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Examining the role of phosphate in glycosyl transfer reactions of *Cellulomonas uda* cellobiose phosphorylase using D-glucal as donor substrate

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ABSTRACT

Cellobiose phosphorylase from *Cellulomonas uda* (*Cu*CPase) is shown to utilize D-glucal as slow alternative donor substrate for stereospecific glycosyl transfer to inorganic phosphate, giving 2-deoxy-α-D-glucose 1-phosphate as the product. When performed in D_2O, enzymatic phosphorolysis of D-glucal proceeds with incorporation of deuterium in equatorial position at C-2, implying a stereochemical course of reaction where substrate becomes protonated from below its six-membered ring through stereoselective *re* side attack at C-2. The proposed catalytic mechanism, which is supported by results of docking studies, involves direct protonation of D-glucal by the enzyme-bound phosphate, which then performs nucleophilic attack on the reactive C-1 of donor substrate. When offered D-glucose next to D-glucal and phosphate, *Cu*CPase produces 2-deoxy-β-D-glucosyl-(1→4)-D-glucose and 2-deoxy-α-D-glucose 1-phosphate in a ratio governed by mass action of the two acceptor substrates present. Enzymatic synthesis of 2-deoxy-β-D-glucosyl-(1→4)-D-glucose is effectively promoted by catalytic concentrations of phosphate, suggesting that catalytic reaction proceeds through a quaternary complex of *Cu*CPase, D-glucal, phosphate, and D-glucose. Conversion of D-glucal and phosphate presents a convenient single-step synthesis of 2-deoxy-α-D-glucose 1-phosphate that is difficult to prepare chemically.

1. Introduction

Cellobiose phosphorylase (CPase; EC 2.4.1.20) catalyzes reversible phosphorolysis of cellobiose into α-D-glucose 1-phosphate and D-glucose.[1] Like the majority of known disaccharide phosphorylases, CPase is an inverting enzyme that forms a glycosidic product having the opposite anomeric configuration as the substrate.[2] The stereochemical course of cellobiose conversion involves a β-to-α change in anomeric configuration, proposed to result from a single displacement-like reaction that produces an equatorial-to-axial substitution at the anomeric carbon.[3] The canonical catalytic mechanism of enzymatic glycosyl transfer with inversion (used by glycoside hydrolases, for example) involves a pair of acidic residues, typically Asp or Glu, that are positioned in the active site so that one can function as the general catalytic acid and the other as the general catalytic base.[4,5] CPase is thought to employ a modified version of this mechanism, in which only one catalytic residue (Asp) is used, and this has the role of a general acid.[2] Since the acceptor substrate, phosphate, is already ionized at physiological pH, it does not need to be activated by deprotonation from a catalytic base, therefore explaining the absence of a corresponding residue in the CPase active site. CPase crystal structures reveal a distinct phosphate recognition site in the enzyme, rich in positively charged amino acids (Fig. 1b). Once accommodated at this site, phosphate is optimally positioned for direct nucleophilic attack on the glycosidic carbon of cellobiose (Scheme 1).

In an original classification based on sequence analysis, CPase was classified as a member of the glycosyltransferase families and further categorized into family GT-36. This classification, which is in accordance with CPase catalytic function of glucosyl transfer to and from phosphate, as also reflected in the EC number, needed to be revised when the first crystal structure of an enzyme from the CPase group, *Vibrio proteolyticus* chitobiose phosphorylase (*Vb*ChPase), became available.[6] Evidence that *Vb*ChPase was structurally similar to certain glycoside hydrolases, in particular to maltose phosphorylase and glucoamylase from glycoside hydrolase family GH-65 and GH-15, respectively, necessitated reclassification of CPase as a glycoside hydrolase, forming a new family GH-94.[6] It is worth noting therefore that CPase enzymes usually show no or just scant glycoside hydrolase activity. As of today, family GH-94 consists of four principal members, CPase (EC 2.4.1.20), cellodextrin phosphorylase (EC 2.4.1.49), ChPase (EC 2.4.1.-), and cyclic β-1,2-glucan synthase (EC 2.4.1.-) (http://www.cazy.org/CAZY). Among individual enzymes belonging to family GH-94, CPase from *Cellvibrio gilvus* (*Cg*CPase) is the one most extensively studied.[7–9] Two crystal structures of *Cg*CPase are available, one containing

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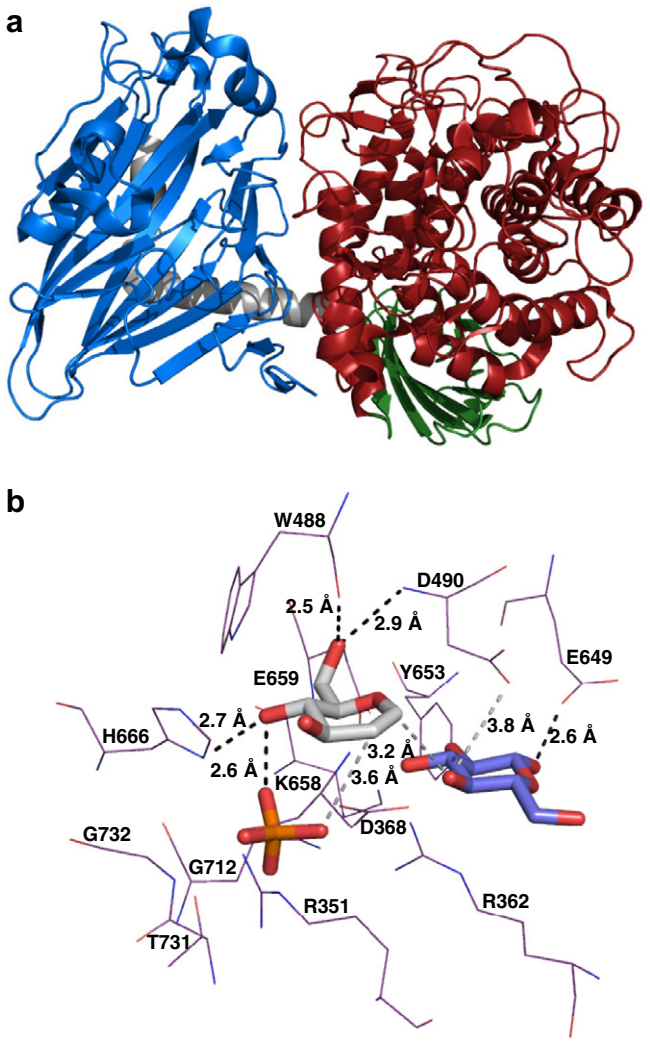
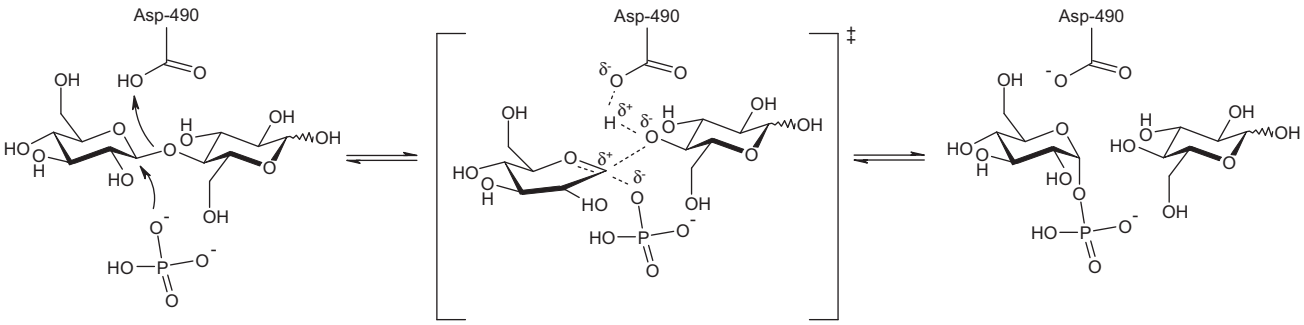


