Promotion of Enzyme Flexibility by Dephosphorylation and Coupling to the Catalytic Mechanism of a Phosphohexomutase

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Background: Enzyme function involves both specific conformational change and intrinsic flexibility of proteins. Results: Dephosphorylation of a catalytic phosphoserine causes a global increase in flexibility of a phosphohexomutase. Conclusion: Increased flexibility following phosphoryl transfer is critical to catalytic mechanism. Significance: Other phosphotransfer enzymes may undergo changes in flexibility linked to dephosphorylation.

The enzyme phosphomannomutase/phosphoglucosmutase (PMM/PGM) from Pseudomonas aeruginosa catalyzes an intramolecular phosphoryl transfer across its phosphosugar substrates, which are precursors in the synthesis of exoproducts involved in bacterial virulence. Previous structural studies of PMM/PGM have established a key role for conformational change in its multistep reaction, which requires a dramatic 180° reorientation of the intermediate within the active site. Here hydrogen-deuterium exchange by mass spectrometry and small angle x-ray scattering were used to probe the conformational flexibility of different forms of PMM/PGM in solution, including its active, phosphorylated state and the unphosphorylated state that occurs transiently during the catalytic cycle. In addition, the effects of ligand binding were assessed through use of a substrate analog. We found that both phosphorylation and binding of ligand produce significant effects on deuterium incorporation. Phosphorylation of the conserved catalytic serine has broad effects on residues in multiple domains and is supported by small angle x-ray scattering data showing that the unphosphorylated enzyme is less compact in solution. The effects of ligand binding are generally manifested near the active site cleft and at a domain interface that is a site of conformational change. These results suggest that dephosphorylation of the enzyme may play two critical functional roles: a direct role in the chemical step of phosphoryl transfer and secondly through propagation of structural flexibility. We propose a model whereby increased enzyme flexibility facilitates the reorientation of the reaction intermediate, coupling changes in structural dynamics with the unique catalytic mechanism of this enzyme.

Many enzymatic mechanisms entail multiple chemical steps, and such reactions must overcome a number of challenges to achieve efficient catalysis. The binding of structurally variable ligands may need to be accommodated, and multiple transition states may need to be stabilized (1, 2). Often different steps of the reaction are accompanied by conformational or dynamic rearrangements of the enzyme (3). The multistep reaction of the α-d-phosphohexomutases, which requires a dramatic reorientation of a reaction intermediate for completion of catalysis, poses the additional challenge of accommodating ligand dynamics within the active site (4). Factors that facilitate the release and subsequent reorientation of this intermediate in the midst of the catalytic cycle of these enzymes are not well understood.

The α-d-phosphohexomutases are ubiquitous in the three kingdoms of life and play essential roles in carbohydrate metabolism where they catalyze the reversible conversion of 1-to-6-phosphosugars (5). The reaction entails two phosphoryl transfers: first from enzyme to substrate and second from the bisphosphorylated sugar intermediate back to enzyme (Fig. 1). Between the two catalytic steps is a mandatory 180° reorientation of the intermediate, a mechanism confirmed by structural characterizations of enzyme-ligand complexes (6–9). For two proteins in the superfamily, kinetic studies indicate that the multistep reaction is processive (i.e. occurs without dissociation of the intermediate from the enzyme) (4, 10). Crystal structures of multiple proteins in the superfamily reveal a common theme of flexibility in the orientation of the C-terminal domain (11), which is correlated with ligand binding and presumably also occurs during product release and to permit reorientation of the reaction intermediate. The non-chemical steps (conformational change and/or ligand binding) appear to be rate-limiting in the steady state reaction (4).

Here we report the use of HDXMS2 and SAXS to probe the solution flexibility of one member of this enzyme superfamily, phosphomannomutase/phosphoglucosmutase (PMM/PGM) from

2 The abbreviations used are: HDXMS, hydrogen-deuterium exchange by mass spectrometry; PMM/PGM, phosphomannomutase/phosphoglucosmutase; SAXS, small angle x-ray scattering; X1P, xylose 1-phosphate; apo-P, active phosphoenzyme; apo-deP, inactive dephosphoenzyme; X1P-P, phosphoenzyme in complex with the substrate analog xylose 1-phosphate; FT, Fourier transform.

