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Hydrolase and Sialyltransferase Activities of *Trypanosoma cruzi trans*-Sialidase Towards NeuAc-α-2,3-Gal-β-*O*-PNP†

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Abstract—NeuAc-α-2,3-Gal-β-*O*-PNP has been synthesised and its ability to act as a substrate for the hydrolase and transferase activities of *Trypanosoma cruzi trans*-sialidase have been investigated. The turn-over of this compound shows marked differences from the behaviour of NeuAc-MU. In addition, distinct differences in the action of *T. cruzi trans*-sialidase and *Clostridium perfringens* neuraminidase on NeuAc-α-2,3-Gal-β-*O*-PNP were apparent. © 2001 Elsevier Science Ltd. All rights reserved.

The South American trypanosome, *Trypanosoma cruzi*, is the etiological agent responsible for Chagas' disease, a debilitating and often fatal condition prevalent in South and Central American populations.[2] This motile, blood-borne parasite needs to invade mammalian cells to undergo cell division and hence complete its life cycle.[3] To do this, the parasite must first adhere to the surface of host cells. This it achieves by the generation of a negatively charged glycopeptide coat on its surface. The charged moieties concerned contain sialic acid (NeuAc). However, the parasite does not in fact produce sialic acid itself; with the aid of a cell surface *trans*-sialidase, it scavenges this sugar from mammalian glycoconjugates and transfers it onto mucin glycopeptides on the parasite cell surface in a regio- and stereo-controlled manner (i.e. it specifically uses and makes α-2,3-linked sialosides).[4] As such, *T. cruzi trans*-sialidase represents a target for therapeutic intervention.

The aim of this study reported was to identify features of the structure and/or mechanism of *trans*-sialidase that mark it out as different from the purely hydrolytic sialidases, which have been well studied and for which there are several crystal structures.[5] From a biological perspective, the structural and functional properties of *trans*-sialidase have been reviewed.[6] More recent studies employing site-directed mutagenesis and selective peptide deletions have suggested a role for two protein domains in the sialyltransferase activity of *trans*-sialidase[7] and have led to proposals about the location of potential galactose binding sites on the enzyme.[8] The recently reported crystal structure of the *T. rangeli* sialidase,[9] which is approx. 70% identical to the core globular region of *T. cruzi trans*-sialidase, supports the presence of a distinct acceptor substrate binding site in *trans*-sialidase.

Assays for sialidase activity routinely rely on the cleavage of *para*-nitrophenyl (PNP) or 4-methylumbelliferyl (MU) sialosides, which give rise to UV–vis active and fluorescent products, respectively. We have been unable to determine reliable kinetic parameters for the hydrolysis or transfer of NeuAc from NeuAc-α-PNP by *trans*-sialidase.[10] However, we note that this substrate, which is straightforward to prepare on a gram scale, is very effective for milligram-scale biotransformations, which proceed with high efficiency when stoichiometric acceptor is present.[11] A more detailed account of this observation can be seen in the recent work of Crout and co-workers.[12] We note that Scudder and co-workers reported the rate of NeuAc transfer to acceptor from NeuAc-PNP was some 25-fold less than from NeuAc-α-2,3-lactose.[13] We were therefore drawn to consider the development of a *trans*-sialidase assay that would monitor cleavage of the NeuAc-α-2,3-Gal glycosidic linkage, but

