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Note

A cellobiose phosphorylase from *Cellvibrio gilvus* recognizes only the β-D-form of 5a-carba-glucopyranose

Motomitsu Kitaoka [a], Seiichiro Ogawa [b] and Hajime Taniguchi [a,1]

[a] *National Food Research Institute, 2-1-2 Kannondai, Tsukuba, Ibaraki 305 (Japan)*
[b] *Department of Applied Chemistry, Faculty of Science and Technology, Keio University, 3-14-1 Hiyoshi, Kohoku-ku, Yokohama 223 (Japan)*

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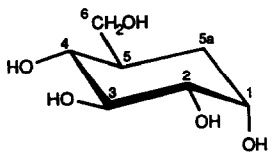
Cellobiose phosphorylase is an enzyme that catalyzes the reversible phosphorolysis of cellobiose to form α-D-glucose-1-phosphate (G-1-P). The enzyme is present in several microorganisms, such as *Clostridium thermocellum*[1], *Ruminococcus flavefaciens*[2], *Cellvibrio gilvus*[3], *Cellulomonas*[4,5], and *Fomes annosus*[6]. The enzyme from *Cellvibrio gilvus* was the first to be purified to an electrophoretically homogeneous state[7]. The authors found that its reaction followed an ordered bi-bi mechanism[8]. Contrary to a previous report[7], Mg^{2+} is not required for activity. In the reverse reaction, the enzyme can utilize some monosaccharides as the glucosyl acceptor instead of D-glucose[9]. Its specificity for the acceptor molecule was studied and the results suggested that the enzyme recognized the β-anomeric hydroxyl group of the acceptor D-glucose molecule[9]. However, the experiment could not clarify whether the enzyme also utilized α-D-glucose to any extent, because of mutarotation[9]. The experimental proof using D-glucose is not unequivocal because of the difficulty in estimating the anomeric ratio of D-glucose. Recently, Tsumuraya et al.[10] reported that a maltose phosphorylase reacted with only the α-pyranose of D-glucose, the reaction was performed at 0°C to minimize the effect of mutarotation. We have chosen a different approach to determine the anomeric specificity on the *C. gilvus* cellobiose phosphorylase.

Diastereoisomers of 5a-carba-aldohexopyranose (5-hydroxymethyl-1,2,3,4-cyclohexanetetrol) are carbocyclic analogues of aldohexopyranoses, the ring oxygen atom of which are substituted by a methylene group[11]. If these compounds act as substrate analogues of the respective aldohexopyranoses in the reverse reaction of the cellobiose phosphorylase, results would not be influenced by mutarotation. In

Correspondence to (present address): Dr. Motomitsu Kitaoka, Central Technical Research Laboratory, Nippon Oil Co. Ltd., 8 Chidori-cho, Naka-ku, Yokohama 231, Japan.
[1] Present address: Iwate Prefecture Brewing and Food Research Institute, 26 Tsushida, Morioka 020, Japan.



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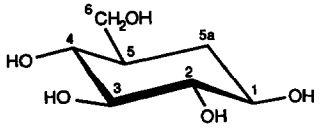

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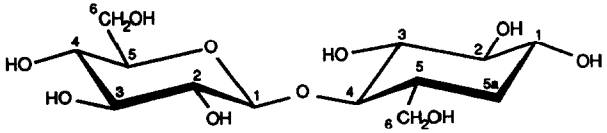

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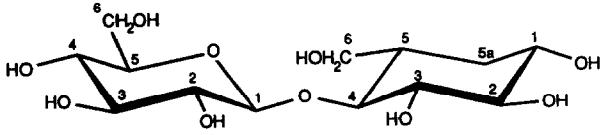

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the present paper, 5a-carba-glucopyranoses have been successfully used to determine the specificity of the anomeric hydroxyl group of the acceptor molecule on the cellobiose phosphorylase.

Preparations of 5a-carba-α-DL-glucopyranose [**1**, DL-(1,2,4/3,5)-5-hydroxymethyl-cyclohexanetetrol] and 5a-carba-β-DL-glucopyranose [**2**, DL-(1,3,5/2,4)-5-hydroxymethyl-cyclohexanetetrol] were previously reported[12]. 5a-Carba-β-D-glucopyranose[13] was prepared starting from an optically active intermediate to give **2**. The cellobiose phosphorylase from *Cellvibrio gilvus* was purified by the method of Kitaoka et al.[8] The enzymatic reaction of the cellobiose phosphorylase was done in 50 mM Tris · HCl buffer (pH 7.0) containing 0.02% bovine serum albumin at 37°C. One unit of activity was defined as the amount of enzyme which produced 1 μmol G-1-P from 10 mM cellobiose and 10 mM orthophospate (P_i). G-1-P was assayed by the phosphoglucomutase–glucose-6-phospate dehydrogenase system[14]. The initial rate in the reverse reaction (acceptor activity) was assayed by measuring the amount of released P_i from 10 mM G-1-P with a suitable concentration of a glucosyl acceptor using the method of Lowry and Lopez[15].

