Aspergillus fumigatus is the causative agent of aspergillosis, a frequently invasive colonization of the lungs of immunocompromised patients. GNA1 (β-glucosamine-6-phosphate N-acetyltransferase) catalyses the acetylation of GlcN-6P (glucosamine-6-phosphate) to GlcNAc-6P (N-acetylglucosamine-6-phosphate), 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. In the present study, 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.

Key words: Aspergillus fumigatus, inhibitor design, kinetics, mutagenesis, protein structure, UDP-GlcNAc biosynthesis, X-ray crystallography.

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 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 U.S.A., 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 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 β(1,3)-glucan core bound to chitin, galactomannan and β(1,3-1,4)-glucan, embedded in an amorphous cement composed of α(1,3)-glucan, galactomannan and polygalactosamine [7]. Chitin is a β(1,4)-linked polymer of GlcNAc (N-acetylglucosamine), deposited by chitin synthase. Chitin, although a minor component of the A. fumigatus cell wall, is essential for cell viability and mother–daughter cell separation [8]. Chitin biosynthesis, which requires 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 GPI (glycosylphosphatidylinositol) 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, GFA1 (glutamine:fructose-6-phosphate amidotransferase), is bifunctional, converting fructose-6-phosphate and glutamine into GlcN-6P (glucosamine-6-phosphate) [11]. The second enzyme in the pathway is GNA1 (β-glucosamine-6-phosphate N-acetyltransferase), which converts AcCoA (acetyl-CoA) and GlcN-6P into GlcNAc-6P (N-acetylglucosamine-6-phosphate) [11]. The third enzyme, GlcNAc phosphomutase, converts GlcNAc-6P into GlcNAc-1P, employing glucose-1,6-bisphosphate as a co-factor [12]. The final enzyme, UDP-N-acetylglucosamine pyrophosphorylase, converts UTP and GlcNAc-1P into UDP-GlcNAc and pyrophosphate [11,13]. GNA1 belongs to the superfamily of GNATs (GCN5-related N-acetyltransferases), 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 α-N-myristoyltransferase and the FEM family of acetyltransferases [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 α/β fold [14].

Abbreviations used: AcCoA, acetyl-CoA; GlcNAc, N-acetylglucosamine; GlcNAc-6P, N-acetylglucosamine-6-phosphate; GlcN-6P, glucosamine-6-phosphate; GNA1, β-glucosamine-6-phosphate N-acetyltransferase; AF/GNA1, Aspergillus fumigatus GNA1; GNAT, GCN5-related N-acetyltransferase; GST, glutathione transferase; HsGNA1, human GNA1; IPTG, isopropyl β-D-thiogalactoside; PEG, poly(ethylene glycol); RMSD, root mean square deviation; ScGNA1, Saccharomyces cerevisiae GNA1; WT, wild-type.

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The co-ordinates and structure factors have been deposited in the PDB with the following entry codes: 2H2U and 2O2B for apo-HsGNA1 and the HsGNA1-CoA–GlcNAc-6P complex respectively, and entry code 2VXX for the AFGNA1–CoA–GlcNAc-6P complex.
The key role that GNA1 plays in eukaryotes was first highlighted by a report of \textit{Saccharomyces cerevisiae gna1} disruption, producing a lethal phenotype [15]. It was later reported that \textit{Candida albicans} lacking \textit{gna1} was viable only in medium containing high concentrations of \textit{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 \textit{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 \textit{A. fumigatus} GNA1 that would be exploitable for selective antifungal drug design. In the present study, we have investigated such potentially exploitable differences in the active sites of \textit{HsGNA1} (human GNA1) and \textit{AfGNA1} (\textit{A. fumigatus} GNA1) 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 \textit{AfGNA1} and \textit{HsGNA1} 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.

