The Reaction of Phosphohexomutase from \textit{Pseudomonas aeruginosa} \\
\textbf{STRUCTURAL INSIGHTS INTO A SIMPLE PROCESSIVE ENZYME}\textsuperscript{*}

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The enzyme phosphomannomutase/phosphoglucomutase (PMM/PGM) from \textit{Pseudomonas aeruginosa} catalyzes the reversible conversion of 1-phospho to 6-phospho-sugars. The reaction entails two phosphoryl transfers, with an intervening 180° reorientation of the reaction intermediate (e.g. glucose 1,6-bisphosphate) during catalysis. Reorientation of the intermediate occurs without dissociation from the active site of the enzyme and is, thus, a simple example of processivity, as defined by multiple rounds of catalysis without release of substrate. Structural characterization of two PMM/PGM-intermediate complexes with glucose 1,6-bisphosphate provides new insights into the reaction catalyzed by the enzyme, including the reorientation of the intermediate. Kinetic analyses of site-directed mutants prompted by the structural studies reveal active site residues critical for maintaining association with glucose 1,6-bisphosphate during its unique dynamic reorientation in the active site of PMM/PGM.

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\textsuperscript{*}This work was supported in part by National Institutes of Health Grant GM59653. The enzyme PMM/PGM\textsuperscript{4} from \textit{Pseudomonas aeruginosa} belongs to the \textalpha-D-phosphohexomutase enzyme superfamily, members of which are found in all organisms from \textit{Escherichia coli} to humans (1). Enzymes in this family catalyze the reversible conversion of 1- to 6-phospho-sugars via a bisphosphorylated sugar intermediate. Previous studies of members of this enzyme family, including structural characterization of PMM/PGM in complex with its substrates and products (2), have demonstrated that a dramatic 180° reorientation of the intermediate is necessary to complete the reaction. Although dynamic processes such as conformational changes are important to many enzymes, the reorientation of the bisphosphorylated sugar intermediate as a required step in the catalytic reaction of PMM/PGM and related enzymes is unique.

The reaction of PMM/PGM, which can utilize either glucose or mannose-based phospho-sugars as substrates, has been characterized (3–6) and entails two phosphoryl transfer reactions (Fig. 1a). The first phosphoryl transfer proceeds from a phosphoserine residue on the enzyme to bound substrate and produces a bisphosphorylated intermediate (e.g. glucose 1,6-bisphosphate or G16P). G16P must then reorient by ~180° to be in position for a second phosphoryl transfer, from the intermediate back to the enzyme.

Recently, transient state kinetic techniques have been used to characterize the reaction of PMM/PGM (6). The non-chemical steps of the reaction (e.g. ligand binding) were found to be the rate-limiting steps of the pathway, whereas phosphoryl transfers were rapid. Single-turnover experiments show that G16P is a transient intermediate in the catalytic cycle but also indicate that it exists in two states; one that is competent to partition to substrate or product and another that is not. This has led to the suggestion that the reorientation of G16P occurs via one or more metastable states during which it is not available to proceed along the reaction pathway. In addition, an isotope trapping experiment has shown that the reorientation of G16P occurs without dissociation from the enzyme (6) and can, thus, be considered a simple example of processivity, as defined by multiple rounds of catalysis without release of substrate (7).

Although a large number of processive enzymes has been structurally characterized, many of these systems are quite complex, often involving multiprotein complexes and large polymeric substrates, such as DNA (for review, see Ref. 7). In contrast, PMM/PGM, which has been well characterized both structurally and mechanistically, presents a uniquely simple system for addressing structural questions about processive reactions. For example, how does the enzyme specifically bind and maintain association with the substrate while also permitting its movement between catalytic steps? What types of interactions and structural features in the enzyme are used to accomplish this? Does conformational change of the enzyme play a role?

Here we present a structural analysis of PMM/PGM in complex with its reaction intermediate G16P. Two distinct complexes, one with dephosphorylated enzyme and the other with phosphorylated enzyme, have been characterized. These two structures, which represent both a productive (on pathway) and non-productive (off pathway) complex, reveal two distinct binding positions for G16P in the active site of PMM/PGM. One of these, the non-productive complex, unexpectedly shows the intermediate bound in a position in which it appears to be partway through its reorientation in the active site. Analysis of the enzyme-intermediate interactions in these PMM/PGM-intermediate complexes followed by mutagenesis and kinetic characterization has identified several key residues that are responsible for maintaining association with the intermediate during its 180° reorientation in the active site. This work is important for understanding all members of the \textalpha-D-phosphohexomutase superfamily and the many biosynthetic reactions they catalyze. Moreover, it has implications for understanding other multistep enzymatic reactions that undergo cycles of catalysis with intervening movement of the substrate.

