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Analysis of the Structural Determinants Underlying Discrimination between Substrate and Solvent in β -Phosphoglucomutase Catalysis^{†,‡}

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Abstract

The β -phosphoglucomutase (β -PGM) of the haloacid dehalogenase enzyme superfamily (HADSF) catalyzes the conversion of β -glucose 1-phosphate (β G1P) to glucose 6-phosphate (G6P) using Asp8 of the core domain active-site to mediate phosphoryl transfer from β -glucose 1,6-(bis)phosphate (β G1,6bisP) to β G1P. Herein we explore the mechanism by which hydrolysis of the β -PGM phospho-Asp8 is avoided during the time that the active site must remain open to solvent in order to allow the exchange of the bound product G6P with the substrate β G1P. Based on structural information, a model of catalysis is proposed in which the general acid/base (Asp10) side chain moves from a position where it forms a hydrogen bond to the Thr16-Ala17 of the domain-domain linker, to a functional position where it forms a hydrogen bond to the substrate leaving-group O and a His20-Lys76 pair of the cap domain. This repositioning of the general acid/base within the core domain active site is coordinated with substrate-induced closure of the cap domain over the core domain. The model predicts that Asp10 is required for general acid/base catalysis and for stabilization of the enzyme in the cap-closed conformation. It also predicts that hinge residue Thr16 plays a key role in productive domain-domain association, that hydrogen bond interaction with the Thr16 backbone amide NH is required to prevent phospho-Asp8 hydrolysis in the cap-open conformation, and that the His20-Lys76 pair plays an important role in substrate-induced cap closure. The model is examined via kinetic analyses of Asp10, Thr16, His20, and Lys76 site-directed mutants. Replacement of the Asp10 by Ala, Ser, Cys, Asn, or Glu resulted in no observable activity. The kinetic consequences of the replacement of linker residue Thr16 with Pro include a reduced rate of Asp8 phosphorylation by β G1,6bisP, a reduced rate of cycling of the phosphorylated enzyme to convert β G1P to G6P, and an enhanced rate of phosphoryl transfer from phospho-Asp8 to water. The X-ray structure of the T16P mutant at 2.7 Å resolution provides a snapshot of the enzyme in an unnatural cap-open conformation where the Asp10 side chain is located in the core-domain active site. The His20 and Lys76 site-

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[‡]Coordinates for the T16P mutant of β -Phosphoglucomutase have been deposited in the Protein Data Bank with accession code 3FM9.

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¹Abbreviations used are: α -PGM, α -phosphoglucomutase; α -PGM/PMM, dual specificity α -phosphoglucomutase/ α -phosphomannomutase; β -PGM, β -phosphoglucomutase; E, β -PGM -Mg²⁺; E-P, phospho- β -PGM-Mg²⁺; β G1P, β -D-glucose 1-phosphate; β G1,6bisP, β -D-glucose 1,6-(bis)phosphate; α G1P, α -D-glucose 1-phosphate; α G1,6bisP, α -D-glucose 1,6-(bis)phosphate; PEP, phosphoenol pyruvate; SA, specific activity.

directed mutants show reduced activity in catalysis of the Asp8-mediated phosphoryl transfer between β G1,6bisP and β G1P but no reduction in the rate of phospho-Asp8 hydrolysis. Taken together, the results support a substrate induced-fit model of catalysis in which β G1P binding to the core domain facilitates recruitment of the general-acid/base Asp10 to the catalytic site and induces cap closure.

Keywords

β -glucose 1-phosphate; β -glucose 1, 6-bisphosphate; β -phosphoglucomutase; HAD superfamily; general acid and base catalysis, induced fit, domain movement, substrate recognition, single turnover reaction; rapid quench; *Lactococcus lactis*; aspartylphosphate

Phosphomutases catalyze the movement of a phosphoryl group from one position in a phosphorylated metabolite to another as needed to initiate or to continue a catabolic pathway (examples include α -phosphoglucomutase (1) and phosphoglycerate mutase (2) of the glycolysis pathway; β -phosphoglucomutase (3) of the trehalose/maltose degradation pathways) or to form the precursor to a biosynthetic pathway (examples include α -phosphomannomutase (4), α -phosphoglucoseamine mutase (5,6) and α -phosphoglucomutase (7) which function in the biosynthesis of membrane bound, cell wall, or capsular glycans). The mechanisms by which phosphomutases orchestrate the transfer of the phosphoryl group between substrate positions have intrigued scientists for decades (5,8-10).

Phosphomutases have evolved within several different enzyme superfamilies including the histidine phosphatase (11), alkaline phosphatase (12), haloacid dehalogenase (HAD) (13), and phosphohexomutase superfamilies (14), yet they share a common catalytic strategy: the use of an active-site nucleophile (His, Ser, Asp, and Ser, respectively) to mediate the transfer of the phosphoryl group. Catalysis in the phosphomutases of the histidine phosphatase, HAD, and phosphohexomutase superfamilies proceeds via a bisphosphate intermediate (11,15-17) whereas catalysis in the phosphoglycerate mutase of the alkaline phosphatase family proceeds via a glycerate intermediate. Each mutase requires that the intermediate reorient in the active site. The glycerate reorients in the active site of the phosphorylated phosphoglycerate mutase by flipping (18). The reorientation of the bisphosphate intermediate in the spacious active site of the phosphohexomutase superfamily mutases also occurs by flipping whereas the confined active sites of the HAD superfamily (HADSF) mutases require that the intermediate must dissociate into solvent and then bind in the opposite orientation (15-17).

The topic of this paper is β -phosphoglucomutase (β -PGM) of the HADSF. The HADSF is comprised mostly of phosphohydrolases (phosphatases and ATPases), which conserve a Rossmann-fold core domain (Figure 1A) (19). The core domain catalytic scaffold consists of four peptide segments or loops on which the Mg^{+2} cofactor-binding residues and the catalytic residues, which constitute the core domain active site, are located (Figure 1B). The conserved Asp nucleophile (Asp8 in β -PGM) of loop 1 mediates phosphoryl transfer in all HADSF phosphotransferases. HADSF phosphatases and mutases conserve a second Asp (Asp10 in β -PGM) located two positions downstream of the Asp nucleophile to participate in general-acid/base catalysis (20).

