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Biology, mechanism, and structure of enzymes in the α -D-phosphohexomutase superfamily

Kyle M. Stiers, Andrew G. Muenks, and Lesa J. Beamer

Biochemistry Department, University of Missouri, Columbia, MO 65211

Abstract

Enzymes in the α -D-phosphohexomutases (PHM) superfamily catalyze the reversible conversion of phosphosugars, such as glucose 1-phosphate and glucose 6-phosphate. These reactions are fundamental to primary metabolism across the kingdoms of life, and are required for a myriad of cellular processes, ranging from exopolysaccharide production to protein glycosylation. The subject of extensive mechanistic characterization during the latter half of the twentieth century, these enzymes have recently benefitted from biophysical characterization, including X-ray crystallography, NMR, and hydrogen-deuterium exchange studies. This work has provided new insights into the unique catalytic mechanism of the superfamily, shed light on the molecular determinants of ligand recognition, and revealed the evolutionary conservation of conformational flexibility. Novel associations with inherited metabolic disease and the pathogenesis of bacterial infections have emerged, spurring renewed interest in the long-appreciated functional roles of these enzymes.

Keywords

Isomerase; phosphosugar; phosphorylation; crystal structure; enzyme mechanism; conformational change; protein flexibility; oligomers; inherited disease; missense variants

1. Introduction

The α -D-phosphohexomutases (PHMs) comprise an ancient and ubiquitous enzyme superfamily, and are found in all three kingdoms of life. These proteins play key roles in many cellular processes, including carbohydrate metabolism, sugar nucleotide biosynthesis, as well as glycolipid, exopolysaccharide, and peptidoglycan synthesis. All organisms, even simple bacteria, appear to encode at least one member of the superfamily in their genomes. Enzymes in the superfamily share highly conserved catalytic residues and a four-domain structural organization. Nevertheless, overall sequence diversity within the superfamily is high, reflecting its ancient evolutionary origins, and consistent with adaptation of these enzymes for widely varied biological roles (Whitehouse, Tomkins, Lovegrove, Hopkinson, & McMillian, 1998).

*Corresponding author: Lesa J. Beamer, Office: 573-882-6072, Fax: 573-884-4812, beamerl@missouri.edu.

Enzymes in the PHM superfamily are typically cytoplasmic and catalyze a reversible, intramolecular phosphoryl transfer on their phosphosugar substrates (Shackelford, Regni, & Beamer, 2004). Often this interconverts the 1- and 6-phospho forms of hexoses,

although one branch of the family prefers pentose phosphosugars as substrates (Maliekal, Sokolova, Vertommen, Veiga-da-Cunha, & Van Schaftingen, 2007). Active enzyme is phosphorylated on a conserved serine residue and binds a divalent metal ion, usually Mg^{2+} . As the name implies, enzymes in the superfamily are highly specific for the conversion of the α -anomers of D-phosphosugars, and are structurally and mechanistically unrelated to the β -D-phosphoglucosmutases, which are part of the haloacid dehalogenase superfamily (Burroughs, Allen, Dunaway-Mariano, & Aravind, 2006).

Unlike some other enzyme superfamilies, where the same structural scaffold has been adapted for differing catalytic mechanisms (Rauwerdink & Kazlauskas, 2015), the sequence diversity of the PHMs is primarily associated with different preferences/specificities for their phosphosugar substrates. Four major sub-groups are distinguished that utilize the related phosphohexoses of glucose, mannose, glucosamine, and *N*-acetylglucosamine (Shackelford et al., 2004). The diversity of these phosphosugar substrates/products allows these enzymes to participate in a multitude of metabolic pathways in microorganisms, plants, and animals.

Here we provide an overview of the biology, mechanism, and structure of this widespread enzyme family, with an emphasis on recent studies. In addition to intriguing mechanistic features, several proteins in the superfamily have emerged as model systems for understanding fundamental relationships between protein structure and function, including the role of conformational change and protein flexibility in catalysis. Moreover, recent biological and clinical studies have shed new light on the importance of PHMs to human health and disease, in bacterial pathogenesis, and for efforts such as metabolic engineering.

2. Biology

The biological roles of the PHMs are as varied as the organisms in which they are found, and too numerous to describe comprehensively. Specific functional roles are determined by their substrate preferences, which in turn define the various sub-groups of the superfamily. Sec. 2.1 presents an overview of the sub-groups and is followed by highlights of two topics: known roles for these enzymes in bacterial pathogenesis (Sec. 2.2) and the five PHM paralogs in the human genome and their relevance to inherited disease (Sec. 2.3).

2.1 Sub-groups of the superfamily

The four main sub-groups of the PHM superfamily are traditionally distinguished according to their substrate preferences (Table 1). Four related phosphosugars are utilized as substrates: glucose, mannose, glucosamine, and *N*-acetylglucosamine (Shackelford et al., 2004). These sugars are structurally similar, differing only in the substituent at the 2'-position of the sugar ring (Fig. 1A). Enzymes in the phosphoglucosmutase (PGM) sub-group have high specificity

for glucose-based phosphosugar substrates, while those in the phosphomannomutase/phosphoglucomutase (PMM/PGM) sub-group have dual substrate specificity for glucose and mannose phosphosugars. The phosphoglucosamine mutase (PNGM) sub-group uses glucosamine-based phosphosugars, while the *N*-acetylglucosamine phosphomutases (PAGM) prefer *N*-acetylglucosamine phosphosugars. While each sub-group has distinct substrate preferences, overlap in substrate usage between the sub-groups is also apparent. For example, in addition to the PGMs, it appears that enzymes in the other three sub-groups are also able to utilize glucose-based phosphosugars as substrates, at least to some extent (Boles, Liebetrau, Hofmann, & Zimmermann, 1994; L Jolly et al., 1999; Kato, Mueller, Wessely, Lan, & Christensen, 2005) (Sec. 4.3.6).

Categorization of proteins in the superfamily into one of the four sub-groups can be challenging, especially without supporting biochemical data. However, as more sequences for the superfamily have become available and more proteins have been studied, some sequence and structural features have begun to emerge that can assist with categorization. One of these is the sugar-binding loop, a key region in the active site of the PHMs, along with the catalytic phosphoserine and metal-binding loop (Fig. 1B and 2). Unlike the latter two regions, which are nearly identical across the superfamily, the sugar-binding loop tends to vary in sequence according to sub-group (Table 1) due to contacts between residues in this loop and the sugar ring of the substrates (Sec. 4.3.3). Phylogenetic distribution and sequence length also can help differentiate among the sub-groups. A discussion of these distinguishing features is included in Sec. 2.1.1-4.

Several other less well-characterized sub-groups in the PHM family are also known, including the eukaryotic phosphopentomutases [not to be confused with the phosphopentomutases related to alkaline phosphatase (Galperin, Koonin, & Bairoch, 1998)] and the glucose 1,6-bisphosphate synthases. More information on these two sub-groups is found in Sec. 2.3. A number of other superfamily members that either lack catalytic activity (Moiseeva, Belkin, Spurr, Koteliensky, & Critchley, 2004; Müller et al., 2002) or are highly divergent in sequence have been reported (Mitra, Cui, Robbins, & Samuelson, 2010), but are not discussed further herein.

2.1.1 Phosphoglucomutases—The PGM sub-group is the most widely distributed evolutionarily, with representatives easily identified in bacteria and eukaryotes, including plants and animals. PGMs appear to be quite specific for glucose, even relative to the closely related mannose, which differs only in the stereochemistry of its 2'-hydroxyl group (Fig. 1A). Rabbit PGM, for example, shows a 6000-fold preference for glucose phosphosugars over their mannose counterparts (Lowry & Passonneau, 1969). Along with a preference for glucose-based substrates, other features common to PGM enzymes are a sequence length of ~550 residues and the -GEESF- sequence motif of the sugar-binding loop (Table 1 and Fig. 2).

