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Research paper

Efficient chemoenzymatic oligosaccharide synthesis by reverse phosphorolysis using cellobiose phosphorylase and cellodextrin phosphorylase from *Clostridium thermocellum*

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ABSTRACT

Inverting cellobiose phosphorylase (CtCBP) and cellodextrin phosphorylase (CtCDP) from *Clostridium thermocellum* ATCC27405 of glycoside hydrolase family 94 catalysed reverse phosphorolysis to produce cellobiose and cellodextrins in 57% and 48% yield from α-D-glucose 1-phosphate as donor with glucose and cellobiose as acceptor, respectively. Use of α-D-glucosyl 1-fluoride as donor increased product yields to 98% for CtCBP and 68% for CtCDP. CtCBP showed broad acceptor specificity forming β-glucosyl disaccharides with β-(1→4)- regioselectivity from five monosaccharides as well as branched β-glucosyl trisaccharides with β-(1→4)-regioselectivity from three (1→6)-linked disaccharides. CtCDP showed strict β-(1→4)-regioselectivity and catalysed linear chain extension of the three β-linked glucosyl disaccharides, cellobiose, sophorose, and laminaribiose, whereas 12 tested monosaccharides were not acceptors. Structure analysis by NMR and ESI-MS confirmed two β-glucosyl oligosaccharide product series to represent novel compounds, *i.e.* β-D-glucopyranosyl-[(1→4)-β-D-glucopyranosyl]$_n$-(1→2)-D-glucopyranose, and β-D-glucopyranosyl-[(1→4)-β-D-glucopyranosyl]$_n$-(1→3)-D-glucopyranose (n = 1–7). Multiple sequence alignment together with a modelled CtCBP structure, obtained using the crystal structure of *Cellvibrio gilvus* CBP in complex with glucose as a template, indicated differences in the subsite +1 region that elicit the distinct acceptor specificities of CtCBP and CtCDP. Thus Glu636 of CtCBP recognized the C1 hydroxyl of β-glucose at subsite +1, while in CtCDP the presence of Ala800 conferred more space, which allowed accommodation of C1 substituted disaccharide acceptors at the corresponding subsites +1 and +2. Furthermore, CtCBP has a short Glu496-Thr500 loop that permitted the C6 hydroxyl of glucose at subsite +1 to be exposed to solvent, whereas the corresponding longer loop Thr637–Lys648 in CtCDP blocks binding of C6-linked disaccharides as acceptors at subsite +1. High yields in chemoenzymatic synthesis, a novel regioselectivity, and novel oligosaccharides including products of CtCDP catalysed oligosaccharide oligomerisation using α-D-glucosyl 1-fluoride, all together contribute to the formation of an excellent basis for rational engineering of CBP and CDP to produce desired oligosaccharides.

1. Introduction

Oligosaccharides are very important both as tools in investigations of the relationship between structure and function of carbohydrate-active enzymes and for a wide range of applications in medicine, nutrition and cosmetics. Challenges in chemical synthesis even of rather simple oligosaccharides make the chemoenzymatic approach a strategy of choice for cost-effective production of oligosaccharides. Useful catalysts include the cellobiose and cellodextrin phosphorylases (CBP, EC 2.4.1.20 and CDP, EC 2.4.1.49) of the inverting glycoside hydrolase family 94 (GH94) [1], which

Abbreviations: CBP, cellobiose phosphorylase; CDP, cellodextrin phosphorylase; CtCBP, *Clostridium thermocellum* ATCC27405 cellobiose phosphorylase; CtCDP, *Clostridium thermocellum* ATCC27405 cellodextrin phosphorylase; DP, degree of polymerization; ESI-MS, electrospray ionization-mass spectrometry; ESI-MS, electrospray ionization-mass spectrometry; GH, glycoside hydrolase family; α-Glc1P, α-D-glucose 1-phosphate; α-Glc1F, α-D-glucosyl 1-fluoride; HPAEC-PAD, high-performance anion-exchange chromatography equipped with a pulsed amperometric detector; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance.

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reversibly catalyse conversion of cellobiose [β-D-glucopyranosyl-(1→4)-D-glucopyranose] and cellodextrins to α-D-glucose 1-phosphate (α-Glc1P), glucose and cello-oligosaccharides, respectively. CBP and CDP were first described from *Clostridium thermocellum* [2,3] and more bacterial enzymes have been reported from *Cellulomonas* [4–6], *Cellvibrio* [7,8], *Clostridia* [9–13], *Fibrobacter* [14,15], *Fomes* [16], *Prevotella* [17], *Ruminococcus* [18–20], and *Thermotoga* [21,22], which as part of the energy catabolism have the capacity to degrade cellulose and metabolize the soluble cellodextrins [5,6,9,11–13,15,16,18,20,21]. A number of GH94 CBP and CDP have been cloned, expressed and characterised [11,12,21–23]. The GH94 in addition includes chitobiose phosphorylase [24,25] and cyclic-1,2-glucan synthase [26] (http://www.cazy.org/CAZY [1]). Three-dimensional structures are reported of *Vibrio proteolyticus* chitobiose phosphorylase [27] and *C. gilvus* CBP [28] and they have an $(\alpha/\alpha)_6$-barrel fold similar to GH15 glucoamylase [29] and GH65 maltose phosphorylase [30], which constitute clan GH-L [1].

Chemoenzymatic synthesis using CBP and CDP takes advantage of the mechanism of inverting phosphorylases that resembles that of inverting glycoside hydrolases [27,28,31] involving a general acid/base-catalysed direct displacement reaction [32]. Nucleophilic attack by phosphate in phosphorylases (Fig. 1A) and by hydroxide derived from water deprotonated by the general base catalyst in hydrolases (Fig. 1B) is assisted by proton donation from the general acid catalyst to the glycosidic oxygen and leading to release of product with inverted anomeric configuration. In contrast to hydrolysis, phosphorolysis is a highly reversible reaction conferring the enzymes with ability to catalyse glycoside synthesis (Fig. 1C). In the case of GH94 this has been coupled with high β-(1→4)-regioselectivity [9,10,13,24,33,34]. The specificity for the donor bound at subsite −1 is narrow [2], whereas acceptor specificity for subsite +1 was broader as reported for a number of CBPs producing various β-(1→4)-linked glucosyl-oligosaccharides by reverse phosphorolysis [6,9,21,28,35–42]. In contrast, knowledge on acceptor specificity for both CDP and chitobiose phosphorylase of GH94 lags much behind [25,33,43,44].

An unprecedented efficient chemoenzymatic approach is the glycosynthase reaction introduced in 1998, in which a hydrolytically inactive catalytic nucleophile mutant of the retaining GH1 β-glucosidase from *Agrobacterium* sp. produced oligosaccharides from α-D-glycosyl 1-fluoride (α-Glc1F) of the opposite anomeric configuration of the natural substrate and various carbohydrate acceptors (Fig. 1D) [45]. The glycosynthase approach has been established for several retaining glycoside hydrolases [32] and recently a related strategy was developed for members of the inverting GH8 [46] and GH95 [47] (Fig. 1D) in which the Hehre resynthesis-hydrolysis mechanism was applied to catalytic base mutants [48,49]. Hehre and co-workers also used β-D-glucosyl 1-fluoride as donor instead of β-D-glucose 1-phosphate in reverse phosphorolysis catalysed by inverting GH65 maltose phosphorylase [50]. Recently, GH94 CBP from *Cellulomonas uda* was shown to use α-Glc1F as donor [6,51] and glucose as acceptor to generate cellobiose with high efficacy, as opposed to reverse phosphorolysis in which phosphate released from α-Glc1P acts as general base catalyst and thus causes oligosaccharide degradation (Fig. 1E).

The present work explores acceptor specificity and regioselectivity of *C. thermocellum* ATCC27405 GH94 CBP (CtCBP) and CDP (CtCDP) in reverse phosphorolysis using α-Glc1P as donor with a series of mono- and disaccharides as potential acceptors. CtCBP was demonstrated to catalyse synthesis of 8 β-glucosyl oligosaccharides in good yields with β-(1→4)-regioselectivity. Furthermore, the little investigated CtCDP is shown to produce two new types of β-linked glucosyl oligosaccharides of degree of polymerization (DP) 3–9 with sophorose and laminaribiose as acceptors with β-(1→4)-regioselectivity, as demonstrated by NMR. Finally,

