Conformational Cycling in β-Phosphoglucomutase Catalysis: Reorientation of the β-D-Glucose 1,6-(Bis)phosphate Intermediate†

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ABSTRACT: Activated *Lactococcus lactis* β-phosphoglucomutase (βPGM) catalyzes the conversion of β-D-glucose 1-phosphate (βG1P) derived from maltose to β-D-glucose 6-phosphate (G6P). Activation requires Mg^{2+} binding and phosphorylation of the active site residue Asp8. Initial velocity techniques were used to define the steady-state kinetic constants $k_{cat} = 177 \pm 9\ s^{-1}$, $K_m = 49 \pm 4\ \mu M$ for the substrate βG1P and $K_m = 6.5 \pm 0.7\ \mu M$ for the activator β-D-glucose 1,6-bisphosphate (βG1,6bisP). The observed transient accumulation of [^{14}C]βG1,6bisP (12% at ∼0.1 s) in the single turnover reaction carried out with excess βPGM (40 μM) and limiting [^{14}C]βG1P (5 μM) and βG1,6bisP (5 μM) supported the role of βG1,6bisP as a reaction intermediate in the conversion of the βG1P to G6P. Single turnover reactions of [^{14}C]βG1,-6bisP with excess βPGM were carried out to demonstrate that phosphoryl transfer rather than ligand binding is rate-limiting and to show that the βG1,6bisP binds to the active site in two different orientations (one positioning the C(1)phosphoryl group for reaction with Asp8, and the other orientation positioning the C(6)phosphoryl group for reaction with Asp8) with roughly the same efficiency. Single turnover reactions carried out with βPGM, [^{14}C]βG1P, and unlabeled βG1,6bisP demonstrated complete exchange of label to the βG1,6bisP during the catalytic cycle. Thus, the reorientation of the βG1,6bisP intermediate that is required to complete the catalytic cycle occurs by diffusion into solvent followed by binding in the opposite orientation. Published X-ray structures of βG1P suggest that the reorientation and phosphoryl transfer from βG1,6bisP occur by conformational cycling of the enzyme between the active site open and closed forms via cap domain movement. Last, the equilibrium ratio of βG1,6bisP to βG1P plus G6P was examined to evidence a significant stabilization of βPGM aspartyl phosphate.

Phosphoglucomutases catalyze the interconversion of D-glucose 1-phosphate (G1P)[1] and D-glucose 6-phosphate (G6P). Operating in the forward G6P-forming direction, this reaction links polysaccharide phosphorolysis to glycolysis. In the reverse direction, the reaction provides G1P for the biosynthesis of exo-polysaccharides (*2*). There are two classes of phosphoglucomutases, the α-phosphoglucomutases (αPGM, EC 5.4.2.2), ubiquitous among eucaryotes and procaryotes, and the β-phosphoglucomutases (βPGM, EC 5.4.2.6), present in certain bacteria and protists. The two classes of mutases are distinguished by their specificity for α- and β-D-glucose phosphates and by their protein-fold family. The rabbit muscle αPGM (*3*) and the closely related *Pseudomonas aeruginosa* αPGM/αPMM (*4*) are members of the phosphohexomutase enzyme superfamily (*5*), whereas βPGM (*6*) belongs to the haloalkanoic acid (HAD) enzyme superfamily (*7*). The four-domain αPGM and αPGM/αPMM (∼50 kDa) are approximately twice the size of the two-domain βPGM (∼25 kDa).

In αPGM (and in αPGM/αPMM), phosphoryl transfer is mediated by an active site serine which forms a stable phosphate ester linkage (half-life in water is ∼7 years) (*8*). The catalytic cycle begins with the binding of αG1P to the active site of the phosphorylated enzyme, followed by phosphoryl transfer to the C(6)O ($k = 1000\ s^{-1}$) (see Figure 1). The α-glucose 1,6-bisphosphate (αG16bisP) thus formed, and tightly bound (*8*, *9*, *11*), must become reoriented in the active site so that the C(1)phosphate can be transferred to the active site nucleophile to yield the G6P product. It does so by rotating 180° while still associated with the enzyme (*10*).

In this paper, we examine the chemical pathway of the *Lactococcus lactis* βPGM-catalyzed conversion of βG1P to G6P.[2] This reaction is mediated by an active site aspartate (Asp8), which forms an acyl phosphate as the covalent enzyme intermediate (*12*). Kinetic methods were used to demonstrate the intermediacy of βG16bisP in G6P formation

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[1] Abbreviations: α-PGM, α-phosphoglucomutase; α-PGM/PMM, duel specificity α-phosphoglucomutase/α-phosphomannomutase; βPGM, β-phosphoglucomutase; E, βPGM-Mg^{2+}; E-P, phospho-β-PGM-Mg^{2+}; βG1P, β-D-glucose 1-phosphate; βG16bisP, β-D-glucose 1,6-(bis)-phosphate; αG1P, α-D-glucose 1-phosphate; αG16bisP, α-D-glucose 1,6-(bis)phosphate; NADP, adenine dinucleotide 3′-phosphate; K^+-Hepes, potassium salt of 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid; HPLC, high-performance liquid chromatography.

