Biosynthesis of UDP-GlcA, a key metabolite for capsular polysaccharide synthesis in the pathogenic fungus *Cryptococcus neoformans*

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UDP-glucose dehydrogenase catalyses the conversion of UDP-glucose into UDP-GlcA, a critical precursor for glycan synthesis across evolution. We have cloned the gene encoding this important enzyme from the opportunistic pathogen *Cryptococcus neoformans*. In this fungus, UDP-GlcA is required for the synthesis of capsule polysaccharides, which in turn are essential for virulence. The gene was expressed in *Escherichia coli* and the 51.3-kDa recombinant protein from wild-type and five mutants was purified for analysis. The cryptococcal enzyme is strongly inhibited by UDP-xylose and NADH, has highest activity at pH 7.5 and demonstrates K_m (app) values of 0.1 and 1.5 mM for NAD^+ and UDP-glucose respectively. Its activity was significantly decreased by mutations in the putative sites of NAD^+ and UDP-glucose binding. Unlike previously reported eukaryotic UDP-glucose dehydrogenases, which are hexamers, the cryptococcal enzyme is a dimer.

Key words: capsule, *Cryptococcus neoformans*, pathogenic fungi, UDP-GlcA, UDP-glucose dehydrogenase.

INTRODUCTION

UDP-glucose dehydrogenase (UDP-GlcDH) is a critically important enzyme throughout biology, both for its direct role in generating UDP-GlcA (UDP-glucuronic acid), and because this product can be decarboxylated to yield UDP-Xyl (UDP-xylose). The formation of UDP-GlcA is initiated by NAD^+-dependent oxidation of UDP-glucose (UDP-Glc), followed by nucleophilic attack of a catalytic cysteine on the resulting C_6 aldehyde, transfer of the remaining hydride to a second molecule of NAD^+ to form a thioester intermediate, and hydrolysis of this intermediate to form UDP-GlcA (literature recently reviewed in [1]). The resulting nucleotide sugar is utilized in processes ranging from capsule synthesis in bacteria and fungi to glycosaminoglycan synthesis and the hepatic glucuronidation of potential toxins in mammals. Additionally, UDP-GlcA may be converted into UDP-Xyl by UDP-GlcA decarboxylase [2]. UDP-Xyl is a key metabolite for the synthesis of structures including mammalian proteoglycans, plant xylans and xyloglucans and capsule polysaccharides in pathogenic fungi.

The enzyme responsible for UDP-GlcA synthesis was described and enriched from bovine liver in 1954 [3] and later purified from the same source [4], followed by extensive studies of its sequence [5–10] and unusual mechanism (literature reviewed in [1]). Crystal structure data from *Streptococcus pyogenes* further support the proposed mechanism of action, and demonstrate how a single active site can perform a 2-fold oxidation [1]. Many examples of the importance of UDP-GlcDH have been explored, including its role in normal embryonic development in *Drosophila* [7–9], *Caenorhabditis elegans* [11] and zebrafish [12], its differential expression in various plant tissues [13] and its requirement for virulence in bacterial systems [6,14,15].

Our interest in this enzyme stems from its role in providing biosynthetic precursors for the capsule of *Cryptococcus neoformans*, an opportunistic fungal pathogen [16,17]. This organism is distinguished among pathogenic yeasts by its polysaccharide capsule, an impressive structure that surrounds the cell wall and aids in thwarting the host immune response [18]. Although the capsule is not required for the growth of *C. neoformans* in culture, it is essential for its virulence. The more abundant of the two polysaccharides that comprise the capsule contains 15–20 % GlcA [19], depending on the specific strain, and both polymers are heavily xylosylated [17]. Thus UDP-GlcDH is required both directly and indirectly to generate capsule precursors, and capsule in cells without this activity, if made at all, should lack both GlcA and xylose. Furthermore, it has been shown that disruption of the gene encoding UDP-glucuronate decarboxylase (*UXS1*; [2]) results in cells with capsule that lacks xylose; these cells are completely avirulent [20]. To examine further the pathways required for the synthesis of capsular polysaccharides in *C. neoformans*, we have cloned, expressed and characterized the cryptococcal UDP-GlcDH.

