Characterization of sialyltransferase mutants using surface plasmon resonance

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Sialyltransferases are enzymes responsible for the important sialylation of glycoconjugates. Since crystal structures are not available, other tools are needed to study enzymatic mechanisms. As a model, we used human α2,6-sialyltransferase. A putative acceptor-binding domain containing the small and the very small sialyl motifs was randomly mutated. This resulted in enzymes with altered enzymatic activity. Affinity chromatography demonstrated that their binding to donor substrate was maintained. To illustrate the role of the mutated domain in acceptor binding, a method based on surface plasmon resonance was set up. Only at low salt and high acceptor concentration was association of wild-type ST6GalI with asialofetuin demonstrated. As expected, this interaction was affected by cytidine 5′-monophospho-N-acetylneuraminic acid, the donor substrate, which proves the specificity of the interaction. Different types of mutants were found. For some, the drop in activity could be explained by loss in affinity for the acceptor. For others, the catalytic center, but not the acceptor-binding site, was affected. Neither acceptor binding nor catalytic activity were limited to the sialyl motifs. To our knowledge, this is the first example in which surface plasmon resonance is successfully used to demonstrate the binding of a glycosyltransferase to its natural acceptor.

Key words: CMP-NeuAc/Glycoconjugate/Surface plasmon resonance/Sialyltransferase/ST6GalI

Introduction

So far, 15 different kinds of sialyltransferases have been characterized in vertebrates (Tsuji, 1999). They differ in substrate specificity and/or the linkage formed (Tsuji, 1996). Moreover, their ability to transfer sialic acid seems to depend on the molecule to which the carbohydrate structure is attached (glycolipids vs. glycoproteins) and the way they are linked (N- vs. O-glycans). They all transfer sialic acid from the common nucleotide donor cytidine 5′-monophospho-N-acetylneuraminic acid (CMP-NeuAc) to the nonreducing end of specific carbohydrate structures. The presence of this particular sugar on macromolecules was shown to be essential in many processes, including differentiation, cell-cell interaction, and malignant transformation (Varki, 1993). Therefore it is not surprising that the expression of both the modification and the enzymes is highly regulated (Wang et al., 1990, 1993; Harduin-Lepers et al., 1995).

All cloned glycosyltransferases, including sialyltransferases, have the same type II membrane topology. A short cytoplasmic tail precedes a transmembrane domain, a proline-rich stem region, and a large carboxy-terminal domain responsible for the catalytic activity in the lumen of the Golgi apparatus (Paulson and Colley, 1989; Kitazume-Kawaguchi et al., 1999). Apparently, only the latter domain is needed for full enzymatic activity. A soluble, active form of the protein can also be found in serum, resulting from the proteolytic cleavage in the stem region. Two isoforms of ST6GalI that differ in only one amino acid in the catalytic domain show different catalytic activity and are differently processed (Ma et al., 1997).

In contrast to what can be expected from this common structure, little sequence homology is found among the different sialyltransferases. Three sequence motifs with high homology were described within the catalytic domain (Wen et al., 1992; Datta and Paulson, 1997). They are called the long, the small, and the very small sialyl motifs. Degenerated primers based on the sequences of these motifs allowed the cloning of several new sialyltransferases (Tsuji, 1996; Giordangen et al., 1997; Samyn-Petit et al., 2000). The presence of these common motifs in all cloned eukaryotic sialyltransferases points at an essential role in the structure or specific activity of the enzyme. The involvement of certain residues within the long sialyl motif in donor binding has been clearly demonstrated (Datta and Paulson, 1995). Since all sialyltransferases known so far use the same donor substrate, the existence of a common motif is not surprising. Based on mutation analysis, the small sialyl motif was related to both donor and acceptor binding (Datta et al., 1998). Different sialyltransferase types use different acceptors. Therefore, a common motif involved in the binding with the acceptor substrate is not expected. In this latter study, researchers were unable to analyze one of their mutants with much lowered activity because of the lack of appropriate tools. Indeed, classical kinetic studies that determine $K_m$ values for the donor and acceptor substrates are only possible with enzymatically active proteins. Little is known about the amino acids essential for the specific activity of sialyltransferases or for the interaction with their substrates.

