12-2-2004

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APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 2004, p. 1563–1569 Vol. 70, No. 3
0099-2240/04/$08.00+0 DOI: 10.1128/AEM.70.3.1563–1569.2004

Kinetics and Relative Importance of Phosphorolytic and Hydrolytic Cleavage of Cellodextrins and Cellobiose in Cell Extracts of *Clostridium thermocellum*

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Received 2 September 2003/Accepted 2 December 2003

Rates of phosphorolytic cleavage of β-glucan substrates were determined for cell extracts from *Clostridium thermocellum* ATCC 27405 and were compared to rates of hydrolytic cleavage. Reactions with cellopentaose and cellobiose were evaluated for both cellulose (Avicel)- and cellobiose-grown cultures, with more limited data also obtained for cellotetraose. To measure the reaction rate in the chain-shortening direction at elevated temperatures, an assay protocol was developed featuring discrete sampling at 60°C followed by subsequent analysis of reaction products (glucose and glucose-1-phosphate) at 35°C. Calculated rates of phosphorolytic cleavage for cell extract from Avicel-grown cells exceeded rates of hydrolytic cleavage by ≥20-fold for both cellobiose and cellopentaose over a 10-fold range of β-glucan concentrations (0.5 to 5 mM) and for cellotetraose at a single concentration (2 mM). Rates of phosphorolytic cleavage of β-glucosidic bonds measured in cell extracts were similar to rates observed in growing cultures. Comparisons of V_{max} values indicated that cellobiose- and cellodextrin-phosphorylating activities are synthesized during growth on both cellobiose and Avicel but are subject to some degree of metabolic control. The apparent K_m for phosphorolytic cleavage was lower for cellopentaose (mean value for Avicel- and cellobiose-grown cells, 0.61 mM) than for cellobiose (mean value, 3.3 mM).

Anaerobic cellulolytic bacteria play an important role in the carbon cycle and are also of potential utility for processing cellulosic biomass to fuels and chemicals (29, 30). *Clostridium thermocellum* is a gram-positive, thermophilic, anaerobic eubacterium that can rapidly utilize cellulose, with ethanol, acetic acid, and lactic acid as products of fermentative catabolism (31). This organism produces a large extracellular cellulase complex (termed a cellulosome), which can contain more than 20 distinct polypeptides and mediates hydrolysis of cellulose and some other polysaccharides (10, 11, 39, 41).

In *C. thermocellum* (as well as in several other species of cellulolytic bacteria), intracellular enzymes are capable of cleaving soluble, β-linked glucans via either phosphorolytic or hydrolytic reactions. Phosphorolytic cleavage is catalyzed by cellobiose phosphorylase (CbP) (EC 2.4.1.20) and cellodextrin phosphorylase (CdP) (EC 2.4.1.49) according to the following reactions:

$$G_2 + P_i \overset{\text{CbP}}{\Leftrightarrow} G + \text{G1P} \quad (1)$$

$$G_n + P_i \overset{\text{CdP}}{\Leftrightarrow} G_{n-1} + \text{G1P} \quad (2)$$

where G_n denotes a glucan oligomer of length i, P_i denotes inorganic phosphate, and G1P denotes glucose-1-phosphate. CbP and CdP have been purified from *C. thermocellum* (4–7, 40), and the presence of one or both of these enzymes has also been documented in other cellulolytic bacteria, including *Ruminococcus flavefaciens* (9), *C. gilvus* (36), *R. albus* (27), *Prevotella ruminicola* (26), *C. stercorarium* (34) and *Thermotoga neapolitana* (45), and *T. maritime* (33). Although *C. thermocellum* grows at ~60°C, specific activities of CbP and CdP have been measured at 37°C in prior studies (6–8, 43). In addition, prior assays for CbP and CdP activities have been carried out by measuring phosphate release in the direction of chain lengthening with glucose-1-phosphate as the glucosyl donor and xylose as the glucosyl acceptor (6, 7) rather than chain shortening, which occurs during catabolism on cellodextrins and cellulose.

