A New Sialidase Mechanism

**BACTERIOPHAGE K1F ENDO-SIALIDASE IS AN INVERTING GLYCOSIDASE**

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Bacteriophages specific for *Escherichia coli* K1 express a tailspike protein that degrades the polysialic acid coat of *E. coli* K1 that is essential for bacteriophage infection. This enzyme is specific for polysialic acid and is a member of a family of endo-sialidases. This family is unusual because all other previously reported sialidases outside of this family are exo- or trans-sialidases. The recently determined structure of an endo-sialidase derived from bacteriophage K1F (endoNF) revealed an active site that lacks a number of the residues that are conserved in other sialidases, implying a new, endo-sialidase-specific catalytic mechanism. Using synthetic trifluoromethylumbelliferyl oligosialoside substrates, kinetic parameters for hydrolysis at a single cleavage site were determined. Measurement of *k*_cat/*K*_m* at a series of pH values revealed a dependence on a single protonated group of *pK*_a* 5. Mutation of a putative active site acidic residue, E581A, resulted in complete loss of sialidase activity. Direct ¹H NMR analysis of the hydrolysis of trifluoromethylumbelliferyl sialotrioside revealed that endoNF is an inverting sialidase. All other wild type sialidases previously reported are retaining glycosidases, implying a new mechanism of sialidase action specific to this family of endo-sialidases.

The important role played by sialylated glycoconjugates (1) in both homeostasis (2, 3) and disease states (4–7) has led to the extensive study of the enzymes responsible for the addition and removal of sialic acid. With the exception of the β-linked CMP donor sugar (8), naturally occurring sialic acid glycosides are found in the α-configuration, their syntheses being catalyzed by sialyltransferases. All of the sialyltransferases are thus inverting enzymes (9). The corresponding wild type sialidases (both exo- and trans-sialidases) that have been studied all share a very similar set of active site residues and cleave the terminal α-linked sialic acid residue by the same catalytic mechanism (10–13). This conserved active site includes a tyrosine acting as the nucleophilic catalyst, two aspartate and/or glutamate residues as the general acid/base, and a trio of arginines associated with the sialic acid carboxylate. Hydrolysis occurs via an acid/base-catalyzed double-displacement mechanism involving a covalent sialyl-enzyme intermediate, resulting in overall retention of configuration at the anomeric center.

A noteworthy exception to this mechanism comes from the sialidases of family GH-58, which are the only class of enzymes found to hydrolyze within a sialic acid polymer (14) (endo-sialidase) because all others reported cleave only the terminal sialic acid residue (exo-sialidase). The x-ray crystal structure of a member of this family, derived from an *Escherichia coli* K1 bacteriophage, was recently solved revealing a putative active site that is similar in geometry to those of exo-sialidases but missing the tyrosine nucleophilic catalyst, one of the two acid catalysts, and one of the three arginines (15). This stark contrast in active site composition implies a new, endo-sialidase-specific mode of action.

The natural substrate of these endo-sialidases is polysialic acid (PSA) (16). PSA has recently been implicated in modulating leukocyte immune responses (17) but is found predominantly in the mammalian brain on the neural cell adhesion molecule (18–21). The polysialylated structure of neural cell adhesion molecule has been implicated in a host of neurological roles including neural plasticity (22), neural cell-cell interaction (23, 24), and growth (25). PSA has also been found on some tumor cell types (26–30) and has been suggested as a marker in identifying tumor oncdevlopment (31, 32). Some bacterial pathogens have also been shown to express PSA on their cell surfaces (33–38), conferring tolerance by the host immune systems because of the presence of PSA on neural cell adhesion molecule in the brain (39). Polysialylation also confers viable passage across the blood brain barrier (40) and can lead to infection causing neonatal sepsis and meningitis, of which *E. coli* K1 is one of the main protagonists (41–43). The PSA capsular coat surrounding *E. coli* K1 protects the bacterium from degradations by the host immune system, but it also acts as an anchor point for bacteriophage infection, and a range of bacteriophages specific to this bacterium have been reported (44–47). These phage all contain an essential tailspike endo-sialidase (48–52), and this enzyme is required for attachment of the bacteriophage onto the bacterium and for subsequent degradation.