We generated a collection of mutant human ST6GalI to search for residues involved in acceptor binding. The frame for mutagenesis was the C-terminal part, including the small and the very small sialyl motifs, immediately following the long sialyl motif. This region was chosen as a first target because the only amino acids involved in acceptor binding were

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previously shown to be situated there. Because several mutants expressed very low or no activity, tools other than classical measurement of $K_v$ values were optimized. For the first time, surface plasmon resonance was successfully exploited in directly determining the binding of a glycosyltransferase to its acceptor substrate.

**Results**

*Expression of wild-type (wt) and mutant ST6GalI and comparison of their enzymatic activity*

For recombinant expression of wt and mutant ST6GalI, pCAGGS was used. The strong promoter-enhancer combination allows good expression in higher eukaryotic host cells, such as COS1 and HEK293. The vector contained the catalytic domain of ST6GalI linked to the secretion signal of interferon-$\gamma$ as described previously (Colley et al., 1989). After transfection, the proteins were expressed in soluble form in the culture medium. The E-tag was linked C-terminally to allow easy detection and quantification of the expressed product.

Because mass screenings were planned, transfection protocols for COS1 and HEK293 cell lines were optimized for their use in 96-well microplates. HEK293 cells were chosen, because they gave the best results as to yield and reproducible transfection efficiency. Moreover, no endogenous sialyltransferase activity could be demonstrated in the extracellular medium after transfection with blank vector.

Homology studies between different sialyltransferases revealed the sialyl motifs (Wen et al., 1992; Datta and Paulson, 1997). Only in the small motif, amino acids involved in acceptor binding were described. Therefore we chose a window for random mutagenesis, including this region. In the final expression product, the C-terminal 139 amino acids, including the small (from P321 to F343) and the very small sialyl motifs (from G367 to V379), were potentially mutated. The introduction of mutations was done under conditions of error-prone polymerase chain reaction (PCR). High Taq DNA polymerase and Mg$^{2+}$ concentrations and trace amounts of Mn$^{2+}$ were responsible for both transversion and transition mutations. In the presence of 0.2 mM MnCl$_2$, the best yield in single mutants was obtained. By exchanging the desired mutated PCR fragment in the expression vector containing wt ST6GalI, a mutant library was created. Each mutant vector was amplified separately after transformation with competent E. coli cells. Subsequently, each of them was transfected to HEK293 cells present in the wells of 96-well microplates.

The culture medium of two subsequent expression rounds was combined (400 $\mu$l total). To check the transfection efficiency, a dot-blot immunoassay was performed. The presence of expression product was confirmed using anti-E-tag antibody, a peroxidase-conjugated secondary antibody, and a luminescent substrate. Quantification of the signal was possible using LumiImager. Because the affinity tag is situated C-terminally, the proteins were expressed in soluble form in the culture medium, other buffer systems (100 mM HEPES pH 7.2; 100 mM sodium cacodylic acid pH 7.2; 20 mM sodium acetate pH 5 and 6; 20 mM Tris-HCl pH 8 and 9.5), addition of cytidine 5'-diphosphate (CDP), cytidine 5'-monophosphate (CMP), or organic solvents (acetonitrile, methanol, DMSO). Because all were unsuccessful, we dropped this option.

Alternatively, the proteins present in the partially purified culture medium were immobilized on a CM5 sensor chip after concentration and buffer change. As a control, equally treated culture medium of cells transfected with empty vector was directly determining the binding to a glycosyltransferase to its acceptor substrate. With that of wt enzyme. Mutants with lowered activity were isolated. Improved activity was not found. Sequencing of the mutated fragment revealed the mutations responsible for altered activity. Those mutants with a single mutation were selected for further investigation (Table I). Many reasons for the inactivating effect of the mutation can be expected. Affinity for both donor and acceptor substrate may be affected, the catalytic domain may be hit, or a conformational change may have occurred.