\textbf{MATERIALS AND METHODS}

\textit{Complex Formation and Structure Determination}—Formation of the dephospho-PMM/PGM-G16P complex was done using an S108D mutant; the carboxylate side chain of this residue substitutes as a ligand for the active site metal ion while simultaneously preventing phospho-
rylation at position 108. Initial production of the phospho-PMM/PGM-G16P complex was inadvertent due to incomplete dephosphorylation of the protein sample used for crystallization. Purification and crystallization of apoPMM/PGM and the S108D mutant was carried out as previously described from 50–70% saturated sodium/potassium tartrate and 100 mM Na-Hepes, pH 7.5 (2, 8). The proteins crystallized in space group P2₁2₁2₁, with differences in the unit cell axes of less than 10% relative to wild-type protein (Table 1). This is consistent with previously characterized PMM/PGM structures that all crystallize in the same space group but show variations in cell axes depending on the form of the protein (apo, ligand-bound, or mutant).

G16P was purchased from Sigma and dissolved at 50 mM in a solution of ~70% (v/w) polyethylene glycol 4000 and 100 mM Na-Hepes, pH 7.4. Crystals were soaked for about 15 min in the ligand solution and flash-cooled without further cryoprotection for data collection at −180 °C. Diffraction data were collected at beam line SBC 19-ID at the Advanced Photon Source, Argonne National Laboratory (phosphoenzyme) and at beam line MBC 4.2.2 at the Advanced Light Source, Lawrence Berkeley National Laboratory (dephosphoenzyme) and processed with DENZo and SCALePACK (9) or D*TREK (10).

Structure Solution and Refinement—Phases for the dephospho-PMM/PGM-G16P complex were determined by molecular replacement with CNS (11) using a PMM/PGM-substrate complex (1P5D) as the search model. Refinement of the phospho-PMM/PGM-G16P complex began using the apoenzyme structure as a model. Refinement was carried out through iterative cycles of positional refinement using REFMAC 5.0 (12) and manual rebuilding with COOT (13). Five percent of the data set was set aside for cross-validation before refinement. Because of the similarity of the unit cell dimensions between the apoenzyme and phospho-PMM/PGM-G16P complex, the data set was set aside for cross-validation before refinement. The phospho-PMM/PGM-G16P complex was inadvertent due to incomplete dephosphorylation of the protein sample used for crystallization. Purification and crystallization of apoPMM/PGM and the S108D mutant was carried out as previously described from 50–70% saturated sodium/potassium tartrate and 100 mM Na-Hepes, pH 7.5 (2, 8). The proteins crystallized in space group P2₁2₁2₁, with differences in the unit cell axes of less than 10% relative to wild-type protein (Table 1). This is consistent with previously characterized PMM/PGM structures that all crystallize in the same space group but show variations in cell axes depending on the form of the protein (apo, ligand-bound, or mutant).

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The final models (Table 1) extend from residues 9 or 10 to 463 and contain the following heteroatoms: Zn²⁺, G16P, waters, and phosphoserine 108 (phospho-PMM/PGM-G16P complex only). The dephospho-PMM/PGM-G16P complex has one residue modeled in two conformations, and the side chains of 26 residues were truncated to alanine. The phospho-PMM/PGM-G16P complex has three residues modeled in two conformations, and the side chains of 33 residues were truncated to alanine. The occupancy for all residues except those modeled in multiple conformations is 1.0. The models were validated with SFCHECK (15), and PROCHECK (16). Only two residues in each structure had backbone angles in the disallowed region of the Ramachandran plot, residues 108 and 128; this is consistent with previous PMM/PGM structures.

The following programs were used for structural analyses: domain interface surface area by the protein-protein interaction server (17), solvent-accessible surface area of the ligands by AREAIMOL (14), domain rotation by DYNDOM (18), and Cα superpositions by MultiProt (19). Enzyme-ligand hydrogen bonds were determined by CONTACT (14) and are compiled in Table 2. The figures were prepared with PYMOL (20).

