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Review

Engineering of cellobiose phosphorylase for glycoside synthesis

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ABSTRACT

Disaccharide phosphorylases are increasingly applied for glycoside synthesis, since they are very regiospecific and use cheap and easy to obtain donor substrates. A promising enzyme is cellobiose phosphorylase (CP), which was discovered more than 50 years ago. Many other bacterial CP enzymes have since then been characterized, cloned and applied for glycoside synthesis. However, the general application of wild-type CP for glycoside synthesis is hampered by its relatively narrow substrate specificity. Recently we have taken some successful efforts to broaden the substrate specificity of *Cellulomonas uda* CP by directed evolution and protein engineering. This review will give an overview of the obtained results and address the applicability of the engineered CP enzymes for glycoside synthesis. CP is the first example of an extensively engineered disaccharide phosphorylase, and may provide valuable information for protein engineering of other phosphorylase enzymes.

Contents

1. Introduction

The importance of carbohydrates and glycosides in biology is widely known. Many biologically active molecules contain a carbohydrate moiety that determines either their activity or pharmacokinetic properties (Kren and Martinkova, 2001). Glycosylation of drugs can, for example, induce targeting to specific organs and tissues, thus resulting in less side-effects and smaller required doses (Wong and Toth, 2001). Furthermore, glycosylation usually improves their solubility and reduces toxicity (Leu et al., 1999). Carbohydrates, in turn, often display prebiotic effects by selectively stimulating growth of beneficial bacteria in the intestines (Wang, 2009).

The synthesis of glycosides and carbohydrates can be performed by chemical or enzymatic methods. Although chemical synthesis has already been applied successfully, it usually suffers from low yields due to non-specific glycosylation, resulting in product mixtures. Also the use of toxic compounds limits their use for large-scale applications. To overcome these problems, the enzymatic glycosylation approach has attracted much attention. Most studies are based on the use of glycosyltransferases (GTs), glycoside hydrolases (GHs) and transglycosidases for glycoside synthesis. Much less work has been done with glycoside phosphorylases (GPs), although they have some interesting properties (Luley-Goedl and Nidetzky, 2010). For example, the sugar donor – a glycosyl phosphate – is cheaper and more easy to obtain than the activated sugar nucleotides required by GTs. Indeed, since GPs catalyze equilibrium reactions, they can synthesize the glycosyl phosphate donor by phosphorolysis of their natural (disaccharide) substrates.

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Table 1
Overview of characterized GH94 enzymes and their origin.[a]

Enzyme	EC-number	Main substrate	Donor	Organism
Cellobiose phosphorylase	2.4.1.20	Glc(β1–4)Glc	αGlc1*P*	*Cellulomonas uda* *Cellvibrio gilvus* *Clostridium stercorarium* *Clostridium thermocellum* *Thermotoga maritima* *Thermotoga neapolitana*
Cellodextrin phosphorylase	2.4.1.49	Glc[(β1–4)Glc]$_n$	αGlc1*P*	*Clostridium stercorarium* *Clostridium thermocellum*
Chitobiose phosphorylase	2.4.1.–	GlcNAc(β1–4)GlcNAc	αGlcNAc1*P*	*Vibrio furnissii* *Vibrio proteolyticus*
Cyclic β-1,2-glucan synthase	2.4.1.–	Glc[(β1–2)Glc]$_n$	αGlc1*P*[b]	*Agrobacterium tumefaciens* *Brucella abortus* *Sinorhizobium meliloti*

[a] Data obtained from the CAZy database (http://www.cazy.org) (Henrissat and Davies, 1997).
[b] The cyclic β-1,2-glucan synthase catalyzes four types of enzymatic reactions, including β-1,2-glucooligosaccharide phosphorolysis with αGlc1*P* as donor (Ciocchini et al., 2007).

In recent years, glycoside phosphorylases have been applied for glycoside synthesis on kilogram scale. Examples include the synthesis of the cosmetic ingredient glucosylglycerol with sucrose phosphorylase (Goedl et al., 2008) and the synthesis of the prebiotic sugar lacto-*N*-biose with lacto-*N*-biose phosphorylase (Nishimoto and Kitaoka, 2007). However, a major drawback for the general application of disaccharide phosphorylases for glycoside synthesis is their rather narrow substrate specificity. Although sucrose phosphorylase has a relatively broad acceptor specificity, it can only α-glucosylate acceptor molecules. Other phosphorylases can be used for β-glucosylation or β-galactosylation reactions, but have a more narrow acceptor specificity.