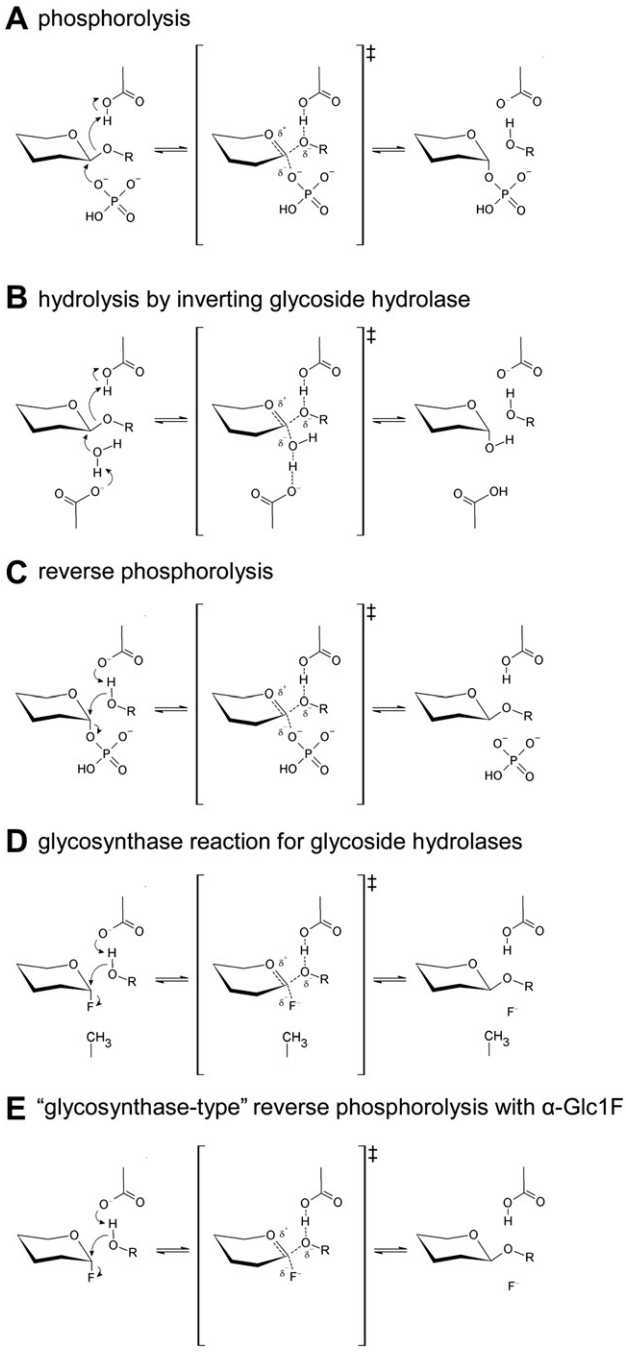

Fig. 1. Schematics of (A) reversible phosphorolysis; (B) hydrolysis by inverting glycoside hydrolase; (C) reverse phosphorolysis with α-Glc1P; (D) glycosynthase reaction by a catalytic nucleophile mutant of *e.g.* retaining GH1 glycoside hydrolase and catalytic base mutants of inverting GH8 and GH95 glycoside hydrolases. The mutated catalytic nucleophile or general base residue for retaining or inverting glycoside hydrolase, respectively, is shown as Ala; (E) "glycosynthase-type" irreversible phosphorolysis by *e.g.* inverting GH94 CBP with α-Glc1F. R indicates a carbohydrate moiety. See text for further explanation.

both CtCBP and CtCDP could use α-Glc1F to catalyse the irreversible glycosynthase-type reaction with accumulation of oligosaccharides in high yields. This study provides the first analysis on GH94 CDP acceptor specificity and use of α-Glc1F for chemoenzymatic oligosaccharide synthesis. Three-dimensional structural elements of CtCBP and CtCDP that control the acceptor specificity are indicated using multiple sequence alignment in combination with a modelled CtCBP structure, that has glucose bound at subsite +1.

2. Materials and methods

2.1. Carbohydrates

Carbohydrates were purchased from Sigma (St. Louis, MO, USA), except for xylose (Carl Roth, Karlsruhe, Germany), xylobiose, mannobiose, and laminaribiose (Megazyme, Bray, Ireland). 2,3,4,6-Tetra-*O*-acetyl-α-D-glucosyl fluoride (Peptides international, Jeffersontown, KY, USA) was deacetylated with sodium methoxide at R.T. for 45 min [52].

2.2. Cloning, expression, and purification of CtCBP and CtCDP

C. thermocellum ATCC27405 ABN51514 (GenBank accession no. gi:125713022) and ABN54185 (gi:125715693) were cloned by PCR from genomic DNA (kind gift of Preben Nielsen, Novozymes). Expand High Fidelity PCR System (Roche, Basel, Switzerland) was used as DNA polymerase with oligonucleotides constructed from the genomic sequence (completed by US DOE Joint Genome Institute): 5′-AA AGAGCTCAAGTTCGGTTTTTTTGATGATG-3′ and 5′-AAACCATGGCA TTACTAAAGTAACAGCGA-3′ as 5′ forward primers for ABN51514 and ABN54185 containing SacI and NcoI sites (underlined), respectively and 5′-TTTTCTAGATTATCCCATAATTACTTCAAC-3′ and 5′-TTT GCGGCCGCTTTAAACTTAAGAGTCACTATATG-3′ as 3′ reverse primers for ABN51514 and ABN54185 containing XbaI and NotI sites, respectively. The PCR products were purified (QIAquick Gel Extraction Kit; QIAGEN, Germantown, MD, USA), and that of ABN51514 was digested by SacI (Fermentas, St. Leon-Rot, Germany) and XbaI (New England Biolabs, Ontario, Canada) and cloned into pCold I (TaKaRa, Tokyo, Japan), while that of ABN54185 was digested by NcoI and NotI (New England Biolabs) and cloned into pET28a (Novagen, Madison, WI, USA). Plasmids were propagated in *Escherichia coli* DH5a (Novagen), purified (QIAprep Spin Miniprep kit, QIAGEN), and verified by sequencing (MWG Biotech, Ebersberg, Germany).

E. coli BL21(DE3) (Novagen) transformants harbouring the plasmids were grown at 12 °C in 3 L Luria–Bertani medium (1% tryptone, 0.5% yeast extract, 1% NaCl) containing 50 μg/mL ampicillin and 50 μg/mL kanamycin, respectively. Expression was induced by 0.1 mM isopropyl-1-thio-β-galactopyranoside and continued at 12 °C for 24 h. Cells were harvested by centrifugation (9000 × *g*, 10 min, 4 °C), resuspended in 30 mL BugBuster Protein Extraction Reagents (Novagen) containing 4 μL Benzonate Nuclease (Novagen) and incubated 30 min at R.T. followed by centrifugation (19000 × *g*, 15 min, 4 °C). The supernatant was filtered (GE Infrastructure Water & Process Technologies Life Science Microseparations, Trevose, PA, USA) and applied to a 5 mL HisTrap HP column (GE Healthcare, Bedford, UK) equilibrated with 20 mM HEPES pH 7.5, 0.5 M NaCl, 10 mM imidazole followed by washing with 20 mM HEPES pH 7.5, 0.5 M NaCl, 22 mM imidazole. Elution was performed by a linear 22–400 mM imidazole gradient in the same buffer and enzyme-containing fractions were pooled and dialyzed against 20 mM HEPES pH 7.0. All purification steps were performed at 4 °C. Protein concentration was determined spectrophotometrically at 280 nm using extinction coefficients $E^{0.1\%} = 1.42$ (CtCBP) and 2.39 (CtCDP) determined by aid of amino acid analysis. The molecular mass of the purified enzymes was estimated by SDS-PAGE (Coomassie Brilliant Blue staining) and gel filtration (HiLoad™ 200 Superdex™ 16/60 column; flow rate, 0.5 mL/min; ÄKTAexplorer, GE Healthcare) in 10 mM MES pH 6.8, 0.15 M NaCl using Gel Filtration Calibration kit HMW (GE Healthcare) as standards.

2.3. pH and temperature dependence of activity and stability of CtCBP and CtCDP

The pH optimum was determined for 0.16 μM CtCBP or 3.0 μM CtCDP towards 3 mM cellobiose at 37 °C by 10 min reaction in 0.1 M phosphate-citrate pH 5.3–8.5 and quantifying released glucose (glucose oxidase/peroxidase kit; Megazyme). The pH optimum of reverse phosphorolysis (10 min) was determined for 2.8 μM CtCBP and 5.1 μM CtCDP with 50 mM α-Glc1P as donor and 50 mM glucose and 50 mM cellobiose as acceptor, respectively, at 37 °C in 0.1 M sodium acetate pH 2.7–6.1 and 0.1 M Tris–HCl pH 6.5–9.5. Produced oligosaccharides were quantified by peak areas in high-performance anion-exchange chromatography equipped with a pulsed amperomeric detector (HPAEC-PAD; 3 × 250 mm Carbopac PA200 Anion Exchange Column; ICS-3000 Reagent-Free Ion Chromatography System; Dionex, Sunnyvale, CA, USA) using a linear 0–75 mM (CtCBP) or 0–175 mM sodium acetate gradient (CtCDP) in 100 mM NaOH (35 min, flow rate 0.35 mL/min) calibrated with commercial cello-oligosaccharides (degree of polymerization 2–6; Megazyme). pH stability at 4 °C for 24 h was assessed in 50 mM sodium acetate pH 3.1–6.0 and 50 mM Tris–HCl pH 6.1–9.4 for 0.16 μM CtCBP and 1.5 μM CtCDP by the residual phosphorolytic activity towards cellobiose. Temperature optimum of phosphorolysis (10 min) by 0.16 μM CtCBP and 3.0 μM CtCDP was determined in the range 20–90 °C towards 3 mM cellobiose at pH 3.9 and pH 5.4, respectively. Thermostability was expressed as residual phosphorolytic activity after 15 min incubation of 0.16 μM CtCBP or 3.0 μM CtCDP in the interval 20–90 °C.

