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Cellobiose phosphorylase from *Cellulomonas uda*: gene cloning and expression in *Escherichia coli*, and application of the recombinant enzyme in a 'glycosynthase-type' reaction☆

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Abstract

We have cloned and sequenced the gene encoding cellobiose phosphorylase from *Cellulomonas uda* and report high yield production in *Escherichia coli* of a functional recombinant enzyme containing an N-terminal metal affinity fusion peptide. Use of heterologous gene expression increases the space-time yield of active phosphorylase by three orders of magnitude, compared to production of the enzyme with the natural organism. The full-length phosphorylase is a 91.3 kDa protein that consists of 821 amino acids and whose primary structure shares significant residue identity with different members of glycosyltransferase family 36. Purified enzyme was obtained in 39% overall yield by using copper-chelate and hydroxyapatite chromatographies. A comparative steady-state kinetic analysis for enzymatic reactions in the directions of phosphorolysis and synthesis of cellobiose at 30 °C and pH 6.6 demonstrates that the catalytic properties of the natural enzyme are retained completely in the recombinant cellobiose phosphorylase. The ability of the phosphorylase to utilize α-D-glucose 1-fluoride (αG1F) as alternate glucosyl donor in place of α-D-glucose 1-phosphate (αG1P) is exploited for the synthesis of β-1,4-glucosides under thermodynamic control in close to 100% yield.

Keywords: Glycosyltransferase; Glucosyl fluoride; Glycobiotechnology; Glycoside synthesis

1. Introduction

Oligosaccharides fulfil a wide array of physiological functions and are key elements of biological recognition under different conditions [1–3]. Oligosaccharide-based drugs therefore hold great promise for therapy. However, their development has been relatively slow in comparison with other pharmaceutical biomolecules [3,4]. One reason is that oligosaccharide synthesis in good yield and with tightly controlled selectivities is difficult in practice [5]. The availability of defined oligosaccharide structures in sufficient quantities is, therefore, often prohibitive.

Glycosyltransferases (GT; EC 2.4) are nature's enzymes for the synthesis of glycosides whereas, glycosylhydrolases (GH; EC 3.2) have been evolved to degrade them. Ironically, GHs run in one of several possible 'reverse' reaction modes are widely used to synthesize oligosaccharides [6–8]. However, in recognition of intrinsic limitations of GH-based approaches such as poor yield and incomplete regioselectivity, GTs have gained more attention for application in oligosaccharide production [5,9–13].

Among GT and GH classes, the glucosyl phosphorylases (GPs; EC 2.4.1) are special in several respects [14]. GPs catalyze glucosyl transfer to and from phosphate, as shown

Abbreviations: CbP, cellobiose phosphorylase (cellobiose:orthophosphate α-D-glucosyltransferase; EC 2.4.1.20); *Cu*CbP, CbP from *Cellulomononas uda*; his_6-*Cu*CbP, recombinant *Cu*CbP containing an N-terminal metal affinity fusion peptide in which 6 of the 11 amino acid residues are histidines; CHT, ceramic hydroxyapatite; GH, glycosylhydrolase; GT, glycosyltransferase; αG1F, α-D-glucose 1-fluoride; αG1P, α-D-glucose 1-phosphate; IMAC, immobilized metal affinity chromatography; GP, glucosyl phosphorylase; IPTG, isopropyl-β-D-thiogalactopyranoside

☆ The genomic sequence of cellobiose phosphorylase from *Cellulomonas uda* DSM 20108 has been deposited in GenBank under the accession number: AY343322.

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in Eq. (1):

$$\text{Glc-OR} + P_i \leftrightarrow \text{Glc 1-}P_i + \text{ROH} \quad (1)$$

where Glc-OR is an α- or β-D-glucoside and Glc 1-P_i is α- or β-D-glucose 1-phosphate depending on the stereochemical control exercised by the enzyme; RO(H) is a monosaccharide that can be substituted to a varying extent, or it is an oligo- or polysaccharide whose original degree of polymerization has been decreased by 1 sugar unit. (An exception to Eq. (1) is chitobiose phosphorylase [15] which transfers a 2-aminoacetyl-2-deoxy-D-glucosyl residue.)