Hydrolytic cleavage of cellobiose and cellodextrins is catalyzed by β-glucosidase (βG) (EC 3.2.1.21) according to the following reactions:

$$G_2 \overset{\beta\text{G}}{\Leftrightarrow} 2G \quad (3)$$

$$G_n \overset{\beta\text{G}}{\Leftrightarrow} G_{n-1} + G \quad (4)$$

Two intracellular βGs of *C. thermocellum* have been purified, characterized, and cloned (2, 3, 16–20, 38). To our knowledge, extracellular βG has not been reported for *C. thermocellum* (11, 39). Although reactions 1 and 2 may function in the chain-shortening direction (as described above) under cellular conditions, the reaction is nonspontaneous under standard-state conditions ($\Delta G^{o'} = +0.82$ kcal/mol; $K_{eq} = 0.25$) (4). Reactions 3 and 4 are highly spontaneous in the direction of hydrolysis, with $\Delta G^{o'} = -4.18$ kcal/mol for cellobiose hydrolysis (calculated from $\Delta G^{o'}$ values in references 4 and 25).

The simultaneous presence of a variety of extracytoplasmic enzyme activities capable of hydrolyzing β-linked substrates,

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intracellular CbP and CdP, and intracellular βG in *C. thermocellum* as well as in other cellulolytic species suggests that soluble cellodextrin and cellobiose metabolism potentially can occur by several processes: (i) extracytoplasmic hydrolysis with subsequent uptake and catabolism, (ii) direct uptake followed by intracellular phosphorolytic cleavage, and (iii) direct uptake followed by intracellular hydrolytic cleavage. The relative importance of these alternatives in cellulolytic microorganisms is in general not well understood (29). This matter is of interest in a bioenergetic context, because phosphorolytic cleavage provides a potential route to ATP synthesis specific to growth on β-glucan substrates. Evidence that this benefit is realized to at least some extent comes from a positive correlation between cell yield and oligosaccharide chain length observed with both *C. thermocellum* (42) and other cellulolytic bacteria (13, 37, 44). Although the potential importance of intracellular phosphorolysis has been recognized for some time (1), there is no definitive quantitative evaluation in the literature that speaks to the relative importance of phosphorolytic and hydrolytic cleavage of soluble β-glucan substrates in *C. thermocellum*. Moreover, established assay techniques (26, 27) are more readily applied to mesophiles than to thermophiles.

This study was undertaken to gain further insights into the kinetics of CbP and CdP in *C. thermocellum* cell extracts and also into the relative importance of phosphorolytic and hydrolytic cleavage of soluble β-glucan substrates in this organism. Work reported here is differentiated from that reported previously by several factors. These include carrying out enzymatic reactions at the optimal growth temperature (60°C) rather than 37°C, determining kinetic constants in the catabolically relevant chain-shorting direction, and evaluating the relative importance of phosphorolytic and hydrolytic cleavage as a function of β-glucan concentration in a single internally consistent study.

MATERIALS AND METHODS

Source and maintenance of strains. *C. thermocellum* ATCC 27405 was originally a gift from Arnold Demain. Stock cultures were maintained in MTC medium containing Avicel (PH105; FMC Corp., Philadelphia, Pa.) or cellobiose as the carbon source (10 g/liter). Batch cultures on Avicel or cellobiose (10 g/liter) were grown at 60°C in sealable serum vials (Wheaton Scientific, Millville, N.J.) containing 100 ml of broth. Details associated with medium composition and strain maintenance are described elsewhere (32, 46).

Chemicals. All chemicals and enzymes were purchased from Sigma Chemical Co. (St. Louis, Mo.) unless otherwise noted. Cellopentaose and cellotetraose (purity > 99%) were prepared using mixed acid hydrolysis and separated by chromatography as described elsewhere (47).

Cell extract preparation. Culture samples (50 ml) grown on Avicel or cellobiose as indicated were removed from serum vials in late exponential phase and centrifuged for 20 min at 15,000 × *g* and 4°C. The pellet was resuspended with 15 ml of 50 mM PIPES buffer (pH 7.4) and centrifuged again, and the washed pellet was suspended in 15 ml of 50 mM PIPES buffer supplemented with 50 mM dithiothreitol and frozen at −20°C overnight. The frozen suspension was thawed at 4°C and passed through a French press (1,120 kg/cm^2) three times. Cell debris and unbroken cells were removed by centrifugation for 20 min at 15,000 × *g* and 4°C. Supernatant protein concentration was measured by the Bradford method with bovine serum albumin (BSA) as the standard (12).

