Formation and Reorientation of Glucose 1,6-Bisphosphate in the PMM/PGM Reaction: Transient-State Kinetic Studies†

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ABSTRACT: The interconversion of glucose 1-phosphate and glucose 6-phosphate, catalyzed by *Pseudomonas aeruginosa* phosphomannomutase/phosphoglucomutase, has been studied by transient-state kinetic techniques. Glucose 1,6-bisphosphate is formed as an intermediate in the reaction, but an obligatory step in the catalytic cycle appears to be the formation of an enzyme−glucose 1,6-bisphosphate complex that is not competent to form either glucose 1-phosphate or glucose 6-phosphate directly. We suggest that during the lifetime of this complex the glucose 1,6-bisphosphate intermediate undergoes the 180° reorientation that is required for completion of the catalytic cycle. The formation of glucose 1,6-bisphosphate from glucose 1-phosphate is in rapid equilibrium relative to the rest of the reaction, where $K_{eq} = 0.14$. In the opposite direction, glucose 1,6-bisphosphate is formed from glucose 6-phosphate with a rate constant of 12 s^{-1}, and the reverse step occurs with a rate constant of 255 s^{-1}. The interconversion of the productive and nonproductive glucose 1,6-bisphosphate complexes occurs with a rate constant of 64 s^{-1} in one direction and 48 s^{-1} in the other direction. Glucose 1,6-bisphosphate remains associated with the enzyme during reorientation. Isotope trapping studies indicate that it partitions to form glucose 1-phosphate or glucose 6-phosphate 14.3 times more frequently than it dissociates from the enzyme.

In *Pseudomonas aeruginosa*, production of glucose 1-phosphate and mannose 1-phosphate from the corresponding 6-phosphohexoses is catalyzed by the same enzyme, PMM/PGM,[1] which is encoded by the *algC* gene (*1*). The products of the PMM/PGM reaction are important for the infectivity and virulence of the bacteria, which is an opportunistic human pathogen (*2*). Mannose 1-P is a precursor of rhamnolipid, and lipopolysaccharide contains components derived from mannose 1-P and glucose 1-P. Mannose 1-P is also a biosynthetic precursor of alginate, the capsular polysaccharide secreted by *P. aeruginosa* that is implicated in their antibiotic resistance and persistent infectivity (*3*, *4*).

Previous studies have revealed the structure of PMM/PGM (*5*, *6*) and some features of the catalytic mechanism (*7*). The *P. aeruginosa* enzyme is a member of the phosphohexomutase superfamily (*8*), the most extensively studied member of which is rabbit muscle PGM. PMM/PGM is phosphorylated at Ser108 in its resting state. Following substrate binding, the phosphoryl group is transferred from the enzyme to the substrate to generate a bisphosphorylated sugar intermediate. To complete the catalytic cycle, the intermediate must undergo a 180° rotation in the active site to position the phosphoryl group that was originally on the substrate next to Ser108. Phosphorylation of Ser108 yields the observed product and regenerates active enzyme that is poised to bind another molecule of substrate.

Although it has long been postulated that the PGM reaction requires reorientation of the intermediate (*9*, *10*), the mechanism by which it occurs is unknown. Glucose 1,6-bisphosphate has been observed in only a single orientation in the active site of PGM;[2] the reorientation has been inferred from the observation that 1-phospho- and 6-phosphosugars bind in different orientations in the active site, and no direct kinetic evidence has been presented that addresses the rate of reorientation. The physical movement of the intermediate is an unusual feature of the phosphohexomutase catalytic reaction, but in a larger sense, it is a simple example of processivity, whereby the enzyme and its substrate must move relative to each other between chemical steps, and do so without dissociating from each other. By virtue of the relative simplicity of its catalytic reaction, and the wealth of structural information that is becoming available, PMM/PGM is an attractive system for investigating how the physical steps in the catalytic cycle are mediated by the enzyme.

The active site of PMM/PGM lies in a deep cleft, and the enzyme adopts a closed conformation upon binding substrate (*5*). Multiple specific interactions between the enzyme and the substrate and product have been described (*5*); these interactions must be relaxed for the intermediate to reorient.

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[1] Abbreviations: PMM/PGM, phosphomannomutase/phosphoglucomutase; PGM, phosphoglucomutase; glucose 1-P, glucose 1-phosphate; glucose 6-P, glucose 6-phosphate; glucose 1,6-P_2, glucose 1,6-bisphosphate; mannose 1-P, mannose 1-phosphate; mannose 6-P, mannose 6-phosphate; DTT, dithiothreitol; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; EDTA, ethylenediaminetetraacetate.

