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Comparison of the Binuclear Metalloenzymes Diphosphoglycerate-Independent Phosphoglycerate Mutase and Alkaline Phosphatase: Their Mechanism of Catalysis via a Phosphoserine Intermediate

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Contents

I. Introduction

Phosphoglycerate mutases (PGMs) catalyze the interconversion of 3-phosphoglyceric acid (3PGA) and 2-phosphoglyceric acid (2PGA) in the pathways of glycolysis and gluconeogenesis.[1] Alkaline phosphatases (APs) catalyze the hydrolysis of phosphomonoesters, with release of phosphate and an alcohol, although these enzymes can also catalyze a phosphotransferase reaction under some conditions.[2] Recent work has led to the surprising conclusion that one class of PGMs exhibits significant similarity to the AP of *Escherichia coli*, in both the structure of their active sites and their mechanisms of catalysis.[3−4]

The structure of the 2,3-diphosphoglyceric acid (DPG) independent PGM (iPGM) from *Bacillus stearothermophilus*, a binuclear metalloenzyme (defined as an enzyme in which two metal ions share a ligand at the catalytic site) containing manganese, has recently been elucidated by X-ray crystallography, and the mechanism of this enzyme appears to involve a phosphoserine intermediate[3,4] as proposed more than 20 years ago.[5] *B. stearothermophilus* iPGM has a specific and absolute requirement for Mn^{2+} for activity, and the two bound Mn^{2+} ions are involved in catalysis.[3,4,6] *E. coli* AP is also a binuclear metalloenzyme but requires two Zn^{2+} ions for catalysis, while the presence of a third metal (Mg^{2+}) in the AP active site does not seem to be catalytically relevant; catalysis by this enzyme also involves a phosphoserine intermediate.[2] The iPGMs and AP exhibit a similar amino acid sequence in only a stretch of ∼70 amino acids (Figure 1);[7] however, as noted above, a high degree of structural similarity has been found between the active sites of iPGM of *B. stearothermo-*

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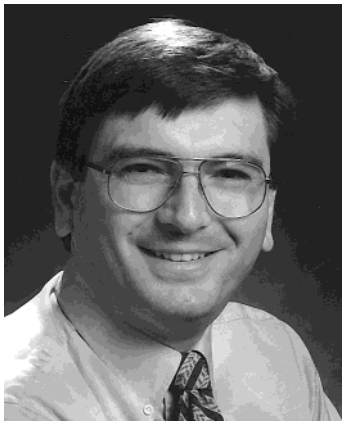

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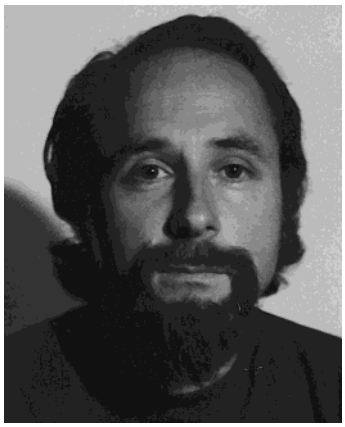

Peter Setlow received his B.A. degree in Chemistry from Swathmore College in 1964 and his Ph.D. degree in Biochemistry from Brandeis University in 1968 and did postdoctoral work in the Biochemistry Department of the Stanford University School of Medicine from 1968–1971. He was appointed an Assistant Professor of Biochemistry at the University of Connecticut in 1971 and is presently Professor and Chair of the Biochemistry Department. He was elected a fellow of the American Academy of Microbiology in 2000. His research interests are biochemical and regulatory aspects of the formation, resistance, and germination of bacterial spores, in particular spores of *Bacillus* species.

philus and *E. coli* AP (Figure 2).[3,4] Strikingly, the residues involved in binding of the two Zn^{2+} ions in AP and the two Mn^{2+} ions in iPGM are conserved among APs and iPGMs from a variety of sources.[3,4,7] The focus of this report is the comparison of structural, functional, and catalytic aspects of the role of the two Mn^{2+} ions in iPGM and the two Zn^{2+} ions in AP. The evolutionary relationship between these two types of enzymes as well as comparisons with other binuclear metalloenzymes with known three-dimensional structures including enolase,[8,9] pyruvate kinase,[10] and serine/threonine protein phosphatase-1[11,12] are also discussed.

