Structural and Kinetic Analysis of Two Covalent Sialosyl-Enzyme Intermediates on Trypanosoma rangeli Sialidase

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Trypanosoma rangeli sialidase is a glycosidase hydrolase (family GH33) that catalyzes the cleavage of α-2→3-linked sialic acid residues from sialoglyconjugates with overall retention of anomeric configuration. Retaining glycosidases usually operate through a ping-pong mechanism, wherein a covalent intermediate is formed between the carbohydrate and an active site carboxylic acid of the enzyme. Sialidases, instead, appear to use a tyrosine as the catalytic nucleophile, leaving the possibility of an essentially different catalytic mechanism. Indeed, a direct nucleophilic role for a tyrosine was shown for the homologous trans-sialidase from Trypanosoma cruzi, although itself not a typical sialidase. Here we present the three-dimensional structures of the covalent glycosyl-enzyme complexes formed by the T. rangeli sialidase with two different mechanism-based inactivators at 1.9 and 1.7 Å resolution. To our knowledge, these are the first reported structures of enzymatically competent covalent intermediates for a strictly hydrolytic sialidase.

Kinetic analyses have been carried out on the formation and turnover of both intermediates, showing that structural modifications to these inactivators can be used to modify the lifetimes of covalent intermediates. These results provide further evidence that all sialidases likely operate through a similar mechanism involving the transient formation of a covalently sialylated enzyme. Furthermore, we believe that the ability to "tune" the inactivation and reactivation rates of mechanism-based inactivators toward specific enzymes represents an important step toward developing this class of inactivators into therapeutically useful compounds.

Sialidases are enzymes that catalyze the hydrolysis or trans-glycosylation (trans-sialidases) of sialic acid residues from various glycoconjugates. Exosialidases (E.C. 3.2.1.18) remove terminal sialyl residues with overall retention of the stereoisomeric configuration of the anomeric carbon. Endosialidases (E.C. 3.2.1.129) on the other hand, break the internal glycosidic bonds present in 2,8-linked sialic acid oligomers or polymers (colominic acid). Although these two enzyme classes share important structural features, including the catalytic β-propeller domain and several residues that interact directly with the sialyl moiety (1, 2), sequence similarity is very low, and they use different mechanisms to catalyze the hydrolysis of the glycosidic linkage (2, 3). The great majority of exosialidases have been classified into three different sequence-based families, GH33, -34, and -83 (4). Naturally sialylated glycoconjugates include a variety of glycoproteins, glycopeptides, glycolipids, and cell-surface oligosaccharides (5). These glycoconjugates mediate a wide range of crucial biological processes, such as cell-cell communication and signal transduction events (6, 7), as well as being involved in a variety of host-pathogen interactions (8). As such, sialidases represent attractive targets for chemical intervention in the treatment of various diseases. In particular, Zanamivir § and Oseltamivir § are two sialidase inhibitors currently on the market for the treatment of human influenza and are of great interest currently as frontline drugs against the threat of pandemic influenza (9–12).

To generate novel inhibitors of sialidases, a detailed understanding of their catalytic mechanism is necessary. The formation of a covalent glycosyl-enzyme intermediate is a trait common to most retaining glycosidases (13–15), wherein a glutamate (or an aspartate) performs the nucleophilic attack. However, the only well located residue in the sialidase active site is a tyrosine, raising concerns as to whether sialidases actually follow the same mechanism as other glycosidases, considering the rather poor nucleophilic tendency of the phenolic Tyr OH group. We have previously reported the first direct evidence supporting a typical ping-pong mechanism for sialidases through mechanistic and structural investigations of a GH33 trans-sialidase from Trypanosoma cruzi (TcTS) § (3, 16). TcTS operates through the transient formation of a covalent sialosyl-enzyme intermediate, demonstrating a direct role of the active site tyrosine as the catalytic nucleophile. Is this a particular trait of TcTS, implying that true, strictly hydrolytic sialidases work through a different mechanism? To answer this question, we recently obtained preliminary evidence using a mechanism-based inactivator in conjunction with mass spectrometry showing that the sialidase from Trypanosoma rangeli also operates through the involvement of a covalent sialosyl-enzyme intermediate formed with the equivalent tyrosine, suggesting that this may be a mechanism common to all exosialidases (17).