Figure 1. (a) Three-dimensional fold of the protein subunit of *Cg*CPase (PDB-entry 2cqt, molecule B). *Cg*CPase consists of an N-terminal domain (blue), linker helices (gray), an α-helix barrel domain (red), and a C-terminal domain (green). (b) Close-up view of the predicted binding of D-glucal (gray) in subsite -1 of *Cg*CPase (PDB-entry 2cqt, molecule B) complexed with phosphate (orange) and D-glucose (purple) in subsite +1. Interactions potentially relevant for catalysis and the reaction are shown as gray-dashed lines, and strong hydrogen bonds stabilizing the quaternary complex are shown as black-dashed lines. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

sulfate (PDB-entry 2cqs) and one containing phosphate (PDB-entry 2cqt) at the catalytic site.[2] The structure of *Cg*CPase monomer is shown in Figure 1a and consists of four distinct regions: an N-terminal domain, a helical linker region, an α-helix barrel domain, and a C-terminal domain.

The substrate specificity of CPase has been examined in a number of biochemical studies using enzymes from different sources.[1,10–12] It was convenient to analyze the reverse direction of reaction where a glycosyl residue is transferred from sugar 1-phosphate donor to acceptor. The various CPases are similar in that relatively relaxed specificity for the acceptor substrate is contrasted with highly restricted use of glycosyl donors.[7,13] Two relevant examples of active donor substrate analogues are α-D-glucose 1-fluoride[14] and α-D-galactose 1-phosphate.[15] Note that ChPase has of course different structural requirements to its donor substrate as compared to CPase where in particular 2-hydroxyl in α-D-glucose 1-phosphate is replaced by 2-acetamido in α-D-*N*-acetylglucosamine 1-phosphate.[6] In a recent paper, Kitaoka and co-workers reported the interesting observation that the endocyclic enitol D-glucal was accepted by *Cg*CPase as donor substrate for 2-deoxy-glucosyl transfer to D-glucose and other sugar acceptors (e.g. D-xylose, D-mannose) used normally in the reaction with α-D-glucose 1-phosphate.[16] They also demonstrated the transfer products to have β-(1→4) glycosidic structure. Using *Cellulomonas uda* CPase (*Cu*CPase), Nidetzky and co-workers had earlier examined reactivity toward D-glucal as donor substrate for glycosyl transfer to phosphate or D-glucose but were unable to detect any.[14] Inspired by Kitaoka's findings, the topic of utilization of D-glucal was therefore revisited herein, and we would like to communicate results of a comprehensive analysis that provide deepened insight into the underlying reaction mechanism. We in particular clarify the role of phosphate in promoting the enzymatic reaction with D-glucal and show that depending on reaction conditions used, D-glucal serves as donor substrate for 2-deoxy-D-glucosyl transfer to phosphate or sugar acceptor. Requirement for phosphate to essentially 'activate' *Cu*CPase reaction with D-glucal explains negative findings of our previous experiments that in direction of disaccharide synthesis were all performed in the absence of phosphate. In addition, the amount of native enzyme (isolated from the natural host *C. uda*) and the analytical method used (TLC) may have been insufficient for identification of D-glucal as donor substrate for phosphorolysis. We herein also show that conversion of D-glucal in the presence of phosphate constitutes a convenient single-step method for preparation of 2-deoxy-α-D-glucose 1-phosphate (**1**) that is difficult to synthesize chemically.

In chemical terms, D-glucal and related glycals are characterized as 1,2-unsaturated derivatives of pentoses or hexoses. These compounds have been widely used as mechanistic probes for study of glycoside hydrolases and phosphorylases.[17–23] Their unique feature of structure and function lies in the half-chair conformation of the typically six-membered ring that contains a highly reactive double bond between C-1 and C-2. It is known from seminal work



Scheme 1. Proposed catalytic mechanism of *Cu*CPase. Asp^{490} is thought to adopt the catalytic role of a general acid and base in direction of cellobiose phosphorolysis and synthesis, respectively. See Ref. 2 for a detailed discussion.

of Hehre, Lehmann, Legler and their co-workers performed as early as the 1970s that glycals are substrates to become hydrated by many glycoside hydrolases.[18,24,25] D-Glucal is utilized by α-glucosidase from *Candida tropicalis* and β-glucosidase from sweet almond.[18] Disaccharide analogues of maltose (maltal) and cellobiose (cellobial) in which D-glucal replaces the original 'reducing-end' D-glucose moiety are hydrated by, respectively, sweet potato β-amylase[19] and *exo*- as well as *endo*-type cellulases.[26] Work of Klein et al. on α-glucan phosphorylase[27] and glycogen phosphorylase[28] provided a first case for reaction of a phosphorylase with D-glucal. Unlike the hydrolases that promoted double bond hydration only, the phosphorylase catalyzed transfer of the 2-deoxy-glucosyl-moiety generated from D-glucal upon protonation at C-2. Depending on relative abundance of acceptor substrates present in the reaction, transfer occurred preferentially to phosphate or α-(1→4)-D-glucosyl oligo/polysaccharide.[27,28] The exocyclic enitol heptenitol (2,6-anhydro-1-deoxy-D-gluco-hept-1-enitol) was applied in the study of different glycoside hydrolases and glycogen phosphorylase. It is particularly relevant for this work that the phosphorylase catalyzed glycosyl transfer to phosphate, forming heptulose 2-phosphate as product.[29]

Mechanistic proposals for enzymatic utilization of glycals share a common initial step that is chemical 'activation' of the glycal by protonation at C-2. Analysis of stereospecifity of this protonation event has therefore been an important component of the mechanistic characterization of different enzymes, including α- and β-glucosidases[18], *exo*-α-glucanases,[30] and glycogen phosphorylase.[27] To examine the stereochemical course of the transformation, reactions were performed in D_2O and their progress analyzed by NMR spectroscopy. The same methodology was applied in this work to monitor *Cu*CPase-catalyzed 2-deoxy-D-glucosyl-transfer to phosphate. We show that deuterium from solvent is incorporated stereospecifically in equatorial position of **1**, revealing that proton attack occurs on the *re* side of C-2, that is from below the ring of D-glucal.