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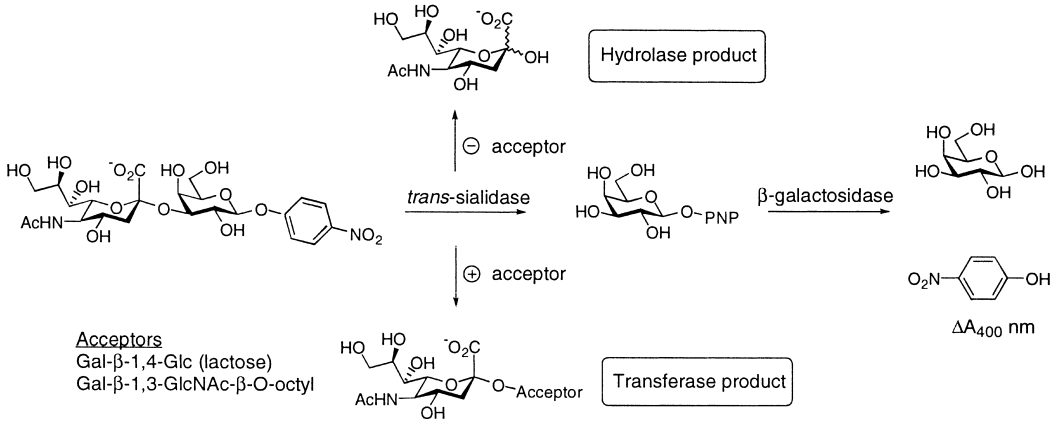
which would give a simple optical readout. This would obviate the need for HPAEC analysis, as required with NeuAc-α-2,3-lactose.[13]

The proposed assay (outlined in Scheme 1) employs NeuAc-α-2,3-Gal-PNP (synthesised as outlined in Figure 1)[14] as a substrate. The basis of the assay relies on *trans*-sialidase to transfer NeuAc to either water (hydrolase) or an acceptor sugar (transferase), with the Gal-PNP released in this step being cleaved in situ by β-galactosidase to liberate PNP, the anion of which can be monitored spectroscopically (ΔA_{400} m; $\varepsilon = 18{,}300\ M^{-1}$).[15,16]

Clearly the choice of β-galactosidase is critical, since it could also cleave β-galactoside acceptors present in the assay. In the first instance, we chose to work with sweet almond β-glucosidase, which has residual β-galactosidase activity but does not cleave inter-sugar glycosidic linkages with any significant efficiency (i.e. lactose is not a substrate for this enzyme).[15,17] However, the pH optimum for this enzyme (5.6)[15] is significantly lower than that of *trans*-sialidase (7.5).[13] As a compromise, assays were conducted at pH 6.5, although it became apparent that NeuAc-α-2,3-Gal-PNP was not particularly stable at this pH. *E. coli* β-galactosidase (pH optimum 7.4)[18] was therefore investigated. Clearly this enzyme is designed to hydrolyse lactose, so an alternative acceptor substrate was required. Gal-β-1,3-GlcNAc-β-*O*-octyl[19] proved effective as a *trans*-sialidase acceptor and was not cleaved by the *E. coli* β-galactosidase.[17] The revised assay, operating at pH 7.5 with the *E. coli* β-galactosidase and Gal-β-1,3-GlcNAc-β-*O*-octyl as the acceptor substrate, was used in further studies.[20]

The addition of acceptor substrate was shown to stimulate the release of PNP in this assay (Figure 2, entries 1 and 2), which contrasts to the situation where NeuAc-MU is used as the donor.[21] In addition, the same assay performed with the hydrolytic *Clostridium perfringens* sialidase showed no such stimulation (Figure 2, entries 4 and 5). In keeping with earlier studies,[22] *trans*-sialidase proved to be insensitive to the standard glycal neuraminidase inhibitor DANA, whilst the *Clostridium* neuraminidase was sensitive (Figure 2, entries 3 and 6).

With a view to indentifying potential *trans*-sialidase inhibitors, we wondered whether *S*-linked NeuAc-Gal disaccharides, with a greater distance between the NeuAc and Gal moieties by virtue of the greater C–S versus C–O bond length, might be inhibitory. Such compounds have previously been investigated as sialidase



Scheme 1. Outline of coupled spectrophotometric assay for *trans*-sialidase.

