Characterization of the 1,3- β -glucan synthase of *Aspergillus fumigatus*

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1,3- β -Glucan synthase activity has been detected in a membrane fraction extracted from the mycelium of the filamentous fungus *Aspergillus fumigatus*. The enzyme was solubilized by CHAPS and stabilized by filtration on a Bio-gel P30 column. Highest activity was obtained in the early exponential phase of growth. Four factors – GTP, NaF, sucrose and EDTA – added during the extraction procedure, were essential for optimal 1,3- β -glucan synthase activity. The soluble enzyme preparation was photolabelled with 5-azido-[³²P]UDP-glucose and 5-¹²⁵I-ASA-UDP-glucose which bind covalently to the enzyme after UV irradiation. These UDP-glucose substrate analogues were competitive inhibitors of the enzyme with a K_i of 1.42 mM and 0.3 mM for 5-azido-UDP-glucose and 5-ASA-UDP-glucose, respectively (K_m for UDP-glucose = 1.9 mM). Potential UDP-glucose-binding polypeptides were identified with molecular masses of 31, 50 and 115 kDa.

Introduction

1,3- β -Glucan is the main polysaccharide constituent of the cell wall of most fungal species (Wessels, 1986). Biosynthesis of fungal 1,3- β -glucan is generally believed to be mediated by membrane-bound glycosyltransferases which use UDP-glucose (UDP-Glc) as a substrate. Although a glucan synthase activity has been detected in several fungi (Larriba & Andaluz, 1991), a UDP-Glc-binding polypeptide responsible for the enzyme activity has not been isolated or even identified in the fungal kingdom.

Attempts to purify 1,3- β -glucan synthase using membrane-dissociating agents followed by chromatographic or electrophoretic procedures have been unsuccessful for several reasons. The half-life of the activity of membrane preparations at 4 °C does not usually exceed a few hours (Szaniszlo *et al.*, 1985). Moreover, glucan synthesis in fungi, as in plants, requires the participation of at least

one catalytic and one regulatory subunit. Separation of one subunit from the other one will result in the suppression of glucan synthase activity (Kang & Cabib, 1986).

Alternative approaches to purify the β -glucan synthase complexes have been reported. Differential centrifugation using glycerol or sucrose density gradients has been used with some success (Bulone *et al.*, 1990; Dhugga & Ray, 1991; Fink *et al.*, 1990; Kamat *et al.*, 1992). Electron microscopy and biochemical studies have demonstrated that the enzyme complex remains bound to the insoluble fibrils of glucan. These observations have led to another type of purification procedure based on a product entrapment method. Whereas this method has been particularly successful in the case of the 1,4- β -glucan synthase of *Acetobacter xylinum* (Lin *et al.*, 1990), only partial purification has been achieved in the case of fungal or plant 1,3- β -glucan synthase (Awald *et al.*, 1993; Bulone *et al.*, 1990; Frost *et al.*, 1990). Another approach has involved the use of polyclonal and monoclonal antibodies which have been most effective with peanut, *Lolium* and *Pisum* glucan synthases (Dhugga & Ray, 1991; Fink *et al.*, 1990; Kamat *et al.*, 1992; Meikle *et al.*, 1991).

Another strategy which has been developed is the use of affinity labelling with substrate analogues to identify the UDP-Glc-binding polypeptides in the synthase complex. The best probes developed to date are analogues of UDP-Glc in which the glucose residue is

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Abbreviations: ADP-Glc, adenosine diphosphoglucose; 5-ASA-UDP-Glc, 5(3-p) azido salicylamine allyl uridine diphosphoglucose; EB, glucan synthase extraction buffer; GDP-Glc, guanosine diphosphoglucose; GTP γ S, guanosine 5' (γ -thio) triphosphate; 5-N₃-UMP, 5-azido-uridine monophosphoglucose; 5-N₃-UDP-Glc, 5-azido-uridine diphosphoglucose; 5-N₃-UTP, 5-azido-uridine triphosphoglucose; RM, glucan synthase reaction mixture; UDP-Glc, uridine diphosphoglucose.

