A kinetic-isotope-effect study of catalysis by *Vibrio cholerae* neuraminidase

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Michaelis–Menten parameters for hydrolysis of seven aryl N-acetyl α-D-neuraminides by *Vibrio cholerae* neuraminidase at pH 5.0 correlate well with the leaving-group pKₐ (Δ pK₃; \( \beta_{\text{deuterium}} (V/K) = -0.73, r = -0.93; \beta_{\text{H}} (V) = -0.25; r = -0.95 \)). The \( \beta\)-deuterium kinetic-isotope effect, \( \beta_{\text{deuterium}} (V) \), for the p-nitrophenyl glycoside is the same at the optimum pH of 5.0 (1.059±0.010) as at pH 8.0 (1.053±0.010), suggesting that isotope effects are fully expressed with this substrate at the optimum pH. For this substrate at pH 5.0, leaving group \(^{18}\text{O}\) effects are \(^{18}(V) = 1.040±0.016\) and \(^{18}(V/K) = 1.046±0.015\), and individual secondary deuterium effects are \( \beta_{\text{deuterium}}(V) = 1.087±0.014, \beta_{\text{deuterium}}(V/K) = 1.037±0.014, \beta_{\text{proton}}(V) = 1.018±0.015, \beta_{\text{proton}}(V/K) = 1.030±0.017\).

INTRODUCTION

Neuraminidases (sialidases; EC 3.2.2.18) cleave sialic acid residues from oligosaccharides and the oligosaccharide moieties of glycoproteins and glycolipids. Biological functions associated with this N-acyl α-D-neuraminosyl-hydrolase activity include receptor recognition and masking, antigenic expression, and processes (including invasion) associated with the action of mammalian pathogens (Schauer, 1985).

A very great deal of structural information is available on influenza neuraminidase of various types, including the crystal structures of the uncomplexed enzyme (Varghese and Colman, 1991; Tulip et al., 1991) and that of the enzyme–N-acetylneuraminic acid complex (Varghese et al., 1992). This last is remarkable in that the sugar ring is bound in the B₄₅₆ conformation rather than the ground-state chair, and that the enzyme binds the α-anomeric form of the sugar, which is only 7.5% of the anomeric mixture at equilibrium (Friebolin et al., 1981).

The influenza enzyme works with retention of the anomeric configuration (Chong et al., 1992), but the crystal structure of the enzyme–N-acetylneuraminic acid complex reveals that there is no suitably placed protein side chain which could act as a nucleophile towards the anomeric centre, as is invariably found for retentive glycoside hydrolases without the substrate carboxylate (Sinnott, 1990). On the basis of site-directed-mutagenesis experiments, it was suggested that Glu-276, Glu-277 and His-274, all on the α-face of the sugar ring, were involved in proton transfer and electrostatic stabilization of an oxocarbonium ion intermediate (Lentz et al., 1987). The mechanistic studies of Chong et al. (1992) establish that the first chemical step in the hydrolysis of 4-methylumbelliferyl N-acetyl-α-D-neuraminic acid is subject to an intrinsic \( \beta\)-(D₂) deuterium kinetic-isotope effect of about 1.15, but that the second chemical step is characterized by an intrinsic effect which is very small and probably slightly inverse. It was therefore proposed that the glycosyl-enzyme intermediate is an oxocarbonium ion. However, it could very well be the α-lactone; we have recently shown that, in the spontaneous hydrolysis of \( p\)-nitrophenyl N-acetyl-α-D-neuraminide, the C-1 carboxylate participates nucleophilically (Ashwell et al., 1992).

In contrast with the influenza neuraminidase, little structural information is available on the *Vibrio cholerae* enzyme, although crystals suitable for X-ray crystallography have been grown (Taylor et al., 1992) and the enzyme has been cloned and expressed in *Escherichia coli* (Vimr et al., 1988). The *V. cholerae* enzyme, though, has been the subject of extensive synthetic organic chemically based studies of substrate specificity (Zbiral et al., 1989; Schreiner et al., 1991) and inhibitor power (Wallmann and Vasella, 1990; Bernet et al., 1990; Czollner et al., 1990; Vasella and Wyler, 1991).

We now report a study of the *V. cholerae* enzyme, using three mechanistic probes applied to aryl N-acetylneuraminide substrates: \( \beta\)-deuterium kinetic-isotope effects, leaving-group \(^{18}\text{O}\) kinetic-isotope effects, and the effect of leaving-group acidity on Michaelis–Menten parameters. These studies indicate that the *V. cholerae* enzyme has a very different mechanism to the influenza enzyme, in particular that the reactive sugar conformation is not the B₄₅₆ conformation seen in the influenza neuraminidase–N-acetylneuraminic acid complex.