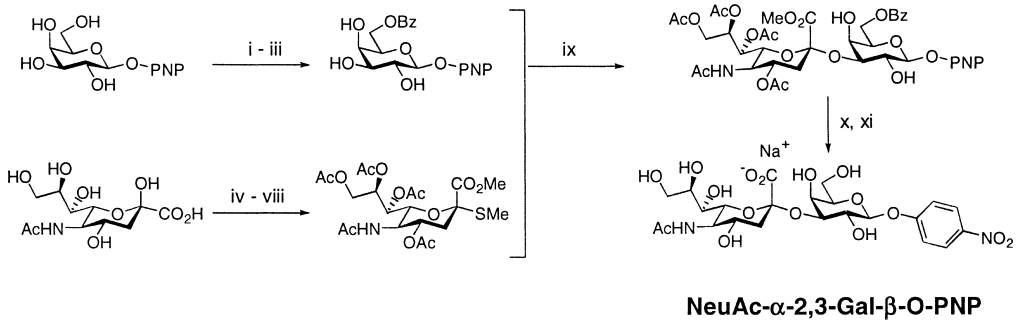
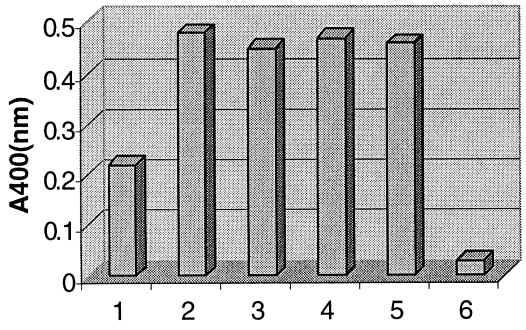


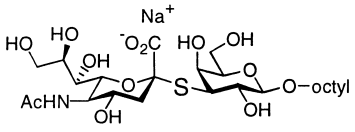
Figure 1. Synthesis of disaccharide donor substrate NeuAc-α-2,3-Gal-β-*O*-PNP. (i) 2,2-Dimethoxypropane, TsOH, rt, 24 h followed by aq TFA/DCM, rt, 10 min; (ii) BzCN, pyr/DCM, ice bath to 10°C, 16 h; (iii) aq TFA/DCM, ice bath, 10 min (65% over 3 steps); (iv) Dowex 50W-X8-200(H^+), MeOH, rt, 2 h, quant.; (v) Ac_2O, pyr, rt, 48 h, quant.; (vi) AcCl, $HCl_{(g)}$, −50 °C to rt, 20 h, quant.; (vii) KSAc, DCM, rt, 20 h, 90%; (viii) Na, MeOH, −40 °C, 40 min, followed by MeI, DMF, rt, 20 h, 91%; (ix) NIS, TfOH, 3 Å mol. sieves, DCM/MeCN, −45° to −20 °C, 4 h, 55%; (x) NaOMe, MeOH, ice bath, 20 h, quant.; (xi) 0.1 M NaOH, ice bath, 4 h, quant.



Acceptor: 1mM Gal-β-1,3-GlcNAc-O-β-octyl
Inhibitor: 1mM 2,3-Dehydro-2-deoxy-NeuAc (DANA)
1. *T. cruzi trans*-sialidase, minus acceptor
2. *T. cruzi trans*-sialidase, plus acceptor
3. *T. cruzi trans*-sialidase, plus acceptor plus inhibitor
4. *C. perfringens* sialidase, minus acceptor
5. *C. perfringens* sialidase, plus acceptor
6. *C. perfringens* sialidase, plus acceptor plus inhibitor

Figure 2. Effect of acceptor substrate and putative inhibitor on the activity of *Trypanosoma cruzi trans*-sialidase and *Clostridium perfringens* sialidase.[20]

inhibitors,[23] and have been found not to serve as substrates for *Vibrio cholerae* sialidase.[24]



NeuAc-α-2,3-S-Gal-β-O-octyl

However, NeuAc-α-2,3-S-Gal-β-*O*-octyl[25] was found to show no significant inhibition of *trans*-sialidase at millimolar concentrations.

For both hydrolase (– acceptor) and transferase (+ acceptor) assays, NeuAc-α-2,3-Gal-PNP gave K_m values in excess of 5 mM, in keeping with data from Horenstein and co-workers for the reaction of *trans*-sialidase with NeuAc-α-2,3-Gal.[26] In the 1–5 mM range, the transfer:hydrolysis ratio for NeuAc-α-2,3-Gal-PNP was approximately 4 (Fig. 3).

