amino acids essential to catalysis, such as Cys-143 (Holmes *et al.* 1995) (Figure 3). This suggested that in the catalytic site of SFT3, the active complex formed would be octahedral with six oxygen ligand groups co-ordinated by a single metal.

In vivo the cation that most likely activates FT3 is Mn^{2+}, and physiological levels of Mn^{2+} are in the micromolar range concentration (Ash & Schramm 1982). Therefore, we have determined fucosyltransferase activity in the presence of lower Mn^{2+} concentrations between 5 μM and 50 mM (Figure 2B). The activation effect of Mn^{2+} was observed in the micromolar range with 1.3-fold activation at 50 μM concentration. Maximal activity of SFT3 was observed in the range of 5–10 mM Mn^{2+} concentration, with 6-fold activation. Concentrations higher than 10 mM Mn^{2+} had an inhibitory effect on fucosyltransferase activity. This could be due to the hydrolysis of the substrate GDP-Fuc by Mn^{2+} as previously described (Murray *et al.* 1996).

In order to be sure that residual levels of the metal ions were not interfering with the activities measured we have performed a control assay with 1 mM EDTA, and we have observed that the specific activity of the enzyme was not altered by the chelator. EDTA efficiently chelates Mn^{2+} with a stability constant $K = 10^{14}\ M^{-1}$ (Fraústo da Silva & Williams 1997).

The enzyme retained approximately 35% of its maximal activity in the absence of metal ions, similarly to that reported for other fucosyltransferases (Beyer & Hill 1980; Murray *et al.* 1996; Kaneko *et al.* 1999) with the exception of FT9 (Kaneko *et al.* 1999). This metal ion activation appears to be a unique feature of these families, since other purified glycosyltransferases that have been studied have either shown an absolute requirement for divalent manganese, such as β4 galactosyltransferase (Tsoponakis & Herries 1978; Witsell *et al.* 1990; Gastinel *et al.* 1999), N-

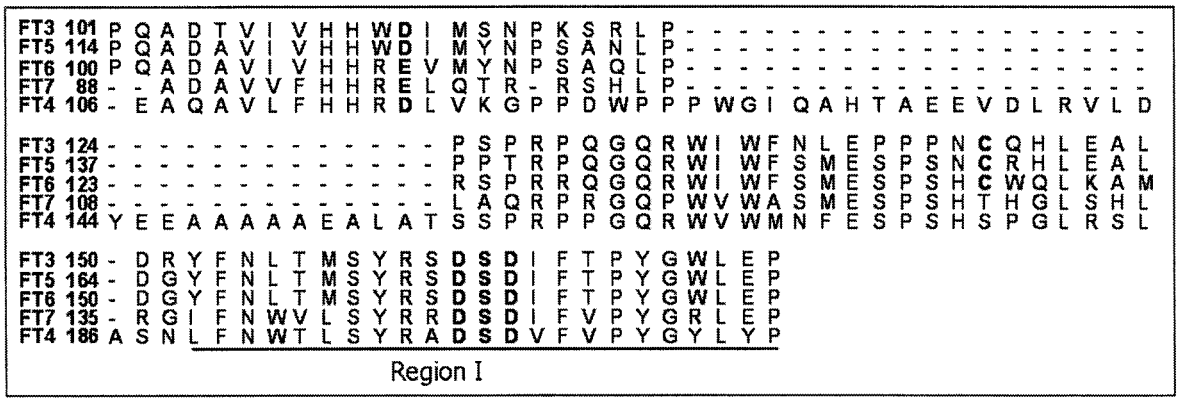

Figure 3. Alignment of Mn^{2+} activated human fucosyltransferases sequences (Pro-101-Pro-174 for FT3) using BLASTP 2.2.3 program (Altschul *et al.* 1997). The catalytic base (Asp-112 for FT3), the GDP-Fuc binding Cys (Cys-143 for FT3), and the Mn^{2+}DxD binding motif (D^{162}SD, for FT3 are highlighted. These sequences had 82 (FT5), 72 (FT6), 47 (FT7) and 34% (FT4) homology compared to FT3.