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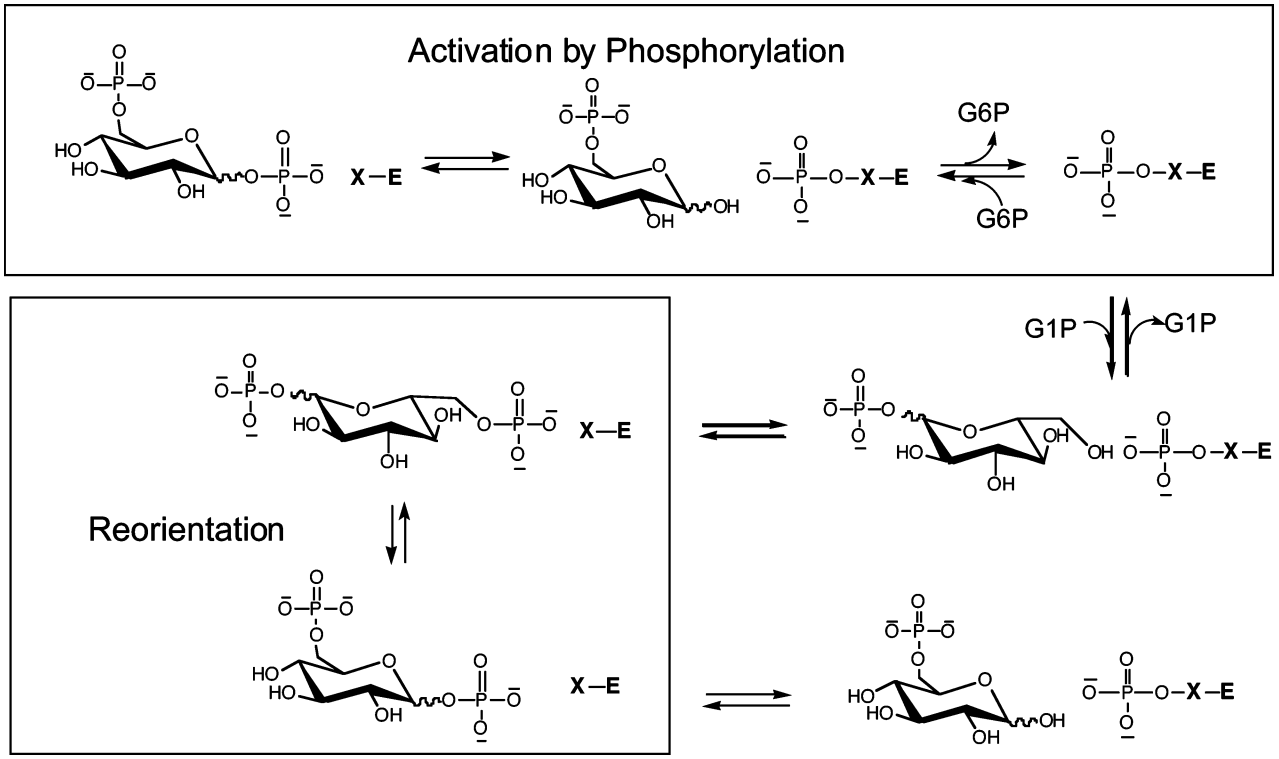

FIGURE 1: The steps associated with phosphoglucomutase (E-X) activation by glucose 1,6-bisphosphate and the subsequent conversion of glucose 1-phosphate (G1P) to glucose 6-phosphate (G6P).

and to show that the βG16bisP intermediate is reoriented in the active site by dissociation into solvent and then binding in the opposite orientation. The rate and equilibrium for phosphorylation of the active site Asp8 by βG16bisP are examined. A model for βPGM catalysis is proposed that is based on cycling of the enzyme between the open and closed conformations observed in the reported βPGM X-ray crystal structures (6, *12*−*14*).

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. [^{14}C-(U)]β-*D*-glucose 1-phosphate and [^{14}C(U)]β-*D*-glucose 1,6-bisphosphate were prepared as described in Supporting Information and reported in reference *15*. Recombinant *L. lactis* β-phosphoglucomutase (βPGM) was prepared according to a published procedure (*16*). Glucose 6-phosphate dehydrogenase (EC 1.1.1.49) type IX from Baker's yeast was purchased from Sigma-Aldrich. G6P, βG1P, NADP, and all buffers were purchased from Sigma-Aldrich.

Activation by Phosphorylation. The steady-state kinetic constants for βPGM activation by phosphorylation were measured by varying the concentration of the substrate βG1P between 0.5- and 5-fold K_m at fixed concentration of activator βG1,6bisP (3, 5, 10, and 20 μM) in 50 mM K$^+$Hepes (pH 7.0 and 25 °C) containing 0.001 μM βPGM, 2 mM $MgCl_2$, 0.2 mM NADP, and 3 unit/mL G6P dehydrogenase. The formation of G6P was monitored by measuring the increase in solution absorbance at 340 nm (ϵ = 6.2 mM^{-1} cm^{-1}) resulting from the G6P dehydrogenase-catalyzed reduction of NADP. Data were computer-fitted to eq 1. Where [A] is

$$V_0 = V_m[A][B]/(K_B[A] + K_A[B] + [A][B]) \quad (1)$$

the βG1P concentration, [B] is the activator concentration, V_0 is the initial velocity, V_m is the maximum velocity, K_A is the Michaelis constant for βG1P, and K_B is the Michaelis constant for activator. The k_{cat} was calculated from the ratio of V_{max} and the enzyme concentration.