We now present the first crystal structures of the covalent intermediates for the strict hydrolytic sialidase TrSA using the fluorinated sialic acid analogues (1) and (2) as mechanism-based inactivators (Fig. 1A). These structures have been determined to 1.95 and 1.7 Å respectively. Fluorinated substrate analogues, such as (1), have proved to be useful compounds for studying the catalytic mechanism of exosialidases (3, 16–17). The introduction of an electronegative fluoride atom adjacent to the anomeric (reaction) center serves to inductively destabilize the formation of a positive charge during the transition states, thereby reducing the rates of glycosylation (k1) and deglycosylation (k2) (Fig. 1B).

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[The on-line version of this article (available at http://www.jbc.org) contains supplemental text, Table 1, and Movie 1.]

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The abbreviations used are: TcTS, T. cruzi trans-sialidase; TrSA, T. rangeli sialidase; KDN, 2-keto-3-deoxy-o-glycero-o-galacto-nonulosonic acid.
The introduction of a good “leaving group” at the anomic position, such as fluoride, increases $k_1$ only, allowing the covalent intermediate to be kinetically accessible (18, 19).

A full kinetic analysis of inactivation and reactivation rates for *T. rangeli* sialidase with the sialic acid analogues (1) and (2) is also reported. This analysis shows how subtle structural modifications to mechanism-based inactivators can be used to dramatically alter their kinetic behavior. We believe that the ability to “tune” the inactivation and reactivation rates of mechanism-based inactivators represents an important step toward the development of therapeutically useful compounds directed to specific glycosidase targets.

**EXPERIMENTAL PROCEDURES**

**Protein Production and Crystallization**

Recombinant TrRSA was overexpressed in *Escherichia coli* TOP10 cells (Invitrogen), purified, and crystallized as described previously (20). Crystals of the covalent intermediates were obtained by soaking TrRSA crystals for 1 h with either compound (1) or (2) at a concentration of 5 mM at 25 °C in the crystallization liquor. Crystals were rapidly passed through 50% glycerol, flash-frozen in liquid N$_2$, and conserved until data collection.

**X-ray Diffraction and Structure Refinements**

Diffraction data were collected on single frozen crystals (100 K) using synchrotron radiation and processed (Table 1) using MOSFLM (version 6.2.4), SCALA (version 3.2.5), and TRUNCATE (version 5.0) of the CCP4 version 5.0.2 software suite (21).

**Complex with Compound (1)—**Having collected several data sets, the best diffracting one in terms of resolution (1.9 Å) was chosen, despite the presence of two ice rings (2.28–2.21 and 1.95–1.9 Å) that were deliberately omitted in the integration of the diffraction intensities. This introduces some distortions in the Wilson distribution of intensities, which results in somewhat unstable isotropic B factor refinement. To confirm the results obtained, a second data set (obtained from a different crystal similarly treated with 2-deoxy-2,3-difluoro-sialic acid) extending to 2.2 Å resolution was also processed and fully refined (supplementary data Table 1; to avoid redundancy, atomic coordinates and structure factors for this second data set were not deposited in the Protein Data Bank but are available on request from A. Buschiazzo). Both crystals were shown to be isomorphous to the previously solved structure of unbound TrRSA (Protein Data Bank (PDB) code 1N1S), hence allowing immediate crystallographic refinement with Crystallography and NMR System software version 1.1 (22) or REFMAC5 version 5.2.0003 (23) using all the data after setting aside 5% of the reflections for $R_{free}$ calculations. Both structures resulted in indistinguishable atomic coordinates and structure factors and were fully refined.

