Structural Basis for Reversible Phosphorolysis and Hydrolysis Reactions of 2-O-α-Glucosylglycerol Phosphorylase*

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Kouki K. Touhara (東原幸起), Takanori Nihira (仁平正則), Motomitsu Kitaoka (北岡本光), Hiroyuki Nakai (中井博之), and Shinya Fushinobu (伏信進矢)

From the †Department of Biotechnology, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, the ‡Faculty of Agriculture, Niigata University, Niigata 950-2181, and the §National Food Research Institute, National Agriculture and Food Research Organization, Tsukuba, Ibaraki 305-8642, Japan

Background: 2-O-α-Glucosylglycerol phosphorylase (GGP) catalyzes the reversible phosphorolysis of glucosylglycerol and the hydrolysis of β-d-glucose 1-phosphate.

Results: Crystal structures were determined for GGP complexed with glucose, isofagomine, and glycerol.

Conclusion: A water-mediated hydrogen bond network that fixes glycerol is the key factor of the bifunctionality.

Significance: The substrate recognition and reaction mechanisms of GGP were clarified in detail.

2-O-α-Glucosylglycerol phosphorylase (GGP) from Bacillus selenitireducens catalyzes both the reversible phosphorolysis of 2-O-α-glucosylglycerol (GG) and the hydrolysis of β-d-glucose 1-phosphate (βGlc1P). GGP belongs to the glycoside hydrolase (GH) family 65 and can efficiently and specifically produce GG. However, its structural basis has remained unclear. In this study, the crystal structures of GGP complexed with glucose and the glucose analog isofagomine and glycerol were determined. Subsite −1 of GGP is similar to those of other GH65 enzymes, maltose phosphorylase and kojibiose phosphorylase, whereas subsite +1 is largely different and is well designed for GG recognition. An automated docking analysis was performed to complement these crystal structures, βGlc1P being docked at an appropriate position. To investigate the importance of residues at subsite +1 in the bifunctionality of GGP, we constructed mutants at these residues. Y327F and Y572F significantly reduced reverse phosphorolytic activity but showed significantly reduced reverse phosphorolytic activity but retained βGlc1P hydrolysis. The mode of substrate recognition and the reaction mechanisms of GGP were proposed based on these analyses. Specifically, an extensive hydrogen bond network formed by Tyr-327, Tyr-572, Lys-587, and water molecules contributes to fixing the acceptor molecule in both reverse phosphorolysis (glycerol) and βGlc1P hydrolysis (water) for a glucosyl transfer reaction. This study will contribute to the development of a large scale production system of GG by facilitating the rational engineering of GGP.

2-O-(α-D-GLucopyranosyl)-sn-glycerol (2-O-α-glucosylglycerol, GG)§ is known as an osmoprotective compound in cyanobacteria (1). GG is also produced by various plants, algae, and bacteria to adapt to salt stress and drought (2) and is found in foods fermented by Aspergillus oryzae (koji mold) (3). GG can be used as a moisturizing agent in cosmetics, a low-calorie sweetener, or a protein stabilizer (4–6). GG has been enzymatically produced and implemented on an industrial scale by employing the side reaction of a glycoside hydrolase (GH) family 13 enzyme, sucrose phosphorylase (EC 2.4.1.7) (7–9) or by a transglycosylation reaction of a commercial α-glucosidase (5). The transglycosylation of α-glucosidase produces a mixture of GG (2R)-1-O-α-glucosylglycerol, and (2S)-1-O-α-glucosylglycerol from maltose and glycerol (5). Alternatively, Thermobacterium brockii GH65 kojibiose phosphorylase can produce (2S)-1-O-α-glucosylglycerol from βGlc1P and glycerol via reverse phosphorolysis (10). However, no enzyme could specifically produce GG as a main reaction product until recently.