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  4 KP...VALIILDGFALRD.E.TYGNAVAQ 27
    ||   : |:|||||  : | | |     |:
 40 KPAKNIILLIGDG..MGDSEITAARNYAE 66


400 ANPDMVGHS 408
    |. |   |.
324 ASIDKQDHA 332

                .
438 IAIITADHGNA 448
    : |:|||| .|
363 LVIVTADHAHA 373


459 QTAHT.T.......NP 466
    |  || .        |
410 Q.EHTGSQLRIAAYGP 424
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Figure 1. Sequence alignment of the metal binding regions of *B. stearothermophilus* iPGM (GB_BA2: AF120091)[3,4] (top) and *E. coli* AP (PDB:1ALK)[40] (bottom). The residues coordinating to metals are highlighted in a bold font. The figure was made using the BestFit feature of the GCG package (1999).[73]

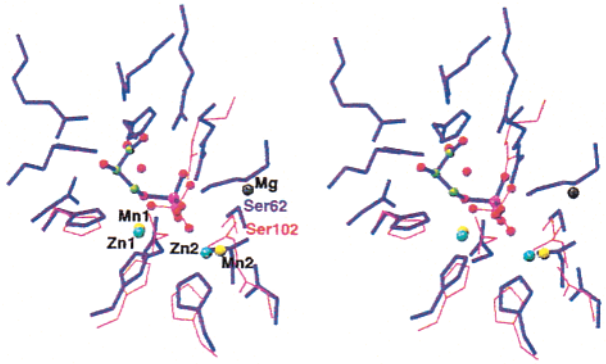

Figure 2. Stereoview of the structural alignment of the active sites of *B. stearothermophilus* iPGM and *E. coli* AP. The coordinates were taken from Jedrzejas et al.[3,4] and from PDB: 1ALK.[40]

II. Manganese and Zinc

A. The Role of Manganese and Zinc Ions in Nature

Manganese is one of the earth's most abundant first-row transition metals, constituting 0.085% of the earth's crust,[13] where it is found in both oxide and carbonate deposits. Its abundance and the fact that manganese can exist in a large number of coordination and oxidation states makes this metal a versatile ion for many biochemical processes. The total concentration of Mn^{2+} in human plasma is 0.11 μM[14] and is 0.25−0.7 μM in rat hepatocytes.[15] These concentrations are >10 times that of Mn^{2+} in seawater,[14] suggesting that life forms may have developed specific requirements for Mn^{2+}. In contrast, zinc's abundance is relatively low, on the order of 0.0001% of the earth's crust,[13] where it is found in numerous minerals, a major one being sphalerite [(ZnFe)S]. Zinc, like manganese, appears to be an extremely important metal biologically and is present in many proteins, including enzymes and DNA binding proteins. Although the concentration of Zn^{2+} inside most human organs is 20−30 μg/g, its concentration in liver, voluntary muscle, and bone reaches 60−180 μg/

Table 1. General Properties of Manganese and Zinc in Metalloenzymes

metal	ionic radius (Å)	oxidation states	common coordination geometry	typical coordination ligands	unique features
manganese	0.80 (Mn^{2+}) 0.65 (Mn^{3+})	−3 to +7 (+2 is most stable)	square pyramidal, trigonal bipyramidal, octahedral, also tetrahedral	carboxylate of Asp, Glu; carboxyamide of Asn, Gln; N of His; H_2O	redox capability
zinc	0.7	+2	tetrahedral	different types	lack of redox behavior

g.[16] Some properties of Zn^{2+} that may be important in the function of this ion in enzymes include a flexible coordination geometry, fast ligand exchange, Lewis acidity, intermediate polarization, and a lack of redox activity. Because Zn^{2+} and Mn^{2+} share some of these properties, either of these ions can be utilized by many enzymes. The major difference between the ions is the redox behavior of Mn^{2+} under biological conditions, a property not shared by Zn^{2+}. The redox behavior of Mn^{2+} is essential in the function of this ion in some enzymes (e.g., superoxide dismutase and catalase) but not in all.[17,18]

