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# Structural and kinetic differences between human and *Aspergillus fumigatus* glucosamine *N*-acetyl transferase

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## Abstract

*Aspergillus fumigatus* is the causative agent of aspergillosis, a frequently invasive colonisation of the lungs of immunocompromised patients. Glucosamine-6-phosphate *N*-acetyltransferase (GNA1) catalyses the acetylation of glucosamine-6-phosphate (GlcN-6P) to *N*-acetylglucosamine-6-phosphate (GlcNAc-6P), a key intermediate in the UDP-GlcNAc biosynthetic pathway. Gene disruption of *gna1* in yeast and *Candida albicans* has provided genetic validation of the enzyme as a potential target. An understanding of potential active site differences between the human and *A. fumigatus* enzymes is required to enable further work aimed at identifying selective inhibitors for the fungal enzyme. Here, we describe crystal structures of both human and *A. fumigatus* GNA1, as well as their kinetic characterization. The structures show significant differences in the sugar binding site, with in particular several non-conservative substitutions near the phosphate binding pocket. Mutagenesis targeting these differences revealed drastic effects on steady-state kinetics, suggesting that the differences could be exploitable with small molecule inhibitors.

## Introduction

*Aspergillus fumigatus* is a filamentous, cosmopolitan and ubiquitous saprophytic fungus [1]. Its natural ecological niche is soil, from which aerosols of conidia are released. If these reach the alveoli in the human lung, they may germinate and start an infection (colonization), leading to invasive or chronic aspergillosis, especially in immunocompromised patients. In these patients, the incidence of invasive infection can be as high as 50%, with a 50% mortality rate [2]. In the USA, aspergillosis is the second most common opportunistic fungal infection in immunocompromised patients, accounting for as many as 20% of fungal infections in patients who have received organ transplants [3]. Amphotericin B has long been the primary drug for the treatment of aspergillosis, although voriconazole has recently been described to represent an improvement against invasive aspergillosis [4], with itraconazole also providing some encouraging results [5]. Nevertheless, new drugs are urgently needed due to the inefficacy and side effects reported for amphotericin, itraconazole and voriconazole [6].

The fungal cell wall is essential for the viability of *Aspergillus fumigatus* and is mainly composed of a branched  $\beta(1,3)$ -glucan core bound to chitin, galactomannan and  $\beta(1,3-1,4)$ -glucan, embedded in an amorphous cement composed of  $\alpha(1,3)$ -glucan, galactomannan and polygalactosamine [7]. Chitin is a  $\beta(1,4)$  linked polymer of *N*-acetylglucosamine (GlcNAc), deposited by chitin synthase. Chitin, although a minor component of the *Aspergillus fumigatus* cell wall, is essential for cell viability and mother-daughter cell separation [8]. Chitin biosynthesis, which requires uridine diphospho-*N*-acetylglucosamine (UDP-GlcNAc), is complex and involves several chitin synthases and ancillary proteins [8]. Normal levels of UDP-GlcNAc are required for chitin biosynthesis and subsequent cell wall assembly and growth [9]. UDP-GlcNAc is also a substrate for the synthesis of glycosylphosphatidylinositol (GPI) anchors of cell wall proteins [10] and the synthesis of *N*-linked and *O*-linked glycans.

The UDP-GlcNAc biosynthetic pathway is formed by four enzymes. The first enzyme, glutamine:fructose-6-phosphate amidotransferase (GFA1), is bifunctional, converting fructose-6-phosphate and glutamine to glucosamine-6-phosphate (GlcN-6P) [11]. The second enzyme in the pathway is D-glucosamine-6-phosphate *N*-acetyltransferase (GNA1), which converts acetyl-CoA (AcCoA) and GlcN-6P to CoA and *N*-acetylglucosamine-6-phosphate (GlcNAc-6P) [11]. The third enzyme, GlcNAc phosphomutase, converts GlcNAc-6P to GlcNAc-1P, employing glucose-1,6-bisphosphate as a co-factor [12]. The final enzyme, UDP-*N*-acetylglucosamine pyrophosphorylase, converts UTP and GlcNAc-1P to UDP-GlcNAc and pyrophosphate [11, 13]. GNA1 belongs to the superfamily of Gcn5-related *N*-acetyltransferases (GNAT), widely distributed in nature, which use acyl-CoAs to acylate their cognate substrates [14]. Examples of key members of this superfamily include: aminoglycoside, serotonin and glucosamine-6-phosphate *N*-acetyltransferases, histone acetyltransferase, mycothiol synthase, protein  $\alpha$ -*N*-myristoyltransferase and the FEM family of acyltransferases [14]. More than 24 crystal structures of members of this superfamily have been solved, and, despite poor sequence conservation, they all have in common a structurally conserved  $\alpha/\beta$  fold [14].

The key role that GNA1 plays in eukaryotes was first highlighted by a report of *Saccharomyces cerevisiae gna1* disruption, producing a lethal phenotype [15]. It was later reported that *Candida albicans* lacking *gna1* was viable only in media containing high concentrations of *N*-acetylglucosamine, and showed significantly reduced virulence in a murine model of candidiasis [16]. Although this appears to genetically validate GNA1 as a potential antifungal drug target, it has also been described that the inactivation of the mouse *gna1* gene is lethal [17], reflecting the essential role of this enzyme in higher eukaryotes. Therefore, it is imperative to identify structural differences between human and *A. fumigatus* GNA1 that would be exploitable for selective antifungal drug design. Here, we have investigated such potentially exploitable differences in the active sites of human GNA1 (*HsGNA1*) and *A. fumigatus* GNA1 (*AfGNA1*) with the aim of providing

further validation of GNA1 as a drug target for the treatment of aspergillosis. We present high resolution crystal structures of *Af*GNA1 and *Hs*GNA1 in complex with products, and the kinetic characterization of these enzymes. By detailed comparison of the active sites of both enzymes, potentially exploitable differences are revealed and validated using site-directed mutagenesis.