The role of PGM in glucose metabolism has been appreciated for decades. In humans, the enzyme PGM1 is the key regulator of cellular glucose homeostasis (Fig. 3A and Sec. 2.3). In higher plants, such as *Arabidopsis*, two (or more) isoforms of PGM exist. The plastidial isoform is essential for starch synthesis, while cytosolic isoform(s) contribute to the

syntheses of sucrose and cell wall components (Malinova et al., 2014). In bacteria, PGM is often also a contributor to cell wall and polysaccharide capsular production (Sec. 2.2).

Our mechanistic understanding of PGM is derived from studies of the enzyme from rabbit muscle (97% identical to human PGM1), which was extensively characterized by Ray and coworkers over several decades [see (W. J. Ray, Burgner, & Post, 1990) and references therein]. These studies remain the foundation for understanding enzyme mechanism of the entire superfamily (Sec. 3). More limited kinetic studies of PGMs from other sources have also been conducted, including from *E. coli* (Lu & Kleckner, 1994), *Acetobacter xylinum* (Brautaset, Petersen, & Valla, 2000), *Entamoeba histolytica* (Ortner, Binder, Scheiner, Wiedermann, & Duchêne, 1997), wheat (Davies, Tetlow, Bowsher, & Emes, 2003), Arabidopsis (Periappuram et al., 2000), and maize (Manjunath, Lee, VanWinkle, & Bailey-Serres, 1998)

2.1.2 Phosphomannomutase/phosphoglucomutase—PMM/PGMs are primarily found in bacteria, although archaeal representatives have also been identified (Akutsu et al., 2005; W. K. Ray et al., 2005). These proteins can utilize both glucose and mannose-based substrates with approximately equal efficiency (L. Naught & Tipton, 2001) and are usually ~450 amino acids in length. The sugar-binding loop of PMM/PGMs often contains a –GEMSG– sequence motif (Table 1 and Fig. 2).

Due to their dual substrate specificity, PMM/PGMs can serve multiple metabolic roles. This is perhaps best exemplified by the case of PMM/PGM from the opportunistic human pathogen, *Pseudomonas aeruginosa*. The product of the AlgC gene, PMM/PGM from this organism plays multiple roles in rhamnolipid, lipopolysaccharide (LPS), and alginate biosynthesis, as well as in the production of Psl and Pel, two critical biofilm matrix exopolysaccharides (Govan & Deretic, 1996; Ma et al., 2012; Rocchetta, Burrows, & Lam, 1999; Ye, Zielinski, & Chakrabarty, 1994). *P. aeruginosa* PMM/PGM provides the common precursor mannose 1-phosphate for alginate and B-band LPS, while providing glucose 1-phosphate for the synthesis of rhamnolipid and the LPS-core. PMM/PGMs from other organisms are similarly involved in the synthesis of polysaccharides and other extracellular polymeric substances (Akutsu et al., 2005; Kim et al., 2003).

Curiously, although PMM/PGMs can effectively utilize both glucose and mannose-based substrates, many bacteria have both a PGM and a PMM/PGM (e.g., for *Escherichia coli* the pgm and manB genes), suggesting either a benefit from this functional redundancy, or that these enzymes occupy different biosynthetic niches within the organism.

2.1.3 Phosphoglucosamine mutase—Enzymes in the PNGM sub-group are generally found in bacteria, although some archaeal proteins also appear to belong to this sub-group (Namboori & Graham, 2008). These proteins are also typically ~450 residues in length, and have a –GE(N/Q)SG– sequence motif in their sugar binding loop (Table 1 and Fig. 2) (Mehra-Chaudhary, Mick, & Beamer, 2011).

PNGMs participate in the cytoplasmic portion of the UDP-*N*-acetylglucosamine biosynthetic pathway, which provides critical precursors for peptidoglycan biosynthesis

(Barreteau et al., 2008). Although eukaryotes also synthesize UDP-*N*-acetylglucosamine, the bacterial pathway differs from the eukaryotic one, making these proteins attractive targets for antimicrobial design (Barreteau et al., 2008; Gautam, Vyas, & Tewari, 2011).

Overall, the PNGMs are less well studied than the other sub-groups of the superfamily. A few have been biochemically characterized, including proteins from *Escherichia coli* (L Jolly et al., 1999), *Helicobacter pylori* (De Reuse, Labigne, & Mengin-Lecreulx, 1997), *S. aureus* (Laure Jolly et al., 1997), *S. gordonii* (Shimazu et al., 2008), *Methanococcus maripaludis* (Namboori & Graham, 2008), and *Mycobacterium tuberculosis* (S. Li et al., 2012).

2.1.4 Phospho-*N*-acetylglucosamine mutase—The PAGM enzymes are exclusively eukaryotic and typically 550 residues in length. They share the highly conserved catalytic and ligand binding residues of the PHM superfamily, but have a sub-group specific circular permutation within domain 1 of the protein (Nishitani et al., 2006). This can make them more difficult to identify as a member of the superfamily based on sequence searches. The sequence motif of the sugar-binding loop in the PAGMs is distinct: –FEANG– (Bandini et al., 2012; Kato et al., 2005) (Table 1 and Fig. 2), with the asparagine reflecting a role in recognizing the *N*-acetyl group of the 1-phospho form of the sugar (Nishitani et al., 2006).

Like the PNGMs, these enzymes are also involved in the synthesis of UDP-*N*-acetylglucosamine, via the distinct eukaryotic pathway (Barreteau et al., 2008). Essential in organisms from fungi to mice (Fang et al., 2013; Greig et al., 2007; Hofmann, Boles, & Zimmermann, 1994), PAGM has been suggested as a target for design of anti-fungal agents. In humans, PAGM is known as PGM3, where it functions in the hexosamine biosynthetic pathway and is associated with an inherited enzyme deficiency (Sec. 2.3).

Only a few PAGM enzymes have been biochemically characterized, including the proteins from *Saccharomyces cerevisiae* (Boles et al., 1994; Hofmann et al., 1994), *Aedes aegypti* (Kato et al., 2005), mice (Greig et al., 2007), and humans (Mio, Yamada-Okabe, Arisawa, & Yamada-Okabe, 2000).

2.2 Roles in bacterial pathogenesis

Bacterial proteins in the PHM superfamily are often involved in the biosynthesis of exopolysaccharides and other exoproducts, such as glycolipids. Because of the importance of these molecules to cell wall properties, capsule production, and biofilm formation, many members of the PHM superfamily are implicated in bacterial pathogenesis (Table 2). The organisms listed include many notorious human pathogens, such as *M. tuberculosis*, *s.*, *Neisseria gonorrhoeae*, and *Staphylococcus aureus*. Effects of these enzymes on the organism were assessed through gene deletion or disruption studies, and demonstrate roles in pathogenesis for three sub-groups of the PHM superfamily (PGM, PNGM, and PMM/PGM). In addition, several bacterial strains with deletions of PGM or PMM/PGM are of interest as vaccine candidates due to their attenuated infectivity (Buchanan et al., 2005; Czibener, Del Giudice, Spera, Fulgenzi, & Ugalde, 2016; Ugalde, Commerci, Leguizamón, & Ugalde, 2003; Ugalde, Czibener, Feldman, & Ugalde, 2000).

Changes to the bacteria caused by lack of PHM activity often manifest as reduced virulence, slowed growth, increased susceptibility to antibiotics, or differences in cell morphology (Table 2). These important roles in infectivity and pathogenesis suggest that enzymes in the family could be targets for antimicrobial inhibitor design, a possibility that has not yet been realized. Effective enzyme inhibitors would be expected to mimic the effects seen on Table 2. Enzymes in the PNGM sub-group are often essential for viability in Gram-negative organisms (e.g., *E. coli*, *H. pylori*, *P. aeruginosa*, etc.), making them particularly attractive targets for inhibitor design (Barreteau et al., 2008; Gautam et al., 2011). In the case of antibiotic resistant bacterial strains, inhibitors of the PHMs might also be helpful as adjuvants to traditional antibiotic therapy (Wright, 2016).