B. General Properties of Manganese and Zinc in Metalloenzymes

Metalloproteins discriminate in their binding of transition metals such as zinc and manganese based on the metal size, charge, and chemical nature (Table 1). Factors contributing to an enzyme's binding of Zn^{2+} versus Mn^{2+} include the different ionic radii, the different ion coordination sphere geometries, and the ion's ability to interact with different protein ligands or water molecules. The approximate ionic radii of Mn^{2+} and Zn^{2+} are 0.80 and 0.74 Å, respectively (Table 1);[13] the radii for the alkaline earth metal ions Mg^{2+} and Ca^{2+} are 0.65 and 0.90 Å, respectively. The oxidation state of Mn can range from −3 to 7 with +2 being the most stable,[13] and the +2 oxidation state is the most common state in active sites of metalloenzymes. Zinc's oxidation state is +2 and is neither oxidized nor reduced in biological reactions (Table 1). The coordination sphere of Mn^{2+} in metalloenzymes is usually square pyramidal, trigonal bipyramidal, or octahedral,[19] while Zn^{2+} usually has a tetrahedral or distorted tetrahedral arrangement of its ligands when bound to enzymes.[20] The Mn^{2+} coordination sphere in enzymes is usually filled with only a few different ligands: the carboxylate groups of Asp or Glu, the carboxyamide of Asn or Gln, or a nitrogen atom of histidine. Zn^{2+} is less discriminating as it interacts with a wide variety of biological ligands. Water molecules also often coordinate with these metal ions in enzymes, and this can decrease water's pK_a value from 15.7 in bulk solvent to as low as 9.0.[20] This is due to the stabilization of the electronegative OH^- group generated via metal-induced ionization of a water molecule by the coordination sphere of the positively charged metal ion. Metal-bound OH^- is very reactive and is often part of reactions catalyzed by metalloenzymes.[20] Coordination with a metal can both position a reactive OH^- group favorably for nucleophilic attack on a substrate and modulate OH^- reactivity.

III. Phosphoglycerate Mutase and Alkaline Phosphatase

A. Comparison of Structures of *B. stearothermophilus* iPGM and *E. coli* AP

PGMs interconvert 3PGA and 2PGA in the processes of glycolysis and gluconeogenesis. In glycolysis, the conversion of 3PGA to 2PGA allows eventual synthesis of ATP by means of enolase and pyruvate kinase.[8,9] There are two different types of PGMs; one is DPG-dependent (dPGM) and the other is DPG-independent (iPGM).[1,3,6] dPGMs are found in vertebrates, yeast, and bacteria, while iPGMs are found in higher plants and bacteria. Interestingly, some bacteria have both types of PGM, although one form appears to be the predominate enzyme.[21,22] The dPGMs and iPGMs have very different structures, as the dPGMs are dimers or tetramers of ∼25 kDa subunits while the iPGMs are monomers of ∼60 kDa; the amino acid sequences of these two groups of enzymes also exhibit no similarity.[1,3,19] Although there is high sequence conservation between dPGMs from different species and between iPGMs from different species, the dPGMs are related to the acid phosphatase[23] and fructose 2,6-bisphosphatase[24,25] family of enzymes while the iPGMs appear to be related to alkaline phosphatases.[3,7]

The structural difference between these two types of enzymes is also reflected in differences in their catalytic mechanisms. dPGM catalysis involves a pair of histidine residues, one of which functions as a crucial acid/base catalyst while the other (His8 in the yeast enzyme) accepts a phosphoryl group from the DPG cofactor releasing 2- or 3-PGA and generating a phosphoenzyme that can readily be isolated.[26−31] The DPG cofactor is then regenerated by transfer of the phosphoryl group from the enzyme back to 2- or 3-PGA. As a consequence, dPGMs catalyze an intermolecular transfer of phosphoryl groups between the two PGA substrates with retention of the configuration at the phosphorus. No metal ions are required for catalysis by the dPGMs.