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 μL of buffer A (50 mM K$^+$Hepes (pH 7.0) and 2 mM $MgCl_2$) containing βPGM and 14 μL of buffer A containing [^{14}C(U)]βG1,6bisP or [1-^{14}C]G6P plus βG1,6bisP or [^{14}C(U)]βG1P plus βG1,6bisP. The reaction was quenched after a specified period of time with 193 μL of 1 M NaOH. The quenched reaction mixture was passed through a 5-kDa filter to remove the enzyme, and then loaded onto a Rainin Dynamax high-performance liquid chromatography (HPLC) system equipped with a CarboPac PA1 (Dionex) column (4 × 250 mm). The column was eluted at a flow rate of 1 mL/min, first with 2 mL of solvent A (54 mM NaOH and 100 mM sodium acetate), then with a linear gradient (15 mL) of solvent A to 53.7% solvent B (75 mM NaOH and 500 mM sodium acetate), and last, with solvent B. Fractions (1 mL) were collected, and their radioactivity was determined by liquid scintillation counting. The retention times of βG1P; G6P; and βG1,6bisP are 8, 15, and 21 min, respectively.

[2] No distinction between the α- and β-anomers of glucose-6-phosphate is offered because the solution epimerization of the G6P C(1)OH is rapid (*1*).

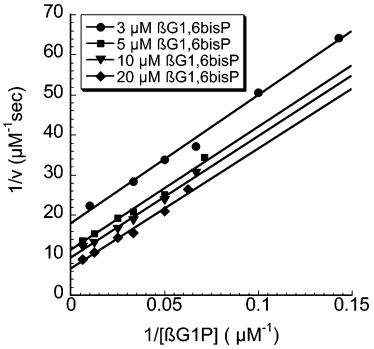

FIGURE 2: Plots of inverse velocity vs inverse βG1P concentration measured at various, fixed phosphoryl donor concentration of βG1,-6bisP, measured at 50 μM βG1P in 50 mM K^+Hepes buffer (pH 7.0) containing 2 mM $MgCl_2$ at 25 °C.

The radioactivity associated with the respective [^{14}C]βG1P; [^{14}C]G6P; and [^{14}C]βG1,6bisP 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 eqs 2 and 3 using the computer program Kaleidagraph. In these equations, k is the first-order

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

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

rate constant; $[P]_t$ and $[S]_t$ are the product and substrate concentrations at time "t", respectively; $[S]_{max}$ is the initial concentration of substrate; and $[P]_{max}$ is the product concentration at equilibrium.

RESULTS AND DISCUSSION

β-PGM Activation by Phosphorylation. To efficiently catalyze the conversion of βG1P to G6P, βPGM must be first phosphorylated at Asp8. In a previous study of *L. lactis* βPGM, we showed that βG1P can serve as the phosphoryl group donor at a turnover rate of 0.8 s^{-1} (*12*). Once formed, the phosphorylated enzyme (E-P) catalyzes the phosphorylation of βG1P to generate βG1,6bisP. The βG1,6bisP is presumed to be the phosphoryl donor in subsequent turnover reactions. In the present study of the *L. lactis* βPGM, activation by synthetic βG1,6bisP was examined by using initial velocity techniques.

Accordingly, the initial velocity of G6P formation was measured as a function of βG1P concentration at changing, fixed βG1,6bisP concentration. Because βG1,6bisP and βG1P bind to separate enzyme forms (E and E-P, respectively), a parallel-pattern, double reciprocal plot, as is typical of a Bi-Bi Ping-Pong kinetic mechanism (*17*), was observed (Figure 2). Data fitting to the rate expression for the Bi-Bi Ping-Pong kinetic mechanism defined a $k_{cat} = 177 \pm 9$ s^{-1}, $K_m = 49 \pm 4$ μM for the substrate βG1P and $K_m = 6.5 \pm 0.7$ μM for the activator βG1,6bisP. The k_{cat} value determined at fixed saturating concentration of βG1,6bisP and varied concentration of βG1P is however typically ~100 s^{-1}.

βPGM Binds βG1,6bisP in Two Different Orientations. The X-ray structure of the βPGM complex of βG1,6bisP is depicted in Figure 3. In this structure, the enzyme assumes a closed conformation with the cap domain bound to the core domain, covering the active site and encapsulating the