In most HADSF members the core domain is linked to a cap domain that, when associated with the core domain, aids in active site desolvation and assists in binding the substrate-leaving group (21-24). The type C1 cap domain of β -PGM (Figure 2A) works in concert with the core domain to catalyze phosphoryl transfer to and from the C(6)O and C(1)O positions of β -glucose 1-phosphate (β G1P) and β -glucose 1,6-bisphosphate (β G1,6bisP) (10,15,25). The catalytic cycle (Figure 2B) consists of the integration of ligand binding and dissociation to and from the

active site of the cap-open conformer and catalysis of phosphoryl transfer to and from the active site Asp8 nucleophile of the cap-closed conformer. β -PGM is distinguished from the HADSF phosphatases and ATPases by the selection of β G1P vs. water to accept the phosphoryl group from the phospho-Asp8. The mechanism by which β -PGM makes the distinction between substrate and solvent as the phosphoryl group acceptor is focus of the present work.

The β -PGM cap-open and cap-closed conformations can be compared by examination of the X-ray structures of the phosphorylated β -PGM (Mg^{+2}) complex (3) and the β -PGM(Mg^{+2}) (β G1,6bisP) complex (25), respectively. The β -PGM cap and core domains are connected by two parallel, solvated peptide segments (Figure 2A). The cap domain rotates by 26° relative to the core domain via a change in the backbone phi/psi angles of linker hinge residues Thr14, Asp15, Thr16, and Ala17 in order to open and close the entrance to the core-domain active site. In the cap-open conformer, the Asp10 side-chain carboxylate group is pinned into a position outside the active site via hydrogen-bond formation with the backbone amide NH groups of hinge residues Thr16 and Ala17 (Figure 3). In the cap-closed conformer the Asp10 side-chain carboxylic acid group is positioned in the core domain active site, where it engages in a hydrogen-bond network that includes the β G1,6bisP C(1)O and a His20-Lys76 cap domain pair. Thus, the two β -PGM structures show that the Asp10 side chain alternates (via a 136° rotation about the C α -C β bond) between the linker site in the cap-open conformation and the catalytic site in the cap-closed conformation.

Herein we propose and evaluate a substrate induced-fit model for the mechanism by which phosphoryl transfer from the β -PGM phospho-Asp8 to water is avoided in the presence of β G1P. The model assumes that Asp10 acts in general-acid/base catalysis and to stabilize the enzyme in the catalytically active cap-closed conformation. Site-directed mutagenesis and kinetic analyses of mutant enzymes were used to demonstrate that Asp10 is indeed essential to β -PGM catalysis. The model also predicts that the linker plays two key roles in catalysis: (1) to pin the Asp10 side chain outside of the active site in the unliganded, phosphorylated enzyme and thus prevent general-base-catalyzed phosphoryl transfer to water and (2) to allow, via main-chain conformational change, the coordinated movement of the cap and core domains that occurs during catalytic cycling. Thr16 was identified as the key mediator of both functions and was accordingly replaced by Pro to demonstrate the consequence of the absence of a functional hinge on β -PGM structure and catalysis. Lastly, the model predicts that the paired cap domain residues His20 and Lys76 collaborate with Asp10 in the formation of a hydrogen-bond network that stabilizes the liganded enzyme in the cap-closed conformation. The kinetic properties of site-directed mutants of His20 and Lys76 show that they too play a vital role in β -PGM catalysis.

Materials and Methods

Enzymes and Reagents

α -D-[^{14}C (U)]-Glucose 1-phosphate (specific activity (SA) = 200 mCi/mmol) and D-[1- ^{14}C]-glucose 6-phosphate (SA= 49.3 mCi/mmol) were purchased from Perkin-Elmer Life Sciences to serve as standards in HPLC analysis. [^{14}C (U)]maltose (SA = 300 mCi/mmol) was purchased from American Radiolabeled Chemicals, Inc. Recombinant *L. lactis* β -PGM was prepared according to the published procedure (27) and recombinant wild-type and mutant *N. meningitidis* β -PGM was cloned, overexpressed, and purified in the same manner with a final yield of 20 mg/g wet cell paste for wild-type and 20-30 mg/g for the D10A, D10N and D10E mutants. Recombinant *L. lactis* maltose phosphorylase was prepared according to the published procedure (28). Fructose 6 - phosphate kinase (EC 2.7.1.11) type VII from *Bacillus stearothermophilus*, pyruvate kinase (EC 2.7.1.40) type III from rabbit muscle, and L-lactate dehydrogenase (EC 1.1.1.27) type XI from rabbit muscle, and glucose 6-phosphate

dehydrogenase (EC 1.1.1.49) type IX from Baker's yeast were purchased from Sigma-Aldrich. ATP, ADP, G6P, β G1P, PEP, NADP, and buffers were purchased from Sigma-Aldrich.

Synthesis of β -D-[$^{14}\text{C}(\text{U})$]glucose 1-phosphate

A 1.7 mL solution of 100 mM phosphate buffer (pH 7.2) containing 0.29 mM [$^{14}\text{C}(\text{U})$]maltose (SA = 300 mCi/mmol), 2 mM MgCl_2 , and 4 μM maltose phosphorylase was incubated for 1 h at 25 °C. The reaction solution was passed through a 10 kDa filter to remove the enzyme and loaded onto a 1.5 $\times$ 25 cm Dowex-1 column. The column was eluted at 25 °C with 100 mL deionized water to elute the maltose and glucose, followed by 130 mL of 0.6 M triethylammonium bicarbonate (pH 8) (flow rate = 200 mL/h). Column fractions (5 mL) were assayed for radioactivity using Ultima Gold liquid scintillation cocktail (Perkin-Elmer) with a Beckman LS 6500 multipurpose Scintillation Counter. The β G1P-containing fractions were combined and concentrated to 5 mL by rotary evaporation at 25 °C. The sample was twice diluted with 30 mL deionized water and concentrated to remove triethylammonium bicarbonate. The resulting pellet was dissolved in 4 mL deionized water to a final volume of 4.5 mL, to give a concentration of 92.6 μM (SA= 150 mCi/mmol) (yield 84%). The sample was stored at -20°C. The radiochemical purity of [^{14}C] β G1P was analyzed by HPLC using a Rainin Dynamax high-performance liquid chromatography system equipped with a CarboPac PA1 (Dionex) column (4 $\times$ 250 mm). The column was eluted at a flow rate of 1 mL/min with 2 mL of solvent A (54 mM NaOH, 100 mM sodium acetate), and then with a linear gradient (15 mL) of solvent A to 53.7% solvent B (75 mM NaOH, 500 mM sodium acetate), and lastly, with solvent B. Fractions (1 mL) were collected and their radioactivity content was determined by liquid scintillation counting. The retention time of the synthetic [^{14}C] β -G1P was 9 min (radioactive purity >95%). Desalting was accomplished by chromatography on a Sephadex G-10 column (1.2 cm $\times$ 120 cm) with deionized water as eluent. The unlabeled β G1P was prepared by the same method, and the concentration of the final solution determined spectrophotometrically using the β -PGM- glucose 6-phosphate dehydrogenase coupled reaction (1 mL of 50 mM K^+ HEPES (pH 7.0) containing 2 mM MgCl_2 , 0.4 mM NADP, 5 U glucose 6-phosphate dehydrogenase, 10 nM β -PGM and 5 μM β G1,6bisP, monitored at 340 nm, 25°C, $\Delta\epsilon = 6200 \text{ M}^{-1}\text{cm}^{-1}$).