Specific activity towards 3 mM cellobiose, cellotriose, sophorose, laminaribiose, gentibiose, and chitobiose was determined for 0.16 μM CtCBP in 0.1 M phosphate-citrate pH 5.3 at 60 °C and for 1.5 μM CtCDP in 0.1 M phosphate-citrate pH 5.7 at 40 °C incubated for 10 min followed by heat inactivation (15 min, 95 °C). Glucose and cellobiose were quantified by glucose oxidase/peroxidase reaction and HPAEC-PAD, respectively (see above). One unit of activity was defined as the amount of enzyme liberating 1 μmol glucose from cellobiose or 1 μmol cellobiose from cellotriose in 1 min at the above conditions.

2.4. Oligosaccharide synthesis by reverse phophorolysis

Donor specificity in reverse phosphorolysis was determined using 50 mM α-glycosyl 1-phosphates, *i.e.* α-Glc1P, α-*N*-acetyl D-glucosamine 1-phosphate, α-D-mannose 1-phosphate, and α-D-galactose 1-phosphate, with 50 mM glucose for 2.8 μM CtCBP or 50 mM cellobiose for 5.1 μM CtCDP in 100 mM Tris–HCl pH 7.0 at 37 °C. The reverse phosphorolysis was examined in detail using 10–200 mM α-Glc1P and 10–200 mM glucose for 2.5 μM CtCBP or 10–200 mM cellobiose for 4.4 μM CtCDP at optimum conditions, 20 mM sodium acetate pH 3.9 at 60 °C (CtCBP) and pH 5.4 at 40 °C (CtCDP). The acceptor specificity was determined at the above conditions and enzyme concentrations for 3 h using 200 mM α-Glc1P and 200 mM monosaccharide (glucose, mannose, galactose, talose, xylose, arabinose, fructose, L-fucose, L-rhamnose, *N*-acetyl-glucosamine, *N*-acetyl-galactosamine, *N*-acetyl-mannosamine), sugar alcohol (sorbitol, xylitol), or disaccharide (cellobiose, sophorose, laminaribiose, gentibiose, lactose, lactulose, melibiose, xylobiose, mannobiose, trehalose, maltose, isomaltose, sucrose, turanose, and arabinobiose). Products were quantified by HPAEC-PAD (see 2.3) using a linear 0–75 mM (CtCBP) or 0–175 mM sodium acetate (CtCDP) gradient in 100 mM NaOH (35 min, flow rate 0.35 mL/min) calibrated with commercial cello-oligosaccharides. Oligosaccharide yields were calculated based on the donor (α-Glc1P) concentration.

2.5. Oligosaccharide synthesis by glycosynthase-type reaction

Progress of oligosaccharide synthesis (0–3 h) from 10 to 50 mM α-Glc1F and either 10–50 mM glucose for 2.9 μM CtCBP or 10–50 mM cellobiose for 5.1 μM CtCDP at 40 °C in 100 mM Tris–HCl pH 7.0 was monitored for removed aliquots after inactivation

(15 min, 95 °C) by thin-layer chromatography (TLC; Silica gel 60 plate; Merck, Darmstadt, Germany) developed by acetonitrile/H_2O of 85:15 (v/v), sprayed by α-naphthol/sulphuric acid/methanol (0.03 : 15 : 85, w/v/v), and tarred at 120 °C. Products were quantified by HPAEC-PAD (see 2.4). Oligosaccharide yields were calculated based on the donor (α-Glc1F) concentration.

2.6. Isolation of oligosaccharides by HPLC

Products for purification were generated from 200 mM α-Glc1P and 200 mM acceptor (see Table 1and Supplementary Table 1) in 20 mM sodium acetate pH 3.9 with 2.5 μM CtCBP at 60 °C for 1 h (glucose as acceptor) or 3 h (other acceptors) or pH 5.4 with 4.4 μM CtCDP at 40 °C for 10 min (for isolation of initial products). Following heat inactivation (15 min, 95 °C) and desalting (Amberlite MB20; Sigma), products were purified by high-performance liquid chromatography (HPLC) equipped with a refractive index detector (Shodex RI-101; Showa Denko, Kanagawa, Japan) and a TSKgel Amide-80 column under a constant flow of 1.0 mL min^{-1} (see Supplementary Table 1 for mobile phase composition and column temperature). Fractions containing products were collected (Foxy Jr. Fraction collector; Teledyne Isco, Lincoln, Nebraska) and the purity verified by TLC. The concentration of each product was determined by the phenol-sulfuric acid procedure using glucose as standard [53].

2.7. Structure determination of oligosaccharides

Oligosaccharide samples (final volume 100 μL) were generated from A. 200 mM α-Glc1P and 200 mM acceptor (see Table 1) in 20 mM sodium acetate pH 3.9 and either 2.5 μM CtCBP at 60 °C for 1 h (glucose as acceptor) or 3 h (other acceptors) and pH 5.4 with 4.4 μM CtCDP at 40 °C for 10 min (for structural analysis of initial products) and B. 50 mM α-Glc1F and 50 mM glucose or 50 mM cellobiose in 100 mM Tris–HCl pH 7.0 and either 2.9 μM CtCBP for 5 h or 5.1 μM CtCDP for 30 min (for structural analysis of initial product) at 40 °C. The produced oligosaccharides were desalted (Amberlite MB20, Sigma) after inactivation (15 min, 95 °C).

Electrospray ionization-mass spectrometry (ESI-MS) analysis was performed using an LTQ XL ion trap MS (Thermo Scientific, San Jose, CA, USA) [54]. Samples were introduced through a Thermo Accela UHPLC system equipped with a Hypercarb column (100 × 2.1 mm, 3 μm; Thermo Scientific) eluted with a gradient of deionised water, acetonitrile and 0.2% trifluoroacetic acid (0.4 mL/min, 70 °C). MS detection was performed in the positive mode using a spray voltage of 4.5 kV, a capillary temperature of 260 °C, and auto-tuned on glucohexaose (*m*/*z* 1013).

Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker DRX 600 spectrometer (Bruker BioSpin AG, Fällanden, Switzerland) in 5 mm NMR tubes at 300 K. Relative amounts were obtained by integration of one-dimensional proton spectra. A series of 2D homo- and hetero-nuclear correlated spectra were obtained by Bruker standard COSY, NOESY, TOCSY, HSQC and HMBC spectra. The following parameters were used: acquisition time 0.4 s, NOESY mixing time 0.8 s, 0.12 s tocsy spinlock and data points 4096 × 512 with zero filling in F1 dimension.

2.8. Homology modelling of CtCBP

The three-dimensional structure of CtCBP was modelled by SWISS MODEL (http://swissmodel.expasy.org/ [55]) using the crystal structure of the *C. gilvus* CBP glucose-complex (PDB, 2QCT [28]) as template. Prediction of acceptor binding to CtCBP was based on superimpositioning of glucose from the complex with the *C. gilvus* CBP onto the modelled CtCBP structure (Deep view/Swiss-PDB viewer V. 3.7). CtCBP, CtCDP, and *C. gilvus* CBP [28] sequences were aligned using ClustalW (http://clustalw.ddbj.nig.ac.jp/top-j.html).

3. Results and discussion

3.1. Production and purification of CtCBP and CtCDP

Recombinant CtCBP (18 mg) and CtCDP (16 mg), were purified from cell lysates of 3 L *E. coli* cultures and migrated in SDS-PAGE as single bands corresponding to 94 kDa and 115 kDa (Supplementary Fig. 1) in agreement with theoretical molecular masses of 96,539 and 115,302, respectively. Gel filtration indicated CtCBP and CtCDP to be dimers of 187 and 218 kDa, respectively. Both recombinant CBP of *C. thermocellum* YM4 [42] and CtCDP purified from a cell lysate of *C. thermocellum* ATCC27405 [10] were also dimers and the crystal structure of *C. gilvus* CBP showed a dimer stabilised through hydrophobic interactions and hydrogen bonds involving conserved residues in GH94 CBP [28]. In contrast, *C. uda* and *C. stercorarium* GH94 CBP were both reported to be monomers in solution [6,11].

3.2. Enzymatic and stability properties of CtCBP and CtCDP

Recombinant CtCBP and CtCDP showed highest apparent phosphorolytic activity at pH 5.3 and 60 °C and at pH 5.7 and 40 °C, respectively (Supplementary Fig. 2A,B,D,E), whereas the reverse phosphorolysis reactions had optima at pH 3.9 and pH 5.4 for CtCBP and CtCDP, respectively (Supplementary Fig. 2A,B). CtCBP and CtCDP retained >95% activity at 4 °C for 24 h in the pH ranges 4.1–8.1 and 4.6–7.0, respectively (Supplementary Fig. 2C), and during 15 min incubation up to 60 °C and 50 °C, respectively (Supplementary Fig. 2F,G). The specific activities towards cellobiose determined at the pH and temperature optima were 11 U/mg and 0.071 U/mg for CtCBP and CtCDP, respectively, while CtCDP showed a specific activity towards cellotriose of 8.7 U/mg. These specific activities of CtCBP and CtCDP were at the same level as reported for the related enzymes from *Clostridium stercorarium* NCIB 11754 [11],

Table 1
Oligosaccharides produced by reverse phosphorolysis catalysed by CtCBP and CtCDP with α-Glc1P[a] or α-Glc1F[b] as donor and various acceptors.