On the basis of a report that the neuraminidases of *Clostridium perfringens* and *Arthrobacter ureafaciens* operate with retention of configuration (Friebolin et al., 1980), it has been widely assumed that all microbial sialidases act with retention. However, classification of glycosidases by hydrophobic-cluster analysis (Henrissat, 1991) puts influenza and *C. perfringens* enzymes in different classes, and it has been shown (Gebler et al., 1992) that the Henrissat classification does indeed

Abbreviation used: f.a.b., fast atom bombardment.

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correlate with the stereochemical course of the catalyst in the reaction. It therefore seemed worthwhile to examine briefly the stereochemical course of the *V. cholerae* enzyme.

**METHODS AND MATERIALS**

Labelled and unlabelled *N*-acetylneuraminides, except for 4-nitrophenyl 2-α-D-N-[^14]O-acetylneuraminide, have been described previously (Ashwell et al., 1992). 4-Nitro-1-[^14]O]phenol was made from 1-fluoro-4-nitrobenzene and H[^14]O by the method of Winkel et al. (1989); fast-atom-bombardment (f.a.b.) m.s. revealed that two separate batches were 85 and 75% ^14O-labelled. The labelled 4-nitrophenol was converted into the labelled *N*-acetylneuraminide (m.p. 130–132 °C) as for the unlabelled compound (Ashwell et al., 1992): f.a.b.m.s. confirmed that label had not been washed out during the alkaline glycosidation reaction.

Affinity-chromatography-purified *Vibrio cholerae* neuraminidase, provided as a solution in 0.15 M NaCl/4 mM CaCl₂, was purchased from Sigma (St. Louis, MO, U.S.A.; batch nos. 121H-4008-1 and 39F-4041-2) and was used without further purification.

Kinetic measurements were made on a Perkin–Elmer Lambda 6 spectrophotometer fitted with an electrically (Peltier-effect) thermostatically controlled cell block. For determination of *k*\text{cat} and *K*₅ values, at least seven measurements of initial rate were made between *K*₅/3 and 3*K*₅, reactions were initiated by addition of enzyme stock solution (10 μl) to substrate (250–1000 μl) and the absorbance was monitored continuously. Wavelengths and absorption coefficients differences (measured as difference between the absorption coefficient of the glycoside and that of the phenol) are given in Table 1. Initial rates were fitted to a rectangular hyperbola using the non-linear least-squares program Kaleidograph (Synergy, PCS Inc.). All rates were standardized on 4-nitrophenyl glycoside, and *k*\text{cat} values were calculated on the assumption that enzyme of the highest activity was 100% pure, fully active enzyme of molecular mass 82 kDa (Galén et al., 1992).

Kinetic-isotope effects on *V*₅ₐ₅, were measured from direct comparison of zero-order rates with [S] = 7*K*m substrate, whereas effects on *V*₅ₐ₅/K₅ were measured from direct comparison of first-order rate constants with [S] = K₅/25; first-order rate constants were calculated using Kaleidograph. For both types of isotope effect, data were compared as paired wise in the sequence light, heavy/heavy, light/light, heavy/etc.

Kinetic data pertain to 0.05 M sodium acetate buffer, pH 5.0, or 0.050 M sodium phosphate buffer, pH 8.0, at 37.0 °C. A literature protocol for assay of *V. cholerae* neuraminidase (Potier et al., 1979) led us to assume that saturation of this Ca²⁺-dependent enzyme could be maintained without additional Ca²⁺ in the assay buffer. The avoidance of excess Ca²⁺ seemed prudent, in the light of the well-known ability of carbohydrates to form complexes with Ca²⁺ and the established effect of this phenomenon on rates of non-enzymic hydrolysis of glycosides (Lönngren and Vesala, 1980). However, after most of the present work had been completed, the findings of Taylor et al. (1992) on the importance of added Ca²⁺ for structural integrity of the enzyme led us to verify this assumption. In fact, addition of enzyme stock solution to assay buffer containing 1.0 mM CaCl₂, at a concentration of 4-nitrophenyl *N*-acetyl-α-D-neuraminide substrate of *K*₅ and 7*K*m, gave rates 2–3% lower than in the absence of added Ca²⁺. Similarly, rates at 7*K*m were strictly proportional to the amount of (Ca²⁺-containing) enzyme stock solution added. We were therefore reassured that our kinetic measurements do indeed pertain to Ca²⁺-saturated enzyme.