Synthesis of ^{14}C - labeled and unlabeled β -D-glucose 1,6-bisphosphate (β G1,6bisP)

A 1.2 mL solution of 100 mM phosphate buffer (pH 7.2), 0.29 mM [$^{14}\text{C}(\text{U})$]maltose (SA = 300 mCi/mmol), 1 mM ATP, 2 mM MgCl_2 , and 50 U/mL fructose 6-phosphate kinase (EC 2.7.1.11) type VII from *Bacillus stearothermophilus* was incubated at 25 °C for 4 h, and then passed through a 10 kDa filter to remove the enzyme. The filtrate was loaded onto a 1.5 $\times$ 25 cm Dowex-1 column, which was eluted at 25 °C using a 400 mL linear gradient of triethylammonium bicarbonate (0 to 0.8 M; pH 8.0) at a flow rate of 200 mL/h. Column fractions (5 mL) were assayed for radioactivity using Ultima Gold liquid scintillation cocktail (Perkin-Elmer) and a Beckman LS 6500 multipurpose Scintillation Counter. The β G1,6bisP-containing fractions (eluted at $\sim$ 0.6 - 0.8 M triethylammonium bicarbonate) were combined and concentrated to 2 mL by rotary evaporation at 25 °C. The sample was diluted with 30 mL of deionized water and concentrated to a solid. This process was repeated several times to remove triethylammonium bicarbonate. The resulting white solid was dissolved in 3 mL deionized water (51 μM [$^{14}\text{C}(\text{U})$] β G1,6bisP of SA = 150 mCi/mmol; >95 % radioactive purity; yield 51%) and stored at -20°C. Complete desalting was accomplished by chromatography on a Sephadex G-10 column (1.2 cm $\times$ 120 cm) and with deionized water as eluent. Unlabeled β G1,6bisP was prepared by the same method. The concentration of β G1,6bisP was determined spectrophotometrically using the β -PGM-glucose 6-phosphate dehydrogenase coupled reaction (*vide infra*).

Preparation of β -PGM Site-Directed Mutants

Mutant genes were prepared using a PCR-based strategy with the pET3a- β PGM clone serving as template and commercial oligonucleotides as primers. The PCR product was purified and following digestion with *Nde* I and *Bam*HI ligated to *Nde* I/*Bam*HI linearized pET3a plasmid. Following confirmation of the mutant gene sequence by commercial DNA sequencing, the purified clone was used to transform competent *E. coli* BL21 (DE3) cells. The mutant proteins were purified to homogeneity (based on SDS-PAGE analysis) from cultured cells using the same method previously described for the preparation of the wild type β -PGM (27).

Steady State Kinetic Constant Determination

All kinetic assays were carried out at 25°C in 50 mM K⁺HEPES (pH 7.0) containing 2 mM MgCl₂, unless stated otherwise. The formation of G6P was monitored by measuring the increase in absorbance at 340 nm ($\epsilon = 6.2 \text{ mM}^{-1}\text{cm}^{-1}$) resulting from the G6P dehydrogenase-catalyzed reduction of NADP. A specified volume of a β -PGM stock solution was added to a 1 mL solution containing β G1P, 50 mM K⁺HEPES (pH 7.0), 2 mM MgCl₂, 5 μ M β G1,6bisP, 0.2 mM NADP and 2.5 units/mL G6P dehydrogenase. The kinetic data were analyzed using equation 1.

$$V_0 = V_m [S] / (K_m + [S]) \quad (1)$$

Where [S] is the substrate concentration, V_0 is the initial velocity, V_m is the maximum velocity, K_m is the Michaelis constant. The k_{cat} was calculated from the V_{max} and the enzyme subunit concentration determined by the Bradford method.

β -PGM Catalyzed β G1,6bisP Hydrolysis

The steady-state kinetic constants of β -PGM-catalyzed β G1,6bisP hydrolysis were determined by measuring the initial velocity of product formation in reaction solutions initially containing wild-type or mutant β -PGM, 2 mM MgCl₂, and various concentrations of β G1,6bisP in 50 mM K⁺HEPES (pH 7.0; 25°C). The formation of G6P was monitored using the G6P dehydrogenase/NADP coupled assay (*vide supra*) whereas the formation of orthophosphate was monitored using the PiBlue Phosphate Assay Kit (Invitrogen) according to manufacturer instructions.

Determination of Time Courses for Single-turnover Reactions

Single-turnover experiments were performed at 25 °C using a rapid-quench instrument from KinTek Instruments equipped with a thermostatically controlled circulator. A typical experiment was carried out by mixing 13 μ L buffer A (50 mM K⁺HEPES (pH 7.0) and 2 mM MgCl₂) containing β -PGM and 14 μ L buffer A containing [¹⁴C(U)] β G1,6bisP or [¹⁴C(U)] β G1P plus β G1,6bisP. The reaction was quenched after a specified period of time with 193 μ L of 1 M NaOH. The quenched reaction mixture was passed through a 5 kDa filter to remove the enzyme, and loaded onto a Rainin Dynamax high-performance liquid chromatography (HPLC) system equipped with a CarboPac PA1 (Dionex) column (4 $\times$ 250 mm). The column was eluted at 1 mL/min with 2 mL of solvent A (54 mM NaOH, 100 mM sodium acetate), and then with a linear gradient (15 mL) of solvent A to 53.7% solvent B (75 mM NaOH, 500 mM sodium acetate), and lastly, with solvent B. Fractions (1 mL) were collected and their radioactivity was determined by liquid scintillation counting. The retention times of β G1P, G6P and β G1,6bisP are 8, 15 and 21 min, respectively. The radioactivity associated with the respective [¹⁴C] β G1P, [¹⁴C]G6P, and [¹⁴C] β G1,6bisP fractions was used to calculate the mole fraction of each species present in the reaction mixture at termination. The observed rate constants for the single-turnover reactions were obtained by fitting the time course data to the first order equations (2) and (3) using the computer program KaleidaGraph.

$$[P]_t = [P]_{\max}(1 - e^{-kt}) \quad (2)$$

$$[S]_t = [S]_{\max} - ([P]_{\max}(1 - e^{-kt})) \quad (3)$$

In these equations, $[P]_t$ and $[S]_t$ are the product and substrate concentrations at time “t”, respectively, $[P]_{\max}$ is the product concentration at equilibrium, $[S]_{\max}$ is the initial concentration of substrate, and k is the first-order rate constant.

