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FOR THE RECORD

A superfamily of metalloenzymes unifies phosphopentomutase and cofactor-independent phosphoglycerate mutase with alkaline phosphatases and sulfatases

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Abstract: Sequence analysis of the probable archaeal phosphoglycerate mutase resulted in the identification of a superfamily of metalloenzymes with similar metal-binding sites and predicted conserved structural fold. This superfamily unites alkaline phosphatase, N-acetylgalactosamine-4-sulfatase, and cerebroside sulfatase, enzymes with known three-dimensional structures, with phosphopentomutase, 2,3-bisphosphoglycerate-independent phosphoglycerate mutase, phosphoglycerol transferase, phosphonate monoesterase, streptomycin-6-phosphate phosphatase, alkaline phosphodiesterase/nucleotide pyrophosphatase PC-1, and several closely related sulfatases. In addition to the metal-binding motifs, all these enzymes contain a set of conserved amino acid residues that are likely to be required for the enzymatic activity. Mutational changes in the vicinity of these residues in several sulfatases cause mucopolysaccharidosis (Hunter, Maroteaux-Lamy, Morquio, and Sanfilippo syndromes) and metachromatic leucodystrophy.

Keywords: alkaline phosphatase; autotaxin; inherited disease; mucopolysaccharidosis; nucleotide pyrophosphatase PC-1; phosphoglycerate mutase; phosphopentomutase; sulfatase deficiency

Phosphoglycerate mutase (EC 5.4.2.1), a key glycolytic enzyme, is found in two forms, which differ in their requirement for 2,3-bisphosphoglycerate and show no detectable sequence similarity to one another (Grana et al., 1992, 1995). Although the 2,3-bisphosphoglycerate–dependent enzyme, found in bacterial, yeast, and animal cells, is relatively well studied (reviewed in Fothergill-Gilmore & Watson, 1989), the information about the structure or catalytic mechanism of the 2,3-bisphosphoglycerate–independent form (iPGM) is limited (Singh & Setlow, 1979a; Blattler & Knowles, 1980; Huang & Dennis, 1995). iPGM has been found in bacteria (Singh & Setlow, 1978; Watabe & Freese, 1979), archaea (Yu et al., 1994), plants (Leadlay et al., 1977; Botha & Dennis, 1986), and in some invertebrates (Carreras et al., 1982). Several bacterial and plant iPGM genes have been sequenced (Grana et al., 1992, 1995; Leyva-Vazquez & Setlow, 1994; Morris et al., 1995). Sequence analysis of the maize iPGM showed significant similarity to alkaline phosphatase (AP), including conservation of several metal-binding residues of the AP active center, and a similar catalytic mechanism for the two enzymes, namely the formation of a phosphoserine intermediate stabilized by divalent cations, has been suggested (Grana et al., 1992). This conclusion, however, has been disputed (Huang et al., 1993) because (1) activity of iPGM from castor bean appeared to be metal independent (Botha & Dennis, 1986), and (2) sequence alignment of several plant iPGMs did not reveal the conserved Asp-Ser-Ala triad, like the one in the AP active center.

When the first complete genome of an archaeon, *Methanococcus jannaschii*, was sequenced, genes for all the enzymes of the lower (tri-carbon) portion of the glycolytic pathway, except PGM, were easily identified (Bult et al., 1996). The PGM-coding gene has been reported missing (Selkov et al., 1997), even though iPGM activity had been experimentally demonstrated in closely related *Methanococcus maripaludis* (Yu et al., 1994). Likewise, no PGM-encoding gene was recognized in the recently sequenced genomes of two other archaea, *Methanobacterium thermoautotrophicum* (Smith et al., 1997) and *Archaeoglobus fulgidus* (Klenk et al., 1997). On the other hand, when the set of *Methanococcus jannaschii* proteins was searched for the closest homolog of known bacterial and eukaryotic iPGMs, a candidate protein, MJ1612 (originally annotated as phosphonopyruvate decarboxylase), has been identified and predicted to possess iPGM activity (Koonin et al., 1997). However, MJ1612 appeared to be related also to phosphopentomutases (phosphodeoxyribomutases) and several other en-

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zymes. This prompted us to investigate the possible relationships between iPGMs and phosphopentomutases.