Donor	Acceptor[c]	Product	Yield(%)[d]
CtCBP			
α-Glc1F	D-Glucose	β-Glc*p*-(1→4)-Glc*p*	98
α-Glc1P	D-Glucose	β-Glc*p*-(1→4)-Glc*p*	57
α-Glc1P	D-Mannose	β-Glc*p*-(1→4)-Man*p*	48
α-Glc1P	D-Xylose	β-Glc*p*-(1→4)-Xyl*p*	30
α-Glc1P	D-Arabinose	β-Glc*p*-(1→4)-Ara*p*	28
α-Glc1P	L-Fucose	β-Glc*p*-(1→4)-L-Fuc*p*	24
α-Glc1P	Xylitol	β-Glc*p*-Xyl-ol[e]	9
α-Glc1P	D-Fructose	β-Glc*p*-Fru*p*[e]	5
α-Glc1P	Gentibiose	β-Glc*p*-(1→4)-[β-Glc*p*-(1→6)]-Glc*p*	73
α-Glc1P	Melibiose	β-Glc*p*-(1→4)-[α-Gal*p*-(1→6)]-Glc*p*	36
α-Glc1P	Isomaltose	β-Glc*p*-(1→4)-[α-Glc*p*-(1→6)]-Glc*p*	26
CtCDP			
α-Glc1F	Cellobiose	β-Glc*p*-[(1→4)-β-Glc*p*]$_n$-(1→4)-Glc*p*	68
α-Glc1P	Cellobiose	β-Glc*p*-[(1→4)-β-Glc*p*]$_n$-(1→4)-Glc*p*	48
α-Glc1P	Sophorose	β-Glc*p*-[(1→4)-β-Glc*p*]$_n$-(1→2)-Glc*p*	41
α-Glc1P	Laminaribiose	β-Glc*p*-[(1→4)-β-Glc*p*]$_n$-(1→3)-Glc*p*	35

[a] CtCBP (2.5 μM) was incubated with 200 mM α-Glc1P and 200 mM acceptor in 20 mM sodium acetate pH 3.9 at 60 °C. CtCDP (4.4 μM) was incubated with 200 mM α-Glc1P and 200 mM acceptor in 20 mM sodium acetate pH 5.4 at 40 °C.

[b] α-Glc1F (10 mM for CtCBP, 50 mM for CtCDP) and acceptor (10 mM glucose for CtCBP, 50 mM cellobiose for CtCDP) were reacted in 100 mM Tris–HCl pH 7.0 with 2.9 μM CtCBP or 5.1 μM CtCDP at 40 °C.

[c] All tested acceptor candidates are listed in 2.4.

[d] Yield was calculated based on the donor concentration using commercial cello-oligosaccharides as standards.

[e] The linkages have not been determined.

C. thermocellum YM4 [33], and *Thermotoga maritima* MSB8 [22]. Sophorose, laminaribiose, gentibiose and chitobiose were not phosphorolysed by CtCBP and CtCDP. CtCBP (Fig. 2A) and CtCDP (Fig. 2B) both produced oligosaccharides by reverse phosphorolysis using 50 mM α-Glc1P as donor and 50 mM glucose and cellobiose as acceptors. α-*N*-acetyl D-glucosamine 1-phosphate, α-D-mannose 1-phosphate, and α-D-galactose 1-phosphate were not donors for CtCBP and CtCDP. These enzymatic properties identify the produced CtCBP and CtCDP as cellobiose and cellodextrin phosphorylase, respectively.

3.3. Reverse phosphorolysis by CtCBP

In reverse phosphorolysis CtCBP catalysed oligosaccharide formation from 200 mM α-Glc1P and 200 mM glucose at the apparent pH optimum of 3.9 (see 3.2.; Supplementary Fig. 2A) as quantified by HPAEC-PAD (Fig. 2C). The β-(1→4) regiospecific synthesis of cellobiose was identified by ^{1}H and ^{13}C NMR (see below) and ESI-MS (*m*/*z* 349 for $C_{12}H_{22}O_{11} + Li^{+}$). The cellobiose yield varied with donor and acceptor concentrations (Supplementary Fig. 3A) from 94% obtained with 10 mM of both α-Glc1P and glucose to 59% corresponding to 117 mM cellobiose formed from 200 mM α-Glc1P and 200 mM glucose.

CtCBP catalysed product formation by reverse phosphorolysis with 10 carbohydrate acceptors (Table 1) of the tested 12 monosaccharides, two sugar alcohols, and 15 disaccharides (listed in 2.4). Yields obtained with mannose (48%), L-fucose (24%), arabinose (28%), and xylose (30%) were highly improved by using CtCBP. Galactose, talose, and L-rhamnose as well as *N*-acetyl-glucosamine, *N*-acetyl-galactosamine, and *N*-acetyl-mannosamine were not acceptors, indicating that CtCBP does not utilize aldohexopyranose with C4 axial hydroxyl, or with an *N*-acetyl amino group at C2. Arabinose and L-fucose have been reported to have $^{1}C_4$ and $^{4}C_1$ chair conformations in solution [40,56]. It is presumed that CtCBP reacted with arabinose and L-fucose in the $^{4}C_1$ form which has equatorial C4 hydroxyl, as was previously reported for *C. gilvus* CBP [40]. Among 15 tested disaccharides (listed in 2.4) CtCBP catalysed trisaccharide formation from the α-(1→6)-linked isomaltose and melibiose and from the β-(1→6)-linked gentiobiose, reflecting a narrow disaccharide acceptor specificity (Table 1; Fig. 3). The produced di- and trisaccharides were purified by HPLC, resulting in pure oligosaccharides obtained in 4–72% yield (Supplementary Table 1).

The structures of products from the above 10 different acceptors and the regioselectivity of the CtCBP catalysed reverse phosphorolysis were elucidated by ESI-MS and two-dimensional NMR (Supplementary Tables 2–1). ESI-MS showed the molecular mass of all products to correspond to the values calculated for Na^{+} and Li^{+} adducts of glucose-acceptor products. The ^{1}H anomeric signal of α-Glc1P was assigned to approx. 5.4 ppm and the ^{13}C signal to 93.6 ppm, which after synthesis changed to approx. 4.5 ppm and 100.7 ppm, respectively, indicating β-linked glucose as confirmed also by a coupling constant at 8 Hz. CtCBP synthesised a single β-linked product with high regioselectivity from each of the 10

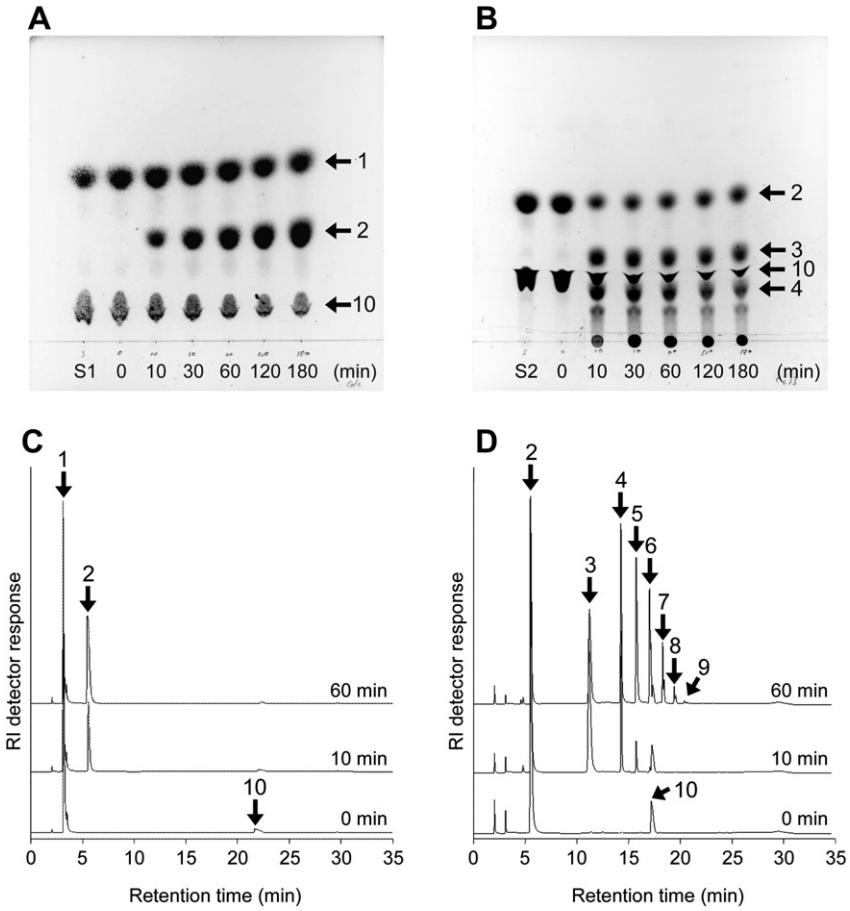

Fig. 2. Monitoring of reverse phosphorolysis with α-Glc1P as donor. (A, B), TLC analysis of cellobiose or cellodextrin synthesis from 50 mM α-Glc1P and glucose by 2.8 μM CtCBP (A) or 50 mM cellobiose by 5.1 μM CtCDP (B). Standards: lane S1, glucose and α-Glc1P; lane S2, cellobiose and α-Glc1P. (C, D), HPAEC-PAD chromatograms of products with 200 mM α-Glc1P and glucose with 2.5 μM CtCBP (C) or cellobiose with 4.4 μM CtCDP (D). Glucose (1), cellobiose (2), cellotriose (3), cellotetraose (4), cellopentaose (5), cellohexaose (6), celloheptaose (7), cellooctaose (8), cellononaose (9), and α-Glc1P (10) are marked by arrows.