In contrast, divalent metal ions are probably required for catalysis by all iPGMs, with these ions likely to be Mn^{2+}, two of which are present in *B. stearothermophilus* iPGM.[1,3,4,6,21,32] iPGM catalysis proceeds via an intramolecular transfer of a phosphoryl group between the 2 and 3 positions of the same PGA molecule, since iPGM does not catalyze exchange of D-glycerate into PGA.[5,30] Despite this intramolecular phosphate transfer, iPGM catalysis is also thought to proceed via a phosphoryl enzyme intermediate based on analysis of flux kinetics,[30] phosphoryl group transfer from PGA substrate analogues to D-glycerate,[5] and the retention of the

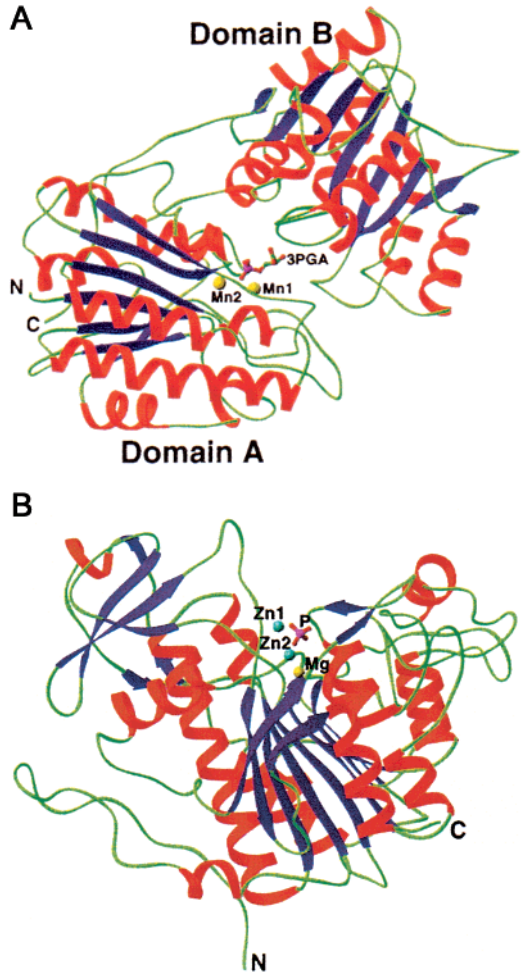

Figure 3. General structure of two binuclear metalloenzymes **(**A**)** iPGM and **(**B**)** AP. The structure of *B. stearothermophilus* iPGM is based on its X-ray structure coordinates,[3,4] and the *E. coli* AP structure is based on the PDB: 1ALK coordinates.[40]

configuration about the phosphorus in the iPGM reaction.[31] However, the phosphoryl enzyme intermediate has never been isolated, and it has been suggested that this intermediate has rather high free energy and binds D-glycerate extremely tightly.[30,31] More recently, studies with the *B. stearothermophilus* iPGM have indicated that iPGM catalysis does indeed proceed via a phosphoryl enzyme intermediate with transfer of the phosphate from the PGA substrate to a specific serine residue on the protein.[3,4]

The crystal structure of the 510 residue *B. stearothermophilus* iPGM has been recently solved at 1.90 Å resolution.[3,4] The protein adopts a compact, globular shape with two domains, A and B (Figures 2B, 3A, and 4), which have similar sizes of approximately 24 × 35 × 31 Å and an α/β type structure similar to that of other glycolytic proteins.[33] For each domain, the parallel and antiparallel β-strands comprise mixed seven-stranded β−sheets which are surrounded by α-helices of various lengths. Domains A and B are connected only by two well-separated (10 Å) short loops (7 and 10 residues long). A well-defined, highly solvent-accessible cleft is present between domains A and B, and this cleft contains the active site of the enzyme and is where the PGA substrate and the two Mn^{2+} ions bind (Figure 4). The

