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Effect of substrate aglycon on enzyme mechanism in the reaction of sialidase from influenza virus

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Abstract The effect of substrate aglycon on enzyme mechanism of sialidase from influenza virus was investigated by kinetic isotope effects using the substrates 4-methylumbelliferyl-*N*-acetyl-α-D-neuraminic acid (Neu5Acα2MU) and *p*-nitrophenyl-*N*-acetyl-α-D-neuraminic acid (Neu5Acα2PNP). The kinetic isotope effect on V_{max} (^{Bp}V), at pH 6.0, as revealed by direct comparison of rates obtained with Neu5Acα2MU and the [3,3-^{2}H]-substituted substrate analogue, was shown to be inverse. This indicates that sialidase-catalysed hydrolysis of Neu5Acα2MU proceeds with substantial positive charge development at the reaction centre in the transition state for the formation of the glycosyl cation-enzyme intermediate. However, no such inverse effect on V_{max} at pH 6.0 was observed when using Neu5Acα2PNP and the [3,3-^{2}H]-substituted substrate. A mechanism by which hydrolysis proceeds through an α-lactone intermediate has been proposed by Guo et al. [8]. We propose that the differences in ^{Bp}V for the substrates investigated are due primarily to the differing properties of the aglycon leaving groups, which may result in influenza virus sialidase catalysing substrate hydrolysis by a similar mechanism with alternative stabilisation of transition state.

Key words: Kinetic isotope effect; Substrate aglycon; Sialidase; Influenza virus; 4-Methylumbelliferyl-*N*-acetyl-α-D-neuraminic acid; *p*-Nitrophenyl-*N*-acetyl-α-D-neuraminic acid; Transition state

1. Introduction

Sialidase (EC 3.2.1.18) catalyses the hydrolysis of terminal sialic acid from a range of glycolipids, glycoproteins and oligosaccharides [1] and has been associated with many important biological functions [2].

The crystal structures of sialidase from influenza virus [3], *Salmonella typhimurium* [4] and *Vibrio cholerae* [5] have recently become available. The application of isotope effects to the mechanistic studies of a variety sialidase has also been reported [6–8].

We [7] and others [8] have previously reported β-deuterium isotope effects on the reaction of sialidase from influenza virus. The present study describes significant differences observed in isotope effects for this enzyme depending on the type of substrate used in the study. Chong et al. [7] observed a significant inverse isotope effect on V_{max} at pH 6.0, using the substrate 4-methylumbelliferyl-*N*-acetyl-α-D-neuraminic acid, indicating that hydrolysis of substrate by sialidase requires the formation of a sialosyl cation–enzyme transition state intermediate. The absence of such an effect observed by Guo et al. [8] using *p*-nitrophenyl-*N*-acetyl-α-D-neuraminic acid as the substrate suggested, on the other hand, that the sialidase-catalysed reaction may equally well occur via hydrolysis of an α-lactone intermediate. In order to clarify these observations for influenza virus sialidase, β-deuterium isotope effects on V_{max} at optimum pH were determined for both the substrates in question. We now report these findings, and propose an explanation which takes into account the leaving group properties of the substrate aglycon.

2. Materials and methods

Influenza virus, A/Tern/Australia/G70c/75 (H11N9) sialidase was provided as a purified enzyme preparation by Dr Jenny Breshkin, CSIRO, Division of Biotechnology.

4-Methylumbelliferyl-*N*-acetyl-α-D-neuraminic acid (Neu5Acα2-MU) and Neu5Acα2MU perdeuterated at C3 ([3,3-^{2}H]Neu5Acα2MU) were prepared as described in [9].

p-Nitrophenyl-*N*-acetyl-α-D-neuraminic acid (Neu5Acα2PNP) and *p*-nitrophenyl-*N*-acetyl-α-D-neuraminic acid perdeuterated at C3 ([3,3-^{2}H]Neu5Acα2PNP) were prepared as described in [10].