acetylglucosaminyltransferase I (Ünligil *et al.* 2000) and α3 galactosyltransferase (Gastinel *et al.* 2001; Zhang *et al.* 2001) or have been unaffected by this ion, such as N-acetylglucosaminyltransferase V (Shoreibah *et al.* 1992). It has been reported that a conserved acidic motif (DxD motif) is present in unrelated glycosyltransferase families, that use different sugar donor and acceptor substrates (Wiggins & Munro 1998; Breton & Imberty 1999; Ünligil *et al.* 2000). Mutagenesis of the residues of the DxD motif demonstrated that this region was essential for activity being involved in Mn^{2+} binding and catalysis (Wiggins & Munro 1998; Zhang *et al.* 2001). Furthermore, the 3D-structures of the four Mn^{2+} dependent mammalian glycosyltransferases solved until present have shown that the Mn^{2+} was in an octahedral geometry co-ordinating six inner oxygen atoms from one or two aspartate residues of the DxD motif, the α and β phosphates of the nucleotide donor sugar and two water molecules (Gastinel *et al.* 1999; Ünligil *et al.* 2000; Pedersen *et al.* 2000; Gastinel *et al.* 2001). This positioned the sugar moiety in a well defined arrangement for interaction with the enzyme. In the α3 fucosyltransferase family two highly homologous regions, named I and II, were detected (Breton *et al.* 1998). An acidic sequence, similar to the acidic DxD motif, was found to be conserved in region I (Breton *et al.* 1998; Wiggins & Munro 1998) and consisted of D^{162}SD for FT3 (Figure 3). The D^{162}N mutation caused an 80% decrease in activity (Pang *et al.* 1998).

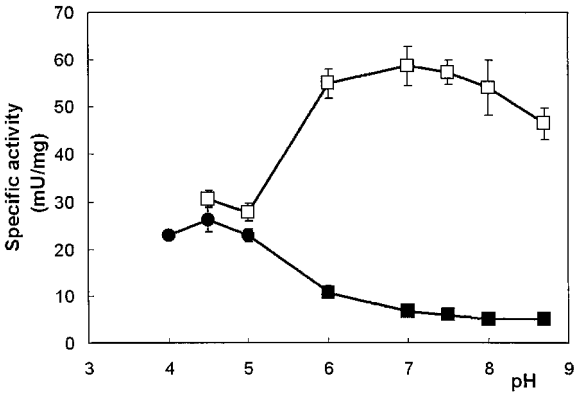

Figure 4. Effect of pH in the specific activity of SFT3 in the presence (opened symbols) or absence (closed symbols) of $MnCl_2$ (20 mM). The α4 FT activity was measured, using 100 mM citrate buffer (●, pH 4.0–6.0 without Mn^{2+}) or 100 mM Mops buffer (■, pH 6.0–8.7 without Mn^{2+}; □, pH 4.5–8.7 with Mn^{2+}). The α4 FT activity was monitored under standard assay conditions. The initial velocity for each pH value was calculated in the linearity range and the SFT3 specific activity (mU/mg) variation was plotted as function of the pH value at 0.54 mM of Galβ3GlcNAc-sp-biotin and 0.05 mM of GDP-Fuc.

SFT3 pH dependency

The SFT3 activity dependence on pH was investigated, in the absence or presence of Mn^{2+} (Figure 4). In the absence of Mn^{2+}, SFT3 had its maximal activity at pH 4.5. At pH 3.0 the enzyme was inactive. By analogy with the SN_2 like mechanism proposed for inverting glycosidases (Sinnott 1991) and based on the identification of a catalytic residue with pKa 4.1 for FT5 (Murray *et al.* 1996), it can be admitted that an acidic amino acid residue would be acting as a catalytic base. This hypothesis is in agreement with the