**Control of the affinity for donor substrate**

We were specifically searching for residues involved in acceptor binding. However, it could not be excluded that mutants were affected in their donor-binding activity. Therefore, affinity purification was achieved using an affinity gel to which CDP-hexanolamine was covalently linked. It was previously demonstrated that this molecule is chemically similar to CMP-NeuAc (Paulson et al., 1977; Weinstein et al., 1982a). Concentrated culture medium was applied to a small column, and fractions were checked for the presence of recombinant ST6GalI using the E-tag. Elution started immediately after the rise of conductivity. All checked mutants showed an elution pattern similar to that of wt enzyme (data not shown). Consequently, we conclude that the donor-binding domain of the mutants was not affected dramatically.

**Control of the affinity for acceptor substrate using surface plasmon resonance**

The most common way to analyze enzyme mutants is measuring apparent $K_v$ values. However, when no or very low remaining catalytic activity is left, this becomes impossible. The only possibility is to look at direct interaction. Surface plasmon resonance is the physical phenomenon used by BIACore to measure such interactions (Fagerstam et al., 1991; Johnsson et al., 1991). However, for the analysis of the interaction between enzymes and their substrates, this is not an obvious technique because of the fast on/off rate of binding. Theoretically, two setups are possible in BIACore, depending on which component is immobilized on the sensor chip. At first sight, immobilization of the acceptor substrate was most obvious. In that way, both wt and mutant enzymes could be analyzed on the same sensor chip. As an acceptor we chose asialofetuin. The N-linked glycans on this protein were previously shown to be good acceptor substrates for ST6GalI (Mattox et al., 1992; Laroy et al., 1997). Using amine coupling, different amounts of this protein (ranging from 2000 to 10,000 response units) were covalently coupled to a CM5 sensor chip. As a negative control, a blank chip was used. After exposure of the sensor chip to culture medium containing wt ST6GalI, no specific binding event could be demonstrated. Different conditions were tried, such as 100-fold concentration of the culture medium, other buffer systems (100 mM HEPES pH 7.2; 100 mM sodium cacodylic acid pH 7.2; 20 mM sodium acetate pH 5 and 6; 20 mM Tris-HCl pH 8 and 9.5), addition of cytidine 5'-diphosphate (CDP), cytidine 5'-monophosphate (CMP), or organic solvents (acetonitrile, methanol, DMSO). Because all were unsuccessful, we dropped this option.
used. Originally, exposure of a sensor chip with wt ST6GalI to different concentrations of asialofetuin was unsuccessful. In these first experiments, HBS (10 mM HEPES pH 7.2; 150 mM NaCl; 0.005% v/v Surfactant P20) was used during binding. However, a specific binding signal appeared only at NaCl concentrations below 25 mM and at asialofetuin concentrations above 400 µg/ml (Figure 1A). Consequently, all further binding assays were done using 0.5 to 1 mg/ml of asialofetuin in 100 mM HEPES (pH 7.2) without salt. The same buffer was used as running buffer. Obviously, regeneration of the sensor chip was easily obtained after injection of 10 µl MNCl.

Under these conditions, binding properties of wt ST6GalI enzyme with asialofetuin were characterized in more detail. No difference in binding was seen in the presence of CMP or CDP. Thus, acceptor binding does not need prior binding of donor substrate. In the presence of CMP-NeuAc, the binding signal was lowered. No more binding was seen at a donor concentration of 10 mM (data not shown). When 2 mM of donor substrate was injected during dissociation, a higher dissociation rate was obtained (Figure 1B). These results prove that the signals obtained are specific for binding between immobilized ST6GalI and asialofetuin. Moreover, these experiments show that sialyltransferase is enzymatically active in the absence of NaCl, which is in agreement with previous results describing assays for this enzyme in saltless buffer (Weinstein et al., 1982a; Datta and Paulson, 1995).