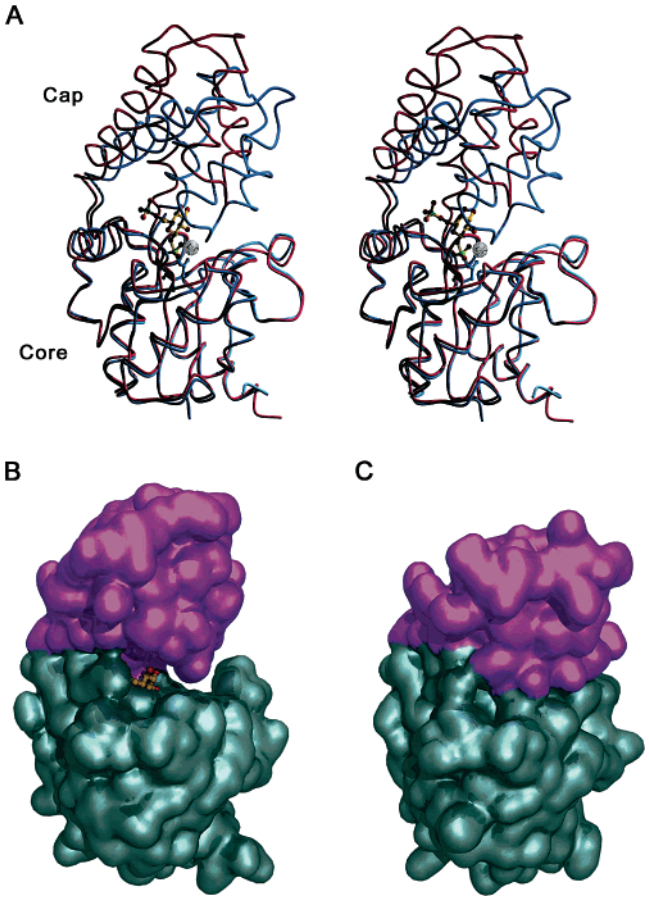

FIGURE 3: (A) Superposition of the phospho-βPGM−Mg^{2+} complex illustrating the cap-open conformation (red; the phosphoryl group is not shown) (*6*) and the βPGM−Mg^{2+}−6-phosphoglucose-1-phosphorane complex (blue) illustrating the cap-closed conformation and the phosphorane intermediate (*13*). (B) Space filling model of βPGM in the open conformation as observed in the structure of the phospho-βPGM−Mg^{2+} complex. (C) Space filling model of βPGM in the closed conformation as observed in the structure of the βPGM−Mg^{2+}−6-phosphoglucose-1-phosphorane complex.

bound ligand (Figure depicts the superposition of the structure of the βPGM(Mg^{2+})(βG1,6bisP) complex illustrating the closed conformation and the structure of the bound phosphorylated βPGM illustrating the open conformation). The βG1,6bisP C(6)phosphate interacts with electropositive residues of the cap domain, and the C(1)phosphate is engaged in covalent bond formation with the core domain Asp8 carboxylate. Thus, the structure provides a snapshot of the enzyme as it moves along the reaction coordinate, in this case one step beyond the Michaelis complex (see reaction scheme in Figure 4A). The phosphorane intermediate shown is formed from the bound βG1,6bisP upon cap closure. We presume that the stability of this reaction intermediate in the crystal is the result of crystal packing forces stabilizing the cap-closed conformation. In solution, the cap domain is free to dissociate, thus, stimulating P−O bond cleavage in the phosphorane to generate either the Michaelis complex of phosphorylated enzyme (depicted in Figure 3A) with G6P bound or the Michaelis complex of unphosphorylated enzyme with βG1,6bisP noncovalently bound.

For βPGM catalysis in the βG1P-forming direction, the βG1,6bisP must be bound with the C(1)phosphate interacting with the cap domain and the C(6)phosphate with the Asp8. This orientation is termed E(βG1,6bisP)-1. For βPGM

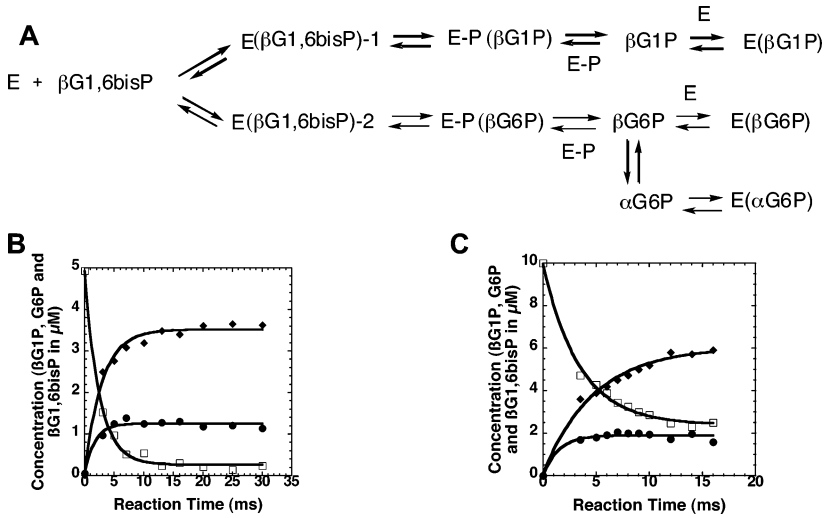

FIGURE 4: (A) The reaction scheme for the single turnover reaction of βPGM and [^{14}C(U)]βG1,6bisP. (B) The time course for the single turnover reaction of βPGM (40 μM) and [^{14}C(U)]βG1,6bisP (5 μM) in 50 mM K^+Hepes containing 2 mM $MgCl_2$ (pH 7.0, 25 °C). k_{obs} = 400 ± 20 s^{-1} for βG1,6bisP consumption; k_{obs} = 600 ± 100 s^{-1} for G1P formation; k_{obs} = 340 ± 40 s^{-1} for G6P formation. (C) The time course for the single turnover reaction of βPGM (20 μM) and [^{14}C(U)] βG1,6bisP (10 μM) in 50 mM K^+Hepes containing 2 mM $MgCl_2$ (pH 7.0, 25 °C). k_{obs}= 280 ± 20 s^{-1} for βG1,6bisP consumption; k_{obs} = 800 ± 500 s^{-1} for G1P formation; k_{obs} = 190 ± 20 s^{-1} for G6P formation. [^{14}C]βG1,6bisP, (□); [^{14}C]βG6P, (♦); [^{14}C]βG1P, (●). The data were fitted to first-order rate eqs 2 and 3.

catalysis in the G6P-forming direction, the βG1,6bisP must be bound with the C(6)phosphate interacting with the cap domain and the C(1)phosphate with the Asp8. This orientation (observed in the structure shown in Figure 3) is termed E(βG1,6bisP)-2. To determine if one orientation is preferred over the other, single turnover reactions between βPGM and limiting [^{14}C]βG1,6bisP were carried out. As is illustrated in Figure 4A, the collision between βPGM and [^{14}C]βG1,-6bisP will form the Michaelis complexes E(βG1,6bisP)-1 and E(βG1,6bisP)-2. Cap closure will produce the corresponding phosphorane intermediate (E(βG1,6bisP)-1* or E(βG1,6bisP)-2*, which upon cap opening will revert to the corresponding Michaelis complex or generate the corresponding product complex E-P(βG1P) or E-P(βG6P). Once formed, the product complexes will dissociate to E-P and the product ligand. If the product ligand binds to the free enzyme which is present in excess, the dead-end complex E(βG1P) or E(βG6P) will be formed. The E-P can bind βG1P or βG6P from the ligand pool and undergo the reverse reaction sequence. In this manner, the reactions shown in Figure 4A can attain equilibrium.