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³ The on-line version of this article (available at http://www.jbc.org) contains supplemental data, including ¹H NMR and MS data for Sia₂-TFMU to Sia₄-TFMU, the method for *pK*_a* determination, the Scatchard Plot analyses, and the method of measurement of rate of mutarotation of α-sialic acid by ¹H NMR spectroscopy.

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⁶ www.cazy.org/fam/GH58.html.
⁷ The abbreviations used are: PSA, polysialic acid; TFMU, α-linked trifluoromethylumbelliferyl glycoside; Siaₙ-TFMU, a sialic acid polymer of length *n* with α-linked TFMU aglycone; HPLC, high pressure liquid chromatography; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid.
of the PSA coat, allowing access to the bacterial cell surface (53). The unique architecture of the endo-sialidase, the lack of information on chemical mechanism, and its ability to manipulate the unusual PSA polymer, as well as its possible utility in therapeutics and tumor diagnosis makes this enzyme an attractive candidate for further study. In this paper we describe the development of substrates for the convenient assay of endo-sialidases and use these reagents to establish that the endo-sialidase follows a completely different chemical mechanism to all other sialidases, resulting in inversion of anomeric configuration.

**EXPERIMENTAL PROCEDURES**

**Materials**—All of the chemicals were of analytical grade purchased from Sigma-Aldrich, unless otherwise stated. Trifluoromethylumbelliferyl α-sialoside was prepared as previously described (54). Sialyltransferase from *Campylobacter jejuni* (Cst-II) (55) and trans-sialidase from *Trypanosoma cruzi* (56) were expressed and purified as previously described. CMP-N-acetyl neuraminic acid was a kind gift from Neose Technologies Inc. All DNA isolations, restriction enzyme digestions, ligations, and transformations were performed as recommended by the supplier. All other enzymes were obtained from New England Biolabs and Sigma-Aldrich.

**Synthesis of Sialic Acid Oligomers**—A buffer (50 mM HEPES, pH 7.5) solution (422 μl total) of trifluoromethylumbelliferyl α-sialoside (10.8 mg,21.1 μmol) containing manganese chloride (10 mM) was incubated at room temperature with CMP-N-acetyl neuraminic acid (67 mg, 105 μmol) in the presence of Cst-II (2.8 mg/ml, 200 μl) and alkaline phosphatase (130 units/μl, 2 μl) for 2 h. After centrifugation (10,000 × g, 2 min), the supernatant was filtered (0.44-μm filter; Millipore), applied to a Biogel-P4 size exclusion column (25 mm × 21.2 mm column, Waters 600 multi-solvent delivery system, and Waters 2487 multi-channel UV-detection system). Oligosaccharides from Sia5-TFMU to Sia3-TFMU were identified by UV (280 nm) and TLC analysis (ethyl acetate/methanol/water/acetic acid, 4:2:1:0.1 ratio mobile phase), were eluted with water (7 ml/h). Product-containing fractions were identified by UV (280 nm) and TLC analysis were pooled and lyophilized.

**Expression of Cst-II**—Genomic DNA was isolated from *E. coli* strain K12 (CGSC 7297) by electroporation. The unique architecture of the Cst-II protein corresponds to amino acids 246–1065 of the full-length protein. Primers for the endo-sialidase were as follows: 5′-GGCGGACATA-TGGCTAAAAGGGATGTTCTACGT (external forward), 5′-GGCGGATGTCAGCACTTCTCTTGGTCAAGGCA-GAAATGC (external reverse), 5′-CCAATTAAAACCATGGGACTTATGGGATAACTCGG (internal reverse), and 5′-CGCTGAGTTATCTCCATAATGGTCATGGTTAAAATGG (internal forward). PCR was performed using Phusion polymerase and the program: 94°C for 5 min, 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 60 s, and finally 72°C for 10 min. DNA was purified using phenol-Tris extraction and ethanol precipitation. Genes digested with NdeI and Sall were ligated into pCWmalE-thrombin (58) and then used to transform *E. coli* AD202 (CGSC 7297) by electroporation. The endo-sialidase E581A mutant was made using the same pull-through PCR method described above using the following primers: 5′-GAGATATGACCCAGATGCGTCAGCGCCGTGCATCAAGTACTATG-3′ (forward) and 5′-CATAGTACTTGTAGCAGC-GGGGTACGCACCACCGGTTCATGTTACATC-3′ (reverse).