Table I. Analysis of human ST6GalI and its mutants

<table>
<thead>
<tr>
<th>Windowa</th>
<th>ST6GalI constructs</th>
<th>Residual activity (%)</th>
<th>Associationb</th>
<th>Dissociacion</th>
</tr>
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<tbody>
<tr>
<td>wt</td>
<td>100</td>
<td>++++</td>
<td>wt</td>
<td></td>
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<tr>
<td>Y270</td>
<td>Mutants</td>
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<td></td>
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<tr>
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<td>ND</td>
<td>ND</td>
<td></td>
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<td></td>
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<tr>
<td>H288L</td>
<td>5–20</td>
<td>++</td>
<td>wt</td>
<td></td>
</tr>
<tr>
<td>Q291R</td>
<td>20–40</td>
<td>++</td>
<td>wt</td>
<td></td>
</tr>
<tr>
<td>K297E</td>
<td>&lt; 5</td>
<td>++++</td>
<td>wt</td>
<td></td>
</tr>
<tr>
<td>E314G</td>
<td>&lt; 5</td>
<td>–</td>
<td>NR</td>
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<td>&lt; 5</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
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<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>N319Y</td>
<td>&lt; 5</td>
<td>–</td>
<td>NR</td>
<td></td>
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<td>wt</td>
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<td></td>
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<tr>
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<td>&lt; 5</td>
<td>+</td>
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<td>wt</td>
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<td>wt</td>
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<tr>
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<td>++</td>
<td>faster</td>
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<td></td>
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<tr>
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<td>++</td>
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<td></td>
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<tr>
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<td>+++</td>
<td>wt</td>
<td></td>
</tr>
<tr>
<td>D389G</td>
<td>40–60</td>
<td>+++</td>
<td>wt</td>
<td></td>
</tr>
<tr>
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<td>40–60</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>K395N</td>
<td>5–20</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>A396S</td>
<td>40–60</td>
<td>+++</td>
<td>slower</td>
<td></td>
</tr>
<tr>
<td>L409</td>
<td>F401L</td>
<td>20–40</td>
<td>++++</td>
<td>wt</td>
</tr>
</tbody>
</table>

Sialyltransferase activity was measured in culture medium of HEK293 cells and compared with wt activity. Association and dissociation of immobilized enzyme with injected asialofetuin were determined in BIAcore experiments.

a Window chosen for random mutagenesis. The positions of the small and very small sialyl motifs are indicated.

b Association kinetics were the same for all mutants, except where mentioned. The relative amount of asialofetuin that bound to immobilized enzyme was compared with wt (ND, not determined; the number of + signs represents the relative amount; –, no binding).

c Dissociation of asialofetuin from immobilized enzyme was compared with wt (ND, not determined; NR, not relevant).

d Mutants that still bind under physiological salt conditions.

Fig. 1. Sensorgram representing the interaction between immobilized wt ST6GalI and asialofetuin. The sensorgrams show the specific binding signal after correction for aspecific binding. (A) 1 mg/ml of asialofetuin was diluted in 100 mM HEPES pH 7.2 with 0 (a), 10, (b), or 25 mM (c) of NaCl. After injection of 20 µl, binding was present only in buffers with less than 25 mM of salt. The arrows indicate the start and the end of injection. (B) Dissociation of bound asialofetuin from the sensor chip in the absence (a) or presence (b) of CMP-NeuAc.
Comparison of the binding properties of mutant ST6GalI with wt enzyme

Culture medium of cells expressing mutant enzymes were treated and immobilized in the same way as for wt ST6GalI. Because only partially purified medium was used, the total amount of immobilized protein could not be used as a measure for the amount of enzyme coupled. Therefore, equal amounts of enzyme, as measured by a dot-blot immunoassay, were sent over an activated sensor chip. Because the mutant and the wt enzyme differ in only one amino acid and a large excess of protein was used during the coupling procedure, it is assumed that comparable amounts of the different proteins are attached to the chip. Binding experiments with asialofetuin were as with wt enzyme (Table I and Figure 2). As expected, several mutants were clearly affected in their acceptor-binding properties. Some enzymatically inactive mutants, such as E314G (Figure 3A), were no longer able to bind asialofetuin. Other inactive mutants, such as F343S, only had lowered binding characteristics (Figure 3B). On the other hand, the inactive mutant K297E interacted as efficiently with asialofetuin as with wt ST6GalI. Injection of CMP-NeuAc during dissociation gave no change in dissociation rate, which confirms the lack of sialyltransferase activity. Because donor binding had also not changed, the catalytic domain was probably affected. The I328N mutant has lowered activity but better binding to asialofetuin (Figure 3B). As mentioned before, binding of the wt enzyme with asialofetuin is only observed at NaCl concentrations below 25 mM. Two mutants (Y355N and Y369N) were found in which binding could still be measured at physiological salt concentrations (Figure 3C). In both cases, a tyrosine was mutated to an asparagine residue. This difference in salt sensitivity of the amino acid–sugar contact suggests a different nature for the interaction.