intact and the uridine residue is modified with a photoreactive group. The first of these probes to be used successfully was a 5-azido [^{32}P]UDP-Glc (5- N_3 -[^{32}P]UDP-Glc; Drake *et al.*, 1989). This compound has been used to label the catalytic subunit of plant and fungal 1,3- β -glucan synthases and of the bacterial 1,4- β -glucan synthase (Frost *et al.*, 1990, 1992; Lawson *et al.*, 1989; Lin *et al.*, 1990). More recently, K. Ng has synthesized a 5(3-p) ^{125}I azido salicylamide allyl UDP-Glc (5- ^{125}I ASA-UDP-Glc) which has been used in combination with monoclonal antibodies to identify a UDP-Glc-binding polypeptide present in the β -glucan synthase complex of *Lolium multiflorum* (Meikle *et al.*, 1991).

We report here the characterization and solubilization of the 1,3- β -glucan synthase of the fungus *Aspergillus fumigatus* and the identification of a UDP-Glc-binding polypeptide by photoaffinity using the two substrate analogues 5- N_3 -UDP-Glc and 5-ASA-UDP-Glc.

Methods

Culture conditions. *A. fumigatus* strain CBS 144-89 was maintained on 2% (w/v) malt (Touzaud & Matignon, Paris) agar slants. Erlenmeyer flasks containing 50 ml of Sabouraud liquid medium (2%, w/v, glucose and 1%, w/v, Mycoseptone; Biokar, Paris) were inoculated and shaken for 3 d at 180–200 r.p.m. at 25 °C. These precultures were then inoculated into a 2 litre Biolaftite fermenter containing 1.2 litres Sabouraud liquid medium. The culture conditions were as follows: temperature 25 °C, agitation 500 r.p.m., aeration 0.5 l air min $^{-1}$. After various growth times, the mycelium was collected by vacuum filtration on a Büchner funnel, extensively washed with water and dried under two pieces of filter paper.

Preparation of cell-free extracts. Mycelium was resuspended (0.25 g wet wt ml $^{-1}$) in 60 ml 1,3- β -glucan synthase extraction buffer containing 50 mM-Tris/HCl pH 7.8, 1 mM-EDTA, 0.2 M-NaF and 1 M-sucrose (EB). The homogenate was disrupted for 5 min in a CO $_2$ -cooled Braun MSK homogenizer with 1 mm glass beads. Complete breakage of the cells was assessed by phase contrast light microscopy. Cell walls were removed by centrifugation at 4000 g for 10 min. The membrane fraction was recovered after centrifugation at 33 000 g for 60 min. The membrane pellet was resuspended in solubilization buffer containing 4 ml EB with 12 mg CHAPS (Sigma) and 20 μM -GTP γS (Boehringer Mannheim). After 20 min at 0 °C, the extract was centrifuged at 33 000 g for 20 min and the supernatant was recovered and applied to a Bio-gel P30 column pre-equilibrated with EB containing 1.5 mg CHAPS ml $^{-1}$. GTP γS (20 μM) was added to the void volume from the column. Preliminary experiments have shown that P30 chromatography stabilized the enzyme preparation without altering its activity. This extract (P30 fraction) was used as the source of enzyme. All fractionation procedures were carried out at 4 °C. To avoid any loss in enzyme activity, all extracts were stored in liquid nitrogen.

The protein concentration of the extract was determined by the Coomassie brilliant blue method (Bradford, 1976), using a Bio-Rad kit and BSA as the standard.

1,3- β -Glucan synthase assay. P30 extract (40–170 μg protein) was added to the reaction mixture (RM) containing EB, 3 mM-cellobiose, 3 mM-glucose 6-phosphate, 3 mM-sodium tartrate, 0.1 mM-CaCl $_2$, 2 mg α amylase ml $^{-1}$ and 2.5 mM-UDP-Glc (containing 2.4 μM UDP [^{14}C]glucose, specific activity 293 nCi (10.8 kBq) nmol $^{-1}$, Amersham) in a

final volume of 25 μl . The assay was incubated for 30 min at 25 °C and the reaction was stopped by the addition of 50 μl ethanol. The resulting homogenate was filtered through a Whatman GF/C glass-fibre filter. The filter was then rinsed with 60 ml 60% (v/v) ethanol and placed in 4 ml of scintillation fluid BCS (Amersham). Radioactivity was estimated in a LKB liquid scintillation counter. The specific activity of the glucan synthase was expressed as nmol UDP-Glc incorporated min $^{-1}$ (mg protein) $^{-1}$.

