Role of non-covalent enzyme–substrate interactions in the reaction catalysed by cellobiose phosphorylase from *Cellulomonas uda*

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Steady-state kinetic studies of the enzymic glucosyl transfer to and from phosphate catalysed by cellobiose phosphorylase from *Cellulomonas uda* have shown that this enzyme operates by a ternary-complex kinetic mechanism in which β -cellobiose binds before phosphate, and β -D-glucose and α -D-glucopyranosyl phosphate are released in that order. α -D-Glucopyranosyl fluoride (but not β -D-glucopyranosyl fluoride) serves as alternative glucosyl donor for β -cellobiose synthesis with a specificity constant that is one-ninth that of the corresponding enzymic reaction with α -D-glucopyranosyl phosphate ($\approx 20000 \text{ M}^{-1} \cdot \text{s}^{-1}$ at 30 °C). The kinetic parameters for a complete series of deoxy and deoxyfluoro analogues of D-glucose have been determined and the data yield estimates of the net strengths of hydrogen-bonding interactions with the non-reacting hydroxy groups of D-glucose at the transition state (0.8–4.0 kcal/mol, where 1 cal $\equiv$ 4.184 J) and enable the prediction of the polarities

of these hydrogen bonds. Each hydroxy group functions as donor of a hydrogen for bonding to probably a charged (at 3-OH) or neutral (at 2-OH and 6-OH) acceptor group on the enzyme. The equatorial 1-OH is essential for enzyme activity. Derivatives of D-glucose in which the 1-OH or the reacting 4-OH were replaced by hydrogen or fluorine have been tested as inhibitors to measure their affinities for the sugar-binding subsite + 1 (numbered from the bond-cleaving/forming site). The data show that hydrogen-bonding interactions between the 1-OH and 4-OH and charged groups on the enzyme stabilize the ground-state ternary complex of the enzymic synthesis of β -cellobiose by 2.3 and 0.4 kcal/mol, respectively, and assist the precise positioning of β -D-glucose for catalysis.

Key words: binding energy, catalysis, deoxy sugar, fluoro sugar, glucosyl transferase.

INTRODUCTION

When classified according to their enzymic reactions, disaccharide phosphorylases constitute a heterogeneous family of glucosyl transferases which catalyse glucosyl transfer to and from phosphate. They use as the natural glucosyl donor a disaccharide of the form D-Glc-OR, where -OR is the leaving group, which can be D-glucose or D-fructose (see eqn 1):

Disaccharide phosphorylases can be separated into two mechanistic classes, based on whether the nucleophilic substitution at the anomeric carbon of D-Glc-OR occurs with retention or inversion of configuration. Invertive disaccharide phosphorylases can be classified further according to whether, in the direction of phosphorolysis of the disaccharide, the preferred orientation of the leaving group is axial or equatorial [1,2]. Cellobiose phosphorylase catalyses the reversible phosphorolysis of cellobiose [β -D-Glcp-(1 $\rightarrow$ 4)-D-Glc] into α -D-glucose 1-phosphate (Glc 1-P) and D-glucose [3–5] in an equatorial-to-axial reaction.

Cellobiose phosphorylase occurs in a number of bacteria capable of degrading cellulose or metabolizing soluble sugars of cellulolysis. It is thought to play an essential role in the energy-efficient catabolism of cellobiose in the cytoplasm [6]. Together with the related cellodextrin phosphorylase, it is a member of family 36 of the glycosyl transferase enzyme families ([7]; <http://afmb.cnrs-mrs.fr/~pedro/CAZY/db.html>). The enzyme is absolutely specific for cleaving and synthesizing β (1 $\rightarrow$ 4) glycosidic bonds, but has a relaxed specificity with respect to

the reducing sugar that functions as a glucosyl acceptor in the reverse reaction catalysed by the enzyme [5,8,9]. At present, structure/function relationships of cellobiose phosphorylase are not well understood, and factors contributing to enzymic catalysis have not been investigated in detail for this glycosyl transferase family.

A major question addressed by the present paper concerns the role of non-covalent interactions between cellobiose phosphorylase and the sugar substrate to achieve a stabilization specific to the transition state of the reaction. A widely held energetic concept of enzyme action invokes the use of interactions with groups on the substrate away from the site of chemical reaction to facilitate the catalytic step ([10], and see [11] for a recent critical discussion). In the case of cellobiose phosphorylase, it would seem that, considering the large number of hydroxy groups present in the substrates, hydrogen-bonding interactions are especially important to bring about specificity. Many of these interactions would be expected to be used by the enzyme to precisely position the substrate at the active site, thereby achieving a selective stabilization of the transition state by reducing the entropic barrier for reaction (for the general case, see [10]). A useful way to obtain estimates of the relative strengths and the polarities of the hydrogen-bonding interactions with individual hydroxy groups of the substrate at different points of the reaction co-ordinate is through kinetic studies with inhibitors and substrate analogues in which one hydroxy group has been replaced by hydrogen or fluorine. The dissociation constant of the protein–inhibitor complex or the catalytic efficiency of the enzymic reaction is measured, and the decrease in binding free energy at the ground state or the loss of transition-state stabilization

Abbreviations used: DCIP, 2,6-dichlorophenol-indophenol; Glc 1-P, α -D-glucose 1-phosphate.

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energy is calculated for each analogue, relative to the corresponding parent compound. The basis of the interpretation of the data is that substitution by hydrogen will remove all hydrogen bonds at the particular position. By contrast, fluorine may serve as weak hydrogen acceptor but not donor of a hydrogen for bonding (for a detailed discussion, see [12]). Therefore, when the fluoro analogue can partially restore interactions that are lost for the deoxy, a likely function of the original hydroxy group is that of a hydrogen-bond acceptor. However, possible electronic effects of the substitutions must be always considered. By using this approach, previous studies have characterized the role of hydrogen-bonding interactions with non-reacting hydroxy groups for reactions catalysed by glycosyl hydrolases [13,14] and glycogen phosphorylase [15].

