Primary ^{13}C and β-Secondary ^{2}H KIEs for Trans-sialidase. A Snapshot of Nucleophilic Participation during Catalysis†

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ABSTRACT: *Trypanosoma cruzi* trans-sialidase catalyzes a novel reaction that involves the transfer of sialic acid between host and parasite glycoconjugates. In this paper, we report kinetic isotope effect studies on recombinant trans-sialidase. β-Dideuterium and primary ^{13}C isotope effects were measured for a good substrate, sialyl-lactose, and a slow substrate, sialyl-galactose, in both acid-catalyzed solvolysis and enzymatic transfer reactions. The β-dideuterium isotope effect for sialyl-lactose in the acid hydrolysis reaction was 1.113 ± 0.012. The primary ^{13}C isotope effects for hydrolysis of sialyl-lactose and sialyl-galactose were 1.016 ± 0.011 and 1.015 ± 0.008, respectively. In the enzymatic transfer reactions, the β-dideuterium and primary ^{13}C effects for sialyl-galactose were 1.060 ± 0.008 and 1.032 ± 0.008, respectively. The isotope effects for hydrolysis describe a dissociative S_N1-like mechanism, and these data are contrasted by the data for the enzyme-catalyzed reaction. The enzymatic deuterium isotope effects are lower by a factor of 2, but the primary carbon isotope effects are higher by a factor of 2. This pattern describes a mechanism involving nucleophilic participation in the rate-determining transition state.

Trypanosoma cruzi, the agent of Chagas' disease, expresses on the parasite surface a series of glycoproteins, some of them with trans-sialidase activity. These trans-sialidases transfer a sialic acid (*N*-acetylneuraminic acid, NeuAc)[1] group from host glycoconjugates to parasite surface glycoconjugates, leading to the formation of α(2→3) linked products (Figure 1) that are thought to be involved in host cell invasion and immune masking (*1, 2*). Sialic acids are involved in a variety of biological processes such as cell adhesion, metastasis of tumor cells, and particularly cell−cell and cell−ligand interactions (*3*−*5*). In the parasite, the transfer of sialic acids by trans-sialidase appears to facilitate the internalization of *T. cruzi* by host nonphagocytes and phagocytes (*6, 7*), and to promote the escape from the vacuole after internalization (*8, 9*). Parasite surface sialic acids are also found to inhibit complement C3bBb assembly of the host immune system (*10*−*12*) and to help protect against antibody-mediated lysis in the absence of complement (manuscript in preparation). Trans-sialidase is therefore implicated in several important roles, suggesting that inhibition of this enzyme might interfere with several steps in the life cycle of the parasite.

Structurally, trans-sialidase is formed by two functionally separable domains. The N-terminal region contains the full catalytic activity. The C-terminal region consists of tandems of 12 amino acid repeats, which were identified as a major

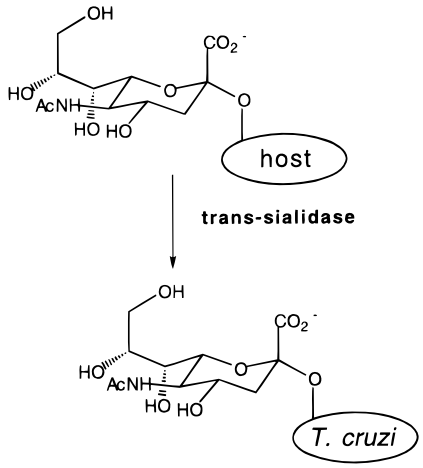

FIGURE 1: The transferase reaction catalyzed by trans-sialidase. The ovals schematically represent either host or *T. cruzi* cell-surface oligosaccharides.

antigen recognized by sera from Chagasic patients in the acute phase. Deletion of the C-terminal region has little effect on the enzymatic activity (*13, 14*). SAPA repeats promote trans-sialidase oligomerization and significantly increase the half-life of trans-sialidase activity in blood in the early stage of infection (*15, 16*). Sequence alignments between trans-sialidase and bacterial neuraminidases revealed some important similarities. Both enzymes have sequences of the amino acids SXDXGXTW (ASP boxes) at similar positions. These sequences are important to form turns in the β-propeller tertiary structure. More importantly, 14 identical or functionally similar amino acid residues in the active site, identified by X-ray crystallography of the *Salmonella typhimurium* enzyme (*17*−*19*), are conserved in the *T. cruzi* enzyme. The conserved residues include the triad of arginine

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[1] Abbreviations: NeuAc, *N*-acetylneuraminic acid; CMP-NeuAc, cytidine monophosphate glycoside of *N*-acetylneuraminic acid; KIE, kinetic isotope effect.

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residues which bind the NeuAc carboxylate group, and Tyr342 (Tyr374 of trans-sialidase), a possible active site nucleophile (*18*−*20*). Mutation of Tyr374 to Thr or Phe, or wild-type trans-sialidase variants which have a His374, are not catalytically active (*17*, *21*). Both *Salmonella typhimurium* sialidase and *T.cruzi* trans-sialidase belong to glycoside hydrolase family 33 in the Henrissat classification system (*22*). Although sharing structural similarities, trans-sialidase and neuraminidases have markedly different functionality. Neuraminidases are hydrolases with no or little transferase activity whereas trans-sialidase is mainly a transferase unless acceptors are not present (*23*). Structural features which confer the transferase activity have been explored through mutagenesis studies (*24*, *25*), but the reasons why the transferase predominates are not understood.