Clinically useful inhibitors would need to avoid compromising the activity of human PHMs (Sec. 2.3). This challenge might be overcome by exploiting the substrate preferences of the sub-groups. For example, while inhibitors of bacterial PGM enzymes would likely also affect human PGM, the possibility of designing inhibitors specific to PMM/PGM or PNGMs is attractive, given the absence of these sub-groups in humans. In such efforts, the structural differences between glucose and mannose, or between glucose and glucosamine, might be co-opted for inhibitor design to provide specificity for enzymes in these two sub-groups.

It is worth noting that a full appreciation for the roles of these bacterial enzymes, even from gene deletion studies as on Table 2, is likely clouded by the existence of multiple members of the PHM superfamily in bacterial genomes. As noted earlier, *E. coli* has genes for a PGM and PMM/PGM; it also encodes for a PNGM (*glmM*). A similar situation exists for the human pathogen *P. aeruginosa*, which has genes for PGM (*pgm*), PMM/PGM (*AlgC*), and PNGM (*GlmM*). The possibility of partial functional substitution (e.g., catalysis with non-preferred substrates) between sub-groups in the superfamily has not been systematically addressed.

2.3 Human paralogs and inherited disease

2.3.1. PGM1—The human genome encodes five members of the PHM superfamily. PGM1 is the major source of phosphoglucomutase activity in most human tissues, particularly in liver and muscles where the metabolic demand is greatest. This enzyme is well known for its role in regulating glucose homeostasis in cells. It acts as the metabolic pivot point between glucose storage in the form of glycogen and glucose utilization for energy (Fig. 3A). New insights into the importance of human PGM1 in metabolism have been gained due to its recent association with an inherited metabolic disease, PGM1 deficiency. In 2014, a landmark study (Tegtmeyer et al., 2014) showed that patients with mutations of PGM1 have characteristics of both glycogen storage disease *and* a congenital disorder of glycosylation (CDG) of types I and II (affecting both sugar processing and assembly). Patient phenotypes are variable, even when comparing siblings with the same genotype, but can include dilated cardiomyopathy, hepatopathy, muscle weakness and exercise intolerance, hypoglycemia, delayed puberty, and developmental abnormalities of the head such as cleft palate (Tegtmeyer et al., 2014). These pleiotropic effects are consistent with the critical roles of human PGM1 in metabolism and its widespread expression in tissues. Given this, it is perhaps surprising that some patients with PGM1 deficiency have only mild symptoms

(Stojkovic et al., 2009). This may be due, at least in part, to multiple paralogs of PGM1 in the human genome, as discussed further below.

Mutations of various types have been associated with PGM1 deficiency, which is autosomal recessive in inheritance. These include nonsense, splicing, and missense variants. In the latter category, ~20 residues out of 562 in the protein are known to have disease-associated mutations (Fig. 3B), but this number is expected to increase as more patients are screened for the condition. Biochemical and structural analyses of PGM1 missense variants have revealed varying origins for enzyme dysfunction, including effects on both enzyme activity and protein folding (Beamer, 2015; Y. Lee, Stiers, Kain, & Beamer, 2014). X-ray crystallographic studies of several disease-related mutations of PGM1 have provided new insights into their effects on enzyme mechanism and structure, and may aid in understanding the complicated disease phenotype (Stiers, Graham, Kain, & Beamer, n.d.; Stiers, Kain, Graham, & Beamer, 2016).

2.3.2. PGM2, 2L, 3 and 5—In addition to PGM1, four other proteins in the PHM superfamily are found in humans, known as PGM2, PGM2L1, PGM3, and PGM5. Based on sequence alignments, each of these paralogs is easily categorized as a member of the superfamily, but has unique features relative to PGM1. These include tissue-dependent differences in expression, as well as differences in substrate preferences and, in one case, a distinct catalytic reaction. Compared to PGM1, all of these proteins are understudied, but recent efforts have begun to unravel their distinct functionalities and cellular roles.

PGM2 and PGM2L1 (for PGM2-like 1) have been identified as a phosphopentomutase and glucose 1,6-bisphosphate synthase, respectively (Maliekal et al., 2007). These two proteins are found primarily in mammals and are closely related in sequence to each other – 60% sequence identity – yet share only 20% sequence identity with PGM1. PGM2 has a preference for pentose phosphosugars, including ribose 1-phosphate and deoxyribose 1-phosphate, and its highest expression levels are found in lung, spleen, and thymus. It contains a conserved -FEEAIG- motif in its sugar binding loop (Whitehouse et al., 1998). PGM2L1 utilizes 1,3-bisphoglycerate and glucose 1- or 6-phosphate to form glucose 1,6-bisphosphate. This reaction is essentially one half of the two-step phosphoryl transfer reaction catalyzed by other members of the superfamily. PGM2L1 is highly expressed in the brain, where glucose 1,6-bisphosphate may play a regulatory role. Both PGM2 and PGM2L1 also have PGM activity, although at a lower level than with their preferred substrates.

Human PGM3 belongs to the PAGM sub-group of the superfamily, and thus has a preference for *N*-acetylglucosamine phosphosugars as substrates. It shares ~20% sequence identity with PGM1 and has relatively high expression in pancreas, heart, liver, and placenta (C. Li, Rodriguez, & Banerjee, 2000). PGM3 is an essential enzyme in the hexosamine biosynthetic pathway required for the production of UDP-GlcNAc (Willems, van Engelen, & Lefeber, 2016). Mutations in PGM3 were recently identified as causing an autosomal recessive immunodeficiency syndrome, combined with CDG phenotypic features (Lundin et al., 2015; Sassi et al., 2014; Stray-Pedersen et al., 2014; Zhang et al., 2014). As is the case with PGM1 deficiency, patient phenotypes in PGM3 deficiency are variable and complex.

Of the four PGM1 paralogs, PGM5 is mostly closely related in sequence (65% identity) to PGM1. It is reported to lack enzyme activity (Moiseeva et al., 2004) and has been most studied for its role as a component of the peripheral membrane cytoskeleton in muscle and some non-muscle cells (Belkin et al., 1994). Its expression is enriched in cardiac, smooth, and skeletal muscle, where it is developmentally regulated. Recently, a detailed study (Molt et al., 2014), established a role for PGM5 (also known as aciculin) in myofibril assembly, remodeling and maintenance, and described its interactions with filamin C (FLNc) and the actin-binding repeat-containing proteins XinA and XinB. PGM5 has also been identified as a binding partner for dystrophin, the protein involved in inherited muscle dystrophy, in skeletal muscle (Belkin & Burridge, 1995).

3. Catalytic mechanism

3.1. Overview

The sequence conservation of the key catalytic and ligand binding residues in the PHMs (Fig. 2) suggests that the intramolecular phosphotransfer reaction is highly conserved. To date, with the exception of the glucose 1,6-bisphosphate synthases, it appears that all superfamily members may utilize the multi-step reaction first proposed and characterized by William Ray, Jr. and coworkers, based on studies of PGM from rabbit muscle. An overview of the mechanism is illustrated in Fig. 4 with glucose 6-phosphate as substrate. (As the reaction is highly reversible, the 1- and 6-phospho forms of the sugars can be considered as either substrate or product). The reaction proceeds through the following steps: 1) binding of glucose 6-phosphate to the active, phospho-enzyme; 2) transfer of the enzyme phosphoryl group from the catalytic phosphoserine to the 1-position of the substrate, creating the bis-phosphorylated sugar intermediate and inactive dephospho-enzyme; 3) diffusional reorientation of the intermediate in the active site placing the phosphoryl group at position 6 near the active site serine; and 4) regeneration of the phospho-enzyme by transfer of the phosphoryl group at position 6 of the sugar back to the catalytic serine, and release of the product, glucose 1-phosphate. This scheme comprises the “exchange” model of the reaction, one of two possibilities initially proposed (Ray, William J, Mildvan, & Long, 1973) and subsequently confirmed by structural studies of a related enzyme (Regni, Naught, Tipton, & Beamer, 2004).

Studies of rabbit PGM span more than three decades, and produced many fundamental insights into enzyme mechanism. PGM was also instrumental in early work on the theory of induced fit (Yankeelov & Koshland, 1965). These seminal contributions to enzymology cannot be adequately reviewed herein; interested readers should refer to the publications of Ray, *et al.* In the following sections, we highlight a few recent developments in understanding the PHM mechanism.

