We prepared [U-14C]cellobiose by cultivating Acetobacter pasteurianus in the presence of [U-14C]glucose and hydrolyzing the [U-14C]cellobiose formed with β-glucosidase-free cellulase from Trichoderma reesei. This 14C-labeled cellobiose was used to investigate the presence of an uptake system for cellobiose in T. reesei. Evidence was obtained for the presence of a high affinity (K_a for cellobiose 0.3 μM) but low activity (2.5 millinits/mg fungal dry weight) cellobiose permease. The permease is formed constitutively, but higher levels are formed after addition of sophorose (glucosyl-β-1,2-diglucoside), a reputed cellulase inducer. The permease appears to be specific for β-diglucosides, as the uptake of [U-14C]cellobiose is inhibited by sophorose, gentiobiose (glucosyl-β-1,3,4-glucoside), and cellobiose. Under these conditions, celloligodextrins (n, 4-7; final concentration, 1 mM) are not inhibitors. Glucose, but no other monosaccharides, inhibits the permease. The hypersecretory mutant T. reesei RUT C-30 exhibits elevated permease activities, whereas in T. reesei QM 9979, a mutant strain defective in the induction of cellulases by cellulose or sophorose, strongly reduced permease activities were demonstrated. The results stress a hitherto not recognized point of control in the uptake of cellulose oligosaccharides.

Despite the progress that has been made in the molecular biology of the Trichoderma reesei cellulase system (Penttila et al., 1991), it is still only poorly understood how the biosynthesis of cellulases is triggered by the extracellular, insoluble polysaccharide cellulose (Kubicek et al., 1993). The available evidence suggests that low levels of constitutively formed cellulases (particularly cellobiohydrolase II) are responsible for the initial attack on the cellulose and thereby release cellobiose (Kubicek et al., 1988; El-Gogary et al., 1989; Messner et al., 1992). Further events in the inductive cascade are still speculative; however, cellobiose, in theory the most logical inducer of cellulases and in fact an inducer of cellulases in other fungi (Bisaria and Mishra, 1989), inhibits cellulase formation in T. reesei (Fritscher et al., 1990). In contrast, the disaccharide sophorose (glucosyl-β-1,2-glucoside) is a strong cellulase inducer (Sternberg and Mandels, 1979). Its formation in vivo has been shown (Vaheri et al., 1979) and may be due to the transglycosylating ability of either β-glucosidase (Schmid and Wandrey, 1987) or endo-β-1,4-glucanase (Claeyssens et al., 1990).

One of the uncertainties within this model is the mode of uptake of cellobiose and sophorose. Uptake of cellobiose has been demonstrated in some yeasts and Escherichia coli mutants (Freer and Greene, 1990; Schnetz et al., 1987) but not yet in filamentous fungi and may not be required for cellobiose metabolism in T. reesei because of the presence of an extra-cellular constitutively formed β-glucosidase (Umile and Kubicek, 1986; Chirico and Brown, 1987; Hofer et al., 1989).

We have previously studied the metabolism of cellobiose by T. reesei and concluded that both a permease as well as β-glucosidase are present and involved (Fritscher et al., 1990). A more detailed study on the putative permease was, however, hampered by the lack of availability of radioactively labeled cellobiose and by the interference of β-glucosidase in the uptake assay.

Here we will describe the preparation of [U-14C]cellobiose from Acetobacter pasteurianus [U-14C]cellobiose, its use in the assay of the presence of a β-linked diglucoside permease of T. reesei, and the characterization of its major properties.

**MATERIALS AND METHODS**

**Trichoderma Strains and Culture Conditions**—The strain used throughout these studies was T. reesei QM 9414, which was obtained from ATCC (No. 26921). In selected experiments (when stated explicitly), the following strains were also used: (a) a recombinant strain T. reesei RLMex40H, which carries an antisense copy of the bgll (β-glucosidase-encoding) gene under the control of the pkil (pyruvate kinase-encoding) promoter and which does not form β-glucosidase during growth on glucose; (b) the hypersecretory strain T. reesei RUT C-30 (ATCC 56765); and (c) the cellulase-negative mutant strain T. reesei QM 9979 (Mandels and Reese, 1960). The strains were maintained on malt agar. Cultures were grown by inoculating 10⁵ spores/ml in Mandels and Andreotti (1978) medium, with glucose (1%, w/v) as a carbon source, in 1-L flasks containing 250 ml of medium using a rotary shaker at 30 °C.