GPs seem to represent an evolutionary 'bridge' between the large GT and GH groups, both in structural and functional terms. They are much alike GHs in a sense that their physiological role is catabolic [14]. However, they bear good resemblance to GTs because in spite of working in the presence of 55 M water they prevent hydrolysis reactions efficiently [14,16,17]. In a widely used sequence-based classification of GTs and GHs, the group of GPs is promiscuous and characterized by memberships to GT and GH families [18,19]. Catalytic mechanisms of enzymic glycosyl transfer by GTs and GHs are different in a sense that the configuration of the substituted anomeric carbon of the substrate may be retained or inverted in the product [14]. GPs belonging to the 'retaining' (α → α) and 'inverting' (β → α, α → β) classes have been described, and so the GP group shares stereochemical diversity of the larger GT and GH groupings [20]. Thermodynamic considerations for Eq. (1) show that GP-catalyzed reactions have equilibrium constants (K_{eq}) that are intermediate between K_{eq} values for reactions of GTs ($K_{eq} \ll 1$) and GHs ($K_{eq} \gg 1$) [14,16,17]. The relatively favorable K_{eq} value and the fact that glucosyl phosphates are less expensive than nucleotide diphosphate sugars required by most GTs make GPs interesting biocatalysts for the stereo- and regiospecific synthesis of glucosides under conditions of thermodynamic control.

We and others have shown that cellobiose phosphorylase (CbP, cellobiose:orthophosphate α-D-glucosyl transferase, EC 2.4.1.20) is highly selective in the synthesis of β-1,4-linked glucosides in which the reducing end part presents considerable structural variation [14,16,21–23]. In a recent study, CbP from *Cellulomonas uda* (*Cu*CbP) was found to utilize α-D-glucose 1-fluoride (αG1F) in place of α-D-glucose 1-phosphate (αG1P) [16]. The reaction of *Cu*CbP with αG1F has been characterized in regard to steady-state kinetics [16], but the very low productivity of active enzyme by *C. uda* prohibited further systematic examination of its utility for preparative synthesis. To overcome limitations in enzyme availability, we report here cloning and high level expression of soluble *Cu*CbP. Recombinant *Cu*CbP has been purified and characterized with respect to kinetic properties. αG1F and αG1P are compared as glucosyl donors for synthesis of β-1,4-linked glucosides by *Cu*CbP, and essential components of the quasi irreversible ('glycosynthase' [24–26]) reaction with αG1F are discussed.

2. Materials and methods

2.1. Enzymes and other materials

Native *Cu*CbP was prepared as described recently [16]. Horseradish peroxidase and glucose oxidase from *Aspergillus niger* were from Roche. Other analytical enzymes have been described elsewhere [16]. Restriction endonucleases, *Taq* DNA polymerase, *Pfu* DNA polymerase, and T4 DNA ligase were obtained from Promega. The plasmid vectors pGEM-T (Promega), pBluescript II SK (+/−) (Stratagene), and pQE 30 (Qiagen) were used. The gel extraction kit Qiaex II, and the Qiaprep Spin Miniprep plasmid purification kit were from Qiagen. The oligolabelling kit was obtained from Amersham Biosciences, and the ABI-PRISM sequencing kit was from Applied Biosystems. α-D-glucose 1-fluoride was a kind gift from Dr. M. Albert (Institute of Organic Chemistry, Graz University of Technology, Austria).

2.2. Molecular cloning and sequencing of the gene encoding *CuCbP*

2.2.1. Preparation of an oligonucleotide probe

Standard techniques of molecular biology were employed [27]. From a multiple sequence alignment of the primary structures of CbPs from *Cellvibrio gilvus* (AB010707), *Clostridium stercorarium* (U56424) and *Clostridium thermocellum* (AB013109) the conserved peptides P_{23}WINYL$_{28}$ and A_{486}DWNDC$_{491}$ (using the amino acid numbering of CbP from *C. gilvus*) were selected. They were reverse translated into the following pair of degenerated oligonucleotide primers for PCR:

- forward primer (p1): 5′-CCKTGGATHAAYTACCTB-3′
- reverse primer (p2): 5′-GRCAGTCRTTCCAGTCSGC-3′

where B = C + T + G, H = A + C + T, K = T + G, R = A + G, S = C + G, and Y = C + T.

Genomic DNA of *C. uda* was prepared by using a standard protocol and polymerase chain reactions were carried out as described recently [28]. Using primers p1 and p2, a 1.4 kbp PCR product was amplified from chromosomal *C. uda* DNA and shown to contain the *Sal*I and *Eco*91I restriction sites that are present in the corresponding region of the gene coding for *C. gilvus* CbP. The amplified fragment was gel-purified and placed into the pGEM-T vector using *Sma*I cleavage and blunt-end cloning. The recombinant vector was transferred into electrocompetent cells of *Escherichia coli* DH10B, and the cloned insert was sequenced.

