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Role of Phosphorolytic Cleavage in Cellobiose and Cellodextrin Metabolism by the Ruminal Bacterium *Prevotella ruminicola*†

J. LOU, K. A. DAWSON, AND H. J. STROBEL*

Department of Animal Sciences, University of Kentucky, Lexington, Kentucky 40546-0215

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In bacteria, cellobiose and cellodextrins are usually degraded by either hydrolytic or phosphorolytic cleavage. *Prevotella ruminicola* B_14 is a noncellulolytic ruminal bacterium which has the ability to utilize the products of cellulose degradation. In this organism, cellobiose hydrolytic cleavage activity was threefold greater than phosphorolytic cleavage activity (113 versus 34 nmol/min/mg of protein), as measured by an enzymatic assay. Cellobiose phosphorylase activity (measured as the release of P_i) was found in cellobiose-, mannose-, xylose-, lactose-, and cellodextrin-grown cells (>92 nmol of P_i/min/mg of protein), but the activity was reduced by more than 74% for cells grown on fructose, L-arabinose, sucrose, maltose, or glucose. A small amount of cellodextrin phosphorylase activity (19 nmol/min/mg of protein) was also detected, and both phosphorylase activities were located in the cytoplasm. Degradation involving phosphorolytic cleavage conserves more metabolic energy than simple hydrolysis, and such degradation is consistent with substrate-limiting conditions such as those often found in the rumen.

Cellulose is the major structural plant carbohydrate and is an important component of ruminant feedstuffs. However, it is resistant to mammalian enzymatic attack, so ruminant animals digest cellulose by relying on a symbiotic relationship with cellulolytic microorganisms harbored in the digestive tract. Although relatively few ruminal bacterial species (e.g., *Ruminococcus albus*, *Ruminococcus flavefaciens*, and *Fibrobacter succinogenes*) exhibit high levels of cellulolytic activity (5), large numbers of noncellulolytic microorganisms are found in ruminants fed fiber-containing diets (6). Fibrolytic bacteria provide noncellulolytic organisms with small-length cellodextrins and cellobiose (20), and this cross-feeding may enhance fiber digestion by removing the products of cellulose hydrolysis (10).

After they have entered bacterial cells, the initial metabolism of soluble cellulose degradation products involves either hydrolytic or phosphorolytic cleavage (21). In general, the metabolic pathways which use phosphorolytic cleavage conserve more energy than those utilizing simple hydrolysis. Since bacterial energy sources in the rumen are often limited and the yield of ATP is generally low in anaerobic microorganisms, efficient utilization of substrate is important for the growth of ruminal bacteria. Thus, it is not surprising that phosphorolytic cleavage mechanisms have been found in several ruminal bacteria (3, 8, 24, 25).

Prevotella ruminicola is one of most numerous ruminal bacteria and uses a wide variety of carbohydrates (9). Although it possesses endoglucanase activity (14), this bacterium cannot grow on cellulose but utilizes cellobiose and soluble cellodextrins as large as celloheptaose (18). An enzyme with β-glucosidase activity has been cloned and characterized in *P. ruminicola* (25). However, there was no work on possible cellobiose phosphorylase activity in this organism and little study of phosphorolytic cleavage of cellodextrins by ruminal bacteria in general. Because bacterial survival and growth in the rumen is largely limited by energy availability and since phosphorolytic degradation of substrates is energetically more efficient, we investigated the role of phosphorolytic cleavage in cellobiose and cellodextrin metabolism by *P. ruminicola.*

MATERIALS AND METHODS

Organisms and cell growth. *P. ruminicola* B_14 was obtained from J. B. Russell, Cornell University, Ithaca, N.Y. The organism was cultured in an anaerobic medium containing the following (per liter): 292 mg of K_2HPO_4, 240 mg of KH_2PO_4, 480 mg of Na_2SO_4, 480 mg of NaCl, 100 mg of $MgSO_4 \cdot 7H_2O$, 64 mg of $CaCl_2 \cdot H_2O$, 600 mg of cysteine, 4 g of Na_2CO_3, 1 g of Trypticase (Becton Dickinson Microbiological Systems, Cockeysville, Md.), 500 mg of yeast extract, 100 mg of resazurin, 22.1 mM acetate, 6.0 mM propionate, 2.4 mM butyrate, 0.68 mM valerate, 0.68 mM isovalerate, 0.81 mM isobutyrate, and 0.66 mM 2-methylbutyrate. The medium was adjusted to pH 6.7 with NaOH and prepared and maintained under a carbon dioxide atmosphere. Carbohydrates were added as separate solutions, and cultures were grown at 39°C.

