Catalytic preference of *Salmonella typhimurium* LT2 sialidase for N-acetylneuraminic acid residues over N-glycolyneyraminic acid residues

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1. Introduction

Sialidases remove sialic acid from sialglycoconjugates and are expressed in many species such as bacteria, viruses, fungi, protozoa, invertebrates and mammals [1-3]. The *Salmonella typhimurium* LT2 sialidase (STSA) cleaves sialic acid residues of glycoproteins and gangliosides efficiently and has kinetic preference for sialyl α2-3 linkages over sialyl α2-6 linkages [4,5]. The catalytic mechanism of STSA for the high specificity toward sialyl α2-3 linkages has been estimated from high-resolution structure analysis by X-ray crystallography [6]. Due to this kinetic preference, STSA has been used for determination of sialyl α2-3 linkage [7] and for detailed investigations regarding the roles of sialyl α2-3-linked oligosaccharides [8].

Many molecular species of sialic acid have been identified, and the two most populous species existing in nature are N-acetylneuraminic acid (Neu5Ac) and N-glycolyneyraminic acid (Neu5Gc) [9-11]. Sialidase, such as those in viruses, bacteria and mammals, generally cleave Neu5Ac residues more preferentially than Neu5Gc residues [12,13]. However, the substrate specificity of STSA in molecular species of sialic acid is poorly understood.

In this study, we compared the enzyme activities toward Neu5Ac and Neu5Gc residues using fluorescent sialidase substrates [14], 4-methylumbelliferyl Neu5Gc (4MU-Neu5Gc) and 4-methylumbelliferyl Neu5Ac (4MU-Neu5Ac), in STSA and sialidase from *Macrobodella decora* (MDSA), *Clostridium perfringens* (CPSA), *Vibrio cholerae* (VCSA) and Arthrobacter urefaciens (AUSA). Since STSA hardly cleaved 4MU-Neu5Gc, cleaving ability of STSA for α2-3 linked Neu5Gc was also investigated using natural substrates such as ganglioside and sialylglycans in equine erythrocytes. We also investigated the catalytic mechanisms of STSA for selective cleavage to molecular species of sialic acid using the kinetic analysis and computer simulations.

2. Materials and methods

2.1. Reagents

The following products were obtained from the vendors or persons indicated: 4MU-Neu5Ac (Nacalai Tesque, Kyoto, Japan); STSA recombinant expressed in *Escherichia coli* (Takara Bio, Shiga, Japan); AUSA...
recombinant expressed in *E. coli* and MDSA recombinant expressed in *E. coli* (Calbiochem, San Diego, CA, USA); Neu5Acα2-3Galβ1-4Glcβ1-1-ceramide (Neu5Ac-GM2), CPSA and VCSA (Sigma–Aldrich, St. Louis, MO, USA); Neu5Gcα2-3Galβ1-4Glcβ1-1-ceramide (Neu5Gc-GM2). Dr. Y. Hirabayashi, Riken BSI, Saitama, Japan), 1,2-diamino-4,5-methylenedioxobenzene (DMB, Dojindo, Kumamoto, Japan), and 2,3-dehydro-2-deoxy-N-acetylneuramic acid (DANA, Dr. K. Ikeda, Hiroshima International University, Hiroshima, Japan).

### 2.2. Synthesis of 4MU-Neu5Gc

The synthetic scheme is shown in Fig. 1. For the synthesis of *N*-substituted sialoside [15], compound 2 [16] was acylated by Boc₂O and 4-dimethylaminopyridine in THF to give N,N-Boc,Ac analogue 3 of sialic acid in 74% yield. Selective N-O-deacetylation of 3 with sodium methoxide gave *N*-Boc derivative 4 in 78% yield, which was deprotected by CF₃CO₂H and subsequently submitted to N-acylation of the resulting free amino group with acetylglucosyl chloride and NEt₃ in MeOH to give the corresponding N-acetylglucosyl glycoside 5 in 69% yield in two steps. Finally, treatment of 5 with 0.1 M NaOH–MeOH (1:1) gave 4MU-Neu5Gc 1 in 49% yield.

