An α2,3-sialyltransferase produced by *Photobacterium phosphoreum* JT-ISH-467 is a bi-functional enzyme showing both α2,3-sialyltransferase and α2,3-linkage specific sialidase activity. To date, the crystal structures of several sialyltransferases have been solved, but the roles of amino acid residues around the catalytic site have not been completely clarified. Hence we performed a mutational study using α2,3-sialyltransferase cloned from *P. phosphoreum* JT-ISH-467 as a model enzyme to study the role of the amino acid residues around the substrate-binding site. It was found that a mutation of the glutamic acid at position 342 in the sialyltransferase resulted in a loss of sialidase activity, although the mutant showed no decrease in sialyltransferase activity. Based on this result, it is strongly expected that the Glu342 of the enzyme is an important amino acid residue for sialidase activity.

**Key words:** bi-functional sialyltransferase; *Photobacterium phosphoreum*; sialidase; α2,3-sialyltransferase; site-directed mutagenesis

Sialic acids are important terminating components of carbohydrate chains in glycoconjugates.1,2 Sialylated carbohydrate chains play important roles in numerous biological events, including the immune response and cell-cell recognition. N-Acetylneuraminic acid (Neu5Ac) is the major sialic acid component of glycoconjugates,3,4 usually four main linkage patterns: Neu5Ac-α(2,6)-linked galactose, Neu5Ac-α(2,3)-linked galactose, Neu5Ac-α(2,6)-linked N-acetylgalactosamine, and Neu5Ac-α(2,8)-linked Neu5Ac.5 These linkage patterns are formed by specific sialyltransferases.5,6 Many sialyltransferases have been cloned from various sources, including mammalian organs, bacteria, and viruses. The sialyltransferases use a common donor substrate, CMP-Neu5Ac.

Sialyltransferases are classified into four families according to the carbohydrate linkages they comprise: β-galactoside α2,3-sialyltransferases (ST3Gal) I–V, β-galactoside α2,6-sialyltransferases (ST6Gal) I, GalNAc α2,6-sialyltransferases (ST6GalNAc) I–IV, and α2,8-sialyltransferases (ST8Sia) I–V.7 They have also been classified into five families based on amino acid sequence similarities in the CAZy database.7 All bacterial sialyltransferases are distributed into four families: (i) glycosyltransferase family (GT) 38, (ii) GT 42, (iii) GT 52, and (iv) GT 80. The genes that encode the sialyltransferases have been cloned from various bacteria, including *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Campylobacter jejuni*, *Escherichia coli*, *Photobacterium damselae*, *Photobacterium phosphoreum*, *Photobacterium leiognathi*, *Vibrio sp.*, *Pasteurella multocida*, *Haemophilus influenzae*, and *Streptococcus agalactiae*.8 Among these, some sialyltransferases are multi-functional enzymes. For example, α2,6-sialyltransferase derived from *P. damselae* has both sialyltransferase and sialidase activities.

Sialidases are also known as neuraminidases (EC 3.2.1.18). They catalyze the cleavage of sialic acid linkages on a variety of glycoconjugates. Sialidases are found in various resources, including humans, other animals, fungi, bacteria, viruses, and other organisms, and are classified into two major classes.21–23 One is the exo-sialidase type that cleaves α2,6-, α2,3-, and α2,8-linkages of terminal sialic acid residues, and the other type is endo-sialidase, which cleaves the α2,8-linkages within a sialic acid polymer. These enzymes are classified into three glycoside hydrolase families (GHs), GH33 (sialidases from most bacteria and mammals), GH34 (sialidases from influenza viruses A and B), and GH58 (endo-sialidases from *E. coli* K1 and bacteriophages).