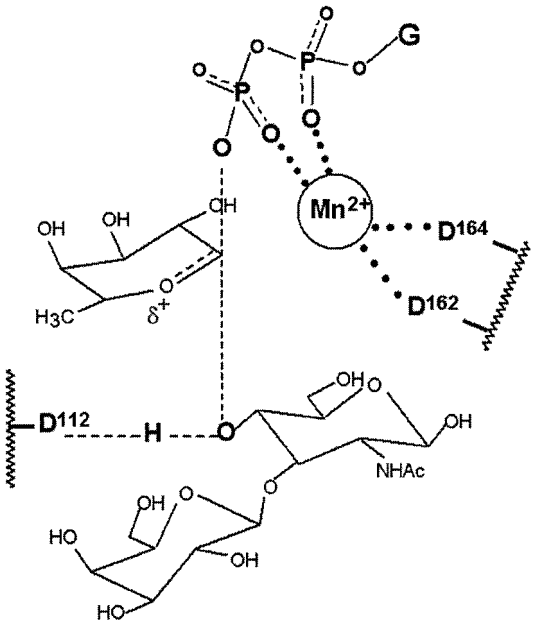

Figure 5. Proposed role of Mn^{2+} in the catalytic site of SFT3. **G**-Guanosine.

acidic optimum pH observed. The inactivating mutation D^{112}-N (Dupuy *et al*. 1999) has suggested that D^{112} (pKa 3.9) would be the catalytic base for FT3.

In the presence of Mn^{2+}, SFT3 had a broad range of maximal activity between pH 6.0 and 8.0 with the optimum at pH 7.0. The divalent cation effect was markedly observed at pH values above 6.0. In the absence of the cation, at pH 7.0, the specific activity was 9-fold lower. Based on these results and in the context of the SN_2 like mechanism, one possible functional role of manganese in the active site of SFT3 is as an electrophilic catalyst, co-ordinated by the phosphate groups from the GDP-Fuc donor substrate. Additionaly, the Mn^{2+} might have an effect in structuring the active site of SFT3 and increase the affinity of the enzyme for the substrates, consequence of its binding to the D^{162}SD motif similarly to that described for N-acetylglucosaminyltransferase I (Ünligil *et al.* 2000) (Figure 5). These two possible effects of Mn^{2+} could have as a consequence the shift in optimal activity towards more basic pH values. We will investigate them in more detail in the next two sections.

GDP-Fuc:Manganese(II) complex

The capability of Mn^{2+} to co-ordinate with the phosphate groups of GDP-Fuc was investigated by nanoESI-MS at pH 7.0 (Figure 6). In the negative