## Materials and Methods

### Cloning of *Af*GNA1 and *Hs*GNA1

The *A. fumigatus gna1* gene was obtained by PCR from genomic DNA using the forward primer 5'-GGATCCATGACCAACGCAACCATTGCTCCGAC-3' and the reverse primer 5'-CTCGAGTCATCAGTACAGTACAGT-3' which contains *Bam*HI and *Xho*I restriction sites. The PCR product was cloned into the pCR2.1 vector (Invitrogen) and subsequently digested with *Bam*HI and *Xho*I and subcloned into the pGEX6P1 vector (which encodes a GST tag and a PreScission protease cleavage site, Amersham Biosciences). Site-directed mutants were generated following the QuickChange protocol (Stratagene), using the KOD HotStart DNA polymerase (Novagene). All plasmids were verified by sequencing (College of Life sciences, University of Dundee). DNA encoding full-length (amino acids 1-184) human GNA1 was amplified by PCR from the Mammalian Gene Collection clone (accession code gi:37620194) and subcloned into a modified pET28a-MHL vector (<http://www.sgc.utoronto.ca/SGC-WebPages/toronto-vectors.php>), downstream of the poly-histidine coding region.

### Expression and purification

pGEX6P1-*Af*GNA1 was transformed into BL21 (DE3) pLysS and grown in LB with 50  $\mu$ g/ml of ampicillin. Cells were grown at 37°C until reaching an optical density of 0.6 at 600 nm, after which the expression of the protein was induced with 0.25 mM isopropyl  $\beta$ -D-1-thiogalactopyranoside

(IPTG) at room temperature for an overnight incubation. The cells were harvested by centrifugation at 3480 g for 30 min and resuspended in a buffer (consisting of 25 mM Tris HCl, 150 mM sodium chloride, pH 7.5), a small spatula of lysozyme and DNase, and one tablet of protease inhibitors cocktail (Calbiochem). The cells were disrupted by sonication and centrifuged at 19000 g at 4°C for 30 minutes. The supernatant was incubated at 4°C with glutathione-sepharose 4B beads (Amersham Biosciences) previously equilibrated with the same buffer for 2 hours and subsequently the GST was cleaved overnight with PreScission protease. The AfGNA1 protein was released from the beads and passed through a pre-equilibrated gel filtration column as the last step of the purification. The protein was concentrated and analysed by SDS-PAGE. Purification yield was 3 mg of the protein per 1 l of culture.

*HsGNA1* was expressed in *E. coli* BL21 (DE3) codon plus RIL strain (Stratagene) in Terrific Broth (TB) in the presence of 50 g/mL of kanamycin. Cells were grown at 37 °C to an optical density of 1.5 and induced by 1 mM of IPTG and incubated overnight at 15 °C. Cells were harvested by centrifugation at 7000 rpm. The cell pellets were frozen in liquid nitrogen and stored at -80 °C. For the purification the cell paste was thawed and resuspended in lysis buffer (50 mM HEPES-NaOH, pH 7.4, 500 mM NaCl, 5 mM imidazole, 2 mM  $\beta$ -mercaptoethanol, 5% glycerol, pH 7.4) with protease inhibitor (0.1 mM phenylmethyl sulfonyl fluoride, PMSF). The cells were lysed by passing through Microfluidizer (Microfluidics Corp.) at 20,000 psi. The crude extract was cleared by centrifugation. The clarified lysate was loaded onto 5 ml HiTrap Chelating column (Amersham Biosciences), charged with Ni<sup>+2</sup>. The column was washed with 10 column volumes 20 mM HEPES-NaOH, pH 7.4, containing 500 mM NaCl and 50 mM imidazole, 5% glycerol, and the protein was eluted with elution buffer (20 mM HEPES-NaOH, pH 7.4, 500 mM NaCl, 250 mM imidazole, 5% glycerol, pH 7.4). The protein was dialyzed against 20 mM HEPES-NaOH, pH 7.4, 500 mM NaCl, 5% glycerol in the presence of TEV protease. The dialyzed protein was passed



through a 5 ml Ni<sup>+2</sup> HiTrap column and loaded on Superdex200 column (26x60) (Amersham Biosciences), equilibrated with 20 mM PIPES buffer, 250 mM NaCl, pH 6.5 at flow rate 4 ml/min. The pooled fractions containing *HsGNA1* were further purified to homogeneity by ion-exchange chromatography on a Source 30S column (10x10) (Amersham Biosciences), equilibrated with 20 mM PIPES pH 6.5, and eluted with a linear gradient of NaCl up to 500 mM concentration (20 column volumes). The purification yield was 25 mg of the protein per 1 l of culture.

### Kinetics

Steady-state kinetics of wild type (WT) and mutant enzymes were determined using a previous described protocol [18, 19] with some modifications. AcCoA and GlcN-6P, and CoA were supplied by Sigma. All measurements were performed in triplicate. Standard reactions consisted of 5 nM *AfGNA1* (200 nM for the *AfGNA1* mutants) or 20 nM *HsGNA1* in 25 mM Tris-HCl, 250 mM NaCl, 2 mM EDTA, 5% (v/v) glycerol, pH 7.5 in a total volume of 50  $\mu$ l, incubated at RT. The reactions were initiated by adding the protein and stopped at different times depending on the enzyme with 50  $\mu$ l of a solution containing 25 mM Bis-Tris-propane, 250 mM NaCl, 2 mM EDTA and 6.4 M guanidine chloride, pH 7.5. 50  $\mu$ l of dithio-bis(2-nitrobenzoic acid) (DTNB) solution (1 mM in 0.1% DMSO) containing 25 mM Tris-HCl, 250 mM NaCl and 2 mM EDTA, pH 7.5 was added and the absorbance at 412 nm determined. Absorbance was quantified using a Spectra max 340 PC (Molecular Devices). The absorbance intensity data were analyzed with non-linear regression analysis using GRAFIT 5 [20], with the default equations for first order reaction rates and Michaelis-Menten steady-state kinetics.