Reaction and enzyme assay conditions. Cleavage reactions were initiated by adding cell extract (100 μl, 0.78 mg protein/ml) to 5 ml (final volume) of a reaction solution containing 50 mM PIPES, 8 mM KH_2PO_4 (pH 7.4), 10 mM dithiothreitol, and substrate (cellobiose, cellotetraose, or cellopentaose) at final concentrations ranging from 0.5 to 5 mM. The reaction mixture was incubated at 60°C. Samples were withdrawn at 5-min intervals and boiled at 100°C for 5 min to stop the reaction. Because of the small volume used, heat-up was very rapid. The sample was divided into two 480-μl subsamples; subsample 1 was used for determination of the combined concentration of glucose and G1P, and subsample 2 was used for determination of the concentration of glucose exclusive of G1P. A total of 50 μl of 200 U of phosphoglucomutase (PGM) solution/ml and 50 mM Mg^{2+} was added to subsample 2, and 50 μl of distilled water was added to subsample 1. Both subsamples were mixed with 480 μl of a twofold-concentrated Infinity glucose kit solution (Sigma kit 18-20) containing ~4.2 mM ATP, ~5 mM NAD^+, ~3 U of hexokinase (HK), and >6.4 U of G-6-PDH (glucose-6-phosphate dehydrogenase). The mixtures were incubated at 35°C for 5 min, and the change in absorbance at 340 nm was determined in a Milton Roy Spectronic 21D spectrophotometer. PGM activity was measured as described by Kotze (23), with modifications including reaction at 60°C, low substrate concentration (0.5 mM), and no added Mg^{2+}.

Rate calculations. Rates of glucose formation (denoted r_{Glu}) and of glucose-plus-G1P formation (denoted $r_{Glu\ +\ G1P}$) were calculated from the slopes of concentration-versus-time curves. It is desired to determine the rate of hydrolytic cleavage of β-glucosidic bonds (denoted r_h) as well as the rate of phosphorolytic cleavage of β-glucosidic bonds (denoted r_p). All rates are defined in units of nanomoles of bonds cleaved/minute/milligram of cell extract (units/milligram of cell extract).

For cellobiose as the substrate, it may be inferred from the stoichiometry of reactions 1 and 3 that

$$r_{Glu} = 2r_h + r_p \tag{5}$$

$$r_{Glu\ +\ G1P} = 2r_h + 2r_p \tag{6}$$

which can be solved to give

$$r_p = r_{Glu\ +\ G1P} - r_{Glu} \tag{7}$$

$$r_h = r_{Glu} - \frac{r_{Glu\ +\ G1P}}{2} \tag{8}$$

The parameter f is defined here as elsewhere (28, 29) as the fraction of β-glucosidic bonds cleaved phosphorolytically:

$$f = \frac{r_p}{r_p + r_h} \tag{9}$$

Thus, with cellobiose as the substrate,

$$f = \frac{2(r_{Glu\ +\ G1P} - r_{Glu})}{r_{Glu\ +\ G1P}} \tag{10}$$

For cellodextrins as the substrate (and considering initial rates so that reactions of hydrolysis products can be neglected), it may be inferred from the stoichiometry of reactions 2 and 4 that

$$r_{Glu} = r_h \tag{11}$$

$$r_{Glu\ +\ G1P} = r_h + r_p \tag{12}$$

Therefore,

$$r_p = r_{Glu\ +\ G1P} - r_{Glu} \tag{13}$$

and

$$f = \frac{r_{Glu\ +\ G1P} - r_{Glu}}{r_{Glu\ +\ G1P}} \tag{14}$$

RESULTS

Cell extracts prepared from *C. thermocellum* cultures grown with either Avicel or cellobiose as the substrate were used to catalyze simultaneous phosphorolytic and hydrolytic cleavage of cellobiose or cellopentaose at 60°C. Samples taken at 5-minute intervals were boiled for 5 min, and HK-G6PDH was used in the presence of PGM to assay either for glucose or for glucose and G1P in combination. Assays involved spectrophotometric measurement of NADH formation and were carried out at 37°C to avoid denaturation of the enzymes (PGM, HK, and G6PDH) used in the assay that originate from mesophiles,