D-Glucopyranosyl fluorides have been instrumental in studies of the catalytic mechanisms of glycosyl hydrolases (reviewed in [1,2,16]), phosphorylases [17–20] and, very recently, nucleotide sugar-dependent glycosyl transferases [21]. The present paper reports experiments that were designed to characterize (i) the utilization of D-glucopyranosyl fluorides as glucosyl donors for the forward and reverse reactions catalysed by cellobiose phosphorylase, and (ii) the hydrogen-bonding interaction of the enzyme with each hydroxy group of the glucosyl acceptor/leaving group (see eqn 1) at the +1 sugar-binding subsite, using the subsite nomenclature of Davies et al. [22].

MATERIALS AND METHODS

Micro-organism, enzymes and other materials

Cellulomonas uda DSM 20108 was obtained from Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Göttingen, Germany). 2-Deoxy-D-glucose, 3-deoxy-D-glucose, 4-deoxy-D-glucose, 6-deoxy-D-glucose and D-glucal were from Chemprosa (Graz, Austria). 5,6-Dideoxy derivatives of D-glucose in which the hydroxy groups at C-5 and C-6 had been replaced by fluorine or hydrogen [23] were gifts from Professor A. E. Stütz (Institute of Organic Chemistry, Technical University of Graz, Graz, Austria). 4-Thio-cellobiose and 1,5-anhydro-D-glucitol were from Toronto Research Chemicals (Toronto, Canada). Glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* and superoxide dismutase were from Sigma (Deisenhofen, Germany). Phosphoglucosyl transferase was from Roche (Mannheim, Germany) and glucose dehydrogenase was obtained from Amano (Milton Keynes, Beds., U.K.). Cellobiose dehydrogenase from *Sclerotium rolfsii* was a partially purified protein preparation lacking any detectable β -D-glycoside hydrolase activity. It was kindly provided by Dr Dietmar Haltrich in our laboratory in Vienna. V_2O_5 was obtained from Sigma. Other chemicals and materials were of the highest purity available from Merck (Darmstadt, Germany), Sigma and Fluka (Buchs, Switzerland).

Enzyme production and purification

Ce. uda was cultivated routinely in media described by the supplier DSMZ. For the production of cellobiose phosphorylase, the medium and the cultivation conditions reported previously by Schimz et al. [24] were used. Cellobiose (5%, w/v) was the sole carbon source. Enzyme purification was carried out at 4 °C using 10 mM Mes buffer, pH 6.8, containing 10 mM $MgCl_2$ and 5 mM dithiothreitol. The procedure used is reported here briefly, and full details can be obtained from the corresponding author (B. N.) upon request. Purification was composed of four steps: (i) anion-exchange chromatography using Fractogel EMD DEAE (S) (Merck); (ii) hydroxyapatite chromatography using a

Macro-Prep ceramic hydroxyapatite type II from Bio-Rad (Hercules, CA, U.S.A.); (iii) hydrophobic interaction chromatography using Fractogel EMD Phenyl I (S) (Merck); and (iv) gel filtration using a column (1.6 cm $\times$ 70 cm) of Superose 12 prep grade (Amersham Pharmacia Biotech). The purified enzyme migrated as a single band in SDS/PAGE, non-denaturing anionic PAGE and analytical gel-filtration chromatography. It was a monomer with an apparent molecular mass of 90 ± 2 kDa.

Synthesis of substrate analogues

Analogues of D-glucose carrying a substitution $OH \rightarrow F$ at positions 2, 3, 4 or 6, the α - and β -anomers of D-glucopyranosyl fluoride and 2-deoxy-2-fluoro- α -D-glucopyranosyl fluoride were synthesized by reported protocols [25]. 2-Deoxy-2-fluoro-Glc 1-P was synthesized by following a general procedure reported previously [26]. All compounds were characterized fully by melting points and analysis of 1H -, ^{13}C - and ^{19}F -NMR spectra. Structures of sugar derivatives used in this study are summarized in Figure 1.

Assays

To assay phosphorylase activity the activity of cellobiose phosphorylase was measured routinely at 30 °C and pH 6.6 (20 mM Mes buffer) in the direction of phosphorolysis using a continuous coupled-enzyme assay, essentially identical with assays described previously for measuring the activities of other phosphorylases [27]. The concentrations of cellobiose and potassium phosphate in this assay were saturating and 50 mM each.

Metabolite concentrations: D-glucose was measured by using an Eppendorf glucoanalyser, model EBIO compact (Eppendorf, Munich, Germany). Phosphate was determined colorimetrically according to Saheki et al. [28]. Glc 1-P was measured enzymically [27]. Cellobiose was determined by using cellobiose dehydrogenase, which oxidizes cellobiose into cellobionolactone and concomitantly reduces electron acceptors such as 2,6-dichlorophenol-indophenol (DCIP). The reaction was carried out at 30 °C in 50 mM sodium acetate buffer, pH 4.0, using 3 mM oxidized DCIP. After a 10-min incubation in the presence of enzyme, the reduced DCIP was monitored by its absorbance at 520 nm. The molar absorption coefficient of DCIP under these conditions is $6.8 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$. The enzymic assay was used to determine concentrations of cellobiose between 0.1 and 1.0 mM. Protein was measured by using the Bio-Rad dye-binding assay.

Substrate specificity and reactions with glucosyl analogues

A screening of alternative glucosyl donors and acceptors was carried out for the forward and reverse reactions catalysed by cellobiose phosphorylase (see eqn 1). This was done at 30 °C in 20 mM Mes buffer, pH 6.6. All reactants were employed at a constant concentration of 20 mM, using a total reaction volume of 300 μ l. They were incubated in the presence of enzyme (≈ 1 unit/ml) for a 24-h reaction time. TLC along with appropriate carbohydrate standards was the primary means of detecting product formation and substrate depletion. In the reverse reaction, release of phosphate from Glc 1-P was also measured and the production of cellobiose was determined enzymically using cellobiose dehydrogenase (see above). The α - and β -anomers of D-glucopyranosyl fluoride were tested as possible glucosyl donors in the forward and reverse reactions, and as glucosyl acceptors in the reverse reaction. The stabilities of D-glucopyranosyl fluorides to spontaneous hydrolysis in aqueous solution were determined in separate control reactions lacking the enzyme and D-glucose, and the uncatalysed formation of D-glucose from the fluorides

