Isotope Trapping and Kinetic Isotope Effect Studies of Rat Liver α-(2→6)-Sialyltransferase†

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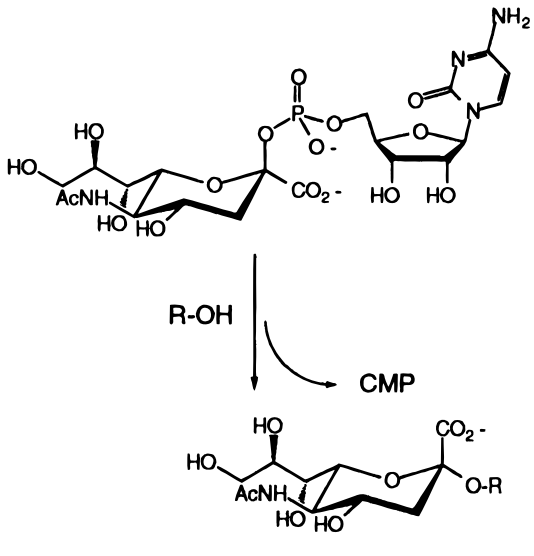
ABSTRACT: A mechanistic study of rat liver α-(2→6) sialyltransferase (ST) is presented that includes isotope trapping experiments and kinetic isotope effects on *V*/*K* for the ST-catalyzed reaction of isotopically labeled CMP−*N*-acetylneuraminate and *N*-acetyllactosamine. The isotope trapping experiments confirmed that the kinetic mechanism is steady-state random, and further analysis indicated that for this sialyltransferase the experimentally observed isotope trapping ratio (product trapped/substrate released) was equivalent to the commitment to catalysis, C_f, the quantity required to correct the kinetic isotope effects. C_f was found to range from 1.0 (at 1.6 mM LacNAc) to 1.7 (at 100 mM LacNAc). After correction for C_f, the isotope effects were as follows: secondary β-dideuterium, 1.04−1.05; anomeric carbon primary ^{14}C, 1.000 ± 0.004; a small ^{3}H binding effect of 1.016 ± 0.007 at C9; and a carboxylate carbon secondary ^{14}C isotope effect of 0.998 ± 0.004. This pattern of KIEs is quite different than observed for solvolysis of CMP-NeuAc [Horenstein, B. A., and Bruner, M. (1996) *J. Am. Chem. Soc. 118*, 10371−10379]. Based on the results of *ab-initio* modeling of isotope effects, a hypothesis is presented which reconciles the unusual pattern of KIEs on the basis of binding interactions at the carboxylate carbon.

Sialyltransferases catalyze the transfer of the *N*-acetylneuraminic acid (NeuAc)[1] portion of CMP-NeuAc with inversion of configuration to acceptor hydroxyl groups at or near the nonreducing terminus of oligosaccharide chains of glycoproteins and glycolipids (*1*, *2*). Diverse roles have been suggested for the biological function of sialic acid-containing glycosides (*3*); one emergent theme has been their role as biological recognition elements. They are implicated or involved in a number of cell−cell interactions such as masking of trypanosomal immunogenicity, viral infection and replication, and cell adhesion (*4*−*7*). CMP-NeuAc is the common sialyl donor, but STs differ in acceptor sugar specificity (*8*, *9*). Members of this family appear to share a conserved region of sequence similarity termed the "sialyl motif" which is associated with the recognition of the CMP portion of CMP-NeuAc (*10*); sialyltransferases do not appear to share sequence similarity with other glycosyltransferases and glycohydrolases, including neuraminidases.



R = glyco-protein,lipid, oligosaccharide

One of the better-characterized sialyltransferases is the α-(2→6) enzyme from rat liver. Obtained as the lumenal C-terminal catalytic domain due to proteolysis during isolation, the enzyme is N-glycosylated with a molecular mass of ∼41 kDa for the protein (*11*). The enzyme has a broad acceptor specificity (*12*−*14*), but prefers sialylation of terminal Gal-β-(1→4)-GlcNAc residues (*15*) and will also tolerate minor modification of the NeuAc residue in the donor substrate CMP-NeuAc (*16*, *17*). The enzyme has no apparent

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[1] Abbreviations: C_f, forward commitment to catalysis; CMP-NeuAc, cytidine monophosphate glycoside of *N*-acetylneuraminic acid; EIE, equilibrium isotope effect; KIE, kinetic isotope effect; LacNAc, *N*-acetyllactosamine; NeuAc, *N*-acetylneuraminic acid; ST, sialyltransferase.

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Mg or Mn requirements,[2] and has a sequential mechanism on the basis of steady-state kinetic studies (*15*, *18*). The enzyme is strongly inhibited by cytidine nucleotides and modestly inhibited by donor or acceptor substrate analogs (*18*, *19*), but potent mechanism-based or transition state inhibitors have not yet been described (*20*).

One question we have concerns what effect the unique structure and reactivity of CMP-NeuAc may have on sialyltransferase catalysis, and a broader concern is the details of glycosyltransferase mechanisms in general, since these enzymes have not been examined as closely as glycosidases (*21*−*23*). The sugar nucleotide CMP-NeuAc and the related CMP-KDO are unusual as glycosyltransferase donor substrates because they are monophosphate glycosides, not pyrophosphate glycosides, and because they contain a carboxylate group immediately adjacent to the anomeric carbon. These compounds are characterized by high reactivity in aqueous solution, even at neutral pH (*24*, *25*). In the most general terms it is reasonable to suppose that there will be some mechanistic similarity between glycosidases and glycosyltransferases, but the differences in substrate structure and reactivity suggest that important differences may exist. For example, the acid-catalyzed solvolysis work on CMP-NeuAc (*26*) has shown that it forms an oxocarbenium ion intermediate in solution, with a lifetime $\sim 10^2$ greater than that of the glucosyl cation. The bulk of the greater stability was attributed to the carboxylate group, and we are curious as to what role the carboxylate group may serve in sialyltransferase enzymology.

In this paper we report the results of isotope trapping experiments for rat liver α-(2→6) sialyltransferase, an analysis of the relationship between experimentally obtained isotope trapping ratios and commitment to catalysis, a multiple KIE study of rat liver α-(2→6) sialyltransferase, and isotope effects calculated by *ab-initio* methods, which explore how ^{2}H and ^{14}C isotope effects may be influenced by electrostatic interactions, hydrogen bonding, and ionization state of the CMP-NeuAc carboxylate group.