**RESULTS AND DISCUSSION**

In Figure 1 are displayed the time courses of u.v. absorbance and optical rotation as p-nitrophenyl *N*-acetyl-α-D-neuraminide (10 mM) reacts with neuraminidase. The cleavage of the glycone–aglycone bond is essentially complete after 3 h, but the rotation changes continue for a further 24 h. The mutarotation of *N*-acetylneuraminic acid is slow (it has a half-life of 80 min at pH 5.4 and 30 °C; Frieboes et al., 1980), and the equilibrium lies 92.5% towards the β-anomer. If *V. cholerae* neuraminidase had reacted with inversion to give initially the β-anomer of *N*-acetylneuraminic acid, therefore, most of the rotation change would have been associated with cleavage of the glycone–aglycone bond, and there would have been little further change as 7.5% of the α-anomer was slowly produced by spontaneous mutarotation. It is clear, though, that less than one-third of the total rotation change is associated with hydrolysis, and that there is a substantial subsequent change after hydrolysis is essentially complete. *V. cholerae* neuraminidase therefore does indeed catalyse hydrolysis with overall retention of configuration.

In Table 1 are set out Michaelis–Menten parameters for hydrolysis of seven aryl *N*-acetyl-α-D-neuraminides by *Vibrio cholerae* neuraminidase, at pH 5.0 and 37.0 °C. A

<table>
<thead>
<tr>
<th>Parent phenol</th>
<th>Wavelength (nm)</th>
<th>ΔA (cm⁻¹·M⁻¹)</th>
<th><em>k</em>₅ (s⁻¹)</th>
<th><em>K</em>₅ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-Nitrophenol</td>
<td>340</td>
<td>1755</td>
<td>107</td>
<td>1.63</td>
</tr>
<tr>
<td></td>
<td>390</td>
<td>661</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-Nitrophenol</td>
<td>340</td>
<td>594</td>
<td>63</td>
<td>1.93</td>
</tr>
<tr>
<td>4-Chlorophenol</td>
<td>262</td>
<td>532</td>
<td>62</td>
<td>1.97</td>
</tr>
<tr>
<td>3,4-Dichlorophenol</td>
<td>280</td>
<td>1130</td>
<td>51</td>
<td>1.91</td>
</tr>
<tr>
<td>3-Chlorophenol</td>
<td>280</td>
<td>781</td>
<td>48</td>
<td>11.0</td>
</tr>
<tr>
<td>4-Chlorophenol</td>
<td>280</td>
<td>1143</td>
<td>22.9</td>
<td>5.08</td>
</tr>
<tr>
<td>Phenol</td>
<td>270</td>
<td>837</td>
<td>21.6</td>
<td>41</td>
</tr>
</tbody>
</table>

**Figure 1** Time courses of (a) absorbance at 390 nm in a 1 mm-path-length cell and (b) optical rotation at 546 nm in a 10 cm-path-length cell of a solution containing p-nitrophenyl *N*-acetyl α-D-neuraminide (5.0 mM) and *V.* cholerae neuraminidase (2.7 × 10⁻⁴ mM) in sodium acetate buffer, pH 5.0, at 37.0 °C.
Table 2  **Kinetic-isotope effects on the hydrolysis of p-nitrophenyl N-acetyl α-β-neuraminide by Vibrio cholerae neuraminidase at 37.8 °C**

Quoted errors are standard deviations on eight pairwise comparisons, except where stated.

<table>
<thead>
<tr>
<th>Site of substitution</th>
<th>(V) at pH 5.0</th>
<th>(V/K) at pH 5.0</th>
<th>(V) at pH 8.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>pro R 1\textsuperscript{4H}</td>
<td>1.037 ± 0.014</td>
<td>1.030 ± 0.017</td>
<td>1.058 ± 0.017</td>
</tr>
<tr>
<td>pro R 3\textsuperscript{4H}</td>
<td>1.018 ± 0.015</td>
<td>1.030 ± 0.017</td>
<td>1.058 ± 0.017</td>
</tr>
<tr>
<td>3\textsuperscript{18O}</td>
<td>1.055 ± 0.010</td>
<td>1.058 ± 0.019</td>
<td>1.053 ± 0.010</td>
</tr>
<tr>
<td>2\textsuperscript{18O}</td>
<td>1.045 ± 0.016*</td>
<td>1.046 ± 0.015 (n = 9)</td>
<td></td>
</tr>
</tbody>
</table>

* The average of two separate determinations with different batches of labelled substrate, which gave effects of 1.035 ± 0.014 (n = 8) and 1.045 ± 0.013 (n = 8).