Characterization of PMM/PGM Mutants—Site-directed mutants of *P. aeruginosa* PMM/PGM with a His₆ affinity tag were produced using the QuikChange mutagenesis kit (Stratagene) and verified by DNA sequencing. All enzymes used in activity assays were purified via Ni²⁺ affinity chromatography to ensure removal of contaminating proteins. Purified proteins were characterized by CD spectroscopy to determine whether gross structural changes in secondary structure resulted from the mutations. The steady state parameters for each mutant protein were quantitated as described previously (4), except that G16P was present at 1.0 mM instead of 0.5 mM. Briefly, the PGM activity of the enzyme was measured in the direction of glucose 6-phosphate formation using a coupled assay with glucose 6-phosphate dehydrogenase. One mutant, R20A, shows no activity under the standard assay conditions, a result that was in conflict with previously published data (4). To confirm our data, the gene was re-sequenced, and the single-codon alteration was verified. The protein was purified twice from independent cultures. The assay was also conducted at 100-fold increased enzyme concentration and 200-fold increased G16P concentration, but activity was still not detectable. An explanation for the prior result is unknown at this time.

The mutant proteins were also characterized in an isotope trapping experiment (6) that determines the rate of dissociation of G16P from the enzyme (Fig. 1b). This experiment was conducted at chemical equilibrium with 10 μM enzyme, 100 μM [¹⁴C]glucose 1-phosphate, 1.73 μM glucose 6-phosphate, 1.0 mM G16P, 1.5 mM MgSO₄, and 1 mM dithiothreitol in 50 mM MOPS, pH 7.4. The ratio of the rate of transfer of label into product (kₔₙ₅₈) and the rate of appearance of label in G16P (kₐ₉₆) provides a direct measure of the frequency of the dissociation of the intermediate. Substrate, product, and intermediate are separated by anion-exchange high performance liquid chromatography and detected and quantitated with a flow-through radioactivity detector. The full details of this method and data analysis can be found in Naught and Tipton (6).

### TABLE 1

| Crystallographic and refinement statistics | Dephospho-PMM/PGM-G16P | Phospho-PMM/PGM-G16P |
|------------------------------------------|-------------------------|----------------------|
| Data collection                          | X-ray source            | Space group          |
|                                          | MBC 4.2.2               | P2₁2₁2₁             |
|                                          | Space group             | a = 70.22            |
|                                          |                         | b = 70.36            |
|                                          |                         | c = 84.39            |
| Resolution, Å                           | 42.83-1.90              | 50.00-2.00           |
| Unique ref./redund.                     | 33,838/6.5              | 33,830/8.1           |
| Rmerge,%                                 | 7.5 (34.6)              | 7.6 (47.0)           |
| Completeness, %                         | 99.9 (99.4)             | 99.7 (100.0)         |

R<sub>merge</sub> = Σ[F<sub>o</sub> - F<sub>c</sub>]/[ΣF<sub>o</sub>], where F<sub>o</sub> and F<sub>c</sub> are observed and calculated structure factors, respectively.

R<sub>free</sub> is the R factor calculated from 5% of the reflections not included in refine-ment. No r<sub>c</sub>-cutoff of the data was used.

r.m.s.d., root mean square deviation.

Most favored/additional allowed regions of the Ramachandran plot.
RESULTS

Overall Structures

Two complexes of PMM/PGM with its reaction intermediate, G16P, have been refined to 1.9 and 2.0 Å (see "Materials and Methods" and Table 1). One of these complexes, with dephospho-PMM/PGM, employs the site-directed mutant S108D, which prevents phosphorylation at this key residue. The other complex, with phospho-PMM/PGM, is formed using wild-type enzyme, phosphorylated at Ser-108. The dephospho-PMM/PGM/G16P complex provides a snapshot of the reaction either immediately after phosphoryl transfer to the intermediate or before phosphoryl transfer back to the enzyme (Fig. 1). The phospho-PMM/PGM/G16P complex, however, is an "off pathway" or non-productive complex, since in the normal course of the reaction G16P is formed only after the phosphoserine has transferred its phosphoryl group to substrate. As was done for previous studies, the enzyme is rendered catalytically inactive by substituting Zn$^{2+}$ for the Mg$^{2+}$ that is required for activity. In both complexes electron density maps show one molecule of G16P bound in a single specific location and orientation in the active site cleft of the monomeric enzyme (Fig. 2).