2. Results and discussion

2.1. Single-step purification of recombinant *Cu*CPase

Using the procedures described under 3.2 enzyme preparation, we obtained *Cu*CPase in a highly purified form. The isolated enzyme migrated as single protein band in SDS PAGE (Supplementary Fig. 1), and its specific activity of 14.4 U/mg was in useful agreement with the value of 19.2 U/mg reported for the same His-tagged *Cu*CPase in a previous paper from this laboratory.[31] It should be noted that *Cu*CPase isolation was possible with only a single purification step via the enzyme's His-tag, using Ni-chelating Sepharose Fast Flow chromatography with a His-Trap HP column. In previous work using Cu-loaded chelating Sepharose CL-4B, a second chromatographic step on hydroxyapatite resin was necessary to obtain highly purified enzyme. Elution of *Cu*CPase occurred at a higher concentration of imidazole (250 mM) using the 'new' method as compared to the published protocol (120 mM imidazole), suggesting that interaction of the His-tagged protein with the chromatographic material was enhanced under the conditions used herein. Overall, the purification of *Cu*CPase was made simpler and more time-efficient as compared to the reported protocol. In some batches of enzyme purified according to literature, we noted the problem of co-purification of phosphorylase and phosphatase activities. The phosphatase hydrolyzed α-D-glucose 1-phosphate into D-glucose and phosphate. It was important to confirm therefore that the purified *Cu*CPase used herein was absolutely devoid of such phosphatase activity.

2.2. Identification of D-glucal as *Cu*CPase substrate for 2-deoxy-D-glucosyl transfer to phosphate

When offered D-glucal (50 mM) and phosphate (50 mM) as substrates, *Cu*CPase (5 μM) catalyzed formation of a single main product that eluted in analytical high performance anion exchange chromatography with pulsed amperometric detection (HPAED-PAD) at a position comparable to that of elution of an authentic α-D-glucose 1-phosphate standard. We determined that product formation took place at the expense of D-glucal (HPAED-PAD). NMR analysis from a reaction mixture in which about 20% of the original substrate had been converted identified the product to be a phosphorylated sugar. Results depicted in Figure 2a establish identity of the phospho-sugar product as **1**. Presence of the phosphate group was indicated by a ^{31}P signal at 2.4 ppm, which showed a $^{3}J_{P\text{-}H}$ coupling in $^{1}H/^{31}P$-HSQC to proton H-1 with a coupling constant of 7.6 Hz. The $^{3}J_{H\text{-}H}$ coupling of 3.0 Hz between H-1 and H2a pointed to α-anomeric configuration at C-1. In Figure 2a the TOCSY trace of protons H-1 is shown, which indicates all protons in the respective spin system. Several scalar couplings can be deduced from additionally shown separated proton signals of **1**. NMR spectroscopic data are summarized in Supplementary Table 1.

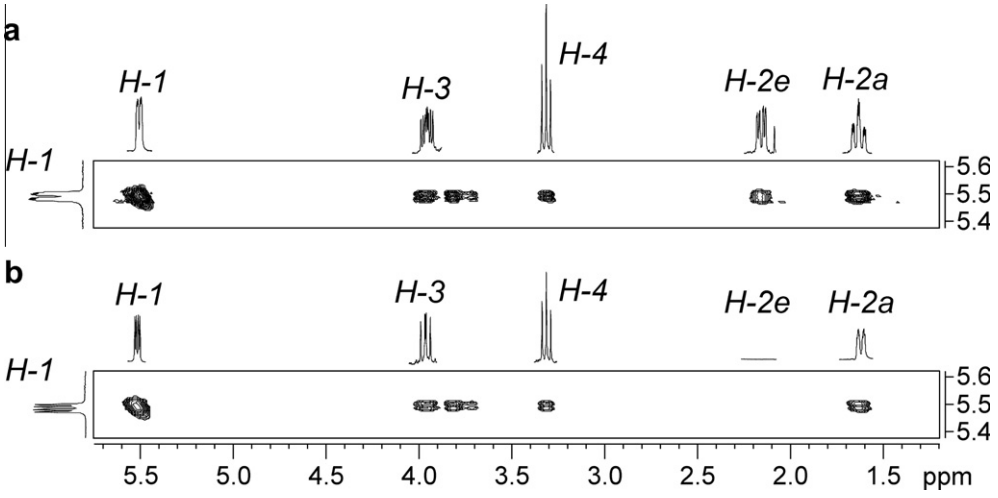


Figure 2. NMR spectroscopy based identification of 2-deoxy-α-D-glucose 1-phosphate (**1**) (panel a) and 2-deoxy-α-D-[(2e)-D]-glucose 1-phosphate (**4**) (panel b). Shown are the 2D TOCSY traces of protons H-1 in f_2-dimension. Additionally, the separated and not overlapped ^{1}H NMR signals are attached to the axes, respectively. Indicative shifts and coupling constants are listed in Supplementary Table 1.

NMR analysis revealed that 2-deoxy-D-glucose (**2**) was a minor additional product of the conversion of D-glucal that was formed in low abundance (⩽2%) relative to **1**. Careful controls performed under otherwise identical reaction conditions in the absence of enzyme showed that D-glucal was stable over the entire timespan of the experiment, eliminating the possibility that spontaneous, non-enzymatic reactions are responsible for the observed product pattern. These results indicate that *Cu*CPase promotes conversion of D-glucal via 2-deoxy-D-glucosyl transfer to phosphate whereby D-glucal serves as donor substrate for phosphorolysis. From time-course data presented in Figure 3 we estimated that the reactivity of D-glucal as substrate for phosphorolysis by *Cu*CPase is roughly three orders of magnitude lower than reactivity of the native donor substrate cellobiose used under otherwise identical conditions (50 mM of each cellobiose and phosphate). A plausible explanation for formation of **2** during phosphorolysis of D-glucal is that slow non-enzymatic hydrolysis of the relatively unstable **1** takes place as a secondary reaction of the overall transformation. The alternative possibility is that *Cu*CPase catalyzes direct hydration of D-glucal at a rate much slower than the phosphorolysis rate. In experiments described later where *Cu*CPase was incubated with D-glucal and D-glucose in the absence of phosphate, the enzyme synthesized tiny amounts of a product that was shown by HPAED-PAD analysis to consist of **2** next to 2-deoxy-β-D-glucosyl-(1→4)-D-glucose (**3**). Product **2** is quite unlikely to have originated from hydrolysis of the trace amounts of **3** formed under these conditions. Direct hydration of D-glucal by *Cu*CPase is therefore supported. The hydration product is assumed to be 2-deoxy-α-D-glucose, which then undergoes mutarotation in solution, yielding the α- and β-anomers of **2** in the reaction mixture, as observed in the NMR analysis.