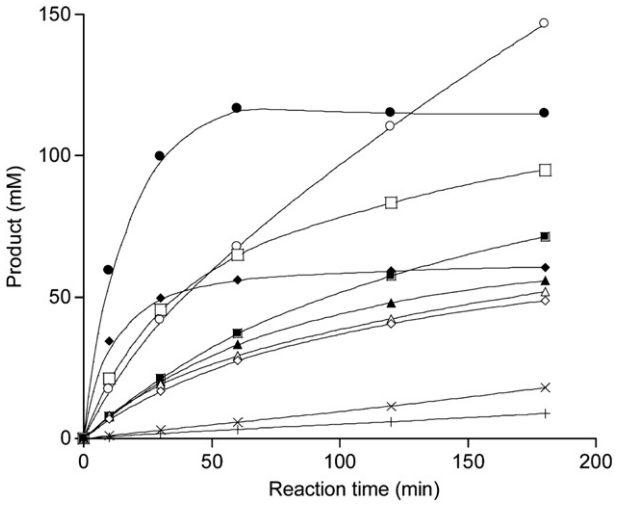

Fig. 3. Acceptor specificity of CtCBP (2.5 μM) in reverse phosphorolysis. The concentration of products from 200 mM α-Glc1P and 200 mM glucose (●), mannose (□), xylose (◆), L-fucose (◇), arabinose (▲), xylitol (×), fructose (+), isomaltose (△), gentibiose (○), and melibiose (■) were calculated from peak areas of HPAEC-PAD calibrated with cellobiose and cellotriose for di- and trisaccharides, respectively.

suitable acceptors (Table 1; Supplementary Tables 2–1). The β-glucose substitution was assigned by 6–10 ppm carbon downfield shifts and confirmed by NOE from the glucose β-anomeric proton to the proton across the new linkage. The monosaccharide substitutions were assigned to be on C4 (Table 1), except for xylitol and fructose, for of which both the formed linkages were not determined due to the low yields (Supplementary Table 1). For all three products obtained with disaccharide acceptors, *i.e.* melibiose, gentiobiose, and isomaltose, substitutions were assigned to occur on C4 of the reducing glucose unit (Table 1). Thus a C6 substituted glucose at the reducing end seems to be required for binding of a disaccharide acceptor to CtCBP at subsite +1 and formation of a branched trisaccharide.

In summarising, CtCBP catalysed synthesis of β-linked di- and trisaccharides by reverse phosphorolysis with β-(1→4) regioselectivity using five monosaccharides and three (1→6) linked disaccharides as acceptors (Table 1). These were previously reported using *C. gilvus* CBP as catalyst [36,38–41], however, the present yields obtained with CtCBP were impressively increased for arabinose, L-fucose, and gentibiose as acceptors to 28%, 24%, and 73% compared to 0.6%, 0.7%, and 17% obtained with *C. gilvus* CBP [40,41], while the yields for CtCBP with isomaltose and melibiose were improved to 26% and 36% here, compared to 10% and 11% [41]. In comparison, CBP from *T. maritima* MSB8 was reported not to use arabinose, L-fucose, and fructose at all as acceptors [22], which further reflects the individual specificity differences emerging amongst CBPs of different origin.

3.4. Structural basis for acceptor specificity of CtCBP

The acceptor recognition was illustrated by superimposing β-glucose from the *C. gilvus* CBP complex [28] at subsite +1 onto the modelled CtCBP structure (Fig. 4A). Direct hydrogen bonds may form by the β-glucose C1 hydroxyl with Glu636, C2 hydroxyl with Tyr640 and Glu646, C3 hydroxyl with Lys645 and Glu646, and C4 hydroxyl with Asp483, which is the general acid catalyst. This model strongly supports the preference of CtCBP for glucose as acceptor (Fig. 3; Table 1). Moreover the recognition of equatorial C2 hydroxyl is in agreement with the preference of CtCBP for glucose

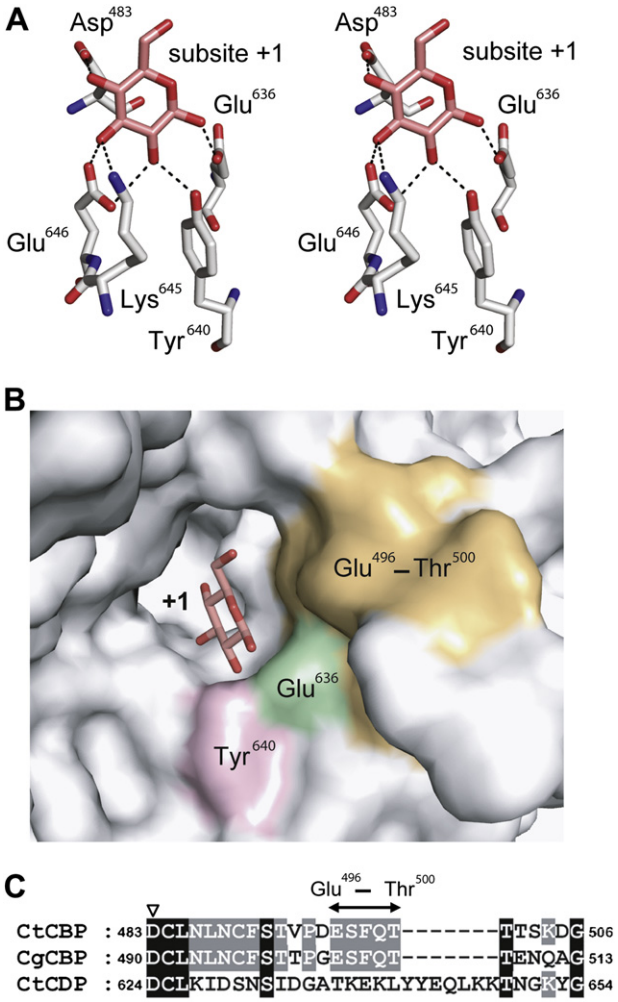

Fig. 4. Acceptor recognition by CtCBP and CtCDP at subsite +1. (A), modelled structure of CtCBP having glucose (pink) of *Cellvibrio gilvus* CBP (PDB, 2QCT [28]) superimposed at subsite +1. Hydrogen bonds are shown as dotted lines. (B), surface representation of the CtCBP active site with glucose at subsite +1. The locations for the loop Glu496-Thr500, Glu636, and Tyr640 are in yellow, green, and pink, respectively. (C), sequence alignment of CtCDP and *C. gilvus* CBP (CgCBP) loops corresponding to loop Asp483-Gly506 of CtCBP. The open triangle marks the general acid catalyst.

as acceptor over mannose. The C6 hydroxyl is solvent-exposed in the modelled structure (Fig. 5B), which is in excellent agreement with previous kinetic analysis of *C. gilvus* and *C. uda* CBP using deoxy-glucose analogues suggesting that the C6 hydroxyl was not involved in hydrogen bond formation, but probably contributed to the binding *via* weak hydrophobic contacts [6,28]. Furthermore, steric clashing with Glu636 situated at the edge of the active site seems to prohibit binding of cellobiose [β-(1→4)], sophorose [β-(1→2)] and laminaribiose [β-(1→3)] (Fig. 4B), whereas the model supports that isomaltose, melibiose, and gentibiose having C6 substituted glucose at the reducing end can bind as acceptor at subsite +1 leading to synthesis of branched trisaccharides (Table 1). Finally, the bulky Tyr640 of which the γOH may form a hydrogen bond with glucose C2 hydroxyl at subsite +1 (Fig. 4A) can clash with an *N*-acetyl amino group at C2 in agreement with *N*-acetyl-galactosamine and *N*-acetyl-glucosamine not being acceptors. In comparison, chitobiose phosphorylase of GH94 from *V. proteolyticus* has a small hydrophobic pocket at subsite +1 formed by Cys493 and Val631, which interact with methyl of the C2 substituent in *N*-acetyl glucosamine [27].