3.2. The “flip”

One of the most mechanistically unique features of the PHM reaction is the required 180° reorientation (flip) of the reaction intermediate, which occurs in between the two phosphoryl transfers (between steps 2 and 3 in Fig. 4). As previously noted (Ray, William J & Roscelli, 1964), two kinetic models are consistent with the required chemistry: 1) The intermediate

could dissociate from the enzyme each time it is formed and rebind in a randomly determined orientation, via a ping-pong mechanism; or 2) the intermediate could reorient without completely losing its association with the enzyme, which could have advantages in terms of efficiency of turnover. The latter model was elegantly demonstrated for *P. aeruginosa* PMM/PGM using isotope trapping experiments (L. E. Naught & Tipton, 2005). This work showed that glucose 1,6-bisphosphate preferentially remains bound to the enzyme throughout the course of the reaction, dissociating only once every 15 catalytic cycles. This result is consistent with prior estimates of the frequency of dissociation of glucose 1,6-bisphosphate from rabbit PGM as occurring once per 20 catalytic cycles (Ray, William J & Roscelli, 1964). Thus, in the two best-studied enzymes in the superfamily, the reaction appears to be a simple example of processivity, as defined by multiple rounds of catalysis without release of substrate. In the case of *P. aeruginosa* PMM/PGM, specific residues in the active site were identified that affect the efficiency of the processive reaction (Regni, Schramm, & Beamer, 2006)

This unique mechanistic pathway poses considerable challenges for enzyme structure and active site evolution. First, it necessitates design of a ligand-binding site that can accommodate both 1- and 6- phosphosugars in *different* orientations (Fig. 1A, far right). Structural studies of *P. aeruginosa* PMM/PGM (Regni et al., 2004) revealed the elegant solution to this problem, which relies on conserved contacts to the phosphate group of bound ligand as well as the specific stereochemistry of glucose-based phosphosugars (Sec. 4.3). Second, conformational change of the C-terminal domain of the enzyme is needed, to close the active site for tight interactions with bound substrate, and open it for release of product. Third, the mechanism also requires that binding interactions between the enzyme and intermediate temporarily relax, in order to encourage reorientation of the intermediate but not its complete dissociation from the enzyme (Regni et al., 2006). Potential insights into this last challenge have emerged from biophysical studies of *P. aeruginosa* PMM/PGM, which demonstrate an intriguing connection between phosphorylation of the active site serine and protein flexibility.

3.2.1. Phosphorylation-dependent protein flexibility in enzyme mechanism—

Due to its amenability to rigorous sample and methodological demands, *P. aeruginosa* PMM/PGM has been the subject of extensive structural and biophysical characterizations. During nuclear magnetic resonance (NMR) studies of this enzyme, which are conducted at relatively high temperatures (37° C), it became apparent that multiple residues existed in two states (populations) in solution (Sarma et al., 2012). Additional work implicated >40 residues as being affected by the phosphorylation state of the protein. Subsequently, the phospho- and dephospho-states of the enzyme were characterized by hydrogen-deuterium exchanged (HDX) mass spectrometry (MS) and HDX-NMR (Y. Lee, Villar, Artigues, & Beamer, 2014; Xu, Lee, Beamer, & Van Doren, 2015). Surprisingly, widespread and significant changes in protein flexibility related to phosphorylation were observed, affecting >100 of the 450 residues in the enzyme, despite the modest chemical difference of a single phosphorylation site. In particular, the dephospho-form of the enzyme, which corresponds to the state of the protein present during the reorientation of the intermediate (steps 2–3 in Fig. 4), was found to be significantly more flexible, based on an overall increase in deuterium

exchange (Y. Lee, Villar, et al., 2014). HDX-NMR studies also show a smaller set of residues with decreased exchange, corresponding to a decrease in flexibility (Xu et al., 2015). In addition to these HDX studies, several traditional biochemical assays further support phosphorylation-dependent changes in protein flexibility, with the dephospho-protein being more flexible overall (Xu et al., 2015).

The correlation between dephospho-enzyme and increased structural flexibility was intriguing, since the dephospho-enzyme is present at the point of the reaction when reorientation of the intermediate occurs (between steps 2 and 3 in Fig. 4). Based on this observation, it was proposed that the increased structural flexibility might facilitate release of high affinity interactions with the bound intermediate, thereby encouraging its reorientation (Y. Lee, Villar, et al., 2014). In other members of the superfamily, similar biochemical evidence is mounting for phosphorylation- dependent differences in protein flexibility (Y. Lee, Stiers, et al., 2014; Stiers, Xu, Lee, & Beamer, n.d.), suggesting that this is a conserved feature of the PHMs, and supporting its functional importance in enzyme mechanism.

3.3 Acid-base catalysis

The chemistry of phosphoryl transfer reactions has been previously studied in many systems (Knowles, 1980), and may proceed via either an associative or dissociative mechanism. In either case, the hydroxyl group that accepts the phosphoryl group must first be deprotonated, a step that is presumably facilitated by a general base. Transfer of the phosphoryl group may also be aided by protonation of the alkoxide formed by departure of the phosphoryl group through participation of a general acid. Fig. 4 shows the putative roles of the general acid-base catalysts in the conversion of glucose 6-phosphate to glucose 1-phosphate. Typically, such residues are expected to meet several criteria (Guillén Schlippe & Hedstrom, 2005), including being within hydrogen bonding distance of the position where proton abstraction or donation occurs. In addition, mutagenesis of the general-acid base is expected to significantly impair enzyme function (i.e., >1000 fold reduction in activity) in the absence of accompanying structural changes.

Until recently, candidates for residues acting as a general acid or base in the PHM mechanism were not forthcoming. However, mutagenesis and kinetic studies of *P. aeruginosa* PMM/PGM showed that a histidine (His329) in the active site is critical for enzyme activity (Y. Lee, Mehra-Chaudhary, Furdui, & Beamer, 2013). Moreover, analyses of protein-ligand complexes show that this residue is appropriately positioned to abstract a proton from the O1/O6 hydroxyl of the phosphosugar substrates, and thus may serve as the general base in the reaction. Histidine is strongly sequence-conserved at this position in the sequence of many proteins in the superfamily, except for the PGM sub-group (purple shading on Fig. 2). In the PGMs, however, a lysine is often conserved at a corresponding structural position (but not corresponding in sequence). Mutation of this lysine in PGM from *Salmonella enterica* (serovar Typhimurium) also results in significant catalytic impairment (Y. Lee et al., 2013). Thus candidate residues for the general base appear to be position-conserved within sub-groups, but may be only structurally conserved across sub-groups (PGM vs. PMM/PGM) (Fig. 2).

One candidate for the general acid is an arginine residue found near the N-terminus of the PHMs (blue star on Fig. 2) (Y. Lee et al., 2013). Although not often found in this catalytic role, some precedent exists in other enzyme systems (Keenholtz et al., 2013; Silva, Schulz, Jahn, Jahn, & Ramos, 2010). In addition to its proximity to the site of phosphoryl transfer, this residue appears to be absolutely conserved across the PHM superfamily (Fig. 2), consistent with a critical functional role (Y. Lee et al., 2013).

4. Three-dimensional structure

4.1. Structures in the PDB

Prior to the year 2000, only one enzyme in the superfamily, rabbit PGM, had been characterized by X-ray crystallography (Dai, Liu, Ray, & Konno, 1992; Lin et al., 1986; Yiwei Liu, Ray, & Baranidharan, 1997). Fortunately, over the past decade, the number of structurally characterized proteins in the PHM superfamily increased significantly. Currently, approximately 20 crystal structures of independent proteins are known, including several from each of the four major enzyme sub-groups (Table 3). In total, these comprise >40 entries in the PDB, representing various versions of the respective proteins (e.g., mutants, ligand complexes, etc.). Fifteen crystal structures are available for *P. aeruginosa* PMM/PGM, which is also the only full-length protein in the family to be characterized by NMR (Sarma et al., 2012; Xu et al., 2015).