MATERIALS AND METHODS

Materials. Buffers and reagents were purchased from Sigma and Fischer. NeuAc aldolase was purchased from Shinko American. Rat liver α-(2→6) sialyltransferase was puchased from Sigma or Boehringer. *N*-Acetylmannosamine isotopomers ([6-^{3}H] and [1-^{14}C-*N*-*acetyl*]) and sodium pyruvate (1-^{14}C and 2-^{14}C) were purchased from New England Nuclear and Moravek. [^{3}H]Acetic anhydride was purchased from New England Nuclear. Cytidine triphosphate (CTP) and cytidine diphosphate (CDP) were purchased from Sigma as the disodium salt with 2.5 equiv of hydration. Liquid scintillation fluid (Scintisafe) was purchased from Fischer. Plasmid pWV200B harboring the expression construct for *Escherichia coli* CMP-NeuAc synthase was a gift from Dr. W. F. Vann at the National Institutes of Health (*27*). CMP-NeuAc synthase was purified from *E. coli* JM109 following the published protocol (*28*) and was judged 90−95% pure based on SDS−PAGE analysis. Amberlite IR120-H^+ form resin used for desalting CMP-NeuAc was first washed with 95% ethanol and then washed extensively with deionized water.

Instruments. ^{1}H-NMR spectra were measured at ambient temperature on a Varian Gemini 300 spectrometer operating at 300 MHz. Spectra were obtained in 99.9% D_2O referenced to the HDO peak (4.80 ppm). NMR analyses of ^{3}H-containing samples were performed in 9-in. flame-sealed tubes. HPLC was performed on a Rainin HPXL gradient unit interfaced to a Macintosh personal computer. A Rainin Dynamax UV-1 detector was employed to monitor separations at 260 nm. 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.

[^{3}H-acetyl]-N-Acetylmannosamine. [^{3}H-*acetyl*]-*N*-Acetylmannosamine was synthesized by acetylation of mannosamine hydrochloride by an adaptation of Roseman's method (*29*). The following reaction was carried out in the original ampule containing the [^{3}H]acetic anhydride. A mixture of 5 mCi [^{3}H]acetic anhydride (0.105 mmol, 47.8 μCi/μmol), 250 mg of damp Dowex 1 × 8-200 resin (CO_3^{2-}), and mannosamine hydrochloride (21.6 mg, 0.1 mmol) in 0.50 mL of H_2O was maintained at 0−4 °C for 2 h. The aqueous solution was carefully removed from the reaction ampule by syringe; the residue in the reaction ampule was washed with 2 × 2 mL of H_2O, and the combined washings and reaction solution were passed through a short pipette containing Amberlite IR120 (H+) resin into a round-bottom flask, which was then fit to a short-path distillation head with a single receiver. The solution was heated to boiling, and then allowed to cool to room temperature. The mixture was then concentrated to dryness *in vacuo*, keeping the bath temperature below 55 °C. The pot residue was resuspended in 2 mL of water, then reconcentrated. The crude [^{3}H-*acetyl*]-ManAc was purified (0.86 mCi, 34% yield) by HPLC (t_R = 7.3 min, C_{18}, 1 × 30 cm, 5% MeOH/H_2O, A_{220}). Aliquots of the purified [^{3}H-*acetyl*]ManAc co-eluted with an authentic sample of ManAc, and unlabeled ManAc which had been prepared by the above method.

Synthesis of CMP-NeuAc isotopomers. [^{3}H-*acetyl*]- and [^{3}H-*acetyl*; 3,3-^{2}H]CMP-NeuAc were synthesized and purified in 34 and 39% yields, respectively, from [^{3}H-*acetyl*]-ManAc as previously described (*26*). We were unable to detect any remaining protium at C3 of [^{3}H-*acetyl*; 3,3-^{2}H]-CMP-NeuAc by ^{1}H-NMR analysis, indicating complete exchange. Other radiolabeled CMP-NeuAc isotopomers used in this study were synthesized and purified as previously described (*26*); however, we wish to emphasize an important point concerning the anion exchange chromatographic purification of CMP-NeuAc. In radioisotopic syntheses, the intermediate radiolabeled NeuAc was not isolated; hence, reactants pyruvate and ManAc are present in the crude CMP-NeuAc. ManAc is easily removed on a MonoQ HR10/10 column, but pyruvate elutes shortly before CMP-NeuAc; if ^{14}C labeled, pyruvate present in the CMP-NeuAc can introduce an artifact in measured KIEs. We recommend use of 75 mM ammonium bicarbonate buffer rather than 100 mM, as we previously reported, and careful monitoring of fractions by absorbance at 220 nm and liquid scintillation counting to confirm the location of the pyruvate peak and its separation from CMP-NeuAc. Both [1-^{14}C]- and [2-^{14}C]

[2] Identical initial velocities were measured in the presence or absence of EDTA, suggesting no involvement of a loosely bound Mg or Mn ion.

CMP-NeuAc employed in KIE experiments were greater than 99.7% pyruvate-free.

Isotope Trapping Experiments. Isotope trapping experiments were carried out at 37 °C; the method for isolation of radioactive sialyl-LacNAc is a modification of a previously reported method (*15*). A 30 μL solution of 40 mM cacodylate buffer, pH 7.0, containing 2 mg/mL BSA, 1mg/mL NaN_3, 0.1% Triton CF-54, 320 μM [9-^{3}H]CMP-NeuAc (0.104μCi/nmol), and 0.0185 nmol α-(2→6)-sialyltransferase (concentration, 0.62 μM) was preincubated at 37 °C for 1 min. A 1.0 mL solution of 40 mM cacodylate buffer, pH 7.0, containing 2 mg/mL BSA, 1mg/ml NaN_3, 0.1% Triton CF-54, 5.5 mM CDP, and the appropriate concentration of LacNAc was incubated at 37 °C for 2 min. The sialyltransferase/CMP-NeuAc solution (28 μL of 30 μL) was added to the 1 mL chase solution with rapid vortexing. After 2−3 s, 980 μL of the solution was applied to a column (1.5 × 10 cm) of Dowex 1 × 2-200 anion exchange resin (P_i form). The column was eluted with 4 mL portions of 1 mM phosphate buffer, pH 7.0, and fractions were collected directly into liquid scintillation vials for counting. A total of 26 fractions were collected (104 mL) until the entire product (sialyl-LacNAc) peak had eluted. The experiment was done in duplicate with both runs yielding the same results within experimental error.

Control experiments demonstrated that 320 μM CMP-NeuAc is sufficient to saturate (≥95%) sialyltransferase; identical amounts of radioactive product formation were noted at 320 or 640 μM CMP-NeuAc. A control to account for post single-turnover activity was also done in which enzyme was added to the final reaction mixture containing both substrates as well as inhibitor (CDP) at their final concentrations; no product was seen in this case, demonstrating that CDP effectively traps free sialyltransferase. An additional control experiment demonstrated that recovery of the product sialyl-LacNAc from the Dowex-1 resin was quantitative, within experimental error. A mock isotope trapping reaction mixture was prepared in the same buffer system used in the isotope trapping experiments described above (including CDP and LacNAc), and 0.0067 nmol of [9-^{3}H]-α-(2→6)-sialyl-LacNAc. The mixture was applied to a 1.5 × 10 cm column of Dowex-1 × 2-200 resin (P_i form) and eluted with 1 mM phosphate buffer, pH 6.8, to afford, quantitatively, 0.0069 nmol of [9-^{3}H]-α-(2→6)-sialyl-LacNAc by liquid scintillation counting. The small (3%) excess over theoretical is reasonable given uncertainty in counting and pipetting.