### 2.3. Measurement of enzyme units

Enzyme units of AUSA and STSA, unless otherwise noted, were determined by incubation of these enzymes in 80 mM sodium acetate buffer (pH 6.0) containing 10 μM 4MU-Neu5Ac, 80 mM NaCl and 0.8 mg/ml BSA at 37 °C. One unit was defined as the amount of enzyme that catalyzed the release of 1 μmol of sialic acid for 1 min.

### 2.4. Comparison of the activities of sialidases from several species toward 4MU-Neu5Gc

4MU-Neu5Gc (0.4 mM) or 4MU-Neu5Ac (0.4 mM) was treated with sialidase (1 μl/ml), adjusted by calculation from commercially labeled enzyme activity) from STSA, MDSA, CPSA, VCSA and AUSA in 50 μl of 100 mM sodium acetate buffer (pH 4.8) containing 2.0 mM CaCl₂ using a 96-well black microplate (Corning, NY, USA) for 60 min at 37 °C. The reaction was terminated by addition of 250 μl of 100 mM sodium carbonate buffer (pH 10.7) in each well. Intensity of 4-methylumbellif erone (4MU)-specific fluorescence (ex/em, 355/460 nm) was measured using the multilabel counter Wallac 1420 ARVOx (PerkinElmer Life Sciences, Waltham, MA) or Infinite M200 (Tecan, Männedorf, Switzerland).

### 2.5. Determination of *Kₘ* and *kₐₕ*

4MU-Neu5Gc (3.13–4000 μM) and 4MU-Neu5Ac (3.13–4000 μM) were treated with 10 and 1 μM/ml STSA, respectively, and 4MU-Neu5Gc (3.1–400 μM) and 4MU-Neu5Ac (3.1–400 μM) were treated with 1 μM/ml AUSA in 50 μl of 80 mM sodium acetate buffer (pH 6.0) containing 80 mM NaCl and 0.8 mg/ml BSA for 60 min at 37 °C. Fluorescence intensities of 4MU were measured after termination of the enzyme reaction with 250 μl of 100 mM sodium carbonate (pH 10.7). *Kₘ* and *Vₘₐₓ* values were calculated using the Lineweaver–Burk plot.

### 2.6. Hydrolysis of ganglioside

Neu5Gc-GM₂ (0.2–2000 μM) or Neu5Ac-GM₂ (0.2–500 μM) was treated with 0.1 μM/ml AUSA or 0.1 μM/ml STSA in 50 μl of 100 mM sodium phosphate buffer (pH 6.0) containing 1.5 mM sodium cholate for 60 min at 37 °C. Then 200 μl of chloroform/methanol (2:1, v/v) was added to the reaction mixture and the mixture was shaken vigorously. After centrifugation at 3000 rpm for 5 min, the aqueous layer containing free sialic acid was collected [17].

### 2.7. Hydrolysis of sialylglycans in erythrocytes

Erythrocytes were prepared from equine blood (Kohjin Bio, Saitama, Japan) by repeated suspension and centrifugation at 2000 rpm for 5 min in phosphate buffered saline (PBS). The erythrocytes (0.5–50%, v/v) were incubated in PBS (100 μl, pH 6.0) containing 1 mM/ml AUSA or 1 mM/ml STSA for 180 min at 37 °C. After centrifugation at 2000 rpm for 5 min at 4 °C, the supernatant containing released sialic acid was collected.