Identification of 1,3- β -glucan synthase reaction product. The reaction product was characterized using an exo-1,3- β -glucanase [laminarinase from mollusc, 32 U (g protein) $^{-1}$, Sigma], and an α amylase (Sigma) as described previously (Beauvais & Latgé, 1989).

To elucidate the chemical nature of the linkages occurring between individual sugars, the reaction product was freeze-dried and incubated overnight at 105 °C in the presence of hydrazine (Pierce). The *de novo* synthesized polysaccharide was recovered by ethanol precipitation, freeze-dried and permethylated. After methanolysis, free hydroxyl groups were acetylated and the position in which sugar residues were glycosylated was identified by GLC (Montreuil *et al.*, 1986).

Characterization of 1,3- β -glucan synthase activity. Characterization of the glucan synthase was performed using P30 fractions from 17-h-old mycelium. The role of each compound of the extraction buffer (sucrose, NaF and EDTA) was determined by removing each of these compounds separately from EB and RM. Optimal NaF concentration was determined with P30 fractions and RM prepared with a range of NaF concentrations from 0 to 0.2 M. The influence of GTP was tested using a P30 fraction without addition of GTP γS .

K_m was determined by incubating in RM at 25 °C for 10 min with different concentrations of UDP-Glc ranging from 0.25 mM to 1 mM. Competition experiments by substrate analogues were performed by adding 0.34–0.68 mM-5-ASA-UDP-Glc or 5- N_3 -UDP-Glc to RM. Reactions in the presence of 5-ASA-UDP-Glc were carried out in darkness.

The role of uridine, uridine mono-, di-, or triphosphate and inorganic pyrophosphate on glucan synthase activity was assessed by addition of these compounds at 2.5 mM and 5 mM in RM. The specificity of UDP-Glc as a substrate for glucan synthesis was investigated by adding ADP-Glc or GDP-Glc in RM containing 2.5 μM UDP-[^{14}C]glucose in the absence of non-radioactive UDP-Glc.

Photoaffinity labelling. 5- N_3 -[^{32}P]UDP-Glc was synthesized as described by Rudominski & Drake (1993) by a procedure slightly modified from Drake *et al.* (1989).

5-ASA-UDP-Glc was synthesized as previously described (Meikle *et al.*, 1991). After iodination, mono and di-iodo derivatives were separated from 5-ASA-UDP-Glc by a modified procedure (K. Ng, unpublished) on PEI cellulose thin-layer chromatography developed with 1 M-NH $_4\text{HCO}_3$. The bands of PEI-cellulose corresponding to the labelled products (visualized by autoradiography) were recovered separately and eluted with 1 M-NH $_4\text{HCO}_3$. The NH $_4\text{HCO}_3$ was removed by repeated lyophilization.

The reaction mixture contained a total volume of 50 μl with the probe and 40 to 150 μg protein (P30 fraction from 17-h-old mycelium) in a buffer with 50 mM-Tris/HCl pH 7.8, 1 mM-EDTA, 200 mM-NaF, 1 M-sucrose, 10 mM-glucose 6-phosphate and 2 mM-cellobiose. 5- N_3 -[^{32}P]UDP-Glc was added at 50 μM final concentration whereas 5- ^{125}I ASA-UDP-Glc was added at 5 μM final concentration in the presence of 5 mM- β -mercaptoethanol.