General KIE Methodology. Kinetic isotope effect experiments for sialyltransferase used ca. 100 000 cpm each of the appropriate pair of ^{3}H- and ^{14}C-labeled CMP-NeuAc isotopomers. A master stock of ^{3}H/^{14}C-labeled isotopomers was prepared from which aliquots were withdrawn for individual reactions and measurement of the ^{3}H/^{14}C ratio at time 0. A master stock containing 3.2 or 200 mM LacNAc and 2× buffer (80 mM cacodylate, 4 mg/mL BSA, 1 mg/mL NaN_3, pH 7) was also prepared to use in the reactions. Reactions were initiated by combining equal volumes of the two master stock solutions (25 μL each) and adding the appropriate amount of enzyme to give 40−60% (*30*) completion in 10−15 min at 37 °C. Unreacted CMP-NeuAc was isolated from the reaction mixture by HPLC, and ^{3}H/^{14}C ratios were determined by liquid scintillation counting as previously described (*26*).

Equations 1 and 2 were used to calculate the ^{3}H and ^{14}C KIEs, respectively. The raw KIEs were then corrected for % conversion with eq 3 to obtain KIE_{exp} (*31*).

$$KIE = (^{14}C/^{3}H)t_o/(^{14}C/^{3}H)_t \quad (1)$$

$$KIE = (^{3}H/^{14}C)t_o/(^{3}H/^{14}C)_t \quad (2)$$

$$KIE_{exp} = \ln(1-f)/\ln[(1-f) \times KIE_{obsd}) \quad (3)$$

Previous control experiments established that the HPLC methodology does not introduce artifactual isotopic fractions, and the same KIEs were determined using this methodology or by direct-rate determinations (*26*). We have measured identical β-^{2}H KIEs with rat liver α-(2→6)-sialyltransferase from different suppliers. KIEs were also measured using CMP-NeuAc containing only the remote radiolabels (e.g., [9-^{3}H]; [1-^{14}C-acetyl]) to control for any binding isotope effect contributions to the observed KIEs.

Calculation of Equilibrium Isotope Effects. Equilibrium isotope effects for interconversion of the structures presented in Figure 3 were calculated (*32*) as previously described (*33*) using Gaussian 94, Revision C (*34*) with the 6−31G* basis to determine energy minima and force constants, and QUIVER for calculation of the isotope effects (*35*). All structures were optimized to tight criteria, and frequencies were scaled by 0.9. Calculations were run on a Silicon Graphics Indigo XZ workstation and an IBM RS6000 SP2 computer.

RESULTS

Kinetic Isotope Effect Experiments. The *V*/*K* KIEs on the enzyme catalyzed sialyltransferase reaction at pH 7 and 37 °C, were measured using the dual-label competitive method (*36*, *37*); the sites of isotopic labeling are identified in Figure 2 and the results are presented in Table 1 (column 3). Entries 1−3 present the results for β-dideuterium KIEs which overall are rather small, ranging from 1.022 to 1.033. A slight increase in KIE was observed when the remote tritium label is moved from the *N*-acetyl group to the 9-position (entries 1 and 2) and a small decrease when the acceptor LacNAc concentration is increased from 1.6 to 100 mM (entries 2 and 3). These observations suggest that a small binding isotope effect may be operative, and as discussed later, the kinetics are steady-state with an associated commitment to catalysis.

A primary ^{14}C KIE of 1.000 ± 0.004 at the C2 anomeric carbon was measured using a mixture of [2-^{14}C]- and [^{3}H-*N-acetyl*]CMP-NeuAc (entry 4). The unity value for this isotope effect was unexpectedly low and will be discussed later in more detail. A unity secondary ^{14}C KIE of 0.999 ± 0.004 at the C1 carboxylate carbon was measured using a mixture of [1-^{14}C]- and [^{3}H-acetyl]CMP-NeuAc (entry 5). Using this same CMP-NeuAc pair of isotopomers we also measured the KIE for solvolysis of CMP-NeuAc at pH 5 in acetate buffer to obtain a value of 1.013 ± 0.004 for the secondary ^{14}C isotope effect at the carboxylate carbon (entry 5, column 6). While this is still a large secondary carbon

Table 1: Kinetic Isotope Effects for Rat Liver α-(2→6)-Sialyltransferase

	CMP-NeuAc pairs	KIE_{exp}[a,b]	$KIE_{intrinsic}$[c]	KIE_{cor}[d]	solvolysis
1	[^{3}H-*acetyl*; 3,3′-^{2}H] [1-^{14}C-*acetyl*]	1.022 ± 0.007 (2)	1.044 ± 0.007	1.044 ± 0.007	1.276 ± 0.008[e]
2	[9-^{3}H; 3,3′-^{2}H] [1-^{14}C-*acetyl*]	1.033 ± 0.007 (4)	1.066 ± 0.007	1.049 ± 0.014	
3	[9-^{3}H; 3,3′-^{2}H] [1-^{14}C-*acetyl*]	1.025 ± 0.007 (4)	1.068 ± 0.007	1.051 ± 0.014	
4	[2-^{14}C] [^{3}H-*acetyl*]	1.000 ± 0.004 (3)	1.000 ± 0.004	1.000 ± 0.004	1.030 ± 0.005[e]
5	[1-^{14}C] [^{3}H-*acetyl*] [^{3}H-*acetyl*]	0.999 ± 0.004 (4)	0.998 ± 0.004	0.998 ± 0.004	1.013 ± 0.004[g]
6	[9-^{3}H] [1-^{14}C-*acetyl*]	1.008 ± 0.007 (4)	1.016 ± 0.007	1.016 ± 0.007	1.002 ± 0.01[e]
7	[^{3}H-*acetyl*] [1-14C-acetyl]	1.003 ± 0.004 (3)	1.006 ± 0.004	1.006 ± 0.004	

[a] KIEs were measured with 1.6 mM LacNAc at pH 7 and 37 °C as described in Materials and Methods. [b] The reported value is the mean ± SD for *n* separate experiments. [c] Corrected for C_f as described in the text. [d] Intrinsic values for KIE experiments which used a [9-^{3}H] remote label were corrected for the binding isotope effect (entry 6), with propagation of error. [e] Reference *26*. [f] KIE measured with 100 mM LacNAc. [g] This work.

isotope effect, we had previously reported (*26*) a greater value of 1.037 for this KIE, which we now consider as erroneous due to contamination with [1-^{14}C]pyruvate.