On the basis of a steady-state k_{cat} = 177 ± 9 s^{-1} for multiple turnover of βG1P by the βG1,6bisP-activated enzyme, we anticipated that a single turnover reaction of [^{14}C]βG1,6bisP would be fast, perhaps too fast to monitor by rapid quench. Nevertheless, the time courses for [^{14}C]βG1P and [^{14}C]βG6P formation from the reaction of [^{14}C]βG1,-6bisP with excess βPGM were measured to determine the rate of phosphoryl transfer from[^{14}C]βG1,6bisP to the Asp8 and to determine the ratio of [^{14}C]βG1P and [^{14}C]βG6P formed as product. This ratio might reflect the relative rates of E-P(βG1P) and E-P(βG6P) formation and, thus, the preference of the enzyme for formation of and catalysis within the Michaelis complexes E(βG1,6bisP)-1 and E(βG1,-6bisP)-2.

The single turnover reaction was carried out using enzyme in large excess to reactant (5 μM [^{14}C]βG1,6bisP reacted with 40 μM βPGM) (Figure 4B) to ensure that the reactant and products are enzyme-bound. For comparison, the reaction was also carried with a 2:1 ratio of enzyme to reactant (10 μM [^{14}C]βG1,6bisP with 20 μM βPGM) (Figure 4C). At the lower enzyme-to-ligand ratio, it is likely that the enzyme−product complexes will release a significant amount of product to solution. The time courses measured under two sets of conditions show that both [^{14}C]βG1P and [^{14}C]βG6P are formed in the reaction. Notably, the [^{14}C]βG1P formation appears to be completed within the first 4 ms time point, at which point the ratio of [^{14}C]βG6P to [^{14}C]βG1P is ~2:1. The remainder of the time course reflects the approach to equilibrium, which is different under the two sets of reaction conditions. This difference can be attributed to the difference in the ratio of the enzyme to ligand, which for the smaller ratio translates into a greater contribution from solvent equilibria of [^{14}C]βG1P; [^{14}C]βG1,6bisP; and [^{14}C]G6P. The time courses of Figure 4B,C are fitted with first-order rate equations to define the k_{obs} values reported in the figure legends. However, we note that the k_{obs} value for [^{14}C]βG1P formation is defined only by the 4 ms time point, and therefore, the data define a minimum value as an estimate for the k_{obs}. The k_{obs} values for [^{14}C]βG1,6bisP (~400 s^{-1}) and [^{14}C]G6P formation are, in contrast, calculated as an average of the rate of the initial turnover and the rate of the subsequent equilibration reactions.

βG1,6bisP Is an Intermediate in the βPGM-Catalyzed Reaction. In the previous section, we reported that the reaction between βG1,6bisP and βPGM generates the active form of the enzyme (E-P) and a mixture of βG1P and G6P. To demonstrate the intermediacy of βG1,6bisP in the βPGM-catalyzed conversion of βG1P to G6P, the accumulation of [^{14}C]βG1,6bisP during a single turnover of the E-P([^{14}C]βG1P) complex was measured. The first reaction was carried out with excess βPGM (40 μM) and limiting βG1,6bisP (5 μM) and [^{14}C]βG1P (5 μM). The reaction sequence is shown in Figure 5 along with the time courses for [^{14}C]βG1P consumption and the formation of [^{14}C]βG1,6bisP and [^{14}C]-G6P. The time course of Figure 5B shows that the E·[^{14}C]βG1,6bisP complex accumulates to a maximum of 12% at ~0.1 s and then declines over the next 0.1 s to an