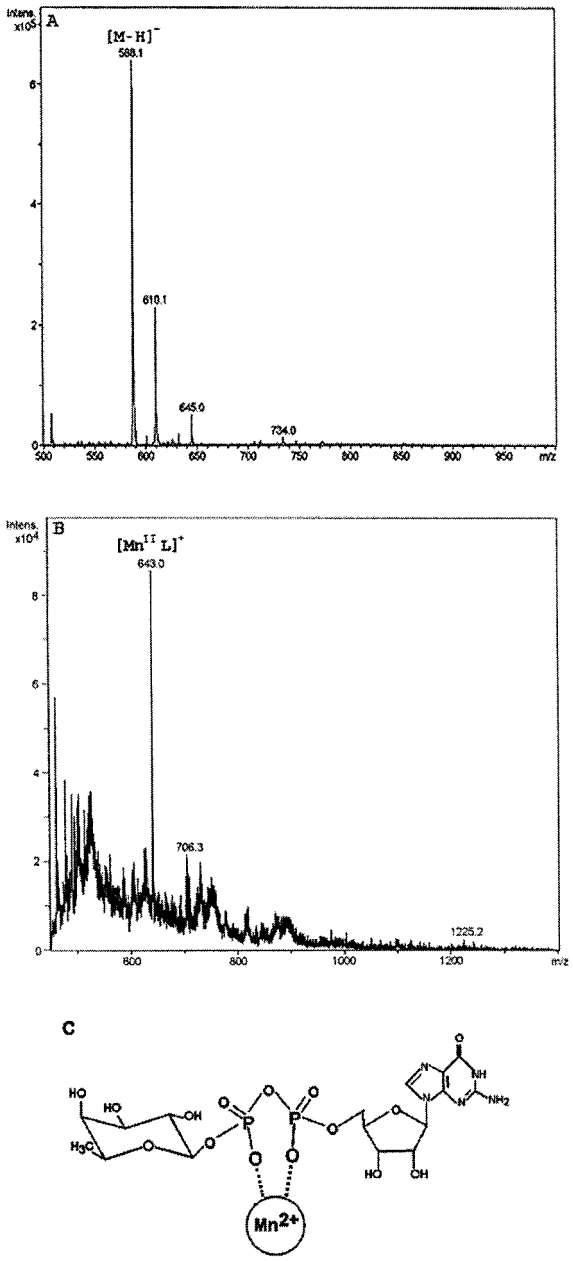

Figure 6. A) ESI mass spectrum of GDP-Fuc (50 μM) in negative ion mode in acetonitrile/water (50:50). B) Nano-ESI mass spectrum of GDP-Fuc (50 μM) in the positive ion mode, in the presence of 5 mM Mn^{2+} at pH 7.0. C) Schematic diagram of the bidentate complex formed between Mn^{2+} and GDP-Fuc in aqueous solution at pH 7.0.

Table 1. Kinetic parameters for SFT3, with the type I Galβ3GlcNAc-sp-biotin acceptor substrate, at pH 4.5 and 7.0 in the presence or absence of Mn^{2+} (5 mM).

Kinetic parameters	pH 4.5		pH 7.0	
	Mn^{2+}		Mn^{2+}	
	−	+	−	+
K_M^{app} (mM)	0.9	1.5	3.3	0.7
$k_{cat}(s^{-1})$	0.3	0.2	0.1	0.2

ion mode, the ESI spectrum of GDP-Fuc showed a peak corresponding to the single charged $(M\text{-}H)^-$ ion at m/z 588.1 (Figure 6A); in the positive ion mode no significant peaks were observed. A signal corresponding to the sodium adduct of GDP-Fuc was also identified at m/z 610.1. When the GDP-Fuc was pre-incubated with Mn^{2+} (5 mM) at 37 °C, a peak was observed at 643 m/z in the positive ion mode (Figure 6B), and no significant peak was observed in the negative ion mode. The molecular mass corresponded to the complex of GDP-Fuc and one Mn^{2+} ion. This indicated that at pH 7.0 the Mn^{2+} formed a complex with GDP-Fuc in a 1:1 stoichiometry (Figure 6C). The concentration dependent-complex formation was tested using 5, 50 and 500 μM Mn^{2+} at pH 4.5 and 7.0. At pH 7.0 the complex was observed already at 50 μM Mn^{2+}.

However, at pH 4.5 the complex was observed only at the higher concentration of 5 mM Mn^{2+}. This was indicative of a competition between the Mn^{2+} and the proton for the negative phosphate groups at pH 4.5. Thus, it is probable that in the active site a bidentate chelation of SFT3 bound Mn^{2+} by the phosphate groups of the GDP-Fuc would occur, thus preventing the electrostatic repulsion developed between the phosphate groups of the donor and the incoming nucleophile, at basic pH values, and facilitating the leaving group departure, similarly to that proposed for FT5 (Murray *et al.* 1996). At acidic pH values the negative charges would be stabilised by the proton (Figure 5).

Manganese and SFT3 kinetic properties

The effect of Mn^{2+} on the kinetic properties of SFT3 at pH 4.5 and 7.0 was investigated. In each case, the data was presented as a plot of $[S]/v_0$ against [S] obtained from the Hanes-Woolf linearization of the Michaelis-Menten equation (Figure 7). The calculated steady-state kinetic parameters are shown in Table 1.

