^{1}H NMR Evidence That *Salmonella typhimurium* Sialidase Hydrolyzes Sialosides with Overall Retention of Configuration

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Abstract: ^{1}H NMR spectroscopy has been used to investigate the mechanism of hydrolysis of the synthetic substrate 2-*O*-(4-methylcoumarin-7-yl)-5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosidonic acid using the bacterial sialidase from *Salmonella typhimurium*. The ^{1}H NMR results clearly demonstrate that the initial product of the substrate hydrolysis is the α-anomer of *N*-acetyl-D-neuraminic acid (Neu5Ac). Release of *N*-acetyl-α-D-neuraminic acid as the first product of substrate cleavage is consistent with previous investigations which conclude that both viral and other bacterial sialidases catalyze the hydrolysis of α-Neu5Ac ketosides with overall retention of anomeric configuration.

Introduction

Sialidases (EC 3.2.1.18) are glycohydrolases that catalyze the release of terminal sialic acids α-ketosidically linked to glycoproteins, glycolipids and polysaccharides.[1] Viral and bacterial sources of sialidases have been isolated and X-ray crystallographic studies of influenza virus, *Vibrio cholerae*, and *Salmonella typhimurium* sialidases have been reported.[2−4] Although these enzymes lack significant sequence homology, the overall topology of the enzymes is quite similar and significant regions of the active sites are conserved. Crystallographic studies of the influenza virus sialidase/Neu5Ac complex have revealed that sialic acid binds to the active site as the α-anomer in a $B_{2,5}$ conformation although the α-anomer is the minor solution equilibrium component (*ca.* 5−10%). Influenza virus sialidase has been used as a drug design target for discovery of anti-influenza drugs.[5−7]

Previous ^{1}H NMR spectroscopy studies have shown that the hydrolysis of a series of α-ketosides of Neu5Ac using sialidases from the bacterial sources *Clostridium perfringens*, *Arthrobacter ureafaciens*, *Vibrio cholerae*, and *Bifidobacterium*[8] and the influenza virus[9] all proceed with the initial release of α-anomer Neu5Ac (**3**) as shown in Scheme 1. Recently it has been reported that *S. typhimurium* sialidase hydrolyzes α-ketosides with overall inversion of anomeric configuration as determined by UV and optical rotation experiments (Scheme 1).[10,11] It was therefore proposed that the mechanism of sialoside hydrolysis must be quite different for *S. typhimurium* sialidase from that proposed for influenza virus sialidase.[12] This result was surprising in light of the X-ray crystallographic studies which revealed very similar active sites for the influenza and *S. typhimurium* sialidases. Herein are presented the results of a ^{1}H NMR spectroscopy time course hydrolysis study which definitively shows that *S. typhimurium* is also a retaining enzyme.

Experimental Section

General Procedures. 2-*O*-(4-Methylcoumarin)-7-yl-5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosidonic acid (Neu5Acα2MeUmb, **1**) and 2-*O*-(*p*-nitrophenyl)-5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosidonic acid (Neu5Acα2PNP, **2**) were prepared according to literature methods.[9,13] *S. typhimurium* sialidase was purchased from both Sigma Chemical Co. and New England Biolabs. The molecular weight and homogeneity of the enzyme, from both suppliers, were checked by SDS-PAGE gel electrophoresis. An isoelectric focusing gel was also run under standard conditions and stained for sialidase activity using **1**. The molecular weight was found to be approximately 41 kD, which is in agreement with literature values.[2,14]