\section*{MATERIALS AND METHODS}

\subsection*{Cloning of \textit{AfGNA1} and \textit{HsGNA1}}

The \textit{A. fumigatus gna1} gene was obtained by PCR from genomic DNA using the forward primer 5'-GGATCCATGGACCAACGCAACATTGTCCGAC-3' and the reverse primer 5'-CTCGAGTCA TCAGTAGTAGTGCGCCA TCTCCAAC-3' which contain BamHI and XhoI restriction sites. The PCR product was cloned into the plcR2.1 vector (Invitrogen) and subsequently digested with BamHI and XhoI and sub-cloned into the pGEX6P1 vector [which encodes a GST (glutathione transferase) tag and a PreScission protease cleavage site; Amersham Biosciences]. Site-directed mutants were generated following the QuikChange \textsuperscript{®} protocol (Stratagene), using the KOD HotStart DNA polymerase (Novagen). All plasmids were verified by sequencing (College of Life sciences, University of Dundee, Dundee, Scotland, U.K.). DNA encoding full-length (amino acids 1–184) \textit{HsGNA1} 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.

\subsection*{Expression and purification}

pGEX6P1-\textit{AfGNA1} was transformed into BL21 (DE3) pLysS and grown in LB (Luria–Bertani) medium with 50 \text{\textmu}g/ml 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 IPTG (isopropyl \textit{\beta}-\text{d}-thiogalactoside) at room temperature (23°C) for an overnight incubation. The cells were harvested by centrifugation at 3480 \text{g} for 30 min and re-suspended in a buffer consisting of 25 mM Tris/HCl and 150 mM sodium chloride (pH 7.5), containing 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 \text{g} for 30 min. The supernatant was incubated at 4°C with glutathione–Sepharose 4B beads (Amersham Biosciences) previously equilibrated with the same buffer for 2 h and subsequently the GST was cleaved overnight with PreScission protease. The \textit{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 litre of culture.

\textit{HsGNA1} was expressed in \textit{Escherichia coli} BL21 (DE3) codon plus RIL strain (Stratagene) in TB (Terrific Broth) in the presence of 50 \textmu g/ml of kanamycin. Cells were grown at 37°C to a \textit{D} of 1.5 and induced by 1 mM IPTG and incubated overnight at 15°C. Cells were harvested by centrifugation at 3480 \text{g} for 30 min at 4°C. 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 Hapes/NaOH (pH 7.4), 500 mM NaCl, 5 mM imidazole, 2 mM 2-mercaptoethanol and 5 % (v/v) glycerol] with protease inhibitor (0.1 mM PMSF). The cells were lysed by passing through a microfluidizer (Microfluidics) at 20,000 lbf/in\textsuperscript{2} (1 lbf/in\textsuperscript{2} = 6.9 kPa). The crude extract was cleared by centrifugation (19000 \text{g} for 30 min at 4°C). The clarified lysate was loaded on to a 5 ml HiTrap chelating column (Amersham Biosciences), charged with Ni\textsuperscript{2+}. The column was washed with 10 column volumes of 20 mM Hapes/NaOH (pH 7.4), containing 500 mM NaCl, 50 mM imidazole and 5 % (v/v) glycerol, and the protein was eluted with elution buffer [20 mM Hapes/NaOH (pH 7.4), 500 mM NaCl, 250 mM imidazole and 5 % (v/v) glycerol]. The protein was dialysed against 20 mM Hapes/NaOH (pH 7.4), 500 mM NaCl and 5 % (v/v) glycerol in the presence of TEV (tobacco etch virus) protease. The dialysed protein was passed through a 5 ml Ni\textsuperscript{2+} HiTrap column and loaded on to a Superdex200 column (25 mm x 60 cm; Amersham Biosciences), equilibrated with 20 mM Pipes buffer and 250 mM NaCl (pH 6.5) at a flow rate of 4 ml/min. The pooled fractions containing \textit{HsGNA1} were further purified to homogeneity by ion-exchange chromatography on a Source 30S column (10 mm x 10 cm; Amersham Biosciences), equilibrated with 20 mM Pipes (pH 6.5), and eluted with a linear gradient of NaCl up to a concentration of 500 mM (20 column volumes). The purification yield was 25 mg of the protein per 1 litre of culture.