Overall, the protein structures in the two PMM/PGM-intermediate complexes are quite similar to those seen in the previously characterized apoprotein and enzyme-substrate complexes (2, 21). However, the two PMM/PGM-intermediate complexes exhibit some differences in overall tertiary structure, specifically in the rotation of domain 4 (residues 369–463) relative to the rest of the enzyme (Fig. 3a). In the dephospho-PMM/PGM-G16P complex, the enzyme adopts a "closed" conformation that creates an interface between domains 1 and 4 of the protein (139 Å$^2$), which is absent in the apoenzyme structure. This domain-domain interaction is essential for forming the invariant phosphate-binding site, a cluster of residues originally observed in the enzyme-substrate complexes that interacts with the phosphate group of each phospho-sugar ligand in the same fashion regardless of the position (1 versus 6) of the substitution (2). Because of the closed conformation of domain 4, G16P is buried deeply in the active site of this complex, with only 7% of its surface area accessible to solvent. The intermediate adopts an extended conformation and spans the active site of the enzyme with its 1-phosphate group adjacent to residue 108 (the conserved phosphoryl transfer site), whereas its 6-phosphate group occupies the phosphate-binding site. Thus, G16P in this complex is appropriately positioned for phosphoryl transfer from the 1-phosphate group to residue 108 of the enzyme to form glucose 6-phosphate.

Relative to the dephospho-PMM/PGM-G16P complex, the complex of phospho-PMM/PGM with G16P exhibits a number of significant structural differences (Fig. 3b). For instance, the enzyme adopts a "half-open" conformation, where the rotation of domain 4 is midway between that observed in the open apoenzyme structure and the closed dephos-
phospho-PMM/PGM-G16P complex. Because of this, the phospho-PMM/PGM-G16P complex has only a small interface between domains 1 and 4 (67 Å²), and also lacks an intact phosphate-binding site. Furthermore, steric conflicts with phosphoserine 108 prevent the intermediate from binding as deeply in the active site; more than 25% of the surface area of G16P is exposed to solvent. The conformation of G16P is more compact than that observed in the dephospho-PMM/PGM-G16P complex; rotation about the C6–O6 bond reduces the distance between the centers of the phosphate groups from 7.7 to 6.9 Å. As seen in the dephospho-PMM/PGM-G16P complex, G16P is also bound in a single orientation in this structure. However, its relative orientation in the active site is reversed: the 6-phosphate group is closest to residue 108, whereas the 1-phosphate group occupies a perturbed version of the phosphate-binding site in domain 4.

**Enzyme-intermediate Interactions**

**Dephospho-PMM/PGM-G16P Complex**—In this structure multiple specific interactions are observed between the protein and G16P, involving residues from all four domains of the enzyme (Fig. 3c and Table 2). Interactions between the 6-phosphate group and residues in the phosphate-binding site include hydrogen bonds from both side chain and backbone atoms of the protein. In the phosphoryl transfer site, the 1-phosphate group is contacted by several side chains, including those of the ionizable residues Lys-118, His-308, His-329, and Arg-247. Hydroxyl groups on the sugar ring of G16P form hydrogen bonds with residues in domains 1–3 of the protein, including Glu-325 and Ser-327, which make similar contacts in the enzyme-substrate complexes (2). Only four solvent molecules contact the intermediate in this structure; two of these make bridging interactions between the protein and G16P.

In many ways the dephospho-PMM/PGM-G16P complex resembles the previously characterized enzyme-substrate complexes (2). Structural superpositions (data not shown) show that phosphate groups of the ligands that occupy the phosphate-binding site have essentially identical environments. Also, the sugar rings of the intermediate and substrates overlap at least partially but not as closely as the phosphate groups. Not surprisingly, nearly all of the residues that contact G16P in the dephospho-PMM/PGM-G16P complex were previously observed to interact with a ligand in one or more of the enzyme-substrate complexes. The single exception to this is a new interaction observed near the phosphoryl transfer site, between Lys-118 and the 1-phosphate group of G16P.

**Phospho-PMM/PGM-G16P Complex**—Despite what appears to be a quasi-stable binding position, a number of specific protein-ligand inter-
actions are also observed in the phospho-PMM/PGM-H18528G16P complex (Fig. 3d and Table 2). Many of these are between the two phosphate groups of G16P and basic residues of the enzyme, including Arg-15, Arg-20, and Arg-421. Presumably, these basic side chains help neutralize the abundance of negative charge in the vicinity of the 6-phosphate group of G16P, which is relatively close to phosphoserine 108 (P-P distance 6.9 Å). The 1-phosphate group of the intermediate occupies a perturbed version of the phosphate-binding site but lacks the otherwise conserved interaction with Tyr-17 of domain 1 and also differs in the number and geometry of the hydrogen bond contacts relative to the enzyme-substrate and dephospho-PMM/PGM-H18528G16P complexes. Only a single direct enzyme contact is made to a sugar hydroxyl of G16P by the side chain of Ser-327. Instead, most hydrogen bonds with the sugar hydroxyls are made by water molecules. Of the 10 water molecules in the active site, 8 form hydrogen bonds to hydroxyl groups of the sugar ring; 4 waters bridge between G16P and side chain or backbone atoms of the protein.