[2] The crystal structure of rabbit muscle PGM complexed with Cd^{2+} and α-D-glucose 1,6-P_2 has been deposited as Protein Data Bank entry 1C47; the structure, which has a resolution of 2.7 Å, has not been described in a publication.

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At the same time, complete release of the intermediate by the enzyme would result in formation of an inactive dephosphoenzyme; indeed, although PMM/PGM is capable of multiple turnovers in the absence of exogenously added glucose 1,6-P_2, its addition stimulates the enzyme, presumably by rephosphorylating dephosphoenzyme that occasionally forms (*11*).

To provide a basis for a deeper understanding of the molecular recognition events that are critical for the PMM/PGM reaction and related enzymes, we have undertaken a detailed kinetic characterization of the catalytic cycle. Using a series of rapid-quench experiments, we have determined the microscopic rate constants for the elementary steps in the catalytic cycle. We also report the results of an isotope trapping experiment, which measured the frequency with which glucose 1,6-P_2 dissociates from the active site during turnover. Perhaps surprisingly, the data suggest that reorientation of glucose 1,6-P_2 is a kinetically significant step in the overall reaction.

MATERIALS AND METHODS

PMM/PGM was purified from *Escherichia coli* strain BL21(DE3) cells harboring the pET-14b vector with *algC* inserted into the multiple cloning site. The expressed protein carried a His_6 tag at the N-terminus, which was not removed after purification. Previous work has established that the His tag has no effect on the kinetic parameters of the PMM/PGM reaction (*7*). The concentration of PMM/PGM was determined by the Bradford assay using bovine serum albumin as the standard. [^{14}C]Glucose 1-phosphate (10 μCi, 300 mCi/mmol) was purchased from American Radiolabeled Chemicals (St. Louis) and used without further purification. $H_3{}^{32}PO_4$ (5 mCi, 9000 Ci/mmol) was purchased from Perkin-Elmer. Unlabeled glucose 1-P was purified chromatographically before use (*12*); ^{1}H NMR analysis of the purified material indicated that it contained only the α-anomer (one anomeric ^{1}H appearing as a doublet of doublets, centered at 5.36 ppm; 3.4 and 7.4 Hz couplings). Stock solutions of glucose 1-P were calibrated by enzymatic end-point assay with PMM/PGM and glucose-6-phosphate dehydrogenase. Glucose 6-P stock solutions were calibrated with glucose-6-phosphate dehydrogenase. Unlabeled compounds were purchased from Sigma; Scintiverse BD and Scintisafe Plus (50%) scintillation fluid were obtained from Fisher Scientific.

Single-Turnover Kinetics. The time course for the PMM/PGM reaction was evaluated under single-turnover conditions using a KinTek RQF-3 rapid-mixing chemical-quench apparatus. The first reactant syringe contained 200 μM PMM/PGM, 3 mM $MgSO_4$, and 2 mM DTT. The second reactant syringe contained 20 μM [^{14}C]glucose 1-P (1 μCi in 0.5 mL). Solutions in both syringes were buffered with 50 mM MOPS (pH 7.4). The quench syringe contained 0.5 M NaOH and 10 mM EDTA. The reaction chamber was maintained at 25 °C with a circulating water bath, and samples were collected after reaction times varying from 0.002 to 0.5 s. An aliquot was removed from each sample, and the radioactivity was determined by liquid scintillation counting to quantitate the dilution that occurred during the rapid mixing experiment. Protein was removed from the remainder of each sample by the addition of 50 μL of $CHCl_3$, vigorous vortexing, and centrifugation.

The components of each sample were separated by HPLC and quantitated by scintillation counting with a β-RAM model 3 (IN/US Inc.) flowthrough radiodetector. Separations were carried out using a Dionex DX500 HPLC system and a CarboPac PA-1 anion exchange column. The column was equilibrated in 16 mM NaOH and operated at a flow rate of 1 mL/min. Following sample injection, the column was washed for 5 min with 16 mM NaOH, and then developed with a linear gradient over 20 min to 16 mM NaOH and 1 M NaOAc.

Dephosphorylation of PMM/PGM. The rate of transfer of the phosphoryl group from Ser 108 to glucose 1-P or glucose 6-P was measured directly by monitoring the disappearance of [^{32}P]PMM/PGM upon mixing with substrate. Labeled PMM/PGM was prepared as follows, based on a modification of the method used for labeling rabbit muscle PGM (*13*). [^{32}P]Glucose 1-P was generated in a solution containing 0.2 M Bis-Tris (pH 7.0), 80 mM sucrose, 12 mM cysteine·HCl, 0.1 mM phosphate (50 μCi of $^{32}P_i$), 10 mM EDTA, and 0.5 unit of sucrose phosphorylase (Sigma) in a total volume of 0.5 mL. After 90 min, 100 μM PMM/PGM was added along with 12 mM $MgSO_4$. The reaction mixture was incubated at room temperature for 15 min, and then dialyzed against three changes of 50 mM MOPS (pH 7.4) containing 1 mM DTT.

