RESEARCH COMMUNICATION

Salmonella typhimurium neuraminidase acts with inversion of configuration

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When the time course of the hydrolysis of identical solutions of *p*-nitrophenyl *N*-acetyl- α -D-neuraminide by *Salmonella typhimurium* neuraminidase is monitored by u.v. and by its optical rotation, the rotation change is synchronous with, or even marginally in advance of, the absorbance change. In experiments under the same conditions with influenza-virus neuraminidase, known to react with retention of configuration [Chong, Pegg, Taylor and von Itzstein (1992) *Eur. J. Biochem.* **207**, 335–343],

the rotation change is much slower than the absorbance change. The inverting, presumably single-displacement, mode of action of the *S. typhimurium* enzyme follows from these observations, and the position (92.5% β) of the slowly established mutarotational equilibrium of *N*-acetylneuraminic acid [Friebolin, Kunzelmann, Supp, Brossmer, Keilich and Ziegler (1981) *Tetrahedron Lett.* **22**, 1383–1386].

INTRODUCTION

Glycosidases can catalyse the hydrolysis of glycosides with either retention or inversion of the anomeric configuration, and clearly any rational consideration of catalytic mechanism must take into account the structure of the product of the catalysed reaction (see, e.g., Sinnott, 1990). Careful studies by ^1H n.m.r. have shown that the neuraminidases from *Arthrobacter ureafaciens*, *Clostridium perfringens* (Friebolin et al., 1980a,b) and influenza virus (Chong et al., 1992) all catalyse the hydrolysis of α -glycosides of *N*-acetylneuraminic acid, and this has led to a widespread assumption that all neuraminidases work with retention of configuration.

^1H -n.m.r.-based determinations of the stereochemical course of action of neuraminidases require comparatively large amounts of enzyme activity, much of which may be lost during the necessary exchange with $^2\text{H}_2\text{O}$. An alternative method which uses less enzyme takes advantage of the fact that mutarotation of *N*-acetylneuraminic acid is slow ($t_{1/2} \sim 80$ min in $^2\text{H}_2\text{O}$ at neutral pH and $\sim 30^\circ\text{C}$) and that the equilibrium lies largely towards the β anomer, which forms 92.5% of the equilibrium mixture (Friebolin et al., 1981). If the sialidase-catalysed hydrolysis of a chromogenic α -D-neuraminide is monitored by both u.v. and polarimetry, the observation of absorbance changes in advance of rotation changes therefore establishes immediately that the catalysed reaction proceeds with retention. The anticipated retentive stereochemistry of the *Vibrio cholerae* enzyme was recently confirmed in this way (Guo and Sinnott, 1993).

In the light of sequence comparisons indicating homology among small and large sialidases, and the existence of a sialidase superfamily (Hoyer et al., 1992; Roggentin et al., 1993), as well as the similarity of the protein-fold and active-site groups of the influenza-virus and *Salmonella typhimurium* enzymes, as indicated in their X-ray crystal structures (Varghese and Colman, 1991; Tulip et al., 1991; Crennell et al., 1993), we had every expectation that the stereochemistry of *S. typhimurium* enzyme would likewise be retention. It is not.

METHODS AND MATERIALS

Recombinant *S. typhimurium* neuraminidase was given by Dr. Eric Vimr, University of Illinois at Urbana-Champaign, and

influenza-virus neuraminidases A and B were given by Dr. Graeme Laver, John Curtin School of Medical Research, Canberra, Australia. *p*-Nitrophenyl *N*-acetyl- α -D-neuraminide was made and purified as described by Ashwell et al. (1992).

The release of aglycone from *p*-nitrophenyl *N*-acetyl- α -D-neuraminide was monitored at 390 nm in 1 mm-path-length cells at 37.0°C in a Perkin-Elmer Lambda 6 spectrophotometer, fitted with the manufacturers' Peltier-effect thermostatically controlled cell block. Optical rotation was monitored at 546 nm in a 10 cm 1 ml-capacity water-jacketed cell in a Jasco DiP 370 polarimeter. Water maintained at $37.0 \pm 0.1^\circ\text{C}$ by a Fischer circulating water bath was circulated through the jacket.