A number of recent structures were targets of structural genomics efforts, and represent sequence-diverse members of the superfamily, such as pdb: 1TUO and 4HJH. These proteins do not segregate cleanly into one of the four major sub-groups, according to the criteria laid out in Sec. 2.1, and so are listed separately on Table 3. Some of the unique features seen in these proteins include PGM-like sequences for their sugar-binding loop (-GEESF-), but in the context of a shorter polypeptide chain (450 instead of the typical 550 residues). Several also have an arginine residue at the position of the putative general base (Sec. 3.3). It seems likely that these proteins represent distinct divisions within the known sub-groups, or perhaps even novel sub-groups of the superfamily. Clarification of these issues awaits further study.

The growing database of 3D structures provides a broad perspective on the structural similarities and differences among these enzymes. Overall, the structures show that the domain architecture and key active site regions of the enzymes have remained remarkably similar throughout evolution (Sec. 4.2-3). In addition, a unifying theme of structural flexibility of, or within, domain 4 is also apparent in the superfamily (Sec. 4.4), although the nature of the conformational change varies. Most strikingly, multiple distinct oligomeric forms have recently been identified for several proteins in the superfamily, some of which appear to be sub-group specific (Sec. 4.4).

In the following sections, we describe the structural architecture, key regions of the active site, the known oligomeric assemblies of the PHMs, and the conserved conformational mobility of domain 4.

4.2. Overall architecture

Despite considerable overall sequence diversity, the structural scaffold of enzymes in the PHM superfamily is remarkably conserved. To date, all of the known structures share a 4-domain architecture, arranged in an overall heart-shape (Fig. 1B and 5A). Each structural domain is of approximately similar size – 100 to 150 residues – and contains both α -helices and β -sheets. The active site is found within a large, positively-charged cleft found at the confluence of these four domains. Residues from each of the four structural domains contribute to the formation of the cleft, and also have roles in catalysis and/or ligand binding. Depending on the enzyme sub-group, additional secondary structural elements may be present, particularly in the longer PGM and PAGM sub-groups. These additional residues generally form secondary structural elements that decorate the periphery of the protein (e.g., pink regions in Fig. 5A).

In the structure of rabbit PGM, it was noted that domains 1-3 (D1-3) share a common topological core (Dai et al., 1992), presumably arising from a series of ancient gene duplications. This common topology for D1-3 occurs in all known structures of the superfamily, regardless of sub-group. The common core consists of a mixed α/β fold, with a central β -sheet flanked on either side by an α -helix. The same loop (between β -strands 3 & 4 of the common core) in D1-3 contributes important residues involved in catalysis or ligand binding (Regni, Tipton, & Beamer, 2002).

As noted previously, proteins in the PAGM sub-group are distinguished by a conserved, circular permutation within domain 1. This effects the sequence order of conserved secondary structural elements (swapped regions in Fig. 2), including the phosphoserine loop (Nishitani et al., 2006), but does not change their 3-dimensional location. The circular permutation of domain 1 appears to be both unique to and characteristic of the PAGM sub-group. However, it has no obvious connection to its substrate preference for *N*-acetylglucosamine phosphosugars, as all of the key catalytic and ligand binding loops are found in similar positions (Nishitani et al., 2006).

The C-terminal domain (D4) of enzymes in the PHM superfamily is structurally unrelated to the fold of D1-3. It has $\alpha+\beta$ topology and contains a TATA-binding protein-like fold, consisting of one β -sheet and two α -helices (Regni et al., 2002). Typically, the β -sheet in D4 is composed of four or more strands, although for proteins in the PNGM sub-group only three strands are found (Mehra-Chaudhary, Mick, & Beamer, 2011). In several members of the superfamily, it has been shown that D4 is an independently folded unit, maintaining a well-ordered structure in the absence of the other three domains (Schramm et al., 2010; Wei et al., 2014) (see also pdb: 1WJW). This appears to be consistent with the conformational variability of this domain relative to the others that has been noted in multiple proteins in the superfamily (Luebbering et al., 2012).

4.3. Key regions of active site

The active site of the PHMs can be segregated into four highly conserved regions that contribute to catalysis and substrate binding (Fig. 2, 5B-C). These regions are: i) the phosphoserine residue that participates in phosphoryl transfer; ii) the metal-binding loop; iii)

a sugar-binding loop; and iv) the phosphate-binding site that interacts with the phosphate group of the substrate. It is important to note the difference between the phosphoryl transfer site (i), which involves the phosphorylated serine of the *enzyme*, and the phosphate-binding site (iv), a group of residues that interacts with the phosphate moiety of the *substrate*. Each of these key regions is found on a loop/turn of the polypeptide chain, and is located in a different structural domain of the enzyme: region (i) in domain 1, region (ii) in domain 2, etc. (Fig. 1B). Thus, the active site relies on residues from all four structural domains for its integrity.

4.3.1. Phosphoserine loop—The catalytic reaction of the PHMs proceeds via transfer of a phosphoryl group (PO_3^{2-}) from a conserved serine in domain 1 to the substrate (Fig. 5B,C). Hence this residue and the geometry of the loop in which it resides are critical for efficient catalysis. Indeed, the residues surrounding this serine (TASHNP) are very highly conserved in the α -D-phosphohexomutase superfamily (region I in Fig. 2). In crystal structures of the PHMs, this serine may be phosphorylated or not (Table 3) depending on the protein and conditions used for crystallization. [The phosphorylation level of the serine is known to be affected by ligands and other experimental variables in several proteins in the superfamily (Y. Lee, Furdui, & Beamer, 2017; Y. Lee, Stiers, et al., 2014; Sarma et al., 2012; Xu et al., 2015)].

The catalytic serine is located in a rare, type II β -turn, with backbone angles that fall in the disallowed region of the Ramachandran plot, and are similar to those of a “nucleophilic elbow”, a structural motif also found in α/β hydrolases (Ollis et al., 1992; Regni et al., 2002). The loop containing the phosphoserine is anchored by extensive inter-residue interactions, which presumably serve to stabilize it in the appropriate conformation for catalysis. Among these interactions are multiple contacts to a highly conserved arginine that participates in a buried salt-bridge with a nearby aspartate (Asp62 and Arg64 in human PGM1) (Beamer, 2015; Yiwei Liu et al., 1997).

4.3.2. Metal-binding loop—All enzymes in the α -D-phosphohexomutase superfamily require a divalent metal ion for maximal activity. In most reports, maximal enzyme activity is achieved with Mg^{2+} , although other metals have been reported to also produce activity, including Mn^{2+} (Akutsu et al., 2005; Namboori & Graham, 2008; Ray, William J, 1969; Ye et al., 1994). The binding site for the metal ion is located proximal to the phosphoserine in the active site (Fig. 5B,C). Three highly conserved aspartate residues (region II in Fig. 2), found in a loop of domain 2, serve as ligands. An oxygen atom from the phosphate group of the phosphoserine also coordinates the metal ion. The positive charge of the metal ion acts as an electron-withdrawing agent, facilitating breakage of the P-O bond necessary for phosphoryl transfer. Hence the phosphoserine and metal-binding loop are intimately connected in the active site, and function together during catalysis.

The metal binding loop is located in a tight turn found deep in the active site cleft. It often includes the sequence motif: -DGDGDR- (Fig. 2). However, new structural representatives have revealed that other residues may take the place of the two glycines (G1 and G2), including alanine and proline for G1, and alanine, phenylalanine, and tyrosine for G2. Regardless of this variation in sequence, the geometry of the loop remains invariant. A

number of crystal structures in the PHM superfamily were found to lack a bound metal (Table 3). This appears to result from the low pH of the crystallization conditions, which protonates the aspartates and interferes with metal binding. Somewhat surprisingly, only a small rearrangement of the aspartate side chains are necessary to accommodate loss of metal; the overall geometry of the loop is unperturbed (Mehra-Chaudhary, Mick, & Beamer, 2011).