^{1}H NMR Experiments. The hydrolysis of 20 mM Neu5Acα2MeUmb (or Neu5Acα2PNP) in the presence of 1 U of *S. typhimurium* sialidase (from both suppliers) 1 U is defined as the amount of enzyme required to catalyze the hydrolysis of **1** μmol of substrate per min) was monitored by ^{1}H NMR spectroscopy over time on a 600 MHz Brüker AMX spectrometer at 25 °C, in 0.6 mL of 0.05 M KD_2PO_4/NaOD (pD 5.5) for 125 min. The optimum pH for this enzyme has been reported to be between 5.5 and 6.0 in phosphate buffer.[14] A spectrum of Neu5Acα2MeUmb (or Neu5Acα2PNP) was also acquired under identical experimental conditions (*i.e.*, 20 mM Neu5Acα2MeUmb (or Neu5Acα2PNP) at 25 °C in 0.6 mL of 0.05 M KD_2PO_4/NaOD (pD 5.5)) to the time course reaction. Spectra were acquired with 16 K data points over a spectral width of 6024 Hz, with a relaxation delay of 2 s and 32 scans.

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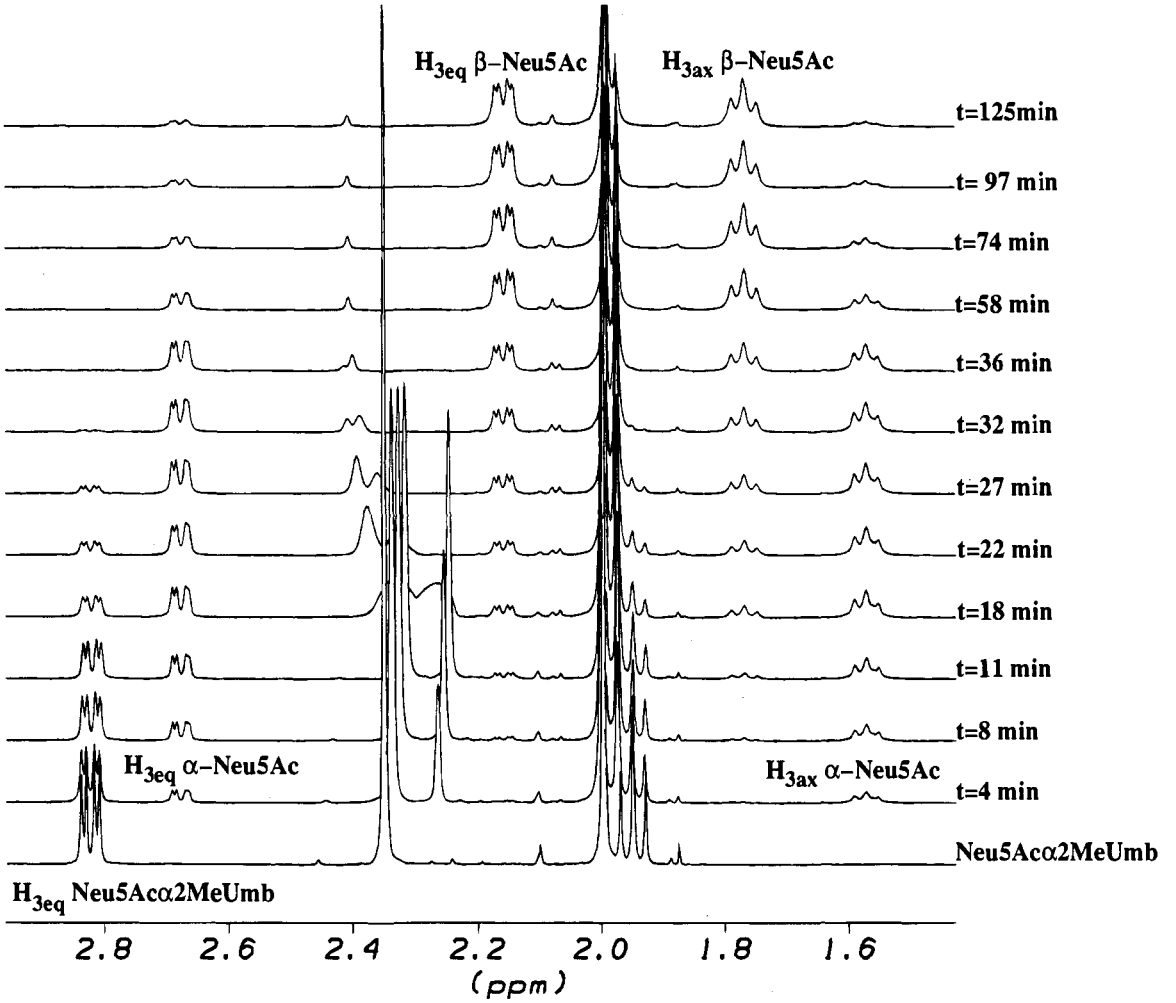
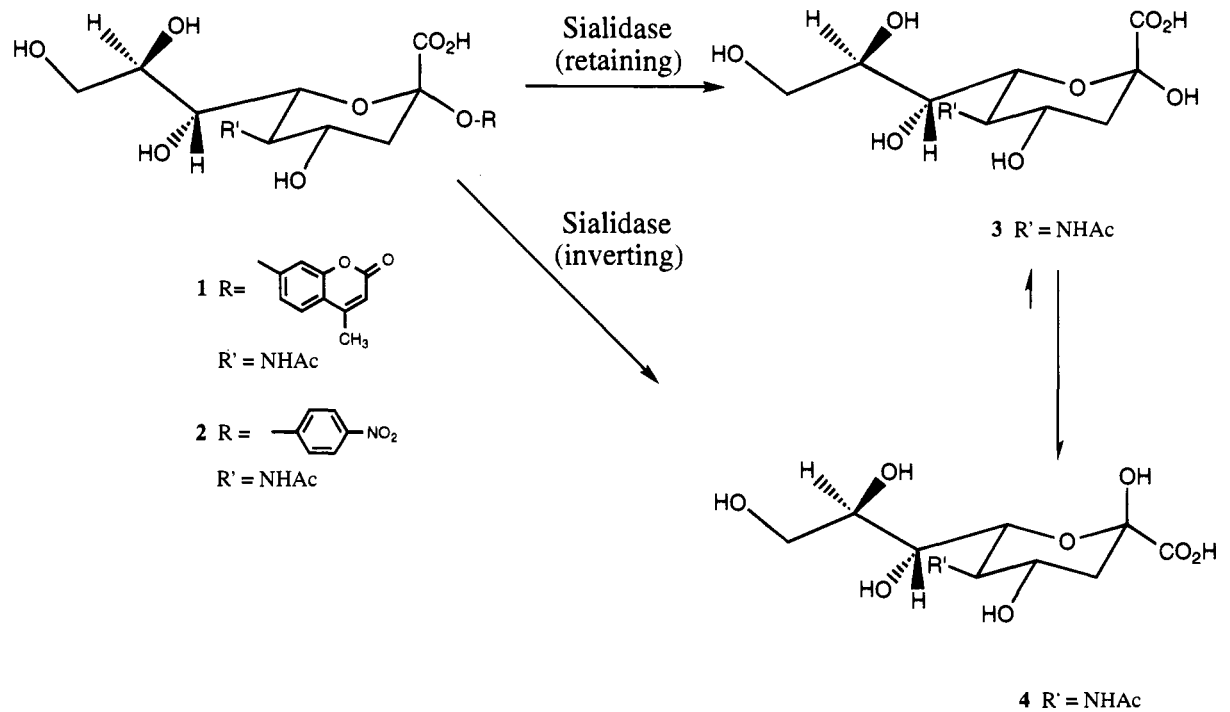


Figure 1. Progress of the *S. typhimurium* sialidase reaction monitored by 600 MHz ^{1}H NMR spectroscopy. Spectral data were acquired at times indicated on the spectra. The reaction was performed with 20 mM Neu5Acα2MeUmb (**1**) in the presence of 1 U of *S. typhimurium* sialidase at 25 °C in 0.6 mL of 0.05 M KD_2PO_4/NaOD (pD 5.5). As the reaction proceeds, 4-methylumbelliferone is released, which precipitates with a concomitant broadening of the methyl resonance of this group in the ^{1}H NMR spectrum.

