The Aspergillus fumigatus Sialidase Is a 3-Deoxy-\(d\)-glycero-\(d\)-galacto-2-nonulosonic Acid Hydrolase (KDNase)

**STRUCTURAL AND MECHANISTIC INSIGHTS**

J. C. Telford, J. H. F. Yeung, G. Xu, M. J. Kiefel, A. G. Watts, S. Hader, J. Chan, A. J. Bennet, M. M. Moore, and G. L. Taylor

From the Biomedical Sciences Research Complex, University of St. Andrews, St. Andrews, Fife KY16 9TJ, United Kingdom, and the Departments of Biological Sciences and Chemistry, Simon Fraser University, Burnaby, British Columbia V5A 1S6, Canada, and the Institute for Glycomics, Griffith University, Gold Coast Campus, Queensland 9726, Australia.

Aspergillus fumigatus is a filamentous fungus that can cause severe respiratory disease in immunocompromised individuals. A putative sialidase from *A. fumigatus* was recently cloned and shown to be relatively poor in cleaving *N*-acetylneuraminic acid (Neu5Ac) in comparison with bacterial sialidases. Here we present the first crystal structure of a fungal sialidase. When the apo structure was compared with bacterial sialidase structures, the active site of the *Aspergillus* enzyme suggested that Neu5Ac would be a poor substrate because of a smaller pocket that normally accommodates the acetamido group of Neu5Ac in sialidases. A sialic acid with a hydroxyl in place of an acetamido group is 2-keto-3-deoxynononic acid (KDN). We show that KDN is a preferred substrate for the *A. fumigatus* sialidase and that *A. fumigatus* can utilize KDN as a sole carbon source. A 1.45-\(\AA\) resolution crystal structure of the enzyme in complex with KDN reveals KDN in the active site in a boat conformation and nearby a second binding site occupied by KDN in a chair conformation, suggesting that polyKDN may be a natural substrate. The enzyme is not inhibited by the sialidase transition state analog 2-deoxy-2,3-dehydro-\(N\)-acetylneuraminic acid (Neu5Ac2en) but is inhibited by the related 2,3-didehydro-2,3-dideoxy-\(d\)-glycero-\(d\)-galacto-nonulosonic acid that we show bound to the enzyme in a 1.84-\(\AA\) resolution crystal structure. Using a fluorinated KDN substrate, we present a 1.5-\(\AA\) resolution structure of a covalently bound catalytic intermediate. The *A. fumigatus* sialidase is therefore a KDNase with a similar catalytic mechanism to Neu5Ac exosialidases, and this study represents the first structure of a KDNase.

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**To whom correspondence should be addressed:** Biomedical Sciences Research Complex, University of St. Andrews, St. Andrews, Fife KY16 9TJ, UK. Tel: 44-1334-467301; Fax: 44-1334-462595; E-mail: gtl2@st-andrews.ac.uk.

Sialic acids comprise the most chemically and structurally diverse carbohydrate family. Over 50 naturally occurring analogs exist, the majority based on 5-acetamido-3,5-dideoxy-\(d\)-glycero-\(d\)-galacto-non-2-ulosonic acid (\(N\)-acetylneuraminic acid, Neu5Ac)\(^*\) (1 in Fig. 1) and 3-deoxy-\(d\)-glycero-\(d\)-galacto-non-2-ulosonic acid (2-keto-3-deoxynononic acid, KDN) (2 in Fig. 1) that only differ at the C5 position (1). KDN, like Neu5Ac, occurs widely in bacteria and vertebrates; is found in almost all types of glycoconjugates including glycolipids, glycoproteins, and capsular polysaccharide; and can be \(\alpha\)2,3-, \(\alpha\)2,4-, \(\alpha\)2,6-, or \(\alpha\)2,8-linked to other carbohydrates (2). KDN was first discovered in the cortical alveolar polysialogloboprotein of rainbow trout eggs as the capping carbohydrate on polysialic acid chains that were resistant to bacterial sialidases (3). In mammals, KDN was first identified in various tissues including human lung carcinoma cells but at a much lower abundances than Neu5Ac (4) and was subsequently found on human red blood cells and ovarian cancer cells (5). The development of linkage-specific KDN antibodies (6, 7) has allowed the identification of \(\alpha\)2,8-linked polyKDN in many mammalian tissues (8), including the human lung (9).