The reaction conditions for 5- N_3 -[^{32}P]UDP-Glc were incubation for 30 s and UV irradiation for 90 s at 4 °C or room temperature and for 5- ^{125}I ASA-UDP-Glc incubation for 60 s and irradiation for 5 min at room temperature in the dark. A hand-held UV lamp was placed 2 cm above the sample. A wavelength of 254 nm is needed for 5- N_3 -UDP-Glc whereas similar labelling was observed with 5-ASA-UDP-Glc at

254, 312 or 356 nm. Control labelling reactions were performed in the absence of UV irradiation or with probes pre-irradiated before addition to the reaction mixture. Reaction was terminated by the addition of 250 μ l 10% (w/v) TCA. After 15 min at 4 °C, the mixture was centrifuged at 13000 r.p.m. for 5 min and the supernatant was carefully removed. The pellet was resuspended in a sample buffer containing 0.25% (w/v) Tris/HCl pH 8.0, 3.72 M-urea, 2.5% (w/v) DTT and 5% (w/v) SDS. Electrophoresis was performed with 7.5 or 10% (w/v) acrylamide gels according to Laemmli (1970). Labelled polypeptides were visualized by autoradiography at -80 °C using Kodak X-Omat-AR or K film with a Cronex intensifying screen. Radioactivity was scanned using a Masterscan (Scanalytics).

The specificity of the photolabelling with the 5-N₃-UDP-Glc and 5-ASA-UDP-Glc probes was verified using two partially purified UDP-Glc utilizing enzymes obtained from Sigma: UDP-glucose pyrophosphorylase and phosphorylase a. Labelling of unique polypeptides of 48 and 97 kDa, respectively, was observed with these two enzymes. No labelling was observed in the absence of UV irradiation. In the presence of UDP-Glc, photoincorporation was decreased.

Results

Characterization of the reaction product of the 1,3- β -glucan synthase assay

The glucan synthase reaction product was completely digested by laminarinase, but not degraded by α -amylase. When the product of laminarinase digestion (3 h at 37 °C) was run on silica gel plates, 64% of the solubilized radioactivity corresponded to glucose and 36% to laminaribiose. After methanolysis of the permethylated

glucan purified after hydrazinolysis of the reaction product, only 1,3-acetyl 2,4,6-methyl glucosides were identified by GLC. These results demonstrated that linear chains of 1,3- β -glucan were the only polysaccharide synthesized by the P30 extract.

Characterization of the glucan synthase of *A. fumigatus*

Fig. 1 shows that the glucan synthase activity [14 nmol UDP-Glc min⁻¹ (mg protein)⁻¹] was highest at the first time point (17-h-old culture) and declined thereafter. At 36 h, 48 h (maximal mycelial growth) and 120 h of culture, the activity was respectively 80%, 65% and 3% of that detected at 17 h.

Incubation of membrane extracts with 0.3% CHAPS resulted in the solubilization of about 70% of the total proteins. In our extraction procedure, CHAPS-solubilized extracts contained 2–4 mg protein ml⁻¹. Increasing the solubilization time or the CHAPS concentration did not enhance protein solubilization. Moreover, CHAPS concentrations higher than 3 mg ml⁻¹ caused a decrease in the glucan synthase activity. Before elution through the Bio-gel P30 column, the solubilized enzyme preparation was very unstable: 26% of the activity was lost after 20 min at 24 °C. In contrast, the fraction eluted in the void volume of the Bio-gel P30 column could withstand several freezing and thawing cycles without any loss of activity. A proportion (50–70%) of the activity was recovered after storage of the P30 fraction for 16 h at 4 °C. The glucan synthase activity of the P30 fraction reached 50–70% of the activity of the membrane extract recovered after the 33000 g centrifugation in the absence of any CHAPS treatment. P30 chromatography increased the activity of the CHAPS-solubilized extract since the activity of the fraction eluted in the void volume of the Bio-gel P30 column is 130% of the activity of the CHAPS extract loaded into the column. The presence of sucrose, NaF and EDTA in the extraction buffer was essential. Absence of sucrose or NaF in EB resulted in 80% and 72% loss of activity, respectively (Fig. 2). Highest activity was obtained with 0.1–0.2 M-NaF. Removal of EDTA from EB caused 46% reduction in the glucan synthase activity (Fig. 2). Solubilization of the membrane proteins in the absence of GTP γ S resulted in a 40% decrease in the glucan synthase activity (Fig. 2).