We previously identified Bsl_e2816 from Bacillus selenitireducens MLS10 as a novel enzyme 2-O-α-glucosylglycerol phosphorylase (GGP, EC 2.4.1.1x) (11). B. selenitireducens is a haloalkaliphilic bacterium that grows optimally in a salinity of 1.5 m NaCl (12). GGP may be involved in biosynthesis of the osmoprotective compound. GGP belongs to GH65 and specifically produces GG from β-D-glucose 1-phosphate (βGlc1P) and glycerol via reverse phosphorolysis. GGP can significantly hydrolyze βGlc1P with a $k_{cat}$ of 2.8 s$^{-1}$ and reversibly phosphorolyze GG with $k_{cat}$ values of 95 s$^{-1}$ (phosphorolysis) and 180 s$^{-1}$ (synthetic reaction) (11). Therefore, the βGlc1P hydrolysis of GGP is a side reaction of the reversible phosphorylase reactions. The βGlc1P hydrolysis is a glucosyl-transfer reaction to an acceptor water molecule that produces an anomer-inverted α-glucose, not a phosphatase-type reaction. The reaction of GH-type inverting phosphorylases, including GH65, GH94, and GH112, is thought to proceed via a single displacement

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†To whom correspondence should be addressed. Tel.: 81-3-5841-5151; Fax: 81-3-5841-5151; E-mail: afushi@mail.ecc.u-tokyo.ac.jp.

‡The abbreviations used are: GG, 2-O-α-glucosylglycerol; GGP, 2-O-α-glucosylglycerol phosphorylase from B. selenitireducens; LbMP, L. brevis maltose phosphorylase; Pi, inorganic phosphate; CsKP, C. saccharolyticus kojibiose phosphorylase; Glc1P, β-D-glucose 1-phosphate; GH, glycoside hydrolase.
Structure of Glucosylglycerol Phosphorylase

mechanism with the assistance of a general acid residue (13), which is probably Glu-475 in the case of GGP (11). To date, 15 bacterial proteins have been characterized among GH65 family with seven known activities: maltose phosphorylase (EC 2.4.1.8) (14, 15), trehalose phosphorylase (EC 2.4.1.64) (16), kojibiase phosphorylase (EC 2.4.1.230) (17), trehalose-6-phosphate phosphorylase (EC 2.4.1.216) (18), nigerose phosphorylase (EC 2.4.1.279) (19), 3-0-alpha-glucopyranosyl-1-rhamnose phosphorylase (EC 2.4.1.282) (20), and GGP. However, the crystal structures of only two enzymes in the GH65 family, maltose phosphorylase (LbMP) in complex with Escherichia coli crystal structures of only two enzymes in the GH65 family, maltose phosphorylase (LbMP) in complex with

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—The expression vectors for C terminally (His)₆-tagged wild-type GGP and its E475Q mutant (11) were introduced into Escherichia coli BL21 Codon-Plus (DE3)-RIL (Stratagene, La Jolla, CA). The transformants were cultured in Luria-Bertani medium containing 50 mg/liter of kanamycin and 100 mg/liter of chloramphenicol at 37 °C for 4 h. Isopropyl 1-thio-galactopyranoside was added to a final concentration of 0.1 mM to induce protein expression. Following an additional incubation at 20 °C for 24 h, the cells were harvested by centrifugation and suspended in 20 mM HEPES-NaOH (pH 7.5) and 500 mM NaCl. Cell extracts were obtained by sonication followed by centrifugation to remove cell debris. The protein was purified to homogeneity using sequential column chromatography involving nickel-nitrilotriacetic acid superflow (Qiagen, Hilden, Germany), MonoQ 10/100 GL, and Superdex 200 pg 16/60 column chromatography (GE Healthcare). The protein concentration was spectrophotometrically determined at 280 nm using a theoretical extinction coefficient of ϵ = 124,110 M⁻¹ cm⁻¹ based on the amino acid sequence. The E475Q mutant was overexpressed and purified in the same way as wild-type GGP.