2.2.2. Southern blot analysis

Genomic DNA from *C. uda* (≈10 μg) was exhaustively digested for up to 4 days with *Sal*I, *Bam*HI, or *Pst*I (10–20 U). DNA fragments were separated by agarose gel (0.8%) electrophoresis, blotted onto a positively charged nylon membrane (Roche) and allowed to hybridize with a

^{32}P-labeled 550 bp probe that was obtained from the cloned 1.4 kbp PCR product by *Sal*I digestion. The main hybridizing fragments from *Bam*HI and *Pst*I digestions were eluted and subjected to PCR screening using a pair of specific oligonucleotide primers which in positive cases, generated a ≈ 1 kbp DNA fragment that contained a *Sal*I restriction site:

- forward: 5′-CCACTTCGAGGCGCGCCACGG-3′
- reverse: 5′-GCCCGCGATCAGCCACAGCGG-3′

Fragments of interest were cloned into pBluescript II SK (+/−) vector via *Bam*HI and *Pst*I restriction sites and used to generate a partial genomic library of *C. uda* DNA. Colony hybridization with the 1.4 kbp PCR probe combined with PCR screening was used to extensively scrutinize this library. However, only a single positive clone (out of 3000 screened) was identified within the mixture of fragments obtained by *Pst*I digestion. It was sequenced in both directions of the DNA at the VBC Genomic Sequencing Facility of the University of Vienna using the 'primer walking' method. It was found to contain the entire open reading frame of the *C. uda cbp* gene. Sequence analysis was carried out with the program VectorNTI (Invitrogen).

2.2.3. Construction of the expression plasmid

The following pair of oligonucleotide primers was used for the amplification of the complete *cbp* gene by PCR using the cloned *Pst*I fragment as the template:

- forward primer (p3): 5′-AGCGGATCCCGTTACGGGCACTTCGACGAC-3′
- reverse primer (p4): 5′-ATTCTGCAGCTAGAGGGTCACGTCGACGC-3′

*Bam*HI and *Pst*I restriction sites are underlined in p3 and p4, respectively, and were used to clone the PCR product into pQE 30 to yield the plasmid expression vector pQE 30-CbP. The recombinant enzyme contains an 11 amino acid-long N-terminal fusion peptide (RGSHHHHHHGS) which provides metal affinity to facilitate purification.

2.3. Production and purification of recombinant CuCbP

2.3.1. Production

The vector pQE 30-CbP was transformed through electroporation into *E. coli* DH10B. The organism was cultivated in 1 l baffled shaken flasks at 37 °C using media that contained 10 g peptone from meat, 5 g yeast extract, 5 g sodium chloride, and 100 mg ampicilline per litre. Expression of the *cbp* gene was induced by adding 0.1 mM isopropyl-β-D-thio-galactopyranoside (IPTG) when the optical density at 600 nm of the culture was about 1.0. Approximately, 30 min before IPTG addition, the growth temperature was decreased to 25 °C and maintained until the end of the culture. Cells were harvested by centrifugation, typically at an optical density of ∼4.5, and disrupted by passing cell suspensions three times through a 9.5 mm French press cell (Thermo Spectronic) operated at a maximum pressure of 138 MPa. The resulting slurry was cleared by ultracentrifugation at 80,000 × *g* and 4 °C for 15 min, and the supernatant was used for further purification of *Cu*CbP.

2.3.2. Purification

All purification steps were carried out with an Äktaexplorer system (Amersham Biosciences) at room temperature and used absorbance at 280 nm for the detection of protein:

- Step 1: Immobilized metal affinity chromatography (IMAC) was carried out using copper-loaded chelating Sepharose CL-4B (Amersham Biosciences). The enzyme was applied to the column (1.6 cm × 4 cm) equilibrated with 50 mM potassium phosphate buffer, pH 7.0, and elution was achieved with a linear gradient of up to 400 mM of imidazole. Under these conditions, *Cu*CbP elutes at 120 mM of imidazole.
- Step 2: Hydroxyapatite chromatography (CHT) was conducted using a prepacked column (1 cm × 5 cm) of CHT (Macro-Prep) ceramic hydroxyapatite type II with a particle size of 20–80 μm from Bio-Rad. The enzyme preparation obtained after step 1 was loaded onto the column, equilibrated with 50 mM potassium phosphate buffer, pH 7.0. Approximately, 4 mg of protein were applied to 1 ml of gel. Elution was carried out at a flow rate of 3 ml/min using a linear gradient in potassium phosphate from 50 to 600 mM. Under these conditions, *Cu*CbP elutes at approximately 500 mM phosphate.

The purification was monitored by SDS-PAGE, non-denaturing anionic PAGE and the measurement of specific enzyme activity. For electrophoreses, precast gradient gels (PhastGel 8-25) were used on a PhastSystem from Amersham Biosciences.