## Crystallization, phasing, and refinement

Two different crystal forms were obtained for AfGNA1. For the first crystal form, AfGNA1 at a concentration of 20 mg/ml was preincubated on ice for 10 minutes with 10 mM CoA and 30 mM GlcNAc-6P. The sitting-drop vapor diffusion method was used to produce crystals by mixing 0.6  $\mu$ l of the protein solution with an equal volume of mother liquor (100 mM HEPES, 200 mM MgCl<sub>2</sub>, 22.5-30% PEG 3350, pH 7.75) at 20 °C. Bi-pyramidal crystals (space group P4<sub>3</sub>2<sub>1</sub>2) grew within 1 day. The second crystal form was obtained when AfGNA1 (17 mg/ml) was co-crystallised with 10 mM CoA and 10 mM GlcNAc-6P. Crystals were produced by mixing 0.6  $\mu$ l of the protein/ligand solution with an equal volume of mother liquor (10-25% PEG 1500; 2.5-15% PEG 1000 and 7.5-22.5% PEG 8000) at 20°C. Bar-like shaped crystals (space group C222<sub>1</sub>) grew within 3 days. The first crystal form was cryoprotected with 30% PEG 3350 and the second crystal form was cryoprotected using 35% PEG 1000 and 10% PEG 8000 in the corresponding mother liquor, and frozen in a nitrogen gas stream cooled to 100 K. A SAD phasing strategy was employed on a P4<sub>3</sub>2<sub>1</sub>2 crystal soaked for 30 min in mother liquor containing 20 mM PbCl<sub>2</sub>, using data collected at the European Synchrotron Radiation Facility (beamline BM14). Data were processed with the HKL suite [21]. Seven lead sites were located and refined with SHELX [22]. This resulted in phases to 2.2 Å with a figure of merit of 0.30, yielding a readily interpretable electron density map. Phases were further improved by solvent-flattening, resulting in an electron density map that showed well-defined density for the protein, and was used for autotracing with warpNtrace [23], which built 143 out of 190 residues. The resulting model was improved by partial iterative refinement with REFMAC5 [24] interspersed with model building with COOT [25]. This partial model was then used to solve the structure of the C222<sub>1</sub> crystal form by molecular replacement with MOLREP [26] against 4 Å data (Table I), giving a single solution (R-factor = 0.372) with one molecule in the asymmetric unit. Further refinement to 1.8 Å with REFMAC5 including TLS, interspersed

with model building with COOT [25] yielded a model with the final statistics shown in Table I. Models for ligands were not included until their conformations were well defined by the unbiased  $|F_o| - |F_c|$ ,  $\phi_{calc}$  electron density maps (Fig. 4). Ligand topologies and coordinates were generated with PRODRG [27]. In the case of the CoA, the last seven atoms including the sulfhydryl group were not well defined by the electron density maps and were omitted from the model. WHAT IF [28] was used to check hydrogen bonds and PyMol [29] was used to generate pictures.

Purified *HsGNA1* (16 mg/ml) was crystallized using the sitting drop vapor diffusion method by mixing 1  $\mu$ l of protein solution with 1  $\mu$ l of the reservoir solution containing 25% PEG4000, 0.2 M ammonium sulfate, 0.1 M Na Acetate, pH 4.6, 0.1 M Yttrium chloride. For the ternary complex, purified *HsGNA1* protein was complexed with CoA and GlcNAc-6P (Sigma) at 1:5:5 molar ratio of protein:CoA:GlcNAc-6P and crystallized using the hanging drop vapor diffusion method by mixing 2  $\mu$ l of protein solution with 2  $\mu$ l of the reservoir solution containing 27% PEG3350, 0.2 M  $MgCl_2$ , 0.1 M Bicine, pH 9.0. Crystals were soaked in the corresponding mother liquor supplemented with 20% glycerol as cryoprotectant before freezing in liquid nitrogen. X-ray diffraction data were collected at 100K at beamline 17ID of Advanced Photon Source (APS) at Argonne National Laboratory. Data were processed using the HKL 2000 software package [21]. The structure of apo-*HsGNA1* was solved by molecular replacement using the program MOLREP [26] with the yeast GNA1 structure (PDB 1I1D [30]) as search model and partially refined. The partial model was then used as a molecular replacement search model against the ternary complex diffraction data. ARP/wARP was used for automatic model building [23] using the initial model phases. Graphics program COOT [25] was used for model building and visualization. Crystal diffraction data and refinement statistics are displayed in Table I.

## Results & Discussion

### Kinetics of *Af*GNA1 and *Hs*GNA1

*Af*GNA1 and *Hs*GNA1 were PCR-amplified and cloned into vectors suitable for overexpression in *E. coli* as GST-fusion and HIS-tagged proteins, respectively. Purification using affinity, ion-exchange and size-exclusion chromatography yielded 4 mg and 25 mg of pure *Af*GNA1 and *Hs*GNA1 per liter of bacterial culture, respectively.

