# Catalysis by Two Sialidases with the Same Protein Fold but Different Stereochemical Courses: A Mechanistic Comparison of the Enzymes from Influenza A Virus and *Salmonella typhimurium*

**Xumin Guo,[†] W. Graeme Laver,[§] Eric Vimr,[‡] and Michael L. Sinnott*,[†]**

*Contribution from the Department of Chemistry (M/C 111), University of Illinois at Chicago, 845 West Taylor Street, Chicago, Illinois 60607-7061, Departments of Veterinary Pathobiology and Microbiology, University of Illinois at Urbana-Champaign, 2001 South Lincoln Avenue, Urbana, Illinois 61801, and John Curtin School of Medical Research, Australian National University, Canberra 2601, Australia*

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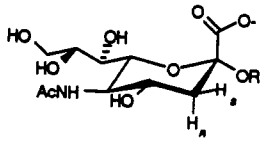
**Abstract:** The protein folds and presumed active site residues of influenza A (Varghese, J. N., McKimm-Breschkin, J. L.; Caldwell, J. B.; Kortt, A. A.; Colman, P. M. *Proteins* **1992**, *14*, 327) and *Salmonella typhimurium* (Crennell, S. J.; Garman, E. F.; Laver, W. G.; Vimr, E. R.; Taylor, G. L. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 9852) neuraminidases are very similar, yet the influenza enzyme works with retention of configuration (Chong, A. K.; Pegg, M. S.; Taylor, N. R.; von Itzstein, M. *Eur. J. Biochem.* **1992**, *207*, 335) and the *S. typhimurium* enzyme with inversion (Guo, X.; Sinnott, M. L. *Biochem. J.* **1993**, *296*, 291). To address the possibility that these two stereochemical outcomes may nonetheless be compatible with an essentially common sialosyl cation-stabilizing protein machinery, we have compared leaving group effects and (geometry-dependent) $\beta$-deuterium kinetic isotope effects for both enzymes. For the influenza enzyme, $\beta_{lg}$ values calculated on $V$ and $V/K$ differ radically (–0.11 and –0.46, respectively), and we could detect neither $\beta$-deuterium nor leaving group $^{18}O$ isotope effects on $V$ for hydrolysis of the *p*-nitrophenyl glycoside at the optimal pH of 6, indicating, as previously found for the 4-methylumbelliferyl compound (Chong et al., 1992) that a step subsequent to glycon–aglycon cleavage determined $V$. Effects on $V/K$ were not fully expressed at pH 6, in accord with the postulation of an isotope-insensitive step preceding bond cleavage. Intrinsic $\beta$-DKIEs on glycon–aglycon cleavage (measured as $^{\beta D}(V/K)$ at pH 9.5) of around 6% for the *pro-R* hydron and 8% for the *pro-S* are compatible with reaction through the $B_{2,5}$ conformation of the sugar ring seen in the X-ray crystal structure of the neuraminidase-*N*-acetylneuraminic acid complex (Varghese et al., 1992). In accord with a single displacement mechanism for the *S. typhimurium* enzyme, the $\beta_{lg}$ values calculated on $V$ and on $V/K$ for the hydrolysis of seven aryl *N*-acetylneuraminides by this enzyme are both strongly negative (–0.53 and –0.80, respectively). $\beta$-DKIEs on $V$ for the *p*-nitrophenyl compound are, however, around 60% of those on $V/K$, as is the leaving group $^{18}O$ effect, when measured at optimal pH of 5.5. When measured at pH 8.0, the $\beta$-dideutero effect on $V$ and on $V/K$ is the same, and the same as that on $V/K$ at pH 5.5. Product release is therefore likely to partly govern $V$ at optimum pH for this excellent ($k_{cat} = 7 \times 10^3$ s$^{-1}$) substrate. The intrinsic leaving group $^{18}O$ and individual *pro-S* and *pro-R* $\beta$-deuterium effects, all ~5%, coupled with the negative $\beta_{lg}$ values, indicate that the single chemical transition state for the *S. typhimurium* enzyme involves little proton donation to the leaving group, probably a sugar ring conformation approximating to ground-state $^2C_5$, and substantial charge development at C2. The catalytic mechanisms of the two enzymes therefore differ radically.

Sialidases catalyze the hydrolysis of $\alpha$-D-*N*-acylneuraminyl residues I from glycolipid, glycoprotein, and oligosaccharide substrates and in so doing play a key role in many important biological functions.[1] Sialidases have been found in viruses and




I

bacteria and seem to occur in most parts of the animal kingdom (including man).[1] The microbial enzymes can be placed in three general classes: the large, bacterial sialidases, such as the *Vibrio cholerae* enzyme, which are $Ca^{2+}$-dependent; the small bacterial sialidases, which do not require $Ca^{2+}$; and the viral enzymes. The activity of the influenza virus is enhanced by $Ca^{2+}$, even though $Ca^{2+}$ is not essential.[2]

Knowledge of the three-dimensional structure of sialidases is confined to two enzymes. Extensive studies of various forms of the influenza virus neuraminidases have revealed an essentially common structure, despite sequence differences.[3] The structure of the *N*-acetylneuraminic acid–influenza A neuraminidase complex has been determined.[4] The enzyme binds the $\alpha$-anomer of this ligand exclusively, despite its equilibrium proportion being only 7.5%,[5] and apparently binds it in the normally disfavored $B_{2,5}$ conformation (II).

* Address correspondence to this author. Tel: (312)-996-6318. FAX: (312)-413-7404.
† University of Illinois at Chicago.
‡ University of Illinois at Urbana-Champaign.
§ John Curtin School of Medical Research.
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(1) (a) Schauer, R. *Trends Biochem. Sci.* **1985**, *10*, 357. (b) Corfield, A. P. *Glycobiology* **1992**, *2*, 509.
(2) Chong, A. K. J.; Pegg, M. S.; von Itzstein, M. *Biochim. Biophys. Acta* **1991**, *1077*, 65.
(3) (a) Varghese, J. N.; Colman, P. M. *J. Mol. Biol.* **1991**, *221*, 473. (b) Tulip, W. R.; Varghese, J. N.; Baker, A. T.; van Donkelaar, A.; Laver, W. G.; Colman, P. M. *J. Mol. Biol.* **1991**, *221*, 487.
(4) Varghese, J. N.; McKimm-Breschkin, J. L.; Caldwell, J. B.; Kortt, A. A.; Colman, P. M. *Proteins: Struct. Funct. Genet.* **1992**, *14*, 327.
(5) Friebolin, H.; Kunzelmann, P.; Supp, M.; Brossmer, R.; Keilich, G.; Ziegler, D. *Tetrahedron Lett.* **1981**, *22*, 1383.