In contrast to the complex of dephospho-PMM/PGM with G16P, many of the interactions in the phospho-PMM/PGM-H18528G16P complex are not observed in other PMM/PGM-ligand structures. Four of the contacting residues, Arg-15, Arg-20, His-109, and Asn-110, make no interactions with ligands in the dephospho-PMM/PGM-H18528G16P complex or any of the enzyme-substrate complexes. Indeed, the side chains of two of these residues, Arg-15 and Arg-20, are partially disordered with high B-factors and/or adopt multiple conformations in other PMM/PGM ligand complexes but are found in single, well defined conformations in this complex with G16P (Fig. 2b). Several of these residues (Arg-20, His-109, and Asn-110) are conserved throughout the-D-phosphohexomutase family (1), suggesting key roles in enzyme function.

Characterization of PMM/PGM Active Site Mutants

Structural analysis of the two PMM/PGM-intermediate complexes raised questions about the functional roles of residues interacting with G16P. To further investigate this, site-directed mutants were created and characterized mechanistically. Residues at various locations in the active site of the enzyme were selected for mutagenesis, including three that make unique contacts in the phospho-PMM/PGM-H18528G16P complex (Arg-15, Arg-20, Asn-110), one involved in the phosphate-binding site (Arg-421), and another residue (Arg-247) with no known role in catalysis. Selected residues were changed to alanine, except for the R421C mutant that was available from previous studies. CD spectroscopy showed no differences in secondary structure between wild-type and mutant proteins.

The kinetic properties of the mutant PMM/PGM proteins are summarized in Table 3. Steady state kinetic parameters for each protein
were determined, and an isotope trapping experiment was conducted to characterize the frequency of dissociation of G16P from the enzyme during catalysis (see "Materials and Methods"). This experiment is performed at chemical equilibrium using [14C]glucose 1-phosphate as substrate and measures the transfer of label to product in the presence of excess unlabeled G16P (Fig. 1b). Under these conditions dissociation of labeled G16P is essentially irreversible, and thus, the appearance of label in G16P measures the rate of its dissociation from enzyme. In the case of wild-type PMM/PGM, this experiment showed that G16P proceeds directly to product 14.3 times more often than it dissociates from the enzyme (6).

Of the five mutant proteins characterized, one (R20A) shows no detectable activity even at 100-fold increased enzyme levels. One other mutant (R421C) shows a significant decrease in apparent $V_{\text{max}}$, with activity 0.3% that of wild-type enzyme, although its apparent $K_{m}$ is similar to wild-type PMM/PGM. The remaining three mutants show unremarkable differences in the apparent $V_{\text{max}}$ and $K_{m}$ compared with wild-type enzyme. However, the isotope trapping experiment reveals striking differences between several of the mutants and wild-type PMM/PGM protein in the frequency of dissociation of the intermediate from the enzyme. R247A shows a modest increase in the rate of loss of G16P to bulk solution; this occurs approximately twice as often as with wild-type enzyme. The R15A and N110A mutants show much larger effects; G16P dissociates from these proteins ~25 times more often than it does from wild-type enzyme, with dissociation occurring at least once per catalytic cycle. In the case of the R421C mutation, which affects a conserved residue in the phosphate-binding site, rate constants describing the transfer of label from substrate to product were not determined due to the low activity of the mutant. However, the slow rate of product formation of this mutant is apparently not related to an increased rate of dissociation of intermediate from the enzyme, since no labeled G16P was detected in solution during the time course of the isotope trapping experiment (data not shown).