Discussion

Glycosyltransferases are enzymes required to build up the glycan structures on glycolipids and glycoproteins (Kleene and Berger, 1993; Field and Wainwright, 1995). As donor substrates, they all need a specific activated nucleotide sugar. Most of them are Golgi-localized. All glycosyltransferases cloned so far have the same topology. N-terminally, a short cytoplasmic tail is oriented toward the cytoplasm. Their transmembrane domain includes a recognition signal for the Golgi apparatus. Oriented to the lumen of this organelle are the protease-sensitive stem region and the tightly folded globular catalytic domain. Only the last domain is needed for full enzymatic activity. A soluble form of the enzyme may be present after proteolytic cleavage in the stem region. Although there is no structural evidence for this model, it can be deduced from the protein sequence of the cloned enzymes. Such structural evidence could only come from crystallization of the enzymes. However, this seems to be a difficult task. From a galactosyltransferase, only the structure of the catalytic domain was solved (Gastinel et al., 1999).

One family of glycosyltransferases is the sialyltransferase family. These enzymes catalyze the transfer of sialic acid from its activated donor substrate, CMP-NeuAc, to the nonreducing end of a growing sugar chain. So far, 15 different kinds of sialyltransferases have been cloned (Tsujii, 1999). They are classified according to the acceptor they sialylate and to the

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Fig. 2. Schematic representation of the mutated C-terminal hST6GalI domain, including the small (P321 to F343) and the very small sialyl motifs (G367 to V379) (both in yellow). The amino acids in the colored boxes under the wt sequence indicate the single mutations with altered activity (the color depends on residual activity as compared to the wt enzyme). The residual binding characteristics toward the wt substrate (ASF) as determined by surface plasmon resonance are represented by the colored lines under the mutation boxes (the number of + signs represents the relative amount of binding as compared to the wt interaction; –, no residual binding).
Analysis of sialyltransferase mutants

The enzyme sequence as deduced from the human cDNA sequence (Grundmann et al., 1990) also suggests the common glycosyltransferase structure. So one could suspect high sequence homology between all sialyltransferase members. However, the opposite is true. Three domains with elevated homology were characterized previously and tentatively called the long (from R183 to T228 in the human enzyme), the small (from P321 to F343), and the very small sialyl motifs (from G367 to V379)(Wen et al., 1992; Tsuji, 1996). It has been reported that the long motif is situated in the donor-binding domain and the small motif is located in both the donor- and acceptor-binding sites (Datta and Paulson, 1995; Datta et al., 1998). Preliminary results suggest that the very small sialyl motif is needed for catalytic activity (Tsuji, 1999). For the amino acid residues outside these motifs, little is known. However, because the members of the sialyltransferase family use different acceptor sugars, the binding site for this substrate may well be located there. ST6GalI was chosen as a model to evaluate the structure–function relationship. Randomly mutated enzymes were checked for their specific activity and their binding to donor and acceptor substrate. Specifically, the binding site for the acceptor substrate was of interest. Asialofetuin was used not only in the activity assay (Laroy et al., 1997) but also in the binding experiments.

