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DIFFERENTIAL REACTIVITY OF TWO TYPES OF *N*-GLYCOLYLNEURAMINIC ACID DIMERS TOWARD ENZYMATIC AND NONENZYMATIC HYDROLYSIS OF THEIR INTERKETOSIDIC LINKAGES

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SUMMARY: The kinetics of acid- and sialidase-catalyzed hydrolysis of the interketosidic linkages of two different disialic acids, Neu5Gcα2→5-$O_{glycolyl}$Neu5Gc and Neu5Gcα2→8Neu5Gc, were studied. The former sequence was recently identified in the polysialic acid chains of a sialic acid-rich glycoprotein isolated from the egg jelly coat of two different species of sea urchins, and the latter was previously found in the cortical alveolar-derived polysialoglycoprotein from rainbow trout eggs. At pH values < 3.8, the rate of hydrolysis of Neu5Gcα2→5-$O_{glycolyl}$Neu5Gc was greater than that of Neu5Gcα2→8Neu5Gc. Paradoxically, however, Neu5Gcα2→5-$O_{glycolyl}$Neu5Gc was more stable than Neu5Gcα2→8Neu5Gc at pH values > 3.8. These findings indicate a greater contribution of intramolecular general acid catalysis to the lability of the α2→5-ketosidic linkage. Neu5Gcα2→5-$O_{glycolyl}$Neu5Gc was a poor substrate for *Arthrobacter ureafaciens*, *Clostridium perfringens*, and *Vibrio cholerae* sialidases, in contrast to Neu5Gcα2→8Neu5Gc. Neu5Gcα2→5-$O_{glycolyl}$Neu5Gc was essentially resistant to hydrolysis by *A. ureafaciens* sialidase.

The α2→8-linked polysialic acid (polySia) glycotope is expressed on surface glycoconjugates in species as evolutionally diverse as neurotropic bacteria and man (1-7). α2→8-linked polySia structures appear to regulate a myriad of biological functions including important cell-cell recognition, cell adhesion and neuroinvasion (3,6,7). Recently, we showed a remarkable diversity in the oligo/polySia chains in polysialoglycoproteins (PSGP) isolated from the eggs of a variety of salmonid fish species (8). This diversity resulted from α2→8-linked homo- or heteropolymers consisting of Neu5Ac or Neu5Gc residues, and the presence of *O*-acetyl or *O*-lactyl substituents

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on these polymers. The structural diversity is even greater if one includes the α2→8-linked oligo/poly(deaminoneuraminic acid or KDN) residues and their *O*-acetylated forms found in glycoproteins isolated from the vitelline envelope and ovarian fluid of *Oncorhynchus mykiss* (rainbow trout) (9–11).

Recently we reported the structure of a novel type of polySia in the jelly coat glycoprotein (polySia-gp) isolated from two different species of sea urchin, *Hemicentrotus pulcherrimus* and *Strongylocentrotus purpuratus* (12). An unexpected feature of polySia-gp was the presence of a new oligo/polySia chain, characterized as $(\rightarrow 5\text{-}O_{glycolyl}Neu5Gc\alpha 2\rightarrow)_n$, where n ranged from 4 to more than 40 Neu5Gc residues. The average degree of polymerization (DP) was about 20 (12). While the structures of the polySia chains from the two species were identical, they differed in molecular weight and in the number of *O*-linked oligosaccharide chains attached to the core protein (12). The function of polySia-gp is not known, but it's polyanionic nature may be important in fertilization and embryogenesis physiologically for maintaining cations, such as Ca^{2+} and basic proteins, around the embryo (12).

This paper describes the kinetics of enzymatic and non-enzymatic hydrolysis of α2→5-$O_{glycolyl}$-linked- and α2→8-linked Neu5Gc dimers. The structure of these dimers is shown in **Fig. 1**. The aim of the present study was to compare the susceptibility of the two interketosidic linkages toward exosialidases and acid-catalyzed hydrolysis. This information will be critical for future studies to elucidate the synthesis and function of this new type of polySia.