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**Fig. 1.** Synthesis of 4MU-Neu5Gc. (a) Boc₂O, DMAP, THF, 60 °C, 3 h, 74%; (b) NaOMe, MeOH, 0 °C, 2 h, 78%; (c) 1. (1) TFA, CH₂Cl₂, rt, 1 h, (2) AcOCH₂CDCl₃, NEt₃, MeOH, 0 °C, 1 h, then rt, 12 h, 69% in two steps; and (d) 0.1 M NaOH, MeOH, 0 °C, 1 h, then rt, 12 h, 49%.

**Fig. 2.** Comparison of sialidase activities toward 4MU-Neu5Gc in several species. (A–E) Enzyme activities of STSA (A), MDSA (B), CPSA (C), VCSA (D) and AUSA (E) were measured with 4MU-Neu5Ac (Ac) and 4MU-Neu5Gc (Gc). Each sialidase (1 ml/l) was incubated with 0.4 mM substrate. Each bar and line represent the mean ± S.E.M. (n = 3). The asterisks indicate significant differences (***P < 0.001; **P < 0.01; *P < 0.05; t-test) from the activity measured with 4MU-Neu5Ac. (F) Enzyme activities toward 4MU-Neu5Gc were compared with those toward 4MU-Neu5Ac among sialidases from the species. N.D.: not detected.
2.8. Quantitative analysis of sialic acid

Fluorometric determination of Neu5Ac and Neu5Gc was performed by high-performance liquid chromatography (HPLC) as previously described [18]. For preclusion fluorescence derivatization, 7.0 mM DMB solution containing 1.0 M β-mercaptoethanol and 18 mM sodium hydrosulfite was added to 10 μl of free sialic acid-containing solutions, and incubated for 2.5 h at 60 °C. The mixtures of DMB-derivatized sialic acid were analyzed by HPLC [LC-2000Plus series, Jasco, Tokyo, Japan; C18 column, Tosoh, Tokyo, Japan; mobile phase, methanol/water (25:75, v/v), flow rate of 1.2 ml/min]. Fluorescence was monitored at excitation and emission wavelengths of 373 and 448 nm, respectively.

2.9. In silico analysis

The 3D structure of STSA for all sequence alignments was generated by homology modeling as a template of the X-ray crystal structure (PDB ID: 2SIM) using the Molecular Operating Environment (MOE) program package (MOE 2011.10, Chemical Computing Group, Montreal, QC, Canada) [19]. Docking simulations of Neu5Ac2en or Neu5Gc2en to the STSA structure were carried out by the MOE-Dock method [20]. The binding affinity was evaluated using the correlated fragment molecular orbital (FMO) calculations at the RI-MP2/cc-pVDZ level [21]. All FMO calculations were performed on 2.93 GHz Nehalem 8Core 7 CPUs (56CPUs) cluster system using the Parallelized ab initio Calculation System based on FMO (PAICS) program (available from http://www.paics.net) [22].

3. Results and discussion

3.1. Comparison of enzyme activities toward 4MU-Neu5Gc among sialidases from several species

The enzyme activities of STSA, MDSA, CPSA, VCSA and AUSA toward 4MU-Neu5Gc were measured and compared with those toward 4MU-Neu5Ac. A calcium ion is required for maximal activity in VCSA [23] but not in STSA [4], MDSA [24], CPSA [25] or AUSA [26]. The optimum pH values of STSA [4], MDSA [24], CPSA [27,28], VCSA [29] and AUSA [26,30] are in the acidic range. Therefore, enzyme activities of sialidase were measured in 0.4 mM 4MU-Neu5Gc and 0.4 mM 4MU-Neu5Ac in a pH 4.8 buffer solution containing 2.0 mM CaCl2 at 37 °C (Fig. 2). Although 4MU-Neu5Ac was cleaved efficiently with STSA, 4MU-Neu5Gc was not cleaved at all with STSA in this condition. In the case of MDSA, CPSA and VCSA, 4MU-Neu5Gc was cleaved with lower efficacy compared to 4MU-Neu5Ac. AUSA cleaved both 4MU-Neu5Gc and 4MU-Neu5Ac efficiently.