It is known that sialidases play important roles in influenza infection.24,25 In addition, they are known to play important roles in decreasing the blood-stream half-life of therapeutic glycoproteins such as erythropoietin.26 To date, several crystal structures of bacterial sialyltransferases have been solved, specifically, the structures...
of α2,3-sialyltransferase (CstI) and α2,3-/α2,8-sialyltransferase (CstII) cloned from C. jejuni,27,28 multi-functional α2,3-sialyltransferase cloned from P. multocida (Δ24PmST1),29,30 α2,6-sialyltransferase cloned from Photobacterium sp. (Δ16pspST6),31 and α2,3-sialyltransferase cloned from P. phosphoreum,32 a NH2-terminal truncated form of which was also reported. Based on these studies, substrate recognition and catalytic mechanisms have been proposed for these enzymes, but to date the roles of the amino acid residues around the catalytic sites of these bacterial sialyltransferases have not been comprehensively studied.

Here we report on a mutational study using 22 amino acids of the NH2-terminal truncated form of bi-functional α2,3-sialyltransferase, cloned from P. phosphoreum JT-ISH-467 (ΔNpp23ST), for further understanding of amino acids related to the activity of both sialyltransferase and sialidase.

Materials and Methods

Materials. CMP-Neu5Ac was purchased from the Japan Food and Alcohol Company (Kotoku, Japan). Lactose was from Wako Pure Chemical Industries (Osaka, Japan). 3′-Sialylactose was synthesized enzymatically using marine bacterial α2,3-sialyltransferase, and purified by a method described by Mine et al.33 All other reagents were of commercially available analytical grade.

Site-directed mutagenesis. Point mutations were introduced into the gene that encodes ΔNpp23ST by polymerase chain reaction with Pyrobest DNA polymerase (Takara Biochemicals, Shiga, Japan) by a method described by Katakta et al.34 The primers used in this study are listed in Table 1. The resulting DNA fragments were inserted between the NcoI and BamHI sites of the pTrc99A vector (Amersham Bioscience, Buckinghamshire, UK). For His-tagged mutants, a His-tag coding sequence was added to the C-terminal ends of the protein sequences.

Expression and purification of the enzymes. Expression of mutant enzymes and ΔNpp23ST was achieved as previously described.35 In brief, expression vectors were introduced into E. coli strain TBI and grown at 30°C in Luria Bertani broth containing 100 μg/mL of tetracycline to an absorbance of 0.4 at 600 nm. Then 1 mM isopropyl-1-thio-β-D-galactopyranoside was added to the culture, which was shaken for a further 4 h. After induction, the cultured cells were harvested by centrifugation and suspended in 20 mM Bis-Tris buffer (pH 6.0) containing 0.3% (v/v) Triton X-100, and disrupted by sonication. The supernatant fractions obtained by centrifugation at 100,000 × g for 60 min were used as crude enzyme solutions. From these crude enzyme solutions, two mutant enzymes (ΔNpp23ST-E342A and ΔNpp23ST-E342A) and ΔNpp23ST were purified by a method described by Tsukamoto et al.13 The His-tagged enzymes were purified with Ni-NTA agarose (Qiagen, Tokyo) following its protocol.

Standard sialyltransferase assay. The reaction mixture (30 μL) consisted of an enzyme sample, 120 mM lactose, 2.3 mM CMP-Neu5Ac (Nakalai Tesque, Kyoto, Japan), 436 Bq CMP-[4,5,6,7,8,9-3H]-Neu5Ac (GE Healthcare UK, Buckinghamshire, UK), 100 mM cacodylate buffer (pH 6.0), and 0.5 mM NaCl. The reaction was carried out at 30°C for 5 min in duplicate. It was stopped by adding 2 mL of 5 mM potassium phosphate buffer (pH 6.8), and the reaction mixture was applied to a column of AG1-X2 resin (200–400 mesh, Bio-Rad) immediately. The eluate (2 mL) was collected and added to the scintillation cocktail. The radioactivity of the mixture was measured directly with a liquid scintillation counter (model TR 1900, Packard, MA). One unit (U) of sialyltransferase activity was defined as the amount of enzyme that transfers 1 μmol of Neu5Ac per min to lactose at pH 6.0 at 30°C.