2.4. Enzyme assays and kinetics

If not indicated otherwise, reported assays for phosphorylase activity and reaction products were used [16]. D-Glucose was determined with glucose oxidase and peroxidase. Initial-rate measurements in the directions of cellobiose phosphorolysis and synthesis were carried out at 30 °C using a 50 mM Mes buffer, pH 6.6. As described elsewhere in more detail [16], discontinuous measurements were used. The reported enzymatic rates are routinely based on measurements of D-glucose (phosphorolysis) and phosphate (synthesis). Protein was determined with the Bio-Rad dye binding assay using BSA as standard.

2.5. Production of cellobiose using different glucosyl donors

A reaction mixture (2 ml) of 30 mM αG1P and 30 mM D-glucose in 80 mM Mes buffer, pH 7.0, was incubated with purified *Cu*CbP (3 U/ml) at 30 °C for 150 min. Mixing (at

700 rpm) and temperature control was achieved by using an Eppendorf thermomixer 5436. Samples of 200 μl were taken at regular times during the reaction and heated to ≈95 °C (for 5 min) to inactivate the enzyme completely. The concentrations of phosphate, D-glucose and cellobiose were quantitated in each sample [16]. In a second experiment, 30 mM αG1F was used in place of αG1P while all other conditions remained unchanged. In samples taken from the incubation with αG1F, concentrations of D-glucose and cellobiose were measured. Due to the formation of HF during the enzymatic reaction (see later), the pH had to be controlled and was maintained at a value of 7.0 by adding manually small volumes (5 μl) of a 2 M sodium carbonate solution. Separate control experiments which lacked the enzyme showed that heat inactivation of *Cu*CbP does not lead to detectable decomposition of αG1F. It was proven that the cellobiose produced enzymatically (using αG1P or αG1F as glucosyl donor) co-migrated and co-eluted with authentic material in TLC and HPLC analyses, respectively. To determine whether *Cu*CbP is capable of degrading cellobiose in the presence of fluoride, 10 U/ml of the enzyme were incubated with 1 M NaF and 30 mM cellobiose in 80 mM Mes buffer, pH 7.0, at 30 °C for 4 days. The formation of D-glucose was quantitated enzymatically, and samples taken from the reaction mixture in regular intervals were analyzed by TLC.

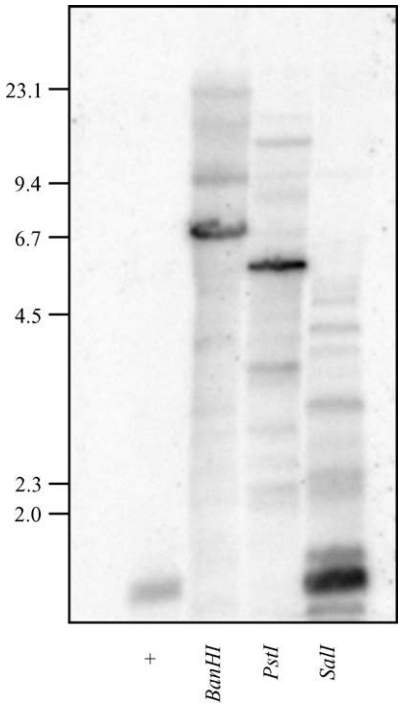


Fig. 1. Southern blot analysis for *C. uda* genomic DNA. Lanes 1–4 of the autoradiogram show the hybridization patterns of the ^{32}P-labeled PCR probe with *C. uda* DNA digested with different endonucleases, as indicated. The lane indicated with + is the control lacking endonuclease addition. Arrows on the left of the blot show the sizes (in kbp) of the main hybridizing DNA fragments. For further details, see the Materials and methods section.

3. Results and discussion

3.1. Cloning and sequencing of the gene encoding CuCbP

Using PCR primers p1 and p2, a 1.4 kbp fragment was amplified from chromosomal *C. uda* DNA and found to be part of a putative *cbp* gene. The [α-^{32}P]dCTP-labeled PCR fragment was used as probe for Southern blot hybridization with *C. uda* genomic DNA digested with different endonucleases (Fig. 1). A strong hybridization to a *Bam*HI fragment of approximately, 7 kbp and a *Pst*I fragment of approximately, 6 kbp was found. These fragments were cloned into pBluescript II SK(+/−). One positive clone of the *Pst*I fragment was identified and sequenced. The sequence contained the whole open reading frame of the *cbp* gene which consists of 2463 bp and encodes a protein of 821 amino acids. The N- and C-terminal parts of *Cu*CbP could be identified clearly using sequence similarity to *C. gilvus* CbP. The molecular mass of *Cu*CbP calculated from primary structure is 91.3 kDa, in good agreement with the experimental value of 90 ± 2 kDa reported for the natural enzyme [16]. Analysis of codon usage in *cbp* genes from *C. uda* and *C. gilvus* reveals that the G + C content is approximately 70% in both cases, i.e., nearly every codon ends with G or C.