*Af*GNA1 and *Hs*GNA1 steady-state kinetics were studied under linear conditions and with no more than 15% of product formation. Both enzymes displayed classical Michaelis-Menten kinetics (Fig. 1). *Af*GNA1 showed a  $K_m$  for AcCoA of  $40 \pm 6 \mu\text{M}$ ,  $K_m$  for GlcN-6P of  $71 \pm 6 \mu\text{M}$  and  $k_{cat}$  of  $38 \pm 3 \text{ s}^{-1}$  (the maximal specific activity was  $107 \mu\text{mol min}^{-1} \text{ mg}^{-1}$  at pH 7.5). Under the same conditions *Hs*GNA1 showed a similar  $K_m$  for AcCoA and GlcN-6P, being  $26 \pm 3 \mu\text{M}$  and  $97 \pm 12 \mu\text{M}$ , respectively. The  $k_{cat}$  of the human enzyme was  $9 \pm 0.2 \text{ s}^{-1}$  (the specific activity was  $25 \mu\text{mol min}^{-1} \text{ mg}^{-1}$ ). Overall, the fungal enzyme ( $k_{cat}/K_m = 0.95 \text{ s}^{-1} \mu\text{M}^{-1}$ ) was 3-fold more catalytically efficient for AcCoA than the human enzyme ( $k_{cat}/K_m = 0.34 \text{ s}^{-1} \mu\text{M}^{-1}$ ). The catalytic efficiency for GlcN-6P was 6-fold higher for *Af*GNA1 compared to *Hs*GNA1 ( $k_{cat}/K_m = 0.53$  and  $0.092 \text{ s}^{-1} \mu\text{M}^{-1}$ , respectively). In terms of the maximal specific activity previously reported for GNA1 from various source, the range is from 0.0054 to  $12.2 \mu\text{mol min}^{-1} \text{ m}^{-1}$ , indicating that *Af*GNA1 would be one of the fastest GNA1s described so far ( $107 \mu\text{mol min}^{-1} \text{ mg}^{-1}$ ) [31, 32].

### *Af*GNA1 and *Hs*GNA1 adopt the GNAT fold

To understand the source of the kinetic differences observed between *Af*GNA1 and *Hs*GNA1 and study potential amino acid differences in their active sites, the crystal structures of these enzymes were solved and refined against high resolution synchrotron diffraction data. An apo-*Af*GNA1 structure was solved using a lead SAD experiment with a  $\text{P4}_32_12$  crystal form (Table I). A partially

refined model from this was then used to solve a C222<sub>1</sub> crystal form of the *Af*GNA1 CoA/GlcNAc-6P complex by molecular replacement, subsequently refined to 1.80 Å resolution with an R-factor of 0.177 ( $R_{free} = 0.215$ , Table I). An apo-*Hs*GNA1 structure was solved by molecular replacement with a C2 crystal form and partially refined. The resulting model was then used to solve a P2<sub>1</sub> crystal form of the *Hs*GNA1 CoA/GlcNAc-6P complex by molecular replacement followed by refinement to 1.8 Å resolution with an R-factor of 0.189 ( $R_{free} = 0.237$ , Table I).

Both *Af*GNA1 and *Hs*GNA1 are homodimers in the crystal (and solution) and adopt essentially the same fold as the first GNA1 structure solved, that of *S. cerevisiae* [30] (Fig. 2A, 0.98 Å on 147 C $\alpha$  atoms and 1.18 Å on 146 C $\alpha$  atoms for *Af*GNA1 and *Hs*GNA1 superposed on *Sc*GNA1 crystal structure, respectively). Thus, the typical signature of the GNAT superfamily is also present: an N-terminal strand ( $\beta$ 1) followed by three  $\alpha$  helices ( $\alpha$ 3- $\alpha$ 4- $\alpha$ 5), three antiparallel  $\beta$  strands, a signature central helix ( $\alpha$ 8), a fifth  $\beta$  strand, two  $\alpha$  helices ( $\alpha$ 9 and  $\alpha$ 10) and a sixth and final  $\beta$  strand (Fig. 2A). The only difference in secondary structure between the human and the fungal enzymes is that *Hs*GNA1 has a short additional  $\beta$  strand ( $\beta$ 6, Fig. 2A). A superposition of *Af*GNA1 and *Hs*GNA1 monomers gives an RMSD of 1.3 Å on 159 C $\alpha$  atoms, suggesting some conformational differences. Most of these differences are located to  $\alpha$ 1,  $\alpha$ 2,  $\alpha$ 5,  $\alpha$ 7,  $\beta$ 2,  $\beta$ 3 and  $\beta$ 6, with  $\alpha$ 7 and  $\beta$ 6 contributing residues to the substrate binding site. Similarly, a sequence alignment between the two GNA1s shows that while these proteins share the four GNAT motifs (Fig. 2B), the sequence identity is only 30%. These significant structural and sequence differences prompted an investigation of whether such differences also extended to the active site.

#### ***Af*GNA1 and *Hs*GNA1 possess different GlcN-6P subsites**

A close inspection of the active sites of both enzymes shows that while the AcCoA binding site is essentially conserved, significant differences are present in the GlcN-6P binding site (Figs. 3,4).

The main differences are located to the residues contacting the  $\alpha$  face of the sugar (Figs. 3,4). These residues are, in *AfGNA1*, Val125, Gly183, Glu185 and Tyr189, with the equivalent residues in *HsGNA1* being Arg116, Glu175, Tyr177 and Arg181, respectively. Arg116, Glu175 and Arg181 of the human enzyme directly contact the sugar product, while Tyr177 lines a pocket just below it (Fig. 4). Interestingly, the two arginines are substituted by smaller, neutral residues in the fungal enzyme, presumably with a reduced ability to interact with the phosphate on the sugar. Furthermore, the substitution of a glutamate (Glu175) with a glycine (Gly183) in the fungal enzyme generates a larger binding cavity. Thus, the *HsGNA1* and *AfGNA1* active sites show significant differences around the sugar binding site.