Dephosphorylation of PMM/PGM was assessed by mixing a solution containing 8 μM labeled PMM/PGM, 1 mM DTT, and 1.5 mM $MgSO_4$ with a second solution containing either glucose 1-P or glucose 6-P, 1 mM DTT, and 1.5 mM $MgSO_4$ in the rapid mixing apparatus. Both solutions were buffered with 50 mM MOPS (pH 7.4); the reactions were quenched with 30% trichloroacetic acid. The samples were collected in tubes that contained 20 μL of $CHCl_3$ and 20 μL of 0.5 M NaOH containing 10 mM EDTA. Each quenched sample was passed through a nitrocellulose filter (Millipore type HA, 0.45 μm), which was rinsed with water, and then dissolved in 4 mL of Scintisafe Plus scintillation fluid. Radioactivity was determined by liquid scintillation counting.

Isotope Trapping of Glucose 1,6-P_2. The rate of dissociation of glucose 1,6-P_2 from the PMM/PGM active site was measured by a modified isotope trapping experiment. A solution containing 0.1 mM [^{14}C]glucose 1-P (0.5 μCi), 1.73 mM glucose 6-P, 1 mM glucose 1,6-P_2, 1.5 mM $MgSO_4$, and 1 mM DTT in 50 mM MOPS (pH 7.4) was prepared in a total volume of 0.5 mL. At time zero, 10 μM PMM/PGM was added, and 40 μL aliquots were removed periodically and placed in sample tubes containing an equal volume of 0.5 M NaOH, 10 mM EDTA, and 20 μL of $CHCl_3$. The samples were vortexed vigorously and centrifuged. Aliquots from the aqueous layer of each sample were analyzed by HPLC as described above.

The flux between the substrate and product pools was measured under conditions identical to those used in the isotope trapping experiment described above, but the reaction was conducted in the rapid mixing chemical-quench apparatus with the first syringe containing PMM/PGM, $MgSO_4$, and glucose 1,6-P_2 and the second syringe containing [^{14}C]-glucose 1-P and glucose 6-P. The reactions were quenched with 0.5 M NaOH containing 10 mM EDTA at times ranging from 0.01 to 20 s. The quenched samples were deproteinized and analyzed as described above.

Data Analysis. The data from the single-turnover experiment were fitted to two alternative models using Dynafit (*14*).

Scheme 1

Model A

$$\text{E-P•G 1-P} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{E•G 1,6-P}_2 \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \text{E-P•G 6-P}$$

Model B

$$\text{E-P•G 1-P} \underset{}{\overset{K_1}{\rightleftharpoons}} \text{E•G 1,6-P}_2 \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \text{E-P•G 6-P}$$

$$k_6 \Vert k_5$$

$$\left[\text{E•G 1,6-P}_2\right]^*$$

The first model represented the PMM/PGM reaction as two consecutive, fully reversible first-order reactions with a glucose 1,6-P_2 intermediate between glucose 1-P and glucose 6-P. The second model differed from the first by including a dead-end reversible pathway from the intermediate glucose 1,6-P_2, which entailed formation of a form of glucose 1,6-P_2 that was not competent to regenerate glucose 1-P or form glucose 6-P (Scheme 1). Other models were evaluated, including one in which substrate binding was explicitly included, and one which included formation of two intermediates sequentially before formation of glucose 6-P, but these models were discarded. The goodness of fit was evaluated from the sum of the squares of the differences between experimental and fitted points, and the standard errors on the values of the microscopic rate constants.

The rates of dephosphorylation of PMM/PGM, dissociation of glucose 1,6-P_2, and transfer of a label between substrate and product pools were determined by fitting the data in each case to a single exponential. The observed rate constant for transfer of a label from glucose 1-P to glucose 6-P, k_{obs}, is the sum of the rate constants for the forward ($k_{1\rightarrow6}$) and reverse ($k_{6\rightarrow1}$) reactions. Since the reaction was at chemical equilibrium, $k_{1\rightarrow6}$ and $k_{6\rightarrow1}$ are equal, and k_{flux}, the rate constant describing the interconversion of the substrate and product, equals the observed rate constant divided by 2 (eq 1). The frequency of dissociation of glucose 1,6-P_2 from the active site of PMM/PGM during turnover is given by the ratio of k_{flux} and the observed rate constant for the appearance of labeled glucose 1,6-P_2.

$$k_{flux} = \frac{k_{obs}}{2} \quad (1)$$

RESULTS

Figure 1 shows the separation that was achieved for glucose 1-P, glucose 6-P, and glucose 1,6-P_2 by high-performance anion exchange chromatography. The facile separation enabled quantitative analysis of the time dependence of each PMM/PGM reaction component during different experimental turnover conditions.