Sialidase activity, using Neu5Acα2MU as substrate, was assayed by a modification [7] of a previously reported fluorometric method [11]. The fluorescence measured was linear with respect to the concentration of 4-methylumbelliferone. β-Deuterium isotope effects, using Neu5Acα2MU as substrate, were measured by direct comparison of initial rates between [3,3-^{2}H]Neu5Acα2MU and non-deuterated Neu5Acα2MU. Kinetic measurements were performed in 50 mM MES, 0.1 M triethanolamine, 20 mM $CaCl_2$, pH 6.0, at 37°C. Kinetic parameters were determined, for both labelled and unlabelled Neu5Acα2MU, using Lineweaver–Burk plots. A data set consisted of 5–7 substrate concentrations (0.3–3 K_m) for both labelled and unlabelled substrate, in duplicate. Substrate concentrations for each data set were calibrated by mild acid hydrolysis. Acid hydrolysis of varying substrate concentrations were carried out in 0.02 M HCl at 84°C for 30 min. After cooling 0.1 M MES pH 6.0 was added and the amount of free 4-methylumbelliferone measured by fluorescence spectroscopy as described above for the fluorometric assay of sialidase activity.

Sialidase activity, using Neu5Acα2PNP as substrate, was assayed in 50 mM sodium acetate, 0.1 mM $CaCl_2$, 0.32 mM NaCl, pH 6.0 at 37°C. Liberated *p*-nitrophenolate was determined spectrophotometrically at 400 nm [8]. β-Deuterium isotope effect on V_{max}, using Neu5Acα2PNP as substrate, were measured by directly comparing zero-order rates of deuterated and non-deuterated Neu5Acα2PNP, as described in [8].

3. Results and discussion

An inverse β-deuterium isotope effect on V_{max} at optimum pH is observed when using labelled and unlabelled Neu5Acα2MU as the substrate (Table 1). This effect was also observed by Chong et al. [7] and is consistent with the proposal that influenza virus sialidase-catalysed hydrolysis of Neu5Acα2MU proceeds with substantial positive charge development at the reaction centre in the transition state for the formation of the sialosyl cation–enzyme intermediate.

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Table 1
Kinetic parameters and kinetic isotope effect on V_{max} for influenza virus sialidase using Neu5Acα2MU and Neu5Acα2PNP as substrate at pH 6.0, 37°C

Sialidase	Neu5Acα2MU			Neu5Acα2PNP		
	$K_{cat}(s^{-1})$	K_m (M)	$^{\beta D}V$	$K_{cat}(s^{-1})$	K_m (M)	$^{\beta D}V$
Influenza virus	230	3.0×10^{-4}	0.93 ± 0.05 0.979 ± 0.007(a)	12.3	1.5×10^{-3}	1.00 ± 0.04 1.0095 ± 0.011(b)

For comparison, value obtained in reference [4].
For comparison, value obtained in reference [5].

Although, it is interesting to note that Craze et al. [12], have suggested that glycosyl–enzyme intermediates need not be long lived, and could escape detection if the intermediate is of an unusually strained nature. The X-ray crystal structure of influenza sialidase–Neu5Ac complex is also compatible with the transition state being a glycosyl cation–enzyme intermediate. It is likely that the positively charged transition state intermediate is stabilised by the carboxylate of the Asp-151 and also by the generally negatively charged environment at optimum pH [7] (Fig. 1).

Interestingly however, the inverse effect on V_{max} at optimum pH was not observed (Table 1) using an alternative substrate, labelled and unlabelled Neu5Acα2PNP. This is consistent with the results obtained by Guo et al. [8]. The lack of an inverse isotope effect led Guo et al. [8] to propose a different mechanism for enzyme catalysis in which substrate hydrolysis proceeded through an α-lactone intermediate (Fig. 2). These authors also proposed that the non-enzymatic hydrolysis of Neu5Acα2PNP proceeded with the nucleophilic participation of the substrate carboxylate to form an α-lactone, even though the reaction centre would of itself give a relatively stable oxocarbocation [13].