Only part of the enzyme was mutagenized for our study. We chose the C-terminal part, including the small and the very small sialyl motifs but not the long one. The study of the homologous motifs suggested that the acceptor-binding site was located there. Single mutants with impaired activity were selected for further investigation. All mutants could be partially purified by affinity chromatography, indicating that the donor-binding site was not affected. To investigate whether the mutation caused a change in acceptor binding, surface plasmon resonance was used. This method was chosen because the measurement of apparent $K_m$ values became impossible for those mutants with low residual activity. Moreover, in the previously mentioned study of the small sialyl motif (Datta et al., 1998), at least one mutant was excluded from kinetic analysis for this reason.

Actual measurement of the interaction of sialyltransferase with asialofetuin using BIAcore technology was only possible in one configuration. Mutant or wt enzyme had to be immobilized on the sensor chip. Subsequent injection of asialofetuin only revealed a clear binding signal when this was done in low or salt-free buffers and at high acceptor concentrations. The first aspect indicates highly hydrophilic interaction; the latter demonstrates low-affinity binding. The conditions used had the consequence that mass transport effects and aspecific binding of the injected asialofetuin to the chip could not be excluded, making it impossible to determine adequate $k_{on}$ and $k_{off}$ rates. A control experiment, in which equally treated proteins of expression medium without sialyltransferase are immobilized to the chip, is essential to correct for these effects.

Upon injection of asialofetuin in the presence of CMP-NeuAc; the overall binding levels dropped. In that case, bound asialofetuin is sialylated by immobilized enzyme and released immediately. Alternatively, CMP-NeuAc can be injected after applying the acceptor to the sensor surface, resulting in a higher dissociation rate. The same explanation as before can be

![Fig. 3. Analysis of ST6GalI mutants and comparison with wt enzyme. The sensorgrams show the specific binding signal after correction for aspecific binding. An arrow indicates the beginning and the end of the injection. (A) Comparison of mutant E314G (b) with wt enzyme (a), demonstrating loss of binding characteristics of the mutant. Binding was done with 0.5 mg/ml asialofetuin in 100 mM HEPES pH 7.2. (B) Difference in binding characteristics between mutant F328N (b), F343S (c) and wt enzyme (a). Binding conditions as in (A), except for asialofetuin concentration (1 mg/ml). (C) Binding of asialofetuin (1 mg/ml) to the immobilized Y369N mutant in 100 mM HEPES pH 7.2 with 0 (a), 50 (b), 150 (c) or 200 mM (d) of NaCl, demonstrating the higher salt resistance of the binding as compared with wt enzyme.](image-url)
given. Both experiments prove that the signals obtained after correction for aspecific binding are specific for the interaction between the enzyme and its acceptor substrate. Moreover, they show that the enzyme is still active after immobilization and under the salt-free conditions used.

For wt enzyme, no difference in binding to acceptor was seen in the presence of CMP or CDP. We conclude that acceptor binding does not require prior donor binding. In contrast, acceptor binding is not influenced by the presence of CDP, a potent inhibitor of CMP-NeuAc. This means that also those mutants with impaired donor binding could be analyzed using this method.

Comparing the sensorgrams of mutant enzymes with those of wt enzyme led to the characterization of different types of mutants (Figure 2). For some mutants (such as E314G and N319Y) the impaired activity could be explained by the lowered or disappeared capability to bind to the acceptor. However, this was not always the case. Mutants K297E and Y281H for example, have no residual activity and lowered residual activity, respectively, but bind asialofetuin as efficiently as wt enzyme. In the former, this binding is not affected by the presence of CMP-NeuAc as with the wt enzyme. This clearly indicates that the mutation inactivates the catalytic center of the molecule. This is the first time that such a mutant is shown. For some other mutants, such as I328N and F401L, the lowered activity was associated with higher binding properties. Probably the improved binding has a negative influence on the catalytic activity. Two mutants in which a tyrosine was changed to an asparagine (Y355N and Y365N) had almost equal binding characteristics in the absence of NaCl. However, this binding is more resistant to higher salt concentrations. Both mutants have impaired activity. Without a three-dimensional structure, a conclusive explanation for this phenomenon is difficult to give.