EXPERIMENTAL

Cloning *UGD1*

Using TRIzol® reagent (Gibco BRL, Gaithersburg, MD, U.S.A.) as described in [2], total RNA was isolated from a 200 ml culture of *C. neoformans* Cap67 in YPD medium (10 g/l yeast extract, 20 g/l Bacto-peptone and 20 g/l dextrose). RNA was reverse-transcribed to cDNA using 1 mM Clamp-oligo(dT) primer (BD Biosciences Clontech, Palo Alto, CA, U.S.A.) and 200 units of Superscript II reverse transcriptase (Gibco BRL). The resulting product was incubated with 2 units of RNase H (Gibco BRL), and used as a template for PCR using high-fidelity Platinum *Taq* DNA polymerase (Gibco BRL), sense primer DH-7 (5′-GGGGATATCATATGCACCATCACCACCATCACGGATCCATGGCCCCCATCACTGTTAAGAAGATCTGC-3′) and antisense primer DH-1 (5′-CAGAAAACTAGCACGAATGTTGGG-3′). The product was gel-purified and cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA, U.S.A.). The coding region and 120 nt of the 3′-untranslated region were excised using *Nco*I and *Eco*RI,

Abbreviations used: DTT, dithiothreitol; UDP-Glc, UDP-glucose; UDP-GlcA, UDP-glucuronic acid; UDP-GlcDH, UDP-glucose dehydrogenase; UDP-Xyl, UDP-xylose.

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Table 1 Primers used to generate mutations to alanine at the indicated positions

All sequences are listed 5′ to 3′.

Mutant	5′-primer	3′-primer
C278A	GGCGTCTGTCGGTTTCGGTGGTAGCGCTTTCCAGAAGGACATTCTCAACTTGG	CCAAGTTGAGAATGTCCTTCTGGAAAGCGCTACCACCGAAACCGACAGACCC
R43A	GACCTCAACCAGCAGGCAATTCACGCCTGGAAC	GTTCCAGGCGTCAATTGCCTGCTGGTTGAGGTC
R262A	GTCGGTAAGGACACGGCAATGGGTTCCAAGTTCC	GGAACTTGGAACCCATTGCCGTGTCCTTACCGAC
K341A	CTTGGTTGGGCCTTCGCGAAGGACACTGGTGAC	GTCACCAGTGTCCTTCGCGAAGGCCCAACCAAG
R348A	GGACACTGGTGACACTGCAGAGTCTCCTTCCATCGGC	GCCGATGGAAGGAGACTCTGCAGTGTCACCAGTGTCC

purified, and subcloned into similarly restricted pET24d (Novagen, Madison, WI, U.S.A.) to form pDH11.1. A C-terminal His_6 tag was incorporated by PCR using DH-His-1 (5′-GAGTTC-CTTGCCGAGGGTACCGC-3′) and DH-His-2 (5′-CCCAAGCT-TGGGTTTTAATGATGATGATGATGATGAATACGGTCGCC-AGTACC-3′) primers and pDH11.1 as template DNA. The resulting PCR fragment was digested with *Hin*dIII and *Kpn*I and used to replace the corresponding fragment of pDH11.1, yielding pDH-His. DNA sequencing (Protein Nucleic Acid Chemistry Laboratory, Washington University School of Medicine, St. Louis, MO, U.S.A.) confirmed correct sequence in each construct, and the genomic and coding sequences were submitted to GenBank® (AAK95561).

Mutagenesis

QuikChange site-directed mutagenesis reagents (Stratagene, La Jolla, CA, U.S.A.) were used to mutate five residues to alanine. Residue positions and primers used are listed in Table 1, and all the mutations were confirmed by DNA sequencing.