It is important to note in this context that 4-methylumbelliferyl aglycon is a poor leaving group in comparison to the *p*-nitrophenyl aglycon. It has been suggested by Craze et al. [12], using nucleophilic reactions on the acetal methoxymethoxy-2,4-dinitrobenzene as a model system, that in the case of a poor leaving group, bond breaking will be well advanced in the transition state. Thus, the resulting positive charge at the anomeric carbon atom may be stabilised by a negatively charged group. In the case of influenza virus sialidase the stabilisation of the sialosyl cation is likely to be provided, in part, by the carboxylate group of Asp-151. In the case of increased leaving group capability, as exists with the *p*-nitrophenyl aglycon through the electron-withdrawing effect of the nitro group, weak bonding of the leaving group (*p*-nitrophenolate) to the anomeric centre with a concomitant stabilisation from a negatively charged group or nucleophile may well reduce the overall positive charge at this centre and therefore markedly affect secondary isotope effects. The notion of weak bonding from good leaving groups in the transition state and the effect on secondary isotope effects has been previously reported [12].

Our results for the two substrates confirm that the differences observed for $^{\beta D}V$ are real. We propose that the differences observed for $^{\beta D}V$, with the two different substrates, are primarily due to the differing leaving properties of the aglycon groups, which may give rise to alternative transition-state stabilisation mechanisms of enzyme-catalysed substrate hydrolysis. In the case of the better leaving group, *p*-nitrophenolate, weak bonding to the anomeric centre will significantly affect secondary isotope effects. The results obtained with Neu5Acα2MU are consistent with a sialosyl cation intermediate, whereas in the case of Neu5Acα2PNP the data are supportive of a less positively charged transition state. The possibility that an α-lactone is an intermediate seems, to us, less likely, because the carboxyl group of the sialic acid substrate forms an energetically favourable salt bridge with an active site arginine [7]. Moreover, it is difficult to rationalise why such lactone intermediate would be formed in preference to the hydration of a sialosyl cation intermediate.

In conclusion, we suggest that careful interpretation of kinetic isotope effect data is required, taking into account potential differences in leaving group properties of aglycons.

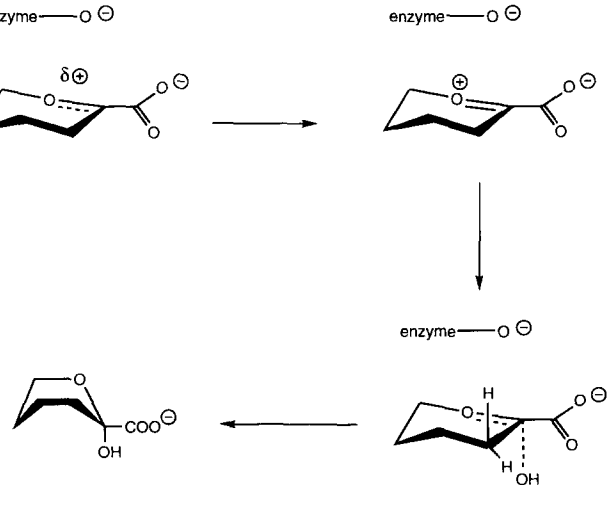

Fig. 1. Glycosyl–enzyme intermediates.

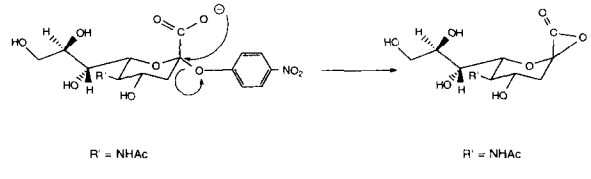

Fig. 2. α-Lactone intermediate.

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