Trans-sialidase utilizes a wide range of glycoproteins, glycolipids, and oligosaccharides as substrates. It strongly prefers that the donor substrates have the presence of a NeuAc unit α(2→3) linked to a galactose residue and that the acceptor substrates have the presence of a nonreducing end terminal β-linked galactosyl residue (*26*). Trans-sialidase is distinct from sialyltransferase enzymes which utilize the sugar nucleotide CMP-NeuAc as the donor substrate. Trans-sialidase catalyzes transfer of NeuAc between saccharides with retention of configuration (*27*), which has some interesting implications in the mechanistic schemes discussed later in this paper. The rates of the glycosyltransfer reactions vary significantly with donor substrates having leaving groups of differing reactivity, which argued against a mechanism featuring a relatively long-lived sialosyl-enzyme intermediate and also indicated that initial glycosidic bond cleavage may be a slow step (*28*). Trans-sialidase displays a temperature dependence in which hydrolysis becomes favorable over the transfer reaction at higher temperatures. In part this is attributed to a lower affinity for the acceptor at higher temperature (*28*). Despite the structural models one may propose for trans-sialidase based on its sequence homology with sialidases of known structure, questions remain regarding the catalytic mechanism of the entire group of enzymes. The retention stereochemistry could be a sign of a double displacement mechanism with a covalent intermediate (*29*). Note that with glycosides of *N*-acetylneuraminic acid, the term "covalent intermediate" as it applies to reaction stereochemistry includes an intermolecular covalent intermediate between enzyme and substrate, and an intramolecular covalent intermediate in which the substrate carboxylate group has formed an α-lactone by bond formation at the anomeric carbon (*30*, *31*). At the other extreme, the mechanism could involve a short-lived oxocarbenium ion intermediate having one face shielded by the enzyme to produce products with retention stereochemistry. The NeuAc oxocarbenium ion has been detected in aqueous solution, raising its existence in an enzyme active site as a real possibility (*32*, *33*). In mechanisms involving covalent intermediates, a further question arises as to the timing of intermediate formation. Does the covalent intermediate arise by capture of an enzyme-bound oxocarbenium ion intermediate, or does it arise by more or less synchronous attack at the anomeric carbon while the bond to the leaving group is broken?

In this paper we report kinetic isotope effect (KIE) studies of the trans-sialidase enzyme. We studied the transferase reactions with the substrate sialyl-lactose, and the alternate slow substrate, sialyl-galactose. We measured the β-dideuterium and primary ^{13}C KIEs for both the acid solvolysis and the enzymatic transfer reactions. The results provide evidence for nucleophilic participation in the transition state of the trans-sialidase-catalyzed glycosyltransfer reaction. The transition state for this reaction is considerably different than the transition state that arises during acid-catalyzed solvolysis of NeuAc glycosides, CMP-NeuAc, and the transition state for sialyltransferase.

MATERIALS AND METHODS

Recombinant trans-sialidase was expressed in *E. coli* TG-1 cells and purified to homogeneity by the published method (*28*) with the following modifications. A Novagen Ni^{2+} affinity column was used instead of a Qiagen Ni^{2+}-NTA-agarose column, and a step gradient of imidazole buffers (150 and 500 mM) was used to elute the affinity column. The purified enzyme showed a single band by SDS−PAGE. ([1-^{14}C]Glc)-Lactose with specific activities of 54.3 and 60 mCi/mmol was purchased from Sigma Chemical Co. [6-^{3}H]-Glucose (27 Ci/mmol) was purchased from ICN Pharmaceuticals, Inc. [1-^{14}C]Galactose (52 mCi/mmol) and [6-^{3}H-(N)]galactose (29.5 Ci/mmol) were purchased from Moravek Biochemicals. Liquid scintillation fluid (ScintiSafe 30%) was purchased from Fisher. Liquid scintillation counting was performed using a Packard 1600 TR instrument which dumped data to a floppy disk for subsequent analysis on a personal computer. Recombinant rat liver α(2→3) sialyltransferase was purchased from Calbiochem−Novabiochem Corp. [2-^{13}C]Pyruvate was purchased from Isotec. NeuAc aldolase was purchased from Toyobo Co., Ltd. Plasmid pWV200B containing the gene for CMP-NeuAc synthase was a gift from Dr. W. Vann of the NIH. CMP-NeuAc synthase was expressed and purified according to the published method (*34*).

N-Acetylneuraminic Acid (NeuAc), Cytidine-5′-monophosphate N-Acetylneuraminic Acid (CMP-NeuAc), and Their Isotopomers. *N*-Acetylmannosamine and pyruvate were the substrates in the synthesis of NeuAc by NANA aldolase (*35*). In this synthesis, [2-^{13}C]pyruvate was utilized to prepare [2-^{13}C]NeuAc. [3,3′-$^{2}H_2$]NeuAc was synthesized by exchanging NeuAc in deuterium oxide at pD 12 (*36*, *37*). CMP-NeuAc and its isotopomers ([2-^{13}C]CMP-NeuAc and [3,3′-$^{2}H_2$-NeuAc]CMP-NeuAc) were synthesized by the reaction of CTP and NeuAc (or its isotopomers [2-^{13}C]NeuAc and [3,3′-$^{2}H_2$]NeuAc), catalyzed by CMP-NeuAc synthase (*32*, *38*, *39*). The yields for CMP-NeuAc and its isotopomers were over 90%. The complete deuterium exchange for [3,3′-$^{2}H_2$]-NeuAc and the retention of deuterium for [3,3′-$^{2}H_2$-NeuAc]-CMP-NeuAc were confirmed by the lack of 3,3′ hydrogen peaks in their ^{1}H NMR spectra.

[6-^{3}H]Lactose. The following one-pot reaction was run in pH 8.6, 100 mM glycine buffer. The reaction mixture contained 20 μCi of [6-^{3}H]glucose (27 Ci/mmol), 7.2 mM UDP-glucose, 5 mM Mn^{2+}, 50 mM KCl, 34 μL of 0.6% (w/v) α-lactalbumin solution (dissolved in pH 8, 50 mM gly-gly buffer), 0.1 unit of UDP-gal-4′-epimerase (dissolved in pH 7, 100 mM citric acid buffer), and 50 milliunits of galactosyltransferase (dissolved in 20 mM Tris-HCl buffer, pH 7.5, 2 mM EDTA, 2 mM 2-mercaptoethanol). The total volume was 1.0 mL. The reaction was run at 37 °C for 6 h

and was monitored by reaction of aliquots with ATP/hexokinase which converted unreacted [6-^{3}H]glucose into [6-^{3}H]glucose 6-phosphate. The separation of [6-^{3}H]glucose 6-phosphate from product [6-^{3}H]lactose on Dowex-1 (formate) columns (4 cm height in a Pasteur pipet) allowed estimation of the fractional conversion. After the fractional conversion had been determined to be 93%, the crude lactose product was chromatographed on a Dowex-1 (chloride) column in a Pasteur pipet which was eluted with deionized water. The silica TLC system $CHCl_3$/*i*-PrOH/H_2O, 2:7:1, was able to cleanly separate glucose and lactose (R_f = 0.32 and 0.1 for glucose and lactose, respectively), and was used to confirm the presence of lactose by its coelution with an authentic standard. Isolation of the glucose and lactose components confirmed the earlier estimate of ca. 90% conversion. The crude ([6-^{3}H]Glc)-lactose was used in the next step without further purification.