Inorganic pyrophosphate (PP_i) and uridine phosphate esters such as UTP, UDP or UMP added to the reaction mixture significantly reduced glucan synthase activity (Fig. 2). Uridine itself did not affect the enzyme activity (data not shown). At 2.5 mM, PP_i was the most effective inhibitor of glucan synthase. Inhibition levels observed with UMP, UDP and UTP were similar to other nucleotides such as ATP, ADP and GDP (data not shown). However, no incorporation of glucose into

Fig. 1. Glucan synthase activity (□) during mycelial growth (■). The enzyme preparation (P30 fraction) was extracted after 17, 36, 48, 72 and 120 h of culture growth and incubated in RM at 24 °C for 30 min (P30 fraction: 100 μ g protein).

Fig. 2. Effect of various chemicals on the activity of *A. fumigatus* glucan synthase. Activity is expressed as percentage incorporation of [14 C]glucose into 1,3- β -glucans. Control in RM displaying 100% activity [$27 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$].

Fig. 3. Competitive inhibition of β -glucan synthase activity by 5-ASA-UDP-Glc and 5- N_3 -UDP-Glc. The value for initial velocities at varying UDP-Glc (0.25–1 mM, □), 5-ASA-UDP-Glc (0.34 mM, ○; 0.68 mM, ▽), 5- N_3 -UDP-Glc (0.34 mM, ◇; 0.68 mM, △) concentrations were obtained in a reaction mixture containing the P30 fraction (169 μg protein) and 2.4 μM -UDP-[14 C]Glc. Only reactions using 5-ASA-UDP-Glc were carried out in the dark.

glucans, nor an inhibition of synthesis were obtained using ADP-Glc or GDP-Glc instead of UDP-Glc.

Glucan synthase incorporated glucose from UDP-Glc into acid-insoluble 1,3- β -glucan in an approximately linear fashion at 24 °C. The apparent K_m for UDP-Glc in the standard enzyme preparation, determined by using Michaelis–Menten plots, was 1.92 mM (Fig. 3). The substrate analogues 5-ASA-UDP-Glc and 5- N_3 -UDP-Glc inhibited the activity in a competitive way (Fig. 3); the slope of double-reciprocal plots increased with

Fig. 4. Coomassie blue-stained 10% gel (lane 1) and its corresponding autoradiographs (lanes 2–4) of the P30 fraction (40 μg protein per lane). Lanes 2 and 3, photolabelling using 5- N_3 -[^{32}P]UDP-Glc. Lane 4: photolabelling using 5- ^{125}I ASA-UDP-Glc. The reaction mixture not illuminated with UV light is shown in lane 2.

increasing concentration of the analogues without change in V_{max} . The apparent K_i was 0.3 mM for 5-ASA-UDP-Glc which was five times more effective than 5- N_3 -UDP-Glc ($K_i = 1.42 \text{ mM}$) (Fig. 3). The affinity of the enzyme for 5-ASA-UDP-Glc was 5 times higher than for 5- N_3 -UDP-Glc.

Photolabelling of the P30 fraction

Fig. 4 shows representative autoradiographs of the CHAPS-solubilized (P30) membrane polypeptides labelled by the two probes 5- N_3 -[^{32}P]UDPG and 5- ^{125}I ASA-UDP-Glc.

Two polypeptides with molecular masses of 31 kDa and 115 kDa were consistently labelled with 5- N_3 -[^{32}P]UDP-Glc. Labelling of the other polypeptides, especially the 50 kDa polypeptide, was not seen in all experiments. One of the labelled polypeptides (64 kDa) is attributed to phosphoglucomutase (see Discussion). The reasons for this variability in the labelling pattern were not explored.

The 31 kDa and 50 kDa polypeptides were most prominently labelled with 5- ^{125}I ASA-UDP-Glc (Fig. 4). The labelled protein at 35 kDa was not always observed in other labelling experiments. In contrast to photolabelling with 5- N_3 -[^{32}P]UDP-Glc, the 64 kDa and 115 kDa polypeptides were not labelled with 5- ^{125}I ASA-

Fig. 5. Inhibition of photolabelling with 5-N₃-[³²P]UDP-Glc (a) and 5-[¹²⁵I]ASA-UDP-Glc (b). P30 fraction (17-h-old mycelium) was photolabelled in the presence of increasing UDP-Glc concentrations (0 mM, lanes a1, b1; 6 mM, lane a2; 12 mM, lanes a3, b2), or in the presence (lanes a4, b1) or absence of GTP γ S (lanes a5 and b3). Lane a6, P30 fraction from a 5-d-old mycelium.