All characterized mutations were randomly distributed along the mutated region. Only some of them were located in the small or the very small sialyl motif. Four mutants were evaluated in the small motif (M325I, I328N, E342G, F343S). All of them had lowered sialyltransferase activity and had changed binding characteristics to acceptor substrate. This is in accordance with Paulson et al. (1977), who link this motif to acceptor binding; they also mention the mutation of the phenylalanine residue on position 343, viz. in rat ST6GalI at position 340 (Datta et al., 1998). However, they could not analyze it because of the low residual activity. We now can demonstrate that this mutant has impaired acceptor binding.

In addition, mutants outside these motifs were analyzed. All types as mentioned above were shown. This demonstrates that neither for acceptor binding nor for catalytic activity are the sialyl motifs sufficient. For the former, this is not surprising because different sialyltransferase types use different acceptor molecules.

These results clearly demonstrate the benefits of using BIAcore technology in the analysis of sialyltransferase mutants. It has hereby become possible to study the binding of mutants with the wild type acceptor without needing any residual enzymatic activity. This is a major advantage as compared to the use of classical studies that calculate $K_\text{M}$ values. Not only a change in affinity for the acceptor could be demonstrated by this method, but also other parameters, such as catalytic activity, could be deduced. Further improvement of the technique applied could even lead to a more detailed kinetic analysis of wt or mutant sialyltransferase. Therefore, more purified enzyme would be needed. Also, the use of the E-tag to immobilize fixed amounts of enzyme could be useful. This technique may also be of use to evaluate other sugar-transferring enzymes and will especially be valuable for enzymes for which three-dimensional structural data are or will become available.

### Materials and methods

#### Construction of mutants

The cDNA of human ST6GalI (E.C. 2.4.99.1) was picked up from a HepG2 cDNA library (LMBP collection Accession No. LIB5 at <http://www.belspo.be/bccm>) using the radio-labeled antisense probe 5'-GGTTGTTGCTGAATCATAT-GAGA-3'. Sequence analysis demonstrated that the sequence was exactly that as previously described (Grundmann et al., 1990). The cytoplasmic tail, the transmembrane domain, and part of the stem region (up to Leu54) were replaced with the interferon-γ signal for secretion, mainly as described previously (Colley et al., 1989). The stop codon was mutated and an FspI recognition site was introduced, allowing attachment of an E-tag (pCANTAB 5E; Pharmacia Biotech, Uppsala, Sweden). This construction was cloned in the vector pCAGGS (Niwa et al., 1991) for expression in HEK293 cells.

Random mutagenesis was achieved using error-prone PCR with the expression plasmid as a template. This method was adapted from a previously described protocol (Leung et al., 1989). The N-terminal primer 5'-GCTGTTACTGCCCAG-GACCAG-3' hybridizes in the coding sequence of the interferon-γ secretion signal, the C-terminal primer 5'-GAAC-AGTCTATGCGGCACGC-3' partially in the coding sequence of the E-tag and partially in the 3'-untranslated region. 2.5 units of Taq polymerase were used in a PCR reaction performed in 20 mM Tris-HCl pH 8.4, 50 mM NaCl and 7 mM MgCl₂, and 30 pmol of each primer. Addition of 0.2 mM MnCl₂ increased the mutation frequency of the polymerase. The latter was determined as the concentration at which 0–1 point mutations per 100 nucleotides are introduced. The reaction conditions were: 94°C for 1 min, 45°C for 1 min, and 72°C for 1 min (30 cycles). The amplified product was purified using agaro gel electrophoresis and QIAquick (Qiagen, Chatsworth, CA). In the original expression vector, the coding sequence between a unique KpnI restriction site (at nucleotide 809 of the original clone) and a unique MunI restriction site in the E-tag (total length of 420 nucleotides) was interchanged with a mutant PCR fragment, prepared after restriction digestion with the same enzymes. Transformation of competent E. coli cells with the ligation mixture allowed the isolation of each mutant separately and their amplification. Purification was achieved as previously described (Birnboim and Doly, 1979) or with Qiagen tips (Qiagen). The mutations were characterized by dideoxy double-stranded sequencing (Sanger et al., 1977). The upstream primer was 5'-CCTAATTGTATGGGACCCA-TCTG-3' just preceding the KpnI restriction site. The downstream primer was the same as the one used for error-prone PCR.
Expression of wt- and mutant-soluble ST6GalI