3.2. Analysis of CuCbP primary structure

We carried out an initial screening of the non-redundant protein data base with the amino acid sequence of *Cu*CbP using the PSI-BLAST program [29] to compile a listing of all related proteins. A BLOSUM62 matrix and an E-threshold value of 10 were selected. With these settings, significant entire-chain similarities and clearly traceable relationships of *Cu*CbP were found with enzymes and uncharacterized open reading frames that have been classified recently into family GT 36 of the glycosyltransferase enzyme families [18–20]. The known enzyme activities of members of family GT 36 include cellobiose phosphorylase, cellodextrin phosphorylase, chitobiose phosphorylase, and cyclic β-1,2-glucan synthase. Fig. 2 shows a multiple sequence alignment using primary structures of *Cu*CbP and five members of family GT 36 that have been characterized at the enzyme level. These GTs provide a representative selection of the typical enzyme activities found to occur within family GT 36. *Cu*CbP is most similar to CbP from *C. gilvus* with a residue identity of 89%. In pairwise comparisons such as that of *Cu*CbP and cyclic β-1,2 glucan synthase from *Sinorhizobium meliloti*, the sequence identity goes down to 26%. Henrissat and co-workers have used threading and other fold recognition techniques to classify some of the currently known 65 glycosyltransferase families into a hierarchical system of currently four clans (or superfamilies; Clan I–IV) and twofolds (GT-A and GT-B) [18,19]. Unfortunately, it has not been possible to extend the sequence-derived classification of family GT 36 to

