Mechanistic variation in the glycosyltransfer of N-acetylneuraminic acid

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Abstract N-acetylneuraminic acid is an acidic nine-carbon amino ketose typically found at the non-reducing terminus of glycoproteins and glycolipids. The presence of a carboxylate group adjacent to the anomeric center suggests that this sugar could have transition states with highly stabilized oxocarbenium ion character during transfer reactions at the anomeric carbon. Kinetic isotope effect (KIE) experiments were used to probe the transition state for solvolysis of UMP-NeuAc, sialyltransferase-catalyzed transfer of UMP-NeuAc to N-acetyl-lactosamine, trans-sialidase catalyzed transfer of α(2→3) Neu-Lac or α(2→3) Neu-Gal, and acid catalyzed hydrolysis of α(2→3) Neu-Lac. The two key positions of isotope substitution in the N-acetyl neuraminic acid residue were the C3' position, di-substituted with deuterium, and the C2' position, substituted with either carbon-13 or carbon-14. The solvolysis reaction had a β-^{2}H KIE of 1.28 and a primary ^{14}C KIE of 1.03. The sialyltransferase-catalyzed reaction had a β-^{2}H KIE of 1.22 and a ^{14}C KIE of 1.03. Trans-sialidase had a β-^{2}H KIE of 1.05 and a primary ^{13}C KIE of 1.03, equivalent to a ^{14}C KIE of 1.06. Solvolysis of the trans-sialidase substrate gave a β-^{2}H KIE of 1.06, and a primary ^{13}C KIE of 1.015. The results indicate a very late transition state for solvolysis of CMP-NeuAc, without nucleophilic participation. The sialyltransferase transition state is similar, but with less charge development. Trans-sialidase has a transition state with diminished charge development and considerable nucleophilic character, which leads to a covalent intermediate. The glycosyltransfer of N-acetylneuraminic acid glycosides is not limited to the classical dissociative mechanism.

Key words CMP-NeuAc • glycosyltransferase • isotope effects • kinetics • sialyltransferase • trans-sialidase • transition-state

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Introduction

The formation and cleavage of glycosidic bonds, "glycosyltransfer", has long been characterized as a process involving formation of transition states with oxocarbenium ion character with possible formation of short-lived oxocarbenium ion intermediates. Kinetic isotope effects (KIE's) provide a sensitive way to detect structural and electronic features of glycosyltransfer transition states for reactions in solution (i.e. hydrolysis) and those catalyzed by enzymes (i.e. hydrolysis or glycoside formation) [14]. The nine carbon amino ketose N-acetylneuraminic acid, NeuAc [12], Figure 1, is an interesting sugar for study of glycosyltransfer due to the proximity of the carboxylate group to the anomeric center. It is close enough to confer substantial electrostatic stabilization of oxocarbenium ions or transition states resembling them; [9] it could be a nucleophilic neighboring group during glycosyltransfer [1, 7], and finally, it could function as an intramolecular general base or acid. Interestingly, all of these possibilities engendered by the structure of NeuAc are parallels of the catalytic functionality identified in glycosyl hydrolase enzymes [18]. We have employed KIE's to characterize three different systems which involve glycosyltransfer of NeuAc. First, the solvolysis of the sugar nucleotides CMP--NeuAc and UMP-NeuAc was studied [4, 10, 11]. The sugar nucleotides are the NeuAc donor substrates for sialyltransferase enzymes [8], and it was of interest to study the

solvolysis chemistry as a point of comparison with the results for sialyltransferases [3, 4]. The sialyltransferases (and other glycosyltransferases in general) have only recently earned the higher level of mechanistic scrutiny previously devoted to glycohydrolases. The third system is the unique enzyme trans-sialidase, from the trypanosome *T. Cruzi* [13]. This enzyme catalyzes the transfer of NeuAc between two oligosaccharides, with retention of configuration. It is therefore distinct from typical glycosyltransferases that use an activated sugar nucleotide as the donor. In addition to measuring the KIEs for the trans-sialidase reaction, we also measured them for acid-catalyzed hydrolysis of the substrates, again, to serve as a point of comparison with the results from the enzyme [17].

Methods

The required double isotope-labeled NeuAc glycosides for use in KIE experiments were synthesized by enzymatic means as previously reported [4, 10, 17]. CMP-NeuAc, UMP-NeuAc, Neuα(2→3) galactose and Neuα(2→3) lactose were synthesized with both stable and radioactive labels for measurement of secondary β-^{2}H KIE's and primary ^{14}C (or ^{13}C) KIEs. Figure 2 presents the structures of the labeled substrates used in KIE experiments. KIE experiments employed the double label competitive method [6] and therefore reported on *V/K* for the enzyme-catalyzed reactions [15]. The details of the KIE methods and control experiments have been previously described [3, 4, 10, 17].

Results

Table 1 summarizes the results of the KIE experiments with UMP-NeuAc as substrate for sialyltransferase and solvolysis reactions. Table 2 presents the results for KIEs with labeled α(2→3) NeuAc lactose or galactose as substrates (SL or SG) for trans-sialidase, and solvolysis of SL.