Here, we show a pattern of sequence conservation between phosphopentomutases, iPGMs, and APs, which suggests conservation of the structural fold and similar reaction mechanisms. In accordance with the recent structural studies (Bond et al., 1997; Lukatela et al., 1998), similar conserved motifs were found in N-acetylgalactosamine-4-sulfatase, cerebroside sulfatase, and several related sulfatases. These findings define a new superfamily of proteins, which we refer to as the alkaline phosphatase superfamily.

The nonredundant protein sequence database at the National Center for Biotechnology Information (Bethesda, Maryland) was searched using the PSI-BLAST (Position-Specific Iterative BLAST) program, which converts local gapped alignment produced by BLASTP into position-specific weight matrices that are then used for iterative database scanning (Altschul et al., 1997). The multiple alignment was constructed using the alignment (-m4) option of PSI-BLAST with subsequent manual refinement on the basis of the structural alignment of AP and sulfatases, which was generated using Dali (Holm & Sander, 1998).

Sequence analysis of MJ1612 showed high similarity to another *M. jannaschii* protein, MJ0010. Corresponding pairs of paralogs were found in genomes of two other archaea, *M. thermoautotrophicum* (Smith et al., 1997) and *A. fulgidus* (Klenk et al., 1997). Sequence database searches confirmed similarity of each of these proteins to a putative phosphonopyruvate decarboxylase from *Streptomyces hygroscopicus* (Lee et al., 1995); they also revealed a highly statistically significant ($P < 10^{-8}$) similarity between all these proteins and iPGMs (Fig. 1). Iterative searches using the PSI-BLAST program resulted in identification of similar conserved regions in phosphopentomutases, APs, and related enzymes, and in several previously uncharacterized proteins (Fig. 1).

Fig. 1. Multiple alignment of the alkaline phosphatase superfamily. The proteins are listed under their unique SWISS-PROT (left column) and GenBank (right column) identifiers; 1160616, human autotaxin (Clair et al., 1997); 927036, Ca^{2+}-ATPase from *Flavobacterium odoratum* (Desrosiers et al., 1996; Peiffer et al., 1996); 1196755, phosphonoacetate hydrolase from *Pseudomonas fluorescens* (Kulakova et al., 1997); 1177864, phosphonate monoesterase from *Burkholderia caryophylli* (Dotson et al., 1996). The numbers indicate distances to the ends of each protein and the sizes of the gaps between aligned segments. Red and blue shading indicate conserved amino acid residues that are involved in metal binding in alkaline phosphatase (1ALK) and sulfatases (1FSU and 1AUK); their positions in mature enzymes are indicated above such residues. Conserved residues identified in this work are colored red and magenta. Black shading indicates the residues that where found mutated in patients with genetic disorders (intermediate or severe forms of hypophosphatasia, mucopolysaccharidosis, or metachromatic leucodystrophy). The references for particular mutations can be found in SWISS-PROT database (Bairoch & Apweiler, 1997). Yellow shading indicates uncharged amino acid residues (A, I, L, V, M, F, Y, or W) with a propensity to form a β-strand. Conserved small residues (G, A, or S) are shown in green, the residues conserved among several protein families are in bold. The consensus includes amino acid residues conserved in all sequences (upper case) and those conserved in the majority of the sequences (lower case). U stands for a bulky hydrophobic residue (I, L, V, M, F, Y, W), O stands for a small residue (G, A, S), - stands for D or E, $ stands for any charged residue (D, E, K, R, N, Q), and dot stands for any residue. In the structure line, α indicates α-helix and β indicates β-strand.

In each case, PSI-BLAST searches using iPGMs or phosphopentomutases as the query produced highly significant sequence alignments ($P < 10^{-6}$) with each other in the second or third iteration, and with APs and sulfatases in the fifth or sixth iteration. Inspection of the multiple alignment of all these proteins showed conservation of the core structural elements of APs and sulfatases (Fig. 1), suggesting that they belong to a distinct superfamily with a common structural fold.

This superfamily includes enzymes with substantially different activities (isomerases, hydrolases, and a putative lyase), which, however, all act on similar phosphocarbohydrate (or sulfocarbohydrate) substrates (Table 1). Remarkably, AP is known to have phosphotransferase activity (Coleman, 1992, and references therein), while iPGM can also function as a phosphatase (Breathnach & Knowles, 1977). Indeed, the conserved region in all these proteins (Fig. 1) contains the amino acid residues that are known to be involved in phosphate binding in AP (Kim & Wyckoff, 1991) and sulfate binding in sulfatases (Bond et al., 1997; Lukatela et al., 1998).