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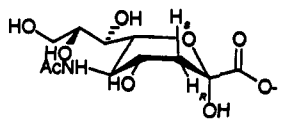


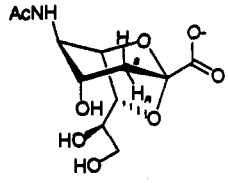
II

The X-ray crystal structure of the *Salmonella typhimurium* LT2 enzyme, a small bacterial sialidase which does not require $Ca^{2+}$, has recently been solved.[6] Despite little sequence similarity with the influenza enzyme, the three-dimensional structures show "remarkable similarity in both the general fold and the spatial arrangement of the catalytic residues".[6]

Data on the primary structures of sialidases are more extensive: a recent comparison of seven sialidase sequences from five different genera and two protozoan sialidase sequences from *Trypanosoma cruzi* suggested homology (common descent) among small and large enzymes and hence the existence of a sialidase superfamily.[7] By contrast, hydrophobic cluster analysis of available sequences puts the influenza enzyme in one glycosidase class and the *S. typhimurium* and small *Clostridium perfingens* sialidases together in another.[8]

Careful studies by $^1H$ NMR have shown that the hydrolysis of $\alpha$-neuraminides by the sialidases of *Arthobacter* ureafacens, the large enzyme from *C. perfringens*,[9] and influenza virus[10] gives *N*-acetyl-$\alpha$-neuraminic acid as first product. Retention was also shown for the *V. cholerae* enzyme by polarimetry.[11] The structural similarities of the sialidases encouraged the supposition that all the sialidases were retaining glycosidases and indeed work by broadly similar mechanisms,[7] although structural similarities can, in principle, also arise from common substrate recognition elements. Our recent discovery that the initial product of the action of the *S. typhimurium* enzyme is the $\beta$-*N*-acetyl-neuraminic acid, i.e., that this enzyme works with inversion of configuration, probably by a single displacement mechanism,[12] indicates that the two enzymes must have radically different mechanisms.

Given the importance of sialidases, direct mechanistic investigations are sparse. Work from this laboratory, based largely on $\beta$-deuterium kinetic isotope and leaving group effects, led to different conclusions about the *V. cholerae* enzyme[11] and the leech sialidase L,[13] which yields 2,7-anhydro-*N*-acetylneuraminic acid (**III**) rather than $\alpha$-*N*-acetylneuraminic acid as product. For



III

both enzymes, glycon–aglycon cleavage is both the rate-determining and the first irreversible step; however, for the leech enzyme, the sugar ring at the transition state is in a conformation derived from the $^{3,6}B \leftrightharpoons {}^{6}S_2 \leftrightharpoons B_{2,5}$ part of the skew-boat pseudorotational itinerary, whereas for the *V. cholerae* enzyme, it is derived from the normal ground-state $^2C_5$ conformation. Moreover, for both enzymes, an intrinsic dideutero $\beta$-deuterium kinetic isotope effect of around 1.07–1.08 was found.

The molecular biology of the *V. cholerae* enzyme is well developed, and its tertiary structure should shortly become available.[14c]

Site-directed mutagenesis experiments on the influenza enzyme, interpreted in the light of the X-ray crystal structure, led to a proposed mechanism in which Glu 276 acts as a general acid and Glu 277 as a counterion to a sialosyl cation intermediate. Glu 277 is on the same face of the pyranosyl cation as the leaving group.[15] Recently, Chong *et al.*[10] measured solvent and di-$\beta$-deuterium kinetic isotope effects for hydrolysis of 4-methylumbelliferyl *N*-acetyl-$\alpha$-D-neuraminide as a function of pH. $^{\beta D_2}(V/K)$ increases from 1.04 ± 0.03 at pH 4 to 1.15 ± 0.04 at pH 9.5, whereas $^{\beta D_2}(V)$ appeared to be 1.00 ± 0.02 up to pH 8, when it also started to increase, to 1.07 ± 0.01 at pH 9.5. $^{D_2O}(V)$ values were essentially pH invariant at 1.9, and the bowl-shaped proton inventory was fit by a second-order polynomial in atom fraction deuterium. A mechanism with the following steps was advanced: (1) reversible change of the conformation of enzyme-bound substrate from $^2C_5$ to $B_{2,5}$ in a process which partly determines $V/K$ at neutral and acid pH; (2) generation of a sialosyl cation by a process involving general acid catalysis by Asp 151 through a water molecule; and (3) general base catalysis by Asp 151 of the attack of water on the sialosyl cation, in a process which determines $V$ at pH 6. The key datum leading to the postulation of a sialosyl cation intermediate was the slightly inverse $^{\beta D_2}(V)$ (0.979 ± 0.007 at pH 6.0 (only)).

We now present a study complementary to the above, which uses leaving group effects and the $\beta$-deuterium kinetic isotope effects for the individual *pro-R* and *pro-S* hydrons at position 3 of *p*-nitrophenyl N-acetylneuraminide to demonstrate directly that glycon–aglycon bond fission in the ES complex cannot occur from the $^2C_5$ conformation, which is favored by the unbound substrate, and most likely occurs from the $B_{2,5}$ conformation seen in the X-ray crystal structure of the N-acetylneuraminic acid–neuraminidase complex. We also present data on the effect of leaving group acidity and $^{18}O$ substitution, to further probe the nature of the transition states and provide confirmatory evidence for the rate-determining step.

The unexpected finding that the *S. typhimurium* enzyme was a single displacement enzyme led us to enquire whether participation by the substrate carboxylate in a double displacement reaction was very finely balanced with direct displacement by a water molecule, as it may be in the nonenzymic reactions.[16] We therefore performed an analogous study with this enzyme.

## Experimental Section

Unlabeled and deuterium-labeled *N*-acetylneuraminides[16] and 4-nitrophenyl 1-[$^{18}O$]-$\alpha$-D-*N*-acetylneuraminide[7] have been described previously.