Cell fractionation. Cells were harvested during logarithmic growth by centrifugation (15,000 × *g* for 10 min at 4°C) and washed twice with either 50 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (pH 6.8)] for cellobiose phosphorylase and β-glucosidase assays or 50 mM Tris-maleate-NaOH (pH 6.8) for cellodextrin phosphorylase assays. Washed cells were passed through a French pressure cell (1,120 kg/cm^2) two times, and debris and whole cells were removed by centrifugation (15,000 × *g* for 10 min at 4°C). Portions of the cell extracts were kept at 4°C for assay the same day. The remaining extract was centrifuged (140,000 × *g* for 30 min at 4°C), yielding cytosol (supernatant) and membrane (pellet) fractions. The membrane pellets were washed with the appropriate buffer. Phosphoglucomutase activity was used as a cytosoplasmic marker and assayed as previously described (12). ATPase activity served as a membrane marker and was assayed as described by Kinoshita et al. (11).

Cellobiose degradation by crude extracts. The total cellobiose cleavage activity was determined in an assay mixture (1 ml at 39°C) which contained 50 mM PIPES (pH 6.8), 33 mM sodium-potassium-phosphate, 3 mM $MgCl_2$, 1 mM ATP, 1 mM NADP, 8 U of phosphoglucomutase, 5 U of hexokinase, 5 U of glucose-6-phosphate dehydrogenase, 20 mM of cellobiose, and 20 to 50 μl of crude extract (120 μg of protein). The phosphorolytic cleavage activity (cellobiose phosphorylase) was measured by omitting ATP and hexokinase from the mixture described above. Hydrolytic cleavage (β-glucosidase) was detected by omitting sodium-potassium-phosphate and phosphoglucomutase from the assay. NADPH formation was monitored at 340 nm, and the cleavage rate was calculated by assuming that one molecule of cellobiose generated two molecules of NADPH in the total cleavage or β-glucosidase assays and one molecule of NADPH in the phosphorylase portion of the assay. In control assays, there was no nonspecific reduction of NADP observed in the absence of cellobiose or crude extract. In addition, endogenous hexokinase and phosphoglucomutase activities in cellular extracts was negligible and did not affect the measurement of cellobiose cleavage activities.

Enzyme assays. Cellobiose phosphorylase and β-glucosidase activities were determined by the NADP-linked assay described above. Cellobiose and cello-

* Corresponding author. Mailing address: 212 W. P. Garrigus Building, Department of Animal Sciences, University of Kentucky, Lexington, KY 40546-0215. Electronic mail address: strobel@pop.uky.edu.

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TABLE 1. Cellobiose phosphorylase activity in *P. ruminicola* grown on different substrates

Growth substrate[a]	Sp act[b]	Relative activity (%)
Cellobiose	163 ± 5	100
Mannose	155 ± 6	95
Xylose	123 ± 8	75
Lactose	103 ± 8	63
Cellodextrins	92 ± 9	56
Fructose	43 ± 3	26
L-Arabinose	33 ± 3	20
Sucrose	13 ± 2	8
Maltose	ND	0
Glucose	ND	0

[a] Cultures were provided with 4 g of carbohydrate per liter and were harvested during logarithmic growth.

[b] Specific activities are given in nanomoles of P_i per minute per milligram of protein. Values represent the means of duplicate cultures ± ranges. ND, not detected (<2 nmol/min/mg of protein).

dextrin phosphorylase activity assays were also performed by measuring P_i formation as described by Alexander (1, 2). Glucose formation by endogenous β-glucosidase activity did not affect the measurement of cellodextrin phosphorylase activity. Hexokinase and phosphoglucomutase activities were determined as described by Kotze (12). All enzyme assays were typically performed at 39°C with duplicate cultures and with at least two replicates per culture.

Analyses. Glucose and cellobiose in cell-free culture supernatant were measured by an enzymatic method as previously described (19). Protein was determined by the Lowry method after the cells were boiled in 0.2 M NaOH for 15 min (13).