UDP-Glc. All bands labelled with the probes could be identified in a protein-stained acrylamide gel (Fig. 4). In P30 fractions with low glucan synthase activity (5-d-old mycelium), the 31 and 50 kDa bands appeared faint in protein-stained gels and were not labelled by both probes. In contrast, labelling could be seen at the level of the 115 kDa band (Fig. 5).

Competition experiments showed that in the presence of increasing concentrations of UDP-Glc in the photolabelling reaction mixture, the intensity of all labelled bands was inhibited with the two probes but was never totally abolished even at 12 mM-UDP-Glc (Fig. 5). The inhibition level was calculated by scanning the bands labelled by the two radioactive probes. In the presence of 6 mM-UDP-Glc, the labelling of the 31, 50 and 115 kDa bands detected by 5-N₃-[³²P]UDP-Glc was inhibited by 41, 31 and 25 %, respectively. After addition of 12 mM-UDP-Glc, inhibition reached 66, 64 and 100 %, respectively, for the 31, 50 and 115 kDa bands. After addition of 5-ASA-UDP-Glc (0.05 mM) the labelling of all bands was completely abolished (data not shown). After addition of 12 mM-UDP-Glc, the labelling of the 31 and 50 kDa polypeptides identified by 5-[¹²⁵I]ASA-UDP-Glc was inhibited by 25 and 43 %, respectively.

These results confirmed the inhibition kinetics studies (Fig. 3) and showed that 5-ASA-UDP-Glc has a higher affinity for the glucan synthase than the substrate UDP-Glc and its analogue 5-N₃-UDP-Glc.

In absence of GTP γ S the labelling of the 31, 50 and 115 kDa bands with 5-N₃-[³²P]UDP-Glc and of the 31 and 50 kDa bands with 5-[¹²⁵I]ASA-UDP-Glc was selectively inhibited (Fig. 5). These extracts had a low glucan synthase activity (Fig. 2). In contrast, the presence of non-competitive inhibitors of enzyme activity like ciclofungin or NaCl, did not decrease photolabelling (data not shown).

Discussion

1,3- β -Glucan synthase activity has been detected in plants and fungi. In plants, glucan synthase is a repair enzyme which is activated under condition of stress such as mechanical damage or pathogen invasion (Delmer *et al.*, 1991). In fungi, it is responsible for the synthesis of the main constituent of the fungal wall and is essential for growth. Both plant and fungal enzymes are membranous and can be solubilized by detergents such as digitonin, octylglucoside or CHAPS (Fevre, 1979; Frost

et al., 1992; Hayashi *et al.*, 1987; Quigley *et al.*, 1988; Lawson *et al.*, 1989). No glucan synthase has been isolated until now.

In *A. fumigatus*, five factors appear to be essential for an optimal glucan synthase activity, namely the age of the culture, GTP, NaF, sucrose and EDTA. Ageing of the mycelium could be accompanied by an increase in the fragility of the membranes or in the amount or specificity of cytosolic proteases which could perturb intact glucan synthase recovery after cell breakage. However, a decrease of glucan synthase activity during growth is not usually the rule amongst fungi. For example, the enzyme activity of CHAPS-solubilized extracts from the zygomycetous *Entomophaga aulicae* increases during the mycelial growth phase (A. Beauvais, unpublished data).

The requirement of GTP for maximal activity is a common property of this fungal enzyme which has been studied in several species belonging to different taxonomic groups (Cabib & Kang, 1987; Szaniszlo *et al.*, 1985). The results obtained with other fungi suggest that the glucan synthase complex consists of at least two subunits: a regulatory GTP-binding subunit and a catalytic UDP-Glc-binding subunit (Kang & Cabib, 1986). In *A. fumigatus* glucan synthase biochemical studies and labelling experiments with substrate analogues confirmed the essential role of GTP: the selective inhibition of the photolabelling due to the removal of GTP γ S would suggest that GTP mediates UDP-Glc binding in the catalytic subunit. The roles of osmotic stabilizer (sucrose or glycerol), phosphatase inhibitor (NaF), disaccharide (cellobiose) or metal chelators (EDTA) vary between membrane preparations of the different fungal species studied (Beauvais & Latgé, 1989; Cabib & Kang, 1987; Cerenius & Söderhäll, 1984; Larriba *et al.*, 1981; Orlean & Ward, 1983; Quigley *et al.*, 1988). However, in the absence of any purified enzyme, the role of all these additives cannot be precisely assessed.