### Catalytic properties of *AfGNA1/HsGNA1* chimeras

To study the contributions to substrate binding of the different residues lining the sugar binding site, *AfGNA1/HsGNA1* chimeras were constructed through site-directed mutagenesis, and their kinetic properties studied (Table II). While all four mutant enzymes show effects in terms of reduced catalytic efficiency, this is, surprisingly, not only due to increases in  $K_m$ , but also decreases in  $k_{cat}$ . Val125Arg and Gly183Glu show the largest effects on  $k_{cat}$ , with 327-fold and 112-fold decrease compared to the *AfGNA1* wild type, respectively (Table II). Val125Arg also shows the largest effects on the  $K_m$ s for AcCoA and GlcN-6P, with a 5-fold and 8.5 fold decrease in the  $K_m$  of AcCoA and the  $K_m$  of GlcN-6P, respectively. Apparently, the arginine present at this position in the human enzyme facilitates binding of the sugar substrate through interaction with the phosphate group (Fig. 4). The precise positioning of the sugar could be important for direct nucleophilic attack mechanism proposed previously [30], and this could explain the concomitant changes in  $k_{cat}$ . A similar explanation of effects on  $k_{cat}$  could be applied to the other three mutants which are likely to have effects on the precise position of the sugar in the binding site. Thus, mutation of the four



non-conserved residues lining the sugar binding pocket suggests that they contributed to the kinetic differences between the *Af*GNA1 and *Hs*GNA1 enzymes.

## Concluding remarks

There is sufficient data at the genetic level to suggest that GNA1 is an attractive antifungal drug target, provided two key issues can be addressed. First, although substrate-based inhibitors for the larger GNAT family, of which GNA1 is a member, have been reported, they do not possess drug-like properties and no significant *in vivo* effects of these molecules have been reported [33, 34, 35]. Second, the issue of selectivity requires careful consideration as a knockout of the mouse enzyme produced a lethal phenotype and GNA1 is generally believed to be essential in eukaryotes [17]. The latter issue was the focus of the present study, using *Af*GNA1 and *Hs*GNA1 as model enzymes. Kinetic analysis of these enzymes revealed differences in their Michaelis-Menten parameters. This was then corroborated by determination of their high-resolution crystal structures, which allowed a direct comparison between the active sites of these enzymes. Surprisingly, this revealed that structural differences between the two enzymes were mostly located to the sugar binding site, whereas the AcCoA binding site appeared to be more conserved. These changes affect not only the electrostatics, but also reveal a more spacious sugar binding site in the *Af*GNA1 enzyme, whereas large side chains at these positions create a tighter pocket in the *Hs*GNA1 enzyme. Probing of these residues using mutagenesis suggests that they are important for sugar binding and positioning. Taken together these data may provide a useful framework for the discovery and/or design of molecules that show selective binding to *Af*GNA1 over *Hs*GNA1.

Although the work reported here appears to provide data to support future drug discovery aimed at identifying *Af*GNA1 inhibitors, it has also uncovered a number of issues that may hamper such endeavours. While selective design of inhibitors may be achievable, such molecules may well

possess undesirable properties. The substrate complexes described here and published previously [30] show that interactions with the sugar are mostly of a polar nature, with almost no hydrophobic/van der Waals interactions. Furthermore, if competition with both sugar and AcCoA (the latter also binding mainly through electrostatic interactions) is sought, the resulting molecules may be of a size outwith the spectrum normally associated with drug-likeness [36]. Indeed, the only well characterized inhibitors known for the GNAT family of enzymes are truncated aminoglycoside-CoA bisubstrate analogues for aminoglycoside 6'-N-acetyltransferase [33], histone H3 peptides conjugated with CoA for GCN5 histone acetyltransferase [35] and the brominated CoA-S-acetyl-tryptamine bisubstrate analogue for serotonin N-acetyltransferase [34]. Nevertheless, this work provides useful structural and kinetic data which may be useful tools in studies towards the discovery of novel GNA1 inhibitors.

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## Table I

Details of data collection & structure refinement. Values between brackets are for the highest resolution shell. All measured data were included in structure refinement.

	<i>Af</i> GNA1+lead	<i>Af</i> GNA1+CoA+GlcNAc-6P	apo- <i>Hs</i> GNA1	<i>Hs</i> GNA1+CoA+GlcNAc-6P
Space group	P4g2 <sub>1</sub> 2	C222 <sub>1</sub>	C2	P2 <sub>1</sub>
Unit cell (Å)	<i>a</i> = <i>b</i> =54.36 <i>c</i> =131.13	<i>a</i> =70.60, <i>b</i> =100.82 <i>c</i> =55.35	<i>a</i> =111.67, <i>b</i> =47.65 <i>c</i> =84.84, $\beta$ =123.52	<i>a</i> =49.19, <i>b</i> =63.76 <i>c</i> =64.74, $\beta$ =91.81
Resolution range (Å)	20.00–1.80 (1.86–1.80)	20.0–1.80 (1.86–1.80)	70.71–2.67 (2.74–2.67)	45.41–1.80 (1.84–1.80)
# Unique reflections	19096 (1839)	18376 (1790)	10659 (1058)	33633 (1834)
Redundancy	10.3 (11.0)	4.6 (4.6)	6.5 (6.6)	3.5 (2.0)
<i>I</i> / $\sigma$ <i>I</i>	10.5 (3.3)	21.2 (10.5)	8.3 (1.7)	10.5 (2.3)
Completeness (%)	99.9 (99.3)	98.2 (98.0)	98.8 (89.1)	89.6 (89.4)
<i>R</i> <sub>merge</sub>	0.094 (0.616)	0.033 (0.150)	0.084 (0.432)	0.045 (0.216)
<i>R</i> , <i>R</i> <sub>free</sub>		0.177, 0.215		0.189, 0.237
RMSD from ideal geometry				
bonds (Å)		0.015		0.012
angles (°)		1.52		1.47
B-factor RMSD (Å <sup>2</sup> )				
(backbone bonds)		0.84		0.87
<B> (Å <sup>2</sup> )				
protein		18.8		27.1
CoA		19.1		29.1
GlcNAc-6P		27.4		30.1
water		26.1		39.4

## Table II

Comparison of the kinetics parameters between the human, the fungal enzyme and the corresponding mutations.  $K_m$ ,  $k_{cat}$ ,  $k_{cat}/K_m$  units are in  $\mu\text{M}$ ,  $\text{s}^{-1}$   $\text{s}^{-1}\mu\text{M}^{-1}$  respectively. All the parameters were obtained at pH 7.5.