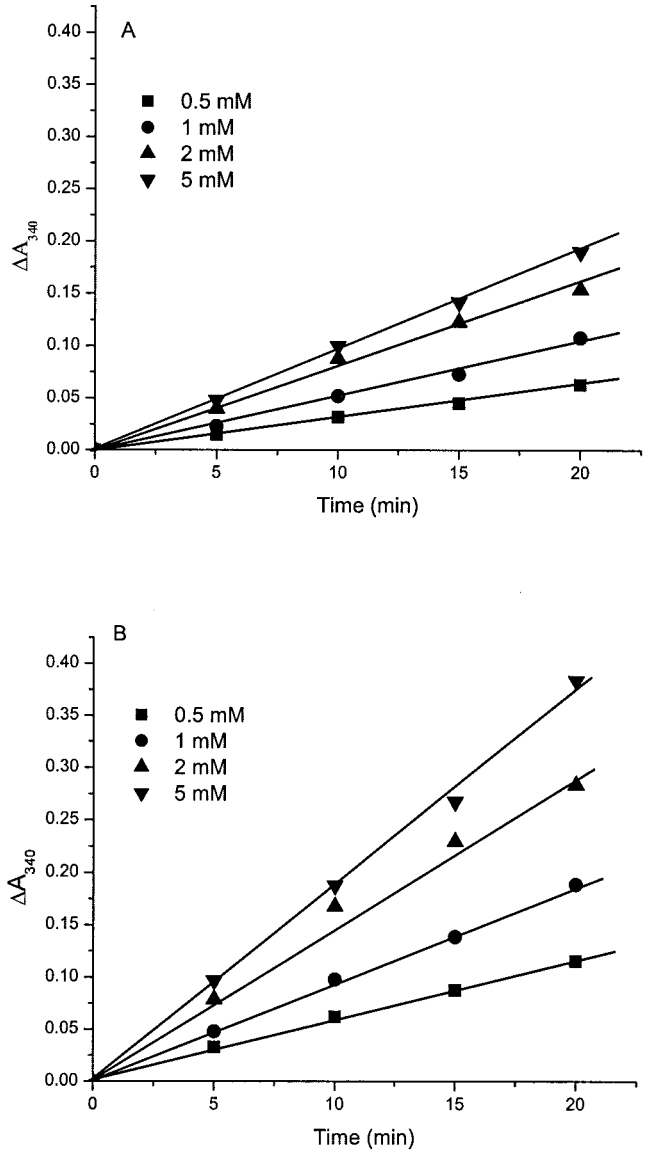

FIG. 1. Absorbance data for cellulose-grown cell extracts used to calculate the rate of glucose formation (r_1) (A) and the rate of glucose-plus-G1P formation (r_2) (B) from cellobiose.