The dual-label competitive method relies on the use of remote reporter labels which ideally should not experience a KIE, as this would require a correction for the observed isotope effect with an associated propagation of error. A KIE of 1.008 ± 0.007 was observed for sialyltransferase with [9-^{3}H; 1-^{14}C-*acetyl*]CMP-NeuAc isotopomers as substrate; experiments with the tritium label moved to the *N*-acetyl group afforded a KIE of 1.003 ± 0.004 (entries 6 and 7). Note that in solution, solvolysis of [9-^{3}H; 1-^{14}C-*acetyl*]CMP-NeuAc gives a KIE of unity. We conclude that a small binding isotope effect is associated with the [9-^{3}H] label. The observed KIEs presented in Table 1 (column 3) were corrected for both the commitment factor as described below (column 4) and binding isotope effect if the experiments used [9-^{3}H] as a remote reporter label (column 5). The corrected β-dideuterium KIEs range from 1.046 to 1.051; the primary ^{14}C KIE at the anomeric carbon is unity, and the corrected secondary ^{14}C KIE at the carboxylate carbon is 0.998 ± 0.004.

Isotope Trapping Experiments. A variant of Rose's isotope trapping experiment (*38*, *39*) was employed to estimate the stickiness factor of the labeled substrate CMP-NeuAc which provides insights on the kinetic mechanism and is required for the interpretation of KIEs (*40*). A binary complex of ST and saturating [9-^{3}H]CMP-NeuAc (320 μM) was allowed to form (Scheme 1, bottom), and then rapidly mixed into a much larger volume containing various concentrations of LacNAc and a fixed, saturating concentration of CDP (*41*), an inhibitor competitive for CMP-NeuAc (*19*). In this experimental design, saturating levels of CDP ensure that upon dissociation of CMP-NeuAc*, free ST is trapped, thus providing single turnover conditions. Control experiments (described in Materials and Methods) established that CDP is an effective quench. The isotope trapping factor is expressed in eq 4

$$\text{trapping ratio} = [P^*]/([E_T] - [P^*]) \qquad (4)$$

where [P*] is the radioactive sialyl-LacNAc formed in a single turnover, and the denominator represents labeled

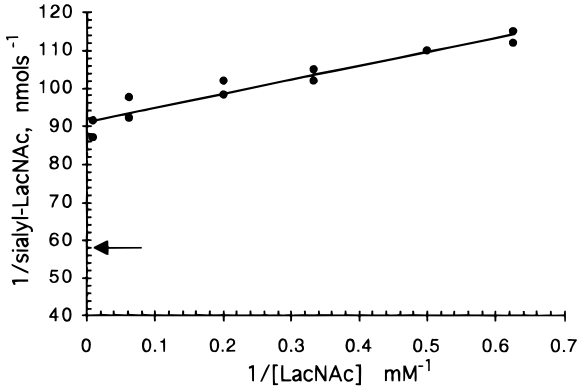


FIGURE 1: Isotope trapping of CMP-NeuAc on sialyltransferase. Experiments were run at 37 °C and pH 7, and employed 0.0173 nmol of ST·CMP-NeuAc which was quenched into a larger volume containing varied amounts of acceptor LacNAc and 5.5 mM CDP. Each data point (●) refers to the amount of radioactive sialyl-LacNAc formed (nmol^{-1}) in a single isotope partition experiment at a given concentration of LacNAc (mM^{-1}). The line was fit to the data by linear regression analysis. The arrow on the ordinate scale represents the maximum amount of trapping theoretically possible.

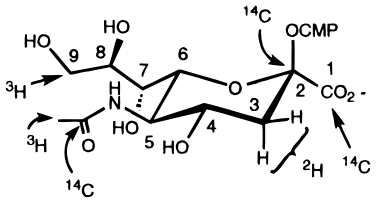
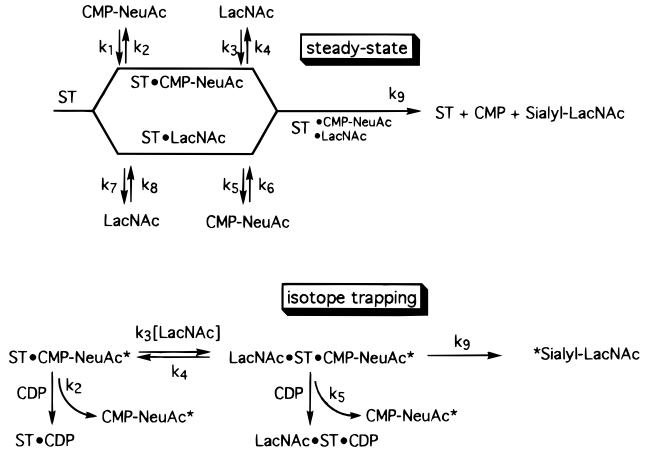


FIGURE 2: CMP-NeuAc isotopomers. The structure shown identifies the identity and location of isotopes incorporated into CMP-NeuAc for the kinetic experiments.

CMP-NeuAc which dissociated from the enzyme. The results of the experiments are presented in Figure 1; the isotope trapping ratio for sialyltransferase was determined to range from 1 to 1.7 between 1.6 and 100 mM LacNAc concentrations. The apparent dissociation constant of LacNAc was 410 ± 35 μM, as determined from the ratio of slope and *y*-intercept.

A Relationship between Isotope Trapping Experiments and Commitment to Catalysis. The isotope trapping ratio (eq 4) obtained from the isotope trapping experiment is conceptually equivalent to commitment to catalysis, but these two quanti-

Scheme 1



ties are not necessarily equal (*42*) since the former is obtained under single turnover conditions. The key physical feature that leads to potential nonequivalence is that in the isotope trapping experiment once the complex of labeled substrate and enzyme dissociates it cannot reform (nor form product) whereas in the steady state conditions described by commitment to catalysis, labeled substrate and enzyme may combine and dissociate many times over the reaction time course. Qualitatively, this difference might be expected to lead to an underestimate of commitment to catalysis in some cases, but our ultimate goal of correcting the observed KIEs for commitment to catalysis required placing this qualitative relationship in an algebraic context, as described below.

For a random kinetic mechanism (Scheme 1), the isotope partition experiment has been described by eq 5 (*43*). The collection of rate constants which comprise the steady-state commitment to catalysis for V/K is defined by eq 6 (*44*). It can be shown algebraically that C_f is related to the isotope trapping ratio by eq 7, where the quantity $[P^*](k_2/k_3[B] + 1)$ must be less than $[E_T]$; B refers to LacNAc in all equations.

$$[P^*]/[E_T] = k_3k_9[B]/[k_2(k_4 + k_5 + k_9) + [k_3(k_5 + k_9)[B]]] \quad (5)$$

$$C_f = k_9/[k_5 + [k_2k_4/(k_2 + k_3[B])]] \quad (6)$$

$$C_f = [P^*][(k_2/k_3[B]) + 1]/[[E_T] - [P^*][(k_2/k_3[B]) + 1]] \quad (7)$$

Consideration of eq 7 will show that if the rate at which the labeled substrate/enzyme binary complex proceeds to the ternary complex ($k_3[B]$) is much faster than the rate of dissociation of the binary complex (k_2), the isotope trapping experiment provides C_f since eq 7 reduces to eq 4. When these conditions do not apply, knowledge of the ratio $k_2/k_3[B]$ may be required to determine C_f from the isotope trapping experiment via use of eq 7.