	$K_m$ AcCoA	$K_m$ GlcN-6P	$k_{cat}$	$k_{cat}/K_m$ AcCoA	$k_{cat}/K_m$ GlcN-6P
<i>Hs</i> GNA1	26 ± 3	97 ± 12	9 ± 0.2	0.34	0.092
<i>Af</i> GNA1	40 ± 6	71 ± 6	38 ± 3	0.95	0.53
<i>Af</i> GNA1-V125R	200 ± 40	600 ± 100	0.12 ± 0.01	0.00058	0.00019
<i>Af</i> GNA1-G183E	100 ± 18	56 ± 8	0.34 ± 0.017	0.0034	0.006
<i>Af</i> GNA1-E185Y	40 ± 7	30 ± 8	1.3 ± 0.07	0.031	0.042
<i>Af</i> GNA1-Y189R	86 ± 11	56 ± 8	1.7 ± 0.04	0.019	0.03

## Figure legends

### 1. GNA1 kinetics.

Initial steady-state velocities are shown for recombinant *Af*GNA1 and *Hs*GNA1 using different concentrations of AcCoA at a fixed (500  $\mu$ M) concentration of GlcN-6P.

### 2. *Af*GNA1 and *Hs*GNA1 fold and sequence conservation.

A: Overall crystal structures of *Af*GNA1 and *Hs*GNA1. Secondary structure is shown in olive (strands) and brown (helices) for one monomer, and blue (helices) and red (strands) for the other monomer. A highly conserved active site tyrosine, that has been proposed to stabilise the leaving thiolate during catalysis [30], is shown as sticks in both crystal structures. CoA and GlcNAc-6P are shown as sticks with green carbon atoms. In the middle of this panel a superposition of the monomers from *Af*GNA1 (blue) and *Hs*GNA1 (red) are shown.

B: Multiple sequence alignment between *Af*GNA1, *Hs*GNA1, *Ce*GNA1, *Sc*GNA1, *Ca*GNA1, *Dm*GNA1, *Mm*GNA1, *Cp*GNA1, *Ae*GNA1. Abbreviations: *Ce*, *C. elegans*, *Ca*, *C. albicans*, *Dm*, *D. melanogaster*, *Mm*, *M. musculus*, *Cp*, *Culex pipiens* and *Ae*, *Aedes aegypti*). Magenta triangles represent the residues from either *Af*GNA1 or *Hs*GNA1 involved in the binding of the sugar. The conserved Tyr is shown as a green triangle. The four different motifs of the GNAT family are represented as yellow boxes.

### 3. Conservation of the substrate binding site.

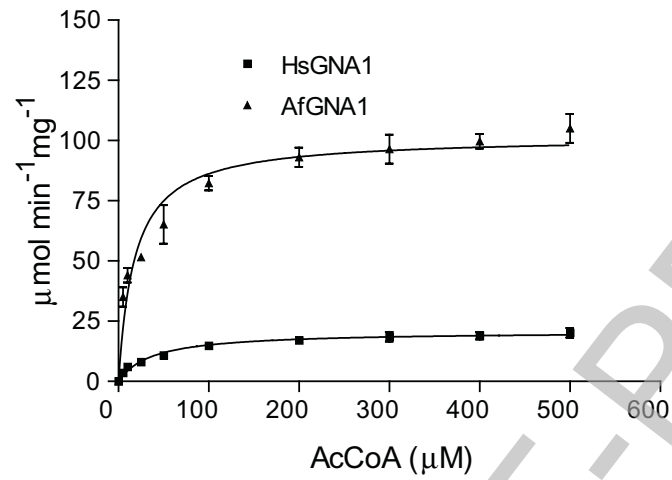
Molecular surfaces of *Hs*GNA1 and *Af*GNA1 are shown, coloured by sequence conservation. Orange/red, purple/pink and grey represent identity, conservative substitutions and non-conservative substitutions, respectively. The ligands are represented as sticks with green