Expression of wt-soluble ST6GalI and separate mutants was obtained after transient transfection of HEK293 cells, using calcium phosphate precipitation (Chen and Okayama, 1987). For mass screening of sialyltransferase mutants, 10,000 cells were transfected in 96-well microplates using 0.1 µg of purified DNA. For surface plasmon resonance experiments, 10 µg of purified DNA was used to transfect 1.5 million cells in medium flasks. After washing the transfected cells, expression was allowed for 48 h in Dulbecco’s minimal essential medium enriched with insulin, transferrin, and selenium. After harvesting, expression was allowed for another 72 h in fresh medium. Both were combined for further experiments.

Analysis of the residual specific activity of ST6GalII mutants

Transfection medium from 96-well microplates was checked for protein expression using dot-blot immunodetection. After immobilization of the proteins on a nitrocellulose membrane (Schleicher & Schuell, Dassel, FRG), incubation with anti-E-Tag antibody (Pharmacia Biotech) as a primary antibody and with peroxidase-conjugated anti-mouse IgG (Sigma Chemical Co., St. Louis, MO) as a secondary antibody allowed luminescent detection with Renaissance luminescence reagent (Du Pont, Wilmington, DE). Quantification was possible using Lum-Imer and its software (Roche Molecular Biochemicals, Basel, Switzerland). The activity of the expressed sialyltransferases was analyzed as described previously (Laroy et al., 1997). Briefly, asialofetuin is used as an acceptor for radiolabeled sialic acid. After precipitation of the reaction product on glass fiber filters present in 96-well filtration plates, excess donor is washed away. The amount of radioactivity left on the filter is a measure for sialyltransferase activity. Both parameters permit us to draw conclusions on the specific activity of the mutants and their comparison with wt enzyme.

Partial purification of expression medium

Transfection medium was concentrated tenfold using micro-concentrators (MWCO 10; Vivascience, Westford, MA). Concentrated medium (500 µl) was supplemented with an equal volume of buffer A (10 mM MES, pH 7.2, 25% glycerol) and applied on a 1-ml glycosyltransferase affinity gel-CDP (Calbiochem-Novabiochem International, San Diego, CA), pre-equilibrated with buffer A. After washing the column with 15 ml buffer A, elution was started using 15 ml buffer B (10 mM MES, pH 7.2, 1 M NaCl, 25% glycerol). All steps were done at a flow rate of 0.2 ml/min. Fractions of 1.5 ml were collected and checked for the presence of E-tag using the manufacturer’s recommendations. Just before immobilization, eluted fractions containing the E-tag were combined. Glycerol was removed, and buffer was brought to 10 mM sodium acetate pH 5 using micro-concentrators (MWCO 10; Vivascience). Dot-blot immunodetection of the E-tag was used to measure the enzyme concentration. Equal amounts of E-tagged protein (i.e., wt or mutated enzyme) were applied to the activated sensor chip. All binding experiments were performed with asialofetuin (500–1000 µg/ml) in 100 mM HEPES, pH 7.2, except where differently mentioned. Per injection, 20 µl was used. Dissociation occurred in 100 mM HEPES, pH 7.2, or in the same buffer with 150 mM NaCl. The flow rate was 10 µl/min. Dissociation experiments were carried out with 2 mM CMP-NeuAc (Sigma Chemical Co.) in 100 mM HEPES pH 7.2. Regeneration of the sensor chip was possible with 10 µl 1 M NaCl.

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Abbreviations

CMP-NeuAc, cytidine 5'-monophospho-N-acetylneuraminic acid; CDP, cytidine 5'-diphosphate; CMP, cytidine 5'-monophosphate; PCR, polymerase chain reaction; wt, wild-type.

References


Analysis of sialyltransferase mutants


Tsui, S. (1999) Molecular cloning and characterization of mouse sialyltrans-