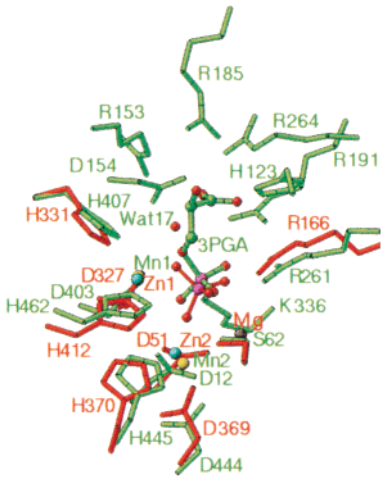

Figure 4. Detailed view of the aligned active sites of both iPGM and AP. Both active sites were defined as residues interacting with the metal ions or the substrate/product moieties in the structures of *B. stearothermophilus* iPGM[3,4] and *E. coli* (PDB: 1ALK).[40]

bound 3PGA is oriented such that the phosphate end is close to domain A and the carboxylate end is near domain B. Both Mn^{2+} ion binding sites are in domain B. The general orientation of 2PGA bound to the enzyme is essentially the same as that of 3PGA.[4]

Mn^{2+} is specifically and absolutely required by the *B. stearothermophilus* iPGM.[6] The situation with other iPGMs is not so clear, although there are data indicating a metal requirement for catalysis of at least some other iPGMs and Mn^{2+} has been detected in the *E. coli* iPGM.[1,21] However, for the bacteria of the *Bacillus* and *Clostridium* species which form endospores, their iPGM's Mn^{2+} requirement appears to play a crucial role in regulating the activity of the enzyme. The binding of Mn^{2+} to the iPGM of these organisms (see below) and thus enzyme activity is exquisitely sensitive to pH at likely physiological concentrations of free Mn^{2+} (<3 μM), with enzyme activity in vitro decreasing >100-fold in going from pH 8 to 6.[34−36] During spore formation in this group of organisms, the pH within the developing spore drops to approximately 6.5, which drastically decreases iPGM activity; this in turn results in accumulation of a large depot of 3PGA in the dormant spore.[34−38] Subsequently, during the first minutes of spore germination, there is a rise in the spore pH to ~8, which restores iPGM's activity and allows utilization of the spore's 3PGA depot. The resultant production of 2PGA results in ATP generation, which in turn serves as a source of energy for biochemical reactions early in spore germination.[38,39] The two enzymes which act in the glycolytic pathway leading to the synthesis of ATP are enolase and pyruvate kinase; enolase catalyses dehydration of 2PGA to form phosphoenolpyruvate (PEP) and pyruvate kinase utilizes PEP to phosphorylate ADP giving ATP and pyruvate. The structures of both enolase and pyruvate kinase have been elucidated and are described in the final part of this paper.

Comparison of the X-ray crystal structures of both *E. coli* AP[40,41] and *B. stearothermophilus* iPGM[3,4] reveals a striking similarity in the active sites of these enzymes (Figures 2B and 4). *E. coli* AP is a

