Communication

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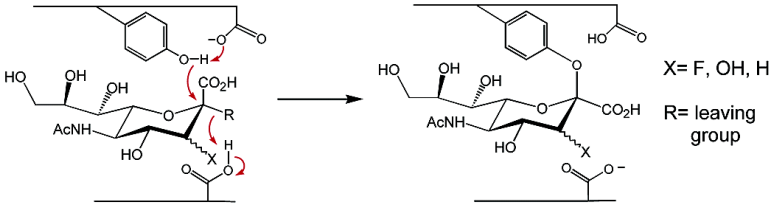
J. Am. Chem. Soc., **2003**, 125 (25), 7532-7533• DOI: 10.1021/ja0344967 • Publication Date (Web): 30 May 2003



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Published on Web 05/30/2003

Trypanosoma cruzi Trans-sialidase Operates through a Covalent Sialyl−Enzyme Intermediate: Tyrosine Is the Catalytic Nucleophile

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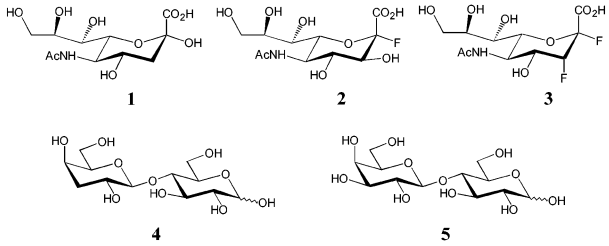
Trans-sialidases are glycosylphosphatidylinositol-anchored surface proteins expressed during the developmental stages of some trypanosomal parasites such as *Trypanosoma cruzi* and *T. brucei*.[1,2] Trans-sialidases belong to the family of sialidases (E.C. 3.2.1.18), but rather than catalyzing hydrolysis,[2] they preferentially transfer α-(2,3) linked sialic acid with retention of anomeric configuration from a terminal galactose of host sialyl glycoconjugates to a terminal galactose on the surface of the parasite.[1,3] *T. cruzi* is the causative agent of Chagas' disease, a parasitic infection that currently affects over 20 million people in Central and South America. Since trypanosomes are incapable of sialic acid biosynthesis, *T. cruzi* trans-sialidase (TcTS) represents a promising target for the development of therapeutics to treat Chagas' disease and has been the subject of extensive structural studies. Recent X-ray crystal structure reports have shed some light on the mode of action of TcTS and detailed kinetic isotope effect studies have been performed.[4−6] However, many fundamental questions about the mechanism remain, especially concerning the identity, or even existence, of a catalytic nucleophile.

A unique Tyr/Glu couple has been suggested to act as the catalytic nucleophile for enzymes belonging to glycosidase family GH 33, of which TcTS is a member, though the exact nature of the role of each residue is yet to be determined.[4] Alternative mechanisms involve a discrete oxocarbenium ion intermediate or an α-lactone involving the substrate's own carboxylate.[7,8] In this Communication we demonstrate that the reaction catalyzed by TcTS proceeds through a covalent glycosyl−enzyme intermediate, a mechanism common to most retaining glycosidases. Furthermore, via LC-MS/MS analysis of peptide digests, we identify Tyr342 as the catalytic nucleophile, the first such example of a retaining glycosidase utilizing an aryl glycoside intermediate.

Trapping of the glycosyl−enzyme intermediates on retaining glycosidases has been achieved using activated glycoside substrates in which a fluorine atom has been substituted adjacent to the anomeric center.[9−11] The electronegative substituent destabilizes the oxocarbenium ion-like transition state, slowing the deglycosylation step. Since sialic acid itself (**1**) bears no electronegative substituent at C3, adjacent to the anomeric center, the introduction of a fluorine atom or even a hydroxyl group at C3 might serve the same end, while incorporation of a fluoride leaving group should render the intermediate kinetically accessible.