Materials. Mixed cellodextrins were prepared and isolated according to the method described by Miller (15), and the composition of the mixture was analyzed by high-pressure liquid chromatography with a Dynamax 60A amino column (4.6 mm by 25 cm) (Rainin Instruments, Woburn, Mass.). The mobile phase consisted of 65% acetonitrile and 35% water at a flow rate of 0.75 ml/min, and compounds were detected by using a refractive index. There was no cellobiose or glucose found in the cellodextrin mixture, and more than 60% of the mixture was composed of cellotriose, cellotetraose, and cellopentaose. The concentration of mixed cellodextrins was determined by an anthrone method (4). All other reagents were obtained from commercial sources.

RESULTS

Cellobiose phosphorolysis versus hydrolysis by crude extracts. By manipulating the assay components, it was possible to demonstrate that cellobiose was phosphorolytically and hydrolytically cleaved by crude extracts from *P. ruminicola*. Hydrolytic cleavage activity was threefold greater than phosphorolytic cleavage activity (113 versus 34 nmol of cellobiose/min/mg of protein), and cleavage activity in the presence of all assay components was slightly greater (167 nmol of cellobiose/min/mg of protein) than the sum of the phosphorolytic and hydrolytic cleavage activities. Exponentially growing cells of *P. ruminicola* utilized cellobiose at a rate that was similar to that for crude extracts (175 versus 167 nmol of cellobiose/min/mg of protein). Hexokinase (101 nmol/min/mg of protein) and phosphoglucomutase (512 nmol/min/mg of protein) activities were also found.

Properties of cellobiose phosphorylase. Enzyme kinetic values were estimated with Lineweaver-Burk double reciprocal plots by the NADP-linked assay using substrate concentrations ranging from 5 μM to 20 mM. The K_m and V_{max} for cellobiose phosphorylase were 0.17 mM for cellobiose and 37 nmol of cellobiose cleaved/min/mg of protein, respectively. Because the phosphate formation assay was more sensitive than the NADP-linked assay, cellobiose phosphorylase activity was determined by following phosphate formation in subsequent experiments. Measurement of cellobiose phosphorylase activity with phosphate formation being used as an indicator usually involves the synthetic reaction in which several monosaccharides can serve as glucosyl acceptors (1). Xylose commonly serves as the acceptor, but if glucose was used instead of the pentose, specific activity was reduced eightfold (173 versus 20 nmol of P_i/min/mg of protein). Alexander (1) noted qualitatively similar results with the cellobiose phosphorylase from *Clostridium thermocellum*.

Regulation of cellobiose phosphorylase. Relatively high levels of cellobiose phosphorylase activity were found in cells grown on cellobiose, mannose, xylose, lactose, and cellodextrins, but the activity was reduced by more than 74% in fructose-, L-arabinose-, sucrose-, maltose-, and glucose-grown cells (Table 1). If a pulse dose of glucose was added to cells fermenting cellobiose, glucose was immediately used while the rate of cellobiose utilization was reduced (Fig. 1a); the specific activity of cellobiose phosphorylase also decreased relative to that of a control which did not receive glucose (Fig. 1b). The activity increased slightly after the depletion of glucose. In the converse experiment, cellobiose was added to cultures growing on glucose (Fig. 2). There was little change in glucose utilization, but cellobiose was not utilized until most of the glucose had been consumed. Cellobiose phosphorylase activity increased after virtually all of the glucose was used, and at this point, the rate of cellobiose utilization began to increase.

Cellodextrin phosphorylase and β-glucosidase activities. Cellodextrin phosphorylase activity was detected in cells grown on cellodextrins (19 nmol of P_i/min/mg of protein) but was not detected when the organism was grown on cellobiose or glucose (data not shown). The growth rates of cellodextrin- and cellobiose-grown cultures (0.53 and 0.62 h^{-1}, respectively) were less than that of glucose-grown cells (0.73 h^{-1}), but growth yields were similar for all three carbohydrates (0.19 g of

