***O*-Glycosylation of Proteins by Membrane Fractions of *Trichoderma reesei* QM 9414**

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In order to investigate *O*-glycosylation of proteins in the fungus *Trichoderma reesei* QM 9414, a membrane preparation was isolated and used to study the glycosylation of endogenous proteins. Exogenously added GDP-[U-¹⁴C]mannose was used to mannosylate both endogenous lipid and protein. The kinetics of mannosylation together with pulse-chase experiments with cold GDP-mannose revealed that lipid was labelled before protein. The lipid was identified as mannosyl phosphoryl dolichol (Dol-P-Man) by TLC together with an authentic standard from yeast. Addition of tsushimycin, a specific inhibitor of Dol-P-Man synthesis, completely blocked transfer of mannose from GDP-[U-¹⁴C]mannose to endogenous lipid. The mannosyl units transferred to endogenous protein could be released by β -elimination, and were shown to consist mainly of tetra-, di- and monomannosyl chains. Mannosylation of endogenous proteins occurred at a lower rate with membranes isolated from glycerol-grown cells. This could be overcome by addition of cold GDP-mannose, suggesting a limitation of endogenous GDP-mannose and/or dolichol phosphate in glycerol-grown (i.e. catabolite-repressed) cells.

INTRODUCTION

Trichoderma reesei is a filamentous fungus which is of considerable interest because of its ability to secrete various proteins in high amounts and at a high rate (i.e. cellulases and hemicellulases; Mandels, 1985). The biochemistry of its secretory pathway, however, is still poorly defined, and little is known about control of secretion. Analysis of enzymes and their genes clearly established that all cellulases so far characterized (cellobiohydrolase I and II, endoglucanase I and III) contain both *N*- as well as *O*-linked carbohydrate (Gum & Brown, 1976; Fägelstam *et al.*, 1984; Salovuori *et al.*, 1987; Enari & Niku-Paavola, 1987; Knowles *et al.*, 1987). Merivuori *et al.* (1985) reported that *N*-glycosylation is not required for secretion of proteins by *T. reesei*, but adds to their stability. Kubicek *et al.* (1987) reported that *O*-linked, but not *N*-linked glycosylation is obligatory for secretion of endoglucanases by *T. reesei*. This was further supported by Messner & Kubicek (1988), by studying the kinetics of accumulation of endoglucanase precursor proteins inside *T. reesei* prior to secretion. Furthermore, *O*-linked carbohydrate may be involved in binding of cellulases to cellulose (Van Tilbeurgh *et al.*, 1986; Tomme *et al.*, 1988).

The pathway of *O*-glycosylation in yeast and other fungi is unique among eukaryotes as it involves dolichol phosphate (Dol-P) and occurs at the endoplasmic reticulum (Tanner & Lehle,

Abbreviations: Dol-P, dolichol phosphate; Dol-P-Man, mannosyl phosphoryl dolichol.

1987). Only a few filamentous fungi have, however, been investigated in this respect (Letoublon & Got, 1974; Gold & Hahn, 1976; Gander *et al.*, 1977; Soliday & Kolattokudy, 1979). In the present paper we describe the introduction of *O*-glycosidically linked mannosyl units into proteins by a membrane fraction of *T. reesei* QM 9414.

METHODS

Organism and growth conditions. *Trichoderma reesei* QM 9414 was maintained on malt agar slants. Inocula were prepared by harvesting 14-d-old conidia in sterile tap water containing 0.1% (w/v) Tween 80. The conidia were added to the medium to give a final concentration of 10^8 conidia l^{-1} . The fungus was grown in shake flasks on a rotary shaker (250 r.p.m.) at 28 °C in the medium described by Mandels & Andreotti (1978), except that the carbon source (glycerol or lactose, as indicated) was used at 0.5% (w/v) and the pH of the medium was kept at 5.0 with phosphate/citrate buffer as described by Labudova & Farkas (1983). Samples (200 ml) of this medium were added to 1 litre wide-mouthed Erlenmeyer flasks.

Cell fractionation. After 22 h of growth, the contents from five flasks were harvested by suction filtration; the mycelia (8–12 g wet weight) were suspended in 150 ml 50 mM-Tris/HCl buffer, containing 15 mM-MgCl₂ and 9 mM-2-mercaptoethanol, pH 7.4, and then homogenized in a Beadbeater with glass beads of 0.5 mm diameter (Biospec Products) as described by Cramer *et al.* (1983). The homogenate was centrifuged at 1000 *g* for 10 min, 14000 *g* for 20 min and then at 40000 *g* for 20 min to yield a membrane fraction. The 40000 *g* pellet was resuspended in homogenization buffer (see above), and was used in all further experiments. Marker enzymes (vanadate sensitive and insensitive ATPase, glucose-6-phosphatase and cytochrome *c* reductase) were assayed as described by Borgeson & Bowman (1983). Protein was determined according to Bradford (1976).