Sialidase assay. The sialidase activity of the mutant enzymes and ΔNpp23ST was measured by two HPLC methods: with pyridylaminated 3′-sialylactose (3′-sialylactose PA), and with 3′-sialylactose. In the HPLC method with 3′-sialylactose PA, the enzymatic reaction mixture (15 μL) consisted of a sample of the enzyme, 1.7 μM 3′-sialylactose PA, 100 mM cacodylate buffer (pH 6.0), and 0.5 mM NaCl. Enzyme reactions were conducted for 17.5 h at 30°C, and then stopped by heat treatment (100°C, 5 min). After the reaction, the reaction mixtures were analyzed by HPLC as previously described.34 In the HPLC method with 3′-sialylactose, the enzyme reaction mixture (150 μL) consisted of a sample of the enzyme, 100 mM 3′-sialylactose, 100 mM Bis-Tris buffer (pH 6.0), and 0.5 mM NaCl at 30°C. The reactions were stopped by heat treatment (100°C, 5 min). Then the reaction mixtures were immediately analyzed by HPLC, as previously described.35

Table 1. Primers Used in This Study

<table>
<thead>
<tr>
<th>Name of primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Universal primer</td>
<td>5′-GGCGTTGATCCTGGACTCTAAGCACAATAACTCAG-3′</td>
</tr>
<tr>
<td>467-23ST-N2-Nco</td>
<td>5′-CTTAGAATGGATCCCTTGCAATACATTATCACC-3′</td>
</tr>
<tr>
<td>467-23ST-C0-Bm</td>
<td>5′-GGGCGATCCTTAGTGAAGTTCAGCTGAGCAGC-3′</td>
</tr>
<tr>
<td>ISH467-23ST-C0-His-BamHI:</td>
<td>5′-GGGCGATCCTTAGTGAAGTTCAGCTGAGCAGC-3′</td>
</tr>
</tbody>
</table>

Mutant primer

| ISH467-23ST-F341A-F | 5′-AAATTTTATTAATAGATACCCACGGTGAACCTCCTAATAATGCTAGTACGC-3′ |
| ISH467-23ST-F341A-R | 5′-GCATCGTACATATTAGAGCGCTTCTGTTCTTCTTATATATTTT-3′ |
| ISH467-23ST-E342A-F | 5′-AAATTTTATTAATAGATACCCACGGTGAACCTCCTAATAATGCTAGTACGC-3′ |
| ISH467-23ST-E342A-R | 5′-GCATCGTACATATTAGAGCGCTTCTGTTCTTCTTATATATTTT-3′ |
| ISH467-23ST-F341AE342A-F | 5′-AAATTTTATTAATAGATACCCACGGTGAACCTCCTAATAATGCTAGTACGC-3′ |
| ISH467-23ST-F341AE342A-R | 5′-GCATCGTACATATTAGAGCGCTTCTGTTCTTCTTATATATTTT-3′ |
| ISH467-23ST-E342A-F | 5′-AAATTTTATTAATAGATACCCACGGTGAACCTCCTAATAATGCTAGTACGC-3′ |
| ISH467-23ST-E342A-R | 5′-TGGTGACCGAACTTAAAATCCAGCCTTCCCTTCCACCGACAC-3′ |

Mutated codons are underlined and restriction sites are double-underlined.
To assess the CMP requirement for sialidase activity, CMP was added to the sialidase assay mixture and the activity was evaluated by HPLC with 3'-sialyllactose PA at 10 equivalents to the enzyme protein concentration.

Protein concentration determination. The protein concentration was determined with Coomassie Protein Assay Reagent (Pierce Chemical, Rockford, IL), with bovine serum albumin as standard.