Figure 1 Structures of substrates and substrate analogues used in this study

D-Glucose (**1**), Glc 1-P (**2**), α-D-glucopyranosyl fluoride (**3**), 2-deoxy-2-fluoro-D-glucose (**4**), 4-deoxy-4-fluoro-D-glucose (**5**), 6-deoxy-6-fluoro-D-glucose (**6**), 2-deoxy-D-glucose (**7**), D-glucal (**8**), 1,5-anhydro-D-glucitol (**9**), cellobiose (**10**) and 4-thio-cellobiose (**11**).

was monitored by TLC and by using the enzymic assay. Under the conditions used significant hydrolysis of both fluorides, but particularly that of the β-anomer, occurred over a time course of 24 h. However, it did not interfere with the determination of initial velocities during a 15-min reaction (see below). Control reactions that lacked D-glucose but contained the enzyme were used to rule out significant enzyme-catalysed hydrolysis of the fluorides. D-Glucal was tested as a possible glucosyl donor of the forward and reverse reactions catalysed by cellobiose phosphorylase. In phosphorolysis the formation of any new product, but particularly that of 2-deoxy-Glc 1-P and the product of spontaneous hydrolysis of the sugar 1-phosphate, 2-deoxy-D-glucose, was monitored by using TLC analysis. Enzymic phosphorolysis of D-glucal to yield 2-deoxy-Glc 1-P has been shown for glycogen phosphorylases [17]. The ability of the enzyme to use D-glucal in place of Glc 1-P was determined in the presence of D-glucose, and transfer product formation was followed by TLC.

Kinetic measurements and calculation of kinetic constants

If not indicated otherwise, all measurements in the forward and reverse reactions catalysed by cellobiose phosphorylase were carried out at 30 °C in 20 mM Mes buffer, pH 6.6. Discontinuous assays were used. The reaction mixture containing substrates and enzyme (approx. 10 µg/ml) had a total volume of 300 µl and was

incubated under gentle mixing in an Eppendorf thermomixer model 5436. Samples (10 µl) were taken after incubation times of 5, 10 and 15 min, and used immediately for measurement by one of the aforementioned assays. The choice of the assay was dependent on the composition of the reaction mixture; however, if not mentioned otherwise, the measurements of D-glucose and phosphate were the basis for the determination of the initial velocities in the forward and reverse reactions, respectively. The initial velocities were calculated from the linear relationship of product concentration against reaction time. Control reactions lacking the enzyme or lacking one of the substrates were carried out in all cases, and the values reported are corrected for blank readings.

Initial velocities were fitted to eqn (2):

$$V = k_{\text{cat}}[E][S]/(K_m + [S]) \quad (2)$$

where V is the initial velocity, E and S are the enzyme and the varied substrate, respectively, k_{cat} is the turnover number and K_m is the Michaelis constant for S . A molecular mass of 90 kDa was assumed for E to calculate k_{cat} .

When inhibition occurred at high $[S]$, initial velocities were fitted to eqn (3):

$$V = k_{\text{cat}}[E][S]/(K_m + [S] + [S]^2/K_{\text{is}}) \quad (3)$$

where K_{is} is the substrate-inhibition constant. The non-linear fitting procedure of the program Sigmaplot 5.0 (Jandel Scientific,

Erkrath, Germany) was used to calculate the kinetic constants. Initial-velocity data obtained from inhibition studies are shown as double-reciprocal plots. Inhibition constants were obtained from non-linear fits of initial velocities to eqn (2), extended to account for competitive, uncompetitive or mixed non-competitive inhibition.

Enzymic reaction with arsenate and in the presence of vanadate

When arsenate replaced phosphate as glucosyl acceptor in the reaction of cellobiose phosphorylase with cellobiose, initial velocities were measured by using a discontinuous assay under otherwise identical conditions as described above. To avoid the uncatalysed reaction of arsenate with sugar hydroxyls to give esters ([29] and references therein), the reactants were mixed immediately before the enzymic reaction was started by adding the phosphorylase. Samples (10 μ l) were taken after incubation times of 5, 10 and 15 min, and the enzymic reaction was stopped by a short heat treatment at approx. 90 °C. The concentration of D-glucose in the samples was measured with the glucoanalyser. The initial velocities were calculated as described above. It was shown that the rate of formation of D-glucose was linearly dependent on the enzyme concentration (5–15 μ g/ml), so that under the conditions used the enzymic reaction limited the observed reaction rate (see the Results section). A stock solution of 20 mM NaH_2VO_4 was prepared by dissolving V_2O_5 in 1 M NaOH and diluting to volume. HCl (1 M) was used to adjust the pH to a value of 7. The yellow colour of the solution indicated that apart from the likely active monomeric vanadate, oligomeric forms of vanadate were also present [30]. The formation of esters of vanadate and sugars such as cellobiose probably occurs at a rate faster than that of the enzymic phosphorolysis of cellobiose [29]. Therefore when vanadate (in equilibrium with the available reactive hydroxy groups of the substrates) was used as inhibitor of the enzymic phosphorolysis of cellobiose, only a qualitative analysis of the inhibition pattern was carried out by using double-reciprocal plots, because the relative proportions of all vanadate species in the assays were not known. Superoxide dismutase (50 units/ml) was added during the determination of Glc 1-P to avoid possible interference by the superoxide radical [31].

Other analyses

The separation of the anomeric forms of D-glucose and cellobiose was done by HPLC at 4 °C as described recently [20]. TLC [32] and high-performance anion-exchange chromatography [20] were carried out by reported protocols.

RESULTS

Kinetic studies in forward and reverse reactions using the natural substrates

These were carried out because a basic set of kinetic parameters for the enzyme from *Ce. uda* was required, and it was essential to settle unambiguously the order of substrate binding and product release. Kinetic parameters for cellobiose phosphorylase were obtained from fits of eqns (2) or (3) to the initial-velocity data. They are summarized in Table 1. Substrate inhibition was observed when D-glucose or cellobiose was the varied substrate. Cellobiose is a competitive inhibitor against Glc 1-P, and Glc 1-P is a competitive inhibitor against cellobiose (Table 1). D-Glucose is a mixed-type inhibitor against cellobiose ($K_{ic} = 1.7 \pm 0.3$ mM; $K_{iuc} = 4.9 \pm 0.5$ mM; where K_{ic} and K_{iuc} are the competitive and uncompetitive inhibition constants, respectively) at saturating concentrations of phosphate (50 mM). 4-Deoxy-D-

Table 1 Kinetic parameters for the forward and reverse reactions catalysed by cellobiose phosphorylase

Experiments were carried out at 30 °C in 20 mM Mes buffer, pH 6.6. Results are fits of eqn (2) (or eqn 3, *) to the experimental data and are shown as means $\pm$ S.D. K_i values are enzyme–ligand dissociation constants determined from inhibition experiments. K_{is} values are substrate-inhibition constants.