**DISCUSSION**

Previous structural characterization of PMM/PGM with its four subunits showed that the 1- and 6-phospho-sugars bind in two distinct orientations in the active site; the phosphate groups bind in essentially the same place, but the sugar rings are flipped by 180° relative to each other (2). These complexes confirmed a model initially proposed for a related enzyme, rabbit PGM, which necessitated reorientation of the bisphosphorylated sugar intermediate in between the two phosphoryl transfer steps (22). A sequence-structure analysis of the α-δ-phosphohexomutases suggests that all members of this enzyme superfamily utilize a common reaction mechanism, which presumably includes the dramatic reorientation of the reaction intermediate (1). Dissociation of G16P from rabbit PGM, for example, has been estimated to occur only once every 20 catalytic cycles (23). The 180° "on enzyme" reorientation of the intermediate is a unique feature of catalysis for this enzyme family; functionally similar enzymes, such as the α-δ-phosphohexomutases, utilize a ping-pong mechanism, releasing the intermediate into bulk solution in between phosphoryl transfer steps (24).

The interactions observed in these two complexes are consistent with the known binding affinities of PMM/PGM for its intermediate; G16P binds to dephospho-PMM/PGM, with a $K_{d}$ of $\approx$5 mM (5), and acts as an inhibitor for phosphorylated enzyme, with a $K_{i}$ of 1.2 mM in the presence of 10 μM glucose 1-phosphate. The complex of dephospho-PMM/PGM with G16P exhibits many features typical of high affinity enzyme-ligand complexes. For example, G16P is almost completely buried in the active site due to the closed conformation of the enzyme. Also, multiple direct enzyme-intermediate interactions are observed to potential hydrogen-bonding partners in the ligand, including both the phosphate groups and sugar hydroxyls. In contrast, these features are missing in the phospho-PMM/PGM-G16P complex, where G16P is less buried in the active site due to the closed-open conformation of the enzyme. Furthermore, nearly all of the direct enzyme-intermediate contacts are made to phosphate groups of the intermediate in this complex, whereas water molecules generally fulfill the hydrogen bonding potential of the sugar hydroxyls. Analysis of the contacts in the two PMM/PGM-intermediate complexes does not, however, provide a clear rationale for the single binding orientation of G16P in each structure. Presumably, the preferred orientations observed in the crystal structures are due to relatively small differences in binding energies between the two (or more) orientations of G16P in the active site of the enzyme that occur at different points of the reaction.

The formation of the non-productive, phospho-PMM/PGM-G16P complex raises a number of questions. Perhaps most intriguing is why the intermediate could be observed at all, since this off-pathway complex is not representative of any required step in the PMM/PGM reaction. Steric conflicts with phosphoserine 108 preclude the intermediate from occupying the high affinity binding site seen in the dephospho-PMM/PGM-G16P complex. Thus, the phospho-PMM/PGM-G16P complex appears to be the result of inadvertently populating a low affinity binding site for G16P in the active site of the enzyme. However, since the binding of G16P to phosphorylated enzyme is of sufficient affinity and specificity to be observed in a crystal structure, it seemed possible that this non-productive complex might be relevant to some aspect of the multistep reaction. Specifically, we hypothesize that the observed binding site might represent a quasi-stable position for the intermediate during its 180° reorientation. The observed structural features meet many of the expectations for such a complex, including the required loss of multiple high affinity enzyme-ligand interactions relative to the dephospho-PMM/PGM-G16P complex, as would be necessary to allow movement of the intermediate via rotational diffusion. In addition, the rotation of domain 4 to create the half-open conformation of the protein expands the volume of the active site enough to permit rotation of G16P while still sequestering it from bulk solution. This hypothesis is also consistent with the results of the single-turnover experiments that suggest the existence of one or more metastable states for G16P during the catalytic cycle of PMM/PGM (6).

The potential importance of individual residues involved in interactions with G16P was assessed through kinetic characterization of site-directed mutants of PMM/PGM. One mutation, R20A, yielded inactive enzyme. Because this residue is in the vicinity of phosphoserine 108, it may play a key role in phosphoryl transfer, although this was not investigated further. Mutations of several other residues produced relatively insignificant effects on $V_{\text{max}}$ and $K_{m}$ but show moderate (R247A) to profound (R15A, N110A) effects on the ability of PMM/PGM to main-

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**Table 3**

| Protein | $V_{\text{max}}$ | $K_{m}$ | $k_{\text{dissoc.}}$ |
|---------|-----------------|-----------|------------------|
| Wild type | 7.01 ± 0.65 | 27.3 ± 4.5 | 14.3 |
| R15A | 2.85 ± 0.47 | 13.2 ± 5.0 | 0.56 |
| R20A | 0.48 ± 0.03 | 12.7 ± 3.2 | 0.46 |
| N110A | 1.48 ± 0.08 | 19.5 ± 3.9 | 5.9 |
| R247A | 0.02 ± 6.22E-04 | 27.9 ± 2.7 | |
| R421C | |

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**Structural Insights into a Simple Processive Enzyme**

P. Tipton, personal communication.
tain association with the intermediate during the multi-step reaction. In fact, the R15A and N110A point mutations are sufficient to change the fundamental nature of PMM/PGM from a processive enzyme, where the reorientation of G16P occurs most frequently without dissociation from the enzyme, to an enzyme utilizing a ping-pong mechanism, where dissociation and re-binding of the intermediate occurs at least once every catalytic cycle.