\subsection*{Kinetics}

Steady-state kinetics of WT (wild-type) and mutant enzymes were determined using a previous described protocol [18,19] with some modifications. AcCoA, GlcN-6-P and CoA were supplied by Sigma. All measurements were performed in triplicate. Standard reactions consisted of 5 mM \textit{AfGNA1} (200 mM for the \textit{AfGNA1} mutants) or 20 mM \textit{HsGNA1} in 25 mM Tris/HCl, 250 mM NaCl, 2 mM EDTA and 5 % (v/v) glycerol (pH 7.5) in a total volume of 50 \textmu l, incubated at room temperature. The reactions were initiated by adding the protein and were stopped at different times depending on the enzyme with 50 \textmu 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). A 50 \textmu l aliquot of DTNB [diithio-bis(2-nitrobenzoic acid)] 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 was determined. The absorbance was quantified using a Spectra max 340 PC (Molecular Devices). The absorbance intensity data were analysed with non-linear regression analysis using GRAFIT 5 [20], with the default equations for first-order reaction rates and Michaelis–Menten steady-state kinetics.

\subsection*{Crystallization, phasing and refinement}

Two different crystal forms were obtained for \textit{AfGNA1}. For the first crystal form, \textit{AfGNA1} at a concentration of 20 mg/ml was
pre-incubated on ice for 10 min with 10 mM CoA and 30 mM GlcNAc-6P. The sitting-drop vapour-diffusion method was used to produce crystals by mixing 0.6 μl of the protein solution with an equal volume of mother liquor [100 mM Hepes, 200 mM MgCl₂, 22.5–30 % PEG (polyethylene glycol)] 3350 (pH 7.75) at 20°C. Bi-pyramidal crystals (space group P4₁2₁2) grew within 1 day. The second crystal form was obtained when AfGNA1 (17 mg/ml) was co-crystallized with 10 mM CoA and 10 mM GlcNAc-6P. Crystals were produced by mixing 0.6 μl 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 C222₁) 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 method using the initial model phases. The graphics program COOT [25] was used for autotracing with warpNtrace [23], which built 143 out of 190 residues. The resulting model was improved by partial replacement search model against the ternary complex diffraction data and refinement statistics are displayed in Table 1.

### RESULTS AND DISCUSSION

#### Kinetics of AfGNA1 and HsGNA1

AfGNA1 and HsGNA1 were PCR-amplified and cloned into vectors suitable for overexpression in *E. coli* as GST-fusion and His-tagged proteins respectively. Purification using affinity,
The catalytic efficiency for GlcN-6P was 6-fold higher for AfGNA1 compared with HsGNA1. The specific activity was 25 μmol·min⁻¹·mg⁻¹ of the fungal enzyme (kₐ/Kₘ = 0.34 s⁻¹·μM⁻¹).

AfGNA1 and HsGNA1 possess different GlcN-6P subsites

A close inspection of the active sites of both enzymes showed that although the AcCoA-binding site is essentially conserved, significant differences are present in the GlcN-6P-binding site (Figures 3 and 4). The main differences are located to the residues contacting the α face of the sugar (Figures 3 and 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, whereas Tyr177 lines a pocket just below it (Figure 4). Interestingly, the two arginine residues 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 residue (Glu175) with a glycine (Gly183) residue 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 chimaeras

To study the contributions to substrate binding of the different residues lining the sugar-binding site, AfGNA1–HsGNA1 chimaeras were constructed through site-directed mutagenesis, and their kinetic properties were studied (Table 2). Although all four mutant enzymes show effects in terms of reduced catalytic efficiency, this is, surprisingly, not only due to increases in Kₘ, but also decreases in kₐ. V125R and G183E show the largest effects on kₐ, with a 327-fold and 112-fold decrease respectively, compared with the AfGNA1 WT (Table 2). V125R also shows the largest effects on the Kᵣₘ for AcCoA and GlcN-6P, with a 5-fold and 8.5-fold decrease in the Kᵣₘ of AcCoA and the Kₘ of GlcN-6P respectively. Apparently, the arginine residue present at this position in the human enzyme facilitates binding of the sugar substrate through interaction with the phosphate group (Figure 4). The precise positioning of the sugar could be important for the direct nucleophilic attack mechanism proposed previously [30], and this could explain the concomitant changes in kₐ. A similar explanation of effects on kₐ 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 AfGNA1 and HsGNA1 enzymes.
Figure 2  AfGNA1 and HsGNA1 fold and sequence conservation