Phosphorolysis	
k_{cat}^p	$29.1 \pm 2.3 \text{ s}^{-1}$
$K_i^{\text{D-cellobiose}}$	$14.8 \pm 2.3 \text{ mM}$
$K_m^{\text{D-cellobiose}}$	$4.3 \pm 0.5 \text{ mM}$
$K_m^{\text{phosphate}}$	$1.20 \pm 0.10 \text{ mM}$
$K_{is}^{\text{D-cellobiose}}$	$677 \pm 188 \text{ mM}$
Synthesis	
k_{cat}^s	$43.2 \pm 3.0 \text{ s}^{-1}$
$K_i^{\text{Glc1-P}}$	$3.20 \pm 0.32 \text{ mM}$
$K_m^{\text{Glc1-P}}$	$2.15 \pm 0.10 \text{ mM}$
K_m^{Glc}	$2.26 \pm 0.30 \text{ mM}$
K_{is}^{Glc}	$202 \pm 27 \text{ mM}$

glucose is a dead-end inhibitor which inhibits as a competitive inhibitor against D-glucose ($K_{ic} = 4.4 \pm 1.5$ mM, Figure 2A). In the presence of D-glucose in concentrations matching K_m^{Glc} , it inhibits uncompetitively against Glc 1-P with an inhibition constant of 24 ± 1 mM (Figure 2B). Note that there is no detectable synthesis of a disaccharide product by cellobiose phosphorylase when using 4-deoxy-D-glucose as a glucosyl acceptor, even at concentrations of up to 100 mM and reaction times up to 24 h. Vanadate is a competitive dead-end inhibitor against phosphate (Figure 2C) and shows uncompetitive inhibition of cellobiose (Figure 2D). These patterns of inhibition [33] strongly suggest the occurrence of two binary reactant complexes between enzyme and Glc 1-P, and enzyme and cellobiose. Uncompetitive inhibition results from the binding of dead-end inhibitors to these complexes. Therefore, the kinetic mechanism of cellobiose phosphorylase is ordered in the forward and reverse reactions, with cellobiose and Glc 1-P being the substrates binding first, respectively (Scheme 1).

The internal consistency of the kinetic parameters in Table 1 has been checked with the Haldane relationship for an Ordered Bi Bi kinetic mechanism [33]:

$$K_{eq} = (k_{cat}^p K_i^{\text{Glc1-P}} K_m^{\text{Glc}}) / (k_{cat}^s K_i^{\text{cellobiose}} K_m^{\text{P}}) \quad (4)$$

where k_{cat}^p and k_{cat}^s are for phosphorolysis and synthesis of cellobiose, respectively. A value of 0.27 was calculated for K_{eq} by using eqn (4) and the kinetic parameters in Table 1. It is in reasonable agreement with the value 0.22 ± 0.04 , which was determined experimentally at pH 6.6 and 30 °C for the thermodynamic equilibrium constant:

$$K_{eq} = [\text{Glc 1-P}][\text{Glc}] / [\text{cellobiose}][\text{phosphate}]$$

By using the relationships of the kinetic constants to microscopic rate constants [33], such as $k_7 = k_{cat}^s K_i^{\text{Glc1-P}} / K_m^{\text{Glc1-P}}$, the kinetic parameters in Table 1 allow one to calculate a value of 64 s^{-1} for k_7 , which is the rate constant for the dissociation of Glc 1-P. The turnover number in cellobiose phosphorolysis is 29 s^{-1} , which suggests that k_7 is partially rate-limiting for the

Figure 2 Dead-end inhibition in the reaction catalysed by cellobiose phosphorylase

(A and B) Inhibition pattern in the reverse reaction of cellobiose phosphorylase using 4-deoxy-D-glucose as the inhibitor and either D-glucose (A) or Glc 1-P (B) as the varied substrate. When kept constant, concentrations of D-glucose and Glc 1-P were 5 and 20 mM, respectively. The symbols denote the inhibitor concentrations: (A) ●, no inhibitor; ○, 2.0 mM; ▽, 10.0 mM; (B) ●, no inhibitor; ○, 1.4 mM; ▼, 7.1 mM; ▽, 14.2 mM. (C and D) Inhibition pattern in the forward reaction of cellobiose phosphorylase using vanadate as the inhibitor (●, no inhibitor; ○, 1.1 mM; ▼, 6.7 mM) and either phosphate (C) or cellobiose (D) as the varied substrate. When kept constant, concentrations of phosphate and cellobiose were 50 and 20 mM, respectively.

Scheme 1 Ordered kinetic mechanism of cellobiose phosphorylase from *Ce. uda*

overall reaction in this direction. At saturating concentrations of cellobiose and phosphate, 45% of the enzyme will thus be in the enzyme-Glc 1-P complex. An estimate of 100 s^{-1} was obtained for the first-order rate constant of the dissociation of cellobiose (k_2) by using $k_2 = k_{\text{cat}}^{\text{p}} K_1^{\text{cellobiose}} / K_{\text{m}}^{\text{cellobiose}}$. The value of k_{cat} is 43 s^{-1} in this direction. Therefore k_2 appears to be a major rate-limiting step in the overall reverse reaction catalysed by cellobiose phosphorylase. At saturating concentrations of Glc 1-P and D-glucose, 43% of the enzyme will be in the enzyme-cellobiose complex.