**Crystallography**—Crystals of GGP were obtained at 20 °C using the hanging drop vapor diffusion method by mixing 1 μl of protein solution containing 4.3–5.7 mg/ml of protein with an equal volume of reservoir solution containing 28% polyethylene glycol 400, 0.1 M HEPES-NaOH (pH 7.5), and 0.2 M CaCl₂. A protein solution containing 10 mM-glucose was used to obtain wild-type GGP crystals in complex with glucose. The crystals of the E475Q mutant were obtained using a protein solution containing 0.1 mM isofagomine (Toronto Research Chemicals, North York, Canada) and 10% glycerol. The hexagonal crystals grew in ~5 days. The diffraction data were collected at 100 K (λ = 1.000 Å) using a charge-coupled device camera on BL17A at the Photon Factory of the High Energy Accelerator Research Organization (KEK, Tsukuba, Japan) and processed using HKL2000 (23). Molecular replacement was performed using Molrep (24). The search model was prepared using a homology modeling server with CsKP (PDB code 3WIR) as a template. Automated model building and refinement was performed using ARP/wARP (25). Manual model rebuilding and refinement were performed using Coot (26) and Refmac5 (27). The data collection and refinement statistics are provided in Table 1. The molecular interfaces were analyzed using the PDBe PISA server. Molecular graphic images were prepared using PyMol (DeLano Scientific, Palo Alto, CA).

**Docking Simulations**—The ligand molecule (βGlc1P) was generated using PCModel (Serena Software, Bloomington, IN). The crystal structure of GGP in complex with glucose was used for this docking study by removing all water and ligand molecules. The docking studies were carried out using the AUTODOCK 4.2 program (28). Using AutoDockTools, polar hydrogen atoms were added to amino acid residues, and Gasteiger charges were assigned to all atoms of the enzyme. The Van der Waals and electrostatic energy grid maps were prepared using the AUTOGIRD program, with 80 × 80 × 80 points spaced at 0.375 Å distances. The grid box was centered on the C1 atom of glucose bound to subsite −1. The ligand structure was docked with flexible torsion angles, whereas the protein structure was fixed. Eight torsion angles of βGlc1P were rotatable but the sugar ring was fixed in the C₄ conformation. An initial random population size of 150 individuals, a maximum number of 2,500,000 energy evaluations, and a maximum number of generations of 27,000 were used for the Lamarckian genetic algorithm search. At the end of a docking procedure of 256 docking runs, the resulting positions were clustered according to a root mean square criterion of 2.0 Å.

**Construction of Mutants and Kinetic Analysis**—Site-directed mutagenesis was carried out with a PrimeSTAR mutagenesis kit (Takara Bio). The following primers and their complementary strands were used: 5′-caggcttccagggcaggcttctg-3′ (Y327F), 5′-tgcatcgcagcagctgctgct-3′ (Y572F), 5′-tgcatcgcagcagctgctgct-3′ (Y572F), and 5′-tggcagctcg-3′ (K587A). The expression plasmids for the mutants were verified by DNA sequencing. The mutant proteins were expressed, purified, and characterized as described previously (11). Extinction coefficients of ϵ = 122,620 and 118,610 M⁻¹ cm⁻¹ were used to determine the protein concentrations of the Tyr to Phe and Trp to Phe mutants, respectively. The reverse phosphorolytic (synthetic) activity was measured by subtracting the increase in D-glucose (hydrolytic activity) from the increase in P₁ (hydrolytic plus synthetic activities) at 30 °C in the reaction mixture containing 0–100 mM glycerol, 10 mM βGlc1P, and 40 mM HEPES-NaOH (pH 7.5) (11). The hydrolytic activity was measured based on the increase in D-glucose at 30 °C in the reaction mixture containing 0.1–10 mM βGlc1P and 40 mM HEPES-NaOH (pH 7.5) (11). At least 9 different substrate concentrations for measurement of each enzyme and activity were collected. Protein concentrations of 58 mM (wild-type), 1.1 mM (Y327F), 0.12 mM (W381F), 0.14 mM (Y572F for reverse phosphorolysis), 1.3 mM (Y572F for βGlc1P hydrolysis), or 1.5 mM (K587A) were used. The kinetic parameters were calculated by curve fitting the experimental data to the Michaelis-Menten equation \( \nu = \frac{k_{cat} [E]_0 [S]}{K_m + [S]} \).
asymmetric due to the large displacement (>10 Å) of the N-terminal domain (21), whereas the GGP dimer is nearly symmetric. The crystal packing of GGP lacks the disulfide bond-linked dimer interfaces between the N-domains that were observed in the CsKP crystals (Fig. 1C).