The influenza A neuraminidase used was a preparation of the pronase-solubilized "heads" of the subtype N2 described previously.[17] $k_{cat}$ values were calculated on the assumption that the material was 100% pure and 100% active and had a molecular weight per active site of 50 kDa; enzyme activity was standardized on *p*-nitrophenyl *N*-acetylneuraminide for each set of kinetic experiments. Michaelis–Menten parameters were measured on a preparation from a virus isolated in 1957 ($R1/5^+$ X-$7F_1$), isotope effects on an analogous strain isolated in 1967 (A/Tokyo/67); the Michaelis–Menten parameters for *p*-nitrophenyl *N*-acetylneuraminide

(6) Crennell, S. J.; Garman, E. F.; Laver, W. G.; Vimr, E. R.; Taylor, G. L. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 9852.
(7) (a) Hoyer, L. L.; Hamilton, A. C.; Steenbergen, S. M.; Vimr, E. R. *Mol. Microbiol.* **1992**, *6*, 873. (b) Roggentin, P.; Schauer, R.; Hoyer, L. L.; Vimr, E. R. *Mol. Microbiol.* **1993**, *9*, 915.
(8) (a) Henrissat, B. *Biochem. J.* **1991**, *280*, 309. (b) Henrissat, B.; Bairoch, A. *Biochem. J.* **1993**, *293*, 781.
(9) (a) Friebolin, H.; Supp, M.; Brossmer, R.; Keilich, G.; Ziegler, D. *Angew. Chem., Int. Edn. Engl.* **1980**, *19*, 208. (b) Friebolin, H.; Brossmer, R.; Keilich, G.; Ziegler, D.; Summ, M. *Hoppe-Seyler's Z. Physiol. Chem.* **1980**, *361*, 697.
(10) Chong, A. K.; Pegg, M. S.; Taylor, N. R.; von Itzstein, M. *Eur. J. Biochem.* **1992**, *207*, 335.
(11) Guo, X.; Sinnott, M. L. *Biochem. J.* **1993**, *294*, 653.
(12) Guo, X.; Sinnott, M. L. *Biochem. J.* **1993**, *296*, 291.
(13) Sinnott, M. L.; Guo, X.; Li, S.-C.; Li, Y.-T. *J. Am. Chem. Soc.* **1993**, *115*, 3334.
(14) (a) Vimr, E. R.; Lawrisuk, L.; Galen, J.; Kaper, J. B. *J. Bacteriol.* **1988**, *170*, 1495. (b) Galen, J. E.; Ketley, J. M., Fasano, A.; Richardson, S. H.; Wasserman, S. S. and Kaper, J. B. *Infection Immunity* **1992**, *60*, 406. (c) Taylor, G.; Vimr, E.; Garman, E.; Laver, G. *J. Mol. Biol.* **1992**, *226*, 1287.
(15) Lentz, M. R.; Webster, R. G.; Air, G. M. *Biochemistry* **1987**, *26*, 5351.
(16) Ashwell, M.; Guo, X.; Sinnott, M. L. *J. Am. Chem. Soc.* **1992**, *114*, 10158.
(17) Varghese, J. N.; Laver, W. G.; Colman, P. M. *Nature* **1983**, *303*, 35.

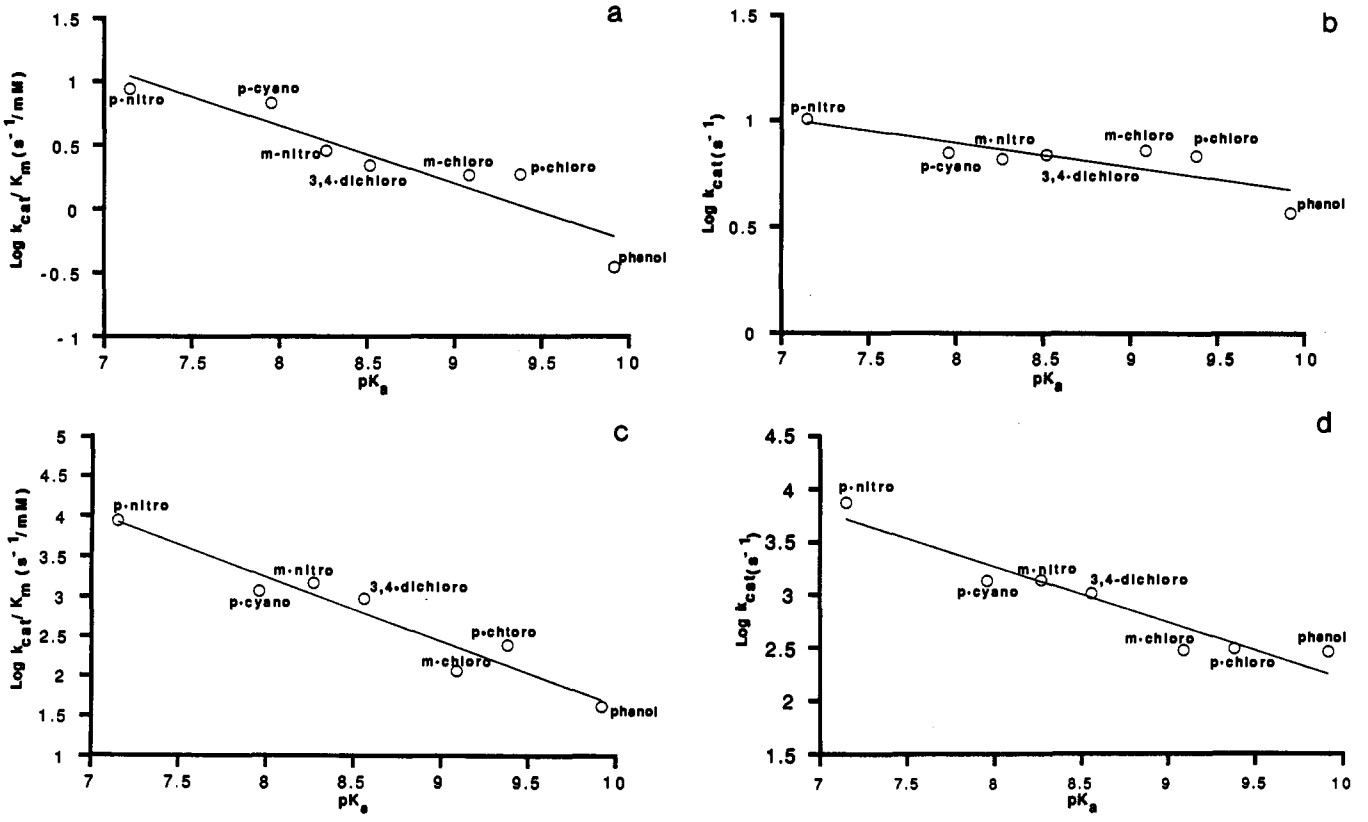



**Figure 1.** (a) Dependence of $V/K$ for influenza neuraminidase-catalyzed hydrolysis of substituted-phenyl *N*-acetyl-α-D-neuraminides on leaving group $pK_a$. (b) Dependence of $V$ for influenza neuraminidase-catalyzed hydrolysis of substituted-phenyl *N*-acetyl-α-D-neuraminides on leaving group $pK_a$. (c) Dependence of $V/K$ for *S. typhimurium* neuraminidase-catalyzed hydrolysis of substituted-phenyl *N*-acetyl-α-D-neuraminides on leaving group $pK_a$. (d) Dependence of $V$ for *S. typhimurium* neuraminidase-catalyzed hydrolysis of substituted-phenyl *N*-acetyl-α-D-neuraminides on leaving group $pK_a$.