Large β-^{2}H KIEs of 1.218 and 1.28 are observed for the sialyltransferase and solvolysis reactions of UMP-NeuAc, respectively. Considerably reduced β-^{2}H KIEs are observed for the SL or SG substrates, around 1.06 for trans-sialidase and 1.11 for solvolysis. The ^{14}C KIEs for UMP-NeuAc are 1.028 and 1.030 for sialyltransferase and solvolysis respectively. The ^{13}C KIEs for SG or SL substrates with trans-sialidase are 1.032 and 1.021, respectively, while solvolysis of SG and SL gave indistinguishable KIEs of 1.015 and 1.016.

Discussion

The two types of isotope effects employed in these studies were β-^{2}H and primary carbon (13 or 14). The ^{2}H isotope effects serve to report on the development of charge at the anomeric carbon in the transition state. This is due to the largely hyperconjugative origin of the isotope effect. The primary carbon KIE provides a measure of the associative/dissociative character of the reaction. In a strictly dissociative process, small carbon isotope effects are observed, whereas in an associative process, large KIEs are observed. The origin of this KIE is related to the change in force constans to the isotopically labeled anomeric carbon between

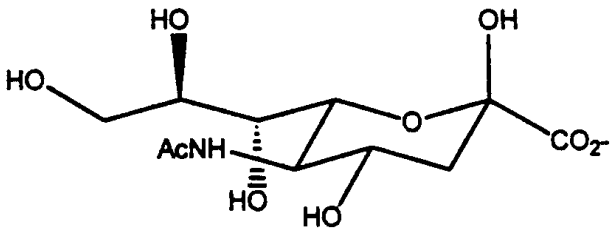

Fig. 1. N-acetylneuraminic acid.

ground and transition states. In a dissociative process the loss of leaving group decreases forces constants to the carbon, but a compensatory stiffening arises due to double bond character of the C-O endocyclic bond. The result is a small KIE (<1.04 for ^{14}C and <1.02 for ^{13}C). In the associative path, a synchronous loss of leaving group and attack of nucleophile results in a greater net loss of vibrational energy at the carbon, with resultingly larger KIEs (>1.06 for ^{14}C and >1.03 for ^{13}C). The β-^{2}H and primary C KIEs also interrelate, since an associative process will have charge reduced relative to a dissociative one so the carbon isotope effect will be larger and the β-^{2}H isotope effect smaller, than in the dissociative pathway.

The KIE data for sialyltransferase and solvolysis with UMP-NeuAc are similar. The β-^{2}H effects are greater than 1.20 in both cases, and this is evidence that both transition states are highly charged at the anomeric carbon. It is noteworthy that solvolysis does have a larger β-^{2}H isotope effect than the sialyltransferase reaction (1.28 *vs.* 1.22) but the reason for the difference is not yet clear. It is possible that the enzyme transition state has less bond breakage to the

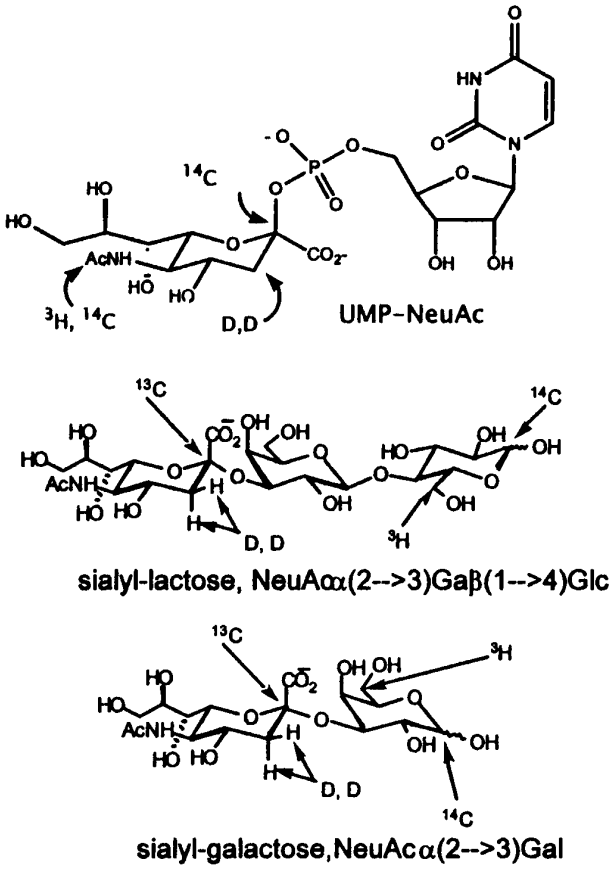

Fig. 2. Labeled substrates used in KIE experiments.

Table 1. Kinetic isotope effects with UMP-NeuAc. The acceptor substrate was N-acetyllactosamine. Data taken from Ref. [4].