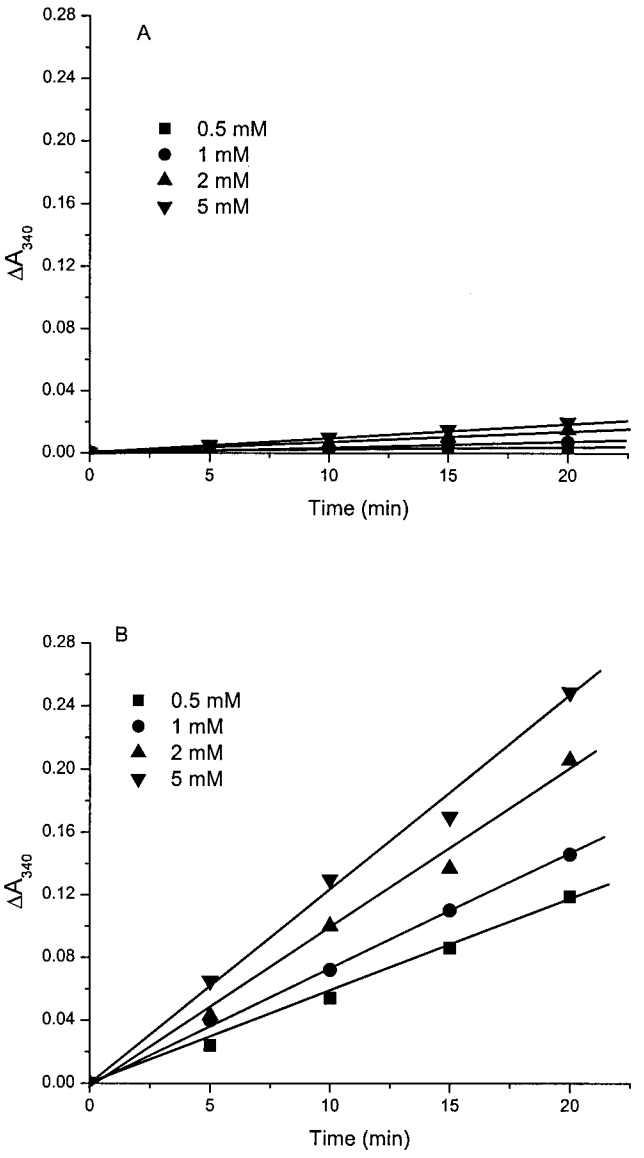

FIG. 2. Absorbance data for cellulose-grown cell extracts used to calculate the rate of glucose formation (r_1) (A) and the rate of glucose-plus-G1P formation (r_2) (B) from cellopentaose.

as would occur with incubation near the optimum temperature for *C. thermocellum*. Control experiments (data not shown) indicated that G1P and G6P were stable during the 5-min boiling period, although CdP, CbP, and βG activities were undetectable after boiling. G1P in boiled samples was only detectable in the presence of added PGM, and no PGM activity was detectable in *C. thermocellum* cell extracts under the conditions used (data not shown).

Absorbance data used to calculate relative rates of phosphorolytic and hydrolytic cleavage mediated by cell extracts from Avicel-grown *C. thermocellum* are presented in Fig. 1 and 2. The reaction of cellobiose to glucose is represented in Fig. 1A, with the reaction of cellobiose to glucose plus G1P represented in Fig. 1B. The reaction of cellopentaose to glucose is represented in Fig. 2A, with the reaction of cellopentaose to glucose plus G1P represented in Fig. 2B. Very little increase in absorbance was observed in Fig. 2A, indicative of correspondingly low rates of βG-mediated glucose formation. Linear trends of absorbance versus time were observed, with r^2 values > 0.99 for the data in Fig. 1 and 2B. P/R ratios $= [G_{n-1}][\text{G1P}]/\{[G_n][\text{P}_i]\}$ ($n = 2$ for cellobiose, $n = 5$ for cellopentaose) at 20 min (the last data point taken) were <0.003 under all conditions. Observed P/R values are thus far less than the equilibrium value of 0.25 for phosphorolytic cleavage, indicating that the chain-lengthening back reaction can be ignored under the conditions studied. Additional experiments were carried out that were identical to those whose results are presented in Fig. 1 and 2 except that cell extracts were prepared from cellobiose-grown cells rather than Avicel-grown cells. Primary data from