Three unique extended loops are present in the catalytic domain of GGP (magenta in Fig. 1A). Loop 1 (388-410, red), loop 2 (567-578, blue), and loop 3 (659-667, green) are shown in Fig. 2A. Loop 1 corresponds to “loop 3” in reports for Lactobacillus acidophilus maltose phosphorylase and CsKP (22, 29), and it is a key determinant of the substrate specificity of these enzymes. These loops completely cover the active site of GGP. Thus, one or more loops must open to accept the substrates. Specifically, loop 1 contains three phenylalanine residues (Phe-395, Phe-396, and Phe-409), which form hydrophobic interactions with glycerol at the active site (discussed below). The three loops are not conserved in the GH65 family members (Fig. 2D). In contrast to GGP, the active site pocket of LbMP is rather open because it contains short loops at these regions (Fig. 2B). The pocket of CsKP is partially closed because a long loop 1 with two antiparallel β-strands covers the active site (Fig. 2C). However, loop 3 is shorter and loop 2 is in an open conformation in CsKP.

**Ligand Binding Pathway of GGP**—To investigate a possible mechanism for substrate entry into the completely closed active site of GGP, we examined the average B-factors of the three loops (Table 1). The average B-factors of loop 1, which contains the three key phenylalanine residues for glycerol binding, are lower than that of the overall structure. The B-factors for loop 2 are not significantly different from the overall average value. In contrast, loop 3 showed significantly higher average B-factor values that are 1.2-1.4 times larger than the overall average value. None of the three loops is affected by crystal packing (data not shown). Loops 1 and 2 are located near the dimer interface but loop 3 is not (Fig. 1A). These results indicate that loop 3 could flexibly move and facilitate ligand binding to GGP. A phylogenetic tree for the GH65 enzymes indicates that GGP, nigerose phosphorylase, kojibiose phosphorylases, trehalose-6-phosphate phosphorylase, and 1,3-α-glucosylrhhamnose phosphorylase diverge from the maltose phosphorylases and trehalose phosphorylases (11). Among the former five enzymes, GGP and nigerose phosphorylase contain three long loops, the kojibiose phosphorylases and trehalose-6-phosphate phosphorylase contain long loops of 1 and 2, and 1,3-α-glucosylrhhamnose phosphorylase contains the shortest loops in all three regions (Fig. 2D). The emergence of the three long loops could be a key event of the molecular evolution of GGP that yielded the specificity for GG phosphorylase.

**GGP-Glucose Structure**—Both monomers in the asymmetric unit of the GGP-glucose structure contain a β-glucose molecule in the active site (subsite −1) with a clear electron density (Fig. 3A, chain B). The glucose forms direct hydrogen bonds with Trp-333, Asp-334, Lys-587, and Gln-588. These four residues stabilize all five hydroxyls of glucose and are perfectly conserved among all characterized bacterial GH65 family enzymes (Fig. 2D). Tyr-327 forms a water-mediated hydrogen bond with glucose. Tyr-327, Thr-381, Glu-475 (catalytic residue), Tyr-572, and Lys-587 (discussed below) form a hydrogen-
bonded water network on the β-face (α-anomer side) of glucose.

**GGP-Isofagomine-Glycerol Structure**—The complex structure with isofagomine and glycerol was determined using a mutant at the catalytic residue (E475Q) (11). Six of the eight chains in the asymmetric unit (A and D–H) contain an isofagomine molecule at the active site (subsite 1). Forty-four glycerol molecules were found in total, and four glycerol molecules are located at the active site (chains D and F–H). Fig. 3B shows a glycerol molecule bound at the acceptor site (subsite 1) of chain D. This glycerol molecule adopts an extended conformation, and the three hydroxyls of isofagomine form hydrogen bonds with Trp-333, Asp-334, Gln-475 (mutated from Glu), and Gln-588 (Fig. 4B). The O2-hydroxyl of glycerol is located at a distance of 3.0 Å from the N1 atom of isofagomine. This is a reasonably close distance to represent the acceptor site of glycerol to produce GG with βGlc1P via the reverse phosphorolysis reaction. The three hydroxyls are oriented in the same direction and fixed by direct hydrogen bonds with Tyr-327, Trp-381, and Gln-475. Two water molecules also form hydrogen bonds with glycerol, forming bridges with Tyr-572 and Lys-587. Furthermore, the hydrophobic side of glycerol is stabilized by interactions with Phe-395, Phe-396, and Phe-409. Although Tyr-327 and Lys-587 are conserved among the bacterial GH65 family enzymes, the other five residues that comprise the acceptor site, Trp-381, Phe-395, Phe-396, Phe-409, and Tyr-572, are not conserved (Fig. 2D). Specifically, Phe-395, Phe-396, and Phe-409 are located in loop 1, which is often lost among GH65 enzymes. CsKP, Clostridium phytofermentans nigerose phosphorylase, and Lactobacillus lactis trehalose-6-phosphate phosphorylase have a long loop in this region, but contain only one or two phenylalanine residues.