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**EXPERIMENTAL PROCEDURES**

**Materials**—Glucosamine 1-phosphate, glucose 1,6-bisphosphate, 99% D$_2$O, and trifluoroacetic acid were purchased from Sigma. Pepsin was purchased from Worthington. Mass spectrometric grade acetonitrile was from Mallinckrodt Baker. Xylose 1-phosphate (X1P) was synthesized by Professor Thomas Mawhinney (University of Missouri, Columbia, MO).

**Protein Preparation**—Expression and purification of *P. aeruginosa* PMM/PGM and its phosphorylation/dephosphorylation were performed as described previously (13, 14). Briefly, phosphorylation and dephosphorylation were achieved by incubating the protein with glucose 1,6-bisphosphate or glucosamine 1-phosphate, respectively, at 6-fold molar excess over enzyme at 4 °C for 18 h. Ligands were removed by extensive dialysis against 50 mM MOPS, pH 7.4 and 1 mM MgCl$_2$. Phosphorylation levels were confirmed by electrospray ionization mass spectrometry (13) at 0 and 93% for the dephosphorylated and phosphorylated samples, respectively.

**HDXMS Conditions**—Prior to deuterium exchange, aliquots of PMM/PGM (3 μl) at 12 mg/ml in 50 mM MOPS, pH 7.4 and 1 mM MgCl$_2$ were preincubated for 10 min with 5 μl of either H$_2$O or 2 mM X1P. To initiate exchange, 82 μl of deuterated buffer (100 mM HEPES in D$_2$O, pH 7.5) was added to the mixture for varying times (0–2720 min) at 4 °C. The quenching reaction and peptic digestion were done as described (15) except cleavage was for 2 min.

**Tandem Mass Spectrometry**—Peptide identification was performed using nondeuterated protein (control samples). Following peptic digestion as indicated above, the resulting peptic peptides were loaded on a reverse phase C18 column (Zorbax C18SB, 1 cm × 0.32 mm, 300 μm; MicroTech Scientific) and desalted for 2.5 min at a rate of 75 μl/min with solvent A (0.05% (v/v) TFA). Peptides were then eluted at a rate of 25 μl/min with the following gradient: 2% solvent B (0.05% (v/v) TFA in acetic acid) for 3 min followed by 20–60% solvent B over 15 min, then 60–90% solvent B over 1 min, and a wash at 90% solvent B for 5 min. Finally, the column was pre-equilibrated with solvent A for 7 min before injection of the next sample. The peptic peptides eluted from the column between 5 and 15 min. Peptide desalting and separation were performed at −2 °C using a custom-built cooling chamber, SAIDE interface, which consistently reduces back-exchange to 17% (16). The mass spectrometer (LTQ FT, ThermoFinnigan) was operated in data-dependent mode to perform one MS scan on the FT at a resolution of 50,000 for an ion at m/z 400 followed by six MS2 scans on the six most intense ions on the ion trap using an
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 exclusion from sample analyses. No other data points were omitted if the 1200-min time point was missing. For presentation of data, five time points (t) were measured using exposure times of 0.5, 1.0, 2.0, and 5.0 s. Scattering curves of protein samples were corrected for background scattering using intensity data from the elution buffer. Data analysis and \( R_g \) determination were performed by scaling and merging the background-corrected high q region from the 5.0-s exposure with the low q region from the 0.5- or 1.0-s exposure using PRIMUS (19). Estimations of \( R_g \) at each protein concentration were achieved by Guinier analysis in PRIMUS by setting the qRg limit to 1.3 or less. The FoXS server (20) was used to calculate \( R_g \) from the relevant crystal structures. The Porod-Debye plot was generated by transforming the scattering data as \( q^2 I(q) \) versus \( q^4 \) using the Porod analysis in PRIMUS (data points 1–170 for all samples). The Porod volume, \( V \), was estimated from the plateau region of the plot in PRIMUS. The pair distribution function \( P(r) \) was calculated using GNOM (21) with the maximum of each curve normalized to 1.0. Plots use data from equivalent midpoint protein concentration that were available for all samples.

