Purification and properties of *Cellvibrio gilvus* cellobiose phosphorylase

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The cellobiose phosphorylase (EC 2.4.1.20) of *Cellvibrio gilvus*, which is an endocellular enzyme, has been purified 196-fold with a recovery of 11% and a specific activity of 27.4 μmol of glucose 1-phosphate formed/min per mg of protein. The purification procedure includes fractionation with protamine sulphate, and hydroxyapatite and DEAE-Sephadex A-50 chromatography. The enzyme appears homogeneous on polyacrylamide-gel electrophoresis, and a molecular weight of 280 000 was determined by molecular-sieve chromatography. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis revealed a single band and mol.wt. 72 000, indicating that cellobiose phosphorylase consists of four subunits. The enzyme had a specificity for cellobiose, requiring P_i and Mg^{2+} for phosphorylation, but not for cellodextrin, gentibiose, laminaribiose, lactose, maltose, kojibiose and sucrose. The enzyme showed low thermostability, an optimum pH of 7.6 and a high stability in the presence of 2-mercaptoethanol or dithiothreitol. The K_m values for cellobiose and P_i were 1.25 mM and 0.77 mM respectively. Nojirimycin acted as a powerful pure competitive inhibitor (with respect to cellobiose) of the enzyme ($K_i = 45 \mu\text{M}$). Addition of thiol-blocking agents to the enzyme caused 56% inhibition at 500 μM -*N*-ethylmaleimide and 100% at 20 μM -*p*-chloromercuribenzoate.

The presence of a cellobiose phosphorylase (EC 2.4.1.20) was first demonstrated in *Clostridium thermocellum* (Sih & McBee, 1955), then in two other bacteria, *Ruminococcus flavefaciens* (Ayers, 1958) and *Cellvibrio gilvus* (Hulcher & King, 1958a). Several properties of the partially purified cellobiose phosphorylase have been reported; the enzyme catalyses the reversible phosphorolysis of cellobiose:

It has specificity for α -D-glucose 1-phosphate, but not the β -form, and it is active towards ten different glucosyl acceptors, including D-glucose, D-xylose and D-mannose (Alexander, 1968). However, very little work has been done on this enzyme, and the purification procedure, physical properties and catalytic properties have yet to be elucidated. In the present paper we report the complete purification of cellobiose phosphorylase from *C. gilvus* and discuss

some relevant structural and catalytic properties of the purified enzyme.

Experimental

Materials

All reagents and biochemicals were obtained from commercial sources and were of the highest purity available. Hydroxyapatite, α -methyl β -glucoside, 6-phosphogluconate and *p*-nitrophenyl β -glucopyranoside were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). DEAE-Sephadex A-50 and Sephadex G-200 were a product of Pharmacia Fine Chemicals (Uppsala, Sweden). Ferritin, catalase, albumin, immunoglobulin G, cytochrome *c*, NADP (sodium salt), glucose 6-phosphate dehydrogenase and phosphoglucose isomerase were purchased from Boehringer (Mannheim, Germany). Protamine sulphate, gentibiose, cellobiose, glucono- δ -lactone, *p*-chloromercuribenzoic acid and *N*-ethylmaleimide were obtained from Wako (Tokyo, Japan). Laminaribiose, kojibiose, α -oxogluconate were provided by one of us (K. K.). Nojirimycin was generously given by Meiji Seika Co. (Tokyo, Japan). Cello-dextrins were prepared as described previously (Sasaki *et al.*, 1979).

Abbreviation used: SDS, sodium dodecyl sulphate.

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Cell growth

Cellvibrio gilvus (A.T.C.C. 13127) was grown at 30°C in a modification of the medium described by Hulcher & King (1958b), containing the following (g/litre): KNO₃, 0.5; NaCl, 0.5; MgCl₂, 0.25; K₂HPO₄, 1; (NH₄)₂SO₄, 0.2; FeSO₄, 0.01; case-amino acids, 5; yeast extract, 0.1; cellobiose, 2; and CaCO₃, 10. Cells were harvested in early stationary phase and washed with 50 mM-Tris/HCl buffer, pH 7.0.