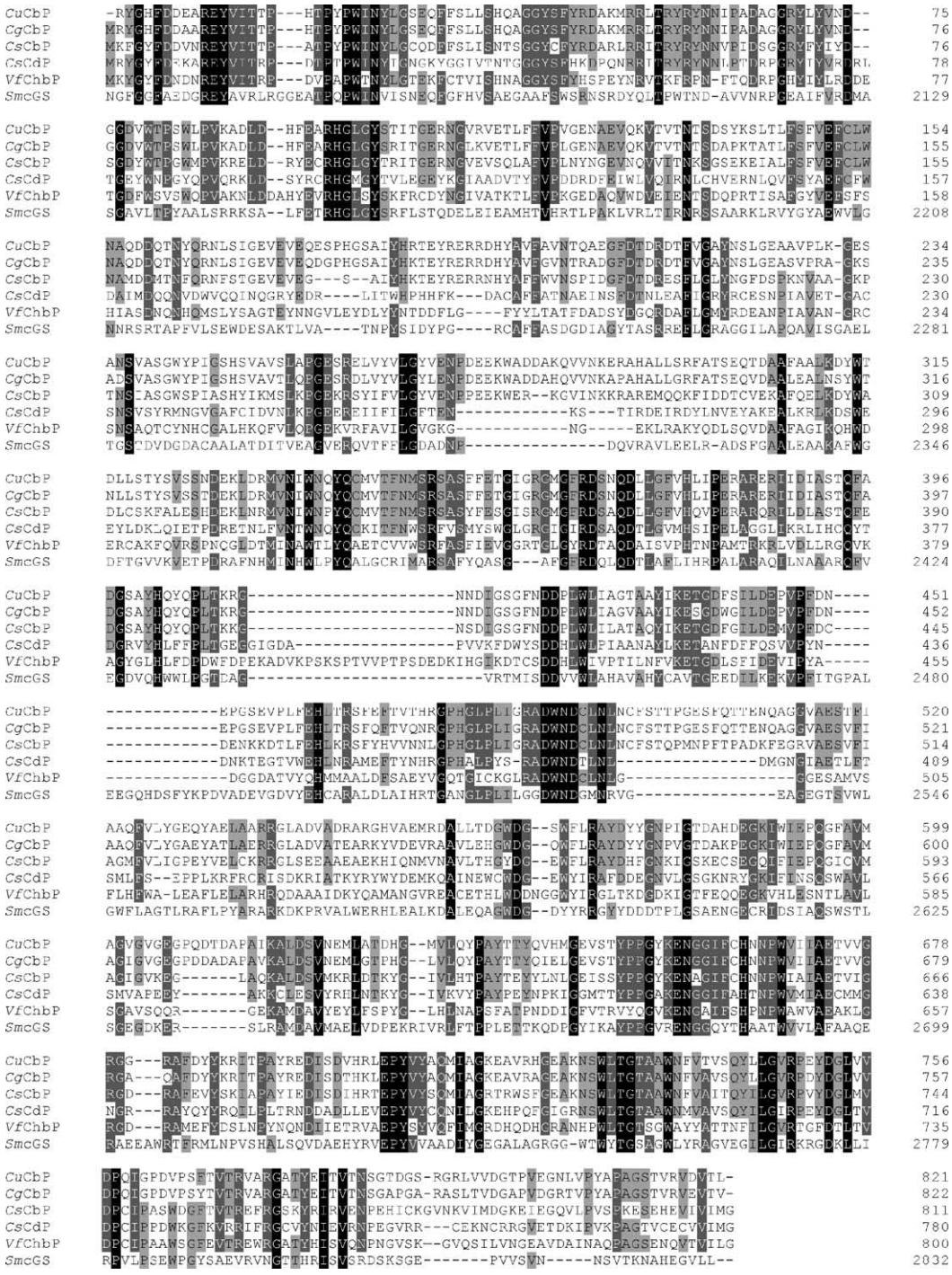


Fig. 2. Comparison of the *Cu*CbP amino acid sequence with sequences of different members of family GT 36. The abbreviations indicate CbPs from *C. gilvus* (*Cg*CbP; AB010707) and *C. stercorarium* (*Cs*CbP; U56424), cellodextrin phosphorylase from *C. stercorarium* (*Cs*CdP; U60580), *N*,*N*′-diacetylchitobiose phosphorylase from *Vibrio furnissii* (*Vf*ChbP; AF230379) and cyclic β-1,2 glucan synthase from *Sinorhizobium meliloti* (*Smc*GS; NC_003047). The alignment was performed with the Vector NTI program using the AlignX-modul with the PAM250 scoring matrix. Conserved amino acids are shaded in black (100% identity), gray (80–100% identity) and light gray (60–80% identity).

achieve a grouping into Clan I–IV, indicating that there is a quite distant relationship of family GT 36 with other glycosyltransferases. There are relatively few well conserved regions revealed by the sequence alignment in Fig. 2. Candidate amino acids for site-directed mutagenesis may be selected from these regions.

3.3. Production of recombinant CuCbP

Using induction with IPTG and the conditions described under Section 2.3, a specific CbP activity of approximately 4.5 U/mg was measured in crude cell extracts of *E. coli* DH10B harboring the expression vector pQE 30-CbP. The

specific activity of pure natural *Cu*CbP is 19.2 U/mg (see later), and comparison of the two values suggests that recombinant *Cu*CbP corresponds to approximately 23% of the total soluble *E. coli* protein. The specific activity in cell extracts of *C. uda* is 0.02–0.08 U/mg, indicating that heterologous gene expression leads to a 56–225-fold increase in enzyme yield, based on total protein present. From the harvested *E. coli* cells of 1 l medium, 3060 U of *Cu*CbP were obtained. This value compares to a value of ≤12 U/l when *C. uda* was used for enzyme production. Apart from the fact that *E. coli* is much easier to cultivate in a reproducible manner than *C. uda*, the overall production time in shaken flask cultures of *E. coli* is about 1/5 of that of *C. uda* under otherwise comparable conditions. Using *E. coli* transformed with pQE 30-CbP, the volumetric productivity of *Cu*CbP activity has therefore been increased by three orders of magnitude, compared to the natural system. The problem of availability of active enzyme for practical applications has thus been eliminated.

3.4. Purification of recombinant CuCbP

We used a two-step protocol to isolate recombinant *Cu*CbP from induced *E. coli* cell extracts, as summarized in Table 1. The purified enzyme migrated as a single protein band in Coomassie-stained PAGE and non-denaturing anionic PAGE (Fig. 3). The overall yield was 39%. Substantial losses of original enzyme activity (≈50%) occurred during IMAC. There is good evidence that imidazole is destabilizing and caused the observed inactivation. If the enzyme (≥1 mg/ml) was frozen at −20 °C in 50 Mes buffer, pH 6.6, containing 200 mM of imidazole, no activity remained after thawing the sample. CbP activity was fully stable under identical conditions when no imidazole was present. The storage stability of *Cu*CbP at 4 °C was reduced substantially (≥10-fold) by imidazole. Therefore, purification by IMAC, particularly the elution step should be optimized further to avoid inactivation of the enzyme. The specific activity of purified recombinant *Cu*CbP was half that of the natural enzyme. We think that the result can be explained on account of co-purification of two CbP fractions, one displaying activity and another that has been inactivated by imidazole. We are not aware of other reports in the literature describing sensitivity of CbP activity towards imidazole. In fact, recombinant CbP from *C. thermocellum* has been purified using IMAC and elution with 150 mM imidazole [30].