Protein expression and purification

For expression of protein, either pDH-His or the empty pET24d vector was transformed into *Escherichia coli* strain BL21 Gold (DE3) pLysS (Stratagene). A single colony from each transformation was grown to an A_{600} of 0.5 at 37 °C in 500 ml Luria–Bertani containing 60 μg/ml kanamycin and 34 μg/ml chloramphenicol. The culture was transferred to 25 °C for 1 h and then induced with 0.1 mM isopropyl β-D-thiogalactoside at 25 °C overnight. Cells were collected by centrifugation, washed once with cold deionized water, resuspended in 15 ml of lysis buffer [50 mM Tris/HCl, pH 8.8/10 % (v/v) glycerol/1 mM EDTA/1 mM DTT (dithiothreitol)/0.5 mM PMSF] and stored at − 80 °C in 3 ml aliquots for convenient small-scale enzyme preparations. For use, one aliquot of cells was thawed, adjusted to 100 μg/ml lysozyme and supplemented with protease inhibitors (final concentrations of 5 μg/ml antipain, trypsin inhibitor and leupeptin; 1 μg/ml *o*-phenanthroline; 0.1 mM aminocaproic acid; 1.6 μg/ml benzamidine). The suspension was incubated for 30 min at 30 °C with vortex-mixing every 10 min, and sonicated with a microtip sonicator on ice for 3 × 1 min intervals alternating with 1 min rest. Whole cells and cell debris were pelleted (SS34 rotor; 13000 rev./min for 30 min at 4 °C), and the supernatant fraction was removed and immediately used for UDP-GlcDH assays or purification.

For purification, 1.5 ml of the supernatant fraction from sonication was mixed with an equal volume of binding buffer (50 mM NaH_2PO_4/300 mM NaCl, pH 7.0) and passed over a 2.5 ml column of TALON Superflow metal affinity resin (Clontech #8908-2), pre-equilibrated in the same buffer. The column was washed three times with 3 ml of binding buffer containing 10 mM imidazole, and proteins were eluted with 3 × 1 ml of binding buffer containing 250 mM imidazole. Most of the protein eluted in the second elution fraction. This was supplemented with 5 mM DTT and stored at 4 °C; the protein and activity were assayed within 48 h. Purity of this fraction was estimated to be > 95 %.

UDP-GlcDH assay

For assays of UDP-GlcDH, 29 μg of protein was incubated at 25 °C in a final volume of 50 μl of 40 mM Tris/HCl (pH 7.5), 2 mM pyruvate, 4.5 units of L-lactate dehydrogenase (Sigma) and the concentrations of NAD^+ and UDP-Glc indicated. In experiments to determine the K_m value for UDP-Glc, concentrations of up to 5 mM UDP-Glc were tested in the presence of 2 mM NAD^+. Under these conditions, < 30 % of the UDP-Glc was used in the 10 min incubation period. For experiments to determine the K_m value for NAD^+, concentrations of up to 2 mM NAD^+ were tested in the presence of UDP-Glc at levels 5–7-fold above the K_m value for the enzyme preparation being tested: 2 mM UDP-Glc for mutant enzymes and 10 mM UDP-Glc for wild-type enzyme. (Depending on the exact proportion of substrate used at each concentration, the K_m and k_{cat} values may be slightly overestimated.) Reactions were stopped by vortex-mixing with 50 μl of cold phenol (pH 4)/chloroform/deionized water (3:3:1, by vol.) followed by centrifugation (16000 ***g*** for 5 min at room temperature 25 °C). (Here and elsewhere deionized water indicates 18 MΩ water from a Millipore system.) A 25 μl portion of the upper phase was removed, and the lower phase was re-extracted with 80 μl of deionized water. An 80 μl portion of the second upper phase was combined with the initial 25 ml, and 80 μl of that mixture was analysed by HPLC (Waters, Milford, MA, U.S.A.) on a 250 mm × 4.6 mm quaternary amine-silica gel anion-exchange column (Hypersil 5 μ SAX gel; Phenomenex, Torrance, CA, U.S.A.) run at 1.5 ml/min. After injection, the column was washed for 5 min with buffer A (5 mM phosphate buffer, pH 3.2) and eluted with a 20 min linear gradient from 0 to 60 % buffer B (0.6 M KH_2PO_4) as described in [21].