Purification of cellobiose phosphorylase

A 10 g sample of *C. gilvus* was resuspended in 50 ml of a buffer consisting of 50 mM-Tris/HCl, pH 7.6, 1 mM-EDTA and 50 mM-2-mercaptoethanol and disrupted in a Bronson Sonifier (Danbury, CT, U.S.A.) at 4°C. Extracts were centrifuged for 30 min at 15000g and the supernatants were pooled. A 50 µl portion of 3% (w/v) protamine sulphate solution was added per ml of crude extract and the precipitate was removed by centrifugation. The enzyme was recovered by the addition of solid (NH₄)₂SO₄ at 4°C until the solution was 65% saturated. The precipitate was collected by centrifugation, dissolved in 5 ml of 50 mM-Tris/HCl buffer, pH 7.6, containing 10 mM-2-mercaptoethanol, and dialysed overnight against the same buffer. The dialysed fraction was applied to a hydroxyapatite column (2.5 cm × 30 cm) that had been previously equilibrated with 10 mM-KH₂PO₄/K₂HPO₄ buffer, pH 7.5, containing 10 mM-2-mercaptoethanol. The column was washed with about 300 ml of the same buffer and cellobiose phosphorylase was eluted with a linear gradient of KH₂PO₄/K₂HPO₄ buffer from 10 mM to 50 mM at pH 7.5, containing 10 mM-

2-mercaptoethanol. Fractions containing enzyme activity were recovered by the addition of solid (NH₄)₂SO₄ to 80% saturation. The precipitate was dissolved in 50 mM-Tris/HCl buffer, pH 7.5, containing 10 mM-2-mercaptoethanol and was dialysed against the same buffer. The enzyme fraction from the hydroxyapatite column was then applied to a DEAE-Sephadex A-50 column (2 cm × 50 cm) previously equilibrated with 50 mM-Tris/HCl buffer, pH 7.5, containing 10 mM-2-mercaptoethanol. Cellobiose phosphorylase was eluted with a linear gradient of NaCl from 100 mM to 500 mM in the same buffer (Fig. 1). Fractions showing activity were pooled, concentrated, and stored at -20°C.

Enzyme assays

Enzyme activity was assayed by measuring spectrophotometrically the formation of glucose 1-phosphate from cellobiose. The reaction mixture, in a total volume of 1.0 ml, consisted of 50 mM-Tris/HCl buffer, pH 7.5, 5 mM-MgCl₂, 5 mM-P_i, 5 mM-cellobiose, 0.2 mM-NADP, 5 mM-dithiothreitol, 5 µl of glucose 6-phosphate dehydrogenase, 5 µl of phosphoglucosmutase and 10 µl of enzyme solution. The measurements were made in a recording spectrophotometer for 5 min at 20°C. Enzyme activity was also assayed by P_i formation. P_i was determined by the method of Tashima & Yoshimura (1975) after precipitation of the protein with 0.1 M-trichloroacetic acid. One unit of activity was defined as 1 µmol of glucose 1-phosphate formed/min under the specified conditions. Specific activity was expressed as units per mg of protein. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Polyacrylamide-gel electrophoresis of purified enzyme

Polyacrylamide-disc-gel electrophoresis was performed by the method of Davis (1964), with gels containing 7.5% (w/v) acrylamide, and run in 50 mM-Tris/glycine buffer, pH 8.5, at 5 mA/gel tube. Gels were stained for protein with Coomassie Blue, and cellobiose activity was detected by incubating the gel in a solution containing 50 mM-glucose 1-phosphate and 50 mM-glucose for 1 h, washing the gel with water and finally incubating the gel in a solution of 30 mM-CaCl₂ until Ca₃(PO₄)₂ had been precipitated.