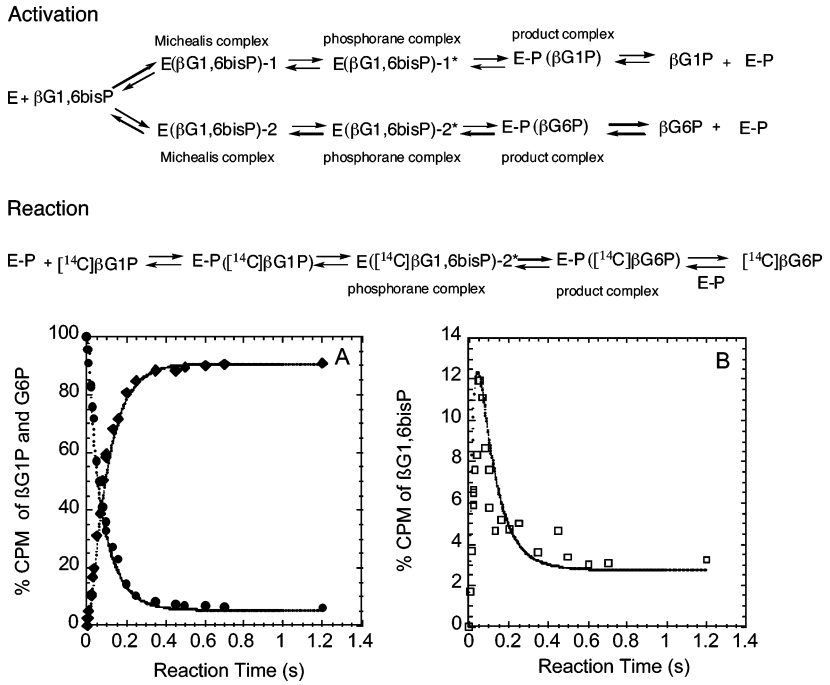

FIGURE 5: Scheme for the activation and catalyzed reaction of βPGM and time course for the single turnover reaction of 40 μM βPGM with 5 μM βG1,6bisP and 5 μM [^{14}C]βG1P in 50 mM K$^+$Hepes (pH 7.0, 25 °C) containing 2 mM $MgCl_2$. (A) Time course for [^{14}C]βG1P (●) consumption and [^{14}C]G6P (♦) formation. (B) Time course for [^{14}C]βG1,6bisP formation and consumption.

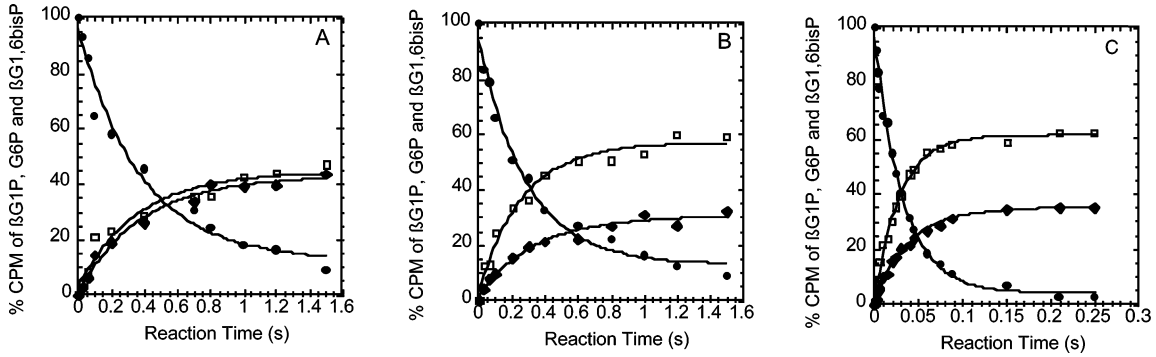

FIGURE 6: Time course of single turnover reactions of (A) 10 μM βPGM, 10 μM [^{14}C]βG1P, and 20 μM βG1,6bisP; (B) 10 μM βPGM, 10 μM [^{14}C]βG1P, and 40 μM βG1,6bisP; and (C) 40 μM βPGM, 5 μM [^{14}C]βG1P, and 50 μM βG1,6bisP in solutions containing and 2 mM $MgCl_2$ in 50 mM K$^+$Hepes (pH 7.0, 25 °C). [^{14}C]βG1,6bisP, (□); [^{14}C]βG6P, (♦); [^{14}C]βG1P, (●). The rate data were fitted to first-order rate eqs 2 and 3.

equilibrium level of 3%. These results indicate that the [^{14}C]βG1P is first converted to [^{14}C]βG1,6bisP which in turn is converted to [^{14}C]G6P.

βG1,6bisP Reorientation. For the βPGM to complete a full catalytic cycle, the βG1,6bisP formed from the reaction of βG1P and E-P must become reoriented in the active site of the free enzyme so that the C(1)phosphate group can phosphorylate the Asp8. The question addressed in this section is whether the βG1,6bisP "flips" while still contained within the active site or the βG1,6bisP dissociates into solvent and then binds to the vacated active site in the opposite orientation. To test the mechanism of reorientation, a single turnover of [^{14}C]βG1P catalyzed by excess E-P was carried out in the presence of unlabeled βG1,6bisP. If there is no exchange of the [^{14}C]βG1,6bisP with the unlabeled βG1,-6bisP, then the radioactivity will be localized in the G6P fraction and none will appear in the G1,6bisP fraction. If, for every turnover, the [^{14}C]βG1,6bisP must leave the enzyme and mix with the unlabeled βG1,6bisP in the solvent, the ^{14}C-label will become distributed between the βG1,6bisP and the G6P. Specifically, the ratio of ^{14}C-label in the G6P and βG1,6bisP fractions will be equal to the molar ratio of the [^{14}C]βG1P and βG1,6bisP used in the reaction.[3]

The distribution of radiolabel from [^{14}C]βG1P to βG1,-6bisP and the G6P was measured using three different reaction conditions (see Figure 6). First, 10 μM βPGM; 20 μM unlabeled βG1,6bisP; and 10 μM [^{14}C]βG1P were reacted. The amount of radiolabel in the βG1,6bisP versus G6P fractions should be 1:1, consistent with the 1:1 molar ratio of βG1,6bisP and βG1P used in the reaction. A 1:1 ratio of radiolabel is evidenced by the time course shown in Figure 6A. Second, 10 μM βPGM; 40 μM unlabeled βG1,-6bisP; and 10 μM [^{14}C]βG1P were reacted. The amount of

[3] This relationship accounts for the fact that the E-P is formed from free enzyme plus the unlabeled βG1,6bisP added, and hence, the amount of βG1,6bisP in the solution is equal to the βG1,6bisP added minus the enzyme added.