Incorporation of [¹⁴C]mannose into Dol-P. Mannosyl transfer from GDP-[U-¹⁴C]mannose to endogenous Dol-P was measured in a 40 µl reaction mixture containing 50 mM-Tris/HCl, pH 7.4, 125 mM-MgCl₂ (or MnCl₂, where indicated), 70000 c.p.m. GDP-[U-¹⁴C]mannose [specific activity 146 mCi mmol⁻¹ (5.4 GBq mmol⁻¹)], 0.125% (w/v) Triton X-100 and 0.15–0.6 mg enzyme protein. After incubation at 30 °C for 60 min (unless otherwise indicated) the reaction was stopped by the addition of 2 ml chloroform/methanol (3:2, v/v). Formation of Dol-P-Man was measured as described by Sharma *et al.* (1974).

Characterization of the mannosyl lipid. The chloroform/methanol extract was subjected to mild alkaline hydrolysis (see below) and the remaining lipid fraction chromatographed on a DEAE-Sephadex column (0.8 × 10 cm) as follows. After applying the sample, the column was washed with 10 ml each of: (a) chloroform/methanol (2:1, v/v); (b) 10 mM-ammonium acetate in (a); (c) 50 mM-ammonium acetate in (a); (d) 100 mM-ammonium acetate in (a). The desired fractions were evaporated to dryness, the residue was dissolved in chloroform, and ammonium acetate was removed by extraction with water. Fractions containing radioactivity were subjected to TLC on Silica Gel G plates using either chloroform/methanol/water (65:25:4, by vol.) (solvent A) or di-isobutyl ketone/acetic acid/water (60:45:6, by vol.) (solvent B) as mobile phases. Radioactive material was localized by autoradiography, and compared with authentic standards.

Transfer of mannosyl residues from [U-¹⁴C]mannosyl phosphoryl dolichol to endogenous protein. [U-¹⁴C]Mannosyl phosphoryl dolichol (Dol-P-Man) (50000 c.p.m.) was dried under nitrogen, resuspended in 30 mM-Tris/HCl pH 7.4, containing 9 mM-MgCl₂, 7 mM-MnCl₂ and 0.28% (w/v) Triton X-100. Enzyme protein was then added and the mixture was incubated for 60 min at 30 °C. The reaction was stopped by addition of 2 ml chloroform/methanol (3:2, v/v). After centrifugation (3000 *g*, 4 °C, 15 min) the supernatant was carefully removed and the pellet thoroughly washed with chloroform/methanol (3:2, v/v), then with chloroform/methanol/water (10:10:3, by vol.) and finally with chloroform/methanol/4 mM-MgCl₂ (10:3:3, by vol.). Radioactivity incorporated into protein was measured as described by Sharma *et al.* (1974).

Characterization of the glycoprotein. The protein pellet was subjected to β-elimination essentially as described by Gold & Hahn (1976), and then lyophilized; the carbohydrates released were identified by TLC on DC-cellulose plastic plates in butanol/ethyl acetate/acetic acid/water (40:30:25:40, by vol.). Distribution of radioactivity was identified with a TLC scanner or by autoradiography.

For characterization of glycoproteins by electrophoresis, the protein pellets were washed twice with 1 ml ethanol, and then taken up in 0.5 ml 0.1 M-Tris/HCl buffer, pH 6.8, containing 3% (w/v) SDS and 5% (w/v) 2-mercaptoethanol, and heated in a boiling water-bath for 10 min. After centrifugation in an Eppendorf centrifuge, the supernatants were subjected to SDS-PAGE (Laemmli, 1970). Following electrophoresis, the gels were either prepared for autoradiography as described previously (Kubicek, 1987), or blotted onto nitrocellulose paper and stained for cellobiohydrolase I by means of a monoclonal antibody (Mischak *et al.*, 1989).

Materials. GDP-[U-¹⁴C]Mannose [100–200 mCi mmol⁻¹ (3.7–7.4 GBq mmol⁻¹)] was from Amersham. C₉₅-Dol-P was obtained from the collection of polyprenols of the Institute of Biochemistry and Biophysics, Warsaw, Poland. TLC plates were from Merck. All other chemicals were of analytical grade and were obtained from commercial sources. Tsushimycin was a gift from Dr A. D. Elbein (The University of Texas, San Antonio, USA).

Fig. 1

Fig. 2

Fig. 1. Time dependence of the transfer of mannose from GDP-[U-¹⁴C]mannose to endogenous lipid (□) and protein (■).

Fig. 2. Effect of Dol-P concentration on the incorporation of mannose from GDP-[U-¹⁴C]mannose into lipid (a) and protein (b).