4.3.3. Sugar-binding loop—Domain 3 contains another key region in the active site of the PHMs, the sugar-binding loop (Fig. 5B,C). Residues in this loop are expected to make direct hydrogen bonds with the sugar moiety of the substrate, and have been proposed to play a role in substrate specificity (Shackelford et al., 2004). This loop contains five highly conserved residues that vary somewhat according to sub-group (Table 1; region III in Fig. 2), but typically includes a 3-residue sub-motif of Glu-X-Ser (or Asn in PAGM). For the two proteins in the superfamily with available high-resolution enzyme-ligand complexes, *P. aeruginosa* PMM/PGM and *C. albicans* PAGM, the conserved Glu is seen to make a bidentate interaction with the O3 and O4 hydroxyls of the sugar ring (Fig. 5B,C), while the Ser (or Asn) makes additional contacts to these hydroxyls, depending on whether the 1- or 6-phosphosugar is bound (Sec. 4.3.5).

4.3.4. Phosphate-binding loop—Contacts between the protein and the phosphate group of bound substrate are largely provided by residues in a loop of D4 (region IV in Fig. 2), although one or more residues in domain 1 may also be involved (e.g., see the tyrosine in Fig. 5B). The phosphate-binding site appears designed to anchor bound substrate in the active site cleft via multiple interactions with the phosphate group. Other conserved residues that contact the phosphate include an arginine, serine and threonine (Fig. 5B,C). These protein-phosphate contacts are nearly identical regardless of whether 1- or 6-phosphosugars are bound (Regni et al., 2004). The phosphate-binding site appears to be a key determinant of ligand recognition, as enzyme-ligand complexes adopting a half-closed conformer (with ligand partially exposed to solvent) retain most of the contacts to phosphate, even while those to the sugar hydroxyls are lost (Regni et al., 2006).

Often, binding of ligand and formation of the phosphate-binding site requires motion of, or within, D4. This changes the active site from an open cleft to a deeply buried pocket that encloses substrate and positions it for catalysis. In some members of the superfamily, a rotation of D4 is involved in creating the phosphate-binding site (Regni et al., 2004); in others, movement of the phosphate-binding loop appears to be sufficient (Müller et al., 2002; Nishitani et al., 2006).

4.3.5. Binding of 1- vs. 6-phospho sugars—As noted in Sec. 3, the exchange mechanism for the PHMs raises questions about how a single catalytic site can accomplish phosphoryl transfer to both the 1- and 6-hydroxyls of the sugar. Enzyme ligand complexes for both 1-phospho and 6-phospho forms of the sugar substrates, first determined for *P. aeruginosa* PMM/PGM (Regni et al., 2004) and subsequently for *C. albicans* PAGM (Nishitani et al., 2006), showed that this occurs through binding of the sugar ring in two different orientations (Fig. 1A, far right). In this superposition, which shows these two sugars as found in the active site of their respective complexes with *P. aeruginosa* PMM/

PGM, it can be seen that the phosphate groups of glucose 1-phosphate and glucose 6-phosphate are in nearly identical positions, resulting in a 180° difference in the orientation of the sugar ring.

One key to recognition of the two orientations of the sugar ring is a conserved set of contacts to O3 and O4 hydroxyls (Regni et al., 2004). Because these hydroxyls are both equatorial in their stereochemistry, these atoms exchange places when the sugar ring is rotated by 180°. This allows the contacting residues to simply switch from O3 to O4 and vice versa, depending on whether the 1- or 6-phosphosugar is bound. A similar exchange could not occur for sugars where these hydroxyls are in axial positions (e.g., galactose), since they would occupy very different positions after reorientation. It appears that these exchangeable contacts with O3 and O4 are another key to recognition of substrates in the entire PHM superfamily, as the O3 and O4 hydroxyls and their stereochemistry are common to four major hexose substrates of the PHMs (Fig. 1A), and the key residues in the sugar-binding loop (Sec. 4.3.3) are also conserved.

4.3.6. Sub-group specific substrate specificity—One consequence of the two binding orientations of the 1- and 6-phospho versions of substrate is that the substituent at the 2'-position of the sugar occupies two very different positions depending on which form is bound (two green arrows on glucose 1-phosphate/glucose 6-phosphate superposition in Fig. 1A). As can also be seen on this figure, it is the substituent at the 2'-position of the sugar ring that differs among these four hexose substrates (glucose, mannose, glucosamine and *N*-acetylglucosamine) (Shackelford et al., 2004). The design of the PHM active site, which allows structural variation at the 2'-position of the sugar, appears to be advantageous for two reasons. First, it accommodates the dual binding mode of the 1- and 6-phospho sugars that is required by enzyme mechanism (Fig. 4). Second, it facilitates use of these four related sugars as substrates, which is key to the functional diversity of the superfamily.

The known crystal structures suggest that the ancestral PHM was designed for glucose-based substrates, or possibly glucose/mannose. With a PGM-like active site as a starting point, only small changes would then be necessary over the course of evolution to relax glucose specificity and create preferences for the related sugars of glucosamine and *N*-acetylglucosamine. This model is consistent with biochemical data showing that most enzymes in the superfamily characterized to date have PGM activity, regardless of sub-group (Sec. 2.1).

4.4. Quaternary structure & conformational mobility of domain 4

Until recently, enzymes in the PHM superfamily were only known to function as monomers. However, crystal structures of proteins in the PNGM and PGM sub-groups revealed apparent dimers (Fig. 6), which were subsequently confirmed in solution (Mehra-Chaudhary, Mick, Tanner, & Beamer, 2011; Mehra-Chaudhary, Mick, Tanner, Henzl, & Beamer, 2011). These observations led to a search for other potential oligomers in the superfamily, which also identified tetramers for PHMs from *P. horikoshii* (pdb: 1WQA) and *S. tokodaii* (pdb: 2F7L) (Luebbering et al., 2012).

For the PGM sub-group, a dimeric arrangement was verified for the *S. typhimurium* enzyme (pdb: 3NA5), the interface of which is formed by an N-terminal extension of the protein (interface A on Fig. 6). Sequence searches confirmed the presence of a similar motif in a sub-group of bacterial species (Mehra-Chaudhary, Mick, Tanner, Henzl, et al., 2011). For the two PNGM proteins, both known structures (pdb: 3I3W and 3PDK) share a similar dimeric arrangement, despite only moderate pairwise sequence identity between the proteins (39%). This suggests that many other PNGMs may share this assembly (Mehra-Chaudhary, Mick, Tanner, & Beamer, 2011). The dimers found in the PGM and PNGM sub-groups are structurally distinct and appear to have evolved independently (Fig. 6). However, the tetramer comprises two dimers with the PNGM arrangement, suggesting an evolutionary relationship between these two assemblies.

Examination of the confirmed oligomers of the PHMs shows that they are all assembled in a manner that permits conformational freedom of D4 (Fig. 6). This is consistent with the known functional importance of D4 motion, or of loops therein, in several proteins in the superfamily (e.g., for substrate binding/product release and during reorientation of the intermediate). Moreover, normal mode analyses of the various assemblies show that predicted low frequency global motions contribute to the conformational variability of this domain (Luebbering et al., 2012). Hence, it appears that the PHM superfamily has conserved not only residues essential for catalysis, but also the conformational flexibility of its C-terminal domain, despite considerable sequence diversity and even across varying oligomeric states. This makes the PHM superfamily an interesting case of evolutionarily conserved protein dynamics.

5. Conclusions and future directions

Recent years have witnessed tremendous gains in our knowledge and appreciation of the PHM superfamily. This includes new connections to inherited human disease, which should grow rapidly due to the availability of sequence information on disease-associated mutations. Another future area of development for these enzymes includes metabolic engineering, which was not addressed herein but is an active area of research (Anasontzis, Kourtoglou, Villas-Boas, Hatzinikolaou, & Christakopoulos, 2016; K. S. Lee et al., 2011; Yanfeng Liu et al., 2014; Nic Lochlainn & Caffrey, 2009; Rodríguez-Díaz, Rubio-del-Campo, & Yebra, 2012; Wang et al., 2016; Zabala, Braña, Flórez, Salas, & Méndez, 2013). New biochemical and structural data summarized in this review have shed light on intriguing mechanistic features of the PHMs that were previously unappreciated. These insights should facilitate future investigations of other proteins in the superfamily.