Molecular-weight determination

The molecular weight of cellobiose phosphorylase was determined by the gel-filtration method of Andrews (1964). Cellobiose phosphorylase was passed through a Sephadex G-200 column (1 cm × 90 cm) previously equilibrated with 50 mM-Tris/HCl, pH 7.5, containing 10 mM-2-mercaptoethanol. The column was calibrated with 2 mg each of ribo-

Fig. 1. Elution profile of cellobiose phosphorylase from *C. gilvus* on DEAE-Sephadex A-50

The column (2.0 cm × 50 cm) was equilibrated with 50 mM-Tris/HCl buffer (pH 7.5)/10 mM-2-mercaptoethanol. The enzyme was eluted by using a linear gradient (----) of 0.1–0.5 M-NaCl in the same buffer at a constant flow rate of 20 ml/h. Fractions (2.5 ml) were collected and assayed for activity (●) and protein (A_{280} ,).

nuclease, chymotrypsinogen, ovalbumin, fructose-bisphosphate aldolase, catalase and ferritin dissolved in equilibrating buffer. The elution volume was plotted against the known molecular weight in a semilogarithmical plot and the molecular weight of enzyme was calculated as described by Andrews (1964).

Subunit molecular weight by gel electrophoresis

Polyacrylamide-gel electrophoresis in the presence of 0.1% SDS was performed by the method of Weber & Osborn (1969). A 10 µg sample each of bovine serum albumin, catalase, ovalbumin, fructose-bisphosphate aldolase and chymotrypsinogen were used as standards.

Results and discussion

Purification procedure and purity of the enzyme

The results of the purification procedure are summarized in Table 1. An overall yield of about 11% of the initial activity was obtained with an increase in specific activity of 196-fold. This enzyme was found to be quite labile during the latter stages

Fig. 2. Disc electrophoresis of cellobiose phosphorylase. The enzyme (20 µg) was electrophoresed within 7.5% polyacrylamide gel as described in the Experimental section. (a) Protein was stained with Coomassie Blue and scanned at 410 nm. (b) Cellobiose phosphorylase activity was detected by the formation of a calcium phosphate precipitate.

of purification. In the case of cellobiose phosphorylase from *Clos. thermocellum*, it was pointed out that occasionally large losses in activity occurred during purification (Alexander, 1961). The use of thiol reagent like 2-mercaptoethanol or dithiothreitol during purification was necessary to obtain acceptable recoveries in these steps. The storage of the purified enzyme at -20°C without thiol reagent could cause an appreciable loss of activity within 2 weeks. The purity of enzyme was monitored by polyacrylamide-gel electrophoresis. Electrophoresis in the presence of 2-mercaptoethanol showed a single band after staining with Coomassie Blue, which was coincidental with the band of enzyme activity (Fig. 2).

Physical properties of cellobiose phosphorylase

The relative molecular mass (M_r) of cellobiose phosphorylase estimated by chromatography on Sephadex G-200 was approx. 280 000 (Fig. 3). SDS/polyacrylamide-gel electrophoresis revealed the presence of a single band of protein and a molecular weight of 72 000 (Fig. 4), indicating that the enzyme consists of four subunits. The absorption spectrum of pure cellobiose phosphorylase showed a protein absorption maximum at 280 nm, but no sign of the presence of pyridoxal 5'-phosphate, which has an absorption in a region from 300 to 450 nm. This suggests that cellobiose phosphorylase does not contain pyridoxal 5'-phosphate as a cofactor. It is noteworthy that sucrose phosphorylase (Graves & Wang, 1972) and maltose phosphorylase (Kamogawa *et al.*, 1973) also gave negative results, although all the α-glucan phosphorylases so far isolated from animals, plants and micro-organisms contain firmly bound pyridoxal 5'-phosphate and are totally inactive without it (Graves & Wang, 1972). The enzyme showed low thermostability and was inactivated above 40°C. Complete inactivation occurred after a 10 min inactivation at 60°C.

Catalytic properties of cellobiose phosphorylase

Purified enzyme activity of cellobiose phosphorylase was assayed over the pH range 6.4–8.6 in 0.2 M-Tris/malate buffer. Optimum activity was found at pH 7.6, with a rapid decrease in activity both below and above this range. This value is higher

Table 1. Purification of cellobiose phosphorylase from *Cellvibrio gilvus*

Cellobiose phosphorylase activity is expressed as µmol of glucose 1-phosphate formed/min. The reaction was performed as indicated in the Experimental section.