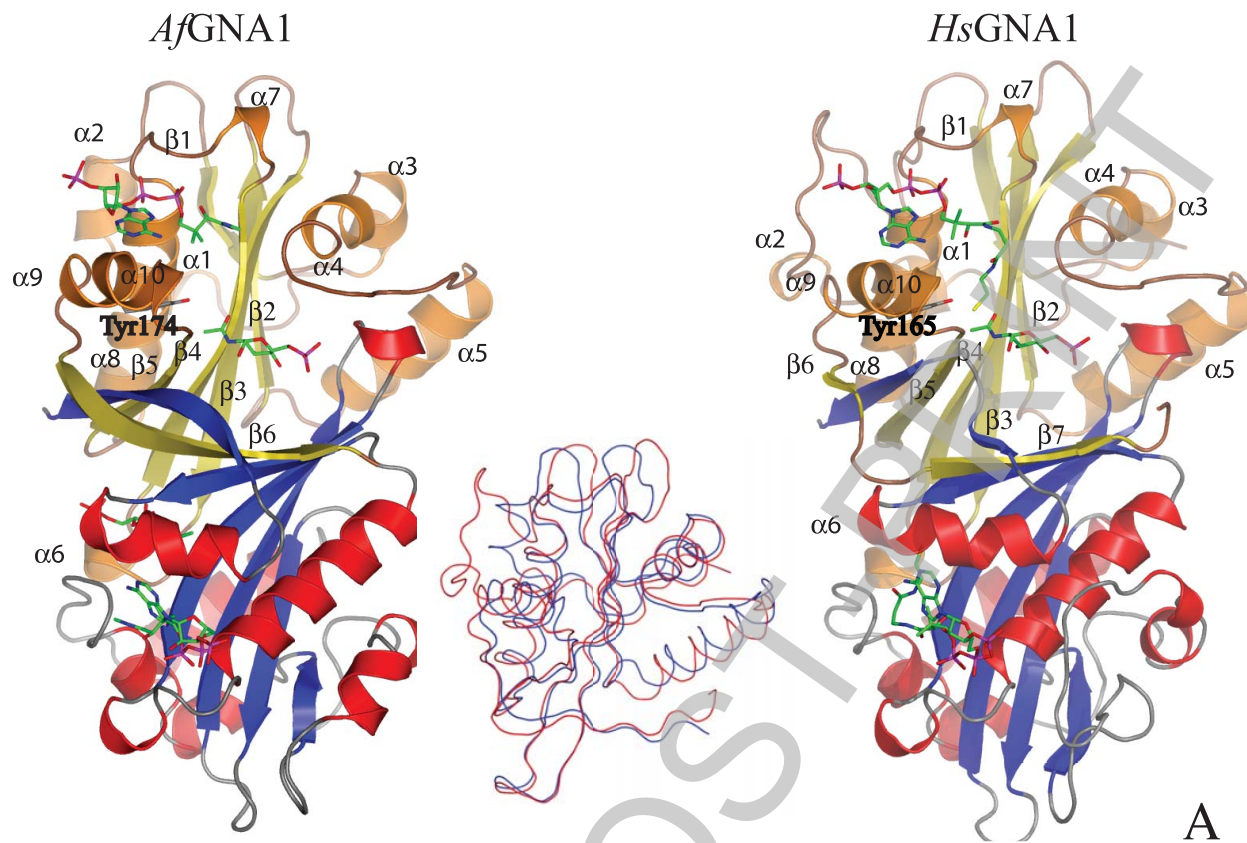


carbons.

#### 4. Comparison of the *Af*GNA1 and *Hs*GNA1 active sites.

Stereo views of the structures of *Af*GNA1 (A) and *Hs*GNA1 (B) in complex with CoA and GlcNAc-6P, and detailed comparison of the phosphate binding sites (C). The protein backbone is shown as a grey ribbon. Residues lining the active site are shown as sticks with grey carbon atoms (conserved residues) or orange carbons (non-conserved residues). Ligands are shown as sticks with green carbon atoms. In the *Af*GNA1 structure, the terminal atoms of CoA, including the thiol, were omitted from the model due to a lack of electron density. Protein-ligand hydrogen bonds are shown as dotted green lines. Unbiased (i.e. before inclusion of any ligand model) (for *Af*GNA1) and a simulated annealing omit (for *Hs*GNA1)  $|F_o| - |F_c|$ ,  $\phi_{calc}$  electron density maps are shown at  $2.5\sigma$ . Water molecules involved in hydrogen bonds with the ligand or the protein are shown as blue spheres. In panel C, protein-phosphate distances (Å) are shown in black.





A

	1	10	20	30	40	50	60	70	80
AfGNA1	MTNAT	IAPTTT	AAPVTKSV	DAPTAD	ENTPLF	SPSLIS	PDVLAV	IPAD	.....
HsGNA1	.....	.....	MKPD	ETPMF	DP	SL	KEVD	WSQNT	ATFS
CeGNA1	.....	.....	MSHI	FDASV	LA	.....	PH	IPSN	LPDN
ScGNA1	.....	.....	.....	.....	.....	.....	.....	.....	.....
CaGNA1	.....	.....	.....	.....	.....	.....	.....	.....	.....
DmGNA1	.....	MVQL	TEET	TYLY	DP	NL	LKLD	FHR	SPAN
MmGNA1	.....	.....	MKP	DET	PMF	DP	SL	KEVD	WSQNT
CpGNA1	.....	MGAS	I	EQELS	LYNSS	I	LANL	NFAG	SPAT
AeGNA1	.....	MGAS	HPE	QE	LSLY	DR	SL	LDL	DFSR

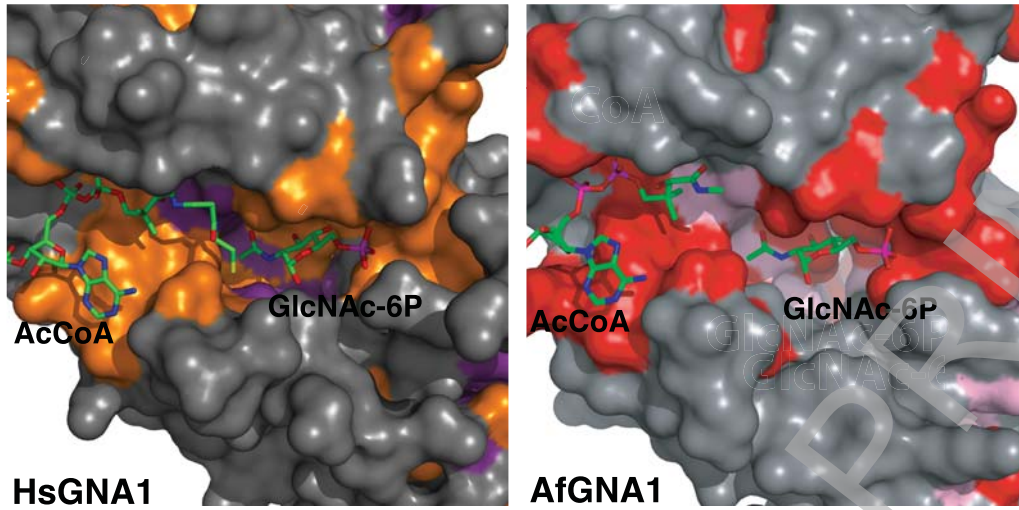
	80	90	100	110	120	130	140	147
AfGNA1	QWNS	RYEW	I	RARS	DE	.....	.....	.....
HsGNA1	QFMK	SEHM	K	SG	.....	.....	.....	.....
CeGNA1	AFEK	REAM	R	T	SVP	.....	.....	.....
ScGNA1	SFSK	LTKY	WNEA	T	VW	ND	NED	KK
CaGNA1	DFTE	L	VNH	WS	LS	PS	I	.....
DmGNA1	QFLT	RF	SQ	MK	ASG	.....	.....	.....
MmGNA1	QFMK	SEHM	K	SG	.....	.....	.....	.....
CpGNA1	QFLN	RAQ	M	R	ASG	.....	.....	.....
AeGNA1	QYLNR	RAQ	M	R	ASG	.....	.....	.....