Antibody production and immunoblotting

A large-scale preparation of UDP-GlcDH for antibody production was generated by first inducing a 0.5 litre culture ($A_{600} \sim 0.6$) of BL21(De3)pLysS cells harbouring pDH11.1 with 1 mM isopropyl β-D-thiogalactoside for 3 h at 30 °C. Cells were washed with water and resuspended in lysis buffer as described above. The entire quantity of cells was then lysed in a French Pressure cell (7.6×10^6 Pa). After two successive centrifugation steps (6000 and 20000 ***g***) the pellet, enriched with inclusion bodies, was solubilized in 1 ml of inclusion body extraction buffer (0.1 M Tris/HCl, pH 9.1/2 % sarcosyl/0.5 mM PMSF/2 mM DTT; 1 or 2 ml of initial packed cells) and incubated for 3 h at room temperature with slow rocking. The sample was then brought to 0.25 mM L-cystine from a 50 mM stock dissolved in 0.1 M NaOH

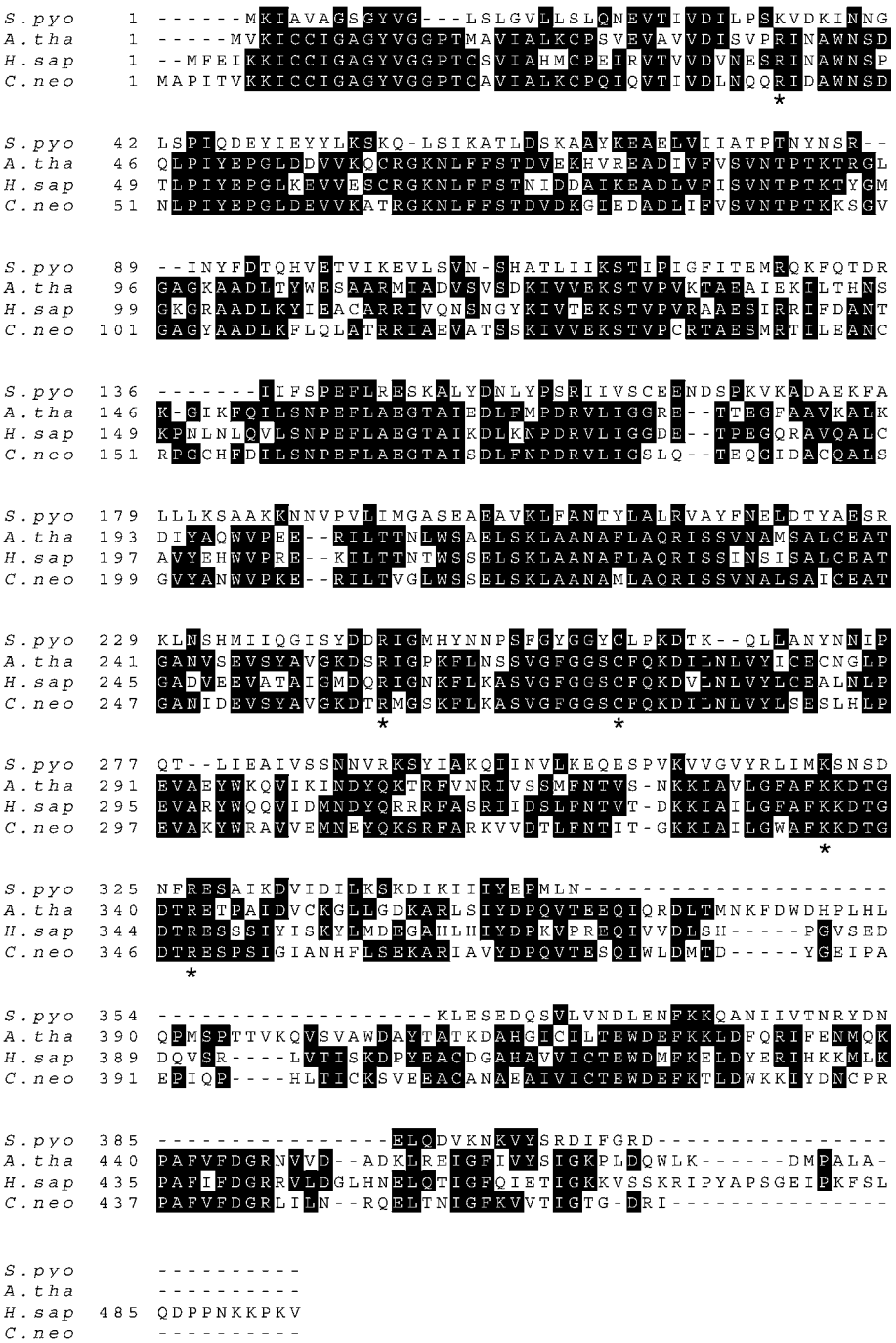

Figure 1 Alignment of sequences encoding UDP-GlcDH

An alignment of sequences from *S. pyogenes* (GenBank® accession number Q07172), *Arabidopsis thaliana* (NP_197053), *Homo sapiens* (AAC36095) and *C. neoformans* (AAK95561) is shown, with filled boxes indicating identical residues. Asterisks under the cryptococcal sequence indicate positions that were mutagenized to alanine.

and incubated for an additional 3.5 h. Protein was dialysed and concentrated by ultrafiltration (Millipore, Billerica, MA, U.S.A.) to 3 mg/ml for use in polyclonal antibody production in rabbits (Covance Research Products, Denver, PA, U.S.A.).