Single-Turnover Kinetics. Single-turnover conditions for PMM/PGM were established by conducting the reaction with a 10-fold excess of enzyme over glucose 1-P. The large excess of enzyme over substrate also enabled us to exclude substrate binding and product release as explicit steps in the analysis, and treat the overall reaction as a series of first-order reactions. Formation of glucose 1,6-P_2 was rapid, reaching a maximal concentration after 10 ms (Figure 2). The data were fitted reasonably well by model A, the simpler

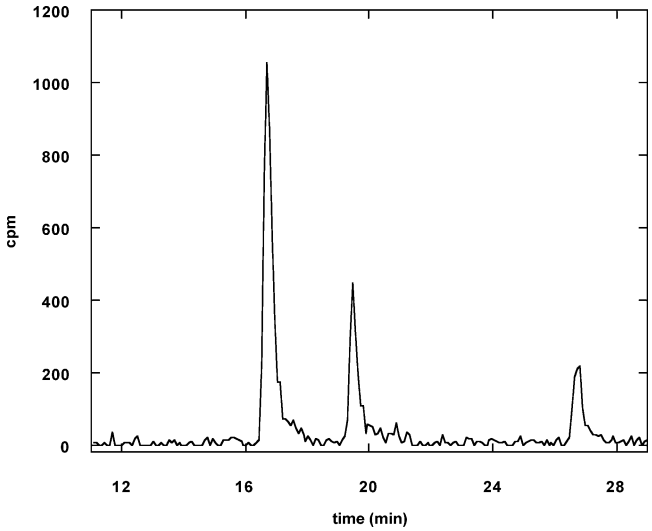


FIGURE 1: Separation of [^{14}C]glucose 1-P, [^{14}C]glucose 6-P, and [^{14}C]glucose 1,6-P_2 from a quenched PMM/PGM reaction by high-performance anion exchange chromatography. Glucose 1-P elutes at 17 min. Glucose 6-P elutes at 19 min. Glucose 1,6-P_2 elutes at 27 min. Conditions for the separation are given in the text. The compounds were detected using a flow-through radiodetector.

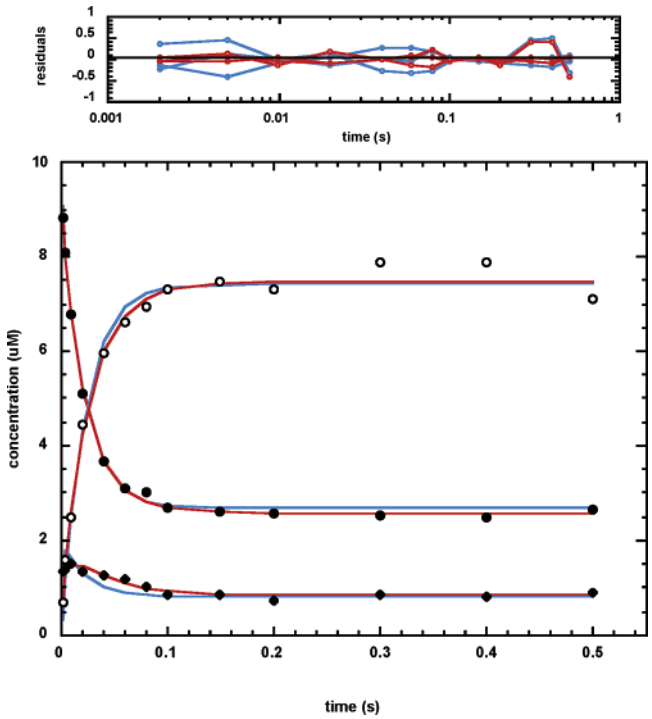


FIGURE 2: Time course for the PMM/PGM reaction under single-turnover conditions. The reaction mixture contained 100 μM PMM/PGM, 1.5 mM $MgSO_4$, 1 mM DTT, and 10 μM [^{14}C]glucose 1-P in 50 mM MOPS (pH 7.4). Experimental points are glucose 1-P (●), glucose 6-P (○), and glucose 1,6-P_2 (◆). The fit obtained with model A in Scheme 1 is shown in blue, and the fit obtained with model B is shown in red. The values for the rate constants that were obtained from the fits are given in the text. At the top are residuals for the fits to the two models. Empty circles represent data for glucose 6-P. Filled circles represent data for glucose 1-P. Filled diamonds represent data for glucose 1,6-P_2. The residuals from the fit to model A are colored blue, and the residuals from the fit to model B are colored red. Note that the time scale has been placed on a logarithmic axis to facilitate visualization of the fits at early times.