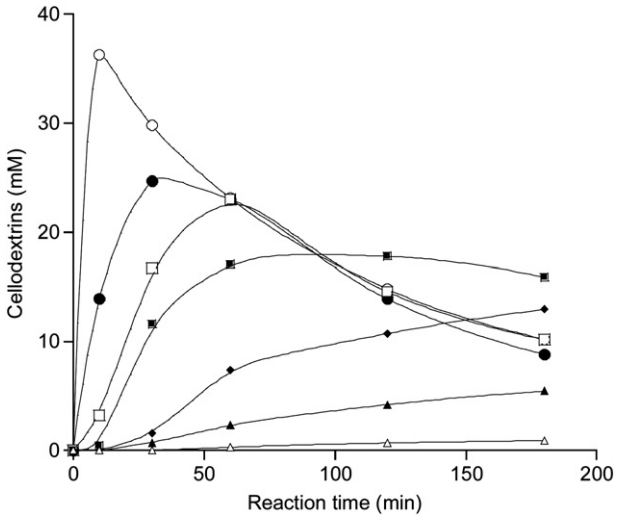

Fig. 5. HPAEC-PAD quantification of cellodextrins (DP 3–9) produced by CtCDP (4.4 μM) reverse phosphorolysis from 200 mM α-Glc1P and 200 mM cellobiose was quantified by HPAEC-PAD: cellotriose (○), cellotetraose (●), cellopentaose (□), cellohexaose (■), celloheptaose (◆), cellooctaose (▲), cellononaose (△).

3.5. Reverse phosphorolysis by CtCDP

CtCDP formed a series of cellodextrins with different degree of polymerization (DP 3–9) from α-Glc1P and cellobiose (Fig. 2D), cellotriose being formed initially and extended to the higher DP cellodextrins (Fig. 5). The highest estimated yield of cellodextrins from 10 to 200 mM α-Glc1P and cellobiose (see 2.4) was 61% obtained with 10 mM of both donor and acceptor (Supplementary Fig. 3B). At 200 mM of both donor and acceptor 75 mg/mL cellodextrins corresponding to a yield of 48% were obtained after 60 min. This is comparable with a reported yield of 54% obtained from 80 mM α-Glc1P and 20 mM cellobiose as catalysed by CtCDP purified from a cell lysate of *C. thermocellum* ATCC27405 [10].

While specificity of CBPs in reverse phosphorolysis has been described for a series of candidate acceptors [6,9,21,28,35–42], very little was reported about the acceptor specificity of CDP. The present CtCDP is subjected to scrutinising acceptor specificity analysis employing 29 mono- and disaccharide candidates with high concentration of CtCDP and long reaction time (see 2.4). CtCDP, however, utilized only three disaccharides, *i.e.* cellobiose (Fig. 2B,D), sophorose [β-(1→2)], and laminaribiose [β-(1→3)] (Supplementary Fig. 4), resulting in products with DP 3–9, which were detected by HPAEC-PAD (Fig. 2D and Supplementary Fig. 4C,D). The yields were 35–48% (Table 1) and increased in the order laminaribiose < sophorose < cellobiose. Neither gentibiose [β-(1→6)] nor any of the monosaccharides (listed in 2.4) were acceptors. The trisaccharides as initial products from cellobiose, sophorose, and laminaribiose were purified by HPLC, resulting in pure trisaccharides obtained in 12–17% yield (Supplementary Table 1). ESI-MS confirmed the calculated molecular mass of Na^+ adducts of the initial trisaccharide products (*m/z* 527 for $C_{18}H_{32}O_{16} + Na^+$). For linkage analysis, chemical shifts of the products were assigned based on two-dimensional NMR spectra (Supplementary Tables 2–2) showing that CtCDP synthesised linear β-D-glucopyranosyl-[(1→4)-β-D-glucopyranosyl]$_n$-(1→4)-D-glucopyranose (cellodextrins), β-D-glucopyranosyl-[(1→4)-β-D-glucopyranosyl]$_n$-(1→2)-D-glucopyranose, and β-D-glucopyranosyl-[(1→4)-β-D-glucopyranosyl]$_n$-(1→3)-D-glucopyranose [n = 1–7, shown by HPAEC-PAD (Supplementary Fig. 4C,D)] from cellobiose, sophorose, and laminaribiose, respectively, with strict β-(1→4) regiospecificity. The products obtained with sophorose and laminaribiose represent novel types of oligosaccharides (Supplementary Fig. 5). Previously, CDP from *C. thermicellum* YM4 was shown to catalyse synthesis of mixed glucosyl and xylosyl oligosaccharides up to DP of 4 in individual yields of 4.8–44% [44] and CDP from *C. thermicellum* DSM 1237 was used to synthesise chain-extended thio-oligosaccharide inhibitors of 1,3:1,4-β-D-glucanase from *Bacillus licheniformis* in 69% yield [33,43].

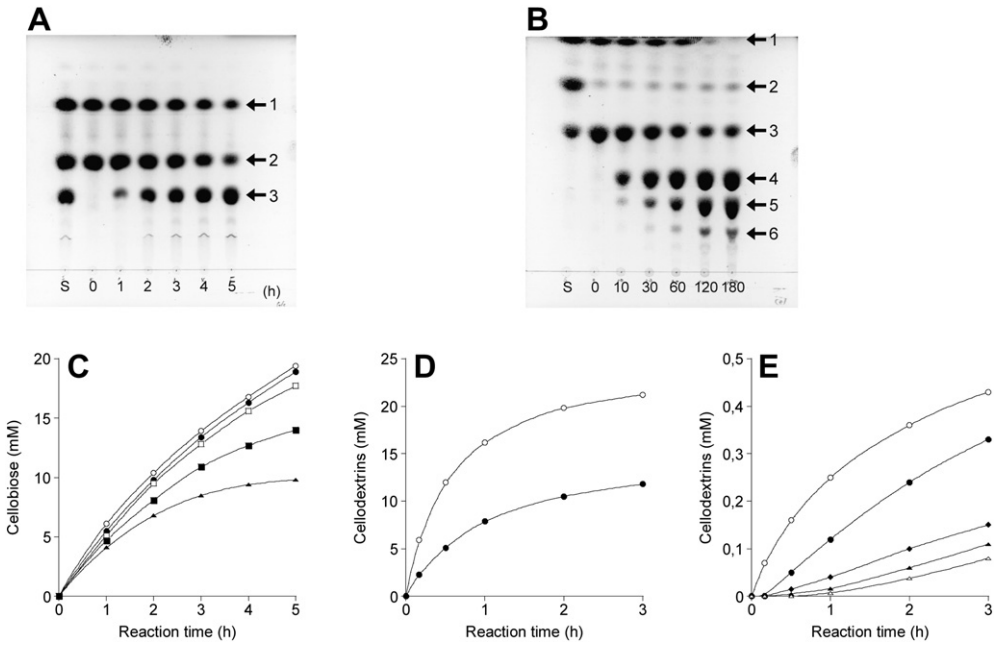

Fig. 6. Irreversible phosphorolysis using α-Glc F as donor. (A, B), TLC analysis of the reaction with 50 mM α-Glc1F and 50 mM glucose for 2.9 μM CtCBP (A) or 50 mM cellobiose for 5.1 μM CtCDP (B). Standards: lane S, α-Glc1F, glucose, and cellobiose. The compounds, α-Glc1F (1), glucose (2), cellobiose (3), cellotriose (4), cellotetraose (5), cellopentaose (6) are marked by arrows. (C, D), HPAEC-PAD quantification of (C) cellobiose from α-Glc1F and glucose: 10 mM (◆), 20 mM (■), 30 mM (□), 40 mM (●), 50 mM (○) and (D, E) cellodextrins of DP 3–9 produced with 50 mM α-Glc1F and cellobiose: cellotriose (○), cellotetraose (●), cellopentaose (□), cellohexaose (■), celloheptaose (◆), cellooctaose (▲), cellononaose (△).

3.6. Structural basis for distinct acceptor specificities of CtCBP and CtCDP

CtCDP produced linear oligosaccharides of DP 3–9 from sophorose, laminaribiose, and cellobiose as acceptors, whereas CtCBP synthesised a branched trisaccharide from each of isomaltose, melibiose, and gentibiose (Table 1) without further elongation. To understand the structural basis for the variation in acceptor specificity between CtCBP and CtCDP, we focused on the subsite +1 region. CtCBP Glu636 was predicted by modelling to recognize the C1 hydroxyl of β-glucose at subsite +1 (Fig. 4A) and to prohibit accommodation of cellobiose, sophorose, and laminaribiose as acceptors at subsites +1 and +2 (Fig. 4B). CtCDP Ala800, corresponding to CtCBP Glu636, allows accommodation of the three disaccharide acceptors at subsites +1 and +2 leading to production of linear oligosaccharides due to the larger space available at the binding site and the lack of hydrogen bond partner of the β-glucose C1 hydroxyl at subsite +1. In the *C. gilvus* CBP such recognition of the β-anomeric hydroxyl was reported to be essential for acceptor binding at subsite +1 as observed in the structure to occur *via* interaction with Glu649 [28,57], that corresponds to Glu636 in CtCBP. These structural features are in accordance with the experimental finding that no monosaccharides acted as acceptor in CtCDP catalysed reverse phosphorolysis. CtCDP in addition has a longer Thr637-Lys648 loop (Fig. 4C), compared to Glu496-Thr500 in CtCBP, forming the edge of the active site next to the C6 hydroxyl of glucose bound at subsite +1 (Fig. 4B). In case of CtCBP this C6 hydroxyl group appears exposed to solvent (Fig. 5B), whereas the counterpart Thr637-Lys648 loop in CtCDP most probably blocks for binding of the C6 substituted disaccharides isomaltose, melibiose, and gentibiose, hence not acting as acceptors for CtCDP (Table 1).