For immunoblotting, *C. neoformans*, *Tremella mesenterica* or *Saccharomyces cerevisiae* were resuspended in lysis buffer, mixed with an equal volume of glass beads (Biospec Products, Bartlesville, OK, U.S.A.) and vortex-mixed vigorously for 3 × 1 min, alternating with 1 min on ice. Total protein (10 mg) from fungal preparations or *E. coli* cells (expressing empty vector or pDH11.1) was mixed with an equal volume of 2× SDS/PAGE sample buffer and boiled (5 min) before subjecting to SDS/PAGE (12% gel). Immunoblotting was performed by standard methods using 1:5000 dilutions of both primary antiserum and secondary antibody (anti-rabbit IgG conjugated with horseradish peroxidase; Amersham Biosciences, Piscataway, NJ, U.S.A.), and the Western Lightning Chemiluminescence Reagent (Applied Biosystems, Foster City, CA, U.S.A.) for detection.

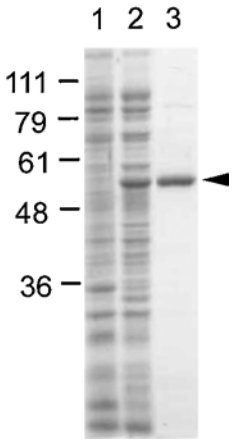

Figure 2 UDP-GlcDH expression and purification

Bacterial proteins analysed by SDS/PAGE and stained with Coomassie Blue. Standards (in kDa) are shown on the left. Lane 1, 20 μg of soluble protein from *E. coli* expressing plasmid only; lane 2, 20 μg of soluble protein from *E. coli* expressing His-tagged UDP-GlcDH; lane 3, 2 μg of UDP-GlcDH purified by cobalt chromatography. A minor contaminant is barely visible at approx. 90 kDa.

Gel filtration

For gel filtration, purified His-tagged protein at 0.4, 2 or 4 mg/ml was analysed on a Sephacryl S300 HR column equilibrated with 20 mM Tris/HCl (pH 7.0), 100 mM NaCl, 1 mM DTT and 10 % glycerol. The column was calibrated with samples from a molecular-mass standard kit (Sigma), run individually; elution volumes for the standards fit well ($R^2 = 0.99$) when plotted against molecular mass.