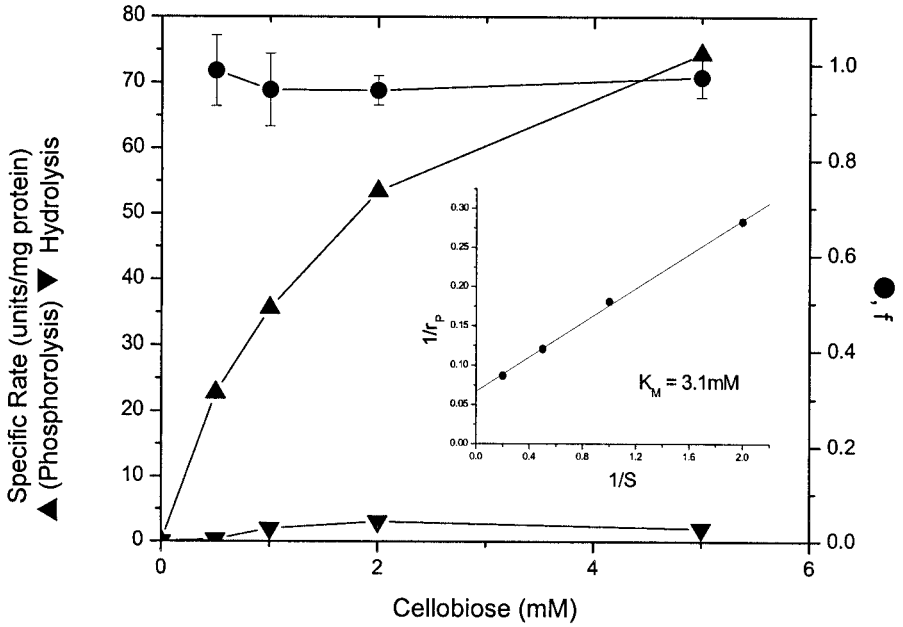

FIG. 3. Rates of β-glucosidic bond cleavage by phosphorolytic and hydrolytic mechanisms in Avicel-grown cell extracts of *C. thermocellum* with cellobiose as the assay substrate and the fractions of bonds cleaved phosphorolytically. Inset: double-reciprocal plot of inverse phosphorolytic cleavage rates versus inverse substrate concentrations.

these additional experiments are not shown, although summary results are presented subsequently (see Table 2).

Rates of phosphorolytic and hydrolytic cleavage are presented in Fig. 3 and 4 as functions of substrate concentration for cellobiose and cellopentaose, respectively. It may be observed that the rate of phosphorolytic cleavage greatly exceeds that of hydrolytic cleavage for both substrates over the range of concentrations tested. The fraction of reactions due to phosphorolytic cleavage, f (also shown in Fig. 3 and 4), is >0.9 for all conditions tested, with mean values of 0.963 for cellobiose and 0.952 for cellopentaose. For both substrates, rates of phosphorolytic reaction exhibit a linear trend on Lineweaver-Burke

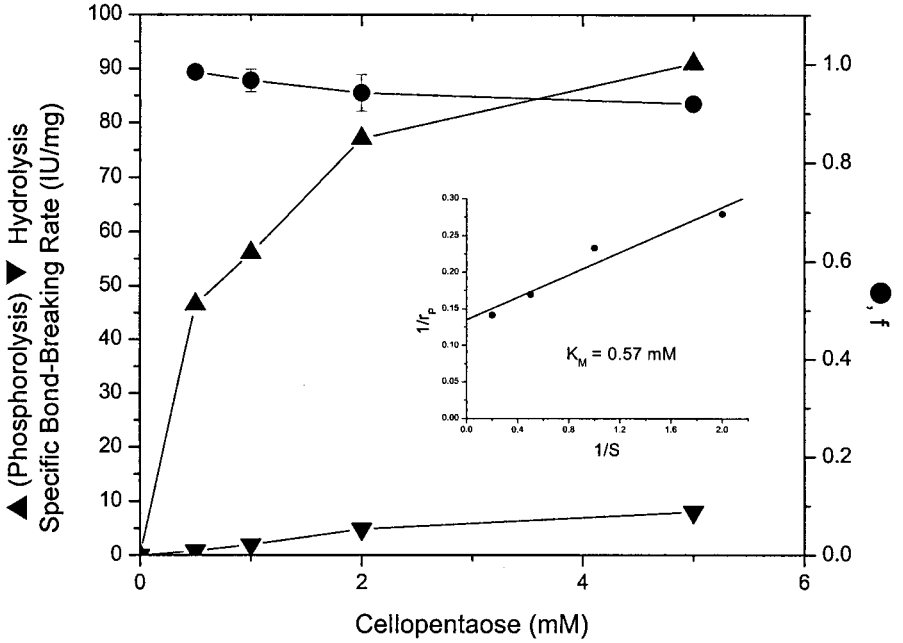

FIG. 4. Rates of β-glucosidic bond cleavage by phosphorolytic and hydrolytic mechanisms in Avicel-grown cell extracts of *C. thermocellum* with cellopentaose as the assay substrate and the fractions of bonds cleaved phosphorolytically. Inset: double-reciprocal plot of inverse phosphorolytic cleavage rates versus inverse substrate concentrations.