Synthesis of ([6-^{3}H]Glc)-, ([1-^{14}C]Glc)-, ([2-^{13}C]NeuAc, [1-^{14}C]Glc)-, and ([3,3′-$^2H_2$]NeuAc, [1-^{14}C]Glc)-Sialyl-lactose Isotopomers. The general procedure for sialyl-lactose synthetic reactions involved reaction of the appropriate CMP-NeuAc isotopomer and 10 μCi of ^{3}H- or ^{14}C-radiolabeled lactose (27 and 60 mCi/mmol, respectively) in 40 mM cacodylate buffer with 0.2 mg/mL BSA, 0.2% Triton CF-54, at pH 6.8, catalyzed by 10 milliunits of recombinant rat liver α(2→3) sialyltransferase.

The following concentrations of CMP-NeuAc and final reaction mixture volumes were employed. For the ([6-^{3}H]-Glc)-sialyl-lactose synthesis, 1.8 mM CMP-NeuAc was used in a reaction volume of 250 μL. For the ([1-^{14}C]Glc)-sialyl-lactose synthesis, 1.8 mM CMP-NeuAc was used in a reaction volume of 100 μL. For the ([2-^{13}C]NeuAc, [1-^{14}C]-Glc)-sialyl-lactose synthesis, 5 mM [2-^{13}C]CMP-NeuAc was used in a reaction volume of 120 μL. For the ([3,3′-^{2}H]-NeuAc, [1-^{14}C]Glc)-sialyl-lactose synthesis, 3.7 mM [3,3′-$^2H_2$]CMP-NeuAc was used in a reaction volume of 135 μL.

The sialyl-lactose synthetic reactions were run at 37 °C for 3 days. Reaction progress was followed by fractionation of reaction mixture aliquots on Dowex-1 (formate) mini-columns (4 cm height in Pasteur pipets). Initial washing with deionized water eluted unreacted lactose, and product sialyl-lactose was eluted with 200 mM ammonium formate, pH 6.6, which was then quantified by liquid scintillation counting. After the reactions had ceased to progress, the product was isolated by chromatography on a Dowex 1 × 8 (formate form) column (0.7 × 8 cm). The column was first washed with water, followed by pH 7.4, 5 mM ammonium bicarbonate buffer. The fractions containing sialyl-lactose were concentrated and desalted with Amberlite IR120(H^+) resin, and then further purified by anion exchange HPLC (MonoQ HR10/10, 0−5 mM NH_4HCO_3, 15% MeOH). Sialyl-lactose fractions were collected and desalted as described above. The final sialyl-lactose isotopomers were greater than 99.9% free of radioactive lactose, with yields ranging from 52 to 76% (Table 1). Sialyl-lactose prepared in this way comigrated with an authentic standard by silica TLC (EtOH/*n*-BuOH/pyridine/H_2O/HOAc, 100:10:10:30:3, v/v; visualized by heating a plate dipped in H_2SO_4/MeOH). The sialyl-lactose so obtained consisted of the two anomers at the Glc C-1. The ^{1}H NMR (300 MHz, pH 7, room temperature) spectrum of sialyl-lactose prepared by this method agreed with reported data (*40*, *41*) and also with standard sialyl-lactose purified from colostrum in this lab: δ = 1.8 (apparent t, J = 12.1, H3a), 2.02 (s, H of *N*-acetyl), 2.75 (d−d, J = 4.7, 12.4, H3e), 3.28 (t, J = 8.6, 0.6 H), 4.11 (d−d, J = 3.3, 10, 1H), 4.54 (d, J = 7.9, 1.5H), 5.28 (d, J = 3.4, 0.3H). The negative-ion FAB-MS (glycerol) of sialyl-lactose prepared by this method gave a molecular ion of 632.2039 (calculated 632.2038).

Synthesis of ([1-^{14}C]Gal)-, ([6-^{3}H]Gal)-, ([2-^{13}C]NeuAc, [6-^{3}H]Gal)-, and ([3,3′-$^2H_2$]NeuAc, [6-^{3}H]Gal)-Sialyl-galactose Isotopomers. Galactose is a poor substrate for α-(2→3) sialyltransferase with a high K_m of 268 mM (*42*). Therefore, the method for sialyl-lactose synthesis was modified for synthesis of sialyl-galactose. Reaction mixtures (∼50 μL) contained 70−100 milliunits of recombinant rat liver α(2→3) sialyltransferase and 5 units of alkaline phosphatase in 50 mM Tris-HCl, pH 7.5, containing 0.2 mg/mL BSA and 0.2% Triton CF-54. The reactions were typically conducted for 4 days at 30 °C. The same purification method used for sialyl-lactose was used to purify sialyl-galactose. The final sialyl-galactose isotopomers were greater than 99.9% free of radioactive galactose. The yields ranged from 75 to 82% and are presented in Table 1. The sialyl-galactose so obtained consisted of the two anomers at the Gal C-1. The ^{1}H NMR (300 MHz, pH 7, room temperature) of sialyl-galactose synthesized by this method agreed with reported (*40*) data: δ = 1.79 (apparent t, J = 12.1, H3a), 1.81 (apparent t, J = 12.1, H3a), 2.02 (s, Me of *N*-acetyl), 2.73, 2.75 (apparent overlapping d−d, J = 4.66, 12.34, J = 4.4, 12.2), 3.52 (d−d, J = 7.8, 9.7, 0.6H), 4.07 (d−d, J = 3.3, 9.8, 1H), 4.32 (d−d, J = 3.1, 10.3, 0.3H), 4.64 (d, J = 7.9, 0.6H), 5.28 (d, J = 4.0, 0.25H). The negative-ion FAB-MS (glycerol) of sialyl-galactose prepared by this method gave a molecular ion of 470.1508 (calculated 470.1510).