A. pasteurianus was obtained from Dr. W. Steiner, Institute of Biotechnology, Graz, Austria.

**Preparation of [U-14C]Cellobiose**—To prepare 14C-labeled cellobiose, we cultivated Acetobacter pasteurianus, a cellulase excreting bacterium, in 0.1-L Erlenmeyer flasks containing 26 ml of glucose-yeast extract-peptone medium (Porng et al., 1989) for 3 days at 25 °C at 250 rpm. 1 ml of this culture was then used to inoculate 10 ml of the same medium in 10-ml flasks, yet contained only 2 μl of glucose and 2 μCi/ml of [U-14C]glucose. Incubation was carried out...
Demonstration of a Cellobiose Permease in T. reesei—When T. reesei was pregrown on glucose as a carbon source, the mycelia harvested, washed, replaced to succinate buffer, and [U-14C]cellobiose added, the 14C-label was taken up by the mycelium (Fig. 1a). Using a mycelial density between 0.7 and 1 mg of dry weight/ml, the rate of uptake was linear for at least 15 min. Control experiments with isolated cell walls of T. reesei showed no binding of radioactive label, which proved that the uptake of [U-14C]cellobiose was not the result of physical adsorption (data not shown). The uptake rate displayed a biphasic dependence on the cellobiose concentration (Fig. 1b). We assumed that this may be due to an interference from the fungus' plasma membrane bound β-glucosidase. Since this enzyme has a Km of 1.5 mM for cellobiose and a Vmax of 0.080 units/mg fungal dry weight (Umile and Kubicek, 1986), it competes with the uptake system at higher cellobiose concentrations. To prove this assumption, several experiments were carried out: (a) classical glucose trapping by adding hexokinase/Mg-ATP to the uptake assay (De La Fuente and Solis, 1962) (the rationale behind this experiment was that [U-14C]glucose, formed by β-glucosidase from [U-14C]cellobiose, would be converted to [U-14C]glucose 6-phosphate, which is not taken up by T. reesei); (b) addition of purified β-glucosidase to the uptake assay to increase the total β-glucosidase activity present; (c) carrying out the uptake experiments with a recombinant of T. reesei, which contains an antiense-bgl-1-DNA under the T. reesei phi (pyruvate kinase) promoter and therefore does not form any β-glucosidase in the presence of glucose; (d), finally, using an inhibitor of β-glucosidase, delta-gluconolactone, in these experiments (Table I) provided evidence for an interference of β-glucosidase with the cellobiose transport assay at higher cellobiose concentrations (0.1 mM). The only 2-fold increase in uptake by 2 units/ml of extra β-glucosidase at low cellobiose concentrations makes it unlikely that the low activities of constitutively present β-glucosidase activity of T. reesei interfere with the assay under these conditions. This concentration was thus used routinely in all further experiments.

Specificity of T. reesei Cellobiose Uptake—We made use of competition experiments to investigate the substrate specificity of T. reesei cellobiose permease (Table I). They suggest that the permease is specific for cellobiose, laminariniose, and sophorose and has no affinity for oligodextrins of higher chain length. Other disaccharides (sucrose, lactose, and xylobiose) had no effect on the transport of [U-14C]cellobiose even in 102-fold higher concentrations, suggesting that they are not substrates of the permease. We thus propose the name “β-linked diglucose permease” for it and will use this term throughout the paper.

Glucose, but not other monosaccharides such as galactose or mannose, inhibited [U-14C]cellobiose uptake with a KI of 4.0 mM. To rule out the possibility that the β-linked diglucose permease can transport glucose, we assayed [U-14C]glucose uptake in the presence of 1 mM cellobiose.

Properties of the β-Linked Diglucose Permease—The uptake of cellobiose exhibited a pronounced pH dependence displaying the highest uptake rates at pH 5. To investigate whether cellobiose uptake was due to facilitated diffusion or to carrier-mediated transport, the effect of inhibitors of ATP formation (NaN3, 2,4-dinitrophenol) and of an ionophore (carbonyl cyanide p-trifluoromethoxyphenyldrazone) were studied (Table III). The data obtained suggest that cellobiose uptake requires an active ATP gradient over the plasma membrane, which is directly coupled to H⁺.

Stimulation of Cellobiose Uptake by Sophorose—In the-
The \( \beta \)-linked diglucoside permease activity is formed before cellulase (assayed as cellobiohydrolase I, which accounts for the major portion of the cellulolytic enzyme system) activity (Fig. 2b). From these results we conclude that sophorose stimulates the formation of cellobiose permease in \( T. \) reesei either by induction or by inducing an activator. Maximal stimulation was observed at 0.2 mM sophorose (Fig. 2c).