nonspecific phosphomonoesterase that proceeds via a phosphoserine intermediate whose hydrolysis releases inorganic phosphate; the enzyme-bound phosphoryl group can also be transferred to other alcohol acceptors.[2,42−44] AP is found in prokaryotes as well as in eukaryotes, and it often exists in multiple isoforms in eukaryotes (four in humans); the APs from all species appear to be related.[40] The structure of *E. coli* AP was originally solved at 2.8 Å resolution,[41] but the resolution was then increased to 2.0 Å.[40] AP is a homodimeric metalloenzyme with 449 residues per monomer, a molecular mass of 94 kDa, and a size of 100 × 50 × 50 Å (Figure 3B). Each subunit shows α/β topology with a central 10-stranded β-sheet surrounded by 15 α-helices and a separated minor 3-stranded antiparallel β-sheet together with an additional small helix. There is one active site per monomer, and this uses residues of only a single subunit. The two active sites in the dimer are approximately 30 Å apart. The active site is located on the surface of the molecule at the carboxyl end of the central β-sheet and consists of an amino acid triad Asp101-Ser102-Ala103, Arg166, two Zn^{2+} and one Mg^{2+} ions, and residues interacting with these ions (Figures 3B and 4).

B. Coordination Geometry of Zinc in AP and Manganese in iPGM

The coordination spheres around the Zn^{2+} ions in *E. coli* AP and the Mn^{2+} ions in *B. stearothermophilus* iPGM have many common features as well as some differences (Figure 4). The three metal binding sites in AP, designated 1, 2, and 3, are occupied by two Zn^{2+} ions and one Mg^{2+} ion, denoted as Zn1Zn2Mg3 (where 1, 2, and 3 denote appropriate metal binding site) (Figure 4); the coordination geometry of the Mn^{2+} ions in iPGM is illustrated in Figure 5A. The AP data are believed to represent the phosphoryl enzyme, and the iPGM data are from the structure of the 3PGA−enzyme complex. The data for the 2PGA−iPGM complex were also obtained from the crystal structure of the complex.[4] The distances from the coordinating ligands to the metal ions for all three structures are provided in Table 2. The coordination of the Zn^{2+} ions in AP is tetrahedral, but the Zn1 coordination sphere is distorted to pseudo-tetrahedral by additional coordination with the two carboxylate oxygen atoms of Asp372 that occupy one of the coordination places. Both Zn^{2+} ions are bridged through the phosphate group and its two oxygen atoms (Figure 5B). The iPGM has two Mn^{2+} ions in the active site, denoted as Mn1 and Mn2. Mn1 has a distorted square pyramidal coordination geometry with the weakest interaction with the ester oxygen atom of the 2PGA or 3PGA substrate. Mn2 also has a distorted square pyramidal coordination geometry with the interaction with the OD2 oxygen atom of Asp12 being the weakest (Figure 4). The distorted square pyramidal arrangement is a characteristic

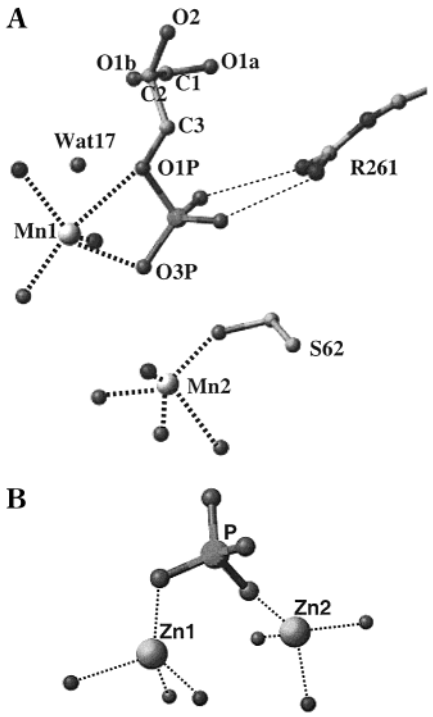

Figure 5. Coordination spheres of Mn^{2+} and Zn^{2+} ions in **(A)** iPGM and **(B)** AP. In A, the square pyramidal geometry around both catalytic Mn^{2+} ions is shown for *B. stearothermophilus* iPGM at neutral pH.[3] In B, the two Zn^{2+} ions in *E. coli* AP at neutral pH are in tetrahedral geometry; the bridging of the two Zn^{2+} ions through a phosphate group is also shown (PDB: 1ALK).