Circular Dichroism—Thermal denaturation was monitored via circular dichroism using an Aviv 62DS spectrometer with a 0.1-cm quartz cuvette. Samples of phosphorylated and unphosphorylated PMM/PGM at 8 \( \mu \)M and in 10 mM MOPS buffer, pH 7.4 were heated from 25 to 95 °C. Ellipticity was monitored at 222 nm. Thermal denaturation of PMM/PGM is not reversible due to precipitation of the protein at high temperature; thus, the apparent \( T_m \) reports on both thermal stability and kinetics of unfolding.

Structure Determination of Unphosphorylated PMM/PGM—PMM/PGM was dephosphorylated as described above. For crystallization, the N-terminal His tag was removed using the Thrombin CleanCleave\textsuperscript{TM} kit (Sigma-Aldrich). Cleaved protein was collected in the flow-through using His-Select Ni\textsuperscript{2+} affinity resin (Sigma-Aldrich); dialyzed into 50 mM MOPS, pH 7.4, 1 mM MgCl\textsubscript{2}, and 0.05 mM NaCl and concentrated to 5 mg/ml for crystallization. Mass spectrometric analysis indicated full cleavage of the affinity tag and 0% phosphorylation. Crystals were grown at 20 °C using hanging drop vapor diffusion and microseeding techniques from 1.3 to 1.4 M sodium/potassium tartrate and 100 mM HEPES, pH 7.5 (13, 22). For cryoprotection, crystals were transferred gradually into a solution of 70% (w/v) PEG 4000 with 0.1 M HEPES, pH 7.5.

X-ray diffraction data were collected using a Rigaku RU H3R rotating anode and R-AXIS IV++ image plate system under cryocooling conditions. The space group and unit cell (Table 1) were isomorphous with crystals of the phosphorylated enzyme (22). Data were processed with HKL2000/Scalepack (23), and refinement was performed with REFMAC 5.5.0109 (24).
starting model for refinement was that of phospho-PMM/PGM (Protein Data Bank code 1K35) excluding the phosphoryl modification of serine 108 and water molecules. Progress of the refinement was monitored by following \( R_{\text{free}} \) 5% of the data set was set aside for cross-validation prior to refinement. The structure was refined to convergence through iterative cycles of refinement and manual rebuilding with Coot (25). Water molecules were placed automatically with Coot in peaks \( >3.0 \sigma \) in \( F_o - F_c \) maps and within reasonable hydrogen bonding distance of oxygen or nitrogen atoms. The final model contains a single bound metal ion, modeled as \( \text{Zn}^{2+} \) in the active site, coordinated by three aspartate residues as in the phosphoenzyme structure (Protein Data Bank code 1K35). A tartrate molecule from the crystallization buffer also coordinates the metal. Data collection and refinement statistics are summarized in Table 1.

## RESULTS

**HDXMS Overview**—The structural flexibility of *P. aeruginosa* PMM/PGM in solution was probed using HDXMS (“Experimental Procedures”). Overall sequence coverage of the peptide mapping for the three samples ranged from 87 to 97% (Table 2). Approximately 15 time points per sample were measured. **Supplemental Fig. S1** shows heatmaps of exchange for the entire amino acid sequence (463 residues). The data used for comparative analyses consist of a set of 23 peptic fragments for each of the three samples. Data for five time points (from 1 to \(~1200 \text{ min}\) are summarized in a histogram (Fig. 3A) where each peptide has a *triplet of bars* corresponding to the three samples, and the *bars* are colored in segments according to time.

During the course of the experiment, the three samples achieved final overall deuteration levels of 28–47% with those of individual peptides ranging from 5 to 82% (Table 2). The apo-deP sample reached the highest level, and its peptides also exchanged most quickly, especially at the earliest time points (e.g. peptide 294–303 in Fig. 3A). Twelve peptide fragments from this sample reached deuteration levels >50%. For the apo-P sample, the final overall deuteration content was reduced by 11% relative to the apo-deP sample with nearly all of its peptides staying below 50%. The X1P-P sample showed the least overall incorporation. Comparing structural domains D1–D4, deuteration exchange was lowest for D1 and highest for D4 (Fig. 3 and supplemental Fig. S2). The differences among domains were smallest for the X1P-P sample. For all three samples, peptides near domain boundaries often exhibited the highest incorporation levels. The entire time course of exchange is presented for the three samples on the crystal structure of PMM/PGM as animations (supplemental Movies 1–3).