TABLE 1. Comparative kinetics with cellopentaose and cellotetraose as substrates[a]

Assay substrate	r_p (IU/mg)	r_h (IU/mg)	f
G_5	81.9	6.1	0.941 ± 0.017
G_4	85.9	5.6	0.958 ± 0.033

[a] All data represent results for Avicel-grown cells and a 2 mM cellodextrin concentration.

plots (see insets in Fig. 3 and 4), indicating that the reaction can be described by Michaelis-Menten kinetics.

Table 1 presents comparative results for reactions of cellotetraose and cellopentaose (both at an initial concentration of 2 mM) mediated by cell extracts prepared from Avicel-grown cells. It may be observed that rates of phosphorolytic cleavage, rates of hydrolytic cleavage, and f values are very similar for both substrates.

Average f values for the range of substrate concentrations examined, as well as values for V_{max} and K_m, are presented in Table 2 for reactions with cellobiose and cellopentaose mediated by cell extracts prepared from Avicel and cellobiose-grown cells. The average value of f is >0.95 for all conditions tested, corresponding to the rate of phosphorolysis exceeding the rate of hydrolysis by ≥20-fold. The V_{max} value for phosphorolytic cleavage of cellobiose is more than 2.2-fold higher for extracts prepared from cellobiose-grown cells than that for cell extracts prepared from Avicel-grown cells. The V_{max} value for phosphorolytic cleavage of cellopentaose is 26% lower for extracts prepared from cellobiose-grown cells than that for extracts prepared from Avicel-grown cells. Substantially greater affinity is exhibited for phosphorolytic cleavage of cellopentaose than for that of cellobiose.

It is of interest to compare the V_{max} values measured for cell extracts to the specific rates of substrate utilization observed in vivo (q) when both of these parameters are expressed in common units. For cultivation of *C. thermocellum* under the conditions investigated here, we have observed a maximum growth rate (μ_{max}) of 0.125 h^{-1} and a cell yield ($Y_{X/S}$) of 0.08 g of cells/g of substrate for growth on cellobiose and a μ_{max} of 0.10 h^{-1} and a $Y_{X/S}$ of 0.09 g of cells/g of substrate for growth on Avicel. On the basis of these data, values for $q = \mu/Y_{X/S}$ are found to be 1.56 g of substrate/g of cells^{-1} h^{-1} for cellobiose and 1.11 g of substrate·g of cells^{-1} h^{-1} for Avicel. After unit conversion with 0.5 g of protein/g of cells (31), these values correspond to 144 nmol of β-glucosidic bonds cleaved/min/mg of protein for cellobiose and 102 nmol/min/mg of protein for Avicel. These in vivo rates of phosphorolytic β-glucosidic bond cleavage are similar to the values observed in vitro using cell extracts: 214 nmol/min/mg of protein for cellobiose and 95 nmol/min/mg of protein for cellopentaose.

TABLE 2. f, V_{max}, and K_m values for extracts from Avicel- and cellobiose-grown cultures with cellobiose and cellopentaose as assay substrates

Growth substrate	Assay substrate	f[a]	V_{max} (nmol/min/mg of protein)	K_m (mM)
Avicel	G_2	0.963 ± 0.055	97	3.10
Cellobiose	G_2	0.992 ± 0.059	214	3.47
Avicel	G_5	0.952 ± 0.014	95	0.57
Cellobiose	G_5	0.923 ± 0.026	70	0.64

[a] Average f value for initial substrate concentrations from 0.5 to 5 mM.

DISCUSSION

We have shown that for both cellobiose and cellopentaose over a 10-fold range of β-glucan concentrations (0.5 to 5 mM) and for cellotetraose at a single concentration (2 mM), rates of phosphorolytic cleavage in Avicel-grown *C. thermocellum* cell extracts exceed rates of hydrolytic cleavage by ≥20-fold. The observation that the phosphorolytic activity observed in vivo is similar to that observed in the in vitro experiments reported in this paper is consistent with (although it does not unequivocally establish) cell extract data being relevant to what actually occurs in growing cells.