Table 2. Metal−Ligand Distances for Coordination Spheres of Zn^{2+} and Mn^{2+} Ions in *E. coli* AP and *B. stearothermophilus* iPGM[a]

AP coordination in crystal structure			iPGM coordination in crystal structure with 3PGA			iPGM coordination in crystal structure with 2PGA	
metal	ligand	distance (Å)	metal	ligand	distance (Å)	ligand	distance (Å)
Zn1	OD1 (D327)	2.1	Mn1	OD1(D403)	2.0	OD1(D403)	2.1
	OD2 (D327)	2.4		NE2 (H407)	2.1	NE2 (H407)	2.2
	ND1 (H331)	2.0		NE2 (H462)	2.2	NE2 (H462)	2.2
	NE1 (H412)	2.0		O1P (PO_4)	2.6	O1P (PO_4)	2.6
	O1 (PO_4)	2.1		03P (PO_4)	2.3	03P (PO_4)	2.3
Zn2	OD1 (D51)	2.1	Mn2	OD1 (D12)	2.2	OD1 (D12)	2.0
	OD1 (D369)	1.8		OD2 (D12)	2.7	OD2 (D12)	2.9
	ND1 (H370)	2.0		OG (S62)	2.2	OG (S62)	2.3
	O2 (PO_4)	2.1		OD1 (H444)	2.2	OD1 (H444)	2.2
				NE2 (H445)	2.2	NE2 (H445)	2.3
Zn1	Zn2	3.9	Mn1	Mn2	4.8	Mn2	4.9

[a] The data for *E. coli* AP were averaged from the two monomers of the AP dimer based on the X-ray diffraction structure (Brookhaven Protein Data Bank (PDB): 1ALK).[40] The data for the 3PGA−iPGM complex and the 2PGA−iPGM complex are from crystal structure coordinates.[3,4]

coordination geometry for Mn^{2+} ions which are in either square pyramidal or trigonal bipyramidal coordination geometries in biological complexes.[45] There is no bridging of the Mn^{2+} ions through the phosphate group in the case of the iPGM complexed with either 2PGA or 3PGA. The distance between the two Zn^{2+} atoms in AP is 3.9 Å (an average of the distances from two monomers, 3.9 and 3.8 Å) and 4.8 or 4.9 Å for the Mn^{2+} ions in iPGM complexed with 3PGA or 2PGA, respectively (Table 2). Mutagenesis of a number of the metal coordinating residues in the *B. stearothermophilus* iPGM resulted essentially in complete inactivation of the enzyme.[3]

C. iPGM and AP as Metalloenzymes

The stoichiometry of the metal ions for AP has been investigated in more detail than that of iPGMs.[46−50] The general conclusion is that there are two Zn^{2+} ions per monomer of AP and both are necessary for full catalytic activity. The crystal structure of *E. coli* AP indicates the presence of two Zn^{2+} ions and one Mg^{2+} ion per AP molecule (Zn1, Zn2, and Mg3), and varying the binding of these metal ions can have little to significant effects on enzyme activity. With only two Zn^{2+} ions, Zn1 and Zn2, the enzyme is fully active,[51] while AP with Zn1, Mg2, and Mg3 in the metal binding sites exhibited normal phosphotransferase activity,[47] but this enzyme was compromised in its hydrolysis of the phosphoserine intermediate.[52,53] These results suggest that the Mg^{2+} in binding site 3 has little effect on the activity of *E. coli* AP and that the Zn^{2+} binding in sites 1 and 2 is more essential. However, the situation may be different for mammalian APs which are much more sensitive to Mg^{2+} activation. Substitution of Zn^{2+} by Cd^{2+}, Co^{2+}, or Mn^{2+} in *E. coli* AP resulted in significantly compromised activity[2] with the Cd^{2+}-substituted enzyme having the least activity. The Mn^{2+}-substituted enzyme was compromised primarily in the rate of hydrolysis of the phosphoenzyme intermediate,[54] possibly due to strong binding of the phosphate group of phosphoserine by Mn^{2+}. One can speculate that this is perhaps why Zn^{2+} is used in AP in which the phosphoenzyme intermediate is hydrolyzed while Mn^{2+} is used in iPGM in which the phosphoenzyme intermediate is not hydrolyzed.