(A) Overall crystal structures of AfGNA1 and HsGNA1. 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 residue, that has been proposed to stabilize 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 AfGNA1 (blue) and HsGNA1 (red) are shown. (B) Multiple sequence alignment between AfGNA1, HsGNA1, CeGNA1 (Caenorhabditis elegans GNA1), ScGNA1, CaGNA1 (C. albicans GNA1), DmGNA1 (Drosophila melanogaster GNA1), MmGNA1 (Mus musculus GNA1), CpGNA1 (Culex pipiens GNA1), AeGNA1 (Aedes aegypti GNA1). Magenta triangles represent the residues from either AfGNA1 or HsGNA1 involved in the binding of the sugar. The conserved tyrosine residue is shown as a green triangle. The four different motifs of the GNAT family are represented as yellow boxes.
Table 2 Comparison of the kinetic parameters between the human and the fungal enzyme and the corresponding mutations

<table>
<thead>
<tr>
<th>Enzyme form</th>
<th>$K_m$ AcCoA ($\mu$M)</th>
<th>$K_m$ GlcN-6P ($\mu$M)</th>
<th>$k_{cat}$ ($s^{-1}$)</th>
<th>$k_{cat}/K_m$ AcCoA ($s^{-1} \cdot \mu$M$^{-1}$)</th>
<th>$k_{cat}/K_m$ GlcN-6P ($s^{-1} \cdot \mu$M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HsGNA1</td>
<td>26 ± 3</td>
<td>97 ± 12</td>
<td>9 ± 0.2</td>
<td>0.34</td>
<td>0.092</td>
</tr>
<tr>
<td>AfGNA1</td>
<td>40 ± 6</td>
<td>71 ± 6</td>
<td>38 ± 3</td>
<td>0.95</td>
<td>0.53</td>
</tr>
<tr>
<td>AfGNA1-V125R</td>
<td>200 ± 40</td>
<td>600 ± 100</td>
<td>0.12 ± 0.01</td>
<td>0.00058</td>
<td>0.00019</td>
</tr>
<tr>
<td>AfGNA1-G183E</td>
<td>100 ± 18</td>
<td>56 ± 8</td>
<td>0.34 ± 0.017</td>
<td>0.0034</td>
<td>0.006</td>
</tr>
<tr>
<td>AfGNA1-E185Y</td>
<td>40 ± 7</td>
<td>30 ± 8</td>
<td>1.3 ± 0.07</td>
<td>0.031</td>
<td>0.042</td>
</tr>
<tr>
<td>AfGNA1-Y189R</td>
<td>86 ± 11</td>
<td>56 ± 8</td>
<td>1.7 ± 0.04</td>
<td>0.019</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Figure 3 Conservation of the substrate-binding site

Molecular surfaces of HsGNA1 and AfGNA1 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 carbon atoms.

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 [33–35]. Secondly, 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 AfGNA1 and HsGNA1 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 AfGNA1 enzyme, whereas large side chains at these positions create a tighter pocket in the HsGNA1 enzyme. Probing of these residues using mutagenesis suggests they are important for sugar binding and positioning. Taken together these results may provide a useful framework for the discovery and/or design of molecules that show selective binding to AfGNA1 over HsGNA1.

Although the work reported in the present study appears to provide data to support future drug discovery aimed at identifying AfGNA1 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 undesir-
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 beyond 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, the present study provides structural and kinetic data which may be useful tools in studies towards the discovery of novel GNA1 inhibitors.

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