Stereoselectivity of the Chinese hamster ovary cell sialidase: sialoside hydrolysis with overall retention of configuration

Yung-Hsiang Kao, Laura Lerner and Thomas G. Warner

Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, CA 94080, USA

Introduction

Sialidases are a family of glycohydroltic enzymes that cleave sialic acid residues from glycoproteins and glycolipids. The sialidases from viral and bacterial sources have been well studied, and the three-dimensional structure of several of these proteins has been determined (Crennell et al., 1993, 1994; Janakiraman et al., 1994). Although there is only a moderate degree of primary amino acid sequence identity (~35%) between these enzymes, the overall fold of the molecules and organization of key amino acids at the active sites are remarkably similar.

An additional feature shared by bacterial and viral sialidases is the stereochemical course of enzymatic hydrolysis of the sialyl-ketosidic bond. 1H NMR studies of the reaction catalyzed by sialidases from Clostridium perfringens, Arthrobacter ureafaciens, Vibrio cholerae, Bifidobacterium (Friebolin et al., 1981), Salmonella typhimurium LT 2 (Wilson et al., 1995), and influenza A (Chong et al., 1992) have revealed that all of these enzymes cleave a variety of sialyl conjugate substrates by releasing the alpha anomer of sialic acid as the initial product. In all cases, enzyme hydrolysis proceeds with an overall retention of configuration at the anomerionic center (C-2) of the sialic acid molecule, presumably as a result of a double displacement type reaction.

The finding that members of the sialidase family share topological features at their active sites and that they catalyze with a similar stereoselective outcome is consistent with the general observations made with other glycohydrolases such as B-1,4 xylanases and β-1,4 glucanases. Groupings of these glycanases based on a common fold or primary amino acid sequence similarities within their catalytic domains have also been shown to have a concomitant identity of their stereoselectivity of catalysis (Henrissat, 1991). Protein members within a given classification are either all inverting enzymes or they are all retaining enzymes. From this analysis it is apparent that the topological organization of the catalytic site dictates the stereochemistry of hydrolysis.

In contrast, mammalian sialidases have not been characterized with the same level of detail as the microbial enzymes. Only recently have the cytosolic sialidase from rat muscle (Miyagi et al., 1993) and a soluble sialidase from Chinese hamster ovary cells been purified (Ferrari et al., 1994) and their c-DNAs isolated. There are no extensive regions of amino acid sequence identities between the mammalian and microbial sialidases. Sequence similarities are only found in "Asp box" motifs (Roggentin et al., 1989). These motifs consist of about 5-12 amino acid residues with about 5 highly conserved residues flanking an aspartic acid residue. It would be of great interest to determine whether the mammalian enzymes share a similar topology and catalytic mechanism with their microbial counterparts. This data might provide additional insight into the genetic or evolutionary relationship between the microbial and mammalian proteins. Unfortunately, three-dimensional structures of the mammalian sialidases are not yet available. It is, however, possible to examine and compare the stereoselectivity and hydrolysis mechanism of microbial and mammalian sialidases by 1H NMR. We report in this article an NMR investigation of the stereochemical course of the sialidases from Chinese hamster ovary cells and Salmonella typhimurium LT 2.

Results and discussion

Monitoring the stereochemical course of sialidase hydrolysis with 1H NMR

The stereoselectivity of sialidase hydrolysis can readily be monitored by employing 1H NMR for determining the anomeric configuration of the sialic acid product that is released by
enzymatic cleavage of sialyl-glycoside substrates (Friebolin et al., 1980a). The resonance signals arising from the axial and equatorial protons at the C-3 position of the liberated sialic acid molecule for both the alpha and beta anomers are distinct from one another, and they are clearly resolved from the H-3 signals of the substrate. This method of analysis is possible because the beta anomer of sialic acid is the predominant conformer in solution and because interconversion between the alpha and beta anomers (mutarotation) is a slow process relative to enzyme hydrolysis reaction, particularly when the amount of enzyme utilized for catalysis is sufficient to complete the reaction within several minutes. Under these conditions, if the alpha anomer of sialic acid is the conformer initially released by the enzyme it can be readily identified before mutarotation takes place to a significant degree (Figure 1).