Temperature and pH dependence of sialyltransferase activity. The pH profiles for the sialyltransferase activity of the mutant enzymes and ΔNpp23ST were determined by the standard sialyltransferase assay described above, except that the 100 mM cacodylate buffer (pH 6.0) was replaced with 100 mM 2-morpholinoethanesulfonic acid (MES) buffer (pH 5.0, 5.5, or 6.0), 100 mM Bis-Tris buffer (pH 6.0, 6.5, or 7.0), 100 mM phosphate buffer (pH 7.0, 7.5, or 8.0), 100 mM N-Tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS) buffer (pH 8.0, 8.5, or 9.0), 100 mM 2-N-cyclohexylamino ethanesulfonic acid (CHES) buffer (pH 9.0, 9.5, or 10.0), or 100 mM 3-(cyclohexylamino)-1-propane sulfonic acid (CAPS) buffer (pH 10.0, 10.5, or 11.0). Temperature profiles were evaluated from 10°C to 50°C by standard sialyltransferase assay. All assays were performed in triplicate.

Kinetic studies of mutant α2,3-sialyltransferases. To determine the kinetic parameters for CMP-Neu5Ac and lactose, sialyltransferase activities were measured using purified enzymes under various conditions. The kinetic parameters for CMP-Neu5Ac and lactose were calculated by Lineweaver–Burk plot.

Modeling of open conformation of sialyllactose complexes. Two molecular models of open conformation with sialyllactose were constructed using Coot and MOE. All protein atoms were fixed during minimization. Molecular models of open conformation with sialyllactose were placed into the open conformation of Δ24PmST1 based on superposition with the Δ24PmST1-CMP-lactose complex structure using Coot. Energy minimization was done using MOE. All protein atoms were fixed during minimization. Sialyllactose was placed into the open conformation of ΔNpp23ST based on superposition with a constructed model of the Δ24PmST1-sialyllactose complex structure using Coot. Energy minimization was performed similarly.

Results and Discussion

Sialidase activity of the mutant enzymes derived from ΔNpp23ST

In a previous study, the Phe341, Glu342, and Ser360 amino acid residues of ΔNpp23ST were found to be associated with the donor substrate. Hence we expressed several mutant enzymes that had mutations at the amino acid residues in the donor substrate-binding site of ΔNpp23ST to obtain more information. The sialidase activity of the mutant enzymes was first measured by the HPLC method with 3'-sialyllactose PA. When the sialidase activity of the sample was low, we applied more enzyme protein and reaction time was elongated to confirm activity. Remarkably, by this method, we confirmed that three mutant enzymes, ΔNpp23ST-E342A (substituted Phe at position 341 of Npp23ST for Ala) with a His-tag displayed sialidase activity weakly. To confirm the loss of sialidase activity in mutant enzymes more quantitatively, the HPLC method with 3'-sialyllactose PA was used. In this method, it is possible to increase the substrate concentration higher than the HPLC method with 3'-sialyllactose PA. No sialidase activity was detected for ΔNpp23ST-E342A with a His-tag or for ΔNpp23ST-F341A/E342A with a His-tag (Fig. 1) when the enzyme reaction time was extended to 72 h. The specific activity (sialidase activity) of each purified enzyme (described in “Materials and Methods”) is summarized in Table 2, right. This result confirms that the mutant enzymes lost sialidase activity. Based on these results, it was concluded that Glu342 and Ser360 of ΔNpp23ST are important for sialidase activity. With and without a His-tag, the enzymes showed almost the same specific activity as sialidase (data not shown).

<table>
<thead>
<tr>
<th>Specific activity</th>
<th>Sialyltransferase (mU/mg)</th>
<th>Sialidase (mU/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔNpp23ST1</td>
<td>2.7 × 10^3</td>
<td>2.4 × 10^3</td>
</tr>
<tr>
<td>ΔNpp23ST-F341A</td>
<td>4.2 × 10^3</td>
<td>3.1 × 10^3</td>
</tr>
<tr>
<td>ΔNpp23ST-E342A3</td>
<td>1.9 × 10^4</td>
<td>3.0 × 10^-2</td>
</tr>
<tr>
<td>ΔNpp23ST-F341A/E342A4</td>
<td>1.1 × 10^4</td>
<td>2.0 × 10^-2</td>
</tr>
<tr>
<td>ΔNpp23ST-S360A5</td>
<td>2.9 × 10^2</td>
<td>1.0 × 10^-1</td>
</tr>
</tbody>
</table>