The alignment on Figure 1 demonstrates that all the amino acid residues that interact with Zn1 (Asp-327, His-331, and His-412) and Zn2 (Asp-51, Asp-369, and His-370) in AP (Kim & Wyckoff, 1991) are absolutely conserved in phosphocarbohydrate-binding proteins of the AP superfamily (Fig. 2). On the other hand, Mg binding residues of AP are much less conserved, as Glu-322 is substituted by Asn in phosphopentomutases and iPGMs, while Asp-153 and Thr-155 (Fig. 2) do not seem to be conserved at all. As noted earlier (Bond et al., 1997; Lukatela et al., 1998), the residues that coordinate Zn2 in AP are also conserved in sulfatases (Fig. 1).

The strong conservation of metal-binding residues in both phosphopentomutase and iPGM indicates that both these enzymes are metal dependent. Indeed, phosphopentomutase from *Escherichia coli* requires Mn^{2+}, Ni^{2+}, or Co^{2+} for activity, binding two metal atoms per enzyme molecule (Hammer-Jespersen & Munch-Petersen, 1970; Hammer-Jespersen, 1983). Similar data were reported for the rat liver enzyme (Barsky & Hoffee, 1983).

Table 1. *Properties of the enzymes of the alkaline phosphatase superfamily*

Enzyme (EC No.)	SWISS-PROT symbol	pH optimum	Metal requirements	Human disease caused by a mutation	Refs.
Phosphoglycerate mutase (EC 5.4.2.1)	PMGI_ECOLI	7.7–9.5	Mn	—[a]	1, 2[b]
Phosphopentomutase (EC 5.4.2.7)	DEOB_ECOLI	8.0–8.5	Mn or Co	Not known	3
Alkaline phosphatase (EC 3.1.3.1)	PPB_ECOLI PPBT_HUMAN	8.0–10.5	Zn + Mg	Hypophosphatasia	4, 5
Streptomycin-6-phosphatase (EC 3.1.3.39)	STRK_STRGR	n.d.[c]	n.d.	—	6
Alkaline phosphodiesterase/ nucleotide pyrophosphatase (EC 3.1.4.1/3.6.1.9)	PC1_HUMAN	8.0–9.0	Mn, Mg, or Ca	Not known; increased activity in type II diabetes	7
Phosphoglycerol transferase (EC 2.7.8.20)	MDOB_ECOLI	8.9	Mn	—	8
Ca^{2+}-ATPase	—	8.0	Ca	—	20
Phosphonopyruvate decarboxylase[d]	BCPC_STRHY	n.d.	Mg	—	9
Phosphonoacetate hydrolase (EC 3.11.1.1)	—	7.7–9.0	Zn, Mn, or Co	—	10
Phosphonate monoesterase	—	8.5–9.0	Mn	—	11
Arylsulfatase (EC 3.1.6.1)	ASLA_ECOLI ARSE_HUMAN	n.d.	n.d.	Chondrodysplasia punctata	12
Steroid sulfatase (EC 3.1.6.2)	STS_HUMAN	6.0–7.5	n.d.	X-linked ichthyosis	13
N-acetylgalactosamine 6-sulfatase (EC 3.1.6.4)	GA6S_HUMAN	3.5–4.0	n.d.	MPS[e] type IVA (Morquio A syndrome)	14
Cerebroside sulfatase (EC 3.1.6.8)	ARSA_HUMAN	4.8–6.2	Mg	Metachromatic leucodystrophy	14
N-acetylgalactosamine 4-sulfatase (EC 3.1.6.12)	ARSB_HUMAN	n.d.	Ca?	MPS type VI (Maroteaux-Lamy syndrome)	16
Iduronate 2-sulfatase (EC 3.1.6.13)	IDS_HUMAN	4.0–5.7	n.d.	MPS type II (Hunter syndrome)	17
N-acetylglucosamine 6-sulfatase (EC 3.1.6.14)	GL6S_HUMAN	3.9–5.7	n.d.	MPS type IIID (Sanfilippo D syndrome)	18
N-sulfoglucosamine sulfatase (EC 3.10.1.1)	SPHM_HUMAN	3.9–4.1	n.d.	MPS type IIIA (Sanfilippo A syndrome)	19

[a]Absence of 2,3-bisphosphoglycerate-dependent PGM in humans causes myopathies.