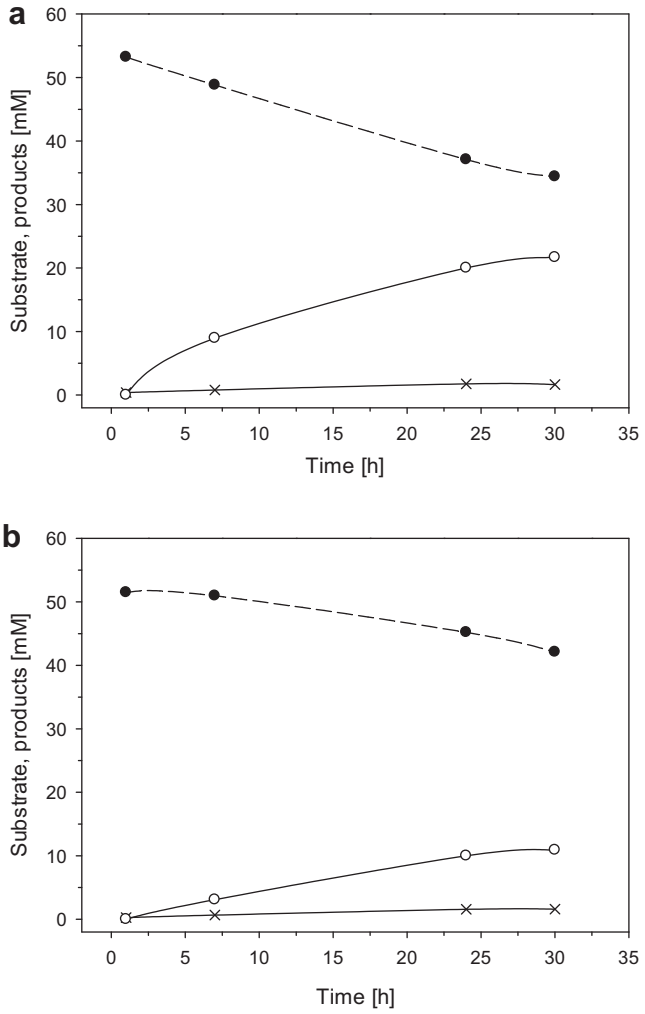


Figure 3. Time-courses for 2-deoxy-α-D-glucose 1-phosphate (**1**) (○) and 2-deoxy-D-glucose (**2**) (x) production from 50 mM D-glucal (●) in the presence of 250 mM phosphate (a) and 50 mM phosphate (b). The enzyme concentration was 5 μM. Lines show trend of the data.

2.3. Enzymatic synthesis of 1 from D-glucal

Figure 3a shows time courses of conversion of 50 mM D-glucal in the presence of the same molar concentration of phosphate and a fivefold molar excess of phosphate. Reaction rate was enhanced (twofold) by increasing the initial phosphate concentration from 50 mM to 250 mM. The final concentration of **1** obtained in the enzymatic conversion was doubled from 10 mM to around 20 mM by the same increase in phosphate concentration, indicating a mass action effect. The maximum conversion of D-glucal under the conditions used was around 40%. Despite this rather modest yield, single-step production by *Cu*CPase appears to be the currently most convenient synthesis of **1**. We will show later under 2.5 enzymatic reaction with D-glucal in the presence of D-glucose and phosphate, that the presence of D-glucose further stimulates catalytic formation of **1**, making the synthesis even more efficient.

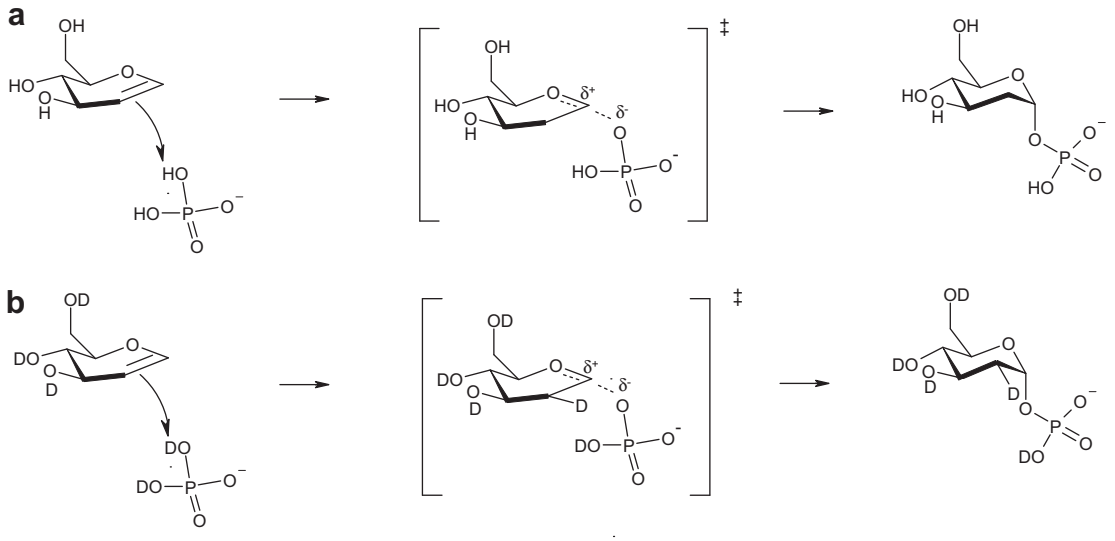
2.4. Stereochemical course of enzymatic reaction with D-glucal

A plausible mechanism of utilization of D-glucal for 2-deoxy-D-glucosyl transfer to phosphate involves substrate activation by protonation at C-2 prior to or concomitant with nucleophilic attack of phosphate oxygen on C-1. To probe the stereoselectivity of the protonation event, we performed enzymatic reaction in D_2O using 50 mM of each D-glucal and phosphate, and analyzed with NMR the incorporation of deuterium in the resulting **1** obtained after 24 h of incubation. The obtained NMR spectrum identified the formed product as 2-deoxy-α-D-[(2e)-D]-glucose 1-phosphate (**4**). This compound has the same anomeric configuration as **1** and shows a ^{31}P signal at 2.5 ppm. It shows the proton signal of H-2a, while no signal of H-2e is detectable (Fig. 2b). Concomitant the signal of H-1 and H-3 show only scalar couplings to H-2a as well as to H-2a and H-4, respectively. Furthermore, C-2 has a typical $^{1}J_{C\text{-}D}$ coupling (data not shown). NMR spectroscopic ^{1}H and ^{13}C data of **4** are summarized in Supplementary Table 1.

Our results indicate that protonation of D-glucal by *Cu*CPase is highly stereoselective. They also suggest a stereochemical course of the protonation where the proton is delivered from below the six-membered ring of substrate in a *re* side attack on C-2 (Scheme 2B). We will discuss later with the help of molecular docking that the most plausible mechanistic scenario is that enzyme-bound phosphate serves as proton donor in the reaction.