([1-^{14}C]Gal)-Sialyl-galactose. CMP-NeuAc (1.8 μmol) and [1-^{14}C]Gal (20 μCi, sp act. 52 mCi/mmol) were reacted with 100 milliunits of sialyltransferase to afford the title compound in 80% yield after HPLC purification.

([6-^{3}H]Gal)-Sialyl-galactose. CMP-NeuAc (1.8 μmol) and [6-^{3}H]Gal (30 μCi, sp act. 60 mCi/mmol) were reacted with 70 milliunits of sialyltransferase to afford the title compound in 75% yield after HPLC purification.

([2-^{13}C]NeuAc, [6-^{3}H]Gal)-Sialyl-galactose. [2-^{13}C]CMP-NeuAc (3.0 μmol) and [6-^{3}H]Gal (30 μCi, sp act. 20 mCi/mmol) were reacted with 70 milliunits of sialyltransferase to afford the title compound in 82% yield after HPLC purification.

([3,3′-$^2H_2$]NeuAc, [6-^{3}H]Gal)-Sialyl-galactose. [3,3′-$^2H_2$]-CMP-NeuAc (3.0 μmol) and [6-^{3}H]Gal (20 μCi, sp act. 20 mCi/mmol) were reacted with 100 milliunits of sialyltransferase to afford the title compound in 75% yield after HPLC purification.

Trans-sialidase Kinetic Experiments. Initial velocities were measured at 26 °C, pH 7.0, in a buffer system containing 20 mM HEPES and 2 mg/mL ultrapure BSA. For glycosyl-transfer reactions, the concentration of the acceptor substrate lactose was 100 mM. About 60 000 cpm of ([1-^{14}C]Glc)-sialyl-lactose (54.3 mCi/mmol) or ([1-^{14}C]Gal)-sialyl-galactose (52 mCi/mmol) was added into each reaction mixture (final volume 50 μL). Reactions were initiated by addition of 125 ng of trans-sialidase. Three time point aliquots (15 μL) were withdrawn within the initial velocity range. Aliquots were quenched into 1 mL of cold deionized water, and 960 μL of the quenched mixture was applied to

a Dowex 1 × 8 (formate) mini-column (4 cm height, Pasteur pipet). Product (radioactive lactose or galactose) was collected in the water fractions and quantified by liquid scintillation counting.

Control for Isotopic Fraction of Sialyl Glycoside on Dowex-1 (Formate) Resin. Individual controls were performed to test for isotopic fractionation of sialyl-galactose or sialyl-lactose on chromatography on Dowex-1 resin. Master $^3H/^{14}C$ mixtures of ([6-^{3}H]Gal)-sialyl-galactose and ([1-^{14}C]Gal)-sialyl-galactose, or ([6-^{3}H]Glc)-sialyl-lactose and ([2-^{13}C]NeuAc, [1-^{14}C]Glc)-sialyl-lactose were prepared. Each mixture was divided into two equal volume fractions. One fraction was transferred directly into an LSC vial with 4 mL of 200 mM ammonium formate (pH 6.6) added into the vial. The other fraction was loaded onto a 5 cm column of Dowex 1 × 8 (formate) in a Pasteur pipet which was then washed with 200 mM ammonium formate buffer to allow recovery of the substrates. Fractions (4 mL) were collected directly into LSC vials to which was added 16 mL of liquid scintillation fluid. Care was taken to collect the entire peak of eluted sialyl-lactose. All vials were counted 10 min each for 10 cycles.

KIE Experiment Accuracy Control. Mixtures of ([6-^{3}H]-Glc)-sialyl-lactose and ([2-^{13}C]NeuAc, [1-^{14}C]Glc)-sialyl-lactose were made to give $^3H/^{14}C$ ratios that would correspond to an actual KIE of 1.025. This was done by first individually measuring the cpm per unit mass of solutions of the ^{3}H- and ^{14}C-labeled sialyl-lactose isotopomers on an analytical balance. The masses of both isotopomer solutions required to give a KIE of 1.025 were then calculated and measured on the same analytical balance. The "KIE" was measured by the method described below, and the prepared solutions were treated as if they were actual KIE reaction mixtures. The measured KIE was compared with the expected KIE to provide a measurement of the accuracy of the KIE method.

Determination of the Extent of the Hydrolysis Reactions under the Conditions for the KIE Experiments. (a) Sialyl-lactose Reaction. A mixture of carrier-free ([9-^{3}H]NeuAc)-sialyl-lactose and ([1-^{14}C]Glc)-sialyl-lactose was included in a reaction mixture with 0.8 mM cold lactose and a buffer system containing pH 7.0, 20 mM HEPES and 2 mg/mL ultrapure BSA. The reaction was initiated by the addition of trans-sialidase. The reaction was allowed to proceed and then quenched in 0.5 mL of ice-cold deionized water. The reaction mixture was separated on an anion-exchange HPLC MonoQ column with a 0−5 mM ammonium bicarbonate gradient. ([1-^{14}C]Glc)-Lactose, ([9-^{3}H]NeuAc)-sialyl-lactose, ([1-^{14}C]-Glc)-sialyl-lactose, and [9-^{3}H]NeuAc peaks were collected into the LSC vials (4 mL/vial) which were assayed by liquid scintillation counting.

(b) Sialyl-galactose Reaction. Carrier-free ([3,3′-^{2}H]-NeuAc, [6-^{3}H]Gal)-sialyl-galactose and ([1-^{14}C]NeuAc)-sialyl-galactose were mixed in pH 7.0, 20 mM HEPES buffer with 2 mg/mL BSA and either 0.8 mM cold lactose or 100 mM cold lactose. The procedure described above for the sialyl-lactose reaction was followed. Reactant glycosides, product glycosides, and product galactose were separated from hydrolytic NeuAc and counted in the liquid scintillation counter as described above.

