CELLOBIOSE METABOLISM: A PATHWAY INVOLVING ADENOSINE 5'-TRIPHOSPHATE-DEPENDENT CLEAVAGE OF THE DISACCHARIDE

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Summary: Cleavage of cellobiose in Aerobacter aerogenes requires adenosine 5'-triphosphate and two protein fractions, the products of the reaction being equimolar amounts of D-glucose and D-glucose 6-phosphate. The two enzymes that participate in the overall reaction are identified as a β-glucoside kinase and a phospho-β-glucosidase.

Two different pathways for the metabolism of cellobiose, involving either hydrolysis (cellobiose → 2 glucose) or phosphorolysis (cellobiose → glucose + glucose 1-phosphate), are known to occur in various organisms (1-4). This paper presents evidence for a third pathway of cellobiose degradation (cellobiose → glucose + glucose 6-phosphate), which occurs in Aerobacter aerogenes PRL-R3. A unique feature of this pathway is that phosphorylation of the disaccharide utilizes ATP rather than phosphoenolpyruvate.

MATERIALS AND METHODS

Bacteriological procedures. The organism used was a uracil auxotroph of A. aerogenes PRL-R3. The mineral medium (5) was supplemented with 0.005% uracil and 0.5% cellobiose. The methods for cell growth and preparation of cell extracts were those used previously (5).

Assay for cellobiose-dependent conversion of ATP to ADP. The reaction mixture (0.15 ml) contained 10.0 μmoles of glycylglycine buffer (pH 7.5), 1.0 μmole of phosphoenolpyruvate, 0.05 μmole of NADH, 0.5 μmole of ATP, 1.0 μmole of $MgCl_2$, 5.0 μmoles of cellobiose, nonlimiting amounts of crystalline lactate dehydrogenase and pyruvate kinase, and the enzyme to be assayed. Controls to correct for NADH oxidase and adenosine triphosphatase activities contained all

of the reaction components except cellobiose. The rate of ADP formation was constant with time and proportional to the enzyme concentration in the ranges used. In this assay, cellobiose did not stimulate NADH oxidation in the absence of ATP.

Other enzyme assays. Cellobiase (cellobiose glucohydrolase; EC 3.2.1.21) and cellobiose phosphorylase (cellobiose:orthophosphate glucosyltransferase; EC 2.4.1.20) activities were assayed by measuring glucose formation with glucose oxidase (Glucostat reagents from Worthington Biochemicals). The reaction mixtures (0.15 ml) contained 10.0 μmoles of glycylglycine buffer (pH 7.5), 1.0 μmole of $MgCl_2$, 1.0 μmole of cellobiose, 0.01 ml of Glucostat enzyme, 0.01 ml of Glucostat chromogen, and cell extract from cellobiose-grown cells. For phosphorylase assays, the reaction mixture was supplemented with 2.0 μmoles of sodium phosphate, pH 7.5. The increase in optical density was measured at 430 nm. Cellobiose phosphorylase was also assayed by measuring NADPH formation in a phosphoglucomutase-glucose 6-phosphate dehydrogenase-linked reaction.

Analytical procedures. Protein was determined by the method of Lowry et al. (6). Spectrophotometric measurements were made with a Gilford absorbance-recording spectrophotometer thermostated at 25°. Microcuvettes with a 1.0-cm light path were used. Oxidation and reduction of pyridine nucleotide coenzymes were measured at 340 nm. Paper chromatography of sugars and sugar phosphates was done on Whatman No. 3MM (citric acid-washed) paper. The solvent was ethyl acetate-water-glacial acetic acid-formic acid (18:4:3:1, v/v), and the developing agent was ammoniacal silver nitrate. $R_{glucose}$ values were glucose 1-phosphate (0.25), glucose 6-phosphate (0.57), and cellobiose (0.44).

RESULTS

Absence of known pathways of cellobiose breakdown in extracts of A. aerogenes. No cellobiase or cellobiose phosphorylase activities could be detected in fresh extracts (< 0.2 nmole of product formed per minute per milligram of protein).

ATP-Dependent cleavage of cellobiose. The first evidence for a pathway

involving an ATP-dependent cleavage of cellobiose was obtained when an attempt was made to assay for cellobiose hydrolysis by linking the reaction to hexokinase and glucose 6-phosphate dehydrogenase (Fig. 1). With the complete reaction mixture, shown by curve 1, NADP reduction did occur, and it was ATP-dependent, which is consistent with the hydrolytic cleavage of cellobiose. However, as shown with curve 2, when ATP was added after 9 minutes to a control minus ATP, NADP reduction occurred at the same rate as in curve 1. If free cellobiose were being cleaved hydrolytically, D-glucose should have accumulated during the 9-minute incubation without ATP, and the slope of curve 2 should have been that of curve 3, which had both D-glucose and ATP added at 9 minutes. These data indicated that the cleavage of cellobiose was somehow dependent on the presence of ATP.