Fraction	Protein (mg)	Activity (units)	Specific activity (units/mg of protein)	Purification (fold)	Yield (%)
Crude extract	136.5	19.8	0.1	1	100
Protamine sulphate	30.5	17.5	0.6	4.1	88.7
Hydroxyapatite	11.8	15.4	1.3	9.3	78.7
DEAE-Sephadex A-50	0.08	2.2	27.4	195.7	11.2

Fig. 3. Estimation of the molecular weight of the cellobiose phosphorylase by Sephadex G-200 gel-filtration chromatography

The purified enzyme was subjected to gel filtration on a column (1.5 cm $\times$ 90 cm) of Sephadex G-200, equilibrated and eluted with 50 mM-Tris/HCl (pH 7.5)/10 mM-2-mercaptoethanol/5 mM-(NH₄)₂SO₄/1 mM-EDTA. This column was calibrated with: 1, ferritin; 2, catalase; 3, aldolase; 4, bovine serum albumin; and 5, hen's-egg albumin. The elution position of the cellobiose phosphorylase (CP) is indicated (O).

Fig. 4. SDS/polyacrylamide-gel electrophoresis of the cellobiose phosphorylase

The enzyme (50 μ g) was electrophoresed as described in the Experimental section. Protein standards used to derive a molecular-weight estimate for the enzyme were: 1, immunoglobulin G; 2, bovine serum albumin; 3, catalase; and 4, aldolase. Abbreviation used: CP, cellobiose phosphorylase (O).

than that (pH 6.5) for the enzyme from *Clos. thermocellum*. Cellobiose, P_i and Mg²⁺ were required for the phosphorylation. Various cello-oligosaccharides and disaccharides were tested to determine of the substrate specificity of the enzyme. Cellodextrin was not a substrate for cellobiose phosphorylase, neither was there any activity in the crude extract towards this oligosaccharide, indicating that *C. gilvus* does not possess a cellodextrin phosphorylase. No activity was observed with the following disaccharides: gentiobiose (β -1,6), laminaribiose (β -1,3), lactose (β -1,4), maltose (α -1,4), kojibiose (α -1,2) and sucrose (α -1,4). These results show that the cellobiose phosphorylase of *C. gilvus* has a high specificity for cellobiose. The K_m values for cellobiose and P_i were 1.25 mM and 0.77 mM respectively. These values are lower than those (7.3 mM for cellobiose, 2.9 mM for P_i) previously reported (Alexander, 1961).

Inhibition of cellobiose phosphorylase

Table 2 shows the effect of various potential inhibitors on cellobiose phosphorylase. The enzyme activity was inhibited by nojirimycin (D-glucopiperidine) and other were weakly inhibitory. Nojirimycin is known as an inhibitor of β -glucosidase which is produced by *Streptomyces roseochromogenes* (Ishida *et al.*, 1967; Inouye *et al.*, 1968). It

Table 2. Effects of various glucose derivatives on cellobiose phosphorylase activity

The enzyme activity was assayed by measuring the release of P_i. Other details are given in the Experimental section.

Glucose derivative	Concn. (mM)	Inhibition (%)
Nojirimycin	0.5	40.0
	1.0	80.4
α -Oxogluconate	0.5	22.6
Glucono- δ -lactone	0.5	12.2
Methyl α -D-glucoside	0.5	9.6
6-Phosphogluconate	0.5	8.7
p-Nitrophenyl β -glucopyranoside	0.5	5.2

Table 3. Effects of thiol-specific inhibitors on cellobiose phosphorylase

The enzyme activity was assayed by measuring the release of P_i. Other details were as described in the text.

Inhibitor	Concn. (μ M)	Inhibition (%)
p-Chloromercuribenzoate	10	95.4
	20	100.0
N-Ethylmaleimide	50	13.0
	100	21.0
	500	56.5

inhibited approx. 80% of enzyme activity at a concentration of 1.0 mM. The inhibition was competitive with cellobiose as determined from a Dixon (1953) plot, and the K_i value was 45 μ M. Table 3 shows the effect of the thiol-blocking reagents on cellobiose phosphorylase. The enzyme activity with *N*-ethylmaleimide at 500 μ M resulted in a 56% inactivation; the enzyme was also inactivated completely by 20 μ M-*p*-chloromercuribenzoate. These results suggest that there are exposed residues essential for cellobiose phosphorylase activity.

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