**Global Effects of Phosphorylation**—The differential effects of phosphorylation on deuteration exchange in PMM/PGM are further assessed on a butterfly plot (Fig. 3B). Effects are seen at multiple levels, including changes in both content and rate of exchange. These occur throughout the protein sequence with increased incorporation in the apo-deP sample relative to the apo-P sample for nearly all peptides and at most time points. Some of the most striking effects are found in the C-terminal half of the protein where differences of >30% deuteron level are seen in two peptides within 1 min (Fig. 3B, pink bars) and in several other peptides by the final time point (Fig. 3B, blue bars). It is interesting to note that peptides with the greatest differential incorporation by the end of the experiment (in D3 and D4) are distant in sequence from the site of phosphorylation in D1 (Fig. 3, red star).

When viewed on the crystal structure of PMM/PGM (Fig. 4A, *left*), peptides with significant differences in deuteron exchange between the apo-deP and apo-P samples at the final time point are seen across the structure both within and outside of the active site cleft. Peptides with the greatest changes in deuteron level (Fig. 4, *red spheres*, peptides 1, 3, and 4) are found near the interface of D3–D4. Selected time courses of exchange (Fig. 4B) show the increase in deuteron incorporation for the apo-deP sample and highlight variations in rates. For example, in peptide 267–293 of the apo-deP sample, a rapid uptake in deuteron exchange takes place prior to the initial time point (presumably due to increased solvent accessibility/conforma-

## TABLE 1

Data collection and refinement statistics for the crystal structure of unphosphorylated *P. aeruginosa* PMM/PGM Values in parentheses are for data in the highest resolution shell. r.m.s.d., root mean square deviation.

| Data collection | Apo-deP | Apo-P | X1P-P |
|----------------|---------|-------|-------|
| Description    | D Dephosphorylated apoenzyme | Phosphorylated apoenzyme | Complex of phosphoenzyme with X1P |
| Sequence coverage (%) (maximal exchangeable amides) | 97 (391) | 92 (369) | 87 (348) |
| Final deuteron (range) (%) | 47 (9–82) | 36 (9–67) | 28 (5–51) |
tional fluctuations) but is followed by relatively parallel increases in deuterium content for both samples. In contrast, the incorporation rates of peptides 407–429 and 442–463 (both in D4) in the two samples are similar at earlier time points but diverge later with the apo-deP form gaining in relative deuterium content, possibly reflecting differences in folding stability of this domain. As several of the most affected peptides lie near the D3-4 interface (Fig. 2A), a known site of conformational change in the enzyme (7), it seems likely that the apo-P and apo-deP samples differ either in their access to certain conformers or in their equilibrium populations of conformers in solution. The existence of multiple conformers for PMM/PGM is supported by crystal structures (Fig. 2A) (7, 8, 26) and by NMR studies showing that D4 has higher relative mobility in solution than the rest of the protein (14).

A comprehensive view of peptides affected by phosphorylation throughout the time course (not just at the final time point as in Fig. 4) may be seen in supplemental Fig. S3A. Peptides with differential incorporation of >30% in deuterium content at the first (pink) or final time point (blue). However, in the crystal structure of apo-PMM/PGM, the only direct contacts (<3.1 Å) made by phosphoserine 108 are to Lys-118 and the active site metal (Fig. 2B). Hence, most of the changes in deuterium exchange within the active site are not easily attributable to direct effects from phosphorylation (e.g. peptides 267–293, 323–346, and 407–429; supplemental Fig. S3A). These are among several loops with internal motions suggested by NMR (14), hinting at a role for phosphorylation in modifying the conformational flexibility of loops within the active site cleft. We note that a number of the affected peptides are >10 Å away from the site of phosphorylation and outside of the active site (e.g. peptides 134–152, 192–204, and 442–463; supplemental Fig. S3A). Hence, in addition to the more localized effects at the active site, dephosphorylation appears to result in an increase in flexibility at much more distal sites of the protein.