MATERIALS AND METHODS

Materials. *Arthrobacter ureafaciens* sialidase was purchased from Nacalai Tescue Co. (Kyoto, Japan). *Clostridium perfringens* and *Vibrio cholera* sialidases and sialyllactose were purchased from Sigma. Rainbow trout egg polysialoglycoprotein (PSGP) and sea urchin egg polySia-gp were

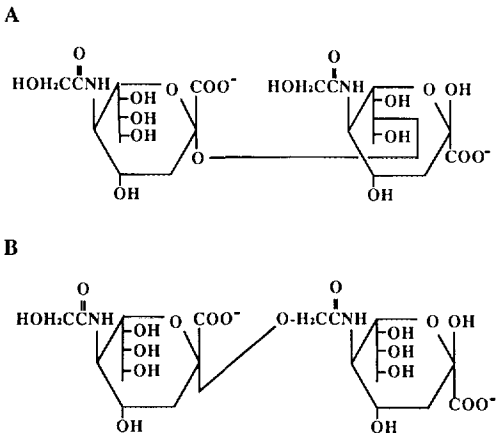

Fig. 1. Structures of **(A)** Neu5Gcα2→8-Neu5Gc and **(B)** Neu5Gcα2→5-$O_{glycolyl}$Neu5Gc.

prepared as previously described (12,13). α2→8-linked- and α2→5-$O_{glycolyl}$-linked Neu5Gc dimers were prepared as previously reported (12,13).

Mild Acid Hydrolysis of Neu5Gc Dimers. α2→8-linked and α2→5-$O_{glycolyl}$-linked Neu5Gc dimers (6 μg each as Neu5Ac) were hydrolyzed in 1.1 ml of McIlvaine-type phosphate-citrate buffer of constant ionic strength of 0.5 M at different pH values (pH 2.2, 2.6, 3.0, 3.4, 3.8, 4.2, 5.0, and 6.0) at 80°C (14). After incubation for varying periods of time, 100 μl aliquots of the reaction mixtures were analyzed by the thiobarbituric acid (TBA) method to quantitate the amount of Neu5Gc released (15,16).

Sialidase Treatment of Sialyloligosaccharides. Six μg of α2→8-linked and α2→5-$O_{glycolyl}$-linked Neu5Gc dimers were separately treated with 0.05 unit of *A. ureafaciens* or *C. perfringens* sialidase at 37°C in 220 μl of 50 mM sodium acetate buffer, pH 5.5. The kinetics of the reaction was followed by analyzing 20-μl aliquots for Neu5Gc at 0, to 180 min by the TBA method.

RESULTS AND DISCUSSION

Kinetics of mild acid hydrolysis of the disialic acids, Neu5Gcα2→5-$O_{glycolyl}$Neu5Gc and Neu5Gcα2→8Neu5Gc. Release of free Neu5Gc from the disialic acids, Neu5Gcα2→5-$O_{glycolyl}$ Neu5Gc and Neu5Gcα2→8Neu5Gc, was measured at different pH values as a function of time (**Fig. 2**).

The hydrolysis of each Neu5Gc dimer followed first-order kinetics, and the apparent rate constants, k_{app} (min^{-1}) were determined at various pH values at 80°C. Under acidic conditions, the k_{app} of Neu5Gcα2→5-$O_{glycolyl}$Neu5Gc was appreciably greater than that of Neu5Gcα2→8Neu5Gc. For example, at pH 2.6, the k_{app} of Neu5Gcα2→5-$O_{glycolyl}$Neu5Gc (0.072) was seven times larger than that of Neu5Gcα2→8Neu5Gc (0.010). This is presumably due to a greater contribution by intramolecular general acid catalysis because of the protonated carboxyl group in Neu5Gcα2→5-$O_{glycolyl}$Neu5Gc, compared to Neu5Gcα2→8Neu5Gc, and the difference in the structure of the