KIE Experiments. The competitive method was used to measure the *V/K* isotope effects (*43*). About 100 000 cpm each of ^{3}H- and ^{14}C-labeled substrates were included in one reaction. Radiolabeled substrates utilized in KIE experiments were greater than 99.9% free of lactose or galactose. A master mixture of $^3H/^{14}C$-labeled substrates was made from which aliquots were withdrawn and the reference $^3H/^{14}C$ ratio at time zero was determined. Reactions were initiated by addition of trans-sialidase for enzymatic reactions or HCl to a final concentration of 0.1 M for acid solvolysis reactions. Prechilled deionized water was added to stop the reaction after 40−60% conversion had been reached (*44*). The reaction mixture was immediately loaded onto Dowex 1 × 8 (formate form) mini-columns (5 cm height in a Pasteur pipet) to separate the unreacted substrate from the product. The column was first eluted with deionized water until all of the radioactive lactose or galactose eluted. The unreacted sialyl glycoside was obtained by elution with 200 mM ammonium formate buffer. Care was taken to collect the entire peak. The eluent (4 mL) was collected into liquid scintillation vials, to which 16 mL of liquid scintillation fluid was added. The $^3H/^{14}C$ ratio of the unreacted substrate was determined by dual-channel liquid scintillation counting as previously described (*45*). The observed KIE was calculated according to eqs 1−3. Equation 1 or 2 is used when the heavy isotope-labeled substrate has a ^{3}H or ^{14}C remote label, respectively. Equation 3 is used to correct the observed isotope effect for fractional conversion (*f*) (*46*). Three independent experiments were conducted for measurement of all KIEs.

$$KIE_{observed} = (^{14}C/^{3}H)_0/(^{14}C/^{3}H)_t \quad (1)$$

$$KIE_{observed} = (^{3}H/^{14}C)_0/(^{3}H/^{14}C)_t \quad (2)$$

$$KIE_{corrected} = \ln(1 - f)/\ln[(1 - f)KIE_{observed}] \quad (3)$$

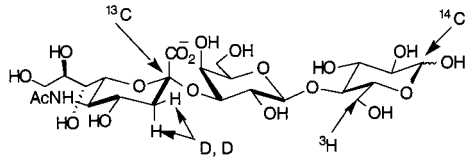
The enzymatic KIE reactions were performed at 26 °C in 20 mM HEPES containing 2 mg/mL BSA at pH 7.0. The typical sialyl-galactose KIE reaction mixture was 50 μL in volume, contained 100 mM lactose, and was initiated by addition of 500 ng of trans-sialidase in a 10 μL volume. Approximately 5 h was required to reach 50% conversion. The typical sialyl-lactose KIE reaction mixture was 50 μL in volume, contained 0.8 mM lactose, and was initiated by addition of 50 ng of trans-sialidase in a 1 μL volume. Approximately 30−60 min was required to reach 50% conversion. For the acid solvolysis KIEs, 0.2 M HCl solution was added into an equal volume of $^3H/^{14}C$-labeled substrate mixture (final volume = 50 μL) to initiate the reactions which were conducted at 37 °C.

RESULTS

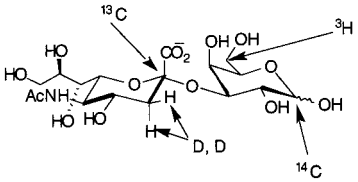
Isotopomer Syntheses. Sialyl-lactose (NeuAcα(2→3)Galβ-(1→4)Glc) and sialyl-galactose (NeuAcα(2→3)Gal) isotopomers have been synthesized with rat-liver α(2→3) sialyltransferase which mediates regiospecific formation of an α-glycosidic bond between carbon 2 of NeuAc and the 3-OH group of a galactose residue. The desired isotopic substitution patterns were obtained by combination of the appropriate CMP-NeuAc and lactose or galactose isotopomers. Table 1 presents the identity and yield of the isotopomers that have been prepared. The α(2→3) sialyltransferase readily accepts

Table 1: Sialyl-lactose and Sialyl-galactose Isotopomers

compound	isotope and position	yield (%)
sialyl-galactose	[3,3′-$^2H_2$]NeuAc, [6-^{3}H]Gal	75
sialyl-galactose	[1-^{14}C]Gal	80
sialyl-galactose	[2-^{13}C]NeuAc, [6-^{3}H]Gal	82
sialyl-galactose	[6-^{3}H]Gal	75
sialyl-lactose	[3,3′-$^2H_2$]NeuAc, [1-^{14}C]Glc	67
sialyl-lactose	[2-^{13}C]NeuAc, [1-^{14}C]Glc	76
sialyl-lactose	[1-^{14}C]Glc	74
sialyl-lactose	[6-^{3}H]Glc	52



sialyl-lactose, NeuAcα(2-->3)Gaβ(1-->4)Glc



sialyl-galactose,NeuAc α(2-->3)Gal

FIGURE 2: Sialyl-lactose and sialyl-galactose structures and positions of isotope labels for compounds utilized in competitive KIE experiments.

lactose as an acceptor substrate; however, galactose is a poor substrate. Syntheses with galactose required longer reaction time and more sialyltransferase. They were further optimized by removal of inhibitory CMP with alkaline phosphatase and maintenance of a slightly alkaline pH and lower reaction temperature to help stabilize the acid-labile CMP-NeuAc.

KIE Methodology for Trans-sialidase. The structures presented in Figure 2 identify the locations of the isotope labels in sialyl-lactose and sialyl-galactose substrates used in the KIE experiments. Remote ^{3}H and ^{14}C labels on the aglycon moieties act as remote reporters for the stable deuterium or ^{13}C isotope labels present on the NeuAc residue. Isotope effects measured with trace radiolabels report on the kinetic parameter *V/K* (*47*). The kinetic isotope effect is manifested as a change of the initial ^{3}H/^{14}C ratio over the course of the reaction, which is detected by liquid scintillation counting of residual substrate, fractionated from reaction mixtures by chromatography on anion-exchange mini-columns.