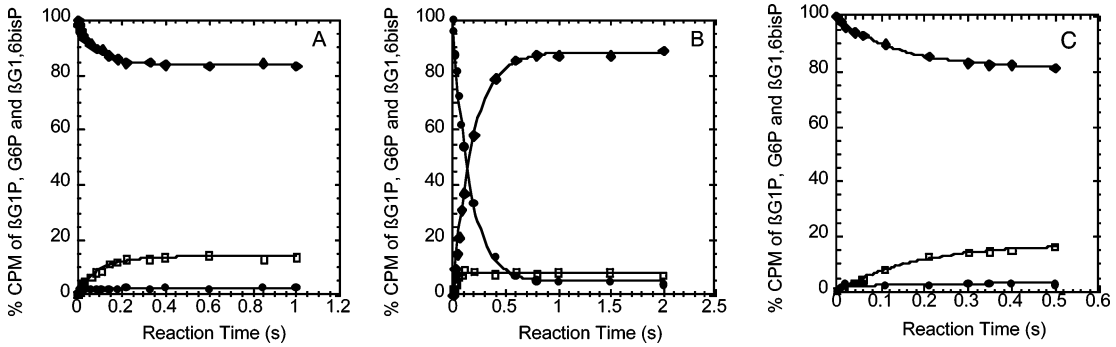

FIGURE 7: (A) Time course for the reaction of 40 μM [1-^{14}C]G6P, 40 μM βG1,6bisP, and 40 μM βPGM; (B) time course for the reaction of 40 μM [^{14}C(U)]βG1P, 40 μM βG1,6bisP, and 40 μM βPGM; and (C) time course for the reaction of 5 μM [1-^{14}C]G6P, 50 μM βG1,6bisP, and 40 μM βPGM in 50 mM K^+Hepes containing 2 mM $MgCl_2$ (pH 7.0, 25 °C). [^{14}C]βG1,6bisP (□); [^{14}C]βG6P, (♦); [^{14}C]βG1P, (●).The curves shown are fits to a first-order rate equation.

radiolabel in the βG1,6bisP versus G6P should be 3:1, consistent with the data shown in Figure 6B. Third, 40 μM βPGM; 50 μM unlabeled βG1,6bisP; and 5 μM [^{14}C]βG1P were reacted. The ratio of radiolabel in the βG1,6bisP versus G6P fractions was 2:1, consistent with the 2:1 molar ratio of βG1,6bisP and βG1P used in the reaction (Figure 6C).

The "diffusion" mechanism for reorientation of the βG1,-6bisP intermediate of βPGM is different from the "flip" mechanism most recently demonstrated for the *P. aeruginosa* αPMM/PGM (*10*). In the αPMM/PGM complex, the αG1,-6bisP intermediate reorients itself by undergoing a 180° rotation while remaining bound to the enzyme, and only once in every 15 catalytic cycles does the intermediate dissociate from the enzyme. The active site of the αPMM/PGM, which is formed at the interface of four domains, can exist in an open or closed conformation depending on domain orientation (*4*, *18*). Tipton and co-workers (*10*) have suggested that an intermediate conformation, which provides ample space for rotation of the αG1,6bisP within the active site, might allow reorientation without dissociation.

In contrast, βPGM consists of only two domains: a core domain that houses the active site and a small cap domain that acts as a lid over the active site. It is evident from the X-ray structure of the enzyme in its catalytically active, fully closed conformation (Figure 3) that little room exists for ligand reorientation. This is also the case for a partially open conformation that has been observed in the inactive complex formed between the unphosphorylated βPGM and α-D-galactose-1-phosphate (the C(6)OH is positioned near the Asp8 and the C(1)phosphate at the domain−domain interface) (*14*). X-ray structures of the apo-native enzyme (*12*) and the apo-phosphorylated enzyme (*6*) illustrate the open conformation in which the cap domain and core domain are dissociated and the active site is open to solvent. Dissociation of the αG1,6bisP intermediate from βPGM in this conformation would account for the diffusion mechanism of reorientation.

Solution and Internal Equilibrium Constant. To determine the equilibrium constant for the conversion of βG1P to G6P under the "βPGM reaction conditions", catalytic amounts of βPGM and βG1,6bisP were reacted with 50 μM [^{14}C]βG1P in 50 mM K^+Hepes (pH 7.0, 25 °C) containing 2 mM $MgCl_2$. The solution equilibrium constant of [βG6P]/[βG1P] was determined by measuring the ratio of radiolabel in the G6P and βG1P fractions to be ∼25 at pH 7.0 and 25 °C. This ratio is comparable to the ratio [G6P]/[αG1P] = 17.3 determined at pH 7.4 and 30 °C (*8*).

The reactions carried out under single turnover conditions employed enzyme concentrations that equaled or exceeded the reactant concentrations. Thus, the ratios of reactant; the product; and the G1,6bisP activator are determined not only by their intrinsic energies but also by the intrinsic energies of E and E-P, and by the binding energy associated with the various enzyme−ligand complexes. To gain insight into this complex set of equilibria, the ratio of βG1,6bisP; βG1P; and G6P was measured for a reaction solution initially containing 40 μM each of βPGM; βG1,6bisP; and [^{14}C]G6P (or [^{14}C]βG1P) in 50 mM K^+Hepes (pH 7.0, 25 °C) containing 2 mM $MgCl_2$. A third reaction solution initially containing 40 μM βPGM, 50 μM βG1,6bisP and 5 μM [^{14}C]G6P was also analyzed. The time courses for approach to equilibrium are shown in Figure 7.