When TcTS was incubated with the hydroxylated sialic acid derivative **2**,[12] only unlabeled protein with mass 71 196 ± 6 Da was observed by ES/MS. Since earlier kinetic analysis had suggested that a ping-pong mechanism was unlikely, it was possible



that acceptor lactose binding was required to promote formation of the intermediate.[13] However, incubation with **2** in the presence of the incompetent 3'-deoxy lactose acceptor analogue **4**[12] also did not lead to accumulation of an intermediate. This indicates either that **2** is not a substrate for TcTS or that hydrolysis of the intermediate is occurring at a rate similar to or greater than that of its formation. Improved accumulation of the intermediate might be expected if a mutant form of TcTS is used in which the putative acid/base residue has been replaced. The presence of the excellent fluoride leaving group obviates the need for acid catalysis, allowing the intermediate to form, but leaving the second step impaired by the absence of general base catalysis. Indeed, incubation of the acid/base mutant of TcTS (D59A) with **2** produced a single species of mass 71 450 ± 6 Da, corresponding to the stoicheometric attachment of a single 3-hydroxy sialyl moiety (306 Da) to the mutated protein.

Alternatively, the use of a sialic acid analogue with a (more electronegative) fluorine atom incorporated at C3 should also lead to a more stable intermediate. Indeed, incubation of wild-type TcTS with the 2,3-difluoro derivative **3**[12] produced a single species of mass 71 500 ± 6 Da. This increase of 304 ± 12 Da is that expected for the covalent attachment of one 3-fluorosialyl moiety (308 Da).

When TcTS, prelabeled by incubation with **3**, was subsequently incubated with the competent acceptor lactose **5** (5 min), labeled protein was no longer observed by LC-MS, indicating turnover of the intermediate. However, little change was observed in the degree of labeling when TcTS was incubated with **3** and the 3′-deoxy lactose acceptor **4**. Importantly, this turnover in the presence of lactose, but not in the presence of the 3′-deoxy analogue, directly demonstrates the catalytic competence and mechanistic relevance of the intermediate trapped.

2,3-Difluorosialic acid, **3**, was found to inactivate wild-type TcTs in a time-dependent manner according to essentially first-order kinetics, although inactivation, particularly at lower inactivator concentrations, did not proceed to completion (Figure 1a). This is consistent with a kinetic model[14] in which turnover (k_{react}) of the sialyl−enzyme is significantly slower than its formation (k_i) at high, but not at low, inactivator concentrations. When freed of excess inactivator by ultrafiltration, followed by incubation in buffer at

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10.1021/ja0344967 CCC: $25.00

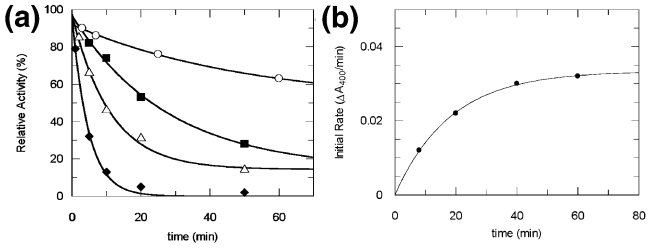

Figure 1. (a) Inactivation of wild-type TcTS with **3**. Enzyme was incubated with the following concentrations of **3**: 1 mM (○), 5 mM (■), 10 mM (△), 20 mM (◆). (b) Spontaneous reactivation of TcTS when freed of excess **3**.

25 °C, a first-order spontaneous recovery of enzyme activity was observed ($k_{cat} = 0.055$ min^{-1}, corresponding to $t_{1/2} = 12.6$ min), again indicating a catalytically competent intermediate that is capable of turnover, but at greatly reduced rates (Figure 1b).

To identify the site of covalent attachment, TcTS (D59A) was incubated with **2** and then immediately subjected to peptic digestion at pH 2 along with a sample of unlabeled protein. The peptic digests from both labeled and unlabeled enzymes were then purified by RP-HPLC coupled with on-line MS analysis. Comparison of the two LC-MS elution profiles revealed that a peptide of mass 1392 Da was observed only in the chromatogram for the labeled sample, with a corresponding peptide of $m/z = 1086$ present in the unlabeled sample. Daughter ion fragmentation analysis of this 1392 Da peptide revealed a sequence of DENSAYSSVL+3OHSial, as shown in Figure 2. Importantly, only the B ion fragments up to and including Tyr342 include the 3-hydroxysialyl label, indicating that the sialic acid is attached at that point. A similar result was obtained for wild-type TcTS which had been labeled with **3**.