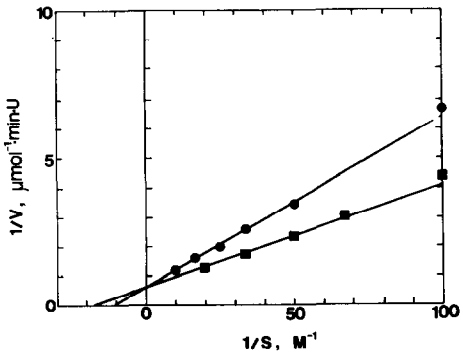

Fig. 1. A double-reciprocal plot of initial velocity versus the initial concentration of 5a-carba-β-DL-glucopyranose or D-glucopyranose: ●, DL-form; ■, D-form.

The acceptor activities of 20 mM both 5a-carba-DL-glucopyranoses were compared with that of equilibrated 10 mM D-glucose. As is expected, the β-form showed 22% acceptor activity compared with D-glucose. The α-form showed no acceptor activity (< 0.1%). Apparent kinetic parameters of the β form were determined to be $K_m = 93$ mM and $V_{max} = 1.6$ μmol/min · U (Fig. 1), whereas those for D-glucose were $K_m = 2.1$ mM and $V_{max} = 1.7$ μmol/min · U.[9] The K_m value increased ~ 44 times, but the V_{max} value did not change. This fact suggests that the substitution of the ring oxygen atom for the methylene group affects binding to the enzyme, but has no effect on enzymatic catalysis. The α-form (50 mM) did not inhibit the reaction with the β-form (5 mM), suggesting that the former did not bind to the enzyme. Kinetic parameters of the β-D-form were determined to be $K_m = 55$ mM and $V_{max} = 1.6$ μmol/min · U (Fig. 1). This suggests that only the D-form of 5a-carba-β-DL-glucopyranose participates in the reaction, because the D-form has a similar V_{max} value and approximately one-half the K_m of the racemate.

The following experiment was done to determine the structure of the product from G-1-P and 5a-carba-β-DL-glucopyranose in the reverse reaction. A reaction mixture (1 mL) containing 200 mM G-1-P, 200 mM 5a-carba-β-DL-glucopyranose, and 0.7 U/mL cellobiose phosphorylase was prepared in 100 mM Tris · HCl buffer (pH 7.0), and incubated at 30°C for 6 h. Concentration of P_i in the reaction mixture reached 67 mM, and a new spot corresponding to a disaccharide appeared on TLC. Then the mixture was boiled to inactivate the enzyme and chromatographed on a column of Toyopearl HW40S (Tohsoh, Tokyo, 2.5 × 40 cm) using distilled water as the solvent. Fractions containing the compound corresponding to a disaccharide were collected and lyophilized into 23 mg of a white powder. This compound showed no reducing power by the Somogyi[16]–Nelson[17] method. Its ^{13}C NMR spectrum was taken in D_2O on a JNM-GX260 spectrometer (Jeol, Tokyo) at 67.8 MHz using dioxane as an internal standard (67.4 ppm). Thirteen signals appeared on the spectrum and all the chemical shifts (Table I)

TABLE I

Assignments of ^{13}C chemical shifts [a] of the disaccharide product **3**.

5a-Carba-glucose residue		Glucosyl residue	
C-1	71.6	C-1	103.7
C-2	77.5	C-2	74.2
C-3	76.0	C-3	76.5
C-4	84.0	C-4	70.2
C-5	40.5	C-5	76.9
C-6	62.2	C-6	61.3
C-5a	32.4		

[a] Values are given in ppm.

corresponded to those of 4-*O*-β-D-glucopyranosyl-5a-carba-β-D-glucopyranose[18,19] (**3**). No signals[18] due to 4-*O*-β-D-glucopyranosyl-5a-carba-β-L-glucopyranose[19] (**4**) or other compounds were observed. From these facts and the kinetic analysis, only the D-form of 5a-carba-β-glucopyranose acts as the glucosyl acceptor of the enzyme.

The above results, obtained by using 5a-carba-glucopyranoses, although indirect, suggest that practically only the β anomer of D-glucose participates in the reverse reaction of the cellobiose phosphorylase. This study is also the first to demonstrate the application of pseudo-sugars to determine anomeric specificity on enzymes. In addition, this is the first example in which a purified enzyme utilizes one pseudo-sugar anomer as a substrate.

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