Studies by Lou et al. (26, 27) of *R. albus* and *P. ruminicola* measured activity at 37°C in the direction of chain shortening with CbP and CdP reaction products converted as they were formed in the presence of added enzymes. We undertook to measure activity at 60°C in the chain-shortening direction so that conditions as similar as possible to those occurring during fermentation of β-glucan substrates by *C. thermocellum* would be maintained. The assay procedure we developed (involving discrete sampling of a reaction carried out at 60°C followed by subsequent enzymatic analysis at 35°C) avoided several difficulties encountered with the use of the assay method of Lou et al. (26, 27) at elevated temperatures. These difficulties included rapid denaturation (half-life < 30 s) of PGM, HK, and G6PDH present in a Sigma glucose kit as well as strong UV absorbance of denatured PGM (data not shown).

The V_{max} values obtained for CbP and CdP activity in cell extracts suggest that these enzymes are synthesized by both cellobiose- and cellulose (Avicel)-grown cells but that they are also subject to some degree of metabolic control. For CbP, V_{max} values were over twofold higher for cellobiose-grown cells (214 nmol/min/mg of protein) than for Avicel-grown cells (97 nmol/min/mg of protein), a finding consistent with the general trend observed previously (6). Observed K_m values were substantially lower for cellopentaose (mean value for Avicel- and cellobiose-grown cells, 0.61 mM) than for cellobiose (mean value, 3.3 mM). These should be regarded as apparent K_m values, since they were measured at a given concentration of inorganic phosphate (8 mM) and it is expected in light of mechanistic considerations that the K_m for β-glucans will be a function of the phosphate concentration (15, 22). As reviewed elsewhere (29), the potential bioenergetic benefit from phosphorolytic bond cleavage is greater, and the bioenergetic cost of substrate transport is smaller, for cellodextins than for cellobiose. Thus, the greater affinity observed for phosphorylation of cellodextrins is consistent with, and may contribute to, preferred utilization of the more bioenergetically advantageous substrate. Our results are generally consistent with those of Alexander and Krishnareddy et al., who used enzymes purified from *C. thermocellum* assayed at 37°C to measure K_m values of 7.3 mM for CbP (6) and 0.8 mM for CdP (7, 24).

By contrast to the rather low K_m values we and Alexander observed for cellodextrins, K_m values for the two intracellular βGs synthesized by *C. thermocellum* are ≥70 mM (2, 3, 20). It

has been proposed that the physiological role of these βGs may be associated with nonfermentative functions (21, 35, 39) such as production of inducers for cellulase synthesis via transglycosylation (14), detoxification of aryl-glucosides (34), and hydrolysis of substrates other than linear β-glucans (34).

There is increasing evidence that a relative dominance of phosphorolytic compared to hydrolytic intracellular cleavage of β-glucans is widespread among cellulolytic anaerobic bacteria. Lou et al. (27) found that the rate of phosphorolytic cleavage of cellobiose in the cellulolytic *R. albus* B199 is ninefold faster than the rate of hydrolytic cleavage whereas phosphorolytic cellobiose cleavage in noncellulolytic *P. ruminicola* is threefold slower than hydrolytic cleavage (26). In addition, phosphorolytic cleavage of β-glucans is the only proposed mechanism for the trend of increasing cell yield with increasing oligosaccharide chain length observed with several cellulolytic microbes (29).

Our results provide more comprehensive support than that previously available for the proposition that phosphorolytic cleavage of cellobiose and cellodextrins dominates hydrolytic cleavage in *C. thermocellum*, although this has been suggested previously (28, 29, 42). For the purpose of understanding and modeling bioenergetics of carbohydrate metabolism in *C. thermocellum*, our results (together with those detailed in the prior literature) (28, 29) are consistent with a value for the parameter *f* (corresponding to the ratio of the rate of β-glucosidic bond cleavage to the combined rates of phosphorolytic and hydrolytic cleavage) of 1.

ACKNOWLEDGMENTS

This work was supported by grant DE-FG02-02ER15350 from the Department of Energy.

We are grateful to H. Strobel and P. Weimer for useful discussions.

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