3.2. Kinetic parameters of STSA

It has been reported that Neu5Gc2-3Galβ1-4Glc-pyridylamine was cleaved with a high concentration of STSA [7]. Our preliminary data also showed that 4MU-Neu5Gc was hydrolyzed slightly with 10 μM/1 mM STSA. To measure the kinetic parameters of STSA and AUSA for the hydrolysis of 4MU-Neu5Gc and 4MU-Neu5Ac, both substrates were cleaved with STSA (1–10 μM/ml) and AUSA (1 μM/ml) in a pH 6.0 buffer solution at 37 °C (Fig. 3). The cleavage of 4MU-Neu5Gc with AUSA was inhibited with 300 μM DANA, indicating that 4MU-Neu5Gc measured sialidase activity specifically.

The K_M (mM) and k_cat/K_M (M⁻¹ s⁻¹) values were calculated using the Lineweaver−Burk plot (Table 1). The K_M values of STSA and AUSA for 4MU-Neu5Gc were 8.4-times and 2.5-times higher, respectively, than those for 4MU-Neu5Ac. The K_M value of STSA for 4MU-Neu5Ac in our measurement was 0.37 mM, which is close to the K_M value (0.25 mM) measured by Hoyer et al. under similar conditions [4]. The k_cat/K_M value of STSA for 4MU-Neu5Gc was 110-times lower than that for 4MU-Neu5Ac. On the other hand, the k_cat/K_M value of AUSA for 4MU-Neu5Gc was only two-times lower than that for 4MU-Neu5Ac. These results indicated that STSA had low affinity toward and weak ability for cleaving 4MU-Neu5Gc compared with 4MU-Neu5Ac.

3.3. Enzyme activity of STSA toward Neu5Gc-GM3

Since STSA and AUSA were reported to hydrolyze Neu5Ac-GM3 efficiently [4,31], we tested the sialidase activities of STSA and AUSA toward Neu5Gc-GM3, Neu5Gc-GM3 (0.2–2000 μM) and Neu5Ac-GM3 (0.2–500 μM) were treated with 0.1 μM/ml STSA and 0.1 μM/ml AUSA at 37 °C in a pH 6.0 buffer solution containing 1.5 mM sodium cholate. Neu5Gc-GM3 was hydrolyzed with AUSA but not with STSA even when the concentration of Neu5Gc-GM3 was high (Fig. 4A), while Neu5Ac-GM3 was efficiently hydrolyzed with both STSA and AUSA (Fig. 4B). The results suggested that STSA hardly cleaves Neu5Gc2-3Gal-linkage of GM3.

Additionally, AUSA cleaved Neu5Gc-GM3 (200 μM) and Neu5Ac-GM3 (200 μM) at the rates of 21.9 and 116 pmol/min, respectively. These results are consistent with results of previous studies showing that AUSA preferentially cleaves Neu5Ac-GM3 rather than Neu5Gc-GM3 [12].
Michaelis–Menten and kinetic constants for STSA and AUSA measured with 4MU-Neu5Gc and 4MU-Neu5Ac.

| Substrate | $K_m$ (mM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (M$^{-1}$ s$^{-1}$) |
|-----------|------------|----------------------|----------------------------------|
| STSA      | 3.10       | 40.0                 | 13 100                           |
|           | 0.367      | 532                  | 1 450 000                        |
| AUSA      | 0.123      | 24.0                 | 195 000                          |
|           | 0.0486     | 190.0                | 390 000                          |

Fig. 4. Catalytic preference of STSA for GM$_3$-Neu5Ac over GM$_3$-Neu5Gc. GM$_3$-Neu5Gc and GM$_3$-Neu5Ac were hydrolyzed with 1 ml/mM STSA and 1 ml/mM AUSA. Each point represents the amounts of Neu5Gc (A) and Neu5Ac (B) released from GM$_3$-Neu5Gc and GM$_3$-Neu5Ac, respectively, as the mean ± S.E.M. (n = 3).