Scheme 1



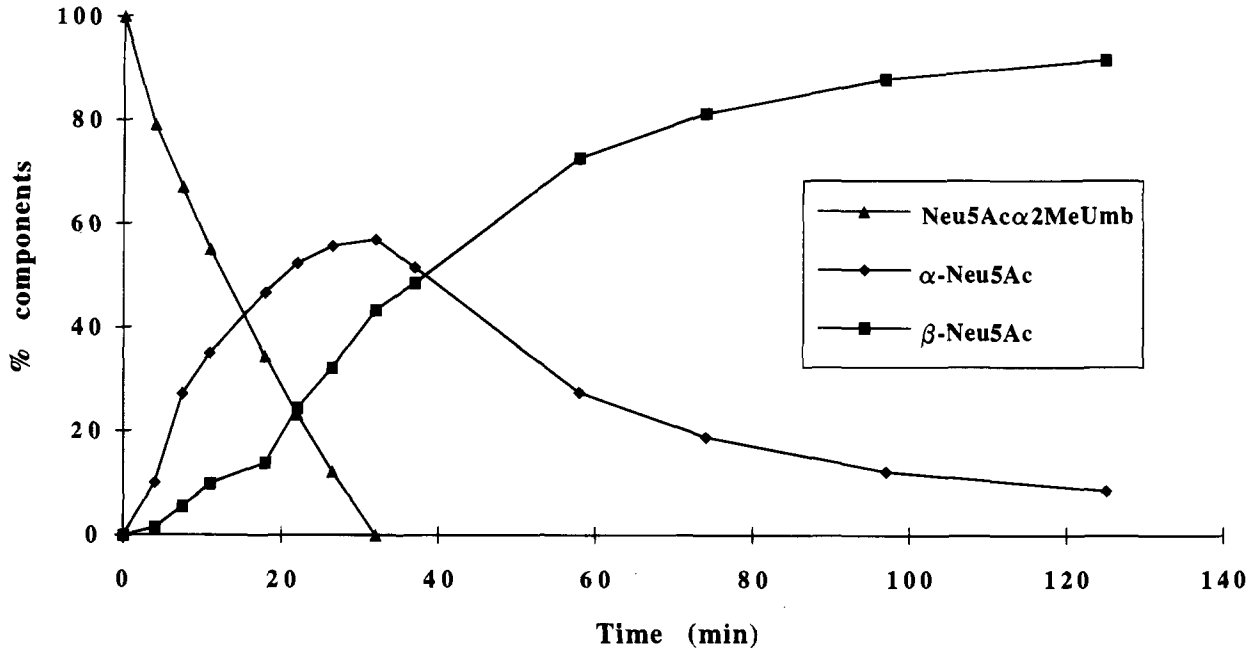


Figure 2. Relative proportions of substrate and catalysis products as monitored over time by ^{1}H NMR. The percentages of substrate and products were calculated from the relative integrals of the H_{3eq} signals of Neu5Acα2MeUmb (**1**), α-Neu5Ac (**3**), and β-Neu5Ac (**4**).