Sialidases, or neuraminidases, catalyze the removal of terminal sialic acids from a variety of glycoconjugates and play an important role in pathogenesis, bacterial nutrition, and cellular interactions. Crystal structures of a growing number of exosialidases that cleave Neu5Ac are available from bacteria (10–16), viruses (17–19), trypanosomes (20, 21), leech (22), and man (23). All sialidases share the same six-bladed \(\beta\)-propeller fold for their catalytic domains, with conservation of key catalytic amino acids (24). The nonviral sialidases have an R(I/L)P motif containing one of the three active site arginines and also have up to five bacterial neuraminidase repeats or Asp boxes ((S/T)XDXTGXT(W/F)). The bacterial neuraminidase repeats occur...
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![Chemical structures of ligands used in this study.](image)

FIGURE 1. Chemical structures of ligands used in this study.

at topologically identical positions in the β-propeller fold, remote from the active site, but any function beyond dictating a structural fold is unknown. Like many glycosyl hydrolases, sialidases can possess carbohydrate-binding modules (CBMs) in addition to the catalytic domain, placed upstream, placed downstream, or even inserted within the β-propeller domain. It has been suggested that the presence of these CBMs increases the catalytic efficiency of the sialidases, particularly in the presence of polysaccharide substrates (25). In the case of sialidases having CBMs, they often recognize sialic acid as has been shown for the sialidases from *Vibrio cholerae* (26), *Clostridium perfringens* (27), and *Streptococcus pneumoniae* (15). Nearly all of the Neu5Ac-specific sialidases are inhibited by 2-deoxy-2,3-dehydro-Ν-acetyl-neuraminic acid (Neu5Ac2en, 3 in Fig. 1), a putative transition state analog.

A sialidase specific for KDN ketosidic linkages was first discovered in the bacterium *Sphingobacterium multivorum*, and this so-called KDNase released KDN from naturally occurring polysaccharides (28), and *Mycobacterium tuberculosis* strain H37Rv (29). The KDNase was, however, inhibited by 2,3-didehydro-2,3-dideoxy-β-d-galacto-γ-D-galacto-nonulosonic acid (KDN2en, 4 in Fig. 1) (30). Further analysis of this enzyme using synthetic KDN analogs suggested that the hydroxyl group at C5 was important for recognition of the inhibitor by the enzyme and that like the Neu5Ac sialidases, the thermodynamically less stable α-form of the product is the first product of cleavage, suggesting a similar catalytic mechanism to the Neu5Ac exosialidases (30, 31).

*Aspergillus fumigatus* is a common soil fungus and is the major species of *Aspergillus* that causes invasive aspergillosis in immunocompromised humans (32). Infection by *A. fumigatus* is mainly through inhalation of airborne conidiospores (conidia) that adhere to lung tissue. It has been shown that *A. fumigatus* conidia have surface sialic acids that may adhere to basal lamina proteins (33) and that pathogenic species of *Aspergillus* have a higher density compared with nonpathogenic species (34). The origin of these sialic acids remains a mystery, because although sialic acid biosynthesis has been reported to occur *de novo* in *A. fumigatus* (34), its genome appears to lack the known Neu5Ac biosynthetic enzymes. In addition, *A. fumigatus* is incapable of utilizing or incorporating exogenous Neu5Ac or ManNAc (35). Nevertheless, *A. fumigatus* does encode a sialidase that has recently been cloned and characterized (36).

The *A. fumigatus* sialidase has a 20-amino acid signal peptide, an NTP motif, and one bacterial neuraminidase repeat and shares 30% sequence identity with the bacterial sialidase from *Micromonaspora viridifaciens* whose structure is known (12). The sequence of the *A. fumigatus* sialidase appears to possess the key active site residues of a sialidase: an arginine triad that interacts with the carboxylic acid group of sialic acids, a nucleophilic tyrosine (Tyr<sup>358</sup>), its associated general acid (Glu<sup>249</sup>), and an acid/base (Asp<sup>84</sup>) (36).