2.5. Enzymatic reaction with D-glucal and D-glucose in the absence and presence of phosphate

Kitaoka et al. have shown exploitation of *Cg*CPase-catalyzed 2-deoxy-β-D-glucosyl transfer from D-glucal to different acceptors (D-glucose, D-xylose, D-mannose, and 2-deoxy-D-arabino-hexose) for synthesis of rare disaccharide analogs of cellobiose.[16] We used *Cu*CPase to perform the same synthesis using D-glucal and D-glucose (50 mM each), but obtained poor yields (⩽4%). Analysis by HPAED-PAD of a sample taken after 30 h of incubation confirmed formation of a disaccharide product that eluted at a position similar, yet not identical to that of elution of authentic cellobiose. However, this product was present in a concentration of only about



Scheme 2. Reaction course for 2-deoxy-D-glucosyl transfer from D-glucal to phosphate catalyzed by *Cu*CPase in H_2O (a) and D_2O (b). In D_2O, all acidic protons are assumed to be exchanged by deuterium.

2 mM (calibrated as cellobiose). We also noticed formation of **2**, presumably resulting from hydration of D-glucal (see Fig. 4f).

The reaction was then repeated in the presence of phosphate. Interestingly and at first unexpectedly, disaccharide synthesis was substantially enhanced under these conditions as compared to the control lacking phosphate. Two main products were formed: a disaccharide whose identity was revealed by NMR as **3**, and **1**, re-confirmed by the NMR data. The molar ratio of synthesis and phosphorolysis products was dependent on the initial phosphate concentration used; it decreased as the phosphate concentration was raised (see Fig. 4). NMR data for the disaccharide product identify the constituent 2-deoxy-β-D-glucosyl group from the typical large coupling constants between the axial protons present in each position, including C-1. The additional equatorial oriented proton on position C-2 shows concomitant smaller $^3J_{H\text{-}H}$ couplings and leads to a high field shift of the C-2 ^{13}C signal. The terminal saccharide is a glucose moiety, present in a typical 2:3 mixture of α- and β-anomers. The interglycosidic linkage has been determined by a weak NOE between the H-1 of the 2-deoxy-β-D-glucosyl moiety and H-4 of the terminal glucose. We isolated **3** from the product mixture, however, only in small amounts that were not sufficient to determine $^3J_{C\text{-}H}$ couplings between the two sugar units. In spite of that, all NMR spectroscopic data (Supplementary Table 2) indicate the disaccharide to be a **3** and are in very good accordance with an earlier reported analysis of this compound.[16]

Figure 4 summarizes time-course data for conversion of D-glucal and D-glucose (50 mM each) in the presence of different phosphate concentrations in the range 1.00–250 mM. A control lacking phosphate is also shown (panel f). Each time course in Figure 4 (panels a–e) was characterized by relatively fast drop of D-glucal concentration in the first 5 h of reaction. Depending on the phosphate concentration used, D-glucose was consumed at roughly the same rate (1 and 5 mM phosphate) or at a rate much lower ($\geqslant$50 mM phosphate) than the D-glucal utilization rate. The products **3** and **1** were formed according to mass balance. At low phosphate concentration, **3** was essentially the only product derived from D-glucal. At phosphate concentrations of 50 mM and greater, the distribution of transfer products varied in dependence of the phosphate concentration. While at 50 mM phosphate the products were formed in almost identical amounts, use of 250 mM phosphate rendered **1** as the predominant reaction product. In each of the time courses in Figure 4 (panels a–e) at 10 h and above, the reaction rate became stalled and the concentrations of substrates and products remained constant over time. Therefore, this suggested that an apparent equilibrium had been reached in each of the reactions. The results in Figure 4 are interesting as they hint a catalytic role of phosphate and D-glucose during conversion of D-glucal into **3** (panels d and e compared to panel f) and **1** (panel a compared to Fig. 3), respectively. A plausible scenario is that quaternary complex between *Cu*CPase, phosphate, D-glucal, and D-glucose shows substantially enhanced activity as compared to the corresponding ternary complexes (enzyme/D-glucal/D-glucose; enzyme/D-glucal/phosphate). Preferred direction of reaction, 2-deoxy-D-glucosyl transfer to D-glucose or phosphate, is determined by prevalence of acceptor substrate for synthesis or phosphorolysis. Reaction of *Cu*CPase with D-glucal therefore appears to occur under thermodynamic control (see later under 2.6 molecular docking studies).

Kinetic analysis of the data in Figure 4 reveals that *Cu*CPase activity was lowest for reaction with D-glucal and D-glucose in the absence of phosphate. An approximate turnover number (k_{app}) of $3.5 \times 10^{-3}\ s^{-1}$ was calculated from data in Figure 4f. Using D-glucal and phosphate in the absence of D-glucose (Fig. 3b), k_{app} was approximately $1.8 \times 10^{-2}\ s^{-1}$. Presence of phosphate (150 mM) and D-glucose next to D-glucal (Fig. 4f compared to Fig. 3b) resulted in a roughly 15-fold increase in k_{app} to a value of $0.27\ s^{-1}$. At 1.00 mM phosphate, k_{app} was still $0.2\ s^{-1}$.

Reaction of CPase with D-glucal is of interest to be applied for synthesis of 2-deoxy-D-glucosyl saccharides, as already demonstrated by Kitaoka and co-workers. This work adds to their previous study showing that **1** can also be synthesized. Generally, deoxy-sugars in which a particular hydroxy group has been substituted by hydrogen have proven to be valuable tools in the biochemical study of substrate binding recognition and catalysis by carbohydrate-active enzymes.[32,33] The OH→H replacement can be viewed as chemical mutagenesis of substrate, resulting in selective removal of hydrogen-bonding interactions that the original hydroxy group had with the enzyme. Considering the central physiological role of α-D-glucose 1-phosphate at the interface of cellular catabolism and anabolism, **1** presents an interesting analog of α-D-glucose 1-phosphate that could be used in the examination of various enzymes utilizing α-D-glucose 1-phosphate as their native substrate (e.g., phosphoglucomutase,