**Table 1**

| Data collection | 3-F-sialic | 3-F-KDN |
|-----------------|-----------|---------|
| ESRF Beamline   | 1D29      | 1D14–2  |
| Space group     | P2$_2$12$_2$ | P2$_2$12$_2$ |
| Cell (Å, degrees) | $a = 74.22$, $b = 96.1$, $c = 106.65$ | $a = 73.67$, $b = 95.57$, $c = 105.81$ |
| Resolution (top shell bin) (Å) | 1.9 (2–1.9) | 1.7 (1.79–1.7) |
| Unique reflections | 55,231 | 81,374 |
| Completeness (top shell) (%) | 91.2 (80.4) | 98.5 (93.8) |
| $R_m$ “(top shell) (%) | 11.1 (30) | 6.6 (24.7) |
| Multiplicity | 2.9 | 3.4 |
| I/0 (top shell) | 10.9 (4.7) | 8.2 (2.8) |
| Refinement | | |
| Resolution range | 29–1.95 | 35–1.7 |
| Reflections used | 47,608 | 81,306 |
| $R$-factor$^a$ (%) | 17.4 | 15.4 |
| $R_m$ “(%) | 21.6 | 18.9 |
| Root mean square bond lengths (Å) | 0.015 | 0.017 |
| Root mean square bond angles (°) | 1.8 | 1.7 |
| Protein atoms | 4,918 | 4,934 |
| Carbohydrate atoms | 21 (3-F-sialic) | 18 (3-F-KDN) |
| Ion atoms | 10 (sulfate) | 10 (sulfate) |
| Water O atoms | 615 | 805 |
| Ramachandran outliers$^d$ (%) | 0.9 | 1.1 |
| PDB code | 2A75 | 2AGS |

$^a$ $R_m = \Sigma_{hkl} |F_{calc}(hkl)|/\Sigma_{hkl} |F_{o}(hkl)|$.

$^b$ $R$-factor $= \Sigma_{hkl} |F_{calc}(hkl)|/\Sigma_{hkl} |F_{o}(hkl)| - |F_{calc}(hkl)|/\Sigma_{hkl} |F_{o}(hkl)|$.

$^c$ The $R_{free}$ was calculated (33) using 5% of the data.

$^d$ Residues in generously allowed and disallowed regions a + s defined in the program PROCHECK (26).
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(see supplemental data). Iterative cycles of model rebuilding were performed with the program O version 8.0 (25) from inspection of σA-weighted 2mFo – DFc and composite omit maps. The presence of a hydrogen bond within the sialyl moiety between the oxygen atom of the N-acetyl group (O-10) and the first oxygen of the glycerol group (O-7) was also accounted for by iteratively adjusting the corresponding non-bonded van der Waals radii of the concerned oxygen atoms, as well as by releasing the restraints that fixed the C-6/C-5/N-5/C-10 dihedral angle to typical values (~120°). At later stages, water molecules were automatically added based on unexplained Fs – Fs* peaks (>3.5σ) and visually inspected for stereochemistry and stability during refinement. The final refined model at 1.95 Å resolution consists of 632 amino acids, one 3-fluoro-sialyl residue, two sulfate molecules, and 615 water molecules. All protein residues are well defined in the electron density map with the exception of the highly exposed loop 403–410, where residues 406–407 could not be modeled. The refined structure displays good stereochemistry, as judged with PROCHECK version 3.5 (26). 99.1% of the non-Pro/non-Gly residues fall in the most favored or the additionally allowed regions of the Ramachandran plot. Protein residues Val-180, Glu-231, Arg-312, and Ser-425 display unfavorable dihedral angles. However, they are well defined in the electron density maps and have similar Φ,ψ values as found in previous unliganded and complexed TrSA structures (20), suggesting that this is a genuine feature of the protein.

Complex with Compound (2)—Diffraction data were collected from a single crystal that diffracted to 1.7 Å. Refinement was performed with REFMAC5 following a similar strategy to that used for the complex with compound (1). The modeling of a 3-fluoro-β-KDN residue covalently bound to Tyr-343 was straightforward. Water molecules were added as described previously. The final refined model at 1.7 Å resolution consists of 634 amino acids, one 3-fluoro-KDN residue, two sulfate molecules, and 805 water molecules. 98.9% of the non-Pro/non-Gly residues fell in the most favored or the additionally allowed regions of the Ramachandran plot. Residues 406 and 407 were modeled as alanines, because the loop is mobile and density is not well defined.