In this study, we report the crystal structure of residues 21–406 of the *A. fumigatus* sialidase (herein after named *AfS*) determined by single wavelength anomalous diffraction from crystals of selenomethionine-derivitized protein. A comparison of the active site with the *M. viridifaciens* sialidase (*MvS*) suggests that KDN might be a better substrate than Neu5Ac for the *A. fumigatus* enzyme, and we show kinetically that this is the case using the fluorescent substrate 4-methylumbelliferon 3-deoxy-d-glycero-α-D-galacto-non-2-ulosononic acid (KDN-MU, 6 in Fig. 1). A 1.45-Å resolution structure derived from a crystal of *AfS* soaked with KDN-MU revealed two KDN molecules bound to the enzyme: one in the active site and one in an adjacent position. *AfS* is not inhibited by Neu5Ac2en but is inhibited by the KDN-related KDN2en (4 in Fig. 1), which we show bound in the active site of *AfS* in a 1.84-Å resolution crystal structure. To complete a set of snapshots of the catalytic cycle, a 1.5-Å resolution structure of a covalent intermediate was obtained with 2,3-difluoro-KDN (2,3F-KDN, 7 in Fig. 1). NMR is used to show that catalysis of KDN-MU by *AfS* proceeds with retention of configuration at the anomeric carbon. Finally, we show that *A. fumigatus* can utilize KDN, and not Neu5Ac, as an effective sole carbon source. These studies suggest a nutritional role for *AfS* in the life cycle of *A. fumigatus* but also stimulate further studies into the potential role of KDN in the pathogenesis of the organism.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of AfS for Structural Studies**—The *AfS* gene from the *A. fumigatus* clinical isolate Af293 was amplified and ligated into the pET28A+ (EMD Chemicals Inc., San Diego, CA) vector and expressed in *Escherichia coli* (DE3) cells as previously described (36). Briefly, *E. coli* expressing the *AfS/pET28A+* vector was grown in LB media with 50 μg/ml kanamycin at 37 °C, shaking at 220 rpm. Once the culture *A<sub>600</sub>* had reached 0.9, *AfS* expression was induced by 0.5 mM isopropyl thiob-β-D-galactopyranoside and incubated overnight. The cells were then harvested by centrifugation at 16,780 × g for 25 min and resuspended in phosphate-buffered saline (50 mM Na<sub>H</sub>PO<sub>4</sub>, 300 mM NaCl, pH 8) with EDTA-free protease inhibitor mixture (one tablet/25 ml of solution; Roche Applied Science) and DNase I (Sigma; final concentration, 20 μg/ml) and sonicated five times for 30 s. The sonicated culture was then centrifuged to remove insoluble cell debris at 37,000 × g for 30 min. The supernatant was then filtered through a 0.22-μm pore syringe-driven filter before being loaded onto a 5-ml HisTrap column (GE Healthcare) equilibrated with PBS. The bound protein was eluted with 5 column volumes of 300 mM imidazole...
in PBS. The eluted fraction was then subjected to size exclusion chromatography by loading onto a 120-ml Sephacryl S-200 column (GE Healthcare). Fractions containing AFS were identified, and purity was assessed by SDS-PAGE and MALDI-TOF MS. Fractions containing high purity AFS were then pooled, concentrated, diluted into 50 mM Tris-HCl, 100 mM NaCl (pH 8), and then stored at −20 °C.

Selenomethionine Incorporation—E. coli expressing the AFS/pET28A+ vector were grown in 150 ml of LB with 50 µg/ml at 37 °C, shaking at 220 rpm overnight. The cells were harvested by centrifugation at 16,800 × g for 20 min at 4 °C. The supernatant was discarded, and the pellet was resuspended in 1 ml of PBS and recentrifuged. The supernatant was discarded, and the pellet was resuspended in 4 ml of PBS and added to 1 liter of sterile selenomethionine medium (17 mM NH₄Cl, 22 mM KH₂PO₄, 6 mM Na₂HPO₄·7H₂O, 25 µM Fe₂(SO₄)₃, 30 µM thiamine, 20% w/v glucose, 0.3% w/v MgSO₄). The addition of 50 mg of selenomethionine and kanamycin (50 µg/ml) was then made to the selenomethionine minimal medium. The culture was incubated at 37 °C shaking at 200 rpm until Aₕ₅₀₅ reached 0.9 when AFS expression was induced by 0.5 mM isopropyl thio-β-D-galactopyranoside and incubated overnight. The cells were harvested, and AFS was extracted and purified as above.