[b]References: 1, Singh and Setlow (1979a); 2, Carreras et al. (1982); 3, Hammer-Jespersen and Munch-Petersen (1970); 4, Henthorn et al. (1992); 5, Murphy et al. (1995); 6, Mansouri and Piepersberg (1991); 7, Oda et al. (1993); 8, Jackson and Kennedy (1983); 9, Nakashita et al. (1997); 10, McGrath et al. (1995); 11, Dotson et al. (1996); 12, Parenti et al. (1997); 13, Alperin and Shapiro (1997); 14, Bielicki and Hopwood (1991); 15, Lukatela et al. (1998); 16, Bond et al. (1997); 17, Bielicki et al. (1990); 18, Freeman and Hopwood (1987); 19, Freeman and Hopwood (1992); 20, Desrosiers et al. (1996).

[c]Not determined.

[d]Phosphonopyruvate decarboxylase activity of BCPC_STRHY has not been demonstrated experimentally and could have been encoded by a different gene; actual function of this protein remains unidentified.

[e]MPS, Mucopolysaccharidosis.

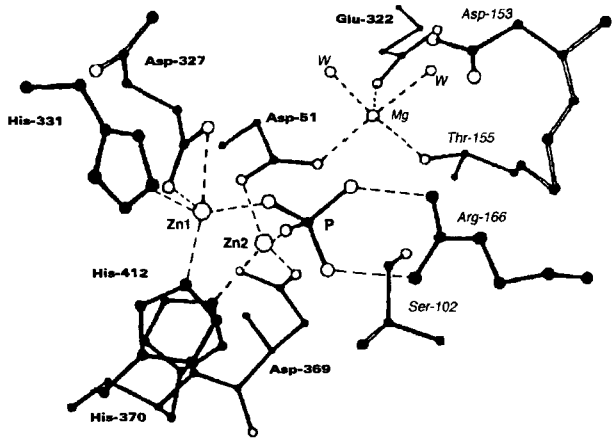

Fig. 2. Conserved residues in the enzymes of the alkaline phosphatase superfamily. The scheme of the active center of the *E. coli* alkaline phosphatase (modified from Kim & Wyckoff, 1991); amino acid residues that are conserved in phosphopentomutases and phosphoglyceromutases (Fig. 1) are labeled in bold; nonconserved amino acid residues are labeled in italic, W indicates water molecules.

Bacterial iPGMs also require Mn^{2+} for activity (Singh & Setlow, 1978, 1979b; Watabe & Freese, 1979; Kuhn et al., 1993). Recently, a detailed study of Mn^{2+} binding by iPGM from *Bacillus megaterium* demonstrated a cooperativity in Mn-dependent activation of iPGM with a Hill coefficient of 2.1 ± 0.1, indicating that two Mn atoms bind per iPGM molecule (Kuhn et al., 1995). Thus, phosphopentomutase and bacterial iPGM each require two Mn atoms for activity.

The metal requirements of the plant iPGM have been a subject of some controversy. It was first reported that iPGM from wheat germ was inhibited by EDTA (Leadlay et al., 1977; Smith & Hass, 1985) and required Mn^{2+} or Co^{2+} for reactivation after denaturation (Smith et al., 1986). In contrast to these data, the activity of castor bean iPGM was reported unaffected by passing the enzyme solution through Chelex resin (Botha & Dennis, 1986). No data were presented, however, and the experimental protocol used has not been shown to completely remove trace metals from the reaction mixture. Based on the high level of sequence similarity between the plant and bacterial iPGMs (Grana et al., 1995; Fig. 1), it would be reasonable to suggest that plant enzymes are also metal dependent.