**Expression and Purification of MalE-EndoNF**—Recombinant *E. coli* strains were grown in LB broth containing ampicillin (150 μg/ml) at 37°C for 2 h. Gene expression was induced with 0.5 mM isopropyl-β-D-galactopyranoside and grown at 30°C for 24 h. The cells overexpressing MalE-EndoNF were resuspended in 20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 2 mM EDTA and lysed by French Press in the presence of a protease inhibitor mixture pellet (Roche Applied Science). The lysate was centrifuged at 27000 × g for 30 min at 4°C to remove cell debris. The supernatant was treated with DNPase (20 μg/ml) and RNase A (10 μg/ml) in 10 mM MgCl2 on ice for 30 min and then centrifuged at 100,000 × g for 60 min at 10°C. The supernatant was applied to amylose resin (New England Biolabs) equilibrated in 20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 2 mM EDTA. MalE-EndoNF was eluted with 10 mM maltose in 20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 2 mM EDTA, and the fractions were analyzed by SDS-PAGE. Fractions with significant amounts of MalE-EndoNF were pooled. Expression and purification of the endo-sialidase E581A mutant was carried out under identical conditions.

**TLC Assays**—A buffer (20 mM phthalate, 50 mM NaCl, pH 4.5) solution (20 μl total) of the oligomer (2 μl in reaction) was incubated with endoNF (2.6 mg/ml, 4 μl). Aliquots were taken over a period of 30 min and subjected to TLC analysis (regular phase silica with an ethyl acetate/methanol/water/acetic acid, 4:2:1:0.1 ratio mobile phase) along with TLC standards of each oligomer and a control carried out in the absence of enzyme.

**Kinetic Analysis by UV Absorbance**—Kinetic analyses were performed at pH 4.5 (20 mM sodium phosphate/citrate buffer, 50 mM NaCl), monitoring the release of the free coumarin at 380 nm (ε = 1580 M−1 cm−1) using a Varian Cary-4000 UV-visible spectrophotometer. The data were analyzed using GraFit software from Erithacus Software. Enzyme stability
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FIGURE 1. Synthesis of oligomers of sialic acid containing a trifluoromethylumbelliferyl aglycone. A range of oligomeric TFMU sialosides was synthesized by oligosialylation, using an oligosialyltransferase (Cst-II), of the chemically synthesized TFMU-sialoside acceptor. Five equivalents of the CMP-sialic acid donor were used to maximize the amount of trimeric substrate formed. The dimer, trimer, and tetramer (n = 1, 2, and 3, respectively) oligosaccharides were isolated by a combination of size exclusion chromatography and C18 reverse phase silica HPLC.

assays were conducted by preincubation of aliquots of EndoNF with the desired buffer (20 mM with 50 mM NaCl) for 30 min at 37 °C before being assayed (at pH 4.5) and compared against a standard incubated at pH 7.5 (20 mM Tris-HCl, 50 mM NaCl) for the same period. Measurements of k_{cat}/K_{m} values at a series of pH values were carried out by the substrate depletion method (59) using citrate/phosphate buffer (20 mM, pH 2.2–6.5, 50 mM NaCl) and phosphate (20 mM, pH 6.0–8.0, 50 mM NaCl).