3.7. Glycosynthase-type reaction with α-Glc1F by CtCBP and CtCDP

α-Glc1F was used as donor (Fig. 1E; Fig. 6) to avoid that phosphate released from α-Glc1P in reverse phosphorolysis acts as base catalyst in phosphorolysis of oligosaccharide products (Fig. 1A,C; Supplementary Fig. 3). Thus from 10 mM α-Glc1F and 10 mM glucose the yield of cellobiose formed by CtCBP was improved to 98% (Fig. 6C; Supplementary Tables 2–3). CtCDP similarly generated cellodextrins (DP 3–9) in an improved 68% yield from 50 mM α-Glc1F and 50 mM cellobiose (Fig. 6D,E; Supplementary Tables 2–3). This introduces reverse phosphorolysis with α-Glc1F for GH94 CDP, while α-Glc1F has been used in case of *C. uda* CBP [6,51].

The irreversible reaction by CtCBP and CtCDP with α-Glc1F (Fig. 1E) is comparable to the glycosynthase reaction reported for catalytic base mutants of inverting glycoside hydrolases of GH8 [46] and GH95 [47] (Fig. 1D). This reaction applies the Hehre resynthesis-hydrolysis mechanism, involving transfer of Glc1F of the opposite anomeric configuration of the natural substrate to an acceptor with release of F^- concomitant with inversion of the anomeric configuration [48,49]. It is an advantage of such an irreversible phosphorylase catalysed chemoenzymatic synthesis using α-Glc1F compared to the glycosynthase reaction that the active wild-type enzyme can be used without mutation of a catalytic amino acid residue. CtCBP and CtCDP thus efficiently generated oligosaccharides from α-Glc1F. It is assumed that this advantageous strategy can be extended to related GH65 and GH94 phosphorylases.

4. Conclusions

Recombinant GH94 CtCBP and CtCDP from *C. thermocellum* ATCC27405 catalysed production of cellobiose and cellodextrins, respectively, by reverse phosphorolysis with α-Glc1P as donor. The high yields obtained were further improved up to 98% and 68% for CtCBP and CtCDP, respectively, by using α-Glc1F as donor instead of α-Glc1P. CtCBP acceptor specificity analysis resulted in synthesis of 8 β-glucosyl di- and trisaccharides with β-(1→4)-regioselectivity. Furthermore, acceptor specificity analysis of CtCDP led to two novel types of β-glucosyl oligosaccharides, β-Glc*p*-[(1→4)-β-Glc*p*]$_n$-(1→2)-Glc*p* and β-Glc*p*-[(1→4)-β-Glc*p*]$_n$-(1→3)-Glc*p* (n = 1–7). These distinctly different acceptor specificities of CtCBP and CtCDP are understood using a modelled structure of CtCBP proposing different recognition of C1 hydroxyl of β-glucose at subsite +1 and loop length variation, near C6 hydroxyl of the glucose at subsite +1 allowing branching for CtCBP as opposed to linear chain extension for CtCDP.

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Appendix. Supplementary data

Supplementary data associated with this article can be found in online version at doi:10.1016/j.biochi.2010.07.013.

References

[1] B.L. Cantarel, P.M. Coutinho, C. Rancurel, T. Bernard, V. Lombard, B. Henrissat, The Carbohydrate-Active EnZymes database (CAZy): an expert resource for glycogenomics, Nucl. Acids Res. 37 (2009) D233–D238.
[2] C.J. Sih, R.H. McBee, A cellobiose phosphorylase in *Clostridium thermocellum*, Proc. Mont. Acad. Sci. 15 (1955) 21–26.
[3] K. Sheth, J.K. Alexander, Purification and properties of β-1,4-oligoglucan: orthophosphate glucosyltransferase from *Clostridium thermocellum*, J. Biol. Chem. 244 (1969) 457–464.
[4] M. Sato, H. Takahashi, Fermentation of C^{14}-labeled cellobiose by *Cellulomonas fimi*, Agric. Biol. Chem. 31 (1967) 470–471.
[5] K.L. Schimz, B. Broll, B. John, Cellobiose phosphorylase of *Cellulomonas*: occurrence, induction and its role in cellobiose metabolism, Arch. Microbiol. 135 (1983) 241–249.
[6] B. Nidetzky, C. Eis, M. Albert, Role of non-covalent enzyme-substrate interactions in the reaction catalysed by cellobiose phosphorylase from *Cellulomonas uda*, Biochem. J. 351 (2000) 649–659.
[7] F.H. Hulcher, K.W. King, Metabolic basis for disaccharide preference in *Cellvibrio*, J. Bacteriol. 76 (1958) 571–577.
[8] T. Sasaki, T. Tanaka, S. Nakagawa, K. Kainuma, Purification and properties of *Cellvibrio gilvus* cellobiose phosphorylase, Biochem. J. 209 (1983) 803–807.
[9] J.K. Alexander, Purification and specificity of cellobiose phosphorylase from *Clostridium thermocellum*, J. Biol. Chem. 243 (1968) 2899–2904.
[10] M. Arai, K. Tanaka, T. Kawaguchi, Purification and properties of cellodextrin phosphorylase from *Clostridium thermocellum*, J. Ferment. Bioeng. 77 (1994) 239–242.
[11] M. Reichenbecher, F. Lottspeich, K. Bronnenmeier, Purification and properties of a cellobiose phosphorylase (CepA) and a cellodextrin phosphorylase (CepB) from the cellulolytic thermophile Clostridium stercorarium, Eur. J. Biochem. 247 (1997) 262–267.
[12] M. Krishnareddy, Y.K. Kim, M. Kitaoka, Y. Mori, K. Hayashi, Cellobiose phosphorylase from *Clostridium thermocellum* YM4 strain expressed in *Escherichia coli*, J. Appl. Glycosci. 49 (2002) 1–8.
[13] Y.H.P. Zhang, L.R. Lynd, Biosynthesis of radiolabeled cellodextrins by the *Clostridium thermocellum* cellobiose and cellodextrin phosphorylases for measurement of intracellular sugars, Appl. Microbiol. Biotechnol. 70 (2006) 123–129.
[14] J.E. Wells, J.B. Russell, Y. Shi, P.J. Weimer, Cellodextrin efflux by the cellulolytic ruminal bacterium *Fibrobacter succinogenes* and its potential role in the growth of nonadherent bacteria, Appl. Environ. Microbiol. 61 (1995) 1757–1762.
[15] C.A. Matheron, M. Delrot, G. Gaudet, E. Forano, *In vivo* ^{13}C NMR study of glucose and cellobiose metabolism by four cellulolytic strains of the genus, *Fibrobacter*, Biodegradation 9 (1998) 451–461.
[16] A. Hütterman, C. Volger, Cellobiose phosphorylase in *Fomes annosus*, Nat. New Biol. 245 (1973) 64.
[17] J. Lou, K.A. Dawson, H.J. Strobel, Role of phosphorolytic cleavage in cellobiose and cellodextrin metabolism by the ruminal bacterium *Prevotella ruminicola*, Appl. Environ. Microbiol. 62 (1996) 1770–1773.