In practice, purification of *Cu*CbP to homogeneity is, of course, not necessary. However, removal of α- and β-D-glycoside hydrolase activities would be important to prevent degradation of αG1F and cellobiose (or another β-1,4-D-glucoside), respectively. Likewise, phosphatase activity should not be present because it could hydrolyze αG1P. With the enzyme preparation used in experiments to be reported below, no evidence of contaminating hydrolase activities has been found.

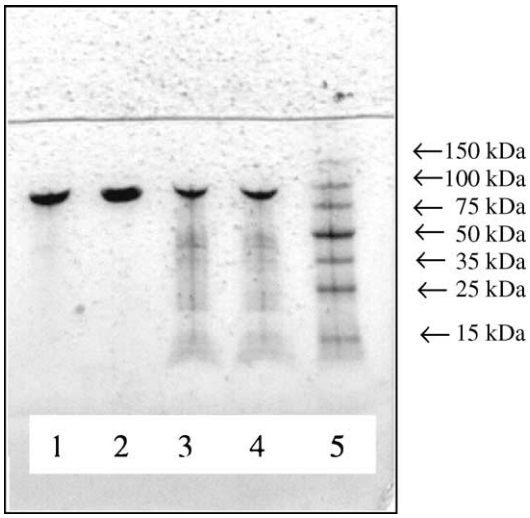


Fig. 3. Purification of recombinant cellobiose phosphorylase from *C. uda* documented by SDS-PAGE. Staining of protein bands was done with Coomassie Brilliant Blue R350. Lanes 1–5, left to right: 1–2, purified his_6-*Cu*CbP; 3–4, crude *E. coli* cell extract; 5, molecular-mass standards. Arrows on the right of the gel indicate molecular masses of the standard proteins.

An estimate of 92 kDa was obtained for the molecular mass of recombinant *Cu*CbP using the SDS gel in Fig. 1. In a column sizing experiment employing gel permeation chromatography on Superose 12, a molecular mass of 90 kDa was measured. Like natural *Cu*CbP [16], the recombinant His-tagged enzyme appears to be a functional monomer.

3.5. Kinetic characterization

Initial-rate measurements for the direction of cellobiose phosphorolysis and synthesis catalyzed by his_6-*Cu*CbP were carried out under conditions in which one substrate concentration was varied and the concentration of the second substrate was constant and saturating (>5K_m) at the steadystate. Data were fitted to Eq. (2):

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

Table 1
Purification of recombinant *Cu*CbP

Purification step	Total volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)
Cell extract	5.5	162	715	4.4	100
IMAC	22.5	37	349	9.4	49
CHT	12.0	35	281	8.0	39

Table 2
Comparison of kinetic parameters for the forward and reverse reactions catalyzed by native and recombinant *Cu*CbP

	Native *Cu*CbP	his$_6$-*Cu*CbP
Phosphorolysis		
k_{cat} (s^{-1})	29.1 ± 2.3	15.4 ± 0.2
K_m (cellobiose) (mM)	4.3 ± 0.5	2.9 ± 0.2
K_m (phosphate) (mM)	1.20 ± 0.10	0.82 ± 0.07
Synthesis		
k_{cat} (s^{-1})	43.2 ± 3.0	27.5 ± 0.6
K_m (αG1P) (mM)	2.15 ± 0.10	3.28 ± 0.19
K_m (Glc) (mM)	2.26 ± 0.30	3.79 ± 0.28
K_{iS} (Glc) (mM)	202 ± 27	255 ± 29

where V is the initial velocity, k_{cat} is the turnover number, [E] and [S] are molar enzyme and substrate concentrations, and K_m is the Michaelis constant for S. Calculation of the catalytic constant k_{cat} was based on a molecular mass of *Cu*CbP of 91.3 kDa. When inhibition occurred at high [S], initial velocities were fitted to Eq. (3):

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

where K_{iS} is the substrate-inhibition constant. Kinetic parameters are summarized in Table 2. The Michaelis constants of his$_6$-*Cu*CbP (this work) and CbP isolated from *C. uda* [16] are closely similar, indicating that the N-terminal His-tag does not appreciably alter the apparent substrate affinities of the enzyme in both directions of the reaction. The k_{cat} values of his$_6$-*Cu*CbP are half that of the corresponding turnover numbers of the natural enzyme. This difference is readily explained by assuming a proportion of ≈50% of inactive protein present in the preparation of his$_6$-*Cu*CbP (see above).