1 S12 ng for sialidase assay, 7.5 μg and 10 min for sialidase assay; 2 322 ng for sialidase assay, 7.5 μg and 30 min for sialidase assay; 3 60 ng for sialidase assay, 8.8 μg and 86.5 h for sialidase assay; 4 72 ng for sialidase assay, 22.3 μg and 52 h for sialidase assay; 1 1.15 ng for sialidase assay, 2.3 μg for 52 h for sialidase assay.
Sialyltransferase activities of mutant enzymes derived from \( \Delta Npp23ST \)

We proceeded to measure the sialyltransferase activities of these mutant enzymes. It was found that mutant enzymes \( \Delta Npp23ST-F341A \) with a His-tag, \( \Delta Npp23ST-E342A \) with a His-tag, and \( \Delta Npp23ST-F341A/E342A \) with a His-tag had sialyltransferase activity. For mutant enzyme \( \Delta Npp23ST-S360A \), almost no sialyltransferase activity was observed. The specific activity for sialyltransferase activity of each of the purified enzymes is summarized in Table 2, left. Based on these results, it is probable that Ser360 of \( \Delta Npp23ST \) is important for both sialyltransferase and sialidase activity. Furthermore, the \( \Delta Npp23ST-E342A \) and \( \Delta Npp23ST-F341A/E342A \) mutants showed increased sialyltransferase activity. The specific activities of \( \Delta Npp23ST \) with and without the His-tag were almost the same (data not shown).

Recently, the reaction mechanism of the sialidase activity and a mutagenesis study to decrease the sialidase activity of \( \Delta 24PmST \), a member of GT80, like \( \Delta Npp23ST \), was reported. It was found by \(^1\)H NMR studies that the mechanism of the \( \omega \)-sialidase activity of \( \Delta 24PmST \) follows a retaining pattern. In addition, it was reported that D141 of \( \Delta 24PmST \) is a possible general acid/base for \( \omega \)-sialidase activity, and H311 is a possible nucleophile for \( \omega \)-sialidase activity, although D141 of \( \Delta 24PmST \) is a general base for \( \omega \)-sialyltransferase activity and H311 is a possible general acid for \( \omega \)-sialyltransferase activity. It has also been reported that increasing the hydrophobicity of the sialidase substrate binding pocket close to the underlying glycan but relatively far from the sialic acid portion by site-directed mutagenesis helped to decrease the \( \omega \)-sialidase activity of \( \Delta 24PmST \) dramatically without affecting \( \omega \)-sialyltransferase activity.

Molecular modeling of the open conformation of \( \Delta Npp23ST \) with sialylactose

In this study, we constructed two molecular models of open conformation with sialylactose. Since both \( \Delta 24PmST \) and \( \Delta Npp23ST \) distribute to GT80, we used \( \Delta 24PmST \) as the base conformation. In one model, sialylactose was placed into the open conformation of \( \Delta 24PmST \) based on superposition with the \( \Delta 24PmST \)-CMP-lactose complex structure using Coot, and in the other, sialylactose was placed into the open conformation of \( \Delta Npp23ST \) based on superposition with a constructed model of the \( \Delta 24PmST \)-sialylactose complex structure using Coot (Supplemental Figs. 1 and 2; see Biosci. Biotechnol. Biochem. Web site). As shown in supplemental Fig. 1, sialylactose is able to locate near E338 (corresponding to E342 of \( \Delta Npp23ST \)) and D141 (corresponding to D148 of \( \Delta Npp23ST \)) of \( \Delta 24PmST \). Based on this model, it is possible to hypothesize that E338 functions as a general base and that D141 functions as a general acid for \( \omega \)-sialidase activity. In the case of \( \Delta Npp23ST \), sialylactose is also able to locate near E342 and D148 in the model (Supplemental Fig. 2). Based on this model, it is also possible to hypothesize that E342 of \( \Delta Npp23ST \) functions as a general base and D148 of \( \Delta Npp23ST \) as a general acid for its sialidase activity.