for the two stocks of enzyme were identical. Kinetic measurements on this enzyme at pH 6.0 were performed in 50 mM sodium acetate containing 0.1 mM $CaCl_2$, 0.32 mM $MgCl_2$, and 60 mM NaCl, and those at pH 9.5 were performed in 50 mM glycine containing 0.1 mM $CaCl_2$, 0.32 mM $MgCl_2$, and 60 mM NaCl. Measurements on the *S. typhimurium* enzyme at pH 5.5 were made in 50 mM sodium acetate buffer, 100 mM in sodium chloride, and those at pH 8.0 were made in 50 mM sodium phosphate containing 100 mM sodium chloride.

Kinetic measurements were made on a Perkin-Elmer Lambda-6 spectrophotometer fitted with an electrically (Peltier-effect) thermostated cell block. For determination of $k_{cat}$ and $K_m$ values, at least seven measurements of initial rate were made between $K_m/3$ and $3K_m$; reactions were initiated by addition of enzyme stock solution (10 μL) to substrate (250–1000 μL) which had been thermally equilibrated in the cell block for 7–8 min, and extinction was monitored continuously. Initial rates were fitted directly to a rectangular hyperbola using Kaleidagraph. Wavelengths and extinction coefficient differences (measured as the difference between the extinction coefficient of the glycoside and that of the phenol) are given in Tables 1 and 3. $K_m$ and relative $k_{cat}$ values are reproducible to within 10%.

The $K_i$ value for binding of fully mutarotated *N*-acetylneuraminic acid to the *S. typhimurium* enzyme was determined from $K_m$ values for the *p*-nitrophenyl glycoside in the presence and absence of 12.5 mM inhibitor: the $V_{max}$ values were identical, indicating competitive inhibition. (Apparent *mixed* inhibition was observed with a sample of enzyme that had been partly degraded in transit.)

Kinetic isotope effects on $V_{max}$ were measured from direct comparison of zeroth-order rates with [S] = $7K_m$ substrate, whereas effects on $V_{max}/K_m$ were measured from direct comparison of first-order rate constants with [S] = $K_m/25$; first-order rate constants were calculated using Kaleidagraph. For both types of isotope effect, data were compared pairwise as obtained in the sequence light, heavy/heavy, light/light, heavy, etc.

## Results and Discussion

**Influenza A Neuraminidase.** This enzyme yields *N*-acetyl-α-neuraminic acid as first product.[10] The retentive stereochemistry strongly suggests[18] that the enzyme turnover sequence involves two chemical steps: glycon–aglycon cleavage and subsequent hydrolysis of a glycosyl–enzyme intermediate. The very different $\beta_{lg}$ values obtained from the data in Table 1 for the dependencies of $V_{max}$ and $V_{max}/K_m$ with leaving group p$K$ (–0.11 ($r$ = –0.81) and –0.45 ($r$ = –0.93), respectively, see Figure 1) suggest that glycon–aglycon cleavage determines $V_{max}/K_m$ at least partly but that at least for the better leaving groups some other process determines $V_{max}$. This is confirmed by the leaving group $^{18}O$ kinetic isotope effects (Table 2) for the best substrate studied, the *p*-nitrophenyl glycoside. There is no effect on $V$ but a significant effect on $V/K$, indicating that the two parameters do refer to different processes, only the first of which involves bonding changes at the leaving group oxygen atom.

The β-deuterium kinetic isotope effects of Table 2 are particularly informative about transition-state geometry and charge development in the transition states involved in the action of this enzyme. β-Deuterium kinetic isotope effects have their origin in two phenomena, hyperconjugation and the inductive effect of deuterium, and can be considered in terms of eq 1:

$$\ln(k_H/k_D) = \cos^2\theta \ln(k_H/k_D)_{max} + \ln(k_H/k_D)_i \quad (1)$$

The first term represents hyperconjugation of the C–L σ orbital with an electron-deficient p orbital on an adjacent carbon atom; θ is the dihedral angle between the C–L bond and this orbital.

(18) Experimental evidence suggests internal return, or $S_Ni$-type, mechanisms for rabbit glycogen phosphorylase (see, e.g.: Palm, D.; Klein, H. W.; Schinzel, R.; Buehner, M.; Helmreich, E. J. M. *Biochemistry* **1990**, *29*, 1099) and for cellobiohydrolase II of *Trichoderma reesei* hydrolyzing the wrong (α) cellobiosyl fluoride (Konstantinidis, A.; Marsden, I.; Sinnott, M. L. *Biochem. J.* **1993**, *291*, 883). All simple retaining glycosidases that have been examined mechanistically in detail to date, though, involve a glycosylated aspartate or glutamate residue as a discrete intermediate.[19]

(19) Sinnott, M. L. *Chem. Rev.* **1990**, *90*, 1171.

**Table 1.** Michaelis–Menten Parameters for Hydrolysis of Aryl *N*-Acetyl-α-D-neuraminides by Influenza Neuraminidase, at pH 6.0 and 37.0 °C

| parent phenol | wavelength (nm) | $\Delta\epsilon$ ($cm^{-1}$ $M^{-1}$) | $k_{cat}$ ($s^{-1}$) | $K_m$ (mM) |
|---|---|---|---|---|
| 4-nitrophenol | 340 | 1931 | 10.1 | 1.17 |
| | 400 | 14 830 | 3.2 | 1.29[a] |
| 4-cyanophenol | 262 | 721 | 6.6 | 2.3 |
| 3-nitrophenol | 340 | 642 | 7.1 | 1.05 |
| 3,4-dichlorophenol | 280 | 1219 | 6.9 | 3.1 |
| 3-chlorophenol | 280 | 912 | 7.3 | 3.9 |
| 4-chlorophenol | 280 | 1251 | 6.8 | 3.7 |
| phenol | 270 | 854 | 3.7 | 10.6 |

[a] At pH 9.5.