For the *B. stearothermophilus* iPGM, the situation is somewhat similar, as this enzyme also requires two metal ions for activity. However, this enzyme requires Mn^{2+} specifically,[6] and this Mn^{2+} requirement was also demonstrated for iPGMs from three related endospore-forming bacteria, *B. subtilis, C. perfringens,* and *Sporosarcina ureae* as well as two organisms that are closely related to *Bacillus* species (*Planococcus citreus* and *Staphylococcus saprophyticus*) but do not form spores.[32] The *C. perfringens* enzyme was also active in the presence of Co^{2+} ions. The activity of the iPGMs from all of these organisms displayed a strong pH dependence, especially at low micromolar Mn^{2+} concentrations where the enzyme exhibits maximum activity at pH values close to 8 and >100-fold less activity at pH 6. For the two nonspore-forming organisms, this pH dependence of iPGM activity may be of no physiological significance but simply a manifestation of the descendance of these two organisms from a spore-forming ancestor.[56]

D. Catalytic Mechanism for *E. coli* AP

E. coli AP catalyzes both hydrolase and transferase activity with phosphomonoester substrates, and the reaction proceeds via a phosphoserine enzyme intermediate involving Ser102 (Figure 6).[40] Phosphate transfer from the substrate to the product has been investigated by oxygen isotope-labeling methods, and the phosphate's configuration during this reaction is maintained.[57] Other than solvent water molecules, other molecules that can accept the phosphate group from the phosphoserine intermediate are generally alcohols, in particular those having an amino group on the carbon adjacent to that with the OH acceptor (e.g., Tris).[58] For values of k_{cat} for AP hydrolysis of selected phosphate monoesters and other kinetic parameters of AP, see ref 2. The catalyzed reaction has several major intermediate steps[2] that are as follows (Figure 6): (a) a phosphate monoester substrate, $ROPO_4^{2-}$ (ROP), forms an intermediate E−ROP during which the ester oxygen coordinates with Zn1 and another phosphate oxygen coordinates to Zn2 (phosphate bridge formed between Zn1 and Zn2). The remaining two phosphate oxygen atoms coordinate with Arg166 in a bidentate fashion, and Ser102 also coordinates to Zn2 (Figure 6, Table 2). (b) The nucleophile Ser102 attacks the phosphorus atom to occupy a fifth coordination site of the phosphate group, forming an enzyme−phosphate (E−P) intermediate; this is followed by the leaving of the RO^- group. During this step the phosphate group maintains its interactions with Zn2 (but not with Zn1) and the bidentate interaction with Arg166. (c) A water molecule then moves in and coordinates with Zn1 in place of the ester oxygen of the substrate. At the alkaline pH values of catalysis, this water molecule dissociates into Zn1−OH^- and a proton. This OH^- group interacts with the phosphate of the E−P intermediate, which leads to the hydrolysis of the phosphoserine bond. The phosphate group moves away from the serine residue, and one of the phosphate oxygen atoms interacts again with Zn1, restor-

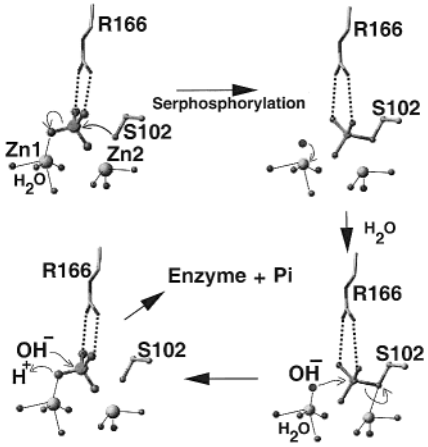

Figure 6. Proposed catalytic mechanism of *E. coli* AP. The catalytic mechanism is as described by Coleman.[2]