**\( \beta \)-Linked Diglucoside Permease Activity in \( T. \) reesei Mutant Strains**—In view of the obvious importance of the \( \beta \)-linked diglucoside permease in the uptake of the inducer of cellulase formation, we have also investigated its activity in two mutant strains, \textit{i.e.} \( T. \) reesei RUT C-30 and QM 9979. The former is a hypersecretory strain, which secretes about 3-fold higher amounts of cellulase protein, whereas the latter mutant strain is unable to form cellulases on cellulose or by addition of sophorose and forms a complete cellulase system during growth on lactose. The permease activity of these two mutant strains deviated significantly from that of \( T. \) reesei QM 9414, whereas that of the hypersecretory strain RUT C-30 was similar.
**β-Diglucoside Permease from Trichoderma reesei**

![Graph](image)

**FIG. 2.** Induction of β-diglucoside permease and cellobiohydrolase I by sophorose. *a*, effect of sophorose and cellobiose on the time-dependent formation of β-diglucoside permease; *b*, 0.02 mM sophorose; *c*, 0.02 mM cellobiose; *m*, 0.02 mM sophorose and 50 mg/liter cycloheximide. *b*, kinetics of sophorose induction of cellobiohydrolase 1. *c*, effect of sophorose concentration on the inducibility of cellobiohydrolase I (Δ) and β-diglucoside permease (○).

about twice as high and that of QM 9979 was below 10% of it (Table IV). The synthesis of β-glucoside permease in mutant QM 9979 could not be induced by sophorose (data not shown).

**DISCUSSION**

In the present study, we provide for the first time direct evidence of the presence of a β-diglucoside permease in *T. reesei*. It has a very high affinity for cellobiose (Km, 0.3 mM) and therefore can successfully compete with the constitutive β-glucosidase for the common substrate cellobiose. Because of the comparatively low Vmax (2.5 versus 80 milliunits/mg fungal dry weight for permease and β-glucosidase, respectively), however, this situation is reversed at higher concentrations. Such properties of the putative permease had previously been anticipated from inhibitor experiments (Fritscher et al., 1990). Hence uptake of cellobiose is preferred to hydrolysis when its concentration is very low. It is tempting to speculate that this situation offers an advantage to the fungus. This appears to be the case during the early phase of contact of *Trichoderma* with cellulase. The activities of the constitutive cellulases are very low (0.025 units/10⁶ conidia; Kubicek et al., 1988) and hence only low concentrations of cellobiose will become available to the fungus at this stage.

To obtain some insights into the putative essential nature of the β-diglucoside permease in cellulase synthesis, we have also compared its activity in two mutant strains of *T. reesei*. The findings of elevated permease activities in strain *T. reesei* RUT C-30 are not unexpected. As this strain has been shown to be hypersecretory (Ghosh et al., 1984) and secretes all components of the cellulase enzyme mixture in elevated amounts, it is of little surprise that β-glucoside permease, which also has to be transported via a secretory pathway, occurs in higher amounts. The strongly decreased activities in mutant *T. reesei* QM 9979 are more interesting; however, this strain cannot be induced to form cellulases by cellulose or sophorose, but it forms a complete cellulase system during growth on lactose. Theoretically, these properties would exactly resemble the phenotype one would expect for a β-glucoside permease negative mutant. However, although reductive of the permease was significant (over 90%) and its induction by sophorose did not occur, the low residual activity was clearly demonstrable. The inability to grow on cellulose therefore cannot be explained by a loss of the permease for the inducer. It may be speculated that the remaining activities are already low enough to enable the accumulation of concen-

**TABLE IV**

| Strain          | Induction | β-Diglucoside permeasea |
|-----------------|-----------|-------------------------|
| T. reesei QM 9414 | −         | 0.483 (±0.021)          |
| T. reesei RUT C-30 | +         | 1.42 (±0.042)           |
| T. reesei QM 9979 | −         | 1.12 (±0.038)           |
|                  | +         | 2.35 (±0.060)           |

*Values are given for mycelia cultivated on glucose for 18 h; induction means activities measured after 3 h of addition of 0.1 mM sophorose as described under "Materials and Methods"; cellobiose concentration was 0.1 mM throughout. Standard deviation is given for at least three separate determinations.

*a*, fungal dry weight.
trations of inducer sufficient for triggering β-glucoside permease and cellulase induction, but this clearly needs other experiments to be proven. The fact that the β-glucoside permease transcript is apparently one of the earliest mRNAs that accumulates upon induction by sophorose offers the possibility to clone its gene by differential hybridization techniques.

The finding that the activity of the β-glucoside permease is inhibited but not repressed by glucose is of particular importance for the regulation of cellulase synthesis. The information of whether cellulase formation is repressed by glucose or not is controversial. Recent findings on the presence of functional creA binding sites in the cbh1 promoter of T. reesei now provide conclusive evidence for glucose repression of cellulase gene transcription. Our data, however, indicate that glucose also interferes with the uptake of the inducer of cellulase biosynthesis, which can be considered as a mechanism that aids in amplifying the negative glucose effect. Inhibition and/or repression of the respective permease have also been observed for other disaccharides whose hydrolases are glucose repressible (Dickson and Barr, 1983; Johnston, 1987).

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