Table 1. Mannosyl transfer from GDP-[U-¹⁴C]mannose into lipid and protein

Conditions for mannosyl transfer are described in Methods. Where indicated, unlabelled GDP-mannose (4 μmol) was added after 60 min and the reaction continued for a further 60 min.

Conditions	[U- ¹⁴ C]Mannose (pmol per assay) in:	
	Lipid	Protein
No GDP-mannose	372	7.9
GDP-mannose	190	52.0
GDP-mannose + 350 μg tsushimycin ml ⁻¹	<2	<2.0

RESULTS

Mannosyl transfer from GDP-[U-¹⁴C]mannose to lipid and glycoprotein fractions

Incubation of the *T. reesei* microsomal fraction with GDP-[U-¹⁴C]mannose at 30 °C resulted in the incorporation of radioactivity into two endogenous acceptors (Fig. 1); the initial rate of formation of the mannosyl-lipid was faster than the initial rate of formation of the mannosyl-protein. This time-course is consistent with the notion that mannose is transferred from GDP-mannose to the protein via a lipid intermediate. Further support for this was obtained by carrying out a pulse-chase with cold GDP-mannose (Table 1): this led to a decrease in label in the lipid fraction with a concomitant increase in label in the protein.

Tsushimycin, a specific inhibitor of the synthesis of Dol-P-Man from GDP-mannose (Elbein, 1981), virtually blocked incorporation of [U-¹⁴C]mannose into both lipid and protein (Table 1). It seems, therefore, that the lipid intermediate is indeed Dol-P-Man and that protein mannosylation occurs via this lipid intermediate.

Fig. 3. Chromatographic characterization of *T. reesei* mannosyl lipid. The samples were separated by TLC using solvent A, and the chromatogram was then autoradiographed. S, standard (Dol-P-Man from yeast); P, sample isolated from *T. reesei* membrane fractions after incubation with GDP-[U- 14 C]mannose as described in Methods.

These initial results implied a mechanism of *O*-glycosylation analogous to that in *S. cerevisiae* (Tanner & Lehle, 1987), in which Dol-P-Man donates the first *O*-linked mannose unit. Direct evidence for such a mechanism came from experiments in which transfer of mannose from Dol-P-Man to endogenous protein, present in the *T. reesei* membrane fractions, was demonstrated (Table 2). Interestingly, mannosyl transfer was significantly higher when membranes were prepared from *T. reesei* grown under conditions of carbon catabolite derepression (lactose as carbon source): this difference was almost abolished when the reaction system was supplemented with exogenous cold GDP-mannose.

The activity of the enzyme transferring mannose from GDP-mannose to Dol-P was also higher in cells grown under conditions of carbon catabolite derepression. Transfer of [U- 14 C]mannose from GDP-[U- 14 C]mannose to lipid as well as to protein increased linearly with increasing amounts of membrane protein up to 0.8 mg protein.

The transfer of mannose to the lipid fraction was strongly increased by addition of exogenous C₉₅-Dol-P (Fig. 2). From these data, the apparent K_m was found to be 2–5 nM.

Characterization of the labelled glycolipid

The mannosyl lipid synthesized by membranes of *T. reesei* was analysed by methods similar to those used to identify Dol-P-Man in other filamentous fungi (Letoublon & Got, 1974; Gold & Hahn, 1976; Gander *et al.*, 1977; Soliday & Kolattokudy, 1979). DEAE-Sephadex chromatography showed that the glycolipid from *T. reesei* cochromatographed with a Dol-P-

Fig. 4. TLC radioactivity scan of oligomannosides, released by β -elimination from endogenous proteins mannosylated in the presence of Mn^{2+} (a) or Mg^{2+} (b). The position of the oligosaccharide markers is indicated.

Table 2. *Transfer of [U - ^{14}C]mannose from [U - ^{14}C]mannosyl C_{95} -dolichol phosphate into endogenous protein*

Membrane fractions were isolated from *T. reesei* grown in a medium containing glycerol (carbon repressed) or lactose (carbon derepressed); the fractions (370 or 390 μ g protein, respectively) were incubated with 56000 c.p.m. [U - ^{14}C]mannosyl C_{95} -dolichol phosphate for 120 min and radioactivity incorporated into protein was measured, all as described in Methods. Where indicated, unlabelled GDP-mannose (4 μ mol) was added after 60 min, and the reaction allowed to proceed for a further 60 min.

Carbon source	Presence/absence of GDP-mannose	[U - ^{14}C]Mannose incorporated into protein (pmol in 120 min)
Glycerol	—	2.4
	+	12.4
Lactose	—	8.5
	+	13.1

Man standard synthesized from GDP-mannose and C_{90} -Dol-P yeast mannosyl transferase. Two different (neutral and acid) solvent systems revealed a single labelled band with R_F values similar to those for yeast Dol-P-Man (Fig. 3).