Effects of Ligand Binding Are Localized Near the Active Site and Domain 4 Interface—The effect of the substrate analog X1P on deuterium incorporation in PMM/PGM was also assessed. In general, the effects were more modest than those due to phosphorylation (Figs. 3A and 4 and supplemental Fig. S2B), although multiple peptides in the enzyme show changes upon ligand binding. Some regions affected by binding of X1P...
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FIGURE 4. Differential deuterium incorporation on structure of PMM/PGM and in peptide time courses. A, difference in percentage of relative deuterium content shown by peptide between apo-deP versus apo-P (left) and apo-P versus X1P-P (right) samples at the final time point of ~1200 min. Color indicates a difference: red, >30%; orange, 30%; yellow, <20%; blue, <10%; gray indicates missing peptide. Peptides with spheres have the greatest difference between sample pairs: left, peptides 267–293, 407–429, and 442–463; right, peptides 294–303, 378–406, and 432–441. The green arrow in the left panel highlights phosphoserine residue. B, time courses of deuterium incorporation for selected peptides from A with contrasting rates and level of incorporation between samples: apo-deP, black circles; apo-P, red circles; X1P-P, blue circles. The maximum value on the y axis corresponds to the total possible number of exchangeable deuteriums. boxed numbers show location of peptides in A and their corresponding time courses in B.

are the same as those affected by phosphorylation (e.g. Fig. 4B, peptides 1 and 3). In these cases, the presence of ligand results in additional protection from exchange relative to the apo-P sample occurring at both short and long time scales depending on the fragment.

A number of the peptides (six of 11 total) with differential exchange in the apo-P and X1P-P samples encompass loops within the active site and include residues that make direct contacts with X1P in the crystal structure of the enzyme-inhibitor complex (supplemental Fig. S3B, highlighted peptides) (27). Hence, differential exchange in these cases can be attributed to steric obstruction of solvent access within the active site due to binding of X1P. This likely also accounts for differential exchange of peptides at the interface of D3-4 (e.g. peptides 267–293, 294–303, and 432–441), which is affected by the domain rotation known to occur upon ligand binding (Fig. 2A) (7). Indeed, one of the peptides with a time course of exchange significantly affected by both phosphorylation and X1P (peptide 267–293; Fig. 4B) includes Lys-285, a key residue that participates in both ligand contacts and a network of co-evolving residues in the D3–4 interface (13).

Other peptides with exchange differentially affected by X1P (supplemental Fig. S3B) do not include residues in the active site (e.g. peptides 169–191, 350–370, etc.). Thus, binding of X1P also results in long range effects but less extensively than those observed due to phosphorylation of the enzyme. One interesting case is peptide 378–406 (Fig. 4B, peptide 2), which is one of two helices in D4 that exhibit contrasting effects in the three samples, that is affected by X1P binding but not by phosphorylation. The other helix, peptide 442–463 (Fig. 4B, peptide 4), shows little effect from X1P but significant effects from phosphorylation. The net result from the combined effects of ligand binding and phosphorylation is significant protection from exchange for D4 (Fig. 4A, red and orange spheres). As the isolated D4 fragment of PMM/PGM is notably well folded in solution (28, 29), this seems most consistent with a decrease in its conformational mobility in the phosphorylated, ligand-bound samples.

SAXS Supports Increased Flexibility of Unphosphorylated PMM/PGM in Solution—To further assess the solution behavior of PMM/PGM, we used SAXS to analyze samples corresponding to those characterized by HDXMS (“Experimental Procedures”). Guinier plots exhibit good linearity and yield radii of gyration \(R_g\) ranging from 24.1 to 25.9 Å (Table 3). Calculations of the pair distribution function \(P(r)\) yielded estimates for \(R_g\) from 24.7 to 26.0 Å with a consistent maximum particle dimension of 90 Å for all samples. Although differences among the samples are small, the trend from both calculations is the same with the apo-deP sample showing the largest \(R_g\). On a \(P(r)\) plot, the apo-deP sample exhibits broadening relative to the others (Fig. 5A); this behavior has been correlated with an increase in conformational flexibility in other systems (30–32).