Kinetic Analysis by Fluorescence Emission—Kinetic analyses were performed at pH 4.5 (20 mM sodium phosphate/citrate buffer, 50 mM NaCl), using a stopped assay. The reaction was initiated by the addition of endoNF (0.5 mg/ml, 5 μl) to a buffered solution (total volume, 25 μl) of Sia_{n}-TFMU substrate. Aliquots of the mixture (5 μl) were taken at 0, 30, 90, and 120 s and diluted into 50 mM CAPS pH 10.0 buffer (995 μl). The fluorescence of the released coumarin at each time point was measured (λ_{exc} = 385 nm, λ_{emit} = 502 nm) using a Varian Cary Eclipse fluorescence spectrophotometer and plotted to give an initial reaction rate at each concentration. The data were analyzed using Grafit software from Erithacus Software.

K_{d} Determination—Aliquots of a solution of Sia_{3}-TFMU (4.0 mM) were added to each of two fluorescence cuvettes: one containing mutant EndoNF (200 μl, 5 mg/ml) in 20 mM sodium phosphate/citrate, 50 mM NaCl pH 4.5 buffer (total volume, 800 μl) and the second containing only buffer. After the addition of each aliquot of substrate followed by thorough mixing and thermal equilibration to 25 °C, the difference in fluorescence (λ_{exc} = 328 nm, λ_{emit} = 495 nm) between the two cuvettes was measured using a Varian Cary Eclipse fluorescence spectrophotometer. After correcting for differences in volume and enzyme fluorescence, a plot of the difference in fluorescence versus concentration was analyzed using GraFit to determine the maximum difference in fluorescence. The data were then analyzed using a Scatchard plot (60) to determine K_{d} and the experiment was repeated twice to determine accuracy.

NMR Determination of Stereochemical Outcome—Sia_{3}-TFMU was dissolved in deuterated buffer (10 mM pthalate pH 4.5, 25 mM NaCl) to give a final substrate concentration of 1.3 mM. 1H NMR spectra were obtained on a Bruker Avance 400/inv spectrometer fitted with 5 mm BBI-Z probe. The data were collected at 298 K over 16 scans at 2-min intervals after the addition of a solution of endoNF in deuterated buffer (40 μl, 5.8 mg/ml in 20 mM Tris-HCl, pH 7.5). NMR experiments were performed using a water suppression protocol by irradiating with a low power (55 dB) continuous wave pulse centered at 4.7 ppm during the relaxation period (d1 = 2 s). Acquisition was performed following a delay (20 μs) after a 90-degree pulse (9.5 μs at 1 dB) 4 μs after the relaxation period. The data were analyzed using ACD software.

RESULTS

The mechanistic and kinetic study of endoNF required a suitable homogeneous substrate with a defined cleavage site that can be easily monitored. Excellent candidates for such substrates would be aryl-oligosaccharides that are cleaved exclusively at the aryl glycoside bond because liberation of the phenol(ate) can be monitored directly by UV-visible or fluorescence spectroscopy. This outcome can be favored if a phenol of relatively low pK_{a} is used, because not only is the rate of cleavage of the aryl glycoside likely to be greater than that of inter-sugar bonds, but also the phenol is likely to be released in a detectable phenolate form at ambient pH. Such a strategy has proved valuable with other endo-glycosidases such as amylases (61, 62) and cellulases (63). In this case a TFMU leaving group was chosen, because its sialoside is relatively stable to spontaneous hydrolysis, and cleavage can be monitored by both UV absorption and fluorescence emission spectroscopy. Previous studies indicated that the minimum substrate length required by endoNF is an oligosialic acid with a degree of polymerization of 5 (i.e. Sia_{8}) (53); thus a range of oligomeric TFMU sialosides was synthesized to find the optimal aryl oligosialoside for kinetic analysis. This was achieved by oligosialylation of a chemically synthesized TFMU sialoside acceptor using an oligosialyltransferase from C. jejuni (Cst-II), which catalyzes the formation of α-2,8-linked sialic acid oligomers (55). Use of five equivalents of the donor CMP-sialic acid (Fig. 1) led to the desired range of oligomers. The oligomeric mixture was separated by size exclusion chromatography, followed by C18 reverse phase silica HPLC.