RESULTS AND DISCUSSION

Conserved peptide domains from plant UDP-GlcDH were used to identify the corresponding partial sequences in translations of *C. neoformans* expressed sequence tag and genomic databases. These sequences guided the primer design for amplification of a cryptococcal cDNA, which encoded a 51.3 kDa protein with extensive sequence homology with known UDP-GlcDH enzymes (Figure 1). Protein alignment indicated that critical amino acids, such as the catalytic cysteine (residue 278 in the *C. neoformans* sequence) and the N-terminal GXGXXG NAD^+-binding motif, were strictly conserved. Examination of the genomic DNA sequence from *C. neoformans* showed that the corresponding gene was composed of 15 exons. No other homologues of the protein were detected in cryptococcal genome sequence.

To facilitate purification of the putative UDP-GlcDH, the gene was modified to incorporate the sequence encoding a C-terminal His_6 tag. Comparison of enzyme activity in crude preparations of cells expressing either tagged or untagged protein showed no detectable effect of the His tag. For further studies, the recombinant protein was expressed at high levels in *E. coli* and purified on cobalt resin (Figure 2). In the presence of NAD^+, the purified protein readily converted UDP-Glc into UDP-GlcA as judged by an HPLC assay (Figure 3), and the gene encoding the enzyme was correspondingly named *UGD1* (for UDP-Glc dehydrogenase). Time-course experiments (results not shown) indicated that the pure enzyme was progressively inhibited by the accumulation of NADH formed during conversion of UDP-Glc into UDP-GlcA. To avoid this and to facilitate kinetic analysis, the assay was modified by the inclusion of pyruvate and lactate dehydrogenase to consume the NADH.

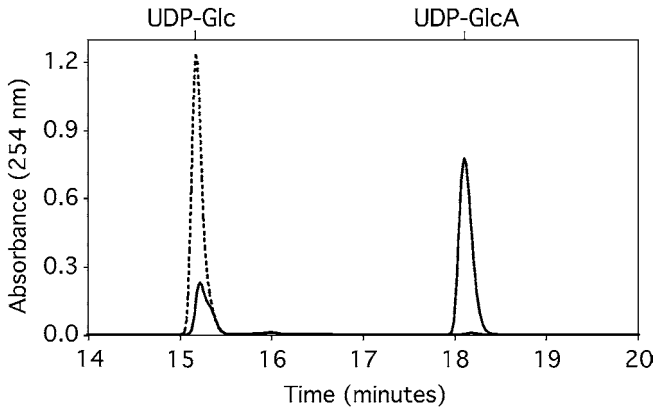

Figure 3 UDP-GlcDH activity of purified protein

Assays were performed and products were analysed by HPLC as described in the Experimental section using 1 mM UDP-Glc and 2 mM NAD^+. ----, 0 min incubation; —, 10 min incubation. Elution position of standards is indicated above the profile.

Table 2 Inhibitors of UDP-GlcDH

Assays were performed in the presence of the indicated compounds, as described in the Experimental section using 1 mM UDP-glucose. The amount of UDP-GlcA formed is expressed as a percentage of an assay with no additions. Results are expressed as means ± S.D. for triplicate samples and are representative for at least four independent experiments.

Addition	Concentration (mM)	% of control
0 time control	–	0
–	–	100 ± 4
NADH*	1	1.3 ± 0.2
UDP-Xyl	1	0.5 ± 0.1
UDP-GlcA†	1	75 ± 5
UDP	1	111 ± 2
UDP-Gal	1	98 ± 6
Mn^{2+}	1	95 ± 5
Mn^{2+}	10	19 ± 2
EDTA	1	110 ± 4

* Lactate dehydrogenase and pyruvate were omitted from this assay.
† The product peak in this reaction was corrected for the amount of UDP-GlcA added.