KIE Method Control Experiments. Chromatography of a ^{3}H/^{14}C mixture of sialyl-lactose on a Dowex-1 (formate) mini-column eluted with 200 mM ammonium formate gave a ^{3}H/^{14}C ratio of 3.387, compared to a value of 3.387 found before chromatography. The results indicate that insignificant isotopic fractionation occurs during chromatography on Dowex-1. The total counts applied to and recovered from the column were found to be 20 675 and 20 431 cpm, indicating that the recovery from the column is >98.8%. A ^{3}H/^{14}C mixture of sialyl-galactose gave a ratio of 2.528 after Dowex-1 (formate) mini-column, compared to 2.525 found before chromatography. The recovery from the column is >99.5%. When mixtures of ^{3}H- and ^{14}C-labeled substrates were prepared to reflect the results anticipated for a KIE of 1.025, chromatography of these mixtures afforded a "mock" KIE of 1.028 ± 0.007. For an enzymatic reaction, the remote labels used in the competitive method may sometimes introduce a binding isotope effect. These were investigated by using two substrates with only the ^{3}H or ^{14}C remote labels. The results (Table 2) showed that for sialyl-lactose, there is a small inverse binding isotope effect; while for sialyl-galactose, there is a 1.024 normal binding isotope effect. Therefore, the measured isotope effects have to be corrected by this effect. For the acid solvolysis reaction, however, the same control experiment yielded a unity KIE of 1.002 ± 0.005. This result was expected because there should be no binding effect in a solvolysis reaction. Due to the reversibility of the trans-sialidase-catalyzed transfer reaction, a large excess of unlabeled acceptor substrate lactose was used in all KIE experiments to prevent isotope scrambling which results if the reverse reaction is allowed to occur. In a control experiment, a mixture of sialyl[6-^{3}H]galactose, [1-^{14}C]-galactose, and a large excess of unlabeled lactose was allowed to proceed to 50% completion. The remaining substrate contained no detectable ^{14}C, which excludes any significant isotopic scrambling under the conditions for carrying out the KIE experiments. Because trans-sialidase catalyzes hydrolysis in addition to its transferase activity, it was necessary to show that no hydrolysis occurs under the conditions used to measure KIEs for the transferase reaction. Utilizing sialyl-lactose or sialyl-galactose with a radiolabel in the NeuAc sugar, hydrolysis would manifest in the production of free radiolabeled NeuAc, whereas transferase activity would not. So long as sufficient acceptor lactose was present, hydrolysis was completely suppressed (Figure 3a–c). The small differences in the retention times of the middle peak in the chromatograms presented in Figures 3a and 3b,c are due to two reasons. First, the middle peak in 3a is composed of sialyl-lactose only, whereas the peak in 3b,c is mainly sialyl-galactose, which has a longer retention time than that of sialyl-lactose. Second, the sample loadings were different. The results are accurate enough to differentiate the NeuAc peak from the sialyl-lactose/sialyl-galactose peaks.

Table 2: Kinetic Isotope Effect Data[a]

location/type of KIE	acid solvolysis	enzymatic transfer	corrected KIE[d]
β-dideuterium	1.113 ± 0.012 (SL)	1.046 ± 0.008 (SL)[b]	1.053 ± 0.010
		1.042 ± 0.01 (SL)[c]	1.049 ± 0.013
		1.085 ± 0.006 (SG)	1.060 ± 0.008
[2-^{13}C] primary	1.016 ± 0.011 (SL)	1.014 ± 0.012 (SL)	1.021 ± 0.014
	1.015 ± 0.008 (SG)	1.056 ± 0.005 (SG)	1.032 ± 0.008
control KIE		0.993 ± 0.008 (SL)	
		1.024 ± 0.006 (SG)	

[a] "SL" = sialyl-lactose; "SG" = sialyl-galactose. [b] 0.8 mM lactose. [c] 8.0 mM lactose. [d] The KIEs in the previous column were divided by the value of the control KIE: 0.993 for transfer reactions with SL, and 1.024 for transfer reactions with SG.

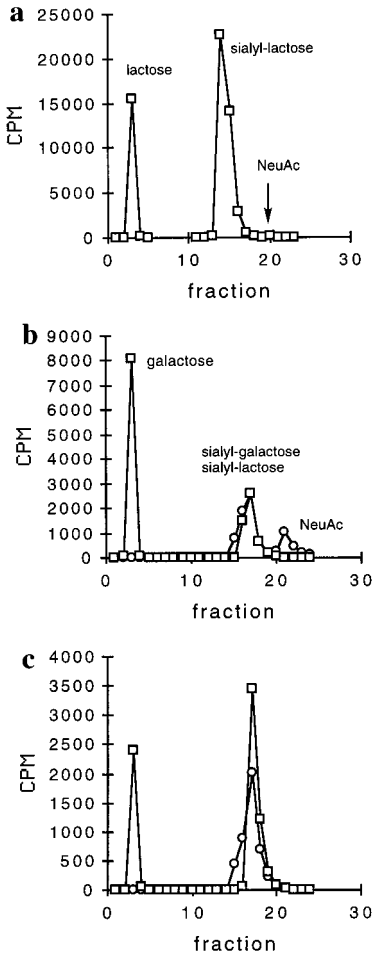


FIGURE 3: (a) Determination of the extent of the hydrolysis reaction of sialyl-lactose under KIE conditions. The anion exchange HPLC chromatogram depicts the elution of lactose, sialyl-lactose, and NeuAc. (b, c) Determination of the extent of the hydrolysis reaction of sialyl-galactose under the conditions for KIE experiments with sialyl-galactose. Carrier-free ([3,3′-$^2H_2$]NeuAc, [6-^{3}H]Gal)-sialyl-galactose and ([1-^{14}C]NeuAc)-sialyl-galactose were mixed in pH 7.0, 20 mM HEPES buffer with 2 mg/mL BSA and either 0.8 mM cold lactose (b, upper curve) or 100 mM cold lactose (c, lower curve). The products were separated by anion exchange HPLC with a 0−5 mM ammonium bicarbonate gradient. Galactose and lactose eluted in fractions 2−4, sialyl glycosides eluted in fractions 15−20, and [1-^{14}C]NeuAc eluted in fractions 20−23. The circles represent the ^{14}C counts; the squares represent the ^{3}H counts.

Thus, it is clear from the results that for transfer between sialyl-lactose and lactose, 0.8 mM lactose was sufficient to ensure that only transferase activity occurred. The poorer substrate sialyl-galactose required much higher concentrations (100 mM) of lactose to saturate the enzyme and eliminate competing hydrolysis.