X-ray Crystallographic Determination of the β -PGM T16P Structure

Crystallization conditions for the β -PGM T16P mutant were identified in hanging-drop, vapor diffusion experiments using the IndexTM Screen (Hampton Research). Although 5 mM β -glucose-1 phosphate and 5 mM β -glucose-1,6 bisphosphate were included in the crystallization buffer in order to allow formation of a Michaelis complex, extensive screening did not yield a liganded complex or crystals of protein in the cap-closed form. Initial screening hits were refined to yield final crystallization conditions using equal parts (1 μ L each) of T16P β -PGM (15 mg/mL) in 50 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT and well solution containing 25% PEG, 0.2 M NaCl, 100 mM BisTris, pH 6.0. Small crystals grew in 7 days to final dimensions of $0.3 \times 0.1 \times 0.05$ mm. X-ray diffraction data were collected on beamline X12C, Brookhaven National Laboratory. The crystals were protected for cryo-crystallography with Paratone N before flash cooling to 100K in a gaseous N_2 stream. Data were collected to 2.7 Å resolution and processed using DENZO and SCALEPACK (29). Data collection statistics are reported in Table 1.

The structure of T16P β -PGM was solved by molecular replacement. Initial trials used the structures of wild-type β -PGM in the cap-open (PDB accession code 1LVH) or cap-closed (PDB accession code 1O08) conformations as search models with the program PHASER (30). Only the cap-open form yielded a correct solution, which was confirmed by subsequent refinement using PHENIX (31). The refinement protocol consisted of one round of rigid body refinement, followed by individual atomic coordinate and isotropic temperature factor refinement, alternating with cycles of model building in COOT (32). Water molecules visible as spherical peaks in $|F_o| - |F_c|$ electron density maps contoured at 3.0σ were added to the model. The quality of the model was assessed by geometry analysis in the program MOLPROBITY (33). Final refinement and model quality statistics are given in Table 1.

The D10N mutant was crystallized from 0.1M Tris HCl, pH 8.5 and 2M ammonium sulfate. Plate-shaped crystals ($0.8 \times 0.2 \times 0.1$ mm) grew in 10-14 days. The cryoprotectant consisted of mother liquor supplemented with 20 % glycerol. The crystals diffracted to 2.1 Å resolution. Although molecular replacement using PHASER gave a successful solution ($R_{\text{factor}} < 40$), the data were incomplete due to crystal decay. Attempts to refine the data set were unsuccessful.

Results and Discussion

Evaluation of the Contribution of Asp10 to β -PGM Catalysis

In order to determine the extent to which Asp10 contributes to catalytic efficiency, Ala10, Ser10, Cys10, Asn10, and Glu10 β -PGM mutants (prepared from either the *N. meningitidis* β -PGM or *L. lactis* β -PGM as dictated by successful gene expression) were evaluated as catalysts of the mutase reaction depicted in Figure 2B. Reaction solutions, initially containing 5 μ M β G1,6bisP, 100 μ M β G1P, 2 mM $MgCl_2$, 50 mM K^+ HEPES (pH 7.0; 25 °C) and 5 μ M β -PGM mutant, were monitored for G6P formation over a 30 min period using the

spectrophotometric assay described in the experimental section. G6P formation was not observed in any of the reactions, indicating a maximum turnover rate that is catalytically insignificant (i.e., $< 1 \times 10^{-3} \text{ s}^{-1}$) (Table 2).

The ability of the β -PGM mutant to catalyze Asp8 phosphorylation with β G1,6bisP was tested by measuring the time course for a single-turnover reaction (Scheme 1). Accordingly, 40 (or 5) μM [^{14}C] β G1,6bisP was reacted with 40 μM *L. lactis* D10N β -PGM in 2 mM MgCl_2 and 50 mM K^+ HEPES (pH 7.0; 25 $^\circ\text{C}$). Following a 90 min incubation period the quenched reaction mixture was analyzed by HPLC to reveal only unconsumed reactant and no [^{14}C]G6P or [^{14}C] β G1P. Similarly, *L. lactis* D10S β -PGM (170 μM) and D10C β -PGM (300 μM) failed to catalyze a single turnover reaction with β G1,6bisP (50 μM). In contrast, the time course for the reaction of 40 μM wild-type *L. lactis* β -PGM with 5 μM [^{14}C] β G1,6bisP (measured by rapid quench) shows that the reaction is complete within 3 ms (the mixing time of the instrument) (Figure 4A). This defines a minimum turnover rate of 400 s^{-1} (Table 2). The lack of observable activity of the Asp10 mutants indicates that Asp10 is required for catalysis, assuming that binding is similar to that observed in the wild-type enzyme.²

The X-ray structure determination of D10N β -PGM was undertaken in hopes of obtaining a snapshot of the liganded mutant enzyme. The crystals that were grown diffracted to 2.1 Å resolution, and an initial molecular replacement solution was achieved. However, the crystal decay that had occurred during data collection resulted in a data set that did not lend itself to satisfactory refinement. Although the success of crystallization and phasing via molecular replacement supports the conservation of native fold in the mutant, the refinement statistics are not sufficient for publication of the structure.

The Hinge Residue Thr16 Plays a Critical Role in *L. lactis* β -PGM Mutase Catalysis

The switch from the cap-open to cap-closed conformers is accompanied by a rotation of the psi/phi dihedrals of -9.0/26.5, -95.1/41.6, and 14.9/-12.1 degrees for the bonds between linker residues Thr14-Asp15, Asp15-Thr16, and Thr16-Ala17, respectively (see Figure 1B and 3A). The program Dyndom (35) was used to identify Asp15 and Thr16 as the residues that undergo the greatest change in dihedral angles. This finding, combined with the observation that in the cap-open conformer, the Asp10 side chain carboxylate group binds to the Thr16 backbone amide NH via a hydrogen bond, prompted the selection of Thr16 for replacement by Pro. Notably the phi/psi angles at Thr16 are in the same portion of Ramachandran space for both cap-open and -closed conformers of the enzyme. Thus, it is expected that the replacement with Pro will limit the dynamic motion by constraint of the phi angle at the mutation site to -60° and thus restrict interconversion between the the cap-open conformer and the catalytically active cap-closed conformer. In addition, it is expected that the removal of the hydrogen bond between Thr16 and Asp10 would impair the enzyme's ability to pin the Asp10 to the linker and hence, outside the active site, in the cap-open conformer. Thus, by examining the structure and the catalytic properties of the T16P mutant the importance of a functional hinge in β -PGM catalysis was probed.