Commitment to Catalysis for Sialyltransferase. The isotope trapping ratios for sialyltransferase at 1.6 and 100 mM LacNAc were 1.0 and 1.7, respectively. One approach to relating these trapping ratios to the sought-after commitment to catalysis would be application of eq 7. The off-rate for labeled CMP-NeuAc in the binary complex (k_2) can be estimated to be 3−4.7 s^{-1} from the isotope trapping data (*43*), but we do not have knowledge of the on-rate for LacNAc (k_3, eq 7). Typical small molecule−protein association rate constants are in the range of 10^6−10^8 M^{-1} s^{-1} (*45*). Numerical models[3] of eq 7 with reasonable values for k_3 (e.g. $\geq 1 \times 10^5$ M^{-1} s^{-1}) showed that the isotope trapping ratios were insignificantly lower than the commitment to catalysis, and therefore useful directly. Also, when [LacNAc] = 100 mM, even very low values for k_3 produced agreement between the isotope trapping results and the commitment to catalysis. Though the results were encouraging, we sought additional support given the uncertainty in k_3.

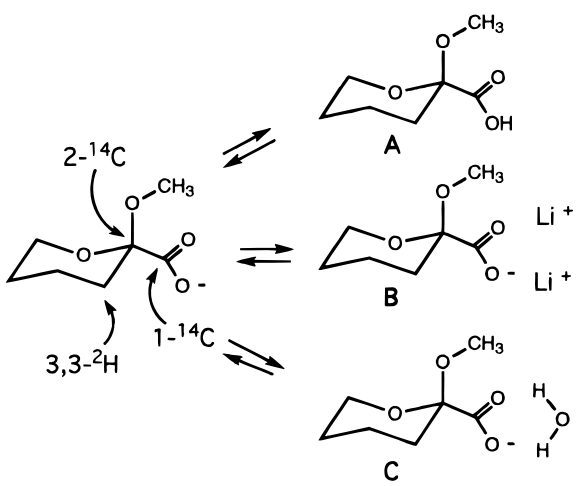
We measured KIEs at low and high concentrations of LacNAc and used the observed isotope trapping ratios at these same concentrations as *assumed* commitments. If the isotope trapping results were indeed equivalent to the commitment, the corrected isotope effects at high and low [LacNAc] should have been identical, and we found this to be the case. When the observed β-^{2}H kinetic isotope effects at 1.6 and 100 mM LacNAc (1.033 and 1.025; Table 1) were corrected[4] by C_f = 1.0 and 1.7 using eq 8 (*44*), nearly identical isotope effects of 1.066 and 1.068 were obtained. This excellent agreement shows that the isotope trapping ratios are reasonable for application as commitment factors, and these values were therefore used for correcting the observed KIEs reported in Table 1.

$$KIE_{obsd} = (KIE_{intrinsic} + C_f)/(1 + C_f) \quad (8)$$

Calculated Equilibrium Isotope Effects. As will be discussed in more detail later, we observed an unusual pattern of KIEs, in particular the unity KIE at the anomeric carbon of CMP-NeuAc. To facilitate the interpretation of the experimentally determined KIEs, we have calculated equilibrium isotope effects for CMP-NeuAc carboxylate group interactions to serve as a model for the differential interactions the carboxylate group might realize when binding to sialyltransferase. The chemical model for equilibrium binding isotope effects is presented in Figure 3. Equilibrium isotope effects were calculated for conversion of a CMP-NeuAc model to species **A**, **B**, and **C**, which represent different active site interactions involving the carboxylate group. The simplified carboxylate interaction partners were selected and considered reasonable for the following reasons. (1) **A**, **B**, and **C**, respectively, represent protonation, ion pairing, and hydrogen bonding at the carboxylate group which are the sort of chemically reasonable interactions we anticipate might exist in the active site. (2) Bonds to the substrate carboxylate group are not covalent (excepting model **A**), and hence the differences in mass between the model interaction partner and the "true" active site interaction partner should have a minimal effect on vibrational modes at isotopic sites in the substrate. (3) Location of the minimum on the potential energy surface was rather slow with a substantial computational overhead, placing a practical

[3] Note that the [P*] and $(k_2/k_3[B] + 1)$ terms of eq 7 are not independent. To arrive at the commitment factor, [P*] was recalculated from eq 5 on the basis of the new value of k_3, while maintaining the K_m for LacNAc by adjusting k_4. Rate constants k_2 = 3−4.7 s^{-1} and k_5 = 1.6 s^{-1} were estimated as described (*43*), and k_9 = 3 s^{-1} (*15*).

[4] We have been unable to observe any reverse reaction using sialyllactose and radiolabeled CMP, indicating that the reaction is effectively irreversible, hence the lack of C_r terms in eq 8.



Equilibrium Isotope Effects

	1-^{14}C	2-^{14}C	3,3-^{2}H
A	0.993	0.986	0.977
B	0.998	0.993	0.962
C	0.999	0.997	0.993

FIGURE 3: Calculated equilibrium isotope effects for carboxylate interactions. The scheme on the left identifies the structures and location of isotopes for the models used to calculate equilibrium isotope effects. The methyl ketal on the left is the reactant state model for CMP-NeuAc. The structures **A**, **B**, and **C** represent protonation, ion pairing, and hydrogen bonding at the carboxylate group. The table on the right presents the calculated equilibrium isotope effects for proceeding from the reactant state to either **A**, **B**, or **C**. The isotope effects for the reactant/model **B** equilibrium were calculated with an aqueous solvation model (dielectric = 78).

limitation on the complexity of the system that could be treated with *ab-initio* calculations.

The results (Figure 3) show that significant inverse isotope effects are predicted at both the anomeric carbon and β-hydrogens for the **A** and **B** carboxylate group interactions, and the carboxylate carbon is relatively insensitive to protonation, which is in agreement with the near unity ^{14}C-fractionation factor observed for ionization of carboxylic acids (*46*). Comparison of bond lengths about the anomeric carbon in models **A** and **B** with those for the reactant state CMP-NeuAc model showed that an overall tightening at the anomeric carbon takes place on formation of **A** or **B**. Interestingly, when we calculated the β-^{2}H isotope effect for the *pro-R* and *pro-S* monodeuterio species, we found that the majority of the inverse β-^{2}H effect resides at the equatorial H3 hydrogen.