As tested by the modified assay, recombinant UDP-GlcDH had an optimum pH of 7.5, with a broad optimum temperature of 25–37 °C. Moderate inhibition of activity was observed in the presence of UDP-GlcA, the immediate product of the enzyme, but there was no inhibition by UDP, UTP or UDP-Gal. In contrast, the enzyme was strongly inhibited by NADH and by the downstream metabolite, UDP-Xyl (Table 2); these effects were similar to those observed in cytosolic extracts of *C. neoformans* [22]. Mn^{2+} at high concentrations was inhibitory, and EDTA slightly enhanced the activity, presumably by chelating bivalent cations.

Biochemical and biophysical studies from Feingold and others (reviewed in [1]) indicate that the functional unit of UDP-GlcDH is a dimer, a conclusion supported by X-ray structure analysis, which indicates that a residue required for the activity (Arg-262) actually occurs in a loop of the second molecule of the enzyme. However, in bacteria the dimer is the basic structural unit of the protein [1,23], whereas in the higher eukaryotes studied the protein is hexameric, occurring as a 'trimer of dimers' [24–27]. Gel-filtration studies of recombinant cryptococcal UDP-GlcDH at various concentrations showed that it elutes in all cases between the expected masses for a monomer and a dimer, with

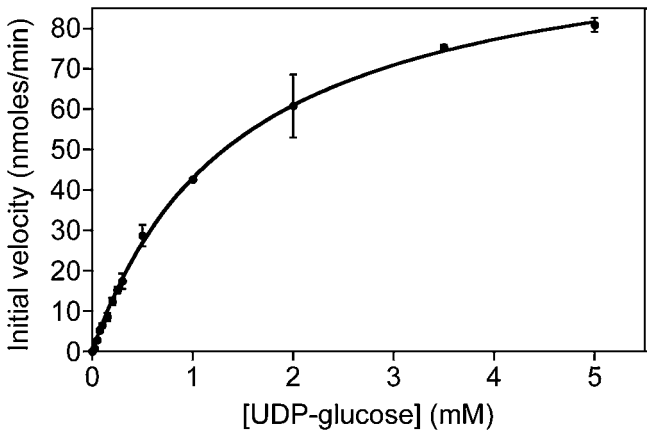

Figure 4 Cryptococcal UDP-GlcDH product formation

Assays were performed in the presence of 2 mM NAD^+ and the indicated concentrations of UDP-Glc. Results were analysed by non-linear least-squares curve-fitting using Kaleidograph software.

Table 3 K_m values of UDP-GlcDH from selected prokaryotic and eukaryotic organisms

	K_m (mM)		
Organism	UDP-Glc	NAD^+	Reference
C. neoformans	1.5 ± 0.1	0.1 ± 0.01	This study
E. coli	1	0.05	[32]
S. pyogenes	0.02	0.06	[33]
Saccharus spp.	0.02	0.07	[34]
Gallus gallus	0.5	0.9	[35]

Table 4 Kinetic parameters of mutant and wild-type UDP-GlcDH from *C. neoformans*

Equal amounts of recombinant protein were used in all assays. K_m values for UDP-Glc were determined in the presence of 2 mM NAD^+, with UDP-Glc varied from 0 to 5 mM; K_m for NAD^+ was determined in the presence of 10 mM UDP-Glc for wild-type and 2 mM UDP-Glc for mutants, with NAD varied from 0 to 2 mM. Results are expressed as means $\pm$ S.E.M.

		K_m (mM)	
Mutation	k_{cat} (s^{-1})	UDP-Glc	NAD^+
None	$2.7 \pm 0.1 \times 10^{-1}$	1.5 ± 0.1	0.1 ± 0.01
C278A	–	–	–
R43A	$5.5 \pm 0.2 \times 10^{-3}$	0.4 ± 0.03	0.8 ± 0.2
R348A	$2.8 \pm 0.1 \times 10^{-2}$	0.4 ± 0.04	0.4 ± 0.04
R262A	–	–	–
K341A	–	–	–