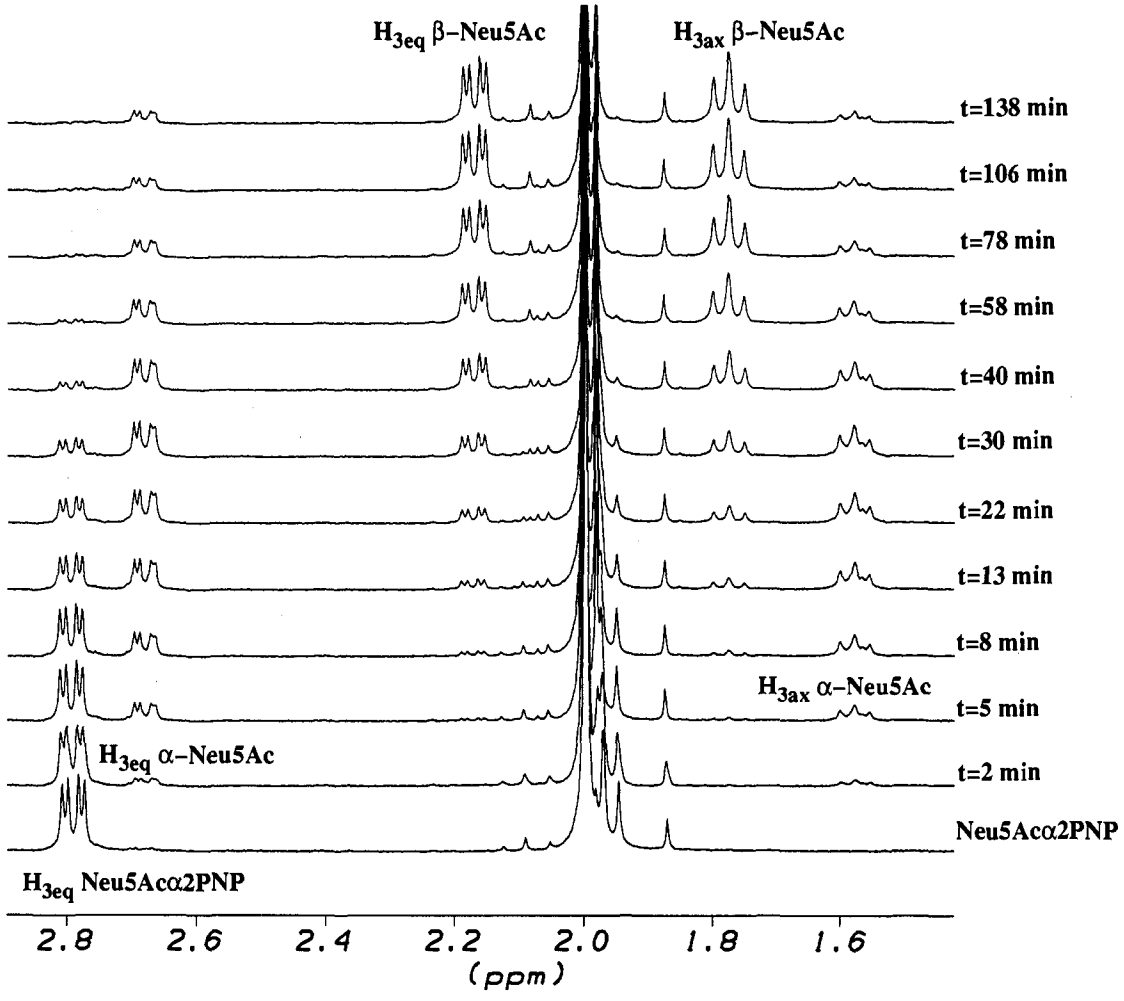


Figure 3. Progress of the *S. typhimurium* sialidase reaction monitored by 600 MHz ^{1}H NMR spectroscopy. Spectral data were acquired at times indicated on the spectra. The reaction was performed with 10 mM 2-*O*-(*p*-nitrophenyl)-5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosidonic acid (**2**) in the presence of 1 U of *S. typhimurium* sialidase at 25 °C in 0.6 mL of 0.05 M KD_2PO_4/NaOD (pD 5.5).