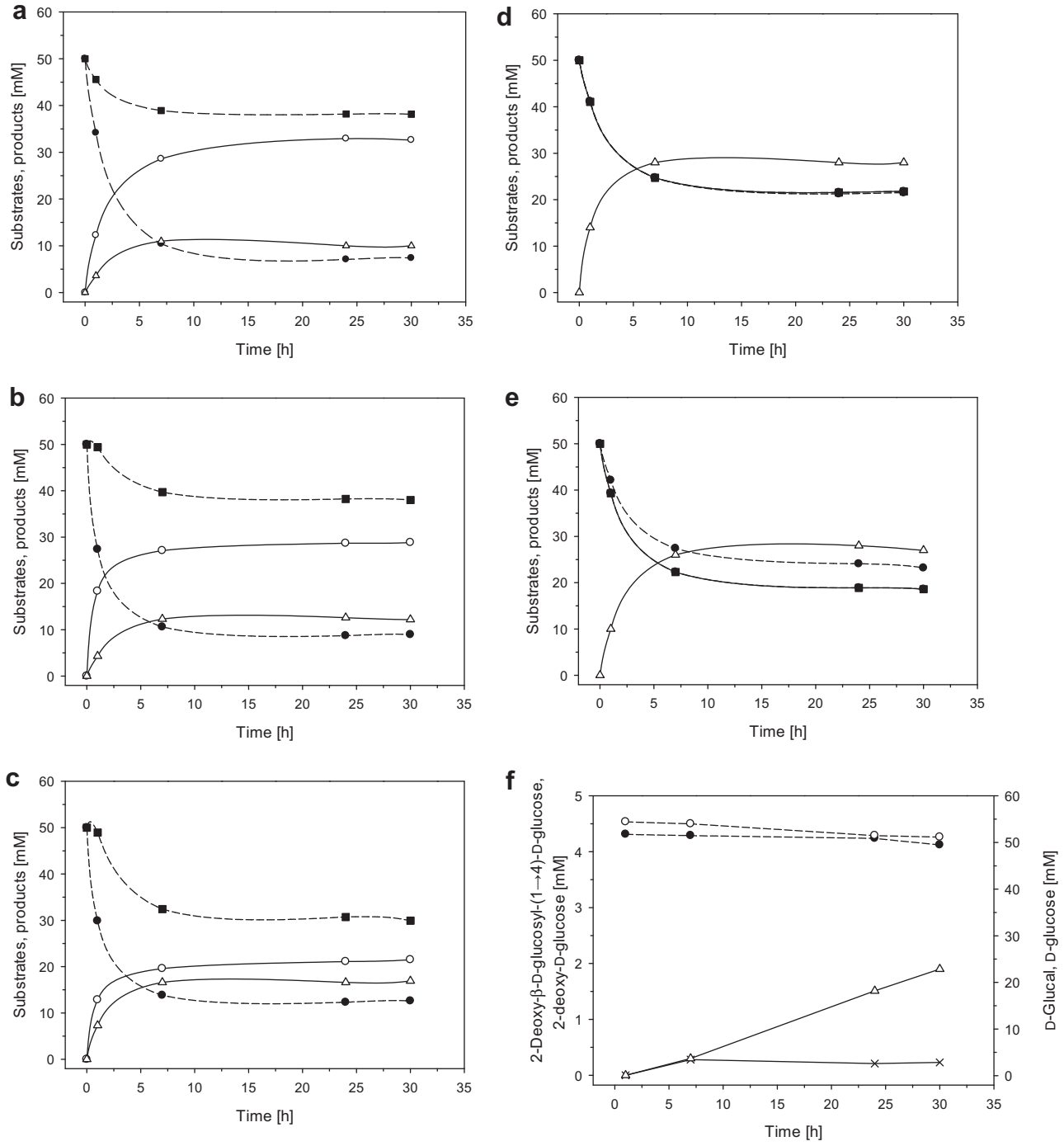


Figure 4. Time-courses for 2-deoxy-β-D-glucosyl-(1→4)-D-glucose (**3**) (△) and 2-deoxy-α-D-glucose 1-phosphate (**1**) (○) production from 50 mM D-glucal (●) and 50 mM D-glucose (■) in the presence of 250 mM phosphate (a), 150 mM phosphate (b), 50 mM phosphate (c), 5 mM phosphate (d) and 1 mM phosphate (e), respectively. (f) shows the time-course of 2-deoxy-β-D-glucosyl-(1→4)-D-glucose (**3**) (△) and 2-deoxy-D-glucose (**2**) (x) production from 50 mM D-glucal (●) and 50 mM D-glucose (■) in the absence of phosphate. Lines show trend of the data.

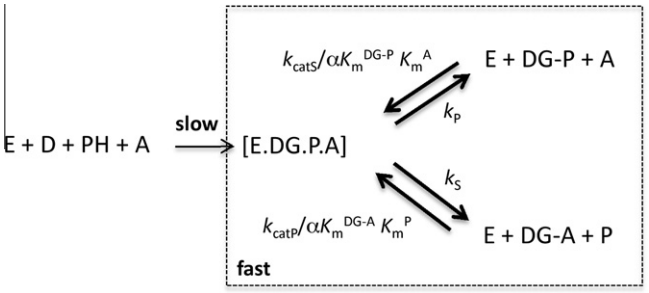
NDP-glucose pyrophosphorylase, phosphatase, phosphorylase). However, applying purely chemical approaches, synthesis of **1** has turned out to be remarkably difficult due to compound instability.[34–38] Percival and Withers used an enzymatic approach that involved five enzymes and proceeded via 2-deoxy-D-glucose 6-phosphate and 2-deoxy-UDP-D-glucose.[39] Thiem and co-workers exploited reaction of glycogen phosphorylase with D-glucal.[40] The enzyme used is special in that its activity is dependent on an α-1,4-oligo-D-glucoside primer. Maltotetraose, which is the smallest primer utilized by the phosphorylase, and starch were used. Applying maltotetraose, authors reported synthesis of **1** in two steps: a 2-deoxy-D-glucosyl polysaccharide having an average degree of polymerization of 20 was prepared first and separated from the reaction mixture; polysaccharide was then used for phosphorolysis, giving **1** in a yield of 50%. An issue of using starch, which is cheaper than maltotetraose, was formation of α-D-glucose 1-phosphate next to **1** and difficulty of separation of the two sugar phosphates. Therefore, synthesis of **1** using *Cu*CPase under conditions as in Figure 4 (panels a and b) is promising.

2.6. Molecular docking studies and proposal for the enzymatic reaction mechanism

The crystal structure of *Cg*CPase (PDB-entry 2cqt) in complex with phosphate and D-glucose (bound at subsite +1) was used for an energy-minimized docking experiment in which D-glucal was rigidly placed into subsite −1 of the enzyme. Inherent ambiguity of the used docking procedure notwithstanding, predicted accommodation of D-glucal at the catalytic subsite −1 of *Cg*CPase appears to be highly plausible. Compared to the PDB structure, no conformational changes were observed in the docked structure. Figure 1b shows the resulting enzyme–ligand interaction map. The docking provides useful insight into formation of and possible reactive group arrangement in a quaternary complex, enzyme/phosphate/D-glucal/D-glucose, that might be catalytically competent. D-Glucal is positioned through a network of hydrogen bonds that involves protein residues (D368; W488, D490; H666), but also phosphate and D-glucose. D-Glucal makes contacts from each of its hydroxy groups, whereby the 4-OH and 6-OH show strong interactions with, respectively, phosphate and the main-chain NH of D490 in particular. Both phosphate and D-glucose (indirectly via D490) are therefore expected to contribute to binding affinity of D-glucal in the quaternary complex.