Stereochemical requirements pertaining to the anomeric hydroxy group

Analysis by HPLC shows that, upon enzymic phosphorolysis of cellobiose, β -D-glucose is produced (Figure 3). The data in Figure 3 also suggest that in an equilibrated mixture of α - and β -cellobiose, the β -anomer is preferentially degraded. To obtain firmer evidence on the specificity of the phosphorylase, solid β -cellobiose was dissolved in buffer immediately before the reaction was started by adding enzyme. The final concentration of cellobiose was 20 mM and saturating. Under these conditions, the production of Glc 1-P was linear with the reaction time and occurred at a rate which matched the reaction rate predicted from the value of k_{cat} . Therefore, the reaction catalysed by cellobiose phosphorylase is:

Enzyme-substrate interactions at the -1 sugar-binding subsite

The α -glycopyranosyl phosphates of D-xylose, D-mannose, D-galactose, D-glucuronic acid and 2-deoxy-2-fluoro-D-glucose are not turned over by the enzyme at measurable rates. α -D-Mannose

Table 2 Kinetic parameters for the reaction of cellobiose phosphorylase with different glucosyl acceptors

Experiments were carried out in 20 mM Mesbuffer, pH 6.6, using a constant saturating concentration of 50 mM Glc 1-P. Results are from non-linear fits of initial velocities to eqn (2), or eqn (3) when substrate inhibition occurred. $\Delta\Delta G^\ddagger$ is the Gibbs energy of activation for k_{cat}/K_m for the enzymic glucosyl transfer to an acceptor, relative to transfer to D-glucose. It was calculated according to $\Delta\Delta G^\ddagger = RT\ln[(k_{cat}/K_m)_{D-glucose}/(k_{cat}/K_m)_{acceptor}]$, whereby R is $1.987\text{ cal}\cdot\text{mol}^{-1}\cdot\text{K}^{-1}$ and T is 303.15 K ; n.d., not determined; n.a., not applicable.

Glucosyl acceptor	k_{cat} (s^{-1})	K_m (mM)	K_{IS} (mM)	k_{cat}/K_m ($\text{M}^{-1}\cdot\text{s}^{-1}$)	$\Delta\Delta G^\ddagger$ ($\text{kcal}\cdot\text{mol}^{-1}$)
D-Glucose	44.2 ± 3.1	2.26 ± 0.3	202 ± 27	19558	—
β -D-Glucose	47.6 ± 4.2	4.7 ± 1.4	108 ± 21	10128	n.a.
2-Deoxy-D-glucose	11.6 ± 1.1	24.3 ± 4.6	n.a.	477	2.24
2-Deoxy-2-fluoro-D-glucose	8.0 ± 0.7	17.2 ± 3.7	n.a.	465	2.25
D-Mannose	6.6 ± 0.6	27.3 ± 14.5	184 ± 4	241	2.65
D-Glucosamine	5.6 ± 0.3	10.8 ± 1.4	241 ± 31	518	2.19
3-Deoxy-D-glucose	0.1 ± 0.02	4.0 ± 0.5	n.a.	25	4.01
3-Deoxy-3-fluoro-D-glucose	n.d.	n.d.	n.a.	2.6	5.38
6-Deoxy-D-glucose	55.3 ± 2.1	10.2 ± 1.1	n.a.	5422	0.77
6-Deoxy-6-fluoro-D-glucose	44.6 ± 1.5	7.4 ± 0.8	n.a.	6027	0.71
D-Xylose	8.7 ± 1.4	15.9 ± 1.2	320 ± 66	547	2.15

Figure 3 Stereochemistry at the anomeric carbon during phosphorolysis of cellobiose by cellobiose phosphorylase

Reactions were carried out in 10 mM Mes buffer, pH 6.6, at 20 °C by using 10 mM phosphate and 33 mM cellobiose as substrates. α - and β -cellobiose were at equilibrium. Samples taken at the times indicated were analysed immediately by HPLC at 4 °C [20].

1-phosphate is a competitive inhibitor of Glc 1-P ($K_i = 2.1\pm 0.6\text{ mM}$).

Cellobiose phosphorylase catalyses the arsenolysis of cellobiose. The enzymic reaction appears to be effectively irreversible under the conditions used, since α -D-glucose 1-arsenate hydrolyses spontaneously to give free D-glucose and arsenate:

Initial velocities for arsenolysis of cellobiose were determined by using arsenate as the varied substrate while the concentration of cellobiose was constant and saturating (20 mM). Kinetic parameters were obtained by fitting eqn (2) to the experimental data. They are: k_{cat} , $13.2\pm 0.1\text{ s}^{-1}$; K_m^{arsenate} , $0.9\pm 0.1\text{ mM}$; $k_{cat}/K_m^{\text{arsenate}}$, $14667\text{ M}^{-1}\cdot\text{s}^{-1}$. Note that the reported k_{cat} refers to half of the observed value of the turnover number based on the release velocity of D-glucose (see eqn 6).

Ground-state binding of O-cellobiose and S-cellobiose

4-Thio-cellobiose, in which the bridging oxygen between the two glucose moieties is replaced by sulphur, was found not to be phosphorolysed by cellobiose phosphorylase. However, it is a relatively weak competitive inhibitor against Glc 1-P. The inhibition constant, $K_i^{\text{S-cellobiose}}$, is $13\pm 3\text{ mM}$. Thio-cellobiose is also a competitive inhibitor against O-cellobiose in the phosphorolysis direction of the reaction catalysed by cellobiose phosphorylase. In the presence of saturating concentrations of phosphate (50 mM), the inhibition constant of S-cellobiose is $12\pm 2\text{ mM}$.

Reactions with D-glucopyranosyl fluorides and D-glucal as alternative glucosyl donors

Neither the α - nor the β -anomer of D-glucopyranosyl fluoride is phosphorolysed by cellobiose phosphorylase to yield Glc 1-P.