The catalytic properties of *L. lactis* T16P β -PGM were evaluated using steady-state and transient-state kinetic methods. Firstly, the time course for the single turnover reaction of [^{14}C] β G1,6bisP was measured by rapid quench to define $k_{\text{obs}} = 0.8 \text{ s}^{-1}$ (Figure 4B; Table 3). Comparison to $k_{\text{obs}} > 400 \text{ s}^{-1}$ for wild-type *L. lactis* β -PGM shows that the mutation causes a

²A reviewer pointed out that because the Asp10 side-chain engages in a hydrogen bond with the C(1)O of the β G1,6bisP (and presumably with the C(6)OH of β G1P) the Asp10 mutants might not be able to bind the substrate. We consider this unlikely, because this hydrogen bond is one among numerous substrate-binding interactions. Moreover, it is likely that the hydrogen bond is preserved in the D10N mutant. The role that Asn10 residue cannot fill is that of a general acid/base catalyst. Notably, our conclusions rest on the assumption that β -glucose 1,6 bisP binds to the D10N variant.

significant reduction in the efficiency of the autophosphorylation step. This indicates that the altered hinge region may disfavor formation of the cap-closed conformer and/or that the alignment of the surfaces at the domain-domain interface of the cap-closed conformer might be affected.

Secondly, we used steady-state techniques to measure the k_{cat} for wild-type and T16P *L. lactis* β -PGM-catalyzed hydrolysis of β G1,6bisP to approximate the rate of hydrolysis of the phospho-Asp8 that occurs in the absence of β G1P. Our working assumption is that β -PGM-catalyzed hydrolysis of β G1,6bisP occurs via the phosphorylation of Asp8 followed by hydrolysis of the phospho-Asp8 to form orthophosphate (P_i) plus G6P and regenerate the Asp8 (Scheme 2).³

For the wild-type β -PGM, the phosphorylation step occurs with a $k_{\text{obs}} > 400 \text{ s}^{-1}$ (Table 3) whereas the k_{cat} value measured for the β G1,6bisP hydrolysis reaction is 0.03 s^{-1} (Table 4). Therefore, we conclude that phospho-Asp8 hydrolysis is the rate-limiting step, and that the $k_{\text{cat}} = 0.03 \text{ s}^{-1}$ approximates the rate constant governing this step. The $k_{\text{obs}} = 0.8 \text{ s}^{-1}$ measured for T16P β -PGM-catalyzed Asp8 phosphorylation with β G1,6bisP is only 2-fold greater than the $k_{\text{cat}} = 0.38 \text{ s}^{-1}$ measured for T16P β -PGM-catalyzed hydrolysis of β G1,6bisP (Table 4). Thus, mutation of the hinge reduces the rate constant governing phosphoryl transfer from β G1,6bisP to Asp8, yet increases the rate constant governing the phosphoryl transfer from phospho-Asp8 to solvent.

How then does the rate constant for phospho-Asp8 hydrolysis compare with the rate constant defining phosphoryl transfer from the phospho-Asp8 to β G1P? The steady-state $k_{\text{cat}} = 175 \text{ s}^{-1}$ (Table 1) measured for the multi-step conversion of β G1P to G6P (Figure 2B) sets the minimum value for this rate constant, and therefore the β G1P vs. water solvent discrimination factor observed for phosphorylated wild-type β -PGM is greater than or equal to $175 \text{ s}^{-1}/0.03 \text{ s}^{-1} = 5,800$. Clearly, in the presence of β G1P, wild-type β -PGM does not lose the phosphoryl group from the phospho-Asp8 to solvent.

The $k_{\text{cat}} = 0.026 \text{ s}^{-1}$ measured for the multi-step conversion of β G1P to G6P catalyzed by T16P β -PGM defines the minimum value for the rate constant for the transfer of the phospho-Asp8 to β G1P. The ratio of the $k_{\text{cat}} = 0.026 \text{ s}^{-1}$ for the full catalytic cycle to the $k_{\text{cat}} = 0.38 \text{ s}^{-1}$ for β G1,6bisP hydrolysis catalyzed by T16P suggests that transfer of the phospho-Asp8 to water might effectively compete with the transfer to the substrate β G1P. To test this possibility, additional kinetic experiments were carried-out. Firstly, continual monitoring of a reaction solution initially containing $5 \mu\text{M}$ T16P, $100 \mu\text{M}$ β G1P and $5 \mu\text{M}$ β G1,6bisP revealed that only $14 \mu\text{M}$ G6P was formed. Given that G6P formation is thermodynamically favored, this result suggested that the mutant enzyme is not able to convert all of the β G1P. Such would be the case if water competes with the β G1P for the phosphorylated enzyme. By carrying out the reaction with [^{14}C] β G1P we were able to monitor its conversion to [^{14}C]G6P as a function of added unlabelled β G1,6bisP. The reaction steps are illustrated in Scheme 3 to show that if a molecule of phosphorylated enzyme loses the phosphoryl group to solvent it must be replaced by consuming an additional molecule of β G1,6bisP. If there is no surplus β G1,6bisP, catalysis will terminate. This is clearly evident in the time course measured for the reaction of $20 \mu\text{M}$ T16P β -PGM with $5 \mu\text{M}$ [^{14}C] β G1P and $5 \mu\text{M}$ β G1,6bisP (Figure 5B). Whereas, the analogous reaction carried out with wild-type β -PGM proceeds to 100 % completion (Figure 5A) the T16P β -PGM-catalyzed reaction proceeds to only 10 % completion. By carrying out the T16P β -PGM-catalyzed reaction in the presence of excess β G1,6bisP ($50 \mu\text{M}$) the reaction is shown

³An alternative mechanism for hydrolysis of the β G1,6bisP cannot, in the absence of experimental proof, be ruled-out. However, such a reaction pathway would require an active-site bound water molecule to compete with the Asp8 for in-line displacement of G6P from bound β G1,6bisP, which we find to be unlikely given the structure and function of the active site catalytic scaffold.

to proceed further towards completion (40%) (Figure 5C). Overall, these results show that water is able to effectively compete with the substrate for phospho-Asp8 in the case of the T16P β -PGM mutant but not in the case of the wild-type enzyme.