of the two models shown in Scheme 1. The fit yielded the following values for the rate constants: $k_1 = 140 \pm 20$ s^{-1},

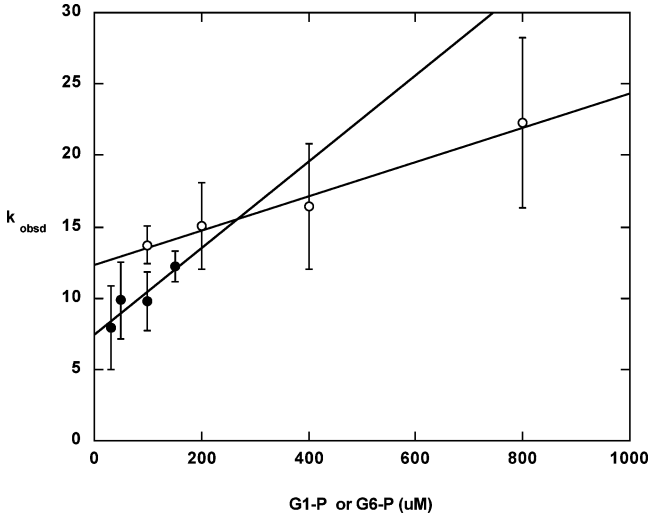


FIGURE 3: Concentration dependence for the observed rate constants for dephosphorylation of PMM/PGM by glucose 1-P (●) and glucose 6-P (○). Experimental conditions are given in the text.

$k_2 = 470 \pm 100\ s^{-1}$, $k_3 = 170 \pm 10\ s^{-1}$, and $k_4 = 19 \pm 2\ s^{-1}$. The sum of the squares of the residuals was 0.0449. However, it is evident that there are systematic deviations from the experimental points using model A; the deviation is most noticeable in the time dependence of glucose 1,6-P_2, where the model predicts a rapid decay of the intermediate, and the data show that it persists somewhat longer. The actual appearance of glucose 6-P also is somewhat slower than that calculated from the model. These deviations disappear when the data are fitted to model B, in which the intermediate glucose 1,6-P_2 can transiently exist as a different form that is not competent to generate either glucose 1-P or glucose 6-P. A satisfactory fit to model B was obtained only when addition of glucose 1-P was treated as rapid relative to the remaining steps in the model. Individual values for k_1 and k_2 could not be obtained; however, the ratio k_1/k_2 was closely constrained by the data to be 0.14, and a satisfactory fit was obtained only when k_1 was greater than 1400 s^{-1}. Under those conditions, the model yielded values for k_3-k_6 of 255 ± 7, 12.3 ± 0.5, 64 ± 12, and 48 ± 11 s^{-1}, respectively. The sum of the squares of the residuals for model B was 0.0195.

The rate of phosphorylation of glucose 1-P and glucose 6-P was measured directly by monitoring the disappearance of ^{32}P from the labeled enzyme. The observed rate constants depend linearly on the substrate concentration (Figure 3). From these data, the rate constant for association of glucose 1-P and PMM/PGM was determined to be 0.033 ± 0.007 $\mu M^{-1}\ s^{-1}$ and the rate constant for dissociation of glucose 1-P from the binary complex was 7.3 ± 1.0 s^{-1}. The rate constant for association of glucose 6-P and PMM/PGM was 0.012 ± 0.001 $\mu M^{-1}\ s^{-1}$, and the rate constant for dissociation was 12.6 ± 0.2 s^{-1}.