In general, glycosidases can be divided into two groups by mode of action: the inverting glycosidase and the retaining glycosidase. Among these enzymes, carboxylate amino acids play important roles as catalytic residues. In the case of inverting glycosidase, it has been found that two carboxylate residues, glutamic acid and aspartic acid, act as catalytic acid and catalytic base respectively. In the case of the retaining enzymes, it has also been found that the two carboxylate residues act as catalytic acid/base and catalytic nucleophile respectively. With regard to the sialidase activity of the enzyme, the mode of action remains unknown, but it is expected that E342 and D148 act as catalytic base and catalytic acid and catalytic base, or as catalytic acid/base and catalytic nucleophile respectively. By this hypothesis, it is possible to explain the results of this study. However, further investigation is needed to fully understand the reaction mechanism of the sialidase activity of \( \Delta Npp23ST \).

CMP requirement for sialidase activity

It has been reported that CMP is not required for the \( \alpha \)-sialidase activity of \( \Delta 15Pd2,6ST \) but enhances the efficiency of the \( \alpha \)-sialidase activity of \( \Delta 15Pd2,6ST \) in a dose-dependent manner. \(^{38} \) Since \( \Delta 15Pd2,6ST \) is the GT80 enzyme and has both sialyltransferase and sialidase activities, like \( \Delta Npp23ST \), we confirmed the effect of CMP on the sialidase activity of \( \Delta Npp23ST-E342A \) with the His-tag and \( \Delta Npp23ST-F341A/E342A \) with the His-tag, but we saw no enhancement of the sialidase activity of \( \Delta Npp23ST-E342A \) or \( \Delta Npp23ST-F341A/E342A \) in the presence of CMP (data not shown).

Enzyme activities of mutant enzymes derived from \( \Delta Npp23ST \) by amino acid substitution at the 342 glutamic acid residue

To confirm the effect of the amino acid residue at position Glu-342 of \( \Delta Npp23ST \), four mutant enzymes substituted the following amino acids for Glu at position 342: \( \Delta Npp23ST-E342H \) (for His), \( \Delta Npp23ST-E342K \) (for Lys), \( \Delta Npp23ST-E342S \) (for Ser), and \( \Delta Npp23ST-E342V \) (for Val) were produced and their sialyltransferase and sialidase activities were assessed using crude extracts prepared from corresponding transformed \( E. coli \) strain TB1. All four mutant enzymes showed sialyltransferase activity, but no sialidase activity (data not shown). The results of this qualitative analysis with crude enzymes indicate that the glutamic acid at this position has an important role in sialidase activity.

General properties of the sialyltransferase activities of \( \Delta Npp23ST-E342A \) and \( \Delta Npp23ST-F341A/E342A \)

We investigated the pH and temperature profile and kinetic parameters of the sialyltransferase activity of \( \Delta Npp23ST \), \( \Delta Npp23ST-E342A \), and \( \Delta Npp23ST-F341A/E342A \). In this case we used the enzymes without the His-tag because the presence of the His-tag showed no effects on sialyltransferase activities. The optimum pH and temperature for \( \Delta Npp23ST \) were pH 6.0 and 25°C (Fig. 2a and d). In contrast, the optimum pH and temperature \( \Delta Npp23ST-E342A \) and \( \Delta Npp23ST-F341A/E342A \) were pH 8.0 and 40°C (Fig. 2b and e) and pH 7.5 and 40°C (Fig. 2c and f).
respectively. These results indicate that the optimum pH and temperature for the mutant enzymes were shifted as compared to those the wild-type enzyme. The optimum pH ΔNpp23ST showed two peaks. The mechanism remains unknown.

Although we introduced mutations in amino acids thought to be involved in CMP-binding, the apparent $K_m$ values for CMP-Neu5Ac were similar to those ΔNpp23ST (Table 3). In contrast, the apparent $k_{cat}$ value ΔNpp23ST-E342A and for CMP-Neu5Ac was approximately 5 times higher than that ΔNpp23ST.