The importance of this tyrosine is revealed by the fact that it is completely conserved in enzymes belonging to family GH 33. Further, recent X-ray crystal structure analysis of TcTS shows that Tyr342 is positioned at a suitable distance from the reaction center to form a covalent linkage,[4] leading us to conclude that Tyr342 is the catalytic nucleophile of TcTS.[15] This is the first case in which tyrosine has been unequivocally shown to play such a role in the catalytic machinery of a retaining wild-type glycoside hydrolase and is also the first example of a glycosidase forming a glycoside rather than a glycosyl ester as a catalytic intermediate.

Interestingly, a number of glycosidases have been shown to use a Glu/Tyr couple as nucleophile, in which the glutamic acid forms the intermediate.[16] However, only three families of glycosidases are proposed to utilize a Tyr/Glu couple as their catalytic nucleophile, GH 33, 34, and 83. As all three of these families are solely responsible for the processing of sialic and neuraminic acids, it is probable that this alternative choice of nucleophile is a direct consequence of the chemical nature of these substrates and it is interesting to speculate on why. For the majority of retaining glycoside hydrolases, a negatively charged catalytic nucleophile directly attacks the anomeric carbon to generate the covalent intermediate, while a neighboring residue (e.g., Tyr) stabilizes the negative charge in the free enzyme. However, as both sialic and neuraminic acids bear a carboxylate group adjacent to the anomeric center, it is likely that unfavorable electrostatic interactions would arise if the Glu were to function as the direct nucleophile. By invoking the Tyr/Glu couple in TS, the tyrosine relays charge from the more remote glutamate residue so that the phenolic oxygen only achieves appreciable negative ion character at the transition state, thereby avoiding this electrostatic repulsion.

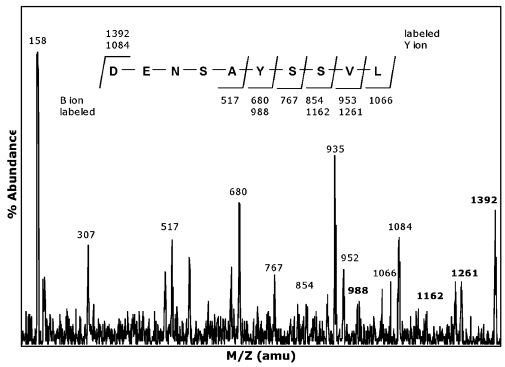

Figure 2. ESI tandem MS daughter ion spectrum of the 3-hydroxysialyl peptide (*m*/*z* 1392). Observed B and Y series fragments are shown below and above the peptide sequence, respectively.

In conclusion, we have shown that TcTS operates through a double displacement mechanism involving the transient formation of a covalent sialyl−enzyme intermediate with Tyr342. The relay of charge required for the generation of this novel intermediate is likely a consequence of the structure of sialic acid. Indeed, some form of charge relay will probably prove to be a common feature among enzymes that process sialic and neuraminic acids.

Acknowledgment. We thank the Human Frontiers Science Program for generous financial support. A.C.F. is an International Research Scholar from the Howard Hughes Medical Center. I.D. is funded by the Carlsberg Fondet.

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(14) In the equation below, E represents free enzyme, I-F the 2,3-difluorosialic acid, E-I the sialyl−enzyme, and I the sugar product. If $k_i > k_{react}$, E-I accumulates and inactivation is observed.

$$E + I\text{–}F \underset{k_i}{\overset{K_i}{\rightleftharpoons}} E{\bullet}I\text{–}F \xrightarrow{F^-} E\text{–}I \xrightarrow[HOH]{k_{react}} E + I$$

(15) *T. cruzi* contains over 100 genes encoding for trans-sialidase, of which about half are catalytically inactive and contain the mutation Y342H (ref 1).
(16) Interestingly, the Glu to Asp mutant of *Agrobacterium* β-glucosidase underwent time-dependent inactivation in the presence of substrate via formation of a stable tyrosyl-glycoside. Gebler, J. C.; Trimbur, D. E.; Warren, R. A. J.; Aebersold, R.; Namchuck, M.; Withers, S. G. *Biochemistry* **1995**, *34*, 14547.

JA0344967