In the experiments here, the stereoselectivity of hydrolysis by the recombinant mammalian sialidase from Chinese hamster ovary cells was compared with the Salmonella typhimurium LT 2 sialidase. The Salmonella enzyme was selected as a control because both sialidases share several properties including nearly identical molecular weights, a preference of 2,3 linked sialosides substrates, and a similar positioning of two "Asp box" motifs in their primary structure (Hoyer et al., 1992). The Salmonella sialidase is also of interest because there is some controversy about its stereochemical course. Initial ultraviolet polarimetry studies of the Salmonella enzyme suggests that catalysis proceeds with inversion of configuration and that the beta anomer of sialic acid is the initial product released as a result of hydrolysis (Guo and Sinnott, 1993; Guo et al., 1994). However, recent experiments using 1H NMR spectroscopy to determine the stereochemistry of the reaction products indicated that the Salmonella enzyme hydrolyzes the substrates with retention of configuration (Wilson et al., 1995). The basis for these conflicting results is not readily apparent. Aside from the different analytical techniques employed, one of the most obvious differences between these experiments was the solvent used for the respective reactions. The polarimetry studies were conducted in 2H2O. In contrast, all NMR studies to date have employed 2H2O as solvent. In addition, the latter experiments were carried out with all exchangeable protons in the buffers, substrates and enzymes substituted with deuterium. This highly deuterated environment may have altered the reaction kinetics so that with the NMR technique the enzyme is observed to function as a retaining catalyst rather than an inverting one. It should be noted that there is no precedence for 2H2O altering the stereochemistry of an enzymatic hydrolytic reaction. However, in order to insure that the deuterated solvent did not effect the reaction, we have monitored, here, the stereochemical course of the CHO cell and Salmonella sialidase reactions using 1H2O as solvent containing only low concentrations of 2H2O (10% by volume) for a deuterium lock in the NMR experiments.

In the studies herein, the synthetic sialoside, 4-methyl umbelliferyl-N-acetyl neuraminic acid (4-MU-Neu5Ac), was employed as substrate in order to compare our results directly with the previous study of Wilson et al. (1995), who also evaluated the stereochemical course of the Salmonella LT2 enzyme with this substrate but in a highly deuterated environment.

4-MU-Neu5Ac hydrolysis by the Salmonella typhimurium sialidase

The reaction course of hydrolysis of the 4-MU-Neu5Ac substrate by the Salmonella enzyme was determined by inspection of the resonance signals arising from the H-3 of the sialic acid product (Figure 2). At the earliest time point of data acquisition after addition of the enzyme (4 min) the presence of the C-3 axial proton of the alpha anomer of sialic acid (1.59 ppm) and the corresponding equatorial proton (2.70 ppm) can be observed. As the enzyme reaction proceeded, these signals continued to increase in intensity up to about 58 min after which they progressively decreased as mutarotation approaches equilibrium and the beta anomer became the predominant species. It is not until after 12-14 min of incubation that the axial (1.79 ppm) and equatorial (2.17 ppm) H-3 of the beta anomer of sialic acid are first detected. Since the beta anomer is the preferred conformer, these signals continue to increase in intensity over the entire course of the experiment. Concomitantly, the intensity of the C-3 axial and equatorial protons (1.97 ppm and 2.84 ppm, respectively) of the substrate decreases up to about 53 min, at which point hydrolysis is complete and these signals are no longer detected.

Under the reaction conditions that we have employed, using 1H2O as solvent containing a minimal amount of 2H2O, the stereoselectivity of the Salmonella sialidase was identical to that found by Wilson and coworkers (1995). Thus, deuteration of the protein or a deuterium solvent effect on the stereochemical course cannot account for the discrepancy between the NMR results and the optical rotation studies (Guo and Sinnott, 1993).
Stereoselectivity of CHO cell sialidase

CHO cell and Salmonella sialidases. As shown in Figure 4, with ~2 U of the CHO cell enzyme the substrate was completely cleaved after about 45 min of incubation. The rapid elimination of the substrate made it possible to accurately determine the mutarotation rate for the interconversion of the alpha and beta anomers under these reaction conditions. The mutarotation rate constants determined using the Salmonella and CHO cell enzymes were $k_f = 4.3 \times 10^{-4}$ s$^{-1}$, $k_b = 4.0 \times 10^{-5}$ s$^{-1}$ and $k_f = 3.6 \times 10^{-4}$ s$^{-1}$, $k_b = 3.7 \times 10^{-5}$ s$^{-1}$, respectively. These rate constants are the average values of four independent fits (i.e., for signals from axial and equatorial protons in alpha and beta anomers). They were similar to those reported by Friebolin et al. (15) ($k_f = 1.3 \times 10^{-4}$ s$^{-1}$, $k_b = 1.1 \times 10^{-5}$ s$^{-1}$), but they are not identical. This may reflect the different reaction conditions used in these experiments. It is clear that under the reaction conditions employed in the experiments here, the conversion of the alpha anomer to the beta anomer is a relatively slow process compared with the rate of formation of product. Also, the values for the rate constants obtained with both enzymes were very close, suggesting that the protein itself does not substantially affect mutarotation of the product.