Protein Crystallization—The optimal AFS concentration for crystal trials was determined as 13.7 mg/ml by precrystallization assay (Hampton Research). Sitting drop vapor diffusion plates held at 20 °C were used for all crystallization experiments. To ascertain the crystallization conditions for AFS, commercial screens Cryo I and II (Emerald Biosystems), Crystal screens I and II and Index (Hampton Research), PEG screen (Qiagen), condition 20% (w/v) PEG 3350 and 0.2M sodium nitrate, and leaving for 20 min. The synthesis of AFS in complex with ligands were refined similarly. The data collection and refinement statistics, together with Protein Data Bank codes of the deposited structures, are given in Table 1.

Sialidase and KDNase Activity Assays—The activity of enzyme preparations was determined by measuring cleavage of the synthetic sialic acid substrate, 4-methylumbelliferyl α-D-N-acetyleuraminide (Neu5Ac-MU, 5 in Fig. 1; Sigma), or KDN-MU (synthesis described in the supplemental materials). Briefly, the reactions were set up in 96-well plates by adding 200 µM Neu5Ac-MU or KDN-MU, purified recombinant enzyme, and the specific reaction buffer for a total volume of 100 µl. The plates were incubated at 37 °C for 10 min followed by the addition of 200 µl of cold stop solution (0.1 M glycine, 0.014 M NaCl, 25% ethanol, pH 10.7). Following the addition of stop solution, the amount of 4-methylumbelliferone released from Neu5Ac-MU or KDN-MU was determined using a fluorescence spectrophotometer at excitation and emission wavelengths of 365 and 450 nm, respectively. Fluorescence produced during the course of the reaction was related to the concentration of Neu5Ac-MU or KDN-MU cleaved by comparison with a standard curve of 4-methylumbelliferone (Sigma). Each reaction was performed in triplicate.

Kinetic Assay—Michaelis-Menten parameters for the AFS and MvS were measured using Neu5Ac-MU or KDN-MU. Each 100–µl reaction mixture was incubated at 37 °C for 1 min prior to the addition of either purified sialidase. The progress of the reaction was continuously monitored for 20 min using a fluorescence spectrophotometer equipped with a temperature controller set to 37 °C and excitation and emission wavelengths of 365 and 450 nm, respectively. For Neu5Ac-MU, kinetic parameters were determined from 19 initial rate measurements using a substrate concentration range of 50–5000 µM. For KDN-MU, the kinetic parameters were determined from 22 initial rate measurements using a substrate concentration range of 10–800 µM. The rate versus substrate concentration data were fitted to the Michaelis-Menten equation using GraphPad (GraphPad Software Inc., San Diego, CA).

pH Profile—The optimal pH of the purified enzyme was determined by measuring its specific activity (nmol·mg⁻¹·min⁻¹) toward KDN-MU in buffers of varying pH. The buffers used were 40 mM sodium formate (pH 3–3.5), 50 mM sodium acetate (pH 4–5), 16 mM sodium tartrate (pH 5.2), 32.5 mM MES (pH 6–7), 40 mM MOPS (pH 6.5), and 50 mM Tris-HCl (pH 7.5–9). The ionic strength of all buffers was maintained at...
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100 mM with NaCl, and all of the buffers were prepared for use at 37 °C.

Temperature Profile—A. fumigatus sialidase activity toward Neu5Ac–MU or KDN–MU was measured in 40 mM sodium acetate at pH 4.0. The activity of the enzyme was evaluated at 10, 15, 20, 25, 30, 37, 40, 45, 50, and 60 °C. Each reaction mixture was incubated for 10 min, and the amount of 4-MU released was determined as described above.

NMR Experiments—1H NMR spectroscopy was used to examine product formation from the hydrolysis of KDN–MU by AfS and to test for trans-sialidase activity. The reaction mixtures included 15 μl of 23 mg/ml AfS, 15 μl of 33 mg/ml KDN in 2H2O. In the case of the trans-sialidase assay, 15 μl of 20 mg/ml galactose or N-acetylgalactosamine in 2H2O was also added. The reaction mixture was then made up to 600 μl with PBS (pH 7) in 2H2O and placed in a 5-mm NMR tube. The reaction mixtures were incubated at room temperature, and the spectra were recorded at 0, 5, 1, 5, 12, and 24 h. All of the NMR spectra were recorded at 298 K using a 500-MHz Bruker spectrometer taking 64 scans with a 2-s relaxation delay. During the relaxation delay, the remaining water signal was suppressed by continuous low power irradiation.