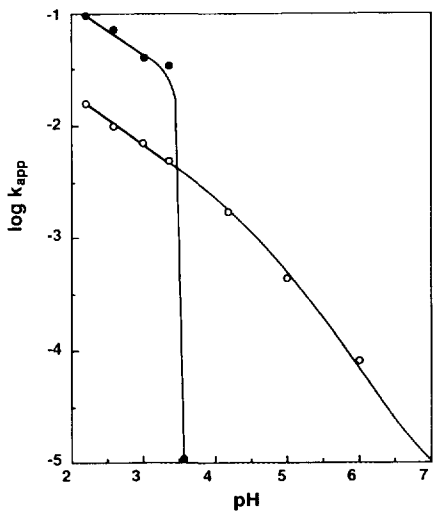

Fig. 2. Kinetics of the mild acid hydrolysis of the disialic acids Neu5Gcα2→5-$O_{glycolyl}$Neu5Gc (●) and Neu5Gcα2→8Neu5Gc (○), at various pH values at 80°C.

ketosidic linkages. Evidence that intramolecular general acid catalysis was an important factor in these differences was obtained by showing that Neu5Gcα2→5-$O_{glycolyl}$Neu5Gc was more stable, paradoxically, at pH values > 3.8 than Neu5Gcα2→8Neu5Gc. The carboxyl group was mostly ionized at pH >3.8 because pKa of this group is approximately 2.3. Thus, Neu5Gcα2→5-$O_{glycolyl}$Neu5Gc was not hydrolyzed at pH > 3.8, while Neu5Gcα2→8Neu5Gc was hydrolyzed at even higher pH. Indeed, Neu5Gcα2→8Neu5Gc was shown to release a small but detectable amount of Neu5Gc even at pH 6. Although the observed difference in lability of the ketosidic linkage near neutral pH between Neu5Gcα2→5-$O_{glycolyl}$Neu5Gc and Neu5Gcα2→8Neu5Gc is interpreted in terms of the difference in the intrinsic electronic property of the ketosidic bonds, a contribution of the term of water catalysis to k_{app} may also be important.

Kinetics of sialidase-catalyzed hydrolysis of the disialic acids, Neu5Gcα2→5-$O_{glycolyl}$Neu5Gc and Neu5Gcα2→8Neu5Gc. Our previous study showed that (→5-$O_{glycolyl}$-Neu5Gcα2→)$_n$ chains in polySia-gp were essentially resistant to *A. ureafaciens* sialidase (12). However, the crude jelly coat fraction containing polySia-gp showed partial susceptibility to hydrolysis by *C. perfringens* sialidase (17). To determine systematically the sensitivity of these polymers to exosialidase, we used *A. ureafaciens*, *C. perfringens*, and *V. cholerae* sialidases to test for enzymatic hydrolysis of Neu5Gcα2→5-$O_{glycolyl}$Neu5Gc.

In **Fig. 3** is shown the kinetics of *A. ureafaciens* and *C. perfringens* sialidase-catalyzed hydrolysis of Neu5Gcα2→5-$O_{glycolyl}$Neu5Gc (●) and Neu5Gcα→8Neu5Gc (○) at 37°C. It is of interest to