In an alternative view of the data, plateaus observed for each of the samples on a Porod-Debye plot (Fig. 5B) are indicative of well folded particles with sharp scattering contrasts. The differing levels of the plateaus, however, suggest particles with discrete conformational behavior. For the apo-P and apo-deP samples, the difference between plateaus corresponds to an increase in molecular volume from 66,510 to 70,106 Å\(^3\). Hence, the SAXS data supports a model of increased conformational flexibility in solution for apo-deP PMM/PGM. The effect of phosphorylation is also reflected in the thermal stability of PMM/PGM with apparent \(T_m\) values of 62 and 66 °C for the apo-deP and apo-P samples, respectively.

TABLE 3
Summary of statistics from SAXS measurements

| Sample     | Apo-P   | Apo-deP | X1P-P   |
|------------|---------|---------|---------|
| \(R_g\) from Guinier (Å) | 25.9 ± 0.2 | 24.7 ± 0.10 | 24.1 ± 0.1 |
| \(R_g\) from \(P(r)\) (Å) | 26.0 ± 0.1 | 25.2 ± 0.3 | 24.7 ± 0.2 |
| \(R_g\) calculated (Å) | 22.1 (4MRQ) | 22.0 (1K35) | 21.6 (2H5A) |
| Porod volume (Å\(^3\)) | 70,106 | 67,510 | 63,775 |

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Dephosphorylation Has Minimal Effects on Crystal Structure of PMM/PGM—The previously determined crystal structure of wild-type apo-PMM/PGM contains phosphorylated enzyme (33). To assess the effect of dephosphorylation on the crystallographic structure of the apoenzyme, the protein was crystallized, and a data set was collected to 1.9 Å (Table 1 and “Experimental Procedures”). Comparison of this structure (Protein Data Bank code 4MRQ) with that of the phosphoenzyme (Protein Data Bank ID code 1K35) shows only minor differences both overall and within the active site cleft (Fig. 5C). The Cα root mean square deviation is 0.51 Å over 455 amino acids, and the calculated Rg is essentially the same as that of the phosphoenzyme (Table 3). Some modest changes in side chain orientations and alternate conformations are present, but because of the differing resolutions (1.9 versus 2.2 Å) of the two structures, these cannot be clearly attributed to dephosphorylation. In short, the increased structural flexibility of the apo-deP enzyme in solution is not reflected in its crystal structure, presumably due to packing constraints of the crystal lattice.

DISCUSSION

Our results show that both ligand binding and phosphorylation of the catalytic phosphoserine affect the overall flexibility of PMM/PGM in solution. Accessibility of amide hydrogens to solvent exchange in the three states of the enzyme is differentially affected in multiple areas of the protein and at varying times. Effects on peptides near the active site cleft and the D3-4 interface are common. Overall and unexpectedly, the differential effects of phosphorylation are greater in magnitude than those due to ligand binding and extend to more distant regions of the protein. The SAXS studies support the notion that the unphosphorylated enzyme has a significant overall increase in conformational flexibility (or in the relative population of more “open” conformers) compared with the phosphorylated state. Of course, phosphorylation as a post-translational modification is well known to exhibit long range effects in regulatory and allosteric proteins, sometimes via changes in protein flexibility (34–40). We are unaware, however, of previous studies showing these affects manifested by a catalytic phosphoryl group involved in a phosphotransfer reaction.

The HDXMS and SAXS data show differential effects potentially related to changes in the dynamics of active site loops, rotational mobility of D4, overall structural compaction, and in the nature of the conformational ensemble(s) present in solution. Some of these effects can be rationalized by previous analyses of the D4 conformational change. Using available crystal structures of PMM/PGM, both principal component and normal mode analyses (11, 14) suggest that the rotation of D4 occurs via a hinge type motion. Therefore, it is simple to envision that the rigid body motion of D4 that produces the closed, ligand-binding conformer of the protein can create a more open conformer as well. The population of this wide open conformer could be enhanced upon dephosphorylation, possibly due to a loss of electrostatic attraction between the phosphate group and positively charged residues in D4 (7). This is consistent with the observed increase in deuterium exchange for D4 and residues near its interface in the dephosphoenzyme. Conversely, in the case of the phosphoenzyme, electrostatic attraction between the phosphate and D4 could decrease the mobility of this domain and increase the overall structural compaction of the enzyme. Binding of the substrate analog X1P appears, generally, to enhance the observed effects of phosphorylation, consistent with a further shift in the conformational ensemble toward a closed, ligand-bound conformer. Both dephosphorylation and X1P binding also result in long range effects (without
clear structural explanations) that could be mediated by energetic or dynamic residue networks (41).