Carbon Source Utilization—To determine the preferred carbon source use by A. fumigatus, 104 A. fumigatus spores were inoculated per well in 96-well plates containing chemically defined media with 25 mM carbon source of glucose, mannose, KDN, or sialic acid, or with no carbon source. All of the carbon sources were tested in triplicate. The KDN contained a maximum of 5% mannose; therefore the K(+0.05 Man) sample, which contains 1.25 mM mannose, was tested to ensure that the small amount of mannose in the KDN sample did not contribute significantly to fungal growth. All of the media were adjusted to an approximate pH level of 6.5 prior to inoculation. A paraffin overlay (50 μl) was added to each well to prevent cross-contamination of spores between wells. All of the plates were placed in a moist chamber and incubated at 37 °C. Growth of the fungus was monitored spectrophotometrically at A620 (Expert Plus Microplate Reader, Biochrom Asys).

RESULTS

Overall Structure—The structure of AfS was solved by single wavelength anomalous diffraction phasing. The asymmetric unit contains two AfS molecules that superimpose with a root mean square deviation of 0.43 Å but do not form any significant association (buried surface area, 400 Å2). The closest structural homolog to AfS is MvS (Protein Data Bank code 1eus), where 311 Cα atoms (chain A) or 309 Cα atoms (chain B) of AfS overlay MvS with an root mean square deviation of 1.52 and 1.44 Å, respectively. AfS has the canonical, six-bladed β-propeller fold of a sialidase with an excursion between strands 2 and 3 of the second propeller blade, involving residues 142–172, that forms a cap region above the active site (Fig. 2). A more extensive excursion at the same topological position exists in the NanA sialidase from C. perfringens (13) and the NanA/NanB sialidases from S. pneumoniae (15, 16). AfS is a basic protein with a theoretical pl of 9.3, and this is reflected in the electrostatic potential surface that shows a predominantly positive surface around the active site and extending in a groove down and below the molecule (Fig. 2). This is in contrast to secreted bacterial sialidases that tend to have a highly acidic surface on
one side that, it is suggested, helps orient the enzyme in the neighborhood of a negatively charged surface (13, 15, 26). Behind the active site and below the cap domain there is a metal-binding site (Fig. 2). The bond lengths are consistent with the binding of sodium or calcium. The metal ion is secured by the side chains of Asp and Asn as well as the backbone carbonyls of Gly and Glu. Two water molecules sit directly above and below the metal ion completing the octahedral stabilization of the ion.

A superposition of the Neu5Ac2en complex (Protein Data Bank code 1eus) onto A/S shows conservation of key residues in the active site (Fig. 3). These include the arginine triad, the nucleophilic tyrosine and its associated glutamic acid, and the acid/base aspartic acid. Neu5Ac2en is not an inhibitor of A/S, and this may be explained, in part, by the presence of Arg in A/S protruding into the hydrophobic pocket that usually accommodates the acetamido group of Neu5Ac2en (Fig. 3). This suggested that A/S recognizes a sialic acid with a smaller group at C5. The only sialic acids with a smaller group at C5 are neuraminic acid, which has an amino group at C5 but does not occur naturally, and KDN, which has a hydroxyl at C5.

Kinetic Analysis Shows A/S Prefers KDN over Neu5Ac—Michaelis-Menten parameters for A/S were measured using Neu5Ac-MU or KDN-MU as substrates. A/S had a $k_{\text{cat}}$ of 5.8 ± 1.7 mM and a catalytic efficiency ($k_{\text{cat}}/K_m$) of 0.86 M$^{-1}$ s$^{-1}$ for Neu5Ac-MU; however, for KDN-MU it had a $K_m$ of 0.23 ± 0.02 mM and a catalytic efficiency of (1.82 ± 0.091) × 10$^5$ M$^{-1}$ s$^{-1}$. MvS has a reported $k_{\text{cat}}/K_m$ of (7.23 ± 0.04) × 10$^6$ M$^{-1}$ s$^{-1}$ (47); therefore, A/S has a similar catalytic efficiency with KDN-MU as MvS has with Neu5Ac-MU. A/S has a pH optimum of between 3.5 and 4.5 with KDN-MU, similar to its optimum with Neu5Ac-MU (36). The optimal temperature range of A/S with KDN-MU was from 30 to 37 °C.