Enzyme Kinetics

Kinetics studies were performed at 30 °C in 50 mM phosphate buffer at pH 6.8. A continuous spectrophotometric assay based on the hydrolysis of 4-nitrophenol-sialic acid was used to monitor enzyme activity by measurement of the rate of 4-nitrophenolate release (λ = 400 nm) using a Varian Cary 4000 spectrophotometer. The time-dependent inactivation of TrSA by 2,3-difluoro-KDN was monitored by incubation of the enzyme under the above conditions in the presence of several concentrations (1, 2.5, 10, and 20 mM) of 2,3-difluoro-KDN. Residual enzyme activity was determined at appropriate time intervals by the addition of an aliquot (10 μl) of the inactivation mixture to an assay solution (800 μl) containing 4-nitrophenol-sialic acid (3 mM), pH 6.8. Pseudo-first-order rate constants at each concentration (kobs) were determined by fitting the residual activity versus time to a single exponential equation using GraFit version 5.0 (27). The inactivation rate constant (k) and the dissociation constant for the inactivator (K) were determined by fitting to the equation kobs = k/[I]/(K + [I]).

The spontaneous reactivation of 2,3-difluoro-KDN-inactivated TrSA was studied as follows. TrSA (100 μl, inactivated as above) was applied to a microspin G-25 size exclusion column (Amersham Biosciences) to remove excess inactivator. Enzyme activity was measured at appropriate time intervals by the addition of an aliquot (10 μl) of eluent to an assay solution (800 μl) containing 4-nitrophenol-sialic acid (3 mM), pH 6.8. Measured activities were corrected for decreases in activity due to denaturation over this time course using data from non-inactivated control samples. The reactivation rate constant (kcat) was determined by fitting the data to a first-order rate equation as described above.

Accession Numbers

Atomic coordinates and structure factors have been deposited in the PDB under codes 2A75 and 2AGS.

RESULTS

The Covalent Glycosyl-Enzyme Structure of TrSA with Difluoro-sialic Acid (1)—The fluorinated sialic acid analogue 2-deoxy-2,3-difluoro-N-acetylenuraminic acid (I) was shown by mass spectrometric analysis to form a covalent sialosyl-enzyme intermediate with TrSA involving the catalytic nucleophile Tyr-343 (17). To perform a detailed structural analysis of this kinetically trapped intermediate, the three-dimensional structure of the complex formed was solved using TrSA crystals that were soaked with (I). Refinement of one data set that extends to a resolution of 1.95 Å, readily allowed the modeling of a β-3-fluoro-sialosyl moiety covalently bound to the side chain of Tyr-343 (Fig. 2). A covalent bond is clearly observed in the 2mFo – DFc electron density map, between C-2 of sialic acid and the phenolic oxygen of the tyrosine. The sialyl pyranose ring is seen to sit in a standard and seemingly unstrained C2′ conformation, similar to that observed previously for the covalent intermediate of (1) with the trans-sialidase from T. cruzi (3).

The 3-fluoro-sialoside intermediate forms many of the H-bond interactions observed in previous complexes of TrSA with its substrates (20). The sialic acid carboxylate forms several electrostatic interactions with the strictly conserved arginine triad of Arg-36, Arg-246, and Arg-315 (Fig. 3A). The glycerol side chain of the sialic acid forms only a weak H-bond with Trp-121. The 4-OH group of the sialic acid forms only a weak H-bond with Trp-121. The 4-OH group forms H-bonds to Arg-54 and Asp-97, whereas the nitrogen atom of the N-acetyl group also forms a single H-bond with Asp-97. The N-acetyl group establishes an intramo-
lecular H-bond through its O-10 atom with the O-7 of the sialyl glycerol group.