**Table 2.** Kinetic Isotope Effects for Influenza Neuraminidase-Catalyzed Hydrolysis of *p*-Nitrophenyl *N*-Acetylneuraminide at 37.0 °C[a]

| site of substitution | effect on V/K | effect on V |
|---|---|---|
| | At pH 6.0 | |
| 3-[$^2$H]$_2$ | 1.087 ± 0.015 | 1.0095 ± 0.011 |
| | (1.09 ± 0.02)[b] | (0.979 ± 0.007)[b] |
| 3-*pro-R*-[$^2$H] | 1.035 ± 0.010 | 1.003 ± 0.015 |
| 3-*pro-S*-[$^2$H] | 1.058 ± 0.006 | 0.999 ± 0.013 |
| 2-[$^{18}$O] | 1.045 ± 0.021 | 1.016 ± 0.006[c] |
| | | (0.998 corrected) |
| | At pH 9.5 | |
| 3-[$^2$H]$_2$ | 1.136 ± 0.018 | 1.076 ± 0.017 |
| | (1.15 ± 0.04)[b] | (1.07 ± 0.01)[b] |
| 3-*pro-R*-[$^2$H] | 1.057 ± 0.010 | |
| 3-*pro-S*-[$^2$H] | 1.078 ± 0.005 | |

[a] For buffer composition and virus strain, see Experimental Section. Quoted errors are standard deviations on at least eight pairwise comparisons. [b] For the 4-methylumbelliferyl compound, taken from ref 10. [c] Result of a direct comparison of rates of appearance of optical density at 390 nm at pH 6.0 and therefore uncorrected for the equilibrium isotope effect on the ionization of *p*-nitrophenol ($^{18}K$ = 1.018).[23]

$\ln(k_H/k_D)_{max}$ is the maximal hyperconjugative effect obtained when the C–L bond and the p orbital are exactly eclipsed, the value and increases as the positive charge on the adjacent carbon atom increases, with the associated weakening of the C–L bond. The second term represents a small, geometry-independent inductive deuterium effect, resulting from the slightly electron-donating inductive effect of deuterium. The inductive effect is thus in the opposite sense to the hyperconjugative effect.

The inductive effect is, however, geometry-independent, so the relative magnitudes of the kinetic isotope effects of the individual diastereotopic β-hydrons enable qualitative information about the reactive conformation to be immediately obtained. Furthermore, an internal check on the measurements is available, since the measured effect from two deuteriums should be the product of the individual single-deuterium effects. With influenza neuraminidase (Table 2), the dideuterio effects are indeed the product of the two single deuterium effects.

Our kinetic isotope effects for the dideuterio *p*-nitrophenyl glycoside are in general agreement with those of Chong *et al.* for the 4-methylumbelliferyl compound,[10] but we can find no evidence for the small inverse effect on the hydrolysis of the glycosyl enzyme at pH 6.0, on which the postulation of a cationic structure for the glycosyl–enzyme intermediate solely rests. Indeed, no substrate isotope effect of any description can be detected on *V* at optimal pH: the null result for the dideuterio compound has not arisen from fortuitous cancellation of direct and inverse effects for the individual diastereotopic hydrons.

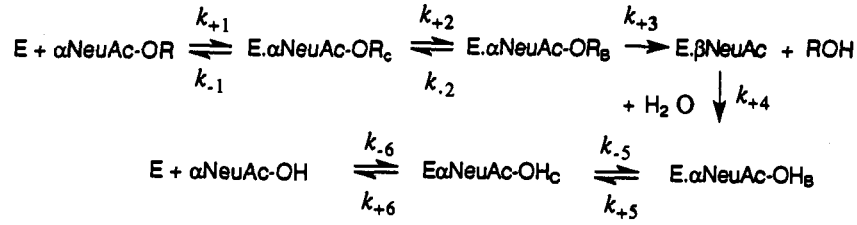
The nature of the rate-determining step for hydrolysis of *N*-acetylneuraminides of more acidic aglycons therefore presents a puzzle. There are two mechanistically realistic possibilities: (i) a noncovalent event which is the near-microscopic reverse of the noncovalent event preceding glycon–aglycon bond cleavage or (ii) the hydrolysis of an α-lactone intermediate. The hydrolysis of an α-lactone intermediate is suggested by the significant solvent deuterium effect ($^{D_2O}(V)$ = 1.9),[10] the absence of a plausible candidate for a nucleophilic carboxylate group from the protein at the enzyme active site, and the precedent of studies on the nonenzymic hydrolysis of neuraminides,[16] in which the participation of the substrate carboxylate (though not necessarily to give a fully covalent α-lactone) was demonstrated. It can be reconciled with the absence of β-deuterium kinetic isotope effects by noting first that in an α-lactone in the $B_{2,5}$ conformation (**II**), the dihedral angle between the fissile C–O bond and both β-hydrogen atoms is approximately 60°, an arrangement which minimizes the hyperconjugative component of the kinetic isotope effect, and second that if the bond had significant ionic character, the change in charge between ground state and transition state would be small, further reducing the isotope effects. It is, however, a notably ineffectual enzyme that hydrolyzes an α-lactone with a rate constant of merely 10 $s^{-1}$.

The case for the penultimate step in the hydrolysis of the *p*-nitrophenyl glycoside being a noncovalent event arises out of a more detailed consideration of the transition state for glycon–aglycon cleavage and the events preceding it. As with the 4-methylumbelliferyl glycoside,[10] our data for the *p*-nitrophenyl compound reveal that isotope effects on (*V*/*K*) are suppressed at optimal pH but increase at the nonoptimal pH of 9.5 (Table 2). In principle, this could be due to nonzero commitments of the first ES complex, but for a substrate with a millimolar $K_m$ and modest $k_{cat}$ (10 $s^{-1}$) to be "sticky" in this way has little precedent. It is far more reasonable to suppose that the isotopically silent step is some noncovalent rearrangement of the ES complex.

That the *pro-S* β-deuterium effect is bigger than the *pro-R* in the cases examined supports the idea that the reactive conformation of the bound substrate is indeed the $B_{2,5}$ conformation of the bound sialic acid seen in the crystal structure of the complex.[4] Reaction through the $^2C_5$ conformation would result in equal effects, but any distortion toward the $^4H_5$ half-chair conformation, which common prejudice suggests would be adopted by a sialosyl cation, would result in the *pro-R* effect becoming bigger than the *pro-S*, as is observed with the *V. cholerae* enzyme[12] and in the nonenzymic hydrolyses.[16] The sugar ring must therefore at some stage change from the normal $^2C_5$ chair to the $B_{2,5}$ boat. In principle, the enzyme could bind the small proportion of the substrate already in the boat conformation in much the same way as leech sialidase L does. However, this latter enzyme exhibits high $K_m$ values and does not show evidence of a chemically silent but kinetically important step in its turnover sequence. It seems more reasonable to identify the isotopically silent step in the influenza enzyme with a conjoint change of enzyme and substrate which puts the sugar ring in the boat conformation.