Crystal Structure of A/S Complexed with KDN—The structure derived from a crystal of A/S soaked in KDN-MU revealed two α-KDN molecules bound per monomer (Figs. 4 and 5). One KDN occupies the active site and is in a boat conformation, and the second occupies a positively charged site (site 2 in Fig. 2) adjacent to the active site such that the distance from the anomic oxygen of the active site KDN is only 6.4 Å from C9 and 7.7 Å from C8 of the second KDN. Intriguingly, in monomer B, there is a glycero keto from the cryoprotectant sitting between the two KDN molecules (Figs. 4 and 5).

Active Site of A/S—KDN in the active site is bound in its $\alpha$-anomeric form, with the ring adopting a distorted $\delta_2$-boat conformation. The O$_2$ hydroxyl of KDN forms a close interaction (2.57 Å) with Asp and also interacts with the bound glycerol (2.71 Å). Although there are a number of significant differences between the active sites of viral, bacterial, and eukaryotic sialidases, a number of key features have been retained throughout their evolution (24) and are shown in Fig. 4. These include a tri-arginal cluster (Arg, Arg, and Arg) that interacts with the carboxylate group of KDN. The position of the first arginine (Arg) is stabilized by a conserved glutamic acid (Glu) and a tyrosine (Tyr) and a glutamic acid (Glu) hydrogen bond with each other and sit beneath and close to the C1–C2 bond of the substrate. A conserved feature of sialidase active sites is the acid/base catalyst, Asp. The O7 and O9 hydroxyls of the KDN glycerol group interact with Glu and Trp, via two water molecules. At its closest approach, Arg is only ~4.5 Å from O5 of KDN. If the structure of the C.
perfringens NanI sialidase in complex with B$_{2,3}$ αNeu5Ac (Protein Data Bank code 2bf6) is superimposed on AfS, then the acetamido methyl group of Neu5Ac would be 2.5 Å from Arg$^{171}$. Therefore, Arg$^{171}$ appears to be a major determinant of AfS being a KDNase. Arg$^{171}$ packs on one side of the indole ring of Trp$^{202}$, with Gln$^{148}$ packing on the other side, and together may define a KDN recognition sequence motif.

**Second KDN-binding Site**—KDN in the second site is also in its α-anomeric form, with the ring adopting a $^2$C$_5$ chair conformation. This KDN is possibly a bound KDN-MU, although no electron density is visible for the methylumbelliferyl moiety. This KDN makes only four direct hydrogen bonds with AfS: O4 makes two interactions with the guanidinium group of Arg$^{323}$, O1B interacts with the backbone amide of Arg$^{322}$, and O5 interacts with Asp$^{376}$. In addition, multiple water-mediated interactions are made involving all the oxygens of KDN apart from O8 and O9. The ring of KDN sits in a cavity sandwiched 4.5 Å above the methylene groups of Arg$^{322}$ and 4.0 Å below Phe$^{378}$ (Figs. 4 and 5). It is unlikely that this second site could bind Neu5Ac because the pocket around O5 is polar and in particular the presence of Arg$^{388}$ would preclude the binding of the acetamido group of Neu5Ac. This hypothesis is supported by repeated failed attempts to visualize Neu5Ac in either KDN-binding site in the crystal.

**KDN2en Complex**—The 1.84-Å resolution structure shows that KDN2en adopts a $^4$H$_5$ half-chair conformation. The distance between the hydroxyl of Tyr$^{358}$ and C2 of the ligand shortens to 2.81 Å, compared with 3.23 Å in the KDN complex. All interactions with AfS, both direct and water-mediated, are identical in the two complexes (Fig. 6).