Isotope Trapping. At chemical equilibrium, the interconversion of glucose 1-P and glucose 6-P occurred with a rate constant of 0.60 ± 0.05 s^{-1}, and glucose 1,6-P_2 dissociated from the enzyme with a rate constant of 0.021 ± 0.005 s^{-1} (Figure 4). From the observed rate constants for interconversion of glucose 1-P and glucose 6-P, k_{flux} is calculated to be 0.3 s^{-1}. Comparing this value to k_{dissoc} for glucose 1,6-P_2 yields a value of 14.3; in other words, conversion of glucose

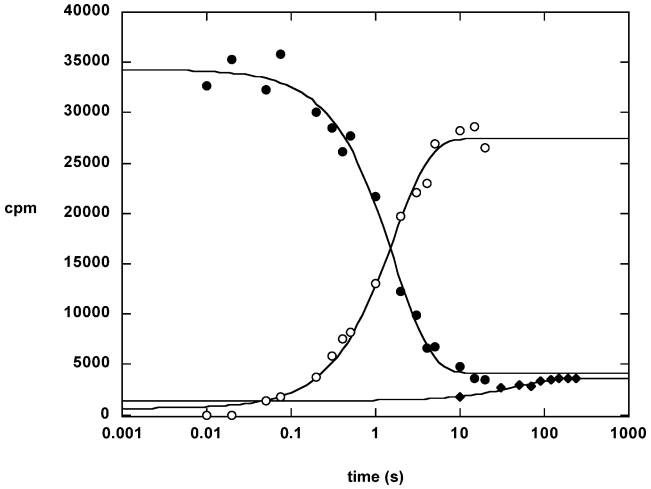


FIGURE 4: Transfer of the ^{14}C label in the isotope trapping experiment with PMM/PGM. Reaction mixtures contained 10 μM PMM/PGM, 100 μM [^{14}C]glucose 1-P, 1.73 mM glucose 6-P, 1 mM glucose 1,6-P_2, 1.5 mM $MgSO_4$, and 1 mM DTT in 50 mM MOPS (pH 7.4). The points are experimental: glucose 1-P (●), glucose 6-P (○), and glucose 1,6-P_2 (◆). The lines show the fits to a single exponential. The rate constant for transfer of the label out of the glucose 1-P pool was 0.60 ± 0.06 s^{-1}. The rate constant for transfer of the label into the glucose 6-P pool was 0.61 ± 0.05 s^{-1}. The rate constant for transfer of the label into the glucose 1,6-P_2 pool was 0.021 ± 0.005 s^{-1}.

1,6-P_2 to product occurs 14 times more frequently than dissociation from the active site.

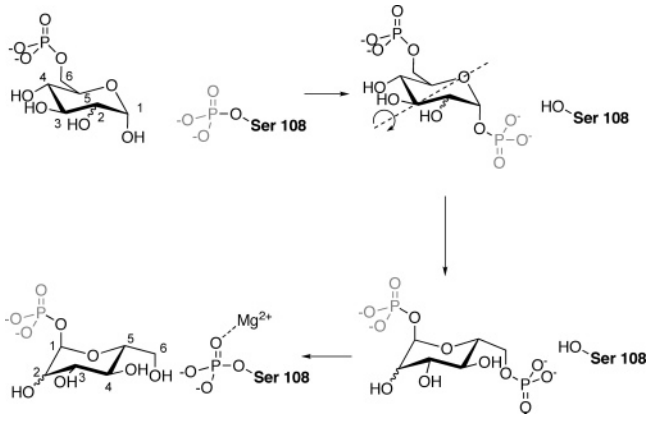
DISCUSSION

PMM/PGM catalyzes the interconversion of mannose or glucose 6-P and mannose or glucose 1-P. The proposal that the reaction proceeds through the intermediacy of a bisphosphorylated species is supported by the following evidence. The resting enzyme is phosphorylated at Ser108, and addition of glucose 1,6-P_2 stimulates the steady-state reaction. PMM/PGM exhibits substrate inhibition with respect to glucose 1-P, and that inhibition is relieved by glucose 1,6-P_2, suggesting that the inhibition arises from binding of the substrate to the dephosphoenzyme, formed when the bisphosphorylated intermediate dissociates from the enzyme instead of completing the catalytic cycle. Finally, as demonstrated here, glucose 1,6-P_2 can be identified as a transient intermediate during turnover.

To complete the catalytic cycle, the intermediate must rephosphorylate the enzyme; to achieve net conversion of the substrate to product, the phosphoryl group that was borne by the substrate must be transferred to the protein. This exchange requires that the bisphosphorylated intermediate reorient by 180° at the active site to place the appropriate phosphate adjacent to Ser108 (Scheme 2). Structural studies have revealed how PMM/PGM accommodates glucose 1-P, mannose 1-P, glucose 6-P, and mannose 6-P at the active site (*5*). In each case, the hydroxyl groups at the 3- and 4-positions form hydrogen bonds to Glu325, and interactions with the phosphate group are conserved as well. It is clear that catalysis does not occur via binding of the substrate in a single orientation that places both the 1- and 6-positions within reach of Ser108.