In the pathway of a double displacement, retaining glycosidase, the pathway to the glycosyl–enzyme intermediate is commonly considered to be the near-microscopic reverse of the pathway to it. If the enzyme has transferase activity, then this is an absolute requirement for, say, the hydrolysis of the methyl glycoside and the methanolysis of the glycosyl–enzyme intermediate. It follows, therefore, that if there is a slow conjoint change of enzyme and substrate on the way to the glycosyl enzyme intermediate, there must similarly be a conjoint change of the enzyme–product complex on the way from it. Such a step, if slow, would not give rise to substrate isotope effects, but would, realistically, result in the breaking of hydrogen bonds and would then account for the solvent isotope effect on *V*, which arises from alteration of the fractionation factors of (at least?) two protons.[10] We favor this, rather than the hydrolysis of a glycosyl–enzyme intermediate of any description, as the process governing *V* for influenza neuraminidase-catalyzed hydrolysis of neuraminides of acidic aglycons. Nucleophilic competition could, in principle, distinguish between these two possibilities, since the addition of a better nucleophile than water would increase $k_{cat}$ in a linear fashion if

**Scheme 1**




hydrolysis of the glycosyl–enzyme were rate-determining, but would have only a modest effect if the rate were limited by a subsequent protein conformation change. Unfortunately, however, transfer to methanol in up to 2 M concentration could be detected neither kinetically (as an increase in $k_{cat}$ for the *p*-nitrophenyl glycoside with [MeOH]) nor by analysis of the products.

The turnover sequence of the enzyme can then be written as in Scheme 1, where the subscripts C and B refer to chair and boat, respectively. Steps 2 and 5 represent the chair–boat interconversion steps, step 3 the loss of aglycon, and step 4 (which is not kinetically accessible) the hydrolysis of the glycosyl–enzyme intermediate.

In addition to the occurrence of step 3 through a conformation close to $B_{2,5}$, the data permit a more detailed description of its transition state. The dihedral angle between the *pro-S* hydron and the electron-deficient p-orbital is $\theta$, so that made by the *pro-R* hydron is $\theta + 120°$. From eq 1 and the intrinsic effect for the *pro-S* hydron of $1.07_8$ and that for the *pro-R* hydron of $105_7$ (Table 2), both $\theta$ and $\ln(k_H/k_D)_{max}$ could be estimated if some estimate of $\ln(k_H/k_D)_i$ (eq 1) were available. If the inductive effect is negligible (as is the case for spontaneous hydrolyses of neuraminides with some nucleophilic participation by the substrate carboxylate[16]), then the physically realistic solution[20] gives $\theta = 23°$, $(k_H/k_D)_{max} = 1.09$. If $\ln(k_H/k_D)_i$ is estimated as about –0.02, a high estimate of the effect in solvolysis reactions,[21] then $\theta$ becomes 25° and $(k_H/k_D)_{max} = 1.12$. Qualitative mechanistic conclusions are thus insensitive to the correction for $(k_H/k_D)_i$ and in accord with a transition state in which the $B_{2,5}$ conformation of the bound substrate has flattened somewhat. The values of $(k_H/k_D)_{max}$ are in accord with a degree of charge development at the transition state similar to that seen in spontaneous hydrolyses of the same molecule when its carboxylate is ionized.[16]

While the $\beta_{lg}(V/K)$ value is a low-resolution mechanistic probe, further complicated in this case by the presence of step 2, its value of –0.4 provides some indication that acid catalysis is being applied to the oxygen. In the absence of such catalysis, $\beta_{lg}$ values around –1 are obtained for both enzymic and nonenzymic reactions.[22] The increase in $^{D_2O}(V/K)$ to ~1.6 for the 4-methylumbelliferyl compound as the pH is increased and substrate isotope effects become more fully expressed[10] is also consistent with some proton donation in step 3.

***S. typhimurium* Neuraminidase.** The most cursory examination of Michaelis–Menten parameters in Table 3, in comparison with those for the influenza enzyme in Table 1, indicates radical differences, the most immediately apparent of which is the much higher efficiency of the *S. typhimurium* enzyme: the $k_{cat}$ value for the *p*-nitrophenyl compound is some 740 times bigger than that for the influenza enzyme. This is unlikely to reflect a role of the influenza enzyme in mediating attachment of the virus to the host cell, since that particular function is carried out by a separate binding protein,[1] since attachment of the more detailed analysis reveals that $\beta_{lg}$ values are much more negative, $\beta_{lg}(V)$ being –0.53 ($r$ = –0.95) and $\beta_{lg}(V/K)$ being –0.80 ($r$ = 0.96). There is evidence that the $\beta_{lg}(V)$ value is probably low because of product release becoming rate-determining for the better substrates (vide inra), so the mechanistically informative parameter is $\beta_{lg}(V/K)$. The value of –0.80 indicates a large buildup of charge on the leaving group oxygen atom, and hence both that the aglycon–aglycon bond is nearly completely cleaved at the transition state and that little or no proton donation to the leaving group is occurring in the chemical transition state. These conclusions are confirmed by the very large value of the leaving group $^{18}O$ isotope effect on $V/K$ (Table 4): the equilibrium effect calculated for complete ionization of *p*-nitrophenyl β-galactopyranoside to a galactosyl cation and a *p*-nitrophenolate anion is 1.0425.[23] Proton donation would reduce the effect because it increases bonding to the substituted atom.[24] Precedent for the $^{18}(V/K)$ effect for the *S. typhimurium* enzyme exists in the effect on the lysozyme-catalyzed hydrolysis of the unnatural substrate

**Table 3.** Michaelis–Menten Parameters for Hydrolysis of Aryl *N*-Acetyl-α-D-neuraminides by *S. typhimurium* Neuraminidase, at pH 5.5 and 37.0 °C

| parent phenol | wavelength (nm) | Δε ($cm^{-1}$ $M^{-1}$) | $k_{cat}$ ($s^{-1}$) | $K_m$ (mM) |
|---|---|---|---|---|
| 4-nitrophenol | 340 | 1844 | $74_{86}$ | 0.87 |
| | 400 | 12 000 | $19_{11}$ | 0.86[a] |
| 4-cyanophenol | 262 | 625 | $135_0$ | 1.17 |
| 3-nitrophenol | 340 | 620 | $137_0$ | 0.94 |
| 3,4-dichlorophenol | 280 | 1150 | 1020 | 1.09 |
| 3-chlorophenol | 280 | 820 | 295 | $2.5_8$ |
| 4-chlorophenol | 280 | 1201 | 306 | 1.25 |
| phenol | 270 | 845 | 287 | 6.9 |
| $K_I$ for fully mutarotated *N*-acetylneuraminic acid | | | | |

[a] At pH 8.0.