The degradation of each of the synthetic oligomers by endoNF was monitored by TLC analysis, revealing that endoNF does not hydrolyze the monomeric (Sia-TFMU) or dimeric (Sia_{2}-TFMU) sialosides significantly over a 2-h period. However, the trimeric (Sia_{3}-TFMU) sialoside is degraded with exclusive cleavage at the coumarin-sialoside bond. The tetramer (Sia_{4}-TFMU) was also hydrolyzed, with formation of two TFMU-containing species, trifluoromethylumbelliferyl one and the TFMU sialoside monomer, indicating multiple cleavage sites (Fig. 2). The pentamer (Sia_{5}-TFMU) could not be purified to homogeneity and was not tested as a substrate. Based upon these data, it appears that endoNF has at least three subsites on the nonreducing side of the cleavage site, and the minimum substrate requirement is for the −1, −2, and −3 subsites (64) to all be occupied. This observation is consistent with the cleavage patterns of other endo-sialidases that have been reported using simple oligosialic acid substrates (53, 65, 66) and particularly with a very recent publication showing that the tetramer is the true minimum substrate (67). Our data also serve to confirm that the enzyme does not work via anexo-sialidase mode from the nonreducing terminus but rather via an endo-mode.
A mutant endo-sialidase bearing the E581A mutation was prepared because previous studies have indicated this is an important residue for catalysis (15). This enzyme behaved like wild type fusion protein in terms of expression level and formation of the catalytic homotrimer. The circular dichroism spectrum of the mutant was identical to the wild type, and melting experiments gave identical temperatures of denaturation, suggesting that the mutant is correctly folded and is as stable as the wild type enzyme. However, when this mutant was tested for sialidase activity using the sialotrioside substrate, no activity was observed, even at substrate concentrations exceeding 10-fold that of the measured $K_m^*$ of the wild type (0.7 mM) and at an enzyme concentration 20-fold (4.6 mg/ml) greater than that used with the wild type enzyme. Nor was any activity above spontaneous hydrolysis observed on the addition of a high concentration of anion (100 mM to 2 M azide, formate, thiolate, acetate) at a range of pH values (pH 5, 7, and 9), in contrast to the rescue of activity that has been observed with mutant retaining glycosidases in which nucleophile or acid/base residues have been replaced (68). To determine whether the absence of sialidase activity was simply caused by a lack of binding of the sialotrioside substrate, a binding constant was determined utilizing the difference in fluorescence between bound and unbound substrate. The data were analyzed using a Scatchard plot (60) (see supplemental information for data plots), yielding a $K_i$ of $10 \pm 2 \mu M$. This shows that the substrate indeed binds but is not degraded, thereby providing further evidence that the protein is correctly folded. This dissociation constant is an order of magnitude lower than the $K_m^{*}$ measured for the wild type enzyme. The tighter binding observed in the mutant may be due to removal of destabilizing Coulombic interactions between Glu581 and the substrate carboxylate group that likely exist in the wild type enzyme. The E581A mutant has no charged group at position 581; thus such electrostatic interactions will not be present, leading to stronger binding.