Label	Type of KIE	$KIE_{sialyltransferase}$	$KIE_{solvolysis}$
[3,3'-$^2H_2$]	β-secondary	1.218±0.010	1.28±0.01
[2-^{14}C]	primary ^{14}C	1.028±0.010	1.030±0.005

departing UMP, or the observed isotope effects could reflect different NeuAc ring conformations that would modulate hyperconjugation between the anomeric carbon and beta-hydrogens. The primary ^{14}C isotope effects are about 1.03, in the range for a dissociative process. The small carbon isotope effect coupled with the large β-^{2}H isotope effects describes transition states that do not involve significant nucleophilic attack, and substantial charge development. In solution, CMP-NeuAc forms a short-lived oxocarbenium ion intermediate after the transition state, as evidenced by trapping experiments [10, 11]. It is not yet known if an oxocarbenium ion intermediate is formed during sialyltransferase catalysis.

The preceding trends observed for UMP-NeuAc do not apply to the chemical and enzymatic glycosyltransfer of NeuAc glycosides. Inspection of the data of Tables 1 and 2 reveals that the degree of charge development is lower than for the transfer reactions of UMP-NeuAc. Acid-catalyzed solvolysis has a moderate β-^{2}H isotope effect of 1.11 and a small ^{13}C primary isotope effect of 1.015, consistent with a moderately dissociated transition state lacking in nucleophilic character. The trans-sialidase catalyzed reaction has much less charge development (β-^{2}H effect of 1.06) and considerable nucleophilic character as evidenced by the large ^{13}C KIE of 1.03. This is equivalent to a ^{14}C KIE of 1.06. Though primary carbon isotope effects on bimolecular replacement reactions can be higher, the observed value is still consistent with a pseudo-bimolecular process in the enzyme active site. For example, ethoxide displacement of 1-bromo-1-phenylethane is a second order process and has a ^{13}C KIE of 1.036 [2]. That the trans-sialidase transition state has nucleophilic character argues for the subsequent formation of a covalent intermediate, given the retention stereochemistry for the reaction overall. The active site of trans-sialidase shows extensive amino-acid conservation with the *Salmonella* sialidase enzyme, whose three dimensional structure is known [5]. Two key features are a triad of Arg residues interacting with the NeuAc carboxylate, and a Tyr residue with its hydroxyl group positioned approximately 3 Å from the anomeric center. The combination of structural information and KIE data point to Tyr as an active site nucleophile, while the NeuAc carboxylate is likely to be tied up in interactions with the Arg triad. In recent work we have trapped a NeuAc/trans-sialidase covalent complex during turnover [16]. The glycosyltransfer of NeuAc proceeds via mechanisms that range from highly dissociative to associative. Despite the potentially stabilizing effect of the anomeric carboxylate group, the trans-sialidase transition state has little oxocarbenium ion character. For NeuAc glycosides, the Lac or Gal group is a poorer leaving group than the UMP group of UMP-NeuAc, and conversely, it may be a better nucleophile (in the reverse reaction). It is possible that trans-sialidase requires nucleophilic assistance to productively expel the departing aglycon. The active site may enforce nucleophilic participation at what would be an otherwise hindered position. Whereas the acid-

Table 2. Kinetic isotope effect data. "SL" = sialyl-lactose; "SG" = sialyl-galactose. Data taken from Ref. [17].

Label	Type of KIE	Corrected KIE[c]	Acid solvolysis
[3,3'-$^2H_2$]	β-dideuterium	1.053±0.010 (SL)[a]	1.113±0.012 (SL)
		1.049±0.013 (SL)[b]	
		1.060±0.008 (SG)	
[2-^{13}C]	primary	1.021±0.014 (SL)	1.016±0.011 (SL)
		1.032±0.008 (SG)	1.015±0.08 (SG)

[a] 0.8 mM lactose.
[b] 8.0 mM lactose.
[c] The KIEs were divided by the value of the control KIE due to the remote reporter labels: 0.993 for transfer reactions with SL, and 1.024 for transfer reactions with SG.

-catalyzed solvolysis of NeuAc-lactose proceeds with little if any nucleophilic participation. Some insight has been obtained from *ab initio* calculations which explored the reaction coordinate for glycosyltransfer reactions (Horenstein BA, manuscript in preparation). In systems with an O-protonated aglycon leaving group, only oxocarbenium ion intermediates and oxocarbenium-ion like transition states were identified. All attempts at identification of transition states having nucleophilic character were unsuccessful. When the leaving group was not protonated and a strong nucleophilie was utilized, *bona fide* "S_N2" transition states were identified. Interestingly, the transition states are somewhat exploded and have shortened endocyclic C-O bonds. Yet, while this would suggest that there is still oxocarbenium ion character, examination of the β C-H bond lenghts reveals that hyperconjugation is lacking. The shortened endocyclic C-O bond could account for the why the ^{13}C KIE is at the low end of the S_N2 range. Work is underway to calculate isotope effects for these S_N2-like systems. The emerging theme from calculations is that sugars prefer to undergo glycosyltransfer reactions via dissociative pathways unless strong nucleophiles and poor leaving groups are present.

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