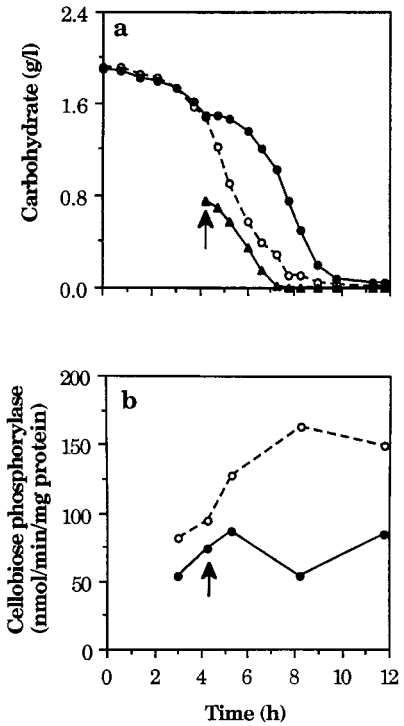

FIG. 1. Effect of a glucose pulse on cellobiose utilization (a) and cellobiose phosphorylase activities (b) in *P. ruminicola*. Glucose (▲) was added at 4.25 h (arrow) to a culture growing on cellobiose (●). A control culture (○) did not receive glucose. Cellobiose phosphorylase activities were determined by following P_i formation (see Materials and Methods).

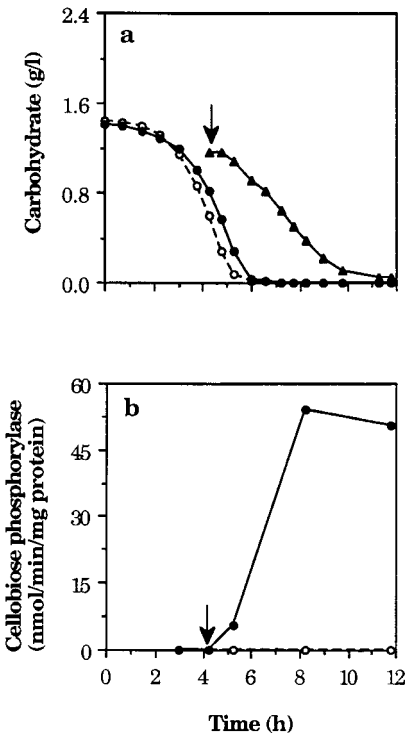

FIG. 2. Effect of a cellobiose pulse on glucose utilization (a) and cellobiose phosphorylase activities (b) in *P. ruminicola*. The cellobiose (▲) was added at 4.25 h (arrow) to a culture growing on glucose (●). A control culture (○) did not receive cellobiose. Cellobiose phosphorylase activities were determined by following P_i formation (see Materials and Methods).

protein/g of carbohydrate). Cellobiose and cellodextrin phosphorylase activities were only detected in cytosol fractions and were not found in membrane preparations (data not shown). β-Glucosidase activity in cellodextrin- and cellobiose-grown cells (>226 nmol/min/mg of protein) was greater than that in glucose-grown cells (23 nmol/min/mg of protein), and more than 85% of the activity was associated with the cell membrane. Recent studies showed that some carbohydrase enzymes in *P. ruminicola* are oxygen sensitive (7), but β-glucosidase, cellobiose phosphorylase, and cellodextrin phosphorylase activities in the present study were not affected if cell harvest and assays were performed under anaerobic conditions (100% nitrogen atmosphere).

DISCUSSION

Information on phosphorolysis of cellodextrins and cellobiose will help elucidate its potential role in cellulolysis and is also necessary for a comprehensive understanding of carbohydrate utilization by ruminal bacteria. Cellulolytic bacteria usually degrade cellulose by the synergistic action of endo- and exo-glucanases (16, 17), but this process is potentially inhibited by soluble end products such as cellodextrins and cellobiose (10). Generally, hydrolytic cleavage by β-glucosidase has been regarded as a mechanism which helps remove inhibitory end products of cellulose degradation, but it is possible that phosphorolysis also contributes to this process. Cellobiose phosphorylase activities have been found in three major ruminal cellulolytic bacteria, namely, *R. flavefaciens* (3, 8), *R. albus* (24), and *F. succinogenes* (25). This study showed that a noncellulolytic bacterium, *P. ruminicola*, also contained phosphorolytic cleavage activities against cellobiose and cellodextrin.