**Kinetic Analysis of the Interaction of (1) with TrSA**—Although the covalent sialyl-enzyme intermediate is now shown to form when compound (1) is allowed to interact with TrSA, time-dependent inactivation could not be observed by removing aliquots over time for assay of residual activity. Nonetheless, the activity actually measured was lower than that of the control reaction containing no inhibitor and was also found to be inversely dependent on inhibitor concentration. This behavior is similar to that observed for the inhibition of some α-glycosidases by 5-fluoro-α-glycosyl fluorides, where apparent tight binding was also observed but found to be due to the accumulation of a glycosyl-enzyme intermediate that turns over on a time scale shorter than the assay time (19). Successful observation of the intermediate by electrospray mass spectrometry (17) presumably depended upon the low pH condition of the analysis decreasing the deglycosylation rates. Several attempts were
made to reduce the rate of inactivation, such that time-dependent inactivation could be observed, by lowering the inhibitor concentration, reducing the temperature of the assay, and incubating (1) with TrSA in the presence of a competitive inhibitor (2-deoxy-2,3-dehydro-N-acetyl neuraminic acid). Time-dependent inactivation could not be observed using any of these methods, quite likely because formation of the intermediate was too fast.

Attempts were therefore made to measure the rate of reactivation by removing excess inactivator from a sample of TrSA labeled with (1) and then measuring the increase in enzymatic activity over time associated with hydrolytic turnover of the intermediate. Unfortunately, an accurate determination of the reactivation rate was not possible, as labeled TrSA had regained ~95% of its initial activity after only 5 min. To perform a more detailed kinetic analysis of the covalent intermediate for TrSA, the generation of a more “long-lived” intermediate was required.

Inactivation and Reactivation Rates of TrSA Labeled with 2,3-Difluoro-KDN (2)—Previous structural and kinetic studies have identified the N-acetyl group of sialic acid to be crucial for substrate binding and, presumably, catalysis by TrSA and TcTS (20, 28). Based upon this knowledge, we chose to investigate the kinetic behavior of the 2,3-difluoro-KDN derivative (2) (in which the N-acetyl group had been replaced with a hydroxyl) toward TrSA. The synthesis of (2) was accomplished following a similar sequence to that used for the synthesis of (1), except that mannose is used in the first step rather than N-acetyl mannosamine (see supplemental data). In contrast to what was seen with (1) and consistent with our expectations, 2,3-difluoro-KDN was observed to inactivate wild-type TrSA at 30 °C in a time-dependent manner according to first-order kinetics (Fig. 4A). The apparent first-order rate constants (k_{obs}) at each concentration of inactivator were fitted to the expression k_{obs} = k_{i}[I]/([I] + K_{i}), yielding the values k_{i} = 0.31 ± 0.02 min^{-1} and K_{i} = 11.4 ± 0.8 mM.

The lifetime of the covalent intermediate was measured by removing excess (2) from an inactivated sample of TrSA by centrifugal dialysis and monitoring the spontaneous increase of enzymatic activity over time (Fig. 4B). A first-order recovery of enzymatic activity was observed with a rate constant k_{cat} = (1.9 ± 0.3) × 10^{-2} min^{-1}, which corresponds to an intermediate half-life of t = 37 min. These results clearly show that the rate constants for inactivation and reactivation have been significantly reduced as a consequence of replacing the N-acetyl group at C-4 with a hydroxyl group, supporting our contention that lifetimes of intermediates on sialidases can be manipulated by modifying enzyme-substrate interactions.

The Covalent Glycosyl-Enzyme Structure of TrSA with (2)—Having successfully manipulated relative rate constants for formation and turnover of the intermediate, we wished to develop some understanding of the physical basis for these effects via crystallographic analysis of the two trapped reaction intermediates.