X-ray Structure Determination of T16P β -PGM

The replacement of hinge residue Thr16 with Pro results in a 500-fold reduction in the rate constant for Asp8 phosphorylation by β G1,6bisP, a 6,700-fold reduction in the “apparent” rate constant for cycling of the phosphorylated enzyme to convert β G1P to G6P, and a 13-fold increase in the estimated rate constant for phosphoryl transfer from the phospho-Asp8 to water. The corresponding structural consequences of the mutation were evaluated by X-ray crystallography.

Exhaustive crystallization screening identified two distinct crystal forms, both of which were consistent with an open conformation of the core and cap domains (based on the initial model obtained via molecular replacement). The form that diffracted to higher resolution (2.7 Å) was used for structure determination. The resulting model was refined to completion to yield the T16P β -PGM(Mg^{+2}) structure presented here. The structure shows good electron density overall, and that observed at the site of mutation is shown in Figure 6. Comparison of the T16P β -PGM(Mg^{+2}) structure with that of the wild-type cap-open conformer observed for the phosphorylated β -PGM(Mg^{+2}) complex reveals that the T16P β -PGM mutant is slightly more “open”, with a 6.5° angle of rotation between the cap and the core domains. The positions of the catalytic residues are conserved in the two cap-open structures with the exception of the Asp10 general-acid/base (Figure 7A), which moves from a position where it forms a hydrogen bond to hinge residues in the wild-type β -PGM to inside the active site in the mutant enzyme. Least-squares fitting of the mutant structure with that of the liganded cap-closed conformer of the wild-type enzyme (*viz.* β -PGM(Mg^{+2})(β G1,6bisP)) (Figure 7B) shows that the respective Asp10 carboxylate groups are in close proximity to one another (Figure 7C).

Together these findings suggest that the removal of the potential for hydrogen bond interaction between the Asp10 carboxylate and the backbone amide NH group at position 16 destabilizes the Asp10 binding interaction with the hinge and that this in turn results in the Asp10 carboxylate group occupying a position in the active site where it can potentially function to activate a water molecule for attack at the phospho-Asp8. This is consistent with the findings of the kinetic analysis, which showed that the rate constant for phosphoryl transfer from the phospho-Asp8 to water is 13-fold larger for the T16P mutant than for wild-type β -PGM.

Kinetic analysis also shows that replacement of Thr16 with Pro impairs Asp8 phosphorylation by β G1,6bisP and phosphoryl transfer from the phospho-Asp8 to the substrate β G1P. Both reaction steps proceed from the cap-closed conformer. We speculate that the restriction imposed on the phi angle at the mutation site might disfavor formation of the cap-closed conformer and/or perturb the alignment of the domain interface. Unfortunately, we did not succeed in generating crystals of the liganded mutant for structure determination of the cap-closed conformer to confirm this hypothesis.

Cap Domain Residues His20 and Lys76 Play a Critical Role in β -PGM Mutase Activity

The hydrogen bond network observed in the crystal structure of the cap-closed conformer of the β -PGM(Mg^{+2})(β G1,6bisP) complex (Figure 3) suggests a cooperative interaction between the reactant C(1)O, the Asp10 general-acid/base, and cap-domain residues His20 and Lys76. This network ensures the proper position of Asp10 in the context of the substrate-mediated closure of the cap domain over the core-domain. In order to test the contributions made by hydrogen bond partners His20 and Lys76 to β -PGM catalysis the kinetic properties of site-directed mutants were evaluated.

Replacement of the His20 side chain with Ala eliminates the potential for formation of this hydrogen bond network. This is anticipated to impair Asp8 phosphorylation by β G1,6bisP and phosphoryl transfer from the phospho-Asp8 to the substrate β G1P. The single turnover rate constant " k_{obs} " measured for H20A β -PGM-catalyzed phosphorylation of the Asp8 with [^{14}C] β G1,6bisP is 2 s^{-1} (Figure 4B, Table 3). The >200-fold reduction in the rate constant for the Asp8 phosphorylation step is no doubt coupled with a comparable reduction in the rate constant governing the phosphoryl transfer from the phospho-Asp8 to β G1P. Indeed, the steady-state $k_{\text{cat}} = 0.026 \text{ s}^{-1}$ measured for the catalyzed conversion of β G1P to G6P in the presence of β G1,6bisP is reduced $\sim 7,000$ -fold in the H20A mutant (Table 3).

The consequence of the reduction in the efficiency of phosphoryl transfer from the mutant phospho-Asp8 was revealed by the time courses measured for the reaction of $40 \mu\text{M}$ H20A β -PGM with $5 \mu\text{M}$ [^{14}C] β G1P and 5 or $50 \mu\text{M}$ β G1,6bisP (Figure 5D and 5E). These time courses show that the reaction proceeds to only 10% or 50 % completion, respectively, which demonstrates that water is successfully competing for the phospho-Asp8. The $k_{\text{cat}} = 0.02 \text{ s}^{-1}$ for H20A β -PGM-catalyzed hydrolysis of β G1,6bisP, which we assume estimates the rate constant for the transfer of the phosphoryl group from the phospho-Asp8 to water, is comparable to the steady-state $k_{\text{cat}} = 0.026 \text{ s}^{-1}$ measured for the catalyzed conversion of β G1P to G6P (Table 4).

The imidazole ring of the H20 residue provides two hydrogen bond donors/acceptors: one for the Asp10 side chain and the other for the Lys76 side chain. In order to explore the impact of substituting His20 with residues that might form one but not both hydrogen bonds we examined the kinetic properties of the H20Q and H20N β -PGM mutants. *In silico* modeling using the X-ray structure of the β -PGM(Mg^{+2})(β G1,6bisP) complex indicated that the Gln side chain can potentially form a hydrogen bond with the Asp10 side chain and that the Asn side chain can potentially form a hydrogen bond with the Lys76 side chain (but not with the Asp10). The steady-state kinetic analysis of *L. lactis* H20Q and H20N β -PGM-catalyzed conversion of β G1P to G6P in the presence of β G1,6bisP revealed an 8- and 300-fold diminution in k_{cat} , respectively (Table 2). The H20Q β -PGM (Figure 5F) is significantly more effective in distinguishing β G1P as the phosphate acceptor than is H20N β -PGM (Figure 5G and 5H), which in turn is more effective than H20A β -PGM (Figure 5D and 5E). The rate constant governing the phosphoryl transfer to water is essentially unchanged in the H20N mutant (the H20Q mutant was not tested) (Table 4).