**Covalent Intermediate**—The 1.5-Å resolution structure clearly shows 3-fluoro-β-KDN covalently linked to Tyr$^{358}$ in an unstrained $^2$C$_5$ conformation (Fig. 7A) as observed in the Trypanosoma cruzi trans-sialidase (48), Trypanosoma rangeli sialidase (49), and C. perfringens NanI (13) covalent complexes. The covalent bond refines to a length of 1.75 Å. The presence of the fluorine at C3, and its close proximity (2.7 Å) to the acid/base...
Asp\textsuperscript{84}, appears to lead to a dual conformation being observed for the side chain of this residue, something that was observed in the NanI complex. The second binding site observed in the KDN complex is here occupied by 2,3F-KDN in the same \textit{2C}\textsubscript{5} chair conformation. Additionally, there are three more 2,3F-KDN molecules bound in the asymmetric unit in a \textit{2C}\textsubscript{5} conformation. Two are at the same site on each monomer of \textit{AfS} (site 3 in Fig. 2), and the third sits at the interface between the monomers (site 4 in Fig. 2). The O4 hydroxyl of 2,3F-KDN in site 3 interacts with the side chain of Ser\textsuperscript{330} and backbone atoms of Lys\textsuperscript{337} and Tyr\textsuperscript{331}, whereas O5 interacts with the side chain of Asp\textsuperscript{332}.

**NMR Monitoring of KDN-MU Hydration by AfS**—The hydrolysis of KDN-MU to form KDN was monitored as a time course reaction using \textsuperscript{1}H NMR spectroscopy in deuterated buffer at a pD of 3.6 (Fig. 8). The H\textsubscript{3eq} proton of KDN-MU has a characteristic doublet of doublets (\(\delta = 2.75\) ppm, \(J_{3e,3a} = 12.7\) Hz, \(J_{3e,4} = 4.7\) Hz). With the addition of \textit{AfS}, cleavage of the umbelliferyl group leads to a shift of the H\textsubscript{3eq} resonance to \(\delta = 2.60\) ppm of the \(\alpha\)KDN product. Over time, \(\alpha\)KDN mutarotates to the thermodynamically more stable \(\beta\)KDN with the appearance of H\textsubscript{3eq} resonance at \(\delta = 2.10\) ppm. No trans-sialidase activity was observed for \textit{AfS} (data not shown).

**A. fumigatus Can Utilize KDN as a Sole Carbon Source**—To determine whether KDN could be a carbon source for \textit{A. fumigatus}, we measured growth in defined medium supplemented with various carbon sources (Fig. 9). The data indicate that \textit{A. fumigatus} is unable to use Neu5Ac as the sole source of carbon; however, it is able to use KDN, although less efficiently on a molar basis compared with glucose. There was also a short lag in growth, suggesting that KDN catabolic enzymes must be induced prior to utilization of this substrate.

**DISCUSSION**

The structure of the sialidase (\textit{AfS}) from \textit{A. fumigatus} presented here represents the first structure of a fungal sialidase and the first structure of a KDN-specific sialidase or KDNase. The KDN complex reveals two KDN molecules bound per monomer (Figs. 4 and 5). The structural details of this complex, the complex of \textit{AfS} with KDN2en, and the covalent intermediate suggest that a similar mechanism for \textit{AfS} as has been described for other Neu5Ac-specific exosialidases, because all of the key catalytic residues are conserved and occupy nearly identical positions (Fig. 3). Although the \(\alpha\)KDN in the active site is the product of KDN-MU hydrolysis, it has its sugar ring in a strained and distorted \(B_{2,5}\) conformation that is reminiscent of the induced sialic acid ring conformation seen in the Michaelis complexes between an aspartic acid mutant of \textit{T. cruzi trans-sialidase} and \(\alpha\)(2,3)-sialyl-lactose (48) and in wild-type NanI sialidase from \textit{C. perfringens} with \(\alpha\)Neu5Ac (13). This conformation of the sugar ring results in a pseudo-axial orientation of the glycosidic bond being cleaved, bringing it within 2.6 Å of the acid catalyst Asp\textsuperscript{84} for proton transfer. The supposed transition state, mimicked by the complex of \textit{AfS} with
The catalytic efficiency of A/S in cleaving the 4-methylumbelliferyl leaving group from KDN-MU is 10^5 times greater than its ability to cleave the same leaving group from Neu5Ac-MU. In addition, A/S is not inhibited by Neu5Ac2en, the transition state analog of Neu5Ac-hydrolyzing sialidases. The specificity of A/S KDNase for KDN lies in the nature of the pocket that accommodates the C5 hydroxyl, as had been predicted (30). Neu5Ac-specific sialidases have a hydrophobic pocket to accommodate the acetamido group at C5; in contrast, A/S has a water-filled, polar pocket where modeling shows that Arg^{171} would clash with the amido group of Neu5Ac. The hydroxyl at C5 of KDN does not interact directly with A/S but makes several water-mediated interactions. Arg^{171}, the second arginine of an EGRR motif, sits one side of Trp^{202}, part of a WD motif, on the other side of which is Gln^{148} that interacts with the O7 and O9 hydroxyls of KDN. Do these three residues define a KDN recognition motif?