The apparent $K_m$ values ΔNpp23ST-E342A and ΔNpp23ST-F341A/E342A for lactose were approximately 3 times and 10 times higher respectively than that ΔNpp23ST. The apparent $k_{cat}$ value ΔNpp23ST-E342A for lactose was approximately 9 times higher than that ΔNpp23ST. The mechanisms of these changes of pH and temperature profiles and kinetic parameters in mutant enzymes remain unknown, and further investigation is required.

Some studies have reported modifications that altered the reaction specificity and/or efficiency of glycosyltransferases.39 For example, human 1,4-galactosyltransferase was converted to 1,4-N-acetyl-galactosaminylationtransferase by a single point mutation. 40 In addition, data have been obtained regarding the structure-activity relationships of sialyltransferases 41 but the roles of the amino acid residues surrounding the catalytic site have not been fully explored. In this study, we obtained information with regard to the roles of the amino acid located in the donor substrate-binding site of ΔNpp23ST. Although the roles of Glu342 and Ser360 and the reaction mechanism of this enzyme as to sialidase activity unknown, we confirmed that two amino acid residues of ΔNpp23ST, Glu342, an important amino acid for its sialidase activity, and Ser360, participated in both sialyltransferase and sialidase.

Fig. 2. Temperature and pH Dependence of the Sialyltransferase Activity of Mutant Enzymes and ΔNpp23ST.

For pH profile (left, a–c), sialyltransferase activity was determined at 30 °C over 1 or 2 min in 100 mM buffer at the indicated pH. The buffers used are described in “Materials and Methods.” Similarly, an enzyme assay for the temperature profile (right, d–f) was done at the indicated temperatures for 1 min at pH 6.0 (ΔNpp23ST) or pH 8.0 (mutant ΔNpp23STs). Activity is shown relative to the maximum value. ΔNpp23ST, a and d; ΔNpp23ST-E342A, b and e; ΔNpp23ST-F341A/E342A, c and f. Variations in relative sialyltransferase activity with (a–c), pH at 30 °C in 100 mM MES buffer (hollow squares), 100 mM Bis-Tris buffer (solid triangles), 100 mM phosphate buffer (diamonds), 100 mM TAPS buffer (solid circles), 100 mM CHES buffer (triangles), or 100 mM CAPS buffer (solid squares), and with (d–f), temperature in 100 mM Bis-Tris buffer.

Table 3. Apparent Kinetic Parameters of ΔNpp23ST and the Mutant Enzymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}$ (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔNpp23ST</td>
<td>8.4</td>
<td>11.2</td>
</tr>
<tr>
<td>ΔNpp23ST-E342A</td>
<td>22</td>
<td>99.7</td>
</tr>
<tr>
<td>ΔNpp23ST-F341A/E342A</td>
<td>85</td>
<td>10.8</td>
</tr>
<tr>
<td>CMP-Neu5Ac</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔNpp23ST</td>
<td>0.72</td>
<td>21.0</td>
</tr>
<tr>
<td>ΔNpp23ST-E342A</td>
<td>0.53</td>
<td>106.3</td>
</tr>
<tr>
<td>ΔNpp23ST-F341A/E342A</td>
<td>1.59</td>
<td>13.3</td>
</tr>
</tbody>
</table>

1 Enzyme reaction was performed at pH 6.0 at 25 °C.
2 Enzyme reaction was performed at pH 8.0 at 40 °C.
activities. In addition, we found that the optimum pH the mutant enzymes was significantly shifted, a feature that should improve the efficiency of the enzyme reaction due to the instability of CMP-Neu5Ac under acidic conditions. Moreover, the data showed an increased apparent $k_{cat}$ value for Npp23ST-E342A. Considering these results, these mutant enzymes, especially Npp23ST-E342A, might provide powerful tools for biosynthetic sialylation in pharmaceutical or industrial carbohydrate synthesis.

The mutagenesis strategy for decreasing sialidase in multifunctional sialyltransferases is becoming better understood. For comprehensive understanding of the amino acids affecting the binding of substrates, further mutational investigation is indispensable.

Acknowledgments

We thank Professor Hiroyuki Wariishi, Kyusyu University, for valuable support.

References