3.6. Application of CuCbP in the synthesis of cellobiose

An equilibrium constant of 0.22 ± 0.04 has been reported for the enzymatic conversion of cellobiose and phosphate into αG1P and D-glucose at pH 6.6 and 30 °C [16]. If *Cu*CbP is used in the reverse direction of the reaction, this value of K_{eq} implies that approximately, 60% of the substrate will be converted into glucosidic product at thermodynamic equilibration, assuming equimolar start concentrations of αG1P and D-glucose. Fig. 4 displays a typical time course of cellobiose production using *Cu*CbP and illustrates limitations in yield that are due to K_{eq}. It has been shown recently that αG1F is about 1/10 as good as substrate as αG1P. Therefore, αG1F could be a practically useful alternate glucosyl donor for *Cu*CbP-catalyzed synthesis of β-1,4-D-glucosides.

Scheme 1 illustrates the experimental approach. The strong hydration of the released (H)F in water leads to a virtually unidirectional ('synthase') reaction whose yield should therefore be close to 100% [31]. In agreement with this notion, we were not able to detect any reaction (i.e., formation of D-glucose and αG1F) between cellobiose

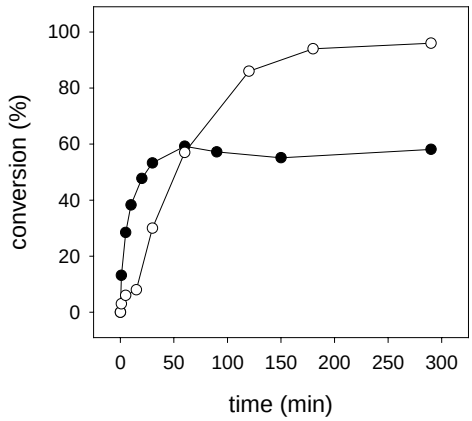
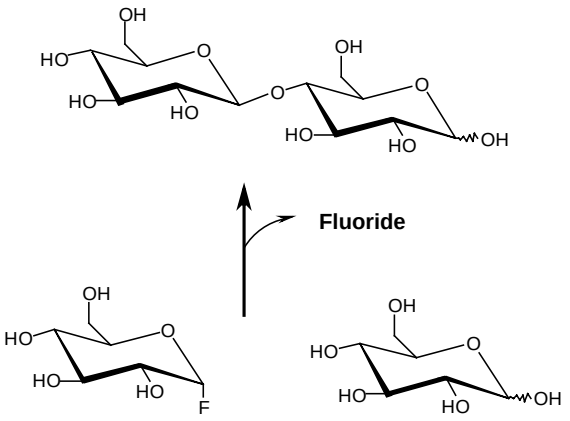


Fig. 4. Synthesis of cellobiose by his$_6$-*Cu*CbP using αG1P or αG1F as glucosyl donor substrate. The reaction conditions are described under Section 2. We used 30 mM of donor and acceptor substrate (D-glucose). Data show the conversion of D-glucose, expressed as percentage. The symbols indicate utilization of αG1P (filled circles) or αG1F (open circles).

(30 mM) and sodium fluoride (1 M) at pH 6.6 when *Cu*CbP was present. The time course of cellobiose synthesis by *Cu*CbP using αG1F and D-glucose as the substrates is shown in Fig. 4. Within the limits of the experimental error (±4%), the conversion of substrates was complete (≈100% yield). The enzymatic reaction with αG1F is slower than the corresponding reaction with αG1P. This is readily explained by differences in specificity constants on the one hand and the pronounced substrate-inhibition by αG1F on the other [16].

In conclusion, our results demonstrate the practical utility of αG1F as an alternate donor substrate for the cellobiose phosphorylase-catalyzed synthetic reaction. Considering that the enzyme is unable to convert the reaction products back into substrates at detectable rates, we can speak of a glycosynthase-type reaction in which modification of the substrate rather than the enzyme catalytic site [24–26] leads to the desired increase in product yield (see Fig. 4). It has been shown that glucosyl fluorides serve as alternative donor substrates of phosphorylases and GTs (reviewed in



Scheme 1. Synthetic reaction of his$_6$-*Cu*CbP using α-D-glucosyl fluoride and D-glucose as substrates. Details are found under Section 2.

[32]; [33–35]). In light of the excellent regiospecificity of phosphorylase-catalyzed synthesis, it will be relevant to explore if the specificity of phosphorylases can be broadened from *glucosyl* to *glycosyl* donor substrates which could be various glycosyl fluorides.

Note added in proof

On the basis of very recent structural evidence currently in press (April 2004), family GT36 has been re-classified as family GH94 belonging to clan GH-L (see the URL address http://afmb.cnrs-mrs.fr/CAZY/index.html for further information).

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