Lastly, we examined the contribution of the conserved cap domain residue Lys76, which positions His20 via a hydrogen bond, and contributes to the hydrogen-bond network. Steady-state kinetic measurements of the conversion of β G1P to G6P in the presence of β G1,6bisP showed that k_{cat} for the *L. lactis* K76A mutant is reduced 100-fold from that of the wild-type β -PGM (Table 2). Nevertheless, the time course for the single-turnover reaction of [^{14}C] β G1P ($5 \mu\text{M}$) plus β G1,6bisP ($5 \mu\text{M}$) catalyzed by K76A β -PGM ($40 \mu\text{M}$) shows complete conversion of the substrate (Figure 5I; Table 5). These results show that Lys76 contributes significantly to β -PGM catalysis, but to a lesser extent than does His20.

Conclusion

β -PGM evolved within an enzyme family that is dominated by phosphatases. It shares with these phosphatases a catalytic mechanism that is built on the use of an Asp to function in nucleophilic catalysis and an Asp to function in general-acid/base catalysis. Whereas the Asp10 general-acid/base of β -PGM moves into the active site when the cap and core domains associate and then moves out of the active site when they dissociate, the Asp general-acid/base of the phosphatase remains in the core domain active site where it is held in place by a nearby electropositive residue (34). Thus, whereas β -PGM relies on substrate-induced cap domain

closure to position the Asp, the phosphatase does not and is therefore able to constitutively activate a water molecule derived from solvent for reaction.

Asp10 was shown to be essential for β -PGM-catalyzed phosphorylation of the Asp8 nucleophile and, although not demonstrated, it is reasonable to assume that it also plays an important role in the ensuing phosphoryl transfer step. By mutation of the hinge we were also able to show that Asp10 will enter the core domain active site in the cap-open conformer. The observed increase in the rate of phospho-Asp8 hydrolysis in the linker mutant is consistent with the greater availability of the Asp10 to bind and activate a water molecule in the cap-open conformer. The mutation of the hinge significantly reduces the efficiency of Asp8 phosphorylation by β G1,6bisP and the efficiency of β G1P phosphorylation by the phospho-Asp8. We were unable to determine the structure of the hinge mutant in the cap-closed conformer and therefore we can only speculate that productive domain-domain association is impaired by the mutation.

The relationship between domain-domain association and catalytic efficiency was further explored by mutating the paired cap domain residues His20 and Lys76 in order to disrupt the hydrogen bond network that includes the β G1,6bisP C(1)O and the Asp10 side chain. The cap domain mutants displayed significantly reduced efficiency in catalysis of Asp8 phosphorylation by β G1,6bisP and of β G1P phosphorylation by the phospho-Asp8 but no change in efficiency of catalyzed phospho-Asp8 hydrolysis. The hydrogen-bond network and the other hydrogen-bond interactions that occur between the G6P moiety and the cap domain are suggested to stabilize the cap-closed conformer and thus form the basis for the substrate-induced cap domain closure.

Taken together these results present a picture that is consistent with the proposal that β -PGM is a very efficient mutase and very inefficient phosphatase because catalysis must occur from the cap-closed conformation, and only β G1P (not solvent water) can stabilize the phosphorylated enzyme in the cap-closed conformation.

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Figure 1. The HASDF phosphotransferase catalytic scaffold. The coloring scheme used to identify the 4 loops is: red (loop 1), green (loop 2), cyan (loop 3) and gold (loop 4). **A.** Schematic of the interactions that occur between a generic phosphoester substrate and the conserved residues of the scaffold. **B.** The position of the conserved catalytic loops on the core Rossmann domain of β -phosphoglucomutase

Figure 2.

Structural and catalytic cycling in β -PGM. **A.** Overlay of the backbone Ca atom positions of the unliganded (pdb code 1LVH, red) and $\beta\text{G1,6bisP}$ complex (pdb code 1O08, gold) with $\beta\text{G1,6bisP}$ shown as ball-and-stick and Mg^{2+} cofactor as a grey sphere **B.** Schematic depiction of the catalytic cycle.

Figure 3.

Changes in the active site between open and closed forms coordinated with ligand binding
A. Stereo view of the overlay of the active sites of unliganded (red) and β G1,6bisP complex (gold) highlighting the hydrogen bond network (black dotted lines) between K76, H20, and D10 in the liganded form. β G1,6bisP is shown as ball-and-stick and Mg^{2+} cofactor as a silver sphere. **B.** Schematic representation of the changes in orientation of D10 between the open (unliganded) and closed (liganded) forms.

Figure 4.

The time course for the single turnover reaction of 40 μ M wild-type β -PGM (A), 20 μ M T16P β -PGM (B), 40 μ M H20A β -PGM (C) with 5 μ M [14 C] β G1,6bisP in 50 mM K⁺HEPES containing 2 mM MgCl₂. [14 C] β G1,6bisP, ($\square$); [14 C] β G6P, ($\blacklozenge$); [14 C] β G1P, ($\bullet$).

G.

H.

I.

Figure 5.

Time course for a single turnover reaction of 5 μM [^{14}C] βG1P in 50 mM K^+HEPES buffer containing 2 mM MgCl_2 and (A) 40 μM wild-type $\beta\text{-PGM}$ plus 5 μM $\beta\text{G1,6bisP}$, (B) 20 μM T16P $\beta\text{-PGM}$ plus 5 μM $\beta\text{G1,6bisP}$, (C) 20 μM T16P $\beta\text{-PGM}$ plus 50 μM $\beta\text{G1,6bisP}$, (D) 40 μM H20A $\beta\text{-PGM}$ plus 5 μM $\beta\text{G1,6bisP}$ (E), 40 μM H20A $\beta\text{-PGM}$ plus 50 μM $\beta\text{G1,6bisP}$ (F) 40 μM H20Q $\beta\text{-PGM}$ plus 5 μM $\beta\text{G1,6bisP}$, (G) 40 μM H20N $\beta\text{-PGM}$ plus 5 μM $\beta\text{G1,6bisP}$, (H) 40 μM H20N $\beta\text{-PGM}$ plus 50 μM $\beta\text{G1,6bisP}$, and (I) 40 μM K76A $\beta\text{-PGM}$ plus 5 μM $\beta\text{G1,6bisP}$. [^{14}C] $\beta\text{G1,6bisP}$, ($\square$); [^{14}C] βG6P , ($\blacklozenge$); [^{14}C] βG1P , ($\bullet$).

Figure 6.

The structure of T16P β -PGM. A. Stereo view of the active site of T16P β -PGM with electron density is shown as magenta cages at the site of mutation and at the general acid/base residue Asp10. The Mg^{2+} cofactor is shown as a silver sphere.