The x-ray structure of the covalent intermediate formed between TrSA and 2-deoxy-2,3-difluoro-KDN (2) was also solved by soaking crystals of the protein with the inactivator (2), and refined to 1.7 Å. The covalency of the intermediate is clearly seen, with a bond length of 1.43 Å between the sialic acid and the catalytic tyrosine (Fig. 5). Once again, the carboxylate group of the inactivator is tightly bound to the arginine triad (Fig. 3B). The OH-4 group conserves the H-bonds with Arg-54 and Asp-97, whereas the OH-5 group, introduced to replace the N-acetyl group, forms a short (2.5 Å) H-bond with Asp-97. Interestingly, Met-96 is seen to adopt a very different position in the intermediate of TrSA with (2), where it “swings” around to fill the hole that is left in the active site upon removal of the N-acetyl group (Fig. 5). This “moved” condition for Met-96 is not observed in the free enzyme (1N1S); hence it is probably actively induced, resulting in van der Waals contact with the engineered OH-5 oxygen, which partially compensates for the loss of several hydrophobic interactions that the two carbon atoms of the N-acetyl group normally establish. The absence of the N-acetyl group also results in a major realignment of the network of crystalline water molecules in the site. The oxygen atom of the N-acetyl moiety (O-10) normally establishes an intramolecular H-bond with O-7 in the glycerol group of the sugar. In KDN, the absence of this feature allows for the appearance of several water molecules, among which especially W360 and W369 strongly interconnect O-5 and O-7 of the sialyl residue with the Ser-120 and Asp-60 of the enzyme, both of which are observed to adopt modified positions with respect to the compound (1) complex, as well as to other previously reported TrSA structures (1N1S, 1N1T). In particular, Asp-60 oxygen 81, which is the acid/base catalyst, moves 0.5 Å away compared with other structures.

Comparison of the Covalent Enzyme Intermediates of TrSA and TcTS—It is interesting to compare the structures of the 3-fluoro-sialosyl-enzyme intermediates formed upon reaction of the TrSA with (1) with the structure of the equivalent intermediate (3) formed by the T. cruzi trans-sialidase enzyme (PDB code 2AH2, see supple-
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FIGURE 5. 3-Fluoro-KDN covalent complex with TrSA. Methionine 96 is observed in three alternate conformations, partially filling the cavity left by the absent N-acetyl group on the sialyl moiety. In the unbound enzyme (PDB code 1N1T) or bound to N-acetyl containing sialic acid derivatives, this Met is observed only in one conformation (corresponding to conformer A in this structure). The water network changes are also highlighted; W360 and W369 interact with important residues in the site. The refined 2mFo – DFc, map contoured at 1σ is shown.

FIGURE 6. Superposition of the structures of 3-fluoro-sialosyl TcTS (cyan), 3-fluoro-sialosyl TrSA (green), and 3-fluoro-KDN TrSA (red) covalent complexes. Numbering of the residues is displayed with TcTS first.

A second correlated effect results from the final position of the glycerol side chain. In TcTS, O-8 would clash against the side chain of Glu-230 (Fig. 3C), if the position of this amino acid were maintained as it is observed in TrSA. This explains why, in TcTS, this residue appears twisted with χ1 changing from −60° to −150°. The position of Glu-230 Oe2, which forms a crucial interaction with the catalytic tyrosine, changes by as much as 1.3 Å between the two structures (TrSA versus TcTS) (Fig. 6).