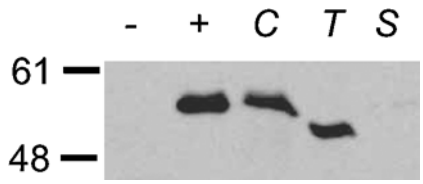

Figure 5 UDP-GlcDH is expressed in several basidiomycetes but not in *S. cerevisiae*

Total cell protein was separated by SDS/PAGE and immunoblotted with polyclonal anti-UDP-GlcDH as described in the Experimental section. –, *E. coli* expressing plasmid alone; +, *E. coli* expressing UDP-GlcDH; C, *C. neoformans*; T, *T. mesenterica*; S, *S. cerevisiae*. Standards (in kDa) are shown on the left. Regions of the gel not shown displayed no specific bands corresponding to UDP-GlcDH.

higher concentrations yielding elution volumes closer to the value predicted for a dimer (results not shown). In no case did we observe data consistent with a higher-order oligomer. The most probable interpretation of these results is that the fungal protein occurs in a monomer–dimer equilibrium, unlike the hexamers of other eukaryotic proteins studied.

To compare the cryptococcal enzyme with UDP-GlcDH of other organisms, we examined its kinetic properties (Figure 4). The K_m (app) of 1.5 mM for UDP-Glc was slightly higher than that reported for other organisms, whereas the K_m value for NAD^+ was in the middle of the reported range; in general, the enzyme appears to function at a similar range of concentrations (Table 3). These values were also close to those measured for this enzyme in the cytosol of *C. neoformans* [22].

To assess whether amino acids implicated in binding and catalysis by the known X-ray structure were critical for cryptococcal UDP-GlcDH activity, we generated site-specific mutations of the residues indicated in Figure 1, converting each into an alanine residue. Mutation of the catalytic Cys-278 yielded a completely non-functional enzyme, as expected (Table 4). Mutation of two residues that are suggested to bind the cofactor (Arg-43 and Arg-348) yielded severely impaired enzymes, with the expected increase in the K_m value for NAD^+. Mutation of two residues with roles in binding UDP-Glc (Arg-262 and Lys-341) caused an almost complete loss of activity. Residue 262, as mentioned above, occurs in a loop of one member of a dimer, yet contributes to the UDP-Glc-binding site of the second. The fact that mutating this amino acid abrogates activity of the protein supports the conclusion from gel-filtration studies that the functional unit of the cryptococcal enzyme is a dimer.

We used polyclonal antiserum raised against recombinant Ugd1p to investigate the protein expression in several fungi. UDP-GlcDH is clearly expressed in *C. neoformans* (Figure 5), and in *T. mesenterica*, a related non-pathogenic fungus, which produces a similar but more highly xylosylated capsule polysaccharide [28–30]. There was no evidence of cross-reacting material in the model yeast *S. cerevisiae*, consistent with the absence of any related protein sequence encoded by the *S. cerevisiae* genome. Wild-type *C. neoformans* cells were also grown under low-iron conditions that induce the formation of larger capsules [31], but there was no apparent alteration of protein expression (results not shown). Presumably, levels of UDP-Glc are sufficiently high to support increased levels of capsule synthesis without additional induction.

UDP-GlcDH in *C. neoformans* shares most of the characteristics of the enzymes from higher eukaryotes, in terms of mass and composition (e.g. amount of cysteine and tryptophan residues [23]). However, unlike the other eukaryotic enzymes that have been studied, it appears to function as a dimer rather than a hexamer. We expect that cryptococcal cells with impaired or absent UDP-GlcDH will be unable to generate UDP-GlcA and UDP-Xyl. The overall effects on cell physiology will depend on which cellular components are modified by these sugars, but the polysaccharide capsule will certainly be greatly altered, as it will lack both GlcA and xylose. On the basis of the results when only xylose is absent from the capsule [20], cells lacking UDP-GlcDH activity are probably avirulent. It remains to be seen whether the

difference between the cryptococcal enzyme and its counterpart in the mammalian host can be exploited for antifungal therapy.

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