Figure 7.

Structural comparison of T16P β-PGM (blue) with unliganded (open, red) and liganded wild-type β-PGM structures (closed, gold) shows that the overall structure of T16P β-PGM (blue) is in the open form. Examination of the active site overlay with the open form (A) and the closed form (B and close up in C) demonstrates that the general acid/base Asp10 is positioned as it is in the wild-type closed form.

Scheme 1.

Scheme 2.

Scheme 3.

Table 1

Crystallographic data collection and refinement statistics.

Data Collection Statistics	
Resolution (last shell) (Å)	50.00 - 2.70 (2.80 - 2.70)
X-ray Source	NSLS X12C
Wavelength (Å)	1.000
Space group	P2 ₁ 2 ₁ 2 ₁
Unit cell dimensions (Å)	a = 54.478 b = 57.656 c = 77.899
Reflections Observed (unique)	259,994 (7,122)
Completeness (%)	99.3
R _{merge} ^a (%)	15.1 (54.7)
I/σ (I)	14.7 (3.3)
Redundancy	5.5 (5.4)
Refinement Statistics	
No. protein/water atoms per asu	1,706/62
No. Mg ions per asu	1
Number of reflections (work/free)	6,778/678
R _{work} /R _{free} (%)	20.1/28.0
Resolution (Å)	39.6-2.70
Average B-factor (Å ²)	32.6
Protein	32.5
Mg ²⁺	40.2
Water	35.3
Wilson B-factor	56.8
Rmsd	
Bond length (Å)	0.008
Bond angle (°)	1.030

^aR_{merge} = $\sum_{hkl} \sum_i |I_{hkl, i} - \langle I_{hkl} \rangle| / \sum_{hkl} \sum_i I_{hkl, i}$, where $\langle I_{hkl} \rangle$ is the mean intensity of the multiple $I_{hkl, i}$ observations for symmetry related reflections.

Table 2

Steady state kinetic constants for wild type and mutant β -PGM measured using assay solutions containing varying concentrations of β G1P, 5 μ M β G1,6bisP, 2 mM MgCl_2 , 0.2 mM NADP, 2.5 U/mL glucose 6-phosphate dehydrogenase in 50 mM K^+ HEPES (pH 7.0 and 25°C).

β -PGM	K_m (μ M)	k_{cat} (s^{-1})	k_{cat} / K_m ($\text{M}^{-1} \text{s}^{-1}$)
<i>N. meningitidis</i> WT ^a	2.6 $\pm$ 0.3	20 $\pm$ 2	8 $\times$ 10 ⁶
<i>N. meningitidis</i> D10A ^a	ND ^b	< 0.001	ND ^b
<i>N. meningitidis</i> D10E ^a	ND ^b	< 0.001	ND ^b
<i>L. lactis</i> WT	31 $\pm$ 2	175 $\pm$ 5	6 $\times$ 10 ⁶
<i>L. lactis</i> D10N	ND ^b	< 0.001	ND ^b
<i>L. lactis</i> D10S	ND ^b	< 0.001	ND ^b
<i>L. lactis</i> D10C	ND ^b	< 0.001	ND ^b
<i>L. lactis</i> T16P	4.8 $\pm$ 0.3	0.026 $\pm$ 0.001	5 $\times$ 10 ³
<i>L. lactis</i> H20Q	45 $\pm$ 1	21.9 $\pm$ 0.1	5 $\times$ 10 ⁵
<i>L. lactis</i> H20N	170 $\pm$ 10	0.62 $\pm$ 0.02	4 $\times$ 10 ³
<i>L. lactis</i> H20A	41 $\pm$ 3	0.026 $\pm$ 0.004	6 $\times$ 10 ²
<i>L. lactis</i> K76A	66 $\pm$ 1	1.56 $\pm$ 0.01	2 $\times$ 10 ⁴

^a Measured using 50 μ M α G1,6bisP in place of 5 μ M β G1,6bisP.

^b ND: Not determined

Table 3

The apparent rate constants of the wild-type and mutant *L. lactis* β -PGM (20–40 μ M) catalyzed single-turnover reactions of 5 μ M [14 C] β G1,6bisP. The k_{obs} values for the formation of [14 C] β G6P or [14 C] β G1P and the consumption of [14 C] β G1,6bisP were obtained by fitting the individual sets of time course data to first order rate equations.

β -PGM (μ M)	k_{obs} (β G1P) (s^{-1})	k_{obs} (G6P) (s^{-1})	k_{obs} (β G1,6bisP) (s^{-1})
Wild-type (40)	> 400	>400	>400
T16P (20)	0.9 ± 0.2	0.8 ± 0.1	0.8 ± 0.1
H20A (40)	4 ± 1	1.9 ± 0.4	2.2 ± 0.4

Table 4

Steady state kinetic constants of *L. lactis* β -PGM-catalyzed β G1,6bisP hydrolysis in 50 mM K⁺HEPES (pH 7.0, 25° C) containing 2 mM MgCl₂ and β G1,6bisP at various concentrations.

β -PGM	K_m (μ M)	k_{cat} (s^{-1})
Wild type	0.63 ± 0.07	0.0298 ± 0.008
H20N	1.49 ± 0.09	0.0303 ± 0.0005
H20A	2.6 ± 0.1	0.0197 ± 0.003
T16P	10 ± 1	0.38 ± 0.01

Table 5

The apparent rate constants of the wild-type and mutant *L. lactis* β -PGM (40 or 20 μ M) catalyzed single-turnover reactions of [14 C] β G1P (5 μ M) in the presence of β G1,6bisP (5 μ M or 50 μ M). The k_{obs} values for [14 C]G6P or [14 C] β G1,6bisP formation and [14 C] β G1P consumption were obtained by fitting the individual sets of time course data to first order rate equations 2 or 3.

β -PGM	[β G1,6bisP] (μ M)	$k_{\text{obs}}(\beta\text{G1P})$ (s^{-1})	$k_{\text{obs}}(\beta\text{G6P})$ (s^{-1})	G6P/ β G1P
Wild-type	5	12.2 ± 0.3	9.3 ± 0.4	10
T16P ^a	50	0.014 ± 0.002	0.014 ± 0.002	0.6
H20Q	5	0.96 ± 0.04	0.78 ± 0.04	10
H20N	50	0.042 ± 0.002	0.018 ± 0.002	10
H20A	50	0.0055 ± 0.0004	0.0029 ± 0.003	1
K76A	5	0.164 ± 0.006	0.157 ± 0.004	10

^aThe enzyme concentration used is 20 μ M.