Characterization of the oligosaccharides transferred to the glycoprotein

To determine the size and linkage of the carbohydrates introduced into membrane protein, the glycoprotein fraction was subjected to β -elimination. Approximately 90% of the label in the protein fraction was released by this procedure. TLC of the released oligosaccharides showed (Fig. 4) that they consisted of mannose, dimannosides and tetramannosides (as checked against appropriate standards). These findings are consistent with the conclusion that *T. reesei* membrane fractions carry out O-glycosylation of endogenous protein acceptors. As described in other systems (Tanner & Lehle, 1987), synthesis of oligomannosides is dependent on the presence of Mn^{2+} ions in the system. In the presence of Mg^{2+} , the released carbohydrate portion consisted mainly of mannose (Fig. 4).

*Demonstration of the presence of CBH I in *T. reesei* membrane fractions*

In order to support the conclusion that *T. reesei* cellulases, which carry O-glycosidically linked mannose chains (Fägerstam *et al.*, 1984; Salovuori *et al.*, 1987; Gum & Brown, 1976), are actually located in the membrane fractions studied here, membrane protein was subjected to SDS-PAGE and the major cellobiohydrolase, CBH I, detected with a monoclonal antibody (Mischak *et al.*, 1989). Immunostaining revealed a band at 60–70 kDa (Fig. 5); this corresponds

Fig. 5. SDS-PAGE/Western blotting and immunostaining of endogenous proteins isolated from *T. reesei* membranes after incubation with GDP-[U- 14 C]mannose. L, membranes isolated from lactose-grown cells; G, membranes isolated from glycerol-grown cells; PM, prestained marker proteins (Sigma).

to the size reported for glycosylated CBH I (Salovuori *et al.*, 1987). CBH I was present in membranes from mycelia grown under carbon-catabolite-repressed (glycerol) as well as derepressed (lactose) conditions. Fluorography of the SDS-PAGE gels revealed diffuse bands of radioactivity in the 60–70 kDa molecular mass area (not shown); hence it cannot be claimed that CBH I was in fact glycosylated in our *in vitro* system.

DISCUSSION

The present report offers evidence that a membrane fraction from *T. reesei* mycelia catalyses the transfer of mannose from GDP-mannose to endogenous lipid acceptors and subsequently to endogenous protein. Pulse-chase experiments, the kinetics of incorporation and the use of exogenously added Dol-P-Man all suggested that Dol-P-Man functions as an intermediate in the transfer of mannose from GDP-mannose to protein; hence, *O*-glycosylation in *T. reesei* is by a mechanism similar to that previously demonstrated in other fungi (Tanner & Lehle, 1987). The mannose incorporated was mainly found in Dol-P-Man, and tsushimycin, which specifically inhibits Dol-P-Man formation, completely inhibited mannosyl transfer in our preparations.

So far, the endogenous mannosyl accepting proteins that are labelled by [U- 14 C]mannose have not been identified. Soliday & Kolattokudy (1979) showed that in *Fusarium solani*, mannosyl residues were incorporated into microsomal proteins that had the same molecular mass as the major secretory proteins of this fungus. Although our membrane fractions contained CBH I protein, we could not demonstrate that this protein was a substrate for glycosylation *in vitro*. This could be due to the fact that CBH I contained in the membranes should already bear both *N*- as well as *O*-glycosidically linked sugar chains, and thus be inaccessible to further glycosylation. However, exogenously added CBH I (purified and deglycosylated) also was not glycosylated, probably because of the lack of translation activity and membrane integrity in our *in vitro* system. Further studies using cell-free translation systems in combination with our membrane fractions may succeed in glycosylating cellulases *in vitro*.

The biological role of *O*-glycosylation in fungi has not yet been elucidated. We recently postulated that *O*-glycosylation of cellulases is one of the parameters controlling their secretion (Kubicek *et al.*, 1987; Messner & Kubicek, 1988). In this paper we have shown that incorporation of mannose into protein from Dol-P-Man is greater in carbon-catabolite-derepressed mycelia than in carbon-catabolite-repressed mycelia. However, this difference was eliminated by adding cold GDP-mannose. This may be interpreted in terms of the presence of lower concentrations of Dol-P-Man and GDP-mannose in carbon-catabolite-repressed mycelia. The previously observed lag in *O*-glycosylation of *T. reesei* endoglucanases during induction of synthesis by sophorose supports this hypothesis (Messner & Kubicek, 1988). The concentration of Dol-P in the endoplasmic reticulum has been shown to be rate limiting to glycosylation in several cell systems (Burton *et al.*, 1979; Schwarz & Datema, 1982). The above findings, together

with the fact that CBH I was clearly present (although in lower concentrations) in microsomes isolated from glucose-grown mycelia, would suggest that O-glycosylation may – at least in part – be involved in catabolite repression of CBH I synthesis.

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