The mechanism by which reorientation of glucose 1,6-P_2 occurs remains unclear. Two kinetic models are consistent

Scheme 2



with the required chemistry. The intermediate could dissociate from the enzyme each time it is formed and rebind with its orientation randomly determined, in a ping-pong mechanism. Although efficiency in turnover would be sacrificed by rebinding in the wrong orientation, the *V*/*K* value for PMM/PGM is several orders of magnitude below the diffusion limit (*11*), so no evolutionary pressure would select against this mechanism. Alternatively, the intermediate could reorient without completely losing its association with the enzyme. This mechanism places a considerable burden of sophistication on the enzyme, since it requires that binding interactions be sufficiently relaxed for the reorientation to occur, without allowing dissociation from the enzyme to take place.

The rate of dissociation of glucose 1,6-P_2 from the active site of PMM/PGM was determined using isotope trapping. Glucose 1-P and glucose 6-P were mixed together in a ratio of 1:17.3 so that the reaction was at chemical equilibrium (*15*). Glucose 1,6-P_2 was present in 100-fold molar excess over PMM/PGM, and glucose 1-P carried a ^{14}C label. Following the addition of the enzyme to the other components of the reaction, the transfer of a label from glucose 1-P into glucose 6-P and glucose 1,6-P_2 was monitored. Since unlabeled glucose 1,6-P_2 was present at a high concentration in solution, the dissociation of labeled glucose 1,6-P_2 from the enzyme active site was irreversible. Because the experiment was conducted with a catalytic amount of enzyme, the amount of labeled glucose 1,6-P_2 that was bound to the enzyme when the reaction was quenched was insignificant compared to the amount in solution. The rate of appearance of the label in glucose 6-P provided a measure of how rapidly the enzyme was interconverting substrate and product. Comparison of the rates of appearance of labeled glucose 1,6-P_2 and labeled glucose 6-P provides a direct measure of how enzyme-bound glucose 1,6-P_2 partitions, whether forward to product or back to substrate, or off of the enzyme. If PMM/PGM followed a ping-pong mechanism, the rate of formation of labeled glucose 1,6-P_2 would have to equal or exceed that of labeled glucose 6-P. In fact, the opposite was observed; the rate of formation of labeled glucose 6-P was 14.3-fold greater than the rate of formation of labeled glucose 1,6-P_2. This result indicates that enzyme-bound glucose 1,6-P_2 preferentially remains bound to the enzyme; only once every 15 catalytic cycles does the intermediate dissociate from the enzyme. The frequency of dissociation of glucose 1,6-P_2 from rabbit muscle PGM, although not measured directly, has been estimated to be approximately once every 20 catalytic cycles (*16*).

The molecular mechanism which allows reorientation of glucose 1,6-P_2 without dissociation is not yet known in detail. The structure of PMM/PGM with substrates bound reveals a number of specific contacts that are formed; indeed, specific interactions must be present to provide binding energy that can be used to accelerate the rate of the reaction, and to account for substrate specificity. The dramatic reorientation that glucose 1,6-P_2 must undergo requires that these interactions be relaxed, and then replaced by new interactions when the intermediate rebinds in the alternate orientation. PMM/PGM forms a closed complex upon substrate binding, which arises primarily through motion of domain 4 relative to the other three. The domain movement results in a decrease in the volume of the active site. One scenario by which reorientation could occur would be for the enzyme to adopt an intermediate conformation between the open and closed forms, which would allow reorientation of glucose 1,6-P_2 without exposing it completely to solvent. More structural information about this aspect of the PMM/PGM mechanism, and data which reveal the roles of individual active site residues in the binding of glucose 1,6-P_2, are becoming available from the structure of a complex of PMM/PGM and glucose 1,6-P_2.[3]

In contrast to the results reported here for PMM/PGM, β-PGM follows a ping-pong mechanism.[4] PMM/PGM and β-PGM do not share significant sequence identity or structural similarity, so perhaps it is not surprising that they are mechanistically unique as well. Since the two enzymes operate on different anomers, they could not utilize the same reorientation mechanism. PMM/PGM forms the α-anomer of mannose or glucose 1-P; the 6-phosphosugars and the corresponding α-anomers of the 1-phosphosugars are related (with regard to the disposition of the phosphate groups) by a 2-fold axis of symmetry that bisects the C3−C4 bond and the ring oxygen. On the other hand, the intermediate formed in the β-PGM reaction needs to rotate around an axis centered in and perpendicular to the carbohydrate ring; recent evidence suggests that the reorientation occurs in bulk solution.[4]