It therefore appears that there may be a polar effect on binding, operating on the aromatic aglycone. The effect is so large that it is unlikely to arise by interactions at the glycosidic centre; rather it is possible to envisage direct interactions of, say, a negatively charged group on the protein with the aromatic π system of the leaving group. Such an interaction would be more sensitive to the electronic nature of the aromatic ring than the pKₐ of the phenol; rates of nucleophilic aromatic substitution, for example, obey the Hammett equation with ρ values of +4, compared with +2 for equilibrium ionization of substituted phenols (Isaacs, 1987). The possible nature of this interaction is discussed below in connection with transition state conformations and the pattern of inhibitory behaviour of sialic acid derivatives (Wallimann and Vasella, 1990; Bernet et al., 1990).

β-Deuterium kinetic isotope effects are particularly informative about transition-state geometry and charge development. They have their origin in two phenomena, hyperconjugation and the inductive effect of deuterium, and can be considered in terms of eqn. (1):

\[ \ln(k_{H}/k_{D}) = \cos^2 \theta \ln(k_{H}/k_{D})_{\text{max}} + \ln(k_{H}/k_{D})_{\text{a}} \]

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. \( k_{H}/k_{D} \) is the maximal hyperconjugative effect obtained when the C–L bond and the p orbital are exactly eclipsed, 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 atoms should be the product of the individual single-deuterium effects. With **V. cholerae** neuraminidase (Table 2), the dideuterium effects are indeed the product of the two single deuterium effects. Moreover, the effect from a proR deuterium is the same as, or somewhat greater than, the effect from a proS deuterium. This immediately rules out reaction from the B₂₉₆ conformation (II), since in this conformation the C-3-proS hydroxyl bond makes a dihedral angle of approx. 180° with the breaking C-OAr bond, whereas the C-3-proR hydroxyl bond makes a dihedral angle of approx. 60° with the breaking C-OAr bond. Reaction from this conformation would thus result in the proS effect being bigger than the proR, and this is not observed.

It is noteworthy that the proS effect is indeed bigger than the proR for the first bond-breaking step of leech sialidase L, a transferase which yields 2,7-anhydro-N-acetylneuraminic acid (III) as product (Sinnott et al., 1993); in this case reaction is occurring from somewhere along the $^4$B $\rightarrow$ $^4$S $\rightarrow$ B₂₉₆ portion of the skew-boat pseudorotational itinerary.
If indeed the complex of influenza neuraminidase with N-acetylmuramic acid in the B₄₅ conformation does reflect the reactive conformation of true substrates, then the influenza and \textit{V. cholerae} enzymes must have radically different mechanisms. The relative magnitudes of the effects for this enzyme are consistent with reaction through the ground-state \( ^1 \text{C}_{4} \) chair (I), perhaps flattened towards the \( ^4 \text{H}_2 \) half-chair (which would result in the \textit{pro}R effect being somewhat bigger than the \textit{pro}S effect, as is observed for non-enzymic hydrolysis (Ashwell et al., 1992). A further difference is the much lower degree of charge development at C-2 in the first chemical transition state. The intrinsic dideutero effect for the \textit{V. cholerae} enzyme (1.06) is much lower than that for the influenza enzyme (1.15; Chong et al., 1992) and is lower even than for spontaneous hydrolysis where the carboxylate participates nucleophilically (1.08; Ashwell et al., 1992).

\[
\begin{align*}
\text{NHAc} & \quad \text{COO}^- \\
\text{OH} & \quad \text{OH} \\
\text{OH} & \quad \text{OH} \\
\text{AcNH} & \quad \text{OH} \\
\end{align*}
\]

We have therefore uncovered two unusual features of this enzyme: low charge development at the reaction centre, and a strong polar effect on the binding of aryl glycosides. A third unusual feature has been discovered by Vasella’s group, that, with certain pairs of inhibitors epimeric at C-2 (e.g. IV; Wallimann and Vasella, 1990), the epimer homologous with substrate, with the carboxylic acid group axial, is bound the less tightly. Their detailed interpretation, in terms of a \( B_{4.5} \)-like transition state in which the carboxylate group made favourable contacts, with, say, a guanidino residue only at the transition state, is not compatible with our kinetic-isotope-effect measurements, for reasons outlined above. However, their basic idea of an active site in which an axial orientation of the substrate carboxylic acid is somehow destabilized will rationalize our results if the destabilization occurs by repulsion of like charges, rather than attraction of opposite ones. If there were a carboxylate group on the enzyme placed approximately as shown in (V), repulsion between like charges would increase the nucleophilicity of the substrate carboxylate group, resulting in less charge development at C-2 at the transition state. The same carboxylate group might also participate in stabilizing interactions with electron-deficient \( \pi \) systems of aryl glycosides.

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\textbf{REFERENCES}


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