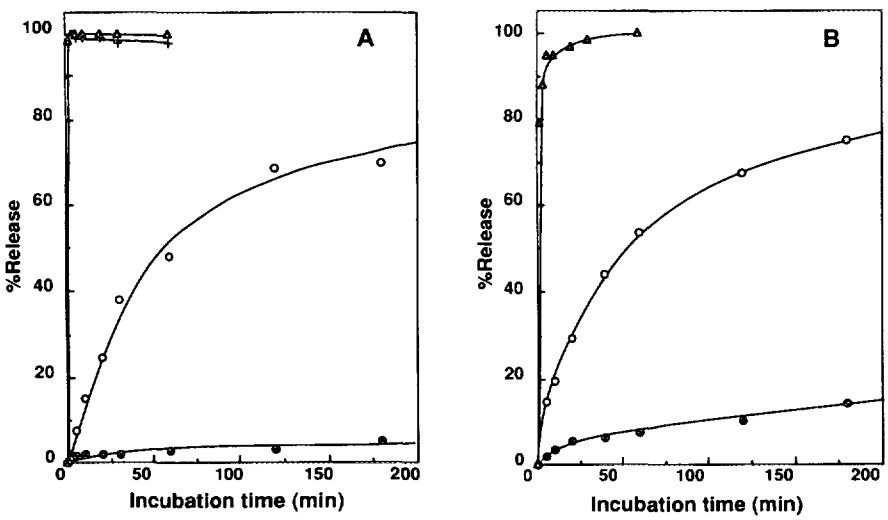

Fig. 3. Kinetics of the (A) *A. ureafaciens* and (B) *C. perfringens* sialidase-catalyzed hydrolysis of the disialic acids, Neu5Gcα2→5-$O_{glycolyl}$Neu5Gc (●) and Neu5Gcα2→8 Neu5Gc (○), α2→3-sialyllactose (△), and Neu5Acα2→OCH_2COOH (+) at 37°C. Samples were treated with enzyme as described under "Materials and Methods". The free Sia released was quantitated by the TBA method (A_{549}).

note that the interketosidic linkage in Neu5Gcα2→5-$O_{glycolyl}$Neu5Gc was extremely resistant to *A. ureafaciens* sialidase, while Neu5Acα2→OCH_2COOH (+) was readily hydrolyzed to Neu5Gc and glycolic acid (**Fig. 3A**). These findings suggest that the sialidase resistance of the Neu5Gcα2→$O_{glycolyl}$Neu5Gc linkage was not due to the intrinsic nature of the ketosidic bond (>C_2-O-CH_2-CO-NH-), but most likely resulted from stereochemical constraint in the Neu5Gcα2→$O_{glycolyl}$Neu5Gc linkage which prevent catalytic attack by the enzyme. It should be also noted that the *A. ureafaciens* sialidase-catalyzed hydrolysis of 4-MU-Neu5Ac was not inhibited by Neu5Gcα2→$O_{glycolyl}$Neu5Gc, even at concentrations 10 times greater than that of the substrate (data not shown). In contrast to the results with *A. ureafaciens* sialidase, the *C. perfringens* sialidase released ca. 10% of the Neu5Gc residues from Neu5Gcα2→5-$O_{glycolyl}$Neu5Gc (**Fig. 3B**). However, the rate of *C. perfringens* sialidase-catalyzed hydrolysis of Neu5Gcα2→5-$O_{glycolyl}$Neu5Gc was still significantly slower than that of Neu5Gcα2→8Neu5Gc (**Fig. 3B**). *V. cholerae* sialidase was also able to cleave Neu5Gcα2→5-$O_{glycolyl}$Neu5Gc (data not shown).

Concluding Remarks. Until now it was generally considered that a slower rate of release of free Sia from presumed Sia-containing polymers at acidic conditions (e.g. 0.1 N TFA), compared to sialyllactose, can be taken as diagnostic of the presence of polySia chains (18-20). While this presumption is still valid for α2→8-linked polySia chains, the results of these studies show that caution must be exercised when applying this criterion to the analysis of potentially new polySia structures, such as was found in the sea urchin polySia-gp. Similarly, the failure to release oligoSia from (→5-$O_{glycolyl}$-Neu5Gcα2→)$_n$ chains in polySia-gp by Endo-N (12) should be also alert us to the limitation of using Endo-N, as a diagnostic reagent for detecting polySia chains, although Endo-N resistance in structures known to contain polySia is still a valid indicator for the absence of α2→8Neu5Ac or Neu5Gc linkages (21).

The observed differential activities of the bacterial sialidases toward (→5-$O_{glycolyl}$-Neu5Gcα2→)$_n$ and Neu5Gcα2→8Neu5Gc chains provides us with an interesting problem to determine the catalytic mechanism of the different sialidases, and also to discover the precise 3-dimensional structure of this new type of polySia.

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