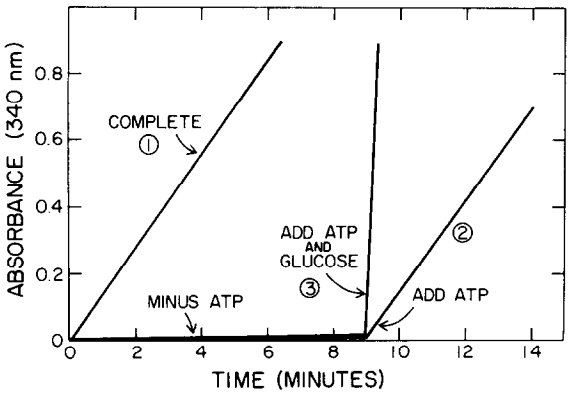

Fig. 1. Dependence of cellobiose cleavage on ATP. The complete reaction mixture (0.15 ml) contained 10.0 μmoles of glycylglycine buffer (pH 7.5), 1.0 μmole of cellobiose, 0.5 μmole of ATP, 0.10 μmole of $MgCl_2$, 0.1 μmole of NADP, crude extract (0.34 mg protein) from cellobiose-grown cells, and non-limiting amounts of hexokinase and glucose 6-phosphate dehydrogenase. In the controls lacking ATP, ATP (curve 2) and ATP + 1.0 μmole of D-glucose (curve 3) were added at 9 minutes. An absorbance value of 1.0 is equivalent to 0.024 μmole of NADPH.

The components responsible for the ATP-dependent cleavage of cellobiose were separated into two fractions by chromatography of the crude extract on Sephadex G-100 (Fig. 2). When individual fractions collected from the column were assayed for the ATP-dependent cleavage of cellobiose by the same method as in Fig. 1, only a small percentage of the original activity was recovered (in

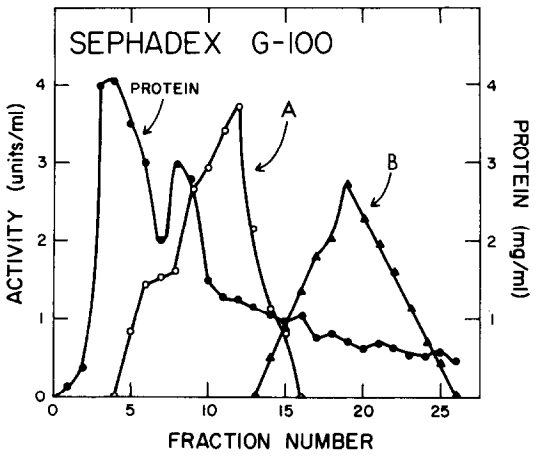

Fig. 2. Separation of the protein which catalyzes ATP-dependent cleavage of cellobiose into two fractions. A crude extract (20 ml containing 400 mg protein) from cellobiose-grown A. aerogenes PRL-R3 was chromatographed on a column (2 X 50 cm) of Sephadex G-100. Fractions (10-ml) were collected during elution with 0.02 M glycylglycine buffer. Component A was located with the assay for cellobiose-dependent formation of ADP from ATP. Component B was then located with the assay for ATP-dependent cleavage of cellobiose (Fig. 1) supplemented with a nonlimiting amount of component A.

fractions 14 and 15 of Fig. 2). However, when fractions on either side of 14 and 15 were combined, activity was restored, suggesting that the ATP-dependent cleavage of cellobiose involved two enzymes. One of the enzymes (component A on Fig. 2) was found to catalyze a cellobiose-dependent conversion of ATP to ADP, and thus its location in the fractions could be determined. The location of the second enzyme (component B on Fig. 2) was then determined by measuring the ATP-dependent conversion of cellobiose to D-glucose 6-phosphate in assays which were supplemented with nonlimiting amounts of component A.

When component B was omitted from the complete reaction mixture and then added in a nonlimiting amount after several minutes, NADP reduction proceeded rapidly until it reached a level equal to that in a cuvette which had the complete reaction mixture added at zero-time. The subsequent rate of NADP reduction was slower and equal to that in the control cuvette. This indicates that component A catalyzes the formation of an intermediate which can accumulate and serve as a substrate for component B.

Identification of reaction products. Fractions of components A and B which were free from glucokinase, phosphoglucomutase, and 6-phosphogluconate dehydrogenase were incubated in a cuvette (0.15 ml total reaction volume) with 0.010 μmole of cellobiose, 10 μmoles of glycylglycine buffer (pH 7.5), 0.5 μmole of ATP, 1.0 μmole of $MgCl_2$, 0.1 μmole of NADP, and a nonlimiting amount of glucose 6-phosphate dehydrogenase. The increase in optical density was equivalent to the formation of 0.010 μmole of glucose 6-phosphate. The further addition of hexokinase resulted in a further optical density increase which was equivalent to the formation of 0.01 μmole of D-glucose. Thus, the stoichiometry of the overall reaction sequence was determined to be the formation of 1 mole each of D-glucose and D-glucose 6-phosphate per mole of cellobiose. Identification of the products as glucose and glucose 6-phosphate in a scaled-up reaction was confirmed by paper chromatography.

DISCUSSION

This paper provides evidence for a new pathway of cellobiose metabolism which functions in A. aerogenes in lieu of, rather than in addition to, previously established pathways. The data show that the cleavage of cellobiose is ATP-dependent, with the products of the reaction being equimolar amounts of D-glucose and D-glucose 6-phosphate. The cleavage reaction requires two protein fractions which are separable on Sephadex G-100. One of the fractions can catalyze the cellobiose-dependent formation of ADP from ATP, with the accumulation of a product that serves as a substrate for the second enzyme. The inability of cellobiose to stimulate pyruvate formation from phosphoenolpyruvate indicates that phosphoenolpyruvate cannot substitute for ATP in the phosphorylation reaction. These data thus indicate that cellobiose is phosphorylated with ATP by a β-glucoside kinase (component A) to yield cellobiose phosphate, and that cellobiose phosphate is then cleaved hydrolytically by a phospho-β-glucosidase (component B) to yield D-glucose and D-glucose 6-phosphate. Data on the properties of these enzymes will be presented elsewhere.

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