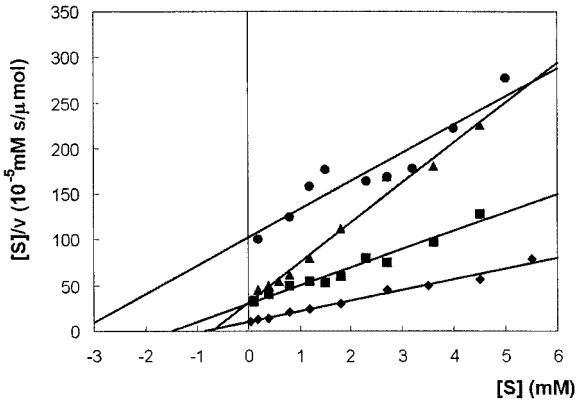

Figure 7. Hanes-Woolf plot analyses of initial rates at pH 4.5 and pH 7.0, in the presence or absence of 5 mM Mn^{2+}, with the type I acceptor Galβ3GlcNAc-sp-biotin. The concentration of the acceptor substrate varied between: (1) 0.056–5.5 mM, at pH 4.5, in the absence of Mn^{2+} (♦), (2) 0.1–4.5 mM, at pH 4.5 (■) and 7.0 (▲), in the presence of Mn^{2+} and (3) 0.2–5 mM, at pH 7.0, in the absence of Mn^{2+} (●) at fixed concentrations of the GDP-Fuc donor substrate, 0.05 mM. The apparent K_M and V_{max} were determined from the y axis intercept (K_M/V_{max}) and from the slope of the straight line ($1/V_{max}$), respectively.

In the absence of Mn^{2+}, at pH 4.5, SFT3 exhibited an apparent K_M for the acceptor substrate Galβ3GlcNAc-sp-biotin of 0.9 mM, 4-fold lower than at pH 7.0. The presence of the divalent cation, at pH 7.0, caused a 5-fold reduction in the apparent K_M for the acceptor. These results indicated that decreasing the pH from 7.0 to 4.5 in the absence of Mn^{2+}, or adding Mn^{2+} to the assay at pH 7.0 increased the affinity of the enzyme for the acceptor substrate.

Furthermore, at pH 7.0 and in the presence of Mn^{2+}, it was observed an initial lag phase, if the enzyme was pre-incubated with the donor (or acceptor) and Mn^{2+}, when the reaction was initiated with the acceptor (or donor, respectively) substrate. This lag-phase was abolished when the enzyme was pre-incubated with Mn^{2+} suggesting that the ion would bind to the enzyme before the other substrates. The solved atomic structures of the Mn^{2+} dependent glycosyltransferases showed a donor-substrate-Mn^{2+} induced conformational change, with the formation of hydrogen and van der Waals bonds that structured a loop sequence and created one side of a deep pocket terminating over the catalytic base (Ünligil *et al.* 2000; Boix *et al.* 2001). It is possible that an additional effect of Mn^{2+} would be to bind to the $D^{162}SD$ deprotonated motif, thus structuring the catalytic site of SFT3 at pH 7.0, as we have discussed above (Figure 5). Supporting this hypothesis is the observed

increase in affinity of SFT3 for the acceptor substrate in the presence of the cation. At pH 4.5 approximately 50% $D^{162}SD$ would be protonated and protons might contribute to the structuring of the catalytic site. Thus, protons might substitute the role of Mn^{2+} in the catalytic site of the enzyme. In this context the determination of the X-ray structure of one member of the FT family, in the presence of Mn^{2+} and the GDP-Fuc donor will be crucial to elucidate the mechanism of FT3 and the physical basis for the activity observed in the absence of Mn^{2+}.

Acknowledgements

We extend thanks to Dr Cláudio Soares and Dr Paulo Martel, ITQB, for valuable discussion. This work was funded by grants BIOTECH/35679 and BCI/38631 from Fundação para a Ciência e Tecnologia (FCT), Portugal. ASP and VAM were recipients of fellowships from FCT.

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