Extending a BLASTP search of the protein database with A/S (36) shows this QX_{24}EGRX_{30}WD motif (bold face letters indicate completely conserved residues; nonformatted letters indicate similar, highly conserved residues; and X_{24} indicates a run of 24 amino acids of any sequence) to be conserved in the fungi Neosartorya fischeri, Aspergillus terreus, Arthrodema glycyeum, Trichophyton verrucosum, Microsporum canis, Chaetomium globosum, and Nectria hematococca, all of which share at least 72% sequence identity with A/S. A degenerate form of this motif, DGRX_{30}W, occurs in proteins annotated as sialidases from Streptomyces bingchengensis and Streptomyces avermitilis. Arg^{171} in A/S sits at the start of the third strand of the second β-sheet of the β-propeller and is seven residues before the start of an Asp box. In the structures of Neu5Ac-sialidases, the equivalent residue occupying the same structural position is a serine (C. perfringens and S. pneumoniae), threonine (human Neu2), or leucine (M. viridifaciens), and the residue forms part of the larger, more hydrophobic pocket accommodating the acetamido group of Neu5Ac. Mutagenesis of Arg^{171} will perhaps show whether this is a determinant of KDN binding.

The discovery of a second KDN-binding site adjacent to the active site is an unusual finding for an exo-sialidase. Many bacterial and parasite sialidases or trans-sialidases possess additional CBMs that often recognize sialic acid (15, 26, 27). The presence of these modules increases catalytic efficiency of the enzyme by targeting the sialidase to appropriate substrates (25). However, these CBMs are distinct domains, and the sialic acid-binding site in the CBM is typically at least 30 Å from the active site. In addition, bacterial sialidases often have an asymmetric charge distribution over their surface with the side opposite the active site being highly negatively charged, and this is thought to assist the orientation of the active site toward negatively charged sialic acid substrates. In contrast, the surface of A/S is mainly positively charged, and the second KDN sits in a basic pocket only 7 Å from the active site KDN. The second KDN is its α-anomeric form, with the ring adopting a low energy C_5 chair conformation and with its anomeric oxygen pointing away from the surface of A/S. There is a positively charged groove running down and underneath the A/S surface beyond this second KDN (Fig. 2). In the complex with a fluorinated KDN, two 2,3F-KDN molecules in a C_5 chair
conformation occupy part of this groove. This is therefore suggestive of polyKDN being a preferred substrate of AFS. A positively charged groove also extends along the surface of the bacteriophage K1F tailspike protein, an endosialidase that recognizes and degrades α2,8-linked polyNeu5Ac (37).

We have shown that the AFS sialidase is a KDNase and may have a preference for polyKDN substrates. Why does A. fumigatus have a KDNase, and where in its life cycle would it encounter KDN or polyKDN? We have shown that A. fumigatus can utilize KDN but not Neu5Ac as a sole carbon source, and this suggests a nutritional role for the KDNase in scavenging KDN from the environment. The few reports on A. fumigatus strain SVQ293, and

Although there is little evidence to date, it may be possible to find sources of KDN in its natural ecological niche in soil where it survives and grows on organic debris.

A. fumigatus is the most prevalent airborne fungal pathogen, causing severe and often fatal invasive infections in immunocompromised hosts (reviewed in Ref. 32). The conidia released by the fungus are small enough to reach the lung alveoli, and the resulting disease called invasive aspergillosis occurs predominantly in the lungs. Intriguingly, polyKDN has been found in the human lung, as well as in other tissues, identified through the use of a monoclonal antibody recognizing α2,8-linked KDN molecules (4, 9). PolyKDN in the lung is a relatively minor component of glycoproteins and glycolipids compared with Neu5Ac and is reported to be associated with a developmentally regulated 150-kDa glycoprotein not present in adults but present in various types of human lung tumors (9). The results presented here from a structural analysis of the A. fumigatus KDNase suggest further explorations into its role in nutrition or pathogenesis.

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