Despite significant progress, many questions remain regarding the PHMs, from the biological to the molecular level. Enzyme representatives from plants remain woefully understudied, despite their critical functional roles. At the cellular level, very little is known about potential protein-protein interactions involving these enzymes, despite their established importance in some systems (Arimura et al., 2009; Belkin & Burridge, 1994, 1995; Gundlach et al., 2015; Molt et al., 2014) and likelihood in others. A role for post-translation modification in the regulation of enzyme activity, localization, or protein-protein interactions is also a potential new horizon (Dey, Bounelis, Fritz, Bedwell, & Marchase,

1994; Fu et al., 1995; Gururaj, Barnes, Vadlamudi, & Kumar, 2004). Structural representatives for the important phosphopentomutase and glucose 1,6-bisphosphate synthase sub-groups are still lacking, as are a number of key enzyme ligand-complexes (e.g., for the PNGMs). Investigations of these poorly studied aspects of the PHMs seem certain to reveal as yet unexpected features/functions of these proteins.

Perhaps the most important underlying issue that remains to be addressed is a systematic characterization of the phosphosugar substrates utilized by the PHMs overall, as well as within and among the various sub-groups of the superfamily. This knowledge would help clarify the issue of potential functional overlap between enzymes in different sub-groups, and may even define additional groupings within the superfamily. Such fundamental information is relevant for the design of inhibitors and also for understanding tissue-specific disease phenotypes. It should also help correct the widespread functional misannotation of these enzymes in databases. This ambitious goal is now feasible using a combination of approaches such as high-throughput substrate profiling and bioinformatic analyses, as has been done with other enzyme families (Burroughs et al., 2006; Huang et al., 2015; Pandya et al., 2013).

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Figure 1. Overview of phosphosugar substrates and structural architecture of the PHMs

(A) Stick figures of the 1-phospho forms of various substrates of the α -D-phosphohexomutases, showing how structural differences are all localized at the 2-position of the sugar (green arrow). Also shown is a superposition of glucose 1-phosphate (white) and glucose 6-phosphate (green) illustrating the two different binding orientations (related by $\sim 180^\circ$ rotation of the sugar ring) for 1- vs. 6-phosphosugars. Superposition based on crystal structures of enzyme ligand complexes of *P. aeruginosa* PMM/PGM (Regni et al., 2004).

(B) Schematics of the 4-domain architecture of the PHMs, highlighting the locations of key catalytic and ligand binding residues. The enzymes are typically 450-550 amino acids in length.

Figure 2. A multiple sequence alignment of one representative from each of the four major PHM sub-groups

Sequences shown are from *B. anthracis* PNGM, *P. aeruginosa* PMM/PGM, human PGM1 and *C. albicans* PAGM. The four (I-IV) highly conserved active site loops are highlighted (Sec. 4.3). Due to the circular permutation of the PAGM proteins relative to the others, the corresponding regions of sequence were swapped to permit the best sequence alignment (sequence between orange triangles was swapped with sequence between blue triangles). See figure key for other symbols.

Figure 3. Metabolic roles and disease-associated variants of human PGM1

(A) The pivotal position of PGM1 determining the storage of glucose as glycogen or its utilization for energy. Also shown is the connection of PGM1 to protein glycosylation, through galactose metabolism. GALT: galactose 1-phosphate uridylyltransferase; GALE: UDP-galactose-4-epimerase.

(B) Ribbon diagram of human PGM1 illustrating residues affected by known missense variants (purple) associated with PGM1 deficiency. The bound metal ion in the active site is shown as an orange sphere.

Figure 4. Detailed reaction mechanism of the PHMs showing the multi-step conversion of glucose 6-phosphate to glucose 1-phosphate

The reaction proceeds via transfer of the enzymic phosphoryl group (blue) to substrate, to form a bisphosphorylated sugar intermediate. Reorientation of this intermediate (gray arrow) places the 6-phospho group (red) of the sugar near the conserved phosphoserine residue, allowing for the second phosphoryl transfer to occur back to enzyme. The product is now phosphorylated at the 1-position with the phosphoryl group (blue) derived from the enzyme. Roles for residues in acid–base catalysis are also indicated.

Figure 5. Overall structure and active site of enzymes in the major PHM sub-groups

(A) A superposition of four crystal structures, one from each major sub-group. Shown are human PGM1 (pdb: 5EPC) in pink, *C. albicans* PAGM (pdb: 3DKA) in blue; *Bacillus anthracis* PNGM (pdb: 3PDK) in green; and *P. aeruginosa* PMM/PGM (pdb: 1K35) in yellow. Domains 1-4 are numbered.

Close-up views of the active site of (B) *P. aeruginosa* PMM/PGM (yellow) in complex with glucose 1-phosphate (pdb: 1P5D) and (C) *C. albicans* PAGM (blue) in complex with GlcNAc-1P (pdb: 2DKD). Side-by-side comparison shows the highly similar arrangements of the two active sites. Divalent metal ion is shown as gray sphere; the catalytic serine is

shown in its two possible states: phosphorylated in (B) and unphosphorylated in (C). Cyan and purple stars mark putative general acid and general base, respectively. Key areas of the active site are labeled. See Fig. 2 for sequence alignments of these two proteins.

Figure 6.

Structures of experimentally confirmed oligomers in the PHM superfamily. Schematics (heart shapes) and ribbon diagrams are shown for each assembly; relevant PDB IDs are noted. Domain 4 is indicated by “4” on each schematic to indicate relative orientation of the protomers within the assembly. Three different protomer-protomer interfaces are indicated by A, B, and C. Two distinct dimers and one tetramer are known. Note how domain 4 is oriented away from the assembly interfaces, permitting conformational mobility. [Reprinted from (Luebbering et al., 2012)].

Summary of known sub-groups in the PHM superfamily

Table 1

Sub-group (EC No.)	Name	Lineage	Preferred substrate(s)	Functional roles	Typical length (No. residues)	Sugar-binding loop sequence motif*
PGM (5.4.2.2)	phosphoglucomutase	Bacteria, eukaryotes	G1P, G6P	Glucose homeostasis, protein N-glycosylation	550	-GE <u>ES</u> F-
PMM/PGM (5.4.2.8)	Phosphomannomutase/phosphoglucomutase	Bacteria	G1P, G6P, M1P, M6P	Polysaccharide/EPS biosynthesis	450	-GE <u>MS</u> G-
PNGM (5.4.2.10)	Phosphoglucosamine mutase	Bacteria	GlcN-1P, GlcN-6P	UDP-GlcNAc pathway, peptidoglycan	450	-GE(n/q) <u>SG</u> -
PAGM (5.4.2.3)	Phosphoacetylglucosamine mutase	Eukaryotes	GlcNAc-6P, GlcNAc-1P	Synthesis of UDP- GlcNAc; nucleotide sugar biosynthesis	550	-FE <u>AN</u> G-
PGM2 (EC 5.4.2.7)	Phosphopentomutase	Animals, fungi	R1P, R5P	Pentose phosphate synthesis/breakdown	600+	-FEE <u>AI</u> G-
PGM2L1 (2.7.1.106)	Glucose 1,6-bisphosphate synthase	Vertebrates	G1P, G6P, 1,3-bisphosphoglycerate	Synthesis of glucose 1,6-bisphosphate	600+	NA

* Residues known or predicted to be involved in direct contacts with the sugar (typically substituents at the 3'- and 4'- position) are underlined.