[18] W. Ayers, Phosphorylation of cellobiose and glucose by *Ruminococcus flavifaciens*, J. Bacteriol. 76 (1958) 515–517.
[19] C.T. Helaszek, B.A. White, Cellobiose uptake and metabolism by *Ruminococcus flavefaciens*, Appl. Environ. Microbiol. 57 (1991) 64–68.
[20] B. Thurston, K.A. Dawson, H.J. Strobel, Cellobiose versus glucose utilization by the ruminal bacterium *Ruminococcus albus*, Appl. Environ. Microbiol. 59 (1993) 2631–2637.
[21] D.A. Yernool, J.K. McCarthy, D.E. Eveleigh, J.D. Bok, Cloning and characterization of the glucooligosaccharide catabolic pathway β-glucan glucohydrolase and cellobiose phosphorylase in marine hyperthermophilic *Thermotoga neapolitana*, J. Bacteriol. 182 (2000) 5172–5179.
[22] E. Rajashekhara, M. Kitaoka, Y.K. Kim, K. Hayashi, Characterization of a cellobiose phosphorylase from a hyperthermophilic eubacterium, *Thermotoga maritima* MSB8, Biosci. Biotechnol. Biochem. 66 (2002) 2578–2586.
[23] A. Liu, H. Tomita, H. Li, H. Miyaki, C. Aoyagi, S. Kaneko, K. Hayashi, Cloning, sequencing and expression of the cellobiose phosphorylase gene of *Cellvibrio gilvus*, J. Ferment. Bioeng. 85 (1998) 511–513.
[24] J.K. Park, N.O. Keyhani, S. Roseman, Chitin catabolism in the marine bacterium *Vibrio furnissii*. Identification, molecular cloning, and characterization of a N, N′-diacetylchitobiose phosphorylase, J. Biol. Chem. 275 (2000) 33077–33083.
[25] Y. Honda, M. Kitaoka, K. Hayashi, Reaction mechanism of chitobiose phosphorylase from *Vibrio proteolyticus*: identification of family 36 glycosyltransferase in *Vibrio*, Biochem. J. 377 (2004) 225–232.
[26] N. Iñón de Iannino, G. Briones, M. Tolmasky, R.A. Ugalde, Molecular cloning and characterization of cgs, the *Brucella abortus* cyclic β(1-2) glucan synthetase gene: genetic complementation of *Rhizobium meliloti* ndvB and *Agrobacterium tumefaciens chvB* mutants, J. Bacteriol. 180 (1998) 4392–4400.
[27] M. Hidaka, Y. Honda, M. Kitaoka, S. Nirasawa, K. Hayashi, T. Wakagi, H. Shoun, S. Fushinobu, Chitobiose phosphorylase from *Vibrio proteolyticus*, a member of glycosyl transferase family 36, has a clan GH-L-like $(\alpha/\alpha)_6$ barrel fold, Structure 12 (2004) 937–947.
[28] M. Hidaka, M. Kitaoka, K. Hayashi, T. Wakagi, H. Shoun, S. Fushinobu, Structural dissection of the reaction mechanism of cellobiose phosphorylase, Biochem. J. 398 (2006) 37–43.
[29] A.E. Aleshin, L.M. Firsov, R.B. Honzatko, Refined structure for the complex of acarbose with glucoamylase from *Aspergillus awamori* var. X100 to 2.4 Å resolution, J. Biol. Chem. 269 (1994) 15631–15639.
[30] M.P. Egloff, J. Uppenberg, L. Haalck, H. van Tilbeurgh, Crystal structure of maltose phosphorylase from *Lactobacillus brevis*: unexpected evolutionary relationship with glucoamylases, Structure 9 (2001) 689–697.
[31] M.R. Stam, E. Blanc, P.M. Coutinho, B. Henrissat, Evolutionary and mechanistic relationships between glycosidases acting on α- and β-bonds, Carbohydr. Res. 340 (2005) 2728–2734.
[32] S.M. Hancock, M.D. Vaughan, S.G. Withers, Engineering of glycosidases and glycosyltransferases, Curr. Opin. Chem. Biol. 10 (2006) 509–519.
[33] E. Samain, C. Lancelon-Pin, F. Ferigo, V. Moreau, H. Chanzy, A. Heyraud, H. Driguez, Phosphorolytic synthesis of cellodextrin, Carbohydr. Res. 271 (1995) 217–226.
[34] M. Hiraishi, K. Igarashi, S. Kimura, M. Wada, M. Kitaoka, M. Samejima, Synthesis of highly ordered cellulose II in vitro using cellodextrin phosphorylase, Carbohydr. Res. 344 (2009) 2468–2473.
[35] J.K. Alexander, Synthesis of 4- *O*-β-glucopyranosyl-D-xylose, 4- *O*-β-glucopyranosyl-D-arabinose, 4- *O*-β-glucopyranosyl-2-deoxy-D-glucose, 4- *O*-β-glucopyranosyl-D-mannose, 4- *O*-β-glucopyranosyl-D-glucosamine by cellobiose phosphorylase from *Clostridium thermocellum*, Arch. Biochem. Biophys. 123 (1968) 240–246.
[36] M. Kitaoka, H. Taniguchi, T. Sasaki, Production of glucosyl-xylose using *Cellvibrio gilvus* cells and its properties, Appl. Microbiol. Biotechnol. 34 (1990) 178–182.
[37] M. Kitaoka, T. Sasaki, H. Taniguchi, Synthetic reaction of *Cellvibrio gilvus* cellobiose phosphorylase, J. Biochem. 112 (1992) 40–44.
[38] M.A. Tariq, K. Hayashi, Synthesis of three hetero disaccharides, 4- *O*-β-glucopyranosyl-6-deoxy-D-glucose, 4- *O*-β-glucopyranosyl-D-mannosamine, 4- *O*-β-glucopyranosyl-D-mannose, and confirmation of their structures by C-13 NMR and MS, Biochem. Biophys. Res. Commun. 214 (1995) 568–575.
[39] M.A. Tariq, K. Hayashi, K. Tokuyasu, T. Nagata, Synthesis and structural analysis of disaccharides of 4- *O*-β-D-glucopyranosyl-D-glucosamine and 4- *O*-D-β-glucopyranosyl-2-deoxy-D-glucose, Carbohydr. Res. 275 (1995) 67–72.
[40] A. Percy, H. Ono, D. Watt, K. Hayashi, Synthesis of β-D-glucopyranosyl-(1→4)-D-arabinose, β-D-glucopyranosyl-(1→4)-L-fucose and β-D-glucopyranosyl-(1→4)-D-altrose catalysed by cellobiose phosphorylase from Cellvibrio gilvus, Carbohydr. Res. 305 (1998) 543–548.
[41] A. Percy, H. Ono, K. Hayashi, Acceptor specificity of cellobiose phosphorylase from Cellvibrio Gilvus: synthesis of three branched trisaccharides, Carbohydr. Res. 308 (1998) 423–429.
[42] Y.K. Kim, M. Kitaoka, M. Krishnareddy, Y. Mori, K. Hayashi, Kinetic studies of a recombinant cellobiose phosphorylase (CBP) of the *Clostridium thermocellum* YM4 strain expressed in *Escherichia coli*, J. Biochem. 132 (2002) 197–203.
[43] V. Moreau, J.L. Viladot, E. Samain, A. Planas, H. Driguez, Design and chemoenzymatic synthesis of thiooligosaccharide inhibitors of 1,3:1,4-β-D-glucanases, Bioorg. Med. Chem. 4 (1996) 1849–1855.
[44] K. Shintate, M. Kitaoka, Y.K. Kim, K. Hayashi, Enzymatic synthesis of a library of β-(1→4) hetero-D-glucose and D-xylose-based oligosaccharides employing cellodextrin phosphorylase, Carbohydr. Res. 338 (2003) 1981–1990.
[45] L.F. Mackenzie, Q.P. Wang, R.A.J. Warren, S.G. Withers, Glycosynthases: mutant glycosidases for oligosaccharide synthesis, J. Am. Chem. Soc. 120 (1998) 5583–5584.
[46] Y. Honda, M. Kitaoka, The first glycosynthase derived from an inverting glycoside hydrolase, J. Biol. Chem. 281 (2006) 1426–1431.
[47] J. Wada, Y. Honda, M. Nage, R. Kato, S. Wakatsuki, T. Katayama, H. Taniguchi, H. Kumagai, M. Kitaoka, K. Yamamoto, 1,2-α-l-Fucosynthase: a glycosynthase derived from an inverting α-glycosidase with an unusual reaction mechanism, FEBS Lett. 582 (2008) 3739–3743.
[48] E.J. Hehre, T. Sawai, C.F. Brewer, M. Nakano, T. Kanda, Trehalase: stereocomplementary hydrolytic and glucosyl transfer ractions with α- and β-D-glucosyl fluoride, Biochemistry 21 (1982) 3090–3097.
[49] S.J. Williams, S.G. Withers, Glycosyl fluorides in enzymatic reactions, Carbohydr. Res. 327 (2000) 27–46.
[50] Y. Tsumuraya, C.F. Brewer, E.J. Hehre, Substrate-induced activation of maltose phosphorylase: interaction with the anomeric hydroxyl group of α-maltose and α-D-glucose controls the enzym's glucosyltransferase activity, Arch. Biochem. Biophys. 281 (1990) 58–65.
[51] B. Nidetzky, R. Griessler, A. Schwarz, B. Splechtna, Cellobiose phosphorylase from *Cellulomonas uda*: gene cloning and expression in *Escherichia coli*, and application of the recombinant enzyme in a 'glycosynthase-type' reaction, J. Mol. Cat. B. Enz. 29 (2004) 241–248.
[52] J. Thiem, M. Wiesner, Alkylation of glycosyl fluorides, Synthesis 2 (1998) 124–127.
[53] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, Colorimetric methods for determination of sugars and related substraces, Anal. Chem. 28 (1956) 350–356.
[54] Y. Westphal, H.A. Schols, A.G.J. Voragen, H. Gruppen, Introducing porous graphitized carbon liquid chromatography with evaporative light scattering and mass spectrometry detection into cell wall oligosaccharide analysis, J. Chromato. 1217 (2010) 689–695.
[55] F. Kiefer, K. Arnold, B. Kunzli, L. Bordoli, T. Schwede, The SWISS-MODEL Repository and associated resources, Nucl. Acids Res. 37 (2009) D387–D392.
[56] C.A. Tarling, S. He, G. Sulzenbacher, C. Bignon, Y. Bourne, B. Henrissat, S.G. Withers, Identification of the catalytic nucleophile of the family 29 α-L-fucosidase from *Thermotoga maritima* through trapping of a covalent glycosyl-enzyme intermediate and mutagenesis, J. Biol. Chem. 278 (2003) 47394–47399.
[57] M. Kitaoka, S. .Ogawa, H. Taniguchi, A cellobiose phosphorylase from *Cellovibrio gilvus* recognizes only the β-D-form of 5a carba-glucopyranose, Carbohydr. Res. 247 (1993) 355–359.