However, α -D-glucopyranosyl fluoride is utilized by the enzyme as an alternative glucosyl donor in the direction of cellobiose synthesis, as shown in eqn (7):

The cellobiose produced by cellobiose phosphorylase according to eqn (7) co-migrates and co-elutes with authentic material in TLC and HPLC, respectively. It was found to be oxidized without lag-time by fungal cellobiose dehydrogenase, which is specific for (i) the β -anomeric form of cellobiose and (ii) $\beta(1 \rightarrow 4)$ glycosidic linkages (U. Baminger and D. Haltrich, personal communication). Initial velocities, based on measuring with cellobiose dehydrogenase the β -cellobiose produced, were determined by using α -D-glucopyranosyl fluoride as the varied substrate while the concentration of D-glucose was constant at 20 mM. Because substrate inhibition was observed at high concentrations of the fluoride, kinetic parameters were obtained by fitting eqn (3) to the experimental data. Values obtained for k_{cat} , K_m and the catalytic efficiency are $7.2 \pm 0.1 \text{ s}^{-1}$, $3.2 \pm 0.7 \text{ mM}$ and $2250 \text{ M}^{-1} \cdot \text{s}^{-1}$, respectively. The substrate inhibition constant is 30 mM. α -D-Glucopyranosyl fluoride is a competitive inhibitor of cellobiose ($K_i = 3.8 \pm 0.2 \text{ mM}$). The β -anomer of glucopyranosyl fluoride is not a glucosyl donor of the reverse reaction catalysed by cellobiose phosphorylase. In the presence of D-glucose it was found not to be converted into cellobiose or another product that would have been detectable by TLC analysis. In addition, the enzyme did not use at measurable reaction rates 2-deoxy-2-fluoro- α -D-glucopyranosyl fluoride as a glucosyl donor in place of Glc 1-P.

The possible reaction of cellobiose phosphorylase with D-glucal is expected to involve first the protonation of the endocyclic double bond (see Figure 1) followed by transfer of the 2-deoxy-glucopyranosyl moiety to phosphate or another acceptor such as D-glucose. This did not occur to a measurable extent (see the Materials and methods section for analyses used).

Enzyme–substrate interactions at the +1 sugar-binding subsite

Alternative glucosyl acceptors

Table 2 reports kinetic parameters for the reaction of cellobiose phosphorylase with different glucosyl acceptors, using Glc 1-P at a constant and saturating concentration. Derivatives of D-glucose in which the hydroxy group at C-2 or C-6 was replaced by fluorine or hydrogen were converted at robust rates. Overall, these deoxy derivatives are poorer substrates than the parent sugar, and the effects expressed in the catalytic efficiencies are distributed between a 7-fold increase in K_m and an ≈ 8 -fold decrease in k_{cat} . Enzymic reactions performed with derivatives of D-glucose that were fluorinated or deoxygenated at C-3 took place with catalytic efficiencies that were 1/4600 and 1/840 times the corresponding values of k_{cat}/K_m for the reaction with D-glucose, respectively. The following derivatives of D-glucose were not detectably utilized as glucosyl acceptors for the reverse reaction of cellobiose phosphorylase: 1,5-anhydro-D-glucitol, α -D-glucopyranosyl fluoride, β -D-glucopyranosyl fluoride, 2-deoxy-2-fluoro- α -D-glucopyranosyl fluoride, 4-deoxy-D-glucose, 4-deoxy-4-fluoro-D-glucose, D-glucal and 5,6-dideoxy-D-glucofuranose. Note that the rates detectable by the experimental procedures set an approximate limit to the second-order rate constant for the reaction of cellobiose phosphorylase with these compounds, and this is equal to or smaller than 0.001 % of k_{cat}/K_m for the reaction of the enzyme with D-glucose. We

Table 3 Enzyme–ligand dissociation constants for the interaction of sugar-binding subsite +1 at the active site of cellobiose phosphorylase

Experiments were carried out in 20 mM Mes buffer, pH 6.6. Results are from inhibition studies in which [D-glucose] was varied and [Glc 1-P] was constant at 20 mM and saturating. $\Delta\Delta G^\circ$ values refer to the difference in binding free energy between the modified inhibitor and the parent compound. They were calculated according to $\Delta\Delta G^\circ = RT \ln[K_i^{\text{inhibition}}/K_i^{\text{Glc}}]$, whereby K_i^{Glc} is the dissociation constant of D-glucose, R is $1.987 \text{ cal} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$ and T is 303.15 K; n.a., not applicable. K_i^{Glc} was calculated by taking values from Table 1 and using the relationship [33], $K_i^{\text{Glc}} = K_i^{\text{Glc}} K_m^{\text{Glc1-P}} K_{\text{cat}}^{\text{P}} / K_i^{\text{Glc1-P}} K_{\text{cat}}^{\text{S}}$, where K_i^{Glc} is K_{Glc} ($4.9 \pm 0.5 \text{ mM}$) determined from inhibition experiments with cellobiose as the varied substrate and phosphate as the constant saturating substrate ($\approx 40 \times K_m^{\text{phosphate}}$).

Inhibitor	K_i (mM)	$\Delta\Delta G^\circ$ (kcal $\cdot$ mol $^{-1}$)
D-Glucose	2.2	—
α -D-Glucopyranosyl fluoride	$30 \pm 5^*$	1.6
β -D-Glucopyranosyl fluoride	$\geq 500^\dagger$	≥ 3.3
1,5-Anhydro-D-glucitol	$102 \pm 29^\ddagger$	2.3
4-Deoxy-D-glucose	4.4 ± 1.5	0.4
4-Deoxy-4-fluoro-D-glucose	$\geq 500^\dagger$	≥ 3.3
D-Glucal	No inhibition ‡	n.a.
5,6-Dideoxy-D-glucose	No inhibition	n.a.
5,6-Dideoxy-5,6-difluoro-D-glucose	No inhibition	n.a.

* Substrate-inhibition constant determined in the direction of cellobiose synthesis (eqn 7).

† Less than 10% inhibition was observed for the concentration range studied (0–50 mM inhibitor).

‡ Varied for the concentration range 0–200 mM.

observed substrate inhibition with D-glucose and a number of analogues of D-glucose (Table 2). The mixture of α - and β -D-glucose at equilibrium showed weaker substrate inhibition than pure β -D-glucose (Table 2).

Enzymic reaction mixtures containing 20 mM Glc 1-P and 20 mM acceptor (Table 2) were incubated until the level of phosphate release indicated that substrate depletion was approx. 10% or greater. Subsequent analysis of the mixtures by TLC showed that a single transfer product, migrating at the position of a disaccharide, had been synthesized by the enzyme in each case. Therefore, the kinetic parameters in Table 2 refer to a well-defined reaction in which only one, most probably $\beta(1 \rightarrow 4)$, glycosidic bond is formed.

Inhibitors

Analogues of D-glucose that were not detectably turned over by the enzyme were tested as inhibitors of cellobiose phosphorylase to determine their binding to the +1 subsite of the enzyme. The results are summarized in Table 3 and compared with the dissociation constant of D-glucose (K_d^{Glc}).