DISCUSSION

Kinetic Mechanism and Commitment to Catalysis. As mentioned, previous kinetic studies of rat liver α-(2→6)-sialyltransferase are consistent with a random sequential mechanism. The results of the isotope trapping experiments contribute two clarifying points. First, the kinetics are steady-state since in a rapid equilibrium system substrate off-rates would be so high as to preclude formation of labeled product. The isotope trapping experiments also provide strong evidence for a random kinetic mechanism. In a strictly ordered mechanism, if CMP-NeuAc were bound first, all of it would be trapped as product at saturating LacNAc, which was not observed (Figure 1); the observation of a finite KIE at saturating LacNAc (Table 1, entry 3) also rules out this ordered pathway (*47*). A strictly ordered mechanism with CMP-NeuAc binding second would not have afforded any trapped product, since the binary complex ST·CMP-NeuAc could not have formed.

Another use of the isotope trapping experiment is to obtain a measure of the commitment to catalysis (*48*), so the observed kinetic isotope effects may be corrected. The results of the isotope trapping experiment may be used to estimate commitment factors directly in a random mechanism with the following considerations. The isotope trapping ratio determined at *saturating* levels of the second substrate will closely approximate C_f because under these conditions the initial binary complex is driven to the ternary complex from which two events can occur: dissociation of the labeled substrate and product formation (trapping). The observed isotope trapping depends solely on the ratio of the rates for these two events. Under steady-state conditions with saturating second substrate, C_f is determined by the same rate constants (*47*), so the experimentaly determined isotope trapping ratio gives a direct value for C_f. Furthermore, in many cases isotope trapping will closely approximate C_f even if the second substrate is not saturating, as long as the labeled substrate off-rate in the binary complex is slow relative to the on-rate for the second substrate (k_3[B], Scheme 1). Isotope trapping will fail as a measure of C_f if the labeled substrate/enzyme binary complex dissociates faster than it can proceed to the productive ternary complex; any observed isotope trapping will result in a significant underestimate of the true value of C_f, which instead may be obtained by eq 7.

In the case of sialyltransferase, the relatively loose binding of LacNAc and the limits placed on the off-rate for CMP-NeuAc (ca. 3−4.7 s^{-1}) argue that the term $[(k_2/k_3[B]) + 1]$ of eq 7 should be very close to 1, but we could not rule out the possibility that k_3 is very slow. However, the substrate concentration dependence of the KIEs is well-fit to isotope trapping ratios of 1.0 and 1.7 for concentrations of LacNAc equal to 1.6 and 100 mM, respectively. As a final point, we cannot yet rule out that an undetected internal commitment to catalysis exists; we hope to directly resolve this question in the near future with a series of CMP-NeuAc analogs that are slow substrates.

Kinetic Isotope Effects. We seek a detailed understanding of sialyltransferase catalysis, which includes a comparison of how it may differ or resemble the acid-catalyzed solvolysis of the sialyltransferase donor substrate CMP-NeuAc. A useful approach to this line of inquiry is to use the results of multiple KIE experiments to model transition state structure (*49*). Since the first applications with lysozyme (*36*), KIEs have been important tools for description of glycosylase and

solvolysis transition states (*22*), and as recently affirmed by X-ray crystallography, can yield an accurate model of enzyme substrate interactions suitable as an aid to inhibitor design (*50*, *51*). Unlike the case for glycosidases, only a few single KIEs have been measured for glycosyltransferases (*52*, *53*), and the potentially more powerful multiple KIE method has not yet seen application.

Measurement of β-^{2}H KIEs for glycosyltransfer reactions is a way to gauge the extent of charge development at the anomeric carbon, information which allows assessment of the degree of bonding at this carbon in the transition state. After correction for commitment and remote-label binding isotope effects, the KIEs for entries 1−3 of Table 1 converge to values of ∼1.05. The observed β-^{2}H kinetic isotope effects are much smaller than observed for solvolysis of CMP-NeuAc (1.276; *26*). The results clearly suggest that the transition state for the sialyltransferase reaction does not have the extensive oxocarbenium ion character found for solvolysis of CMP-NeuAc. The β-^{2}H isotope effects are almost identical to those measured (1.058 ± 0.019) for hydrolysis of the *p*-nitrophenyl glycoside of NeuAc by the neuraminidase from *Vibrio cholerae* (*54*). The low value for this isotope effect was attributed to intramolecular nucleophilic participation by the carboxylate group adjacent to the anomeric carbon. While we consider this specific feature unlikely for sialyltransferase (the neuraminidase is retaining, sialyltransferase is inverting), the same effect on the sialyltransferase β-^{2}H KIE can be realized if the transition state involves significant partial bonding to the leaving group CMP, which would have low charge development.

Finally, we discount conformational issues as the primary basis for the small β-^{2}H KIEs measured for sialyltransferase. The magnitude of a β-^{2}H KIE has an angular dependence (*55*); hyperconjugation between the β C−L bond and the electron deficient p-orbital overlap will be at a maximum at 0 or 180°, and a minimum at 90°. The β-^{2}H KIEs observed for solvolysis of CMP-NeuAc are 4−5 times larger than for sialyltransferase; the solvolysis reaction has essentially full oxocarbenium ion character at the transition state (*26*). We performed simple calculations as outlined by Sinnott (*56*), which show that when the maximum KIE is equal to the value observed for solvolysis of CMP-NeuAc, no ring conformations can result in a β-^{2}H KIE of 1.05, the value for sialyltransferase. To get this low, it is necessary to start with less than full charge development at the anomeric carbon. The mechanistic implication of the small β-^{2}H KIE is that the transition state is dissociative and early.

Carbon KIEs. In carbon transfer chemistry, primary ^{14}C KIEs range from about 1.02 to 1.14; those at the low end are associated with S_N1-like or dissociative transition state character, while isotope effects near the high end are associated with S_N2 processes which are associative in nature (*57*−*59*, *63*). The observed unity primary ^{14}C KIE for sialyltransferase is totally inconsistant with nucleophilic participation in the transition state. Solvolysis of CMP-NeuAc proceeds with a primary ^{14}C KIE of 1.030, typical of a transition state which is extremely late, oxocarbenium ion-like, and without nucleophilic participation. For late dissociative transition states, the near complete loss of the C−O glycosidic bond vibration is nearly offset by partial π bonding of the C−O endocyclic oxocarbenium ion, the reason for the low but non-unity ^{14}C isotope effect. Ready explanations for the small size (1.000) of the analogous primary ^{14}C KIE for sialyltransferase are not obvious. Any bond cleavage at the anomeric carbon should have a normal contribution due to reaction coordinate motion, and at least in a qualitative sense, partial π-bonding may be less important for the sialyltransferase transition state since it has much less charge development than the transition state for solvolysis of CMP-NeuAc. We thus face an unusual primary ^{14}C KIE. One way to account for the results is to postulate that during the catalytic cycle, binding CMP-NeuAc includes a binding isotope effect with an inverse contribution; in such a case a small normal effect could be offset to a near-unity value. We consider and find some support for this hypothesis on the basis of calculations discussed later.