DISCUSSION

In summary, the three-dimensional structures of the two 3-fluoro-sialosyl-enzyme intermediates described here provide the first detailed characterization of the reaction intermediate formed by a "true" strictly hydrolytic sialidase. The results confirm the proposed catalytic chemistry involving the formation of a covalent intermediate with an active site tyrosine nucleophile (17), further supporting the hypothesis that all exosialidases share a common mechanism. The sugar moiety is bound in the same unstrained chair conformation as was seen previously with the trans-sialidase (3). We have previously described the crystal structure of TrSA bound to sialic acid (20), and as seen in trans-sialidase, the substrate binds to the enzyme in a distorted boat-like conformation (PDB code 1N1Y); the energetic cost for this being paid by non-covalent substrate-substrate interactions. This conformation places the leaving group in a pseudo-axial configuration in a position to accept a proton from the general acid/base residue Asp-60. Formation of the sialyl-enzyme intermediate then occurs via an electrophilic migration of the anomeric carbon from the leaving group oxygen to the tyrosine hydroxyl via a sugar conformational change from the boat-like conformation to the chair conformation of the intermediate (supplemental data Movie 1). Hydrolysis of the intermediate then occurs via a process that is essentially the reverse of its formation involving electrophilic migration of the anomeric carbon onto the water oxygen that is positioned appropriately by the acid/base residue, with formation of a sialic...
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Recent work on the sialidase from *Micromonospora viridifaciens* (29, 30) shows that mutation of the catalytic tyrosine frequently results in detectable residual activity. Although in terms of catalytic efficiency ($k_{cat}/K_m$) the best mutant (Y→D) is 70 times less proficient than the wild type (∼10$^{-1}$ times for the worst mutant Y→H, naturally present in trypanosomal members of the family), it is indeed astonishing to find high values of $k_{cat}$ ranging from 400 to only 3.5 times lower rates than that of the non-mutated enzyme. The Y→N mutant is even reported to catalyze hydrolysis >2.5 times faster than the wild type (although under unreliable experimental conditions). However, as the authors stress (31), the high residual activities for the nucleophile mutants might be a trait of this particular bacterial sialidase, because equivalent mutations in other sialidases result in complete inactivation. Furthermore, the catalytic rates of the mutants are critically dependent on the aglycone-leaving group ability in contrast to the behavior of the wild-type enzyme, which shows higher $k_{cat}$ values (>10$^3$) when working with natural substrates, such as α-2,6- and α-2,3-sialyl-lactose (29, 31). In the absence of the naturally selected nucleophilic tyrosine, which is strictly conserved in all known exosialidases, compensation by alternative chemical groups appears to take place on the *M. viridifaciens* mutants, with reaction occurring via several different mechanisms (29, 31). Although artificial, these results are fully consistent with the evolutionary concept of adaptation, whereby mutations on a particular residue become acceptable as soon as alternative reactive groups relieve the selective pressure. Recent structural work on the endosialidase from the bacteriophage K1F provides an exciting example where the chemical peculiarities of the polysialic acid substrate are suggested to be responsible for a mechanism that is no longer dependent on an enzyme-driven nucleophilic attack (2). Instead it is suggested that optimal substrate positioning enhances the pre-existing self-cleavage potential of polysialic acid.

Increasing the Lifetime of the Covalent Sialyl-Enzyme Intermediate—In addition to ground state destabilization, glycosidase catalysis is effected through the stabilization of transition states, both by electrostatic interaction with the developing positive charge and by the provision of suitable hydrogen-bonding interactions to bind the partially planar sugar ring. Substrate analogues that compromise stabilization of the transition state through either or both of these methods will undergo enzymatic reaction more slowly. The replacement of a hydroxyl group adjacent to sites of charge development with a fluorine atom not only serves to inductively destabilize the formation of positive charge during the transition state but also results in the loss of crucial hydrogen bonding interactions required to stabilize the conformation of the sugar ring. In the case of 3-fluoro-sialic acid (1), however, instead of a hydroxyl group, it is a hydrogen atom that is replaced by fluorine. Although the difference in inductive effects (F versus H rather than F versus OH) will be greater (thereby causing a greater rate reduction), there is no loss of any specific hydrogen bonding interactions. To further decrease the turnover rates, we removed key binding interactions elsewhere on the sugar ring, resulting in a more long-lived intermediate. Undoubtedly, the changes observed between the inactivation and reactivation rates for difluoro-KDN (2) compared with difluoro-sialic acid (1) are because of a combination of several electronic, structural, and entropic factors, which are to be the subject of future research. The removal of the N-acetyl group likely results in the loss of key binding interactions required for transition state stabilization and also creates a hydrophobic cavity in the occupied sialyl-binding pocket that causes the repositioning of Met-96 and the concomitant reorganization of several waters in the active site. As well, the modified network of waters on the KDN complex appears to exert important effects on key residues, such as the observed repositioning of the catalytic aspartic acid (Asp-60). Fine-tuning of turnover rates combined with optimization of binding properties appears to be a very promising strategy to conduct rational design of mechanism-based inhibitors that are more likely to hinder the eventual phenomenon of drug-induced resistance (32).

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