DISCUSSION

The main objectives of this study of cellobiose phosphorylase, a family-36 glycosyl transferase [7], were to (i) characterize the utilization of glucopyranosyl fluorides as alternative glucosyl donors of the enzyme-catalysed phosphorolysis and synthesis of cellobiose and (ii) determine the role of non-covalent interactions between cellobiose phosphorylase and its sugar substrate(s) to bring about specificity.

Utilization of D-glucopyranosyl fluorides

Cellobiose phosphorylase catalyses the synthesis of β -cellobiose from α -D-glucopyranosyl fluoride and β -D-glucose. In this newly recognized enzymic reaction the fluoride replaces Glc 1-P, and its steric course is analogous to the natural reverse reaction catalysed

by the phosphorylase. Like Glc 1-P, α -D-glucopyranosyl fluoride binds to the free enzyme and so it is assumed that the order of substrate addition to the enzyme remains unaltered when the fluoride is utilized in place of Glc 1-P. The closely similar dissociation constants of binary complexes of enzyme and α -D-glucopyranosyl fluoride and of enzyme and Glc 1-P suggest that, relative to the fluoride leaving group, phosphate does not provide significant extra stabilization of this ground-state complex. However, there is an approx. 9-fold specificity of cellobiose phosphorylase for the reaction with Glc 1-P over the corresponding reaction with α -D-glucopyranosyl fluoride.

In accordance with the widely held view of a direct displacement mechanism of action of inverting glycosyl transferases [1,2,16,34], cellobiose phosphorylase is expected to assist glycosidic bond cleavage in cellobiose by general acid catalysis, which probably involves a carboxylic active-site group. The attack of phosphate in an equatorial-to-axial reaction may involve general base catalysis by a suitably positioned likely carboxylate ion of the enzyme. In the direction of cellobiose synthesis the roles of the proposed catalytic groups would be reversed according to the principle of microscopic reversibility. In the enzymic reactions with α -D-glucopyranosyl fluoride and Glc 1-P, the two catalytic residues are expected to show a similar functional involvement. Their ability to assist the heterolytic cleavage of the C-1–F bond provides evidence of functional flexibility of the catalytic machinery of cellobiose phosphorylase.

The absence of detectable enzyme activity with β -D-glucopyranosyl fluoride stresses the stereochemical requirements of the phosphorylase pertaining to the interaction with the glucosyl donor. β -D-Glucopyranosyl fluoride and β -D-glucose 1-phosphate do not show detectable binding to the –1 subsite of cellobiose phosphorylase, suggesting that the α -anomeric form of the glucosyl donor is required to position the substrate in the active site. Our results and the results of Tsumuraya et al. [18] with maltose phosphorylase, which can accept β -D-glucopyranosyl fluoride as a substrate in place of β -D-glucose 1-phosphate, suggest that the ability of several inverting glycoside hydrolases to utilize different anomeric forms of the fluoride substrate (yet via very different mechanisms [16]) is probably not found with disaccharide phosphorylases. This conclusion is also supported by the absence of detectable enzyme activity of cellobiose phosphorylase (this study) and maltose phosphorylase [18] towards phosphorylating D-glucal to produce the α - and β -anomers of 2-deoxy-D-glucose 1-phosphate, respectively.

Role of the anomeric hydroxy group of D-glucose for recognition and catalysis

The catalytic competence of cellobiose phosphorylase for glucosyl transfer from phosphate is critically dependent on interactions of the enzyme with the equatorial 1-OH of β -D-glucose. This finding is in agreement with the results of previous studies by Kitaoka et al. [35], who have shown that only the β -D-form of 5a-carbaglucopyranose serves as a glucosyl acceptor of the cellobiose phosphorylase from *Cellibrio gilvus*. Replacing the 1-OH of β -D-glucose by H or F results in a significant decrease in the affinity of the deoxy derivatives for the sugar-binding subsite +1 and a loss of detectable enzyme activity. The relatively weak binding of 1,5-anhydro-D-glucitol ($K_i = 102 \pm 30$ mM) and the much weaker binding of β -D-glucopyranosyl fluoride ($K_i \geq 0.5$ M), compared with the binding of D-glucose ($K_d^{\text{Glc}} = 2.2$ mM), suggest that recognition of D-glucose depends on interactions at the 1-OH, in which the hydroxy group participates as the donor of a hydrogen for bonding as follows. By avoiding the otherwise strongly non-favourable interaction of an electronegative atom (O or F) at C-

1 and a charged functional group on the enzyme, this hydrogen bond is proposed to affect a ≥ 230 -fold stabilization of the ground-state complex of the enzyme, Glc 1-P and β -D-glucose, relative to the corresponding complex in which β -D-glucopyranosyl fluoride replaces β -D-glucose.

In the absence of interactions other than those at the 1-OH, water would clearly out-compete D-glucose because water is present to great excess (55 M) and the capabilities to function as donor of a hydrogen for bonding are expected to be comparable for the 1-OH ($pK_a \approx 12.4$ [36]) and water ($pK_a \approx 16$). The 46-fold stronger binding of D-glucose relative to 1,5-anhydro-D-glucitol (see Figure 1) indicates a 46-fold-higher preference of the functional group on the enzyme to interact with the 1-OH than with 55 M water when 1,5-anhydro-D-glucitol is bound. (We assume that, overall, D-glucose and 1,5-anhydro-D-glucitol bind in an identical fashion to the active site of cellobiose phosphorylase.) Therefore, the estimate of the 'effective concentration' of the 1-OH group is $46 \times 55 \text{ M} = 2530 \text{ M}$ relative to the OH of water [10]. This value for the effective concentration reflects contributions from (i) the precise positioning of 1-OH in the active site by entropic fixation, and (ii) the positioning of 1,5-anhydro-D-glucitol so that it interferes with the ability of bulk water to hydrogen bond to the active-site functional group.