	150	160	170	180	190
AfGNA1	QALD	YVAE	KV	GY	KT
HsGNA1	STLT	LL	SK	KL	NY
CeGNA1	KTLV	SL	GK	SL	GV
ScGNA1	DQLV	T	IG	FDY	GY
CaGNA1	TSLT	KVAE	QEN	GY	KV
DmGNA1	VTVS	LT	AE	EL	GY
MmGNA1	VTVS	LL	SK	KL	NY
CpGNA1	VTVS	LL	SK	KL	NY
AeGNA1	VTVS	LL	SK	KL	NY

B

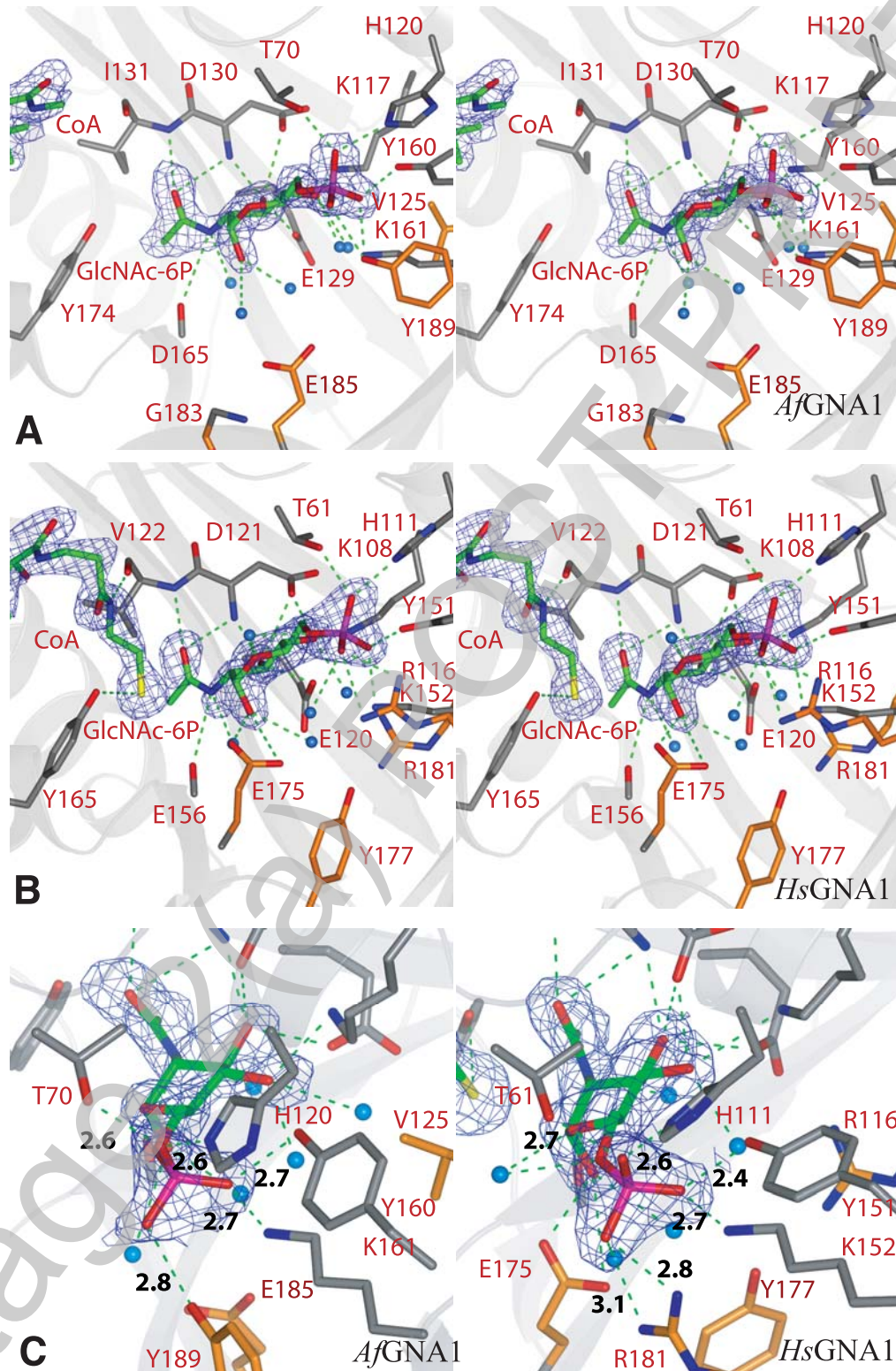
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