To determine the stereochemical outcome of the enzyme-catalyzed hydrolysis, the degradation of Sia$_3$-TFMU was monitored over time by $^1$H NMR spectroscopy. Because both axial and equatorial protons at C-3 of $\alpha$- and $\beta$-sialides display distinct chemical shifts (69), the stereochemical outcome can be readily determined (11, 70). In this case the peaks of interest are at 2.51 ppm, corresponding to the C-3 equatorial proton of the sialic acid residue at the reducing end of the substrate and 2.09 ppm corresponding to the C-3 equatorial proton of the $\beta$-linked hemi-ketal hydrolyzed product (Fig. 4). As the reaction progresses the substrate peak at 2.51 ppm decreases, the intensity of the peak at 2.09 ppm increases. A plot of the intensity of these peaks, as well as the sum of the integrals of both of these peaks, at the various time points is shown (Fig. 5). It is clear that these peak intensities are inversely correlated, indicating that one species is directly converting into the other, (calculated rates being 7.0 min$^{-1}$ for formation of $\beta$-product and $-7.5$ min$^{-1}$ for depletion of $\alpha$-substrate). Furthermore the sum of these peaks is constant, indicating that there are no other intermediates, i.e. the $\alpha$-substrate is converted directly to a $\beta$-product. EndoNF is therefore an inverting glycosidase.
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The minimum substrate length degraded by endoNF is the sialotrioside (Sia₃-TFMU) and this substrate is cleaved exclusively at the coumarin-sialoside bond. This indicates that at least three minus subsites (−1, −2, and −3) must be occupied by sialic acids for efficient catalysis. Hydrolysis of the tetrameric substrate (Sia₄-TFMU) by endoNF occurs at two points, indicating two binding modes. The major binding mode leads to the release of the free coumarin and involves four subsites (−4, −3, −2, and −1) being occupied by sialic acid, with the +1 site being occupied by the coumarin. The minor cleavage event results from the occupation of three minus sites (−1, −2, and −3), with the remaining sialic acid and coumarin occupying the +1 and +2 sites (Fig. 2). This requirement for the minimum occupation of three minus subsites is consistent with previous reports on degradation of oligosialic acids by endoNF (53) and by the endo-sialidase from bacteriophage K1A (65, 66, 71). However, these cleavage patterns are quite different from those observed in spontaneous decomposition, whereby the major cleavage mode of a tetramer of sialic acid produces two sialic acid dimers (72).

The measured kinetic parameters are similar to those reported for simple oligosialosides (66, 67), indicating that the coumarin modification does not significantly affect the binding of these modified oligosialosides to endoNF. The pH profile of endoNF shows that a single ionizable residue with a pKᵦ of ~5 in either the free enzyme or free substrate is essential for catalysis, suggesting a carboxylic acid. Of those residues identified in the putative active site that are required for catalysis (15), only Glu⁵₈₁ contains a side chain carboxylate. However, because the substrate itself contains a number of carboxylic acids, these could also potentially play a role in catalysis. To address this possibility a potentiometric titration of TFMU, sialotrioside was carried out yielding a pKᵦ range, for the sum of these carboxylic acids, of 3.6–3.8 (see supplemental information), consistent with previously determined values of sialic acid dimers and trimers (72). This value is higher than sialic acid itself (2.2–3.0) (73) but lower than that of PSA (3.9–5.5) (72). However, the measured pKᵦ value is substantially lower than that reflected in the pH dependence of k_cat/Kₘ, implying that the ionization of pKᵦ of 5 observed in the pH dependence belongs to an enzymic carboxylic acid, most likely Glu⁵₈₁.

To further study the role of this glutamic acid, a mutant endo-sialidase bearing the E581A mutation was prepared. This mutant was shown to be properly folded and capable of binding substrate but showed no sialidase activity toward the sialotrioside substrate under a range of conditions. This is not entirely unexpected because recent studies on other endo-sialidases have shown that other mutations around the putative active site can have a drastic effect on catalysis without affecting binding (48). The complete ablation of sialidase activity, however, is in apparent contrast to previous studies of the E581A mutant, in which a 5% residual sialidase activity was observed (15). These conflicting results may simply reflect the preference of endo-sialidases for polysialic acid substrates over shorter oligosialosides (53) but may also reflect the somewhat less precise assay used in that study.