When much smaller amounts of enzyme were employed (0.1 unit), as expected, the rate of substrate cleavage was substan-

**Fig. 2.** Time course of hydrolysis of 4-MU-Neu5Ac by the Salmonella typhimurium LT 2 sialidase (1 unit of enzyme) monitored with proton magnetic resonance spectroscopy. Shown are proton signals for: A, H3 equatorial of substrate; B, H3 equatorial of alpha anomer sialic acid product; C, H3 equatorial of beta anomer of product; D, H3 axial of substrate; E, H3 axial of beta anomer of product; F, H3 axial of alpha anomer of product. Incubation times (in minutes) for the enzyme reaction are, from bottom panel to the top: 0 (no enzyme), 4, 10, 15, 21, 33, 49, 72, 92, 127.

4-MU-Neu5Ac hydrolysis by the Chinese hamster ovary cell sialidase

Hydrolysis of 4-MU-Neu5Ac by the CHO cell sialidase followed a reaction course similar to that observed with the Salmonella enzyme except that the overall reaction was slightly slower (Figure 3). This may be the result of the fact that the CHO cell enzyme is extremely thermal labile and its reaction rate may deviate from linearity over the course of the experiment. The resonance signals for the C-3 axial and equatorial protons (1.59 ppm and 2.70 ppm, respectively) of the alpha anomer of the sialic acid product were clearly visible after only 6 min of incubation with the mammalian enzyme. These signals continued to increase in intensity as the enzyme hydrolysis progressed. Their intensity subsequently decreased after about 73 min of reaction as the substrate was depleted and the remaining alpha anomer converted to the corresponding beta anomer. Signals for the H-3 of the beta anomer were detected after about 13 min of incubation. The reaction was nearly complete after about 95 min.

**Experiments with variable enzyme concentrations**

Similar hydrolysis experiments were carried out using both higher (2–3 unit) and lower (~0.1 unit) concentrations of the CHO cell and Salmonella sialidases. As shown in Figure 4, with ~2 U of the CHO cell enzyme the substrate was completely cleaved after about 45 min of incubation. The rapid elimination of the substrate made it possible to accurately determine the mutarotation rate for the interconversion of the alpha and beta anomers under these reaction conditions. The mutarotation rate constants determined using the Salmonella and CHO cell enzymes were $k_f = 4.3 \times 10^{-4}$ s$^{-1}$, $k_b = 4.0 \times 10^{-5}$ s$^{-1}$ and $k_f = 3.6 \times 10^{-4}$ s$^{-1}$, $k_b = 3.7 \times 10^{-5}$ s$^{-1}$, respectively. These rate constants are the average values of four independent fits (i.e., for signals from axial and equatorial protons in alpha and beta anomers). They were similar to those reported by Friebolin et al. (15) ($k_f = 1.3 \times 10^{-4}$ s$^{-1}$, $k_b = 1.1 \times 10^{-5}$ s$^{-1}$), but they are not identical. This may reflect the different reaction conditions used in these experiments. It is clear that under the reaction conditions employed in the experiments here, the conversion of the alpha anomer to the beta anomer is a relatively slow process compared with the rate of formation of product. Also, the values for the rate constants obtained with both enzymes were very close, suggesting that the protein itself does not substantially affect mutarotation of the product.

When much smaller amounts of enzyme were employed (0.1 unit), as expected, the rate of substrate cleavage was substan-

**Fig. 3.** Time course of hydrolysis of 4-MU-Neu5Ac by the Chinese hamster ovary cell sialidase (1 unit of enzyme) monitored with proton magnetic resonance spectroscopy. Shown are proton signals for: A, H3 equatorial of substrate; B, H3 equatorial of alpha anomer sialic acid product; C, H3 equatorial of beta anomer of product; D, H3 axial of substrate; E, H3 axial of beta anomer of product; F, H3 axial of alpha anomer of product. Incubation times (in minutes) for the enzyme reaction are, from bottom panel to the top: 0 (no enzyme), 6, 11, 16, 22, 29, 36, 58, 93, 121.
The enzyme buffer was exchanged with 50 mM phosphate buffer, pH 5.5, containing 100 mM sodium chloride using a microconcentrator filter (Filtron Tech. Inc., Norborough, MA). The Chinese hamster ovary cell sialidase was obtained as a recombinant protein expressed in Sf9 insect cells using a modified nuclear polyhedrosis virus containing the sialidase cDNA (Ferrari et al., 1994). The enzyme was isolated from the culture fluid of infected insect cell cultures using immunoaffinity chromatography employing a heterologous rabbit antibody raised against the purified recombinant protein immobilized on Sepharose 4B. Further purification was carried out with heparin–agarose anion exchange chromatography (Warner et al., 1993). The final enzyme preparation was dialyzed against 50 mM phosphate buffer, pH 6.0. Both enzymes were adjusted to a concentration of about 30 units/ml (1 unit is defined as a micromole of product/min).