The results of the single-turnover experiment are particularly interesting in light of the isotope trapping data. The time course for the reaction clearly reveals that glucose 1,6-P_2 is formed as a transient intermediate in the catalytic cycle. However, the data do not conform best to a simple model where the only fate available to the intermediate is to partition between substrate and product. Rather, the distinct lag in disappearance of glucose 1,6-P_2 seems to require an additional feature in the model, whereby the glucose 1,6-P_2 exists transiently in a state in which it is not competent to form either substrate or product. That state cannot involve any chemical transformation of the glucose 1,6-P_2, since glucose 1,6-P_2 is released upon denaturation of the enzyme. Therefore, the glucose 1,6-P_2 that is detected must be unique only in terms of its disposition with respect to PMM/PGM. We suggest that the slow disappearance of glucose 1,6-P_2 represents the time during which it is reorienting, when neither phosphoryl group is positioned appropriately to

[3] Catherine Regni and Lesa J. Beamer, manuscript in preparation.
[4] Personal communication from Karen Allen and Debra Dunaway-Mariano.

rephosphorylate Ser108. This result was unexpected; rotational diffusion, even if partially constrained by the enzyme, would be too rapid to be able to be detected by our kinetic techniques. Although this has not been proven by the kinetic data, they seem to suggest that the reorientation occurs by populating one or a series of specific, metastable states. The residues that may participate in the reorientation, and the structural changes to PMM/PGM when the intermediate is bound, are explored in more detail in a future manuscript.

The rate constants for transfer of the phosphoryl group from PMM/PGM to substrate depend in a linear fashion on the concentration of glucose 1-P or glucose 6-P. Thus, binding and phosphoryl transfer are not kinetically distinguishable processes, and it is evident that the initial phosphorylation event in the catalytic cycle is quite rapid relative to substrate binding. Subsequent phosphoryl transfers are also rapid, so glucose 1,6-P_2 does not accumulate to a significant extent on the enzyme. Overall, it appears that nonchemical steps such as ligand binding, and the concomitant conformational change, contribute most significantly to rate limitation in the PMM/PGM reaction.

The internal equilibrium constant, that is, the ratio of enzyme-bound glucose 6-P and glucose 1-P, can be calculated from the rapid-quench data. As shown in Figure 2, the components of the reaction have reached a steady state after 150 ms. At that point, and for the remainder of the reaction, the ratio of glucose 6-P to glucose 1-P is 2.9. The overall K_{eq} for the interconversion of α-glucose 1-P and (α+β)-glucose 6-P is 17.3; however, when the anomeric specificity of PMM/PGM is taken into account, the relevant equilibrium involves α-glucose 1-P and α-glucose 6-P, for which K_{eq} equals 7. Thus, PMM/PGM provides differential stabilization of glucose 1-P, relative to its energy free in solution compared to glucose 6-P. The value of K_{eqint} for rabbit muscle PGM is 2.5, quite similar to what we observe with PMM/PGM. Interestingly, the two enzymes differ significantly in the proportion of glucose 1,6-P_2 that is bound to the enzyme. For PMM/PGM, only 8% of the bound ligand is present as glucose 1,6-P_2, whereas it comprises 54% of the ligand bound to rabbit muscle PGM (*15*).

Despite the apparent functional and structural similarities between rabbit muscle PGM and *P. aeruginosa* PMM/PGM (*6*), striking differences are also emerging. Although both enzymes appear to lose glucose 1,6-P_2 from the active site with approximately the same frequency, they differ in their affinity for it with regard to the other enzyme-bound ligands. Also, the turnover number for rabbit muscle PGM is more than 10-fold higher than that for PMM/PGM, and PGM also discriminates strongly against mannose 1-P; on the other hand, PMM/PGM utilizes mannose 1-P and glucose 1-P with equal efficiency. To date, the available structural data for PMM/PGM and PGM have not provided a clear explanation of these differences. It seems apparent that the determinants of catalytic efficiency and substrate specificity for these related enzymes are both subtle and complex, and likely involve the larger issues of domain motion and protein flexibility.

PMM/PGM and rabbit muscle PGM are representatives of two of the four subgroups that make up the phosphohexomutase superfamily (*8*). Other members of the superfamily have not been characterized in detail, despite their importance in metabolism and intriguing differences with the better-studied members of the superfamily. For example, the 20-residue phosphoserine loop in human phosphoacetylglucosamine mutase has been translocated by 120 residues in the primary sequence; the effects of this change on catalytic mechanism and efficiency are unknown. Given the variation in catalytic properties that PMM/PGM and PGM exhibit, it would be interesting to determine whether these differences extend throughout the superfamily.

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