Abbreviations: EPS - extracellular polymeric substances, G1P – glucose 1-phosphate, G6P – glucose 6- phosphate; M1P – mannose 1-phosphate; M6P – mannose 6-phosphate; R1P – ribose 1-phosphate; R5P – ribose 5-phosphate; GlcNAc-6P - N-acetylglucosamine 6-phosphate; GlcN- 1P - glucosamine 1-phosphate; GlcN-6P - glucosamine 6-phosphate; GlcNAc- 1P - N-acetylglucosamine 1-phosphate. NA – not available

Table 2

Roles of PHM superfamily members in bacterial pathogenesis

	Organism	Enzyme	Role	Ref.
Gram positive	<i>N. gonorrhoeae</i>	PMM/PGM	Lipooligosaccharide biosynthesis, pathogenesis	(Sandlin & Stein, 1994; Zhou et al., 1994)
	<i>N. meningitidis</i>	PMM/PGM	Lipooligosaccharide biosynthesis, vaccine target	(Monteiro, Fortuna-Nevin, Farley, & Pavliak, 2003; Zhou et al., 1994)
	<i>S. aureus</i>	PGM	Cell wall envelope, antibiotic resistance	(Gründling & Schneewind, 2007)
	<i>S. aureus</i>	PNGM	Antibiotic resistance	(Jolly et al., 1997)
	<i>S. gordonii</i>	PGM	Fitness, cell wall properties	(Bizzini et al., 2007)
	<i>S. gordonii</i>	PNGM	Growth, biofilm formation, antibiotic sensitivity	(Shimazu et al., 2008; Shimazu, Takahashi, Karibe, Mitsuhashi, & Konishi, 2012; Yajima et al., 2009)
	<i>S. iniae</i>	PGM	Virulence, vaccine target	(Buchanan et al., 2005)
	<i>S. mutans</i>	PNGM	Reduced growth rate, biofilm attenuation, increased autolysis	(Liu, Duan, & Guo, 2009)
	<i>S. pneumoniae</i>	PGM	Virulence, capsule production	(Hardy, Caimano, & Yother, 2000; Hardy, Magee, Ventura, Caimano, & Yother, 2001)
Gram negative	<i>S. coelicolor</i>	PMM/PGM	Chloramphenicol sensitivity; reduced exp. drug efflux pumps	(Rajesh et al., 2012)
	<i>M. tuberculosis</i>	PMM/PGM	Host-pathogen interactions	(McCarthy et al., 2005)
	<i>M. smegmatis</i>	PNGM	Essential	(Kang et al., 2013; Li et al., 2012)
	<i>A. baumannii</i>	PMM/PGM	Biofilm formation	(Sahu, Iyer, Barage, Sonawane, & Chopade, 2014)
	<i>B. bronchisepta</i>	PMM/PGM	Virulence, sensitivity to oxidative stress &	(West et al., 2000)
	<i>B. abortus</i>	PGM	Virulence, resistance to complement, cytotoxicity	(Pei & Ficht, 2004; Ugalde, Comerc, Leguizamón, & Ugalde, 2003; Ugalde, Czibener, Feldman, & Ugalde, 2000)
	<i>B. melitensis</i>	PMM/PGM	Establishment of chronic infections	(Paixão et al., 2009)
	<i>B. suis</i>	PGM	Virulence, vaccine candidate	(Czibener, Del Giudice, Spera, Fulgenzi, & Ugalde, 2016)
	<i>E. coli</i>	PGM	Cell morphology, microbicide sensitivity	(Lu & Kleckner, 1994)
	<i>E. coli</i>	PNGM	Essential for viability	(Mengin-Lecreux & Heijenoort, 1996)
	<i>H. influenzae</i>	PGM	Glycosylation, bacterial adherence to host	(Grass et al., 2003; Swords et al., 2000)
	<i>H. pylori</i>	PNGM	Essential for viability	(De Reuse, Labigne, & Mengin-Lecreux, 1997)
	<i>P. aeruginosa</i>	PMM/PGM	Virulence, growth, clearance from host	(Goldberg, Coyne, Neely, & Holder, 1995; Tang et al., 1996)
	<i>P. aeruginosa</i>	PNGM	Essential	(Tavares, Leitão, & Sá-Correia, 2003)
	<i>S. typhimurium</i>	PGM	Fitness, antibiotic resistance	(Paterson, Cone, Peters, & Maskell, 2009)
	<i>S. typhimurium</i>	PNGM	Viability	(Rathi, Sarangi, & Trivedi, 2009)
	<i>S. maltophilia</i>	PMM/PGM	Virulence, antibiotic resistance	(Liaw, Lee, & Hsueh, 2010; McKay, Woods, MacDonald, & Poole, 2003)

Organism	Enzyme	Role	Ref.
<i>V. furnissii</i>	PMM/PGM	Virulence, O-antigen polysaccharide synthesis	(Kim et al., 2003)
<i>V. cholerae</i>	PMM/PGM	Colonization	(Chiang & Mekalanos, 1999)
<i>Y. pestis</i>	PGM	Decreased autoaggregation, reduced resistance to polymyxin B	(Felek et al., 2010)

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Table 3

Summary of Protein Data Bank depositions for members of the PHM superfamily

Organism	Lineage	UniProt ID	PDB ID	No resi.	Resolution (Å)*	P-Ser	Divalent metal ion	Ligand	Oligomer?
PGM									
<i>S. typhimurium</i>	bacterial	Q8ZQW9	2FUV, 3NA5, 3OLP	546	1.70	N	Mg		dimer
<i>T. thermophilus</i>	bacterial	Q5SLE0	2Z0F	524	2.52	N	No (pH 5.4)		dimer
<i>L. minor</i>	bacterial	Q4QCF1	4QG5	589	3.50		Mg		
<i>H. sapiens</i>	eukaryote	P36871	5EPC, 5F9C, 5HSH	562	1.85	N	Mg		
<i>O. cuniculus</i>	eukaryote	P00949	3PMG, 1VKL, 1JDY, 1LXT, 1C4G, 1C47	561	2.40	Y/N	Mg, Ni, Cd, Co	G1P6V, G16P	
<i>P. tetraurelia</i>	eukaryote	P47244 [‡]	1KFI, 1KFQ	572	2.40	N	Zn, Ca		
PNGM									
<i>B. anthracis</i>	bacterial	Q81VN7	3PDK	448	2.70	N	No (pH 4.5)		dimer
<i>F. tularensis</i>	bacterial	Q5NII8	3I3W	443	2.30	Y	Zn		dimer
PMM/PGM									
<i>B. thailandensis</i>	bacterial	Q2SYG4	3UW2	485	1.95	Y	Zn		
<i>P. aeruginosa</i>	bacterial	P26276	1K35, 1K2Y, 1P5D, 1P5G, 1PCI, 1PCM, 3BKQ, 3C04, 2H4L, 2H5A, 2FKF, 2FKM, 3RSM, 4IL8, 4MRQ	463	1.60	Y/N	Zn, Mn	G1P, G6P, M1P, M6P, R1P, X1P, G16P	
<i>X. citri</i>	bacterial	Q8PGN7	5BMN, 5KLO, 5BMP	468	1.27	Y	Mg	G16P	
PAGM									
<i>C. albicans</i>	eukaryote	Q9P4V2	2DKA, 2DKC, 2DKD	544	1.93	N	No (pH 4.6), Zn	GlcNAc-6P, GlcNAc-1P	
<i>A. fumigatus</i>	eukaryote	B0XP14	4BJU	549	2.35	Y	Mg		
<i>M. musculus</i>	eukaryote	Q9CYR6	1WJW	112	NMR	-	-		
AMBIGUOUS SUB-GROUP									
<i>B. melitensis</i>	bacterial	D0B6G5	4HJH	481	2.10	Y	Mg	G6P	
<i>T. thermophilus</i>	bacterial	Q5SKJ3	1TUO	464	1.70		No (pH 4.4)		
<i>P. horikoshii</i>	archaea	O58651	1WQA	455	2.00	N	Mg		tetramer
<i>S. tokodaii</i>	archaea	Q976E4	2F7L	455	2.80	N	No (pH 5.4)		tetramer

* In cases of multiple structures, only the highest resolution listed.

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Ligand abbreviations: G16P – glucose 1,6-bisphosphate; G1P6V – glucose 1-phosphate-6-vanadate; X1P – xylose 1-phosphate; see also Table 1 legend. Ligands derived from crystallization buffer (e.g., sulfate ions) not included. Oligomers are described in (Luebbert et al., 2012) and references therein

[‡]Reported to lack enzyme activity.