KIE Experiments. Kinetic isotope effects for trans-sialidase-catalyzed transfer of sialyl-lactose and sialyl-galactose to acceptor lactose are presented in Table 2. In the enzymatic transfer reactions, β-^{2}H isotope effects were measured with two lactose concentrations (0.8 and 8 mM) in otherwise identical reaction mixtures with sialyl-lactose as the donor substrate. The measured values were 1.046 ± 0.008 and 1.042 ± 0.01, which gave 1.053 ± 0.010 and 1.049 ± 0.013 after correction for the binding isotope effect and propagation of error. The KIE values for sialyl-galactose reactions are identical to those for the sialyl-lactose reactions within experimental error. The acid solvolysis KIE experiments required relatively mild conditions due to the lability of glycosidic bonds to NeuAc (0.1 N HCl, 37 °C, 10 h). The stabilities of the products lactose and NeuAc were estimated by incubating them under reaction conditions for 30 h, and the structures were confirmed unchanged by NMR. The measured β-dideuterium KIE for sialyl-lactose hydrolysis is 1.113 ± 0.012. The ^{13}C primary KIEs for hydrolysis of sialyl-lactose and sialyl-galactose are 1.016 ± 0.011 and 1.015 ± 0.008, respectively.

Kinetic Parameters for Sialyl-galactose. We were unable to detect saturation of trans-sialidase by sialyl-galactose even at concentrations up to 100 mM. Therefore, the K_m for sialyl-galactose is greater than 100 mM, and is at least 100 times greater than the K_m for sialyl-lactose (*28*). Since saturation of trans-sialidase with sialyl-galactose could not be obtained, no estimate of the V_{max} for this substrate is yet available. We were able to estimate that the k_{cat}/K_m for sialyl-galactose is approximately 200 times smaller than the k_{cat}/K_m for sialyl-lactose.

DISCUSSION

Kinetic Mechanism. The kinetic mechanism for trans-sialidase has not yet been established. One tool to help rule out possibilities is the substrate concentration dependence of measured KIEs (*48*). The KIE data presented in Table 2 help rule out an ordered sequential mechanism involving initial binding of the labeled donor followed by acceptor. Essentially unchanged β-^{2}H KIEs are observed (1.046 to 1.042) at lactose concentrations of ∼10 and 100 times the K_m (*28*). If the kinetic mechanism were ordered with the labeled donor sialyl-lactose binding first, saturating acceptor lactose would trap all of the labeled sialyl-lactose on the enzyme and drive the KIE to unity, which was not observed (*48*).

Kinetic Complexity and the Isotope Effects. Kinetic complexity can mask the full value of a kinetic isotope effect (*49*). When an isotope-sensitive step in a reaction is clearly the slowest in the catalytic sequence and it is irreversible, the *V/K* KIEs measured for the step provide information on the change in structure that occurs between ground state and transition state (*50*). The observed trans-sialidase isotope effects are very similar for the fast substrate sialyl-lactose and the slow substrate sialyl-galactose. That *V/K* is about 200 times smaller for sialyl-galactose indicates very little external commitment to catalysis exists. The pattern of a small deuterium and (relatively) large carbon isotope effect indicates that these are intrinsic isotope effects, free of significant commitment factors. Specifically, if there were a commitment factor in operation, the intrinsic values of the β-^{2}H and primary ^{13}C KIEs would *both* be higher than the observed values, a pattern which is unknown to us. With net retention stereochemistry, trans-sialidase is likely to proceed in two steps with an enzyme-bound intermediate. The isotope effects measured here report on the first step since trans-sialidase rates and isotope effects are dependent on the nature of the leaving group aglycon (*28*).

Transition State and Mechanism. Many glycosyl group transfer reactions have been found to proceed by transition states that are dissociative in character (*51*). By dissociative, we specifically mean that loss of bonding between the leaving group and anomeric carbon has progressed further than bond

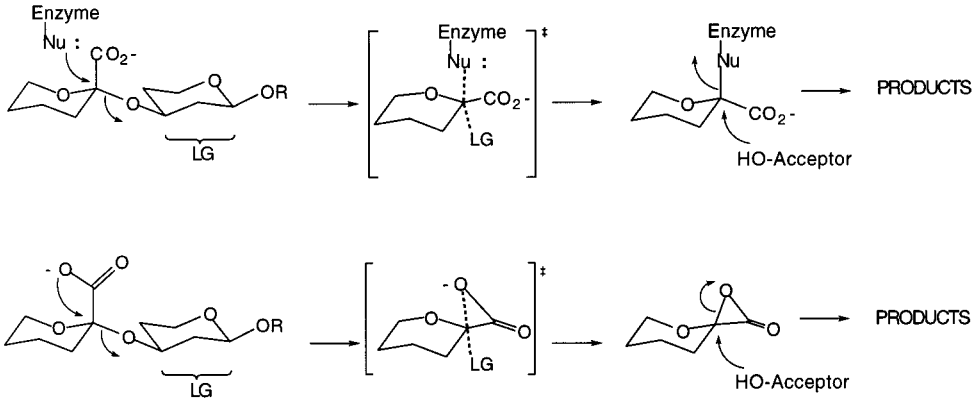


FIGURE 4: Proposed mechanisms for trans-sialidase-catalyzed reaction. -R = glucosyl residue; substituents on the NeuAc and Gal rings have been omitted for clarity. The top equation represents a pathway with a transition state having nucleophilic participation with an enzyme group. The bottom equation represents a pathway involving formation of an intermediate α-lactone.

formation between the nucleophile and the anomeric carbon. In one mechanistic limit, bond formation does not occur at all in the transition state, and glycosyl oxocarbenium ions can form as transient intermediates. Note that covalent intermediates could occur by subsequent attack on the oxocarbenium ion. At the other extreme, the transition state could feature considerable nucleophilic participation. In this case, covalent intermediates are formed directly instead of via oxocarbenium ion intermediates. It is likely that the nature of the leaving group, the inherent stability of the glycosyl donor as an oxocarbenium ion, and contributions from the enzyme active site will determine whether oxocarbenium ion intermediates are formed in enzyme active sites. We have found the cytidine monophosphate glycoside of *N*-acetylneuraminic acid (CMP-NeuAc) reacts in mild acid solution to form a short-lived oxocarbenium ion intermediate, and recent work on sialyltransferase indicates that the enzymatic transition state is very similar to the one in solution (*32*, *33*, *52*, *53*). A key feature of NeuAc is the carboxylate group, which is proposed to stabilize the oxocarbenium ion by short-range electrostatic interactions. On the other hand, aryl glycosides of NeuAc appear to solvolyze under weakly acidic conditions with the carboxylate group functioning as an intramolecular nucleophile, possibly leading to strained α-lactone intermediates (*30*). Another important consideration is the nature of carboxylate−enzyme interactions. Ion pairing between the NeuAc carboxylate and an active site arginine triad is indicated by crystallographic analyses of *Salmonella* sialidase (*18*, *19*). Given the conservation of these residues in trans-sialidase, it is likely that a similar interaction exists. It has been suggested that the carboxylate/arginine triad interaction may dictate the behavior of the carboxylate group during sialidase catalysis, and it is likely that this too is an important consideration for the trans-sialidase mechanism (*30*). These different mechanistic paths for group transfer of the same sugar challenge the idea that solution and enzyme mechanisms are identical, and, as will be presented below, two enzyme transition states for transfer of the same sugar can be considerably different.