Although both plant and fungal 1,3- β -glucan synthases have not been sufficiently characterized, these enzymes seem to belong to different protein families, due to differences in cofactor or activator requirements. In contrast to fungal enzymes, plant enzymes require divalent Ca²⁺ and Mg²⁺ but do not need GTP for activation. Furfuryl glucoside, which stimulates the glucan synthase of mung bean at least 20-fold (Ohana *et al.*, 1992), has a very low effect (29%) on *A. fumigatus* glucan synthase (data not shown).

Because of the instability of all glucan synthase preparations and the necessity of a probable multimeric complex to obtain the activity, the use of UDP-Glc analogues able to bind covalently to the UDP-Glc-binding polypeptides seems to be the best chemical approach to identify the catalytic subunit of glucan synthase. Amongst all probes used to label plant or

bacterial 1,3- β - and 1,4- β -glucan synthases, three substrate analogues [³²P]UDP-Glc, 5-N₃-[³²P]UDP-Glc, and 5-¹²⁵IASA-UDP-Glc seem the most appropriate markers of the UDP-Glc binding domain. All these probes apparently form covalent bonds with amino acids at the binding site after UV irradiation. The exact mechanism of photolabelling with [³²P]UDP-Glc is not clear. A longer UV exposure time (20 min) is required than with azidoanalogues (1–5 min). It is suggested that upon irradiation, free radicals can be generated from the uracil moiety of UDP-Glc or from an aromatic amino acid in the active site of the protein to form covalent bonds between UDP-Glc and UDP-Glc-binding polypeptide (Delmer *et al.*, 1991).

The photoreactivity of 5-N₃-UDP-Glc and 5-ASA-UDP-Glc is due to the azido function which forms a highly reactive short lived nitrene intermediate upon UV irradiation. In the case of *A. fumigatus* glucan synthase, the *K_i* value for 5-ASA-UDP-Glc was lower than that obtained with 5-N₃-UDP-Glc. These results are in agreement with previous studies (Frost *et al.*, 1990; Meikle *et al.*, 1991) and confirm that the affinity of the substrate analogue for its enzyme site is directly dependent upon the hydrophobicity of the probe. However, the binding is due not just to the hydrophobicity of the analogue but also to a direct interaction of the ASA group on the uridine C-5 position with an amino acid on the binding site (K. Ng, unpublished data). Our comparative study with 5-N₃-UDP-Glc and 5-ASA-UDP-Glc on the same enzyme preparation confirms that both substrate analogues can compete efficiently for the same enzymic site. Nevertheless, the use of 5-ASA-UDP-Glc requires the addition of 5 mM-mercaptoethanol to avoid a non-specific labelling of numerous proteins (data not shown). The highly nucleophilic thiol group of mercaptoethanol apparently forms unreactive photolysed products with intermediate long-lived reactive species produced by irradiation of 5-ASA-UDP-Glc which are responsible for non-specific binding of proteins (Meikle *et al.*, 1991).

In some experiments with 5-N₃-[³²P]UDP-Glc, a polypeptide of 64 kDa was labelled. This band was mostly seen in the absence of glucose 6-phosphate and also with pre-irradiated probe. This was consistent with the property of the phosphoglucomutase (Drake *et al.*, 1989). Labelling of proteins with a similar apparent molecular mass has also been detected in other plant systems (Lawson *et al.*, 1989).