An interesting representative is cellobiose phosphorylase (CP), which was first described in crude cell extracts of *Clostridium thermocellum* (Sih and McBee, 1955a,b). CP enzymes catalyze the reversible phosphorolysis of cellobiose into α-glucose 1-phosphate (αGlc1*P*) and glucose. Since its discovery, cellobiose phosphorylases have been identified in numerous bacteria that are directly or indirectly involved in cellulose degradation (Table 1). Its natural role is the energy-efficient metabolism of cellobiose, producing αGlc1*P* in which much of the substrate's energy is conserved. CP belongs to CAZy family GH94, together with chitobiose phosphorylase (ChP), cellodextrin phosphorylase and cyclic β-1,2-glucan synthase (Henrissat and Davies, 1997) (Table 1).

The specificity of cellobiose phosphorylase has been extensively studied, especially for glycoside synthesis (Alexander, 1968; Kitaoka et al., 1992; Nidetzky et al., 2000). However, applications of CP for glycoside synthesis are mainly limited to carbohydrate synthesis. This is due to the relatively narrow acceptor specificity of CP, which accepts mainly glucose, glucose-derivatives and other monosaccharides (Table 2). Although a free C-1 hydroxyl group (preferably in the β-configuration) is an absolute requirement for most CP enzymes (Kitaoka and Hayashi, 2002; Nidetzky et al., 2000), a hyperthermophilic CP from *Thermotoga maritima* was found to accept methyl β-glucoside (Rajashekhara et al., 2002). Several unnatural trisaccharides and disaccharides have been synthesized with *Cellvibrio gilvus* CP using αGlc1*P* as sugar donor and melibiose, gentiobiose, isomaltose, D-arabinose, D-altrose or L-fucose as acceptor molecules (Percy et al., 1997, 1998). Radioactive cellobiose has been synthesized with *C. thermocellum* CP using either radiolabeled αGlc1*P* or glucose in the wild-type synthesis reaction (Ng and Zeikus, 1986). An example of glycosylation with non-carbohydrate acceptors is the synthesis of alkyl β-glucosides using *C. thermocellum* CP (Kino et al., 2008). The authors were able to synthesize the glucosides of alcohols ranging from methanol up to heptanol.

Since phosphorolytic reactions are reversible, full conversion of substrates is not possible unless one of the reaction products is continuously removed from the reaction mixture. An approach resulting in irreversible synthesis reactions with *Cellulomonas uda* CP has been applied using αGlc1*F* as the glucosyl-donor (Nidetzky et al., 2004). This resulted in a 'glycosynthase-like' reaction with 100% conversion of substrates into glycosidic product. Besides αGlc1*P* and αGlc1*F*, also D-glucal was found to function as donor molecule for CP, although the activity was about 500 times lower than with the natural donor (Kitaoka et al., 2006).

2. Structural determinants of substrate specificity in *C. uda* CP

Known CP enzymes consist of around 800 amino acids, corresponding to a molecular mass of about 90 kDa. They often form dimers, although monomeric forms of CP in solution have also been reported (Nidetzky et al., 2000; Reichenbecher et al., 1997). The crystal structure of *C. gilvus* CP is a dimer (Fig. 1) and shows high similarity with the structure of *Vibrio proteolyticus* chitobiose phosphorylase (Hidaka et al., 2006). Two CP crystal structures have been published, one containing sulphate in the active site (PDB 2cqs) and one containing phosphate (PDB 2cqt). While both structures contain glucose at the acceptor site (+1 subsite), only the latter contains a ligand at the donor site (−1 subsite), i.e., the glycerol used as cryoprotectant. Interestingly, the positions of the glycerol atoms superpose almost perfectly with the C-4 to C-6 and O-4 to O-6 atoms of GlcNAc (−1 site) in the chitobiose phosphorylase crystal structure (PDB 1v7x).

At the acceptor site of *C. gilvus* CP, a strong hydrogen bond is formed between residue E649 and the O-1 atom at the reducing end of glucose (Fig. 2). Furthermore, the glucose molecule is exclusively in the β-anomer configuration. The *C. uda* CP substrate preference for β-cellobiose over α-cellobiose has also been observed in activity assays (Nidetzky et al., 2000). This is in contrast to ChP, where no α/β preference is found due to the absence of a hydrogen bond with the O-1 atom of GlcNAc at the acceptor site. A weaker hydrogen bond is formed between O-2 and Y653, while the O-3 hydroxyl group forms a strong hydrogen bond with E659. The O-4 hydroxyl group interacts with the catalytic amino acid D490, which acts as a general acid in phosphorolysis. In the synthesis reaction D490 is likely to assist deprotonation of the O-4 hydroxyl group of the acceptor molecule. The O-6 hydroxyl group does not form hydrogen bonds with the CP enzyme, but instead there is a hydrophobic interaction with the guanidium plane of R362. The fact that the O-6 atom is exposed to the solvent and that there is a large open space