The β-^{2}H KIE's presented in Table 2 clearly indicate that the transition state for solvolysis (KIE = 1.113) and the transition states for the enzymatic transfer of NeuAc to lactose (KIE's 1.049−1.060) are different. The β-^{2}H KIEs derive from hyperconjugation with the anomeric oxocarbenium ion carbon. The amount of charge development will place a limit on the size of the β-^{2}H KIE, and the observed value is further dependent on the dihedral angular relationship that describes the orbital overlap between the CH (or CD) bond and the vacant p orbital of the adjacent oxocarbenium ion carbon (*54*). However, the ^{13}C KIE data discussed below suggest that the difference between the solvolysis and enzymatic β-^{2}H KIEs rests primarily in a difference in charge development, not transition state dihedral bond angles.

The primary ^{13}C KIE of 1.015 shows that the sialyl-lactose hydrolysis transition state is a dissociative one with little nucleophilic participation. This is in contrast to the enzymatic reaction, which has a primary ^{13}C KIE of 1.032 for transfer of sialyl-galactose to lactose. This ^{13}C isotope effect can be converted to the corresponding ^{14}C KIE of 1.062 with eq 4 (*55*). With few exceptions, reported glycosyltransfer primary ^{14}C KIEs fall within the range of 1.01−1.05 (any ^{13}C values were converted to the corresponding ^{14}C values); the reactions encompass enzymatic hydrolyses, phosphorolysis, and solution hydrolyses of nucleosides and fluoro- and alkyl-glucosides (*56*−*64*).

$$(^{12}k/^{13}k)^{1.9} = {}^{12}k/{}^{14}k \qquad (4)$$

The primary ^{13}C KIE observed here for trans-sialidase is too large to allow the mechanism to be classified as the typical dissociative or "S_N1"-like mechanism often associated with glycohydrolases and glycoside hydrolysis reactions (*51*, *65*, *66*). Exceptions to this pattern are found in the large ^{13}C KIE's of 1.032 and 1.028 reported for the aqueous hydrolysis of α-glucosyl fluoride and α-glucosidase-catalyzed hydrolysis of α-D-glucopyranosyl pyridinium bromide, respectively (*67*, *68*). In both of these cases, transition states with nucleophilic participation were proposed. The trans-sialidase primary ^{13}C KIE is just large enough to equal smaller KIEs measured for true S_N2 reactions. For example, displacement of 1-phenyl-1-bromoethane with sodium ethoxide affords a ^{13}C KIE of 1.036 (*69*). This reaction shows a first-order dependence on the concentration of ethoxide and is certainly a bimolecular process. The 1.032 ^{13}C KIE and the 1.060 β-^{2}H KIEs measured for trans-sialidase argue for a transition state with nucleophilic participation and limited oxocarbenium ion character, which must collapse to a covalent intermediate after the transition state as presented in Figure 4. We suggest that for trans-sialidase catalysis, nucleophilic participation is enforced by the architecture of the active site and may facilitate expulsion of the aglycon.

A covalent intermediate in trans-sialidase catalysis could serve more than one purpose. A covalent intermediate would allow transfer of the NeuAc residue to the acceptor hydroxyl with overall retention stereochemistry. Another advantage to the covalent intermediate over a shielded oxocarbenium ion might be related to lifetime. The short lifetime (and high reactivity) of the NeuAc oxocarbenium ion could make it unselective regarding capture by water or by an acceptor saccharide hydroxyl group (*70*). The covalent intermediate might facilitate the selective transfer to a saccharide acceptor over water by chemically sequestering the NeuAc residue until it reaches a geometric relationship with the bound acceptor saccharide that is competent for glycosyltransfer.

The existence of covalent intermediates has been well established in several hydrolase reactions, by their successful trapping and characterization (*71*−*74*). Such intermediates may be reached by collapse of oxocarbenium ion/active site side chain ion pairs, or directly by participation of active site nucleophiles in the initial glycosidic bond breaking step. In the case of trans-sialidase, we have used carbon and deuterium isotope effects to identify the transition-state signature of a forming covalent intermediate. This not only identifies a subsequent covalent intermediate, but also rules out that it originated by capture of an oxocarbenium ion.

At this time the identity of the proposed nucleophile is unproven, leading to the two mechanistic scenarios portrayed in Figure 4. It could be the NeuAc carboxylate group (*30*), or a group in the enzyme active site that acts as a nucleophile in the transition state, possibly Tyr 374 as mentioned previously. The inhibitor 2,3-dehydro-3-deoxyneuraminic acid is a geometric analogue of an oxocarbenium ion-like transition state because of its planarity at the position isosteric with the anomeric carbon of the substrate. This compound shows strong inhibition of many sialidases (*75*). However, it does not inhibit trans-sialidase (*76*), suggesting a different transition state character in the trans-sialidase-catalyzed glycosyl transfer reaction (or a more restrictive active site). It is also interesting to note that nucleophilic participation during glycosyltransfer of NeuAc by sialyltransferase does not occur. The rat liver $\alpha(2\rightarrow6)$ sialyltransferase transition state features nearly full oxocarbenium ion charge development (β-^{2}H KIE = 1.218) and no indication of nucleophilic participation (1° ^{14}C KIE = 1.030; equal to a ^{13}C KIE of 1.016) (*53*). These observations show that glycosyltransfer reactions of the same sugar can span a considerable mechanistic breadth.

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