When the P30 fraction was incubated with 5-N₃-[³²P]UDP-Glc or 5-¹²⁵IASA-UDP-Glc in the presence of increasing concentrations of unlabelled UDP-Glc, photolabelling of all polypeptides decreased. However complete inhibition was not obtained even at 12 mM-UDP-Glc. Unfortunately, a concentration of NDP sugars

above 12 mM would have interfered with UV light penetration. For example, high levels of uridine (100 mM) which do not affect glucan synthase activity reduced the photolabelling obtained with both probes (data not shown). Using the same 5-N₃-[³²P]UDP-Glc probe, Frost *et al.* (1990) have seen only a partial abolition of the labelling of the *Beta vulgaris* callose synthase in the presence of UDP-Glc. For the probe 5-ASA-UDP-Glc, the *K_i* was sixfold lower (and even more for the iodinated 5-ASA-UDP-Glc), than the *K_m* for UDP-Glc. Thus a complete inhibition would not be expected at these UDP-Glc concentrations. Similar results have also been reported by Meikle *et al.* (1991) with the same probe and *Lolium multiflorum* glucan synthase.

UDP, UMP, UTP and, in the case of 5-ASA-UDP-Glc, PPi were also good inhibitors of the photolabelling (data not shown). All these compounds inhibited the glucan synthase activity of *A. fumigatus* to differing extents. Inhibition of other UDP-Glc-binding polypeptides and of UDP-Glc pyrophosphorylase with these compounds has been reported previously (Drake *et al.*, 1989; Meikle *et al.*, 1991). In contrast, glucose does not have any inhibitory effect. These results suggest that UDP-Glc-binding polypeptides recognize the phosphorylated uridine part of the UDP-Glc molecule.

In the plant kingdom, polypeptides labelled with UDP-Glc analogues or with antibodies belong to two families of protein with molecular masses in the range of 30 and 55 kDa. The 30 kDa family includes the 30 and 31 kDa proteins of Italian ryegrass, a 31 kDa protein from soybean and a 34 kDa protein from cotton (Meikle *et al.*, 1991; Fink *et al.*, 1990; Delmer *et al.*, 1991). The 55 kDa family contains a doublet with molecular mass of 54 and 58 kDa in Italian ryegrass, a 57 kDa protein in both carrot and red beet, a 52 kDa protein in cotton and a 55 kDa protein from pea (Delmer *et al.*, 1991; Dhugga & Ray, 1991; Frost *et al.*, 1990; Lawson *et al.*, 1989; Meikle *et al.*, 1991). Two proteins labelled from *A. fumigatus* of 31 and 50 kDa are in the same molecular mass range. As in cotton (Delmer *et al.*, 1991) some variable labelling of the 50 kDa protein in *A. fumigatus* was obtained (Fig. 4). Frost *et al.* (1990) showed that the photolabelling of callose synthase UDP-Glc-binding polypeptides from *Beta vulgaris* by 5-N₃-[³²P]UDP-Glc was greatly influenced by pH. A similar result has been observed in the *Lolium* system using 5-ASA-UDP-Glc; the binding of the probe and catalysis had different pH optima (5.5 and 7.5 respectively) (K. Ng, unpublished data). These results indicated that slight variations in extraction procedure or reaction conditions may be responsible for different photoinjection results.

UDP-Glc-binding proteins with higher molecular masses (83–84 kDa) have been also identified in cotton and in the cellulose synthesizing bacterium *Acetobacter*

xylinum (Delmer *et al.*, 1991; Lin *et al.*, 1990). A high molecular mass (115 kDa) band in *A. fumigatus* membrane extracts was also labelled. Thus UDP-Glc analogues usually label more than one protein in fungal or plant membrane preparations. This is in agreement with the presence of several subunits with different functions in 1,3- β - or 1,4- β -glucan synthase complexes. However further purification will be required together with photolabelling to identify the catalytic subunit of the *A. fumigatus* glucan synthase since other UDP-Glc-binding proteins could be present in the membrane preparations. The use of two structurally different analogues which detect a set of similar proteins (31 kDa, 50 kDa) highlights the usefulness of probing our impure enzyme with different photoreactive analogues of UDP-Glc. Moreover, product entrapment or differential centrifugation using sucrose density gradients did not increase the intensity of any bands after Coomassie staining whereas it increased glucan synthase specific activity (data not shown). The labelled proteins can now be electroeluted and sequenced, so that the amino acid sequence data can be checked against the databank of proteins to see if they are unique and if they are a good candidate for the catalytic subunit of 1,3- β -glucan synthase.

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