The side chain of the Gln-475 residue adopts a conformation different from that of Glu-475 in the GGP-glucose structure. This difference may be an artifact due to the mutation. However, flexibility of the catalytic residue may be required to fulfill its roles as the proton donor (phosphorolysis) and the proton acceptor (reverse phosphorolysis and βGlc1P hydrolysis).

In summary, the acceptor (glycerol-binding) site of GGP contains a hydrophobic side with phenylalanine residues as well as a hydrophilic side with several hydrogen bond-forming residues, and this site is well designed to recognize a glycerol molecule. Importantly, glycerol is a relatively flexible molecule compared with pyranose sugars (e.g., glucose), which are acceptor molecules of other GH65 enzymes. Therefore, GGP features these extensive interactions to fix the acceptor. Most of the residues involved in these interactions (Trp-381, Phe-395, Phe-396, Phe-409, and Tyr-572) are located within or near loops 1 and 2 (Fig. 2D, orange stars).

**Docking Analysis**—We performed an automated docking analysis to obtain probable binding models of βGlc1P. The first-ranked cluster was reasonably large (55 of 256 docking runs) and shows a significantly lower binding energy (−7.52 kcal/mol) compared with other clusters (−6.88 kcal/mol). These features yielded the best result for estimating the binding mode of βGlc1P (Fig. 3C). The glucose moiety of βGlc1P forms hydrogen bonds with Arg-320, Trp-333, Asp-334, Lys-587, and Gln-588. The phosphate moiety forms hydrogen bonds with the side chains of Arg-320, Tyr-327, Lys-587, and Ser-622, and the main chain NH group of Ser-623. In summary, the docked
Glucose moiety of docked structure, Trp-582 is located at a corresponding position, Arg at this site (Fig. 2 and Lys-330 in LbMP and CsKP), but a few members contain that corresponds to Arg-320 is generally Lys in GH65 (Lys-345 highly conserved, but Arg-320 and Tyr-572 are not. The residue

tures. The phosphate moiety of the docked
ement from the glucose molecules in the GGP and CsKP struc-
. Residues that have similarity across the sequences are shown in the
C. phytofermentans, C. phytofermentans, C. trehalose phosphorylase; TbTP, T. brockii trehalose phosphorylase; LIT6pP, L. lactis trehalose-6-phosphate phosphorylases; CpRhaP, C. phytofermentans 3-O-α-glycopyranosyl-β-rihamnose phosphorylase.

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other mutants exhibited no detectable or significantly decreased activities. The $k_{\text{cat}}$ of Y572F was 15 times lower than that of the wild-type.

**Reaction Mechanisms**—Our previous work (11) indicated that the $\beta$Glc1P hydrolysis of GGP can be explained as a glycosyl transfer reaction to a water molecule. In other words, GGP can utilize whether a water molecule or a glycerol as an acceptor for a glycosyl transfer reaction from $\beta$Glc1P. Therefore, the position of the key water molecule for hydrolysis assumedly corresponds to the O2 atom of glycerol (Fig. 3B). This area is the $\alpha$-anomer side of glucose, which features a hydrogen-bonded water network in the GGP-glucose structure (Fig. 3A).