Fig. 5. Weak cleavage ability of STSA for Neu5Gc residues in equine erythrocytes. Neu5Gc-containing glycans in equine erythrocytes were hydrolyzed with STSA (1 ml/mM) and AUSA (1 ml/mM). Each point represents the amount of released Neu5Gc at the mean ± S.E.M. (n = 3). The asterisks indicate significant differences (* P < 0.05, ** P < 0.001; t-test) from the amount of released Neu5Gc with AUSA.

Table 2 Binding scores of Neu5Gc2en and Neu5Ac2en to STSA.

| Binding score [kcal/mol] |
|--------------------------|
| Neu5Gc2en                | −338.4 |
| Neu5Ac2en                | −352.7 |
| Δ                        | 14.3   |

Equine erythrocytes contain Neu5Gc in gangliosides and glycoproteins. Suzuki et al. reported that Neu5Gc was the only molecular species of sialic acid contained in equine erythrocyte membranes [32]. The ganglioside from equine erythrocytes was shown to be composed of Neu5Gc as Neu5Gc-GM$_3$ or 4-O-acetyl-Neu5Gc-GM$_3$ but with little Neu5Ac [33–35]. Neu5Gc-containing glycoproteins were also detected in equine erythrocytes as a 68-kDa protein band by Western blotting analysis [36]. The linkage form of sialic acid in equine erythrocytes was analyzed using lectins, suggesting that equine erythrocytes contained sialic acid (Sia)$_2$-3Gal-linkage abundantly but little Sia$_2$-6Gal-linkage [37]. Thus, equine erythrocytes would contain a large amount of the Neu5Gc$_2$-3Gal structure.

We analyzed the hydrolytic potential of STSA toward Neu5Gc-containing sialylglycans in equine erythrocytes. Equine erythrocytes (0.5–50%, v/v) were treated with STSA and AUSA at 37 °C in a pH 6.0 buffer solution. The amount of Neu5Gc cleaved with STSA was remarkably small compared to the amount cleaved with AUSA, suggesting that STSA hardly hydrolyzes Neu5Gc$_2$-3Gal structure in equine erythrocytes (Fig. 5).

3.5. In silico analysis for the catalytic mechanism of STSA

To explore the origin of substrate specificity of STSA, we performed in silico analysis for the STSA complex with Neu5Ac/Neu5Gc based on first-principles (ab initio) calculations using the FMO method, which can correctly evaluate interactions between a substrate and hydrophilic/hydrophobic amino acid residues in a protein. We docked transition-state analogues, Neu5Gc$_2$en and Neu5Ac$_2$en, to the STSA structure and evaluated the binding affinity of them to STSA. As a result, the binding affinity of Neu5Gc$_2$en is 14.3 kcal/mol more unstable than that of Neu5Ac$_2$en (Table 2). The positions of the hydroxyl and carboxyl groups of Tyr307 and Arg309 were changed by 1.18 and 0.85 Å, respectively, due to steric hindrance of the hydroxymethyl group of Neu5Gc in the binding site (Fig. 6). Tyr307 and Arg309 have been pointed out as the key residues of recognition for the difference between sialyl α2-3 and sialyl α2-6 linkages. Neu5Ac$_2$en can make the salt-bridge interaction with Arg309 more effectively than Neu5Gc$_2$en.

In conclusion, in addition to its kinetic preference for sialyl α2-3 linkage over sialyl α2-6 linkage, STSA has kinetic preference for Neu5Ac residue over Neu5Gc residue. The amino acid residues that recognize sialyl α2-3 linkage in STSA also play crucial roles in selective cleavage for the molecular species of sialic acid. STSA is useful for preferentially removing α2-3-linked sialic acids. For usage of STSA, it is necessary to pay attention to the low ability to cleave Neu5Gc. Our
Neu5Gc.

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