Figure 1b supports a catalytic mechanism for reaction of *Cu*CPase with D-glucal. The proposed mechanism is shown in Scheme 3. D-Glucal is accommodated in a position that allows the bound phosphate molecule to serve as proton donor for stereospecific *re*-face protonation at C-2. From previous studies of inverting glycoside hydrolases and glycosyltransferases,[41–43] the subsequent glycosyl transfer is expected to proceed through an oxocarbenium ion-like transition state. Partial negative charges at the reactive O atoms of phosphate and D-glucose will stabilize this transition state. The ionized side chain of D490 will provide general base catalytic assistance to the reaction through partial deprotonation of the glucose 4-OH. Regioselective 2-deoxy-D-glucosyl transfer to D-glucose is therefore explained. The proposed role of D490 during conversion of D-glucal is analogous to its role in the natural reaction when cellobiose is synthesized.[2]

A kinetic mechanism for reaction of *Cu*CPase with D-glucal is proposed in Scheme 4. 'Activation' of donor substrate to formally generate a 2-deoxy-D-glucosyl cation is thought to be completely rate-limiting and therefore, 2-deoxy-D-glucosyl transfer between phosphate and D-glucose comes to equilibrium at all times during the reaction. The thermodynamic equilibrium constant for conversion of 2-deoxy-cellobiose into **1** at given pH (K_{eq}) is defined in kinetic terms as the ratio of third-order rate constants in direction of phosphorolysis and synthesis (see Scheme 4). It is interesting that glycogen phosphorylase differs from *Cu*CPase in that its reaction with D-glucal is kinetically controlled, where initial 2-deoxy-D-glucosyl transfer occurs exclusively on the carbohydrate acceptor substrate (e.g., maltotetraose).[27,40] Compound **1** is formed only in a subsequent step, as required by reaction thermodynamics. A possible explanation for switch from thermodynamic to kinetic control in two otherwise similar phosphorylase-catalyzed transformations of D-glucal could be that, when compared on the basis of turnover number for reaction with the natural donor substrate α-D-glucose 1-phosphate, glycogen phosphorylase (relative k_{cat}: 0.1–0.4)[27] is by far (⩾100-fold) more reactive toward D-glucal than *Cu*CPase (relative k_{cat}: 0.001). Mechanistic differences between the two phosphorylases, reflected in a different stereochemical course of the reaction catalyzed, could also be responsible. Glycogen phosphorylase is a retaining enzyme that promotes an axial-to-axial substitution at C-1 of the transferred glycosyl residue. Unlike *Cu*CPase that is reactive with D-glucal and phosphate in the absence of sugar acceptor, glycogen phosphorylase *must* form a quaternary complex of enzyme, phosphate, D-glucal, and oligosaccharide acceptor (primer) to become detectably active. Arrangement of reactive groups in this complex may be such that phosphate, which is thought to serve a proton donor function in the reaction with D-glucal analogous to that proposed for *Cu*CPase,[27] cannot directly participate as nucleophilic acceptor of the transferred 2-deoxy-D-glucosyl moiety. Initial formation of oligosaccharide elongated by 2-deoxy-D-glucosyl residues may thus be a necessity of the mechanism utilized. It is interesting that glycogen phosphorylase promotes conversion of heptenitol to heptulose-2-phosphate in the absence of primer molecule,[29] using a reaction course highly similar to that of direct **1** formation by *Cu*CPase from D-glucal and phosphate.

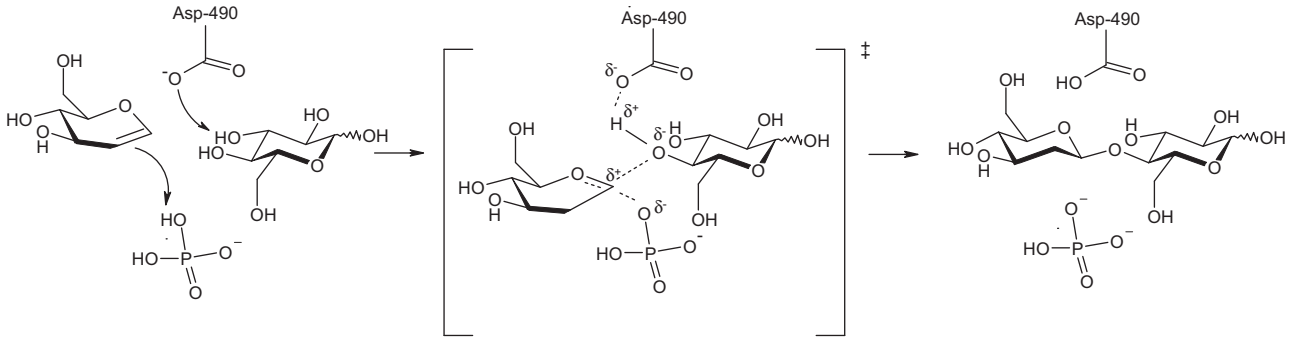


Scheme 4. Proposed kinetic mechanism for reaction of *Cu*CPase with D-glucal. E: *Cu*CPase; D: D-glucal; PH: phosphate, protonated; A: D-glucose; DG: transient 2-deoxyglucosyl oxocarbenium ion (**2**); DG-P: 2-deoxy-α-D-glucose 1-phosphate (**1**); DG-A: 2-deoxy-β-D-glucose-(14)-D-glucose (**3**); P: deprotonated phosphate. [E.DG.P.A] is the quaternary transition state complex. The parameter α is an interaction coefficient describing the effect of binding of one substrate to the enzyme on the binding of the respective other substrate.

3. Experimental

3.1. Materials

Unless otherwise stated, all chemicals used have been described elsewhere.[44] D-Glucal was purchased from Sigma, 2-deoxy-α-D-glucose (**2**) from Roth.



Scheme 3. Proposed catalytic mechanism for reaction of *Cu*CPase with D-glucal, D-glucose and phosphate.

3.2. Enzyme preparation

Recombinant *Cellulomonas uda* CPase (*Cu*CPase) was prepared by a modification of a procedure reported elsewhere.[31] Details of the protocol were as follows. *Escherichia coli* BL21-Gold(DE3) expression cells harboring the pQE30-vector encoding *Cu*CPase were cultivated in 1-L baffled shaken flasks at 37 °C and 110 rpm using LB-media and 115 mg/L ampicillin. When OD_{600} reached 0.9–1.0, temperature was decreased to 22 °C and gene expression was induced with 0.1 mM IPTG for 20 h. Cells were harvested by centrifugation at 4 °C and 5000 rpm for 30 min in a Sorvall RC-5B Refrigerated Superspeed centrifuge. Resuspended cells were frozen at −20 °C, thawed and the suspension was passed twice through a French pressure cell press (Aminco) at 150 bar. Cell debris was removed by centrifugation at 4 °C, 14,000 rpm for 45 min. The resulting supernatant was used for further enzyme purification after it was passed through a 1.2 μm cellulose-acetate filter (Sartorius). As the pQE30-vector encoding *Cu*CPase has a His-tag fused to the N-terminus of the enzyme, *Cu*CPase was purified by using a Ni-chelating Sepharose Fast Flow chromatography (His-Trap HP, GE Healthcare) at 4 °C. All buffers were degassed and filtered using 0.45 μm cellulose-acetate filters (Sartorius). The column was equilibrated with buffer A (20 mM Tris–HCl, 20 mM imidazole, 500 mM NaCl, pH 7.4) at a flow rate of 5 mL/min (5 column volumes). The crude cell extract was applied at a flow rate of 1 mL/min. After washing with buffer A at a flow rate of 5 mL/min (5 column volumes), fractions containing the enzyme (91-kDa band in SDS–PAGE) were eluted with buffer E (20 mM Tris–HCl, 250 mM imidazole, 500 mM NaCl, pH 7.4) at a flow rate of 2 mL/min (6 column volumes). Pooled fractions were concentrated and desalted using a Vivaspin ultrafiltration tube (10 kDa cutoff; Vivascience) and again concentrated to about 40 mg/mL in 50 mM MES buffer, pH 6.6. Stock solutions of *Cu*CPase were stored at −20 °C. The yield of pure protein from one liter culture was about 50 mg. Purification was monitored by SDS–PAGE and the measurement of the specific activity (see Supplementary Fig. 1). To further check the preparation's purity and specifically the absence of a phosphatase related impurity, *Cu*CPase (1 μM) was incubated with 50 mM α-D-glucose 1-phosphate in 50 mM MES buffer, pH 6.6 for 24 h and the reaction mixture was analyzed by high performance anion exchange chromatography with pulsed amperometric detection (HPAED-PAD) (see below). No hydrolytic activity of α-D-glucose 1-phosphate into D-glucose and phosphate could be detected with purified *Cu*CPase prepared with the protocol just described.