The unity (0.998 ± 0.004) secondary ^{14}C KIE at the carboxylate carbon is consistant with the sialyltransferase transition state having only modest charge development. Acid-catalyzed solvolysis of [1-^{14}C; ^{3}H-*acetyl*]CMP-NeuAc affords a secondary KIE at the carboxylate of 1.013 ± 0.004, which can be attributed to a loosening of carboxylate vibrational modes via electrostatic interactions in the oxocarbenium ion-like transition state (*26*, *33*). Comparison of β-^{2}H isotope effects for these two processes demonstrates that there is much less charge development for the sialyltransferase transition state than for solvolysis of CMP-NeuAc, so the size of the secondary ^{14}C carboxylate KIEs are correlated with transition state oxocarbenium ion character. In the case of the solvolytic reaction, with its large transition state charge development, the carboxylate has a clear role in electrostatic stabilization of the dissociative transition state, and while a similar role is likely to be operative in the sialyltransferase transition state, additional active site interactions are likely involved in the apparent shift to an earlier position on the reaction coordinate for cleavage of the CMP-NeuAc glycosidic bond.

Binding Isotope Effects Induced by Carboxylate Binding Interactions. We have observed an unusual pattern of isotope effects for the ST-catalyzed reaction. To reiterate, the β-^{2}H effects are low and argue for partial bonding at the anomeric carbon (which should give a non-unity ^{14}C primary KIE), the ^{14}C primary KIE is unity which clearly rules out an associative S_N2-like process, but is even lower than that for a late S_N1 process (which should have a large β-^{2}H KIE). We were curious as to whether and how binding interactions at the carboxylate group could contribute to the isotope effects. There is no systematic information to allow us to predict the magnitude of binding isotope effects for sialyltransferase; this constraint led us to an *ab initio* modeling approach. Unlike empirical approaches, no assumptions need be made regarding the force matrix used to calculate the isotope effects. An example which underscores our belief in the importance of applying a nonempirical approach to this problem may be found in our observation that the isotope effect at the carboxylate carbon of CMP-NeuAc is complex, arising from changes in a number of different vibrational modes, with both normal and inverse contributions to the net isotope effect (*33*).

The results of the calculations presented in Figure 3 indicate that the process of ligating the CMP-NeuAc carboxylate group (either by a proton or ion pair) can in theory produce binding isotope effects, even at isotopic substitution sites *removed* from the carboxyl group. Given the ubiquity

of carboxylate-substituted enzyme substrates, in future studies it will be of interest to determine the scope of this observation through computational and experimental approaches. In the present case, the inverse isotope effects as large as 0.965 and 0.986 for the β-^{2}H and anomeric carbon ^{14}C isotopomers would have an important contribution to the experimentally observed KIEs, raising the β-^{2}H effect to 1.088 and the primary ^{14}C effect to 1.014. These values could be readily interpreted in terms of an S_N1-like process, with the contradiction of the unity ^{14}C KIE having been removed. Clearly the models we used are unlikely to be an actual portrayal of the sialyltransferase active site, but we suggest that their importance lies in their successful demonstration of the feasibility of the binding isotope effect hypothesis in this system. It is evident that binding isotope effects can and do make significant contributions to observed enzyme KIEs when small isotope effects are being measured (*60*−*62*). We are very interested in the prospects for measuring binding isotope effects between sialyltransferase and CMP-NeuAc.

In conclusion, rat liver α-(2→6)-sialyltransferase has a steady-state random kinetic mechanism with a significant commitment to catalysis for CMP-NeuAc. The kinetic isotope effect data are most consistent with an early dissociative transition state which is unlike the late transition state for solvolysis of the donor substrate CMP-NeuAc. There is no indication of any significant nucleophilic participation by the acceptor substrate LacNAc in the transition state, which is mechanistically equivalent to the results for the solvolytic reaction. The unity ^{14}C primary KIE for the sialyltransferase reaction is unusual, however, and its mechanistic significance is under investigation.