Sequence analysis shows that alkaline phosphodiesterase/nucleotide pyrophosphatase PC-1, a cell surface enzyme, implicated in pathogenesis of cancer and diabetes (Maddux et al., 1995), is also a member of the AP superfamily. Comparison of PC-1 with AP (not shown) shows that Thr-204 of PC1_MOUSE aligns with the active site Ser-102 of AP (Fig. 2); it is similarly phosphorylated during the catalytic cycle of PC-1 (Belli et al., 1995). Autotaxin, a human tumor motility-stimulating protein, very similar to PC-1 (Clair et al., 1997; Fig. 1) has the same conserved region around Thr-208, indicating that it could also be phosphorylated. Divalent cations (Ca^{2+}, Mg^{2+}, or Mn^{2+}) are required for the activity of PC-1 (Oda et al., 1993); they also improve its thermal stability (Belli et al., 1994). Nucleotide pyrophosphatase activity has also been found in *Haemophilus influenzae* (Kahn & Anderson, 1986); it could belong to one of the previously uncharacterized *H. influenzae* proteins shown in Figure 1. Mn^{2+} was also shown to stimulate the activity of three additional members of the AP superfamily (Fig. 1; Table 1), phosphoglycerol transferase (Jackson & Kennedy, 1983), phosphonoacetate hydrolase (McGrath et al., 1995), and phosphonate monoesterase from a glyphosate-degrading bacterium (Dotson et al., 1996). Another unusual member of the AP superfamily is the Ca^{2+}-dependent ATPase that requires two Ca atoms for activity (Desrosiers et al., 1996; Peiffer et al., 1996). Finally, an outer membrane protein YHBX_ECOLI, associated with the adherence of enteropathogenic *E. coli* O157:H7 to human epithelial cells (Zhao et al., 1996), is also a member of this superfamily. The conservation of the predicted catalytic residues (Fig. 1) suggests that this protein possesses phosphatase activity that may be important for pathogenicity.

Several amino acid residues that form the active center of AP (Kim & Wyckoff, 1991) or sulfatases (Bond et al., 1997; Lukatela et al., 1998) are not conserved in phosphopentomutase and iPGM. The structure of the AP active center (Fig. 2) shows that one of these missing amino acid residues, Arg-166, binds the two remaining O atoms of the phosphate group (Kim & Wyckoff, 1991; Coleman, 1992) and thus assists in loosening the bond between the P atom and Zn1-bound O atom of the leaving RO^- group. Neither could we identify a counterpart of the phosphorylated Ser-102 of AP in phosphopentomutase or iPGM, even though iPGM has been suggested to form a phosphoenzyme intermediate (Blattler & Knowles, 1980).

The balance between phosphotransferase and phosphatase reaction may be affected by the difference in the metal specificity between AP, on one hand, and phosphopentomutase and iPGM, on the other hand. Even though AP is maximally active with Zn^{2+} ions, substitution of Mn^{2+} or Co^{2+} for Zn^{2+} still produced an enzyme with detectable activity. The decreased activity of such enzymes was largely due to the lower rate of the enzyme dephosphorylation, caused by a tighter binding of phosphate (Applebury et al., 1970; Coleman, 1992). Such an arrangement favors phosphotransferase reaction, which could be a reason for the Mn^{2+} dependence of phosphopentomutase and iPGM.

The alignment in Figure 1 also shows several highly conserved amino acid residues that have no known role in enzyme activity. These include Asp-346, Thr-367, Thr-413, Asp-437, and Thr-441 of AP and additional Thr residues in phosphopento- and phosphoglyceromutases, which have no counterparts in other enzymes. Examination of the three-dimensional structures of AP and sulfatases shows that in phosphopentomutases and iPGMs, only the equivalents of Thr-367, Thr-413, and Gln-410 of AP are likely to be positioned close enough to the phosphate-binding site to participate in binding of the carbohydrate moiety of the phosphocarbohydrate substrate (Fig. 1).

Asp-346 and Asp-437 of AP, located at ca. 20 Å from the metal-binding site in both AP and sulfatases, could be involved in the maintenance of the structural integrity of these enzymes. It is also possible that they participate in a relay that directs substrates to the active sites of these enzymes. In any case, the absence of activity in D255H and D335V mutants of human cerebroside sulfatase (Hess et al., 1996; Lissens et al., 1996) demonstrates that these conserved Asp residues are required for sulfatase activity, and suggests that they might be important in other enzymes as well. Analysis of genetic disorders that result from mutations in human genes coding for known enzymes may offer additional insight into the organization of their molecules. Several inherited disorders, such as hypophosphatasia, chondrodysplasia, metachromatic leukodystrophy, and various mucopolysaccharidoses are caused by

Table 2. *Phylogenetic distribution of the enzymes of the alkaline phosphatase superfamily*