The observation that mutation of the residues affecting processivity produces generally small effects on the steady state kinetic parameters is consistent with the known rate constants of the PMM/PGM reaction, where non-chemical steps, such as substrate binding and the concomitant conformational change of the enzyme, are rate-limiting (6). Thus, these residues appear to function primarily in maintaining association with the intermediate during its reorientation. In the cell, where substrate may be present at limiting levels, the processivity of PMM/PGM could be critical for the flux of the biosynthetic pathways in which the enzyme participates.

The precise roles of the three residues shown to influence processivity are not completely defined by this study, but several intriguing possibilities exist. First, these residues could participate in specific and required interactions with G16P while it occupies a metastable position during its reorientation. Although a formal possibility, we consider this somewhat unlikely due to the obvious differences between the active sites of the phosphorylated and dephosphorylated forms of the enzyme. An attractive alternative hypothesis is that the primary effect of mutating these residues is alteration of the electrostatic environment of the active site. The flexibility of these side chains may assist in this process. However, other factors are also clearly involved, since Asn-110 is also a critical residue for processivity. No clear rationale for the importance of Asn-110 is apparent from the structures, although in the PMM/PGM-substrate complexes the side chain of this residue participates in hydrogen bond interactions with the backbone carbonyl of Gly-107. Thus, its effect may be an indirect result of small structural changes in the vicinity of the phosphoryl transfer site. The possibility that the R15A and R247A mutations create small structural changes in the active site that indirectly affect processivity also cannot be ruled out at present.

Characterization of the R421C mutant shows no evidence for a role in the processivity of PMM/PGM, but the function of this residue in the reaction is still intriguing. Relative to wild-type enzyme, the activity of this mutant is considerably decreased, yet its $K_m$ is unchanged. Structural studies, on the other hand, have shown that Arg-421 is a key residue in the invariant phosphate-binding site and is too far from the phosphoryl transfer site to be directly involved in the chemistry of the reaction. One possibility that is consistent with our data, although not conclusively proven by it, is that a role for Arg-421 in substrate binding ($i.e.$ $K_m$) may be masked by the numerous other enzyme-ligand interactions observed in the dephospho-PMM/PGM-G16P and enzyme-substrate complexes. However, the effect of this mutation could be much more significant in the context of the phosphate-binding site. Thus, we
propose that the importance of this residue is manifested at a specific stage of the reaction where the interactions between G16P and the phosphate-binding site are critical; that is, formation of the half-open enzyme conformer necessary to permit reorientation of the intermediate. This proposal is consistent with the interactions observed in the phospho-PMM/PGM-G16P complex, where contacts between G16P and many residues in the phosphate-binding site are maintained despite the rotation of domain 4. However, if the side chain of Arg-421 is absent, rotation of domain 4 could cause the phosphate-binding site to lose association with the intermediate. In this scenario, interactions between G16P and residues in the phosphoryl transfer site and residues contacting the sugar hydroxyls would remain, and the intermediate would “stall” in this position, resulting in the observed decrease in V_max.

A synthesis of the structural and kinetic characterization of PMM/PGM from this and previous studies yields a detailed model of its multistep reaction (Fig. 4). A key feature of this model is the rotation of domain 4 as a trigger for removing G16P from its high affinity binding site as found in the closed conformer of the enzyme and, subsequently, permitting its rotation. Based on data from the R421C mutation, effective binding of G16P by residues in the phosphate-binding site must be coupled with rotation of domain 4 to initiate the reorientation (Fig. 4c). Other features of the model are highlighted in Fig. 4, including a summary of the types of enzyme-intermediate interactions and changes in the domain 1–4 interface. Although not shown explicitly, the rotation of domain 4 is also required for substrate binding and product release and, thus, is critical at several different stages of the multistep reaction.