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REFERENCES

1. Comb, D. G., Watson, D. R., and Roseman, S. J. (1966) *J. Biol. Chem. 241*, 5637−5642.
2. Schauer, R. (1982) *Adv. Carbhydr. Chem. Biochem. 40*, 131−234.
3. Varki, A. (1993) *Glycobiology 3*, 97−130.
4. Lowe, J. B. (1994) in *Molecular Glycobiology* (Fukuda, M., Hindsgaul, O., Eds.) pp 163−205, Oxford University Press, New York.
5. Schauer, R., Kelm, S., Reuter, G., Roggentin, P., and Shaw, L. (1995) in *Biology of the Sialic Acids* (Rosenberg, A., Ed.) pp 7−67, Plenum Press, New York.
6. Powell, L. D., and Varki, A. (1995) *J. Biol. Chem. 270*, 14243−14246.
7. Crocker, P. R., Kelm, S., Hartnell, A., Freeman, S., Nath, D., Vinson, M., and Mucklow, S. (1996) *Biochem. Soc. Trans. 24*, 150−156.
8. Brossmer, R., Gross, H.-J., Rose, U., Krause, J. M., Paulson, J. C., Schmid, K., and Feeney, R. E. (1989) *Biochemistry 28*, 7386−7392.
9. Harduin-Lepers, A., Recchi, M.-A., and Delannoy, P. (1995) *Glycobiology 5*, 741−758.
10. Datta, A. K., and Paulson, J. C. (1995) *J. Biol. Chem. 270*, 1497−1500.
11. Weinstein, J., Lee, E. U., McEntee, K., Lai, P., and Paulson, J. C. (1987) *J. Biol. Chem. 262*, 17735−17743.
12. Hokke, C. H., Van derVen, J. G. M., Kamerling, J. P., and Vliegenthart, J. F. G. (1993) *Glycoconjugate J. 10*, 82−90.
13. Wlasichuk, K. B., Kashem, M., Nikrad, P. V., Bird, P., Jiang, C., and Venot, A. P. (1993) *J. Biol. Chem. 268*, 13971−13977.
14. Van Dorst, J. A. L. M., Tikkanen, J. M., Krezdorn, C. H., Streiff, M. B., Berger, E. G., Van Kuik, J. A., Kamerling, J. P., and Vliegenthart, J. F. G. (1996) *Eur. J. Biochem. 242*, 674−681.
15. Weinstein, J., Souza-e-Silva, U., and Paulson, J. C. (1982) *J. Biol. Chem. 257*, 13845−13853.
16. Higa, H. H., and Paulson, J. C. (1985) *J. Biol. Chem. 260*, 8838−8849.
17. Gross, H. J., Rose, U., Krause, J. M., Paulson, J. C., Schmid, K., Feeney, R. E., and Brossmer, R. (1989) *Biochemistry 28*, 7386−7392.
18. Kajihara, Y., Kodama, K., Wakabayashi, T., Sato, K., and Hashimoto, H. (1993) *Carbohydr. Res. 247*, 179−193.
19. Kleineidam, R. G., Schmelter, T., Schwarz, R. T., and Schauer, R. (1997) *Glycoconjugate J. 14*, 57−66.
20. Khan, S. H., and Matta, K. L. (1995) in *Glycoconjugates. Composition, Structure, and Function* (Allen, H. J., and Kisailus, E. C., Eds.) pp 361−378, Marcel Dekker, New York.
21. Sinnott, M. L. (1990) *Chem. Rev. 90*, 1171−1202.
22. Schramm,V. L. (1991) in *Enzyme Mechanisms from Isotope Effects* (Cook, P. F., Ed.) pp 367−388, CRC Press, Boca Raton, FL.
23. Withers, S. G. (1995) *Pure Appl. Chem. 67*, 1673−1682.
24. Beau, J., Schauer, R., Haverkamp, J., Kamerling, J. P., Dorland, L., and Vliegenthart, J. F. G. (1984) *Eur. J. Biochem. 140*, 203−208.
25. Lin, C.-H., Murray, B. W., Ollmann, I. R., and Wong, C.-H. (1997) *Biochemistry 36*, 780−785.
26. Horenstein, B. A., and Bruner, M. (1996) *J. Am. Chem. Soc. 118*, 10371−10379.
27. Zapata, G., Vann, W. F., Aaronson, W., Lewis, M. S., and Moos, M. J. (1989) *J. Biol. Chem. 264*, 14769−14774.
28. Liu, J. L., Shen, G.-J., Ichikawa, Y., Rutan, J. F., Zapata, G., Vann, W. F., and Wong, C.-H. (1992) *J. Am. Chem. Soc. 114*, 3901−3910.
29. Roseman, S., and Ludoweig, J. (1954) *J. Am. Chem. Soc. 76*, 301−302.
30. Duggleby, R. G., and Northrop, D. B. (1989) *Bioorg. Chem. 17*, 177−193.
31. Bigeleisen, J., and Wolfsburg, M. (1958) *Adv. Phys. Chem. 1*, 15−76.
32. Bigeleisen, J., and Mayer, M. G. (1947) *J. Chem. Phys. 15*, 261−267.
33. Horenstein, B. A. (1997) *J. Am. Chem. Soc. 119*, 1101−1107.
34. Gaussian 94, Revision C; Frisch, M. J., Trucks, G. W., Schlegel, H. B., Gill, P. M. W., Johnson, B. G., Robb, M. A., Cheeseman, J. R., Keith, T., Petersson, G. A., Montgomery, J. A., Raghavachari, K., Al-Laham, M. A., Zakrzewski, V. G., Ortiz, J. V., Foresman, J. B., Cioslowski, J., Stefanov, B. B., Nanayakkara, A., Challacombe, M., Peng, C. Y., Ayala, P. Y., Chen, W., Wong, M. W., Andres, J. L., Replogle, E. S., Gomperts, R., Martin, R. L., Fox, D. J., Binkley, J. S., Defrees, D. J., Baker, J., Stewart, J. P., Head-Gordon, M., Gonzalez, C., and Pople, J. A. (1995), Gaussian, Inc., Pittsburgh.
35. Saunders, M., Laidig, K. E., and Wolfsberg, M. (1989) *J. Am. Chem. Soc. 111*, 8989−8994.
36. Dahlquist, F. W., Rand-Meir, T., and Raftery, M. A. (1969) *Biochemistry 8*, 4214−4221.
37. Parkin, D. L. (1991) in *Enzyme Mechanisms from Isotope Effects* (Cook, P. F., Ed.) pp 269−290, CRC Press, Boca Raton, FL.
38. Rose, I. A., O'Connell, E. L., Litwin, S., and Tana, J. B. (1974) *J. Biol. Chem. 249*, 5163−5168.
39. Rose, I. A. (1980) *Methods Enzymol. 64*, 47−59.
40. Northrup, D. B. (1981) *Annu. Rev. Biochem. 50*, 103−131.
41. Rose, I. A. and Iyengar, R. (1982) *Biochemistry 21*, 1591−1597.

42. Cleland, W. W. (1989) *Z. Naturforsch. 44a*, 445−449.
43. Cleland, W. W. (1975) *Biochemistry 14*, 3220−3224.
44. Cook, P. F., and Cleland, W. W. (1981) *Biochemistry 20*, 1790−1796.
45. Fersht, A. (1985) *Enzyme Structure and Mechanism*, pp 150−151, W. H. Freeman and Company, New York.
46. Bayles, J. W., Bron, J., and Paul, S. O. (1976) *J. Chem. Soc., Faraday Trans. 1 7*, 1546−1542.
47. Cook, P. F. (1991) in *Enzyme Mechanisms from Isotope Effects* (Cook, P. F., Ed.) pp 203−230, CRC Press, Boca Raton, FL.
48. Markham, G. D., Parkin, D. W., Mentch, F., and Schramm, V. L. (1987) *J. Biol. Chem. 262*, 5609−5615.
49. Rodgers, J., Femec, D. A., and Schowen, R. L. (1982) *J. Am. Chem. Soc. 104*, 3263−3268.
50. Schramm, V. L., Horenstein, B. A., and Kline, P. C. (1994) *J. Biol. Chem. 269*, 18259−18262.
51. Degano, M., Gopaul, D. N., Scapin, G., Schramm, V. L., and Sacchettini, J. C. (1996) *Biochemistry 35*, 5971−5981.
52. Kim, S. C., Singh, A. N., and Raushel, F. M. (1988) *Arch. Biochem. Biophys. 267*, 54−59.
53. Murray, B. W., Wittman, V., Burkart, M. D., Hung, S. C., and Wong, C.-H. (1997) *Biochemistry 36*, 823−831.
54. Guo, X., and Sinnott, M. L. (1993) *Biochem. J. 294*, 653−656.
55. Sunko, D. E., Szele, I., and Hehre, W. J. (1977) *J. Am. Chem. Soc. 99*, 5000−5005.
56. Ashwell, M., Guo, X., and Sinnott, M. L. (1992) *J. Am. Chem. Soc. 114*, 10158−10166.
57. Melander, L., and Saunders, W. H., (1980) in *Reaction Rates of Isotopic Molecules*, pp 242−246, Kreiger, Malabar, FL.
58. Goitein, R. K., Chelsky, D., and Parsons, S. M. (1978) *J. Biol. Chem. 253*, 2963−2971.
59. Ando, T., Yanabe, H., and Yamataka, H. (1984) *J. Am. Chem. Soc. 106*, 2084−2088.
60. LaReau, R. D., Wan, W., and Anderson, V. E. (1989) *Biochemistry 28*, 3619−3624.
61. Horenstein, B. A., Parkin, D. W., Estupinan, B., and Schramm, V. L. (1991) *Biochemistry 30*, 10788−10795.
62. Rakowski, K., and Paneth, P. (1996) *J. Mol. Struct.: THEOCHEM. 378*, 35−43.
63. Bennett, A. J., and Sinnott, M. L. (1986) *J. Am. Chem. Soc. 108*, 7287−7294.

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