3.3. Assays

Enzyme activity in direction of phosphorolysis of cellobiose was determined at 30 °C using a continuous coupled enzymatic assay with phosphoglucomutase and glucose-6-phosphate dehydrogenase.[14] Protein concentration was measured with the BioRad dye-binding method referenced against BSA.

3.4. Analytical methods

All synthesis products were analyzed by high performance anion exchange chromatography with HPAED-PAD. The analysis was performed using a Dionex BioLC system equipped with a CarboPac PA10 column (4 × 250 mm) and an Amino Trap guard column (4 × 50 mm) thermostated at 30 °C. D-Glucal, **2**, D-glucose, **3,** and **1** were detected with an ED50A electrochemical detector using a gold working electrode and a silver/silver chloride reference electrode by applying the predefined waveform for carbohydrates. Elution was carried out at a flow rate of 1 mL/min with the following method: isocratic flow of 52 mM NaOH for 20 min, followed by an isocratic flow of 100 mM NaOH and 100 mM NaOAc for 5 min, and a linear gradient from 100 mM NaOAc to 400 mM NaOAc applied within 15 min in an isocratic background of 100 mM NaOH. The column was washed 10 min with 52 mM NaOH. Under the conditions applied, the following retention times were obtained: 2.6 min for D-glucal, 5.8 min for **2**, 7.9 for D-glucose, 11.2 min for **3,** and 23.0 min for **1**.

3.5. Reactions with D-glucal and phosphate

D-Glucal was tested as a possible glycosyl donor in phosphorolysis direction catalyzed by *Cu*CPase. 5 μM *Cu*CPase were incubated with 50 mM D-glucal and different concentrations of phosphate (50 mM, 250 mM) in 50 mM MES buffer, pH 6.6. The formation of a new product for *Cu*CPase, **1**, was monitored by HPAED-PAD after the reaction mixture was incubated at 30 °C, 550 rpm for up to 30 h.

To determine the stereochemical course of the reaction, we performed a reaction using 50 mM D-glucal, 50 mM phosphate, 50 mM MES (pH 6.6), and 5 μM *Cu*CPase in 99% (v/v) D_2O. The mixture was incubated at 30 °C, 550 rpm for 24 h and the content of the mixture was analyzed by NMR analysis (see 3.8).

3.6. Reactions with D-glucal and D-glucose in presence and absence of phosphate

To test whether *Cu*CPase still utilizes D-glucal when phosphate and D-glucose is present, 5 μM *Cu*CPase were incubated with 50 mM D-glucal, 50 mM D-glucose, and different concentrations of phosphate (1 mM, 5 mM, 50 mM, 150 mM, 250 mM) in 50 mM MES buffer, pH 6.6, at 30 °C for 30 h. The reaction mixture was analyzed by HPAED-PAD and the synthesis products were identified by NMR-analysis as **3** and **1**. To test the role of phosphate in the reaction, *Cu*CPase was incubated with only D-glucal and D-glucose (without phosphate). 5 μM *Cu*CPase were incubated with 50 mM D-glucal and 50 mM D-glucose in 50 mM MES buffer, pH 6.6, at 30 °C and after 24 h the reaction was stopped and the reaction mixture was analyzed by HPAED-PAD.

3.7. Energy-minimized molecular docking

Yasara V 11.11.21 was used for the determination of energy-minimized structure and the enzyme–ligand docking. PyMOL (http://pymol.sourceforge.net) was used for visualization. The X-ray crystal structure of *Cg*CPase having a D-glucose and phosphate bound in subsite +1 and phosphate binding site, respectively (PDB-entry 2cqt) was used as macromolecule in a rigid molecular docking experiment that employed D-glucal as the ligand. A search space of 15 × 12 × 12 Å was used, spanning the enzyme's active site. The docking algorithm resulted in 4 binding modes with comparable energies. From these, the most accurate model was selected on the basis of the binding mode of *N*-acetylglucosamine in subsite −1 of *Vp*ChPase.[6]

3.8. NMR measurements

NMR spectroscopic measurements were made from crude or partially purified reaction mixtures. Lyophilized samples were dissolved in D_2O (99.95%, 0.7 mL) and transferred into 5 mm NMR sample tubes (Promochem, Wesel, Germany). All spectra were recorded on a Bruker DRX-400 AVANCE spectrometer (Bruker, Rheinstetten, Germany) at 400.13 MHz (^1H), 100.61 MHz (^{13}C), and 161.98 MHz (^{31}P) using the Bruker Topspin 1.3 software. Measurements temperature was 300 ± 0.1 K. For 1D-spectra, 32k data points were acquired using a relaxation delay of 1.0 s and appropriate number of scans. After zero filling to 64k data points and Fourier transformation, spectra with a range of 7200 Hz (^1H),

20,000 Hz (^{13}C), and 16,200 Hz (^{31}P) were obtained, respectively. To determine the 2D COSY, TOCSY, NOESY, HMQC, and HMBC as well as ^{1}H/^{31}P-HSQC spectra, 128 experiments with 8 scans and 1024 data points each were performed. After linear forward prediction to 256 data points in the f_2-dimension and appropriate sinusoidal multiplication in both dimensions, the data were Fourier transformed to obtain 2D-spectra with ranges of 4000 Hz (^{1}H), 20,000 Hz (^{13}C), and 8100 Hz (^{31}P). Chemical ^{1}H and ^{13}C shifts were referenced to external acetone (δ_H 2.225 ppm; δ_C 31.45 ppm) and phosphorus shifts were referenced to external aq 85% phosphoric acid (^{31}P: δ_P 0.00 ppm).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carres.2012.04.003.

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