Results and Discussion

Figure 1 shows the time course reaction of the hydrolysis of the synthetic substrate 2-*O*-(4-methylcoumarin)-7-yl-5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosidonic acid (**1**) catalyzed by *S. typhimurium* sialidase as monitored by ^{1}H NMR spectroscopy. The first spectrum, which corresponds to $t = 0$ min, is Neu5Acα2MeUmb (**1**) at 25 °C in 0.6 mL of 0.05 M KD_2PO_4/NaOD (pD 5.5), *i.e.* experimental conditions identical to those of the time course reaction. The H_{3eq} resonance of **1** is clearly seen at 2.78 ppm, and H_{3ax} at 1.91 ppm, very close to the methyl of the acetamido group of this substrate. The methyl resonance of the 4-methylumbelliferyl aglycon is observed at 2.31 ppm. All subsequent spectra show the time course reaction. At $t = 4$ min, H_{3eq} and H_{3ax} resonances from α-Neu5Ac (**3**) are visible at 2.64 and 1.53 ppm, respectively, together with the signals described for **1**. As the reaction proceeds the H_{3eq} and H_{3ax} resonances for Neu5Acα2MeUmb (**1**) decrease in intensity and finally disappear at 32 min with a concomitant increase in intensity for the resonances of α-Neu5Ac (**3**). This is consistent with the product retaining the same anomeric configuration as the substrate. As expected, mutarotation of the reaction product is also observed in these time course reactions. Resonances due to the H_{3eq} and H_{3ax} of the β-anomer of Neu5Ac (**4**) at 2.12 and 1.73 ppm, respectively, steadily increase as the reaction proceeds and are first observed at about 11 min into the reaction. In fact, after 37 min, the intensities of the α-Neu5Ac (**3**) resonances start to decrease as mutarotation continues to establish final equilibrium values for the anomeric mixture of 95% β- and 5% α-Neu5Ac at ~125 min. These observations are summarized graphically in Figure 2, which shows the percentage values of Neu5Acα2MeUmb (**1**), α-Neu5Ac (**3**), and β-Neu5Ac (**4**) as a function of time.

Recent UV spectroscopy and optical rotation experiments have suggested that *S. typhimurium* sialidase hydrolyzes the α-ketoside of *N*-acetyl-D-neuraminic acid, 2-*O*-(*p*-nitrophenyl)-5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosidonic acid (**2**) to give the β-anomer of Neu5Ac (**4**) as the first product of release from the enzyme reaction.[10,11] This result is contrary to the results presented above for Neu5Acα2MeUmb (**1**), and therefore, the hydrolysis reactions, monitored by ^{1}H NMR spectroscopy, were repeated using **2** for purposes of comparison to that study. As Figure 3 clearly demonstrates, the first product of release for *S. typhimurium* sialidase-catalyzed hydrolysis of 2-*O*-(*p*-nitrophenyl)-5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosidonic acid (**2**) was indeed the α-anomer of Neu5Ac (**3**). Also, because of the concern over this conflicting data, all of the NMR experiments were repeated with an alternative source of the enzyme (see the Experimental Section). There was no difference observed between these data and those collected from the original experiments (results not shown). The homogeneity of the enzyme was investigated and found to be clean with respect to sialidase activity.

Conclusion

The discrepancy between the data from our study and those previously reported[10,11] is not easy to rationalize and may arise because of differences in enzyme purity, pH, or enzyme concentration.[15] However, ^{1}H NMR spectroscopy, although more enzyme intensive, does allow the direct observation of the substrate and its enzymatically-hydrolyzed products. Moreover, this technique provides for the ready identification of each of the reaction components (excluding the enzyme), which is not possible by either UV spectroscopy or optical rotation methods, during the course of the reaction. The data from the present work lead us to propose that under our experimental conditions sialidase from *S. typhimurium* is indeed, at optimum pH, a retaining enzyme and not an inverting enzyme as previously reported. This is consistent with a number of other sialidases from both bacterial and viral sources.

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(15) Interestingly by using a lower enzyme concentration (similar to that reported in ref 10), in a single NMR experiment at pD 5.0 and 37 °C, an ambiguous result was found in which both α- and β-Neu5Ac were observed during the early stages of the reaction. This presumably arises because there is not sufficient accumulation of the α-anomer to be observed by NMR prior to the commencement of the mutarotation process. In fact, at $t = 20$ min a ratio of 1:1 for the two anomers was observed.