The striking overall increase in flexibility of the unphosphorylated enzyme leads us to propose that it plays a role in catalytic mechanism of PMM/PGM and by analogy that of other $\alpha$-D-phosphohexomutases. The active sites of these enzymes are exquisitely designed to accommodate substrate and product in two distinct orientations within a single binding site, an arrangement necessary to complete the two phosphoryl transfers at different positions of the phosphosugar (7). An important unresolved question is what triggers the release of the intermediate from its nearly enclosed binding site (as present immediately following the initial phosphoryl transfer from enzyme) into a loosely associated state that permits its reorientation and allows it to rebind in the opposite orientation and form product. Previous crystallographic characterizations of PMM/PGM led to the suggestion that conformational fluctuations of D4 while it retains its contacts with ligand might help initiate this process (8). Based on the results presented here, we propose that reorientation of the intermediate is facilitated by a global increase in structural flexibility of the unphosphorylated enzyme (including especially D4) that occurs concomitantly with the first phosphoryl transfer reaction. Dephosphorylation may serve in essence to loosen the grip of the enzyme on the intermediate, reducing the energetic penalty of its reorientation. The most compelling feature of this hypothesis is the coupling between phosphoryl transfer and the increase in the structural dynamics of the enzyme at the precise moment it is needed in the catalytic cycle.

One caveat to this proposal is that the apo-deP sample may not be fully representative of the dephosphoenzyme that occurs in the middle of the multistep reaction because kinetic studies have shown that the bisphosphorylated sugar intermediate remains loosely associated with PMM/PGM during catalysis (4). However, the association of the enzyme with reorienting intermediate is necessarily transient, and at some point, all direct contacts between the two must be released. Therefore, we argue that the apo-deP sample is a reasonable approximation of the enzyme during this step of the reaction.

In combination with previous structural information, the HDXMS and SAXS studies help illuminate the conformational landscape of the multistep reaction of PMM/PGM. The accumulated data suggest that the phosphoenzyme fluctuates between two major conformers, which we designate $E^p_{\text{open}}$ and $E^p_{\text{closed}}$. $E^p_{\text{open}}$ has a wide and accessible central cleft, which enables access to the active site for substrate binding and product release (33). In contrast, $E^p_{\text{closed}}$ has a nearly enclosed substrate binding site that is reorganized for phosphoryl transfer (7). The conformers of the phosphoenzyme observed in various crystal structures of PMM/PGM are consistent with the proposed functional roles of $E^p_{\text{open}}$ and $E^p_{\text{closed}}$ although in solution, it is likely that these two conformers are part of a larger ensemble. The dephosphoenzyme, however, appears to occupy different regions of the available conformational landscape as indicated by its global increase in flexibility. This flexible state ($E_{\text{flex}}$) seems optimized to encourage release and reorientation of the intermediate as proposed above. Unlike $E^p_{\text{open}}$ and $E^p_{\text{closed}}$, the unique behavior of $E_{\text{flex}}$ is not apparent from the crystal structure of the dephosphoenzyme but is distinct in both its SAXS and HDXMS profiles. A more detailed view of $E_{\text{flex}}$ may be provided by ongoing NMR studies of PMM/PGM that should add to the insights emerging from the use of orthogonal methodologies on this system.

In summary, we found that the solution flexibility of PMM/PGM is highly affected by the phosphorylation state of its catalytic phosphoserine. This residue is strictly conserved in related proteins, and thus increased flexibility concomitant with phosphoryl transfer may be a theme common to its large enzyme superfamilly. It will also be intriguing to see whether other, unrelated phosphotransfer enzyme families utilize changes in protein flexibility linked to dephosphorylation during their catalytic cycles.

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