**Table 4.** Kinetic Isotope Effects for *S. typhimurium* Neuraminidase-Catalyzed Hydrolysis of *p*-Nitrophenyl *N*-Acetylneuraminide at 37.0 °C[a]

| site of substitution | efefct on V/K | effect on V |
|---|---|---|
| | At pH 5.5 | |
| 3-[$^2$H]$_2$ | 1.097 ± 0.017 | 1.064 ± 0.014 |
| 3-*pro-R*-[$^2$H] | 1.051 ± 0.011 | 1.030 ± 0.014 |
| 3-*pro-S*-[$^2$H] | 1.050 ± 0.014 | 1.030 ± 0.013 |
| 2-[$^{18}$O] | 1.050 ± 0.010 | 1.022 ± 0.010 |
| | At pH 8.0 | |
| 3-[$^2$H]$_2$ | 1.090 ± 0.019 | 1.089 ± 0.018 |

[a] For buffer composition, see Experimental Section. Quoted errors are standard deviations on at least eight pairwise comparisons.

(20) Another solution to these two simultaneous quadratic equations gives $\theta = -31°$. This would be attainable only from the $^2C_5$ conformation distorted to give greater pucker, i.e., *away* from the $^4H_5$ half-chair, and is not physically realistic.

(21) Barnes, J. A.; Williams, I. H. *J. Chem. Soc., Chem. Commun.* **1993**, 1286.

(22) $\beta_{lg}$ values of –1.2, –1.1, and –1.3 are obtained for the water reactions of (aryloxy)tetrahydropyrans (Craze, G. A.; Kirby, A. J. *J. Chem. Soc., Perkin Trans. 2*, **1978**, 354), (aryloxy)tetrahydrofurans (Lönnberg, H.; Pohjola, V. *Acta Chem. Scand.* **1976**, *30A*, 669), and aryl *N*-acetyl-α-neuraminide anions,[16] respectively. A $\beta_{lg}$ ($V/K$) value of –1.0 was estimated for sweet almond β-glucosidase-catalyzed hydrolysis of aryl glucosides (Dale, M. P.; Kopfler, W. P.; Chait, I.; Byers, L. D. *Biochemistry* **1986**, *25*, 2522).

(23) Rosenberg, S.; Kirsch, J. F. *Biochemistry* **1981**, *20*, 3196.

(24) Values for $^{18}k$ of 1.0355 ± 0.015 (at 50 °C) and 1.0386 ± 0.0032 (at 35 °C) were obtained for the acid- and alkaline-catalyzed hydrolyses of *p*-nitrophenyl glucoside.[23] The acid-catalyzed effect is, however, anomalously temperature-dependent, because of a change in the mode of acid catalysis with temperature, and decreases to 1.023 at 75 °C (Bennet, A. J.; Davis, A. J.; Hosie, L.; Sinnott, M. L. *J. Chem. Soc., Perkin Trans. 2* **1987**, 581).

*p*-nitrophenyl 2′-acetamido-2′-deoxy-β-D-cellobioside ($^{18}(V/K)$ = 1.0467 ± 0.0015).[23]

All four isotope effects measured on $V$ at pH 5.5 are smaller than those measured on $V/K$ (Table 4) and (within the limits of error of our experiments) by the same factor: if the $V/K$ effects are intrinsic, then the $V$ effects are around 60% expressed. The very high $k_{cat}$ value for *p*-nitrophenyl *N*-acetylneuraminide (Table 3) makes the hypothesis that loss of one or other of the products (β-*N*-acetylneuraminic acid or *p*-nitrophenol) partly limits $V$ at optimal pH not unreasonable. This hypothesis was tested by measuring the largest effect at the nonoptimal pH of 8.0, where the $k_{cat}$ value was reduced by a factor of 3–4. At this pH, the dideuterio effect on $V$ and $V/K$ was the same and also was the same as that on $V/K$ at pH 5.5. We therefore conclude that the $V/K$ effects at pH 5.5 are indeed intrinsic and that some noncovalent event, most reasonably product loss, partly limits $V$ for substrates with good leaving groups at optimum pH.

Taking the intrinsic *pro-R* and *pro-S* effects as both 1.05 and substituting this into eq 1, as with the influenza enzyme, gives two physically realistic solutions, one of $\theta = 30°$ and one of $\theta = 300°$. The former would arise from a slightly flattened $B_{2,5}$ transition state, much like that for the influenza enzyme, but would result in estimates for $(k_H/k_D)_{max}$ of 1.067 with no inductive correction and 1.094 with an inductive correction of $\ln(k_H/k_D)_i = -0.02$. The other solution gives $(k_H/k_D)_{max} = 1.22$ with no inductive correction and $(k_H/k_D)_{max} = 1.31$ with an inductive correction of $\ln(k_H/k_D)_i = -0.02$. This solution would correspond to a nearly perfect $^2C_5$ chair.

The available data seem to favor reaction through the chair conformation, although reaction through a boat conformation similar to that associated with the action of the influenza enzyme cannot be excluded at present. However, the influenza enzyme binds the α-anomer of *N*-acetylneuraminic acid quite tightly, and the sugar can be diffused into the crystal, whereas it is not possible to diffuse *N*-acetylneuraminic acid into crystals of the *S. typhimurium* enzyme.[25] The $K_i$ value for *N*-acetylneuraminic acid against this enzyme (Table 3) has the same sort of relationship to $K_s$ values for aryl glycosides that is commonly found for glycosidases (where a small hydrophobic contribution to binding of aryl glycosides is unremarkable[17]). It is the influenza, not the *S. typhimurium*, enzyme that is unusual in respect of the binding of parent sugar.