	Phylogenetic distribution												
	Bacteria						Archaea			Eukaryotes			
Enzyme	Ec[a]	Hi	Hp	Sy	Bs	Mg	Mj	Mt	Af	Sc	Plants	Ce	Hs
Cofactor-independent phosphoglycerate mutase[b,c]	yibO	—[d]	HP0974	slr1945	**pgm**	MG430	MJ1612	MTH1591	AF1751	—	P30792	2773203	
Phosphopentomutase[b]	**deoB** yhfW	—	HP1179	—	drm	—	MJ0010	MTH418	AF1425	—			D79585
Alkaline phosphatase	**phoA**	—	—	—	**phoA** **phoB**	—	—	—	—	**YDR481c**			**4 or more isozymes**
Alkaline phosphodiesterase/ nucleotide pyrophosphatase	—	HI0841	—	—	—	—	—	—	—	YCR026c YEL016c	818849	C27A7.1 C27A7.3 1330365	**PC-1** **1160616** **2465540**
Phosphoglycerol transferase	**mdoB**	HI1246	HP0578		yqgS	—	—			—			
Sulfatases	aslA yidJ b1498	—	—	—	—	—	—	—	—	—		1256283 1017806 1125842	**10 or more enzymes**
Uncharacterized	yhbX ybiP yhjW yjdB yejM yijP yigX	HI0275 HI1005 HI1064	HP0022	—	yf1E yfnI yvgJ	—	—	—	AF0247	YJL062w YKL165c YLL031c	P14217	10 or more proteins	

[a] Ec, *Escherichia coli*; Hi, *Haemophilus influenzae*; Hp, *Helicobacter pylori*; Sy, *Synechocystis sp.*; Bs, *Bacillus subtilis*; Mg, *Mycoplasma genitalium*; Mj, *Methanococcus jannaschii*; Af, *Archaeaoglobus fulgidus*; Sc, *Saccharomyces cerevisiae*; Ce, *Caenorhabditis elegans*; Hs, *Homo sapiens*. The proteins are listed under their original authors' gene designations or their GenBank identifiers. Human phosphopentomutase fragment is from the expressed sequence tags (EST) database.

[b] The proteins with experimentally demonstrated enzymatic activity are shown in bold type; other proteins are predicted to possess indicated activities on the basis of sequence similarity to previously characterized proteins (Fig. 1).

[c] Ec, Hi, Sy, Bs, Sc, and Hs have genes coding for 2,3-bisphosphate-dependent phosphoglycerate mutase.

[d] No corresponding gene is present in the complete genome

missense mutations in the genes for sulfatases that belong to the AP superfamily. Some of these mutations result in single amino acid substitutions in the conserved motifs shown on Figure 1 and abolish the enzymatic activity (reviewed in Henthorn et al., 1992; Parenti et al., 1997).

The phylogenetic distribution of the AP superfamily enzymes is unusual. While some bacteria, such as *E. coli* or *Bacillus subtilis*, encode both iPGMs and APs, archaea and eukaryotes usually have only one of these enzymes (Table 2). Thus, iPGM activity has not been found in vertebrates (Carreras et al., 1982); the respective gene is also absent from the yeast genome. Instead, fungi and vertebrates have a different, 2,3-bisphosphoglycerate–dependent form of phosphoglycerate mutase (Fothergill-Gilmore & Watson, 1989). In algae, iPGM is encoded in the chloroplast, while in higher plants it is nuclear encoded and absent from the chloroplast genome. Plant iPGMs thus appear to have chloroplast origin. On the other hand, AP, found in yeast and animal cells, so far has not been described in plants. Conceivably, AP and iPGM could have evolved from a common ancestral enzyme, with selective loss of one of these enzymes in various eukaryotic branches. The pairs of paralogous archaeal proteins (e.g., MJ0010 and MJ1612) have shown significant sequence similarity to all the enzymes of the AP superfamily and may resemble the ancestral phosphomutases.

The distribution of the two classes of PGMs in bacteria is also remarkable. While organisms with larger genomes, such as *E. coli*, *B. subtilis*, and *Synechocystis sp.* have genes coding for both classes of this enzyme (Table 2), the organisms with smaller genomes code for only one of them. Thus, iPGM is the only form of this enzyme that is encoded in the genomes of such human pathogens as *Mycoplasma genitalium, Mycoplasma pneumoniae,* and *Helicobacter pylori,* the causative agents of nongonococcal male urethritis, atypical pneumonia, and gastric ulcer, respectively. The importance of iPGM for the metabolism of these bacteria and its apparent absence in vertebrates (Carreras et al., 1982) suggest that iPGM may be a plausible target for new, specialized antibacterial drugs. The 3D structure of iPGM, once determined, will facilitate the development of such drugs and will help resolve the remaining questions about its catalytic mechanism.

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