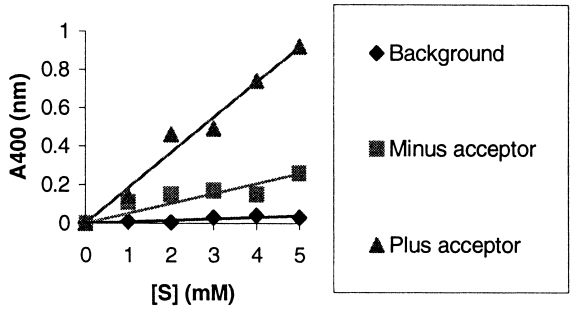


Figure 3. Effect of acceptor substrate Gal-β-1,3-GlcNAc-β-*O*-octyl (1 mM) on the activity of *Trypanosoma cruzi trans*-sialidase towards NeuAc-α-2,3-Gal-PNP.

A number of groups have investigated the mechanism of *trans*-sialidase but it is clear that the conclusions drawn are heavily dependent on the substrates used and the temperature of the reaction, in particular.[13,21,22,26] The ratio of hydrolysis to transfer for NeuAc-α-2,3-lactose (1:13) is substantial.[13] The relative rates of hydrolysis of NeuAc-α-2,3-lactose (1) and NeuAc-MU (10)[21] contrast with the relative rates of transfer of NeuAc from NeuAc-α-2,3-lactose (25), NeuAc-MU (1) and NeuAc-PNP (1).[13] In the current study, we observe a modest hydrolysis:transfer selectivity with NeuAc-α-2,3-Gal-PNP (approx 1:4) which is mid-way between that observed with NeuAc-α-2,3-lactose and NeuAc-MU.

Studies with NeuAc-MU conclude that aglycone release is rate limiting[21,22] since the addition of acceptor does not influence the rate of release of the MU aglycone. In contrast, with NeuAc-α-2,3-Gal-PNP as a donor substrate we have been able to demonstrate that the presence of acceptor does influence the rate of NeuAc transfer. We note that NeuAc-α-2,3-Gal is a poorer *trans*-sialidase substrate than NeuAc-α-2,3-Gal-β-1,4-Glc by some 200 fold.[26] NeuAc-α-2,3-Gal-PNP appears to be recognised and acted upon by *trans*-sialidase better than do 'simple' synthetic substrates (e.g. NeuAc-MU) but less well than 'more natural' substrates (e.g. NeuAc-α-2,3-Gal-β-1,4-Glc). It would appear that comparison of mechanistic information obtained with different types of *trans*-sialidase donor substrate should be made with caution.

In conclusion, we have developed a straightforward spectrophotometric assay capable of monitoring both the hydrolase and sialyltransferase activities of *trans*-sialidase. Whilst NeuAc-α-2,3-Gal-PNP is a useful alternative to radiochemical substrates for routine monitoring of *trans*-sialidase activity, it seems likely that it is not suitable for mechanistic studies aimed at understanding the action of *trans*-sialidase on naturally occurring parasite and mammalian glycoconjugates.

Acknowledgements

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Selected characteristic analytical data for Gal-β-1,3-GlcNAc-β-*O*-octyl: δ_H (D_2O): 2.04 (3H, s, N-Ac), 3.92 (2H, m, 6a,b-H), 4.44 (1H, d, $J_{1,2}$ 7.6 Hz, H-1), 4.57 (1H, d, $J_{11',22'}$ 7.6 Hz); δ_c (D_2O): 11.5, 20.1, 20.4, 23.2, 26.4, 26.6, 29.2, 52.7, 58.8, 59.1, 66.6, 66.8, 68.7, 68.8, 70.6, 73.4, 73.5, 80.6, 99.0, 101.6, 172.6. FAB-MS: Found $[M+H]^+$ 496; $C_{22}H_{42}NO_{11}$ requires 495.6.
20. Typical assay: 30mM HEPES pH 7.5, *E. coli* β-galactoside (80 units), donor substrate (1–5 mM), acceptor substrate (1 mM) and *trans*-sialidase in a total volume of 50 μL. This mixture was incubated at 37 °C for 30 mins, quenched by the addition of 1 mL of Na_2CO_3 (100 mM, pH 10), and the A_{400} measured. Stopped assays proved more reliable than continuous assays.
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