A greater affinity or reactivity of the deoxy sugar, relative to the fluoro, could indicate a loss of hydrogen-bonding interactions, which are available to the fluoro sugar in water but not in the complexes with the enzyme (see [12] for a detailed discussion). However, the hydrogen-bonding capability of fluorine with water is expected to show a relatively small dependence on the position of the substitution $\text{H} \rightarrow \text{F}$ in the sugar. In contrast, the kinetic data for deoxy and deoxyfluoro analogues of D-glucose (see Tables 2 and 3) have revealed a marked positional effect of fluorine, relative to hydrogen, pertaining to weakening the binding at the ground state or transition state of the reaction. That finding provides strong support for the suggestion that when the fluoro sugar has lower affinity or is a poorer substrate than the deoxy sugar, the observed effects are manifestations of true non-favourable interactions of the fluorine with functional groups on the enzyme. We note here also that electronic effects of substitutions $\text{OH} \rightarrow \text{F}$ and $\text{OH} \rightarrow \text{H}$ in D-glucose are quite difficult to predict for the reaction catalysed by cellobiose phosphorylase. Depending on the relative timing of proton removal from the nucleophilic oxygen atom at C-4 versus bond formation, partial negative charge or partial positive charge could develop at this reactive centre at the transition state. Electron-withdrawing substituents such as the fluorine atom near the 4-OH are expected to cause an increase in reaction rate when there is development of partial negative charge, as was observed for a number of β -glycosidase-catalysed reactions [1,2].

The absence of detectable enzyme activity with 1,5-anhydro-D-glucitol could suggest that hydrogen-bonding interactions of cellobiose phosphorylase and the equatorial 1-OH are required for the formation of a functional active site, an induced-fit mechanism. In an alternative interpretation of the data, the interactions at the 1-OH make a large, $\geq 100\,000$ -fold, contribution to specificity, equivalent to ≥ 7 kcal/mol of binding energy stabilizing the transition state ($1 \text{ cal} \equiv 4.184 \text{ J}$). Co-operative hydrogen bonding at the 1-OH involving several charged residues of the enzyme [37], or a low-barrier hydrogen bond [38], could contribute this or an even greater amount of transition-state stabilization energy, as has been shown for enzyme–substrate interactions of other glycosidases/glycosyl transferases [13,14,39]. We prefer this latter interpretation because it simply requires that interactions at the 1-OH which lead initially to the orientation of D-glucose at the active site,

concentrations of 50 mM were used, sets a lower limit of 0.5 M to the value of the binding constant of 4-deoxy-4-fluoro-D-glucose. The dissociation constant for 4-deoxy-D-glucose is closely similar to the dissociation constant for D-glucose. Therefore a hydrogen bond donated from the 4-OH to a probably charged acceptor group appears to chiefly prevent otherwise non-favourable electrostatic interactions between the 4-OH and the enzyme, and overall stabilizes weakly the ternary complex at the ground state by ≈ 0.4 kcal/mol (see Table 3). The most likely candidate suitably positioned for interaction with the 4-OH would be the catalytic base (presumably a carboxylate ion).

(iii) The transition states of the enzymic reactions with 3-deoxy-3-fluoro-D-glucose and 3-deoxy-D-glucose are destabilized by 5.4 and 4.0 kcal/mol, respectively, relative to the transition state of the same reaction with D-glucose. The effect on k_{cat}/K_m for the reaction of the enzyme with 3-deoxy-D-glucose is almost entirely expressed in the value of k_{cat} (see below). The involvement of a charged enzyme group involved as a hydrogen-bond acceptor at the transition state would explain the net strength of the observed interaction at the 3-OH. It could also explain the non-favourable interactions with the fluorine-substituted substrate.

(iv) Replacement of 2-OH by fluorine or hydrogen had a closely similar destabilizing effect on the transition state (≈ 2.2 kcal/mol). Therefore, interactions of the enzyme and this hydroxy group probably involve a hydrogen bond in which the 2-OH is the donor and a neutral group on the enzyme is the acceptor. Removal of this hydrogen bond caused a significant decrease in k_{cat} , by approx. 4- and 7-fold when the turnover numbers for the reaction of cellobiose phosphorylase with 2-deoxy-D-glucose and D-mannose, respectively, were used for comparison. The decrease in k_{cat} brought about by relatively small structural changes of the glucosyl acceptor, together with the fact that all effects on k_{cat}/K_m are expressed to a large extent in the value of k_{cat} , suggests that the chemical interconversion of the ternary enzyme-substrate complex contributes to rate limitation of the synthesis of cellobiose catalysed by cellobiose phosphorylase. Therefore chemical steps (possibly including a pre- and post-catalytic conformational change at the ternary-complex level) and dissociation of cellobiose seem to be partially rate-limiting steps in the overall reverse reaction of the enzyme. Considering rate-limiting chemistry, the results suggest differential hydrogen bonding of the 2-OH to specifically stabilize the transition state. In a 'uniform binding' case [44], all enzyme-bound states would be affected equally by the substitutions OH $\rightarrow$ H or OH $\rightarrow$ F at C-2 and, consequently, the values of k_{cat} remain unaltered relative to k_{cat} for the reaction with D-glucose.

(v) The replacement of the 6-OH by fluorine or hydrogen causes a weak and similar destabilization of the transition state by ≈ 0.8 kcal/mol, suggesting a weak interaction between the 6-OH and a neutral H-bond acceptor group on the enzyme. Upon removal of the hydroxymethyl group at C-6 a marked 36-fold decrease in k_{cat}/K_m is observed, relative to the corresponding catalytic efficiency for the reaction with D-glucose, and that equals a loss of binding energy in the transition state of 2.2 kcal/mol. Therefore, an estimate of 1.4 (2.2–0.8) kcal/mol can be obtained for the contribution of C-6 to a stabilization of the transition state, probably via hydrophobic interactions.

In conclusion, cellobiose phosphorylase utilizes all possible hydrogen-bonding interactions with the hydroxy groups of the substrate bound at the sugar-binding subsite +1, and probably subsite –1, to bring about specificity. Its ability to accept α -D-glucopyranosyl fluoride as alternative glucosyl donor in the reverse reaction is evidence of functional flexibility of the catalytic groups of the enzyme beyond the requirements of microscopic reversibility and points out mechanistic similarities of cellobiose

phosphorylase and maltose phosphorylase [18]. The strict (or at least very large) dependence of the catalytic competence of the enzyme on the presence of the 1-OH group at the reducing sugar is a clear manifestation of the theoretical concept of use of binding energy in enzymic catalysis.

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