Our characterization of the PMM/PGM structure and mechanism reveals some intriguing parallels with other processive or quasi-processive enzymes. For example, several “sliding clamp” proteins show unusual charge distributions, with a negative electrostatic potential overall but positive charge in the cavity that encircles DNA (7). A similar situation is seen with PMM/PGM; although negatively charged overall, its active site has a positive electrostatic potential (21). The positively charged active site cleft may help reduce the energetic cost of substrate binding, whereas the overall acidic character of the protein surface may help keep the bisphosphorylated intermediate localized in the active site during its reorientation. Other enzymes have been shown to undergo conformational changes between a high affinity conformer that specifically recognizes substrate and a low affinity conformer that permits translocation of the substrate. For example, a complex of the restriction enzyme BamH1 bound to non-cognate DNA shows a complete loss of specific protein contacts to the DNA bases and phosphate backbone relative to multiple specific contacts seen in a complex with its target DNA site (25). This parallels many of the differences observed between the two PMM/PGM-intermediate complexes, although some direct DNA site (25). This parallels many of the differences observed between the two PMM/PGM-intermediate complexes, although some direct

REFERENCES

1. Shackelford, G. S., Regni, C. A., and Beamer, L. J. (2004) Protein Sci. 13, 2130–2138
2. Regni, C., Naught, L. E., Tipton, P. A., and Beamer, L. J. (2004) Structure 12, 55–63
3. Naught, L. E., Gilbert, S., Imhoff, R., Snook, C., Beamer, L., and Tipton, P. (2002) Biochemistry 41, 9637–9645
4. Naught, L. E., Regni, C., Beamer, L. J., and Tipton, P. A. (2003) Biochemistry 42, 9946–9951
5. Naught, L. E., and Tipton, P. A. (2001) Arch. Biochem. Biophys. 396, 111–118
6. Naught, L. E., and Tipton, P. A. (2001) Biochemistry 40, 6833–6836
7. Breyer, W. A., and Matthews, B. W. (2001) Protein Sci. 10, 1699–1711
8. Regni, C. A., Tipton, P. A., and Beamer, L. J. (2000) Acta Crystallogr. Sect. D 56, 761–762
9. Otwinowski, Z., and Minor, W. (1997) Methods Enzymol. 296, 307–326
10. Pflgrath, J. W. (1999) Acta Crystallogr. D Biol. Crystallogr. 55, 1718–1725
11. Brünger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gron, F., Grzesiek, S., Pannier, J. W., Wang, C., and Wang, J. (1998) Acta Crystallogr. D Biol. Crystallogr. 54, 905–921
12. Murshudov, G. N., Vagin, A. A., Lebedev, A., Wilson, K. S., and Dodson, E. J. (1999) Acta Crystallogr. Sect. D 55, 247–255
13. Emsley, P., and Cowtan, K. (2004) Acta Crystallogr. D Biol. Crystallogr. 60, 2126–2132
14. Collaborative Computational Project 4 (1994) Acta Crystallogr. Sect. D 50, 760–763
15. Vaguna, A. A., Richelle, J., and Wodak, S. I. (1999) Acta Crystallogr. Sect. D 55, 191–205
16. Laskowski, R. A., McArthur, M. W., Moss, D. S., and Thornton, J. M. (1993) J. Appl. Crystallogr. 26, 283–291
17. Jones, S., and Thornton, J. M. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 13–20
18. Hayward, S., and Berendsen, H. J. (1998) Proteins 30, 144–154
19. Shatsky, M., Nussinov, R., and Wolfson, H. J. (2004) Proteins 56, 143–156
20. DeLano, W. L. (2002) The PyMOL Molecular Graphics System, DeLano Scientific, San Carlos, CA
21. Regni, C., Tipton, P. A., and Beamer, L. J. (2002) Structure 10, 269–279
22. Percival, M. D., and Withers, S. G. (1992) Biochemistry 31, 505–512
23. Ray, W. J., Jr., and Roselli, G. A. (1964) J. Biol. Chem. 239, 1228–1236
24. Zhang, G., Dai, J., Wang, L., Dunaway-Mariano, D., Tremblay, L. W., and Allen, K. N. (2005) Biochemistry 44, 9404–9416
25. Viadiu, H., and Aggarwal, A. K. (2000) Mol. Cell 5, 889–895
26. Parsiegla, G., Reverbel-Leroy, C., Tardif, C., Belaich, J. P., Driguez, H., and Haser, R. (2003) Biochemistry 39, 11238–11246

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Structural Insights into a Simple Processive Enzyme