Moreover, reaction of the *S. typhimurium* enzyme through the $B_{2,5}$ conformation of the substrate requires the conformation changes which limit the rate of influenza enzyme to an ineffectual 10 $s^{-1}$ to be by some mechanism compatible with essentially identical protein machinery, at least 3 orders of magnitude faster with the *S. typhimurium* enzyme. The back-side approach of a nucleophile to an equatorial substituent in a cyclohexane ring, though, is well known to be essentially sterically impossible.[26] Some distortion of the sugar ring may be compatible with measured effects, given their error bars. For example, if the *pro-S* effect were 1.036 and the *pro-R* effect 1.062, then $\theta$ in the absence of an inductive effect would be 305°. Circumstantial evidence in favor of the chair comes from the high value of $(k_H/k_D)_{max}$, indicating a very electron-deficient reaction center, associated with the $\theta = 300°$ solution of the simultaneous equations. This is consistent with the strongly negative $\beta_{lg}$ value and the use of an only weakly nucleophilic water molecule by this inverting enzyme. An extended study of AMP nucleosidase, another inverting glycohydrolase by multiple kinetic isotope effects, led to the conclusion that the bond order at the transition state to the incoming water was only 0.03.[27] In the case of *Aspergillus* glucoamylase, an inverting glycosidase, both α-deuterium kinetic isotope effects and electronic probes of charge development indicated that the charge development at the transition state for the hydrolysis of α-glucopyranosyl fluoride was the same as that in the spontaneous hydrolysis of the same compound.[28] The value of $(k_H/k_D)_{max} = 1.22$ is close to that observed in acid-catalyzed hydrolysis of *p*-nitrophenyl *N*-acetylneuraminide when the carboxylate group is protonated and unable to participate nucleophilically.[16]

**Table 5.** Comparison of Estimated Intrinsic Isotope Effects for the Hydrolysis of *p*-Nitrophenyl *N*-Acetyl-α-D-neuraminide by Various Sialidases at 37 °C and under Nonenzymic Conditions[a]

| reaction | 3-[$^2$H]$_2$ | 3-*pro-R*[$^2$H] | 3-*pro-S*[$^2$H] | 2-[$^{18}$O] | 4-[$^2$H] |
|---|---|---|---|---|---|
| 30 °C, nonezymic, $H_3O^+$ + neutral molecule | $1.10_2$ | $1.06_8$[b] | $1.02_5$[b] | $1.03_2$[c] | $0.97_6$[b] |
| 50 °C, nonenzymic, $H_3O^+$ + anion | $1.08_2$[b] | $1.08_6$[b] | 1.00[b] | $1.02_8$[c] | 1.00[b] |
| 60 °C spontaneous reaction of anion | $1.07_5$[b] | $1.07_3$[b] | 1.00[b] | $1.05_3$[c] | 1.00[b] |
| leech sialidase L[d] | $1.07_5$ | $1.02_0$ | $1.05_4$ | | |
| *V. cholerae* neuraminidase[e] | $1.05_8$ | $1.03_7$ | $1.01_8$ | $1.04_6$ | |
| influenza neuraminidase | $1.13_6$ | $1.05_7$ | $1.07_8$ | 1.06 | |
| *S. typhimurium* neuraminidase | $1.09_7$ | 1.05 | 1.05 | 1.05 | |

[a] For double-displacement sialidases, the effects are the best estimate for the first chemical step. [b] Reference 16. [c] Zhang, Y. Ph.D. Thesis, University of Illinois at Chicago, 1993. [d] Reference 13. [e] Reference 11.

**Summary and Conclusions.** The four sialidases we and others have examined mechanistically turn out to have four radically different catalytic mechanisms; intrinsic isotope effects are summarized and compared with values for spontaneous hydrolyses in Table 5.

(1) *V. cholerae* sialidase.[11] The reactive substrate conformation is derived from the ground-state $^2C_5$; the reaction proceeds with overall retention of configuration (probably via a glycosyl–enzyme intermediate).

(2) Leech sialidase L. The reactive substrate conformation is derived from the $^{3,6}B \Leftrightarrow {}^6S_2 \Leftrightarrow B_{2,5}$ part of the skew-boat pseudorotational itinerary, and the enzyme appears to bind the small proportion of the substrate already in this conformation directly. The reaction proceeds with overall retention of configuration, by internal transfer to O7 of the substrate.

(3) Influenza virus neuraminidase. The reactive substrate conformation is $B_{2,5}$, and this conformation appears to be attained by a kinetically significant step after the substrate is bound. The enzyme works with retention of configuration, and there is significant proton donation to the leaving group.

(4) *S. typhimurium* neuraminidase. The enzyme works with inversion of configuration, through a single chemical transition state derived from the ground-state $^2C_5$ chair, with little proton donation to the leaving group.

Mechanisms 3 and 4 occur with enzymes which have been shown, by high-quality X-ray crystallographic data, to have closely similar tertiary structures. The adoption of very different reaction paths as a consequence of minor changes in the protein may be a feature of reactions (such as glycosyl transfer) which involve intermediates (such as glycosyl cations) on the borderline of a real existence and hence which commonly adopt preassociative pathways in free solution. Small changes in the disposition of nucleophiles, charges, and general acids and bases may therefore be enough to divert the enzymic as well as the nonenzymic reaction from one pathway to another (and even from reaction through one conformation to another), so long as, in the enzymic case, the machinery for stabilizing the intermediate/transition-state structure remains in place. The idea that modest evolutionary change

(25) Taylor, G. L.; Vimr, E., unpublished work, 1993.

(26) So much so that the 2-adamantyl system has been used extensively as a model for "lm" solvolyses with no nucleophilic participation; see, e.g.: Bentley, T. W.; Bowen, C. T.; Morten, D. H.; Schleyer, P. v. R. *J. Am. Chem. Soc.* **1981**, *103*, 5466 and references therein.

(27) Mentch, F.; Parkin, D. W.; Schramm, V. L. *Biochemistry* **1987**, *26*, 921.

(28) (a) Matsui, H.; Blanchard, J. S.; Brewer, C. F.; Hehre, E. J. *J. Biol. Chem.* **1987**, *264*, 8714. (b) Konstantinidis, A.; Sinnott, M. L. *Biochem. J.* **1991**, *279*, 587.

in the enzyme can give rise to radical differences in reaction mechanism receives support from our recent finding that a single evolutionary change in the *ebg* β-galactosidase of *Escherichia coli* causes a large change in transition-state structure.[29]

It seems that in the case of the influenza and *S. typhimurium* sialidases, these subtle changes in the enzyme protein are apparently outside the resolution of even high-quality X-ray crystallographic data: the similarity of the protein folds of the *S. typhimurium* and influenza sialidases presumably arises because both structures have evolved to stabilize bound sialosyl cation-like species, whether by divergent or by convergent processes.

The failure of models based on good experimental enzyme tertiary structures to predict a mechanism in the present case suggests that, in general, caution should be applied to mechanistic conclusions derived from static X-ray electron density maps alone.

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(29) K, S.; Konstantinidis, A.; Sinnott, M. L.; Hall, B. G. *Biochem. J.* **1993**, *291*, 15.