On the basis of the levels and location of enzyme activities, it is possible to describe a hypothetical model of cellobiose and cellodextrin utilization by *P. ruminicola* (Fig. 3). Cellobiose appears to be cleaved by either externally acting, membrane-associated β-glucosidase activity (26) or internally located cellobiose phosphorylase. Relatively low levels of β-glucosidase activity were found in the cytosol fraction, and this activity could have been from membrane contamination. Phosphorolytic cleavage would be supported by the high phosphoglumutase activity found in the cytoplasm. The presence of internal enzymes capable of degrading cellobiose implies that the organism also transports the disaccharide; however, transport of intact cellobiose across the cell membrane has not yet been demonstrated in *P. ruminicola*. The cellobiose consumption rate for crude extracts of *P. ruminicola* was similar to that for growing cells, suggesting that cellobiose cleavage may be a rate-limiting step for growth. If one assumes that cellobiose and glucose share common metabolic steps after disaccharide cleavage, the hypothesis that cellobiose cleavage is rate limiting is supported by the observation that the growth rate of glucose-grown cultures was at least 18% greater than that of cellobiose- or cellodextrin-grown cells.

Since earlier work demonstrated that β-glucosidase was membrane associated and preferentially cleaved cellodextrins rather than cellobiose (26), cellobiose phosphorylase may be important in situations in which both cellobiose and cellodextrins are present in the environment; under such circumstances, cellodextrins could possibly preclude extracellular hydrolytic cleavage of the disaccharide. Since only very small amounts of cytoplasmic β-glucosidase and cellodextrin phosphorylase activities were detected, it appeared that internal cleavage was not the major route of cellodextrin degradation.

The regulation of enzyme synthesis allows bacteria to selectively utilize substrates. Previous work indicated that *P. ruminicola* preferentially uses glucose over cellobiose and that β-glucosidase activity was repressed by the presence of glucose and

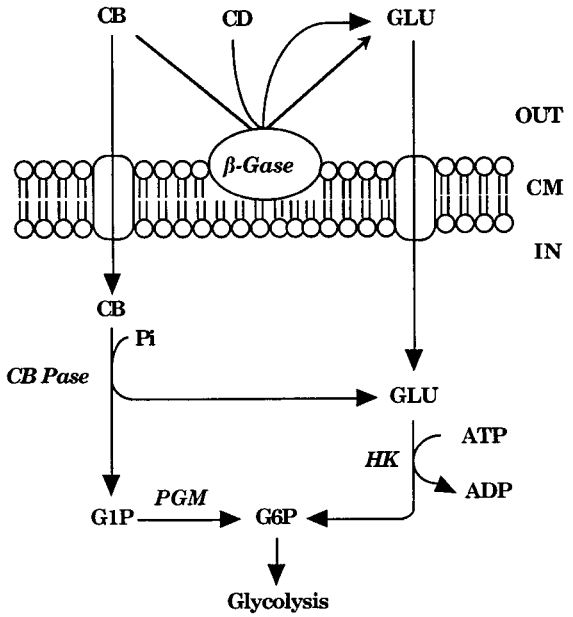

FIG. 3. Scheme for cellobiose and cellodextrin degradation by *P. ruminicola*. Abbreviations: CM, cytoplasmic membrane; CB, cellobiose; CD, cellodextrin; GLU, glucose; G1P, glucose-1-phosphate; G6P, glucose-6-phosphate; β-Gase, β-glucosidase; CBPase, cellobiose phosphorylase; HK, hexokinase; PGM, phosphoglucomutase.

several other substrates which supported high growth rates (23). Similar responses of cellobiose phosphorylase activity to the growth substrate were observed in the present study, and the regulation of both enzymes partially explains why this organism prefers the monosaccharide over cellobiose. However, it is not known whether regulation of these enzymatic activities is genetically linked. The immediacy and severity of the effect that glucose addition had on cellobiose utilization (Fig. 1a) suggest that the activity of enzymes involved in cellobiose metabolism (possibly including transport) was inhibited by glucose. However, the details of these potential interactions remain unresolved.

Phosphorolytic cleavage of cellobiose and longer cellodextrins leads to the formation of phosphorylated glucosyl molecules without the investment of ATP. The longer the size of the cellodextrin, the more energy is conserved, and theoretically higher bacterial growth yields are possible (22). Because of substrate-limiting conditions in the rumen, it is not surprising to find that some ruminal bacteria metabolize cellobiose and cellodextrins through phosphorolysis to obtain more efficient growth. However, the similar cell yields for *P. ruminicola* grown on glucose, cellobiose, and cellodextrins suggested that phosphorolytic cleavage may not be quantitatively important for growth of the organism under certain conditions. Nevertheless, phosphorolytic degradation of cellulose degradation products by this predominant ruminal organism provides an alternative route for the metabolism of soluble cellulose degradation products.

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