The ¹H NMR studies show that an α-linked oligomeric substrate is hydrolyzed directly to a β-hemiketal product, without formation of intermediates. These observations are consistent with an inverting mechanism for endoNF, making the endo-sialidase mechanism unique because all other wild type sialidases that have been reported are retaining glycosidases. The possibility that very rapid mutarotation of the product was obscuring the outcome was discounted by measurement of slow (t₁/₂ = ~60 min) mutarotation of α-sialic acid under the same conditions (see supplemental information). α-Sialic acid was generated in situ by the action of the retaining T. cruzi sialidase (56), on trifluoromethylumbelliferyl α-sialoside and its conversion to the thermodynamically favored β-sialic acid was monitored by ¹H NMR. Indeed, to deal with this a number of pathogen employs a sialic acid mutarotase to increase the rate of the anomalously slow mutarotation of sialic acid (74). Although unlikely, one possibility that cannot be ruled out is...
that the rate of mutarotation of the sialic acid trimer is much greater than that observed for monomeric sialic acid.

The only previous reports of inverting sialidases are those describing mutant enzymes, wherein the catalytic tyrosine nucleophile of a retaining sialidase has been mutated to an alanine (70, 75, 76). Removing the nucleophilic catalyst resulted in a switch from a retaining mechanism in the wild type sialidase to an inverting mechanism in the mutant. An inverting mechanism for endoNF is thus consistent with the absence of a catalytic nucleophile in its active site.

It is known that polysialic acid is an unusual carbohydrate polymer in that it is relatively unstable (77–79) compared with other polysaccharides and that it forms helical structures in solution (80–84). This secondary structure could be responsible for this intrinsic instability by aligning the sialic acid carboxylate groups in a way to provide acid or base catalysis that promotes spontaneous hydrolysis of the polymer. It has also been shown that endoNF preferentially binds sialic acid oligomers in this helical form (85), and the enzyme could provide the second acid or base catalyst, normally required in a glycosidase mechanism, to further increase the rate of depolymerization. This is consistent with the observation that amino acid changes around the putative active site of endo-sialidases drastically change the catalytic efficiency, by locally distorting the polymer in a way that does not promote transition state formation but does not affect binding of the polymer, because there are multiple sugar-binding sites (48).

It has been speculated that polySia provides the acid catalyst and that the conserved Glu581 in endoNF is the base catalyst (15), based on $pK_a$ arguments (72) and the observed 25-fold lower sialidase activity measured in an E581A mutant. However, our study has shown that endoNF is catalytically competent toward a TFMU-sialtrioside with comparable kinetic parameters to those of the natural substrate. The $pK_a$ of the carboxylic acid of this substrate has been measured and is found to be significantly different from the $pK_a$ of the single ionization observed in the pH dependence of catalysis (3.7 versus 4.9, respectively). Thus the sialic acid carboxylate responsible for this ionization cannot be acting as the catalytic acid. An alternative role for the sialic acid carboxylate is that of the general base, and increased rates of glycoside hydrolysis have been attributed to such intramolecular base catalysis (86, 87). If a sialic acid carboxylate is the general base, then the general acid is most likely Glu581, because it is the closest acidic residue identified in the putative active site (Fig. 6). This mechanism would be consistent with the observations of low $k_{cat}/K_m$ values at high pH, where both sialic acid and Glu581 are deprotonated and with the $k_{cat}/K_m$ value increasing with decreasing pH as Glu581 becomes protonated at an approximate pH of 5, thus providing acid catalysis. The complete loss of all sialidase activity of the E581A mutant on the sialotrioside substrate supports the important role of this residue in catalysis, and the lack of anion rescue is consistent with a role as acid catalyst in an inverting mechanism (88).

CONCLUSIONS

A range of oligomeric sialosides containing a trifluoromethyl-lumbelliferyl aglycone have been synthesized by semi-synthetic methods. Of these substrates a sialoside trimer (Sia$_3$-TFMU) was found to be cleaved exclusively at the coumarin-sialoside bond by endoNF. By monitoring the release of the free coumarin of this substrate, endoNF was found to have a pH optimum beneath 4.5, the activity being dependent upon a titratable active site residue with a $pK_a$ of ~5, tentatively assigned to Glu581. The E581A mutant was prepared and found to be catalytically inactive toward the substrate but still able to bind substrate. This sialotrioside substrate was also used to show that endoNF is an inverting sialidase. To date EndoNF is the only inverting wild type sialidase to have been characterized.

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