$\beta$Glc1P hydrolysis must be primarily facilitated via proton extraction by Glu-475, which is located close to the O2 atom of glycerol. According to the proposed reaction mechanism, E475A and E475Q mutants did not show detectable activities for either hydrolysis or phosphorolysis (11). In this study, we focused on the additional water-mediated hydrogen bond,
which holds the O2 atom of glycerol (Fig. 3B). This water molecule is further fixed by Tyr-572 and Lys-587. Interestingly, Tyr-572 is not conserved among the GH65 enzymes. The mutation of residues involved in water-mediated hydrogen bonds (Y572F and K587A) significantly reduced both of the activities of the two hydrogen bonds at the acceptor oxygen site may fix a water molecule to this site and then exert the hydrolysis activity by correctly orienting a lone pair of water molecules to the acceptor molecule, glycerol. Because glycerol does not contain an oxygen atom of P, attacks the anemic C1 atom, and Glu-475 works as a general acid. This reaction mechanism is typical for inverting GH-type phosphorylases (30). For the reverse phosphorolysis reaction, βGlc1P is assumed to bind at the position shown in the docking analysis. The O1 and O3 hydroxyls of glycerol are coordinated with Trp-381 and Tyr-327. However, a water molecule must form a hydrogen bond coordinated by Tyr-572 and Lys-587 to fix the O2 atom of glycerol to the position where the reverse phosphorolysis reaction would occur. The two hydrogen bond fixes and orient the O2 atom of glycerol or a water molecule to the anomeric C1 atom of βGlc1P to facilitate reverse phosphorolysis and hydrolysis reactions.

Recently, a faint hydrolytic activity on α-mannose 1-phosphate was identified for a GH130 β-1,4-mannooligosaccharide phosphorylase (31). GH130 enzymes are inverting phosphorylases that act on β-mannosides. Interestingly, a unique proton-relay reaction mechanism that utilizes a water molecule was proposed for a GH130 4-O-β-D-mannosyl-D-glucose phosphorylase because the distance between the catalytic residue and the glycosidic bond oxygen of the substrate is too large for direct

**TABLE 2**

Kinetic parameters of wild-type and mutants of GGP. Activities were measured in 40 mM HEPES-NaOH (pH 7.5) at 30 °C. Values include statistical error limits of the data fit.

| Enzyme | Reverse phosphorolysis | βGlc1P hydrolysis |
|--------|-------------------------|-------------------|
|        | $K_m$ | $k_{cat}$ | $k_{cat}/K_m$ | $K_m$ | $k_{cat}$ | $k_{cat}/K_m$ |
| Wild-type | 5.9 ± 0.5 | 163 ± 4 | 27 | 0.17 ± 0.009 | 1.7 ± 0.02 | 10 |
| Y327F  | ND | ND | ND | ND | ND | ND |
| W381F  | 20 ± 4 | 0.60 ± 0.03 | 0.030 | 0.68 ± 0.2 | 2.6 ± 0.2 | 3.8 |
| Y572F  | 43 ± 2 | 19.8 ± 0.4 | 0.46 | 0.076 ± 0.001 | 0.11 ± 0.002 | 1.4 |
| K587A  | ND | ND | ND | ND | ND | ND |

*Measured with 0–100 mM glycerol and 10 mM βGlc1P.
*Not detected (less than 1/1000 activity of wild-type).
*Not reliable because the rates of the Pi and D-glucose releases were too close at all of the concentrations of glycerol.
Structure of Glucosylglycerol Phosphorylase

protonation (32). Thus, the presence of water-mediated hydrogen bonds at the catalytic center of GH130 enzymes may also be a key for the faint hydrolytic activity on the glycosyl phosphate substrate.

In this study, a structural basis for substrate recognition and reaction mechanisms of GGP was clarified. Although GGP can efficiently and specifically produce GGP via its reverse phosphorylase activity, a considerably high activity of the side reaction, βGlc1P hydrolysis can be an obstacle for industrial applications. The structure determinations of sugar phosphorylases provided solid foundations for endowing altered substrate specificities and increased thermostability to these enzymes (33–36). Our structural study will also facilitate future protein engineering of GGP to modulate and improve its function.

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