**SDS-PAGE**

The purity of both protein preparations was evaluated using SDS–PAGE under denaturing conditions using the method of Laemmli (1970) staining for protein with Coomassie blue. Both enzymes were nearly homogeneous giving identical molecular weights of about 43 kDa with this analysis.

**Nuclear magnetic resonance**

The sialidase substrate, 4-MU-Neu5Ac, 20 mM, was prepared in 50 mM phosphate buffer, pH 5.5 with 100 mM sodium chloride (substrate for the Salmonella sialidase) or at pH 6.0 without salt (substrate for the CHO cell enzyme) in 90% H2O, 10% D2O. The total sample volume was 0.7 ml. The hydrolysis reaction was carried out at 298 K and monitored by a series of one-dimensional 1H NMR spectra recorded on a Bruker AMX 500 spectrometer. After collecting a spectrum of the substrate, the sample was removed from the probe and the reaction was initiated by mixing sialidase solution with the substrate in the NMR tube. No further tuning or shimming was performed after the sample was quickly placed back in the probe. The first spectrum was taken within 4–5 min after the reaction had begun, and a spectrum was acquired every 3–5 min thereafter for over 2 h. A total of 6192 complex data points and a relaxation delay of 2 s were used in acquiring 16 scans for each of those one-dimensional spectra. A low power pulse of 1.5 s was applied during the relaxation delay to suppress the H2O signal. The total relaxation time between individual scans was 7.9 s, roughly 5 times T1 for the H3 protons. All data were processed with FEI LiX 2.34 (Biosym Technologies, San Diego, CA) on an Indigo 2 work station (Silicon Graphics Inc., Mountain View, CA). The free induction decay of each spectrum was zero-filled to 16,384 complex points and subjected to a sine-bell window function with a phase shift of 40° prior to Fourier transform. All spectra were referenced to H2O at 4.76 ppm.

**Determination of mutarotation rate constants**

Mutarotation rate constants were determined by using a high enzyme concentration (2.1 U for CHO and 3 U for ST) in the hydrolysis reaction. Under these conditions, hydrolysis was completed within ~45 min after the reaction was initiated. However, the mutarotation of the hydrolysis product (sialic acid) continued to occur as the ratio of α and β anomers (—1:1) was still significantly far from its equilibrium value (~1:9). This allows for an accurate determination of mutarotation rate constants. The peak integrals of H3 signals in α and β anomers were taken from all spectra collected at different time points. The plot of peak integral versus time indicates that the substrate signals drop below detection limit after 45 min. Therefore the first six time points were discarded and the remaining points were fitted to the following equations for a two-state kinetic model (Noggle, 1989):

\[
A = A_0 - \frac{\left(2k_+ A_0 + k_{-B}\right)}{k_+ + k_-} (1 - \exp(-(k_+ + k_-) * t))
\]

\[
B = B_0 + \frac{\left(2k_+ B_0 + k_{-A}\right)}{k_+ + k_-} (1 - \exp(-(k_+ + k_-) * t))
\]

In these equations, \(A_0\) and \(B_0\) represent the initial populations (peak integrals) of alpha and beta anomers (i.e., the initial populations at the time when substrate vanishes, \(t = 0\); and \(k_+ (\alpha \rightarrow \beta)\) and \(k_- (\beta \rightarrow \alpha)\) are the mutarotation rate constants. The fitting was done using a nonlinear least-squares procedure with \(k_+\) and \(k_-\) as adjustable parameters.

**Abbreviations**

Neu5Ac, N-acetyl neuraminic acid; 4MU-Neu5Ac, 4-methyl umbelliferyl-N-acetyl neuraminic acid, SDS-PAGE, sodium dodecyl polyacrylamide gel electrophoresis; CHO, Chinese hamster ovary; NMR, nuclear magnetic resonance.
References


Received on September 19, 1996; revised on December 6, 1996; accepted on December 11, 1996