At equilibrium, [^{14}C]G6P ≫ [^{14}C]βG1,6bisP ≫ [^{14}C]βG1P. On the basis of the ratio of [^{14}C]βG1,6bisP to [^{14}C]βG1,-6bisP plus [^{14}C]βG1P, the ratio of phosphorylated enzyme to unphosphorylated enzyme can be estimated to fall between 8:2 and 9:1. The ratio of [^{14}C]G6P to [^{14}C]βG1P, in contrast, was found to approximate the solution equilibrium. Without binding constants, it is not possible to accurately define the ratios of all species in solution. Nonetheless, it is clear from the estimated ratio of E-P/E that the energy of the aspartyl phosphate group in E-P is not as high as the −10.7 kcal/mol suggested by the Gibbs free energy change for hydrolysis of acetyl phosphate (*19*). Thus, the environment of the active site must stabilize the aspartyl phosphate group. The X-ray crystal structure of the phosphorylated βPGM shows some of the mechanisms for such stabilization, the foremost of which are a coordination bond formed between the phosphoryl group of aspartyl phosphate and the Mg^{2+} cofactor, a hydrogen bond formed with the Ser114 side chain, and an ion pair formed with Lys145 (*6*).

Conformational Cycling in β-Phosphoglucomutase Catalysis. β-Phosphoglucomutase catalyzes the conversion of βG1P to G6P in the presence of 2 mM Mg^{2+} (K_m = 270 ± 20 μM) (*12*) with a k_{cat} = 177 ± 9 s^{-1} and K_m = 49 ± 4 μM at pH 7.0 and 25 °C. The catalytic cycle begins with binding the βG1P to the active form of the enzyme, which is phosphorylated at the Asp8. The phosphoryl group is transferred to the C(6)OH to form the βG1,6-bisP, which then dissociates from the enzyme. The βG1,6-bisP binds to

the enzyme in two different orientations and transfers the C(1) phosphoryl group to the Asp8 form G6P or, from the other, orientation, transfers the C(6)phosphoryl group to the Asp8 to reform the βG1P. Following dissociation of the product ligand, the E-P binds another molecule of βG1P to start the cycle again. Therefore, to complete a catalytic cycle, the enzyme must open to bind substrate or βG1,6bisP; close to form βG1,6bisP or product; and then open again to release intermediate or product.

Comparison of βPGM and αPGM (and αPGM/PMM). βPGM and αPGM (and αPGM/PMM) catalyze the same type of chemical reaction but with opposite anomeric specificity. Both enzymes employ covalent catalysis yet different active site residues to perform this catalysis. The Ser residue used by αPGM (and αPGM/PMM) forms a stable phosphate ester intermediate (Gibbs free energy for hydrolysis of a simple phosphate ester is ~−3.5 kcal/mol (*19*)). These enzymes do not catalyze phosphoryl transfer to water, and consequently, αPGM (and αPGM/PMM) can persist in the activated, phosphorylated state. This is not the case with βPGM, which employs an Asp residue in nucleophilic catalysis. The intrinsic energy of this functional group is comparatively high (Gibbs free energy for hydrolysis of acetyl phosphate is −10.7 kcal/mol (*19*)), and its kinetic stability is limited (the half-life of acetyl phosphate in aqueous solution at 25 °C is 21 h (*20*)). Studies have shown that, in the absence of βG1P, the βPGM aspartlyphosphate readily loses it phosphoryl group to water (half-life ~12 s) (*12*). Thus, unlike the αPGM (and αPGM/PMM), it cannot persist in the cell in its activated state. However, to compensate, βPGM is able to autophosphorylate itself with its substrate βG1P. Although this is a slow reaction (k_{cat} = 0.83 ± 0.01 s^{-1} and K_m = 400 ± 40 μM), only a single turnover is required for activation (*12*). Once the phosphorylated enzyme is formed, it generates βG1,6-bisP which can be used to phosphorylate the enzyme in subsequent cycles (k_{cat} = 177 ± 9 s^{-1} and K_m = 49 ± 4 μM).

αPGM (K_d = 0.02 μM) (*8*, *9*) and αPGM/PMM (*11*) (K_m for activation = 0.1 μM) bind the αG1,6-bisP intermediate much tighter than βPGM binds βG1,6-bisP (K_m for activation = 6.5 μM). Whereas αG1,6-bisP remains bound to αPGM and αPGM/PMM during the catalytic cycle, βG1,6-bisP must dissociate from βPGM and rebind to reorient itself.

Comparison of the internal equilibria measured for the βPGM, rabbit muscle αPGM, and *Pseudomonas* αPGM/αPMM indicates that the ratio of phosphorylated to unphosphorylated enzyme is not dictated by the intrinsic energy of functional group but rather by the enzyme. For rabbit muscle αPGM and *Pseudomonas* αPGM/αPMM, the ratio of E-P/E is ~1:1 and ~10:1, respectively (*8*, *10*) (serinephosphate vs G1P or G6P, which in turn are equally populated on the enzyme), compared to the ~10:1 ratio measured for βPGM (aspartyl phosphate vs G6P).

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SUPPORTING INFORMATION AVAILABLE

The procedures for the preparation of [^{14}C]β-glucose 1-phosphate and [^{14}C]β-glucose 1,6-bisphosphate. This material is available free of charge via the Internet at http://pubs.acs.org.

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