Enzyme was added to a thermostatically controlled stock solution, and then portions of this reacting solution were placed in u.v. and polarimeter cells. The first time point was then taken after a small fraction of reaction, so that total absorbance changes or rotation changes were not exactly constant from experiment to experiment.

RESULTS AND DISCUSSION

Figure 1 illustrates the time course of the hydrolysis of *p*-nitrophenyl *N*-acetyl- α -D-neuraminide by *S. typhimurium* neuraminidase, as monitored by absorbance or by optical rotation. The rotation changes parallel to, or if anything very slightly in advance of, the absorbance changes. This is what would be expected were the glycoside hydrolysed directly to β -*N*-acetylneuraminic acid, which is 92.5% of the equilibrium mixture.

Figure 2 illustrates an exactly analogous experiment with the influenza-virus neuraminidase, in the same buffer and at the same temperature. The first product of the action of influenza neuraminidase has been established to be α -*N*-acetylneuraminic acid (Chong et al., 1992). It is quite clear that, for this retaining enzyme, even when liberation of aglycone is noticeably slower than with the *S. typhimurium* enzyme, rotation changes lag markedly behind absorbance changes. Therefore the curves of Figure 1 are not being produced by enzymic hydrolysis with retention, followed by rapid spontaneous mutarotation.

There remains the possibility that the *S. typhimurium* enzyme, or some contaminant therein, catalyses the mutarotation of the neuraminic acid. This possibility was addressed by producing a

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Figure 1 Time courses for the *S. typhimurium* neuraminidase-catalysed hydrolysis of *p*-nitrophenyl *N*-acetyl- α -D-neuraminide (5.0 mM) in 50 mM sodium acetate buffer, pH 5.0, at 37 °C, monitored by rotation (×) and absorbance (○), and expressed as percentage of the total changes on complete reaction

These were 0.482 A unit for absorbance and -135 m-degrees for rotation. The enzyme concentration was 0.4 nM (16.5 ng/ml).

solution of neuraminic acid with the α -anomer in excess of its equilibrium proportion by influenza-neuraminidase-catalysed hydrolysis of its *p*-nitrophenyl glycoside, and adding *S. typhimurium* enzyme (0.7 nM, 29 ng/ml). No interruption of the time course of optical rotation was apparent (results not shown), indicating that any *S. typhimurium* neuraminidase-catalysed mutarotation of *N*-acetylneuraminic acid is negligible for present purposes.

Therefore influenza and *S. typhimurium* neuraminidases must have radically different mechanisms, since they catalyse different reactions (Figure 3). Questions then inevitably arise about the proposal of common ancestry for all neuraminidases on the basis

Figure 2 Time courses for the influenza neuraminidase A-catalysed hydrolysis of *p*-nitrophenyl *N*-acetyl- α -D-neuraminide (5.0 mM) in 50 mM sodium acetate buffer, pH 5.0, at 37 °C, monitored by rotation (·) and absorbance (○), and expressed as percentage of the total changes on complete reaction

These were 0.493 A unit for absorbance and -140 m-degrees for rotation. The enzyme concentration was 10 nM (0.5 μ g/ml).

Figure 3 Reactions catalysed by influenza and *S. typhimurium* neuraminidases

of sequence homologies (Hoyer et al., 1992; Roggentin et al., 1993). It is significant that the hydrophobic cluster analysis (Henrissat, 1991) puts influenza and *C. perfringens* neuraminidases in different classes, and that this type of classification appears to correlate well with stereochemistry (Gebler et al., 1992).

There remains, however, the possibility that the retaining stereochemistry of the influenza enzyme and the inverting stereochemistry of the *S. typhimurium* enzyme represent pathways of comparable energy within an essentially common active site, and that comparatively little change in the protein is required to change from one to the other. It is possible to envisage a protein structure, the primary function of which is to stabilize a sialosylation. The *S. typhimurium* enzyme would then have a site for a pre-associated water molecule on the β face of the sugar ring, whereas nucleophilic participation by the carboxylate group of the substrate, as with the spontaneous hydrolysis of neuraminides (Ashwell et al., 1992) would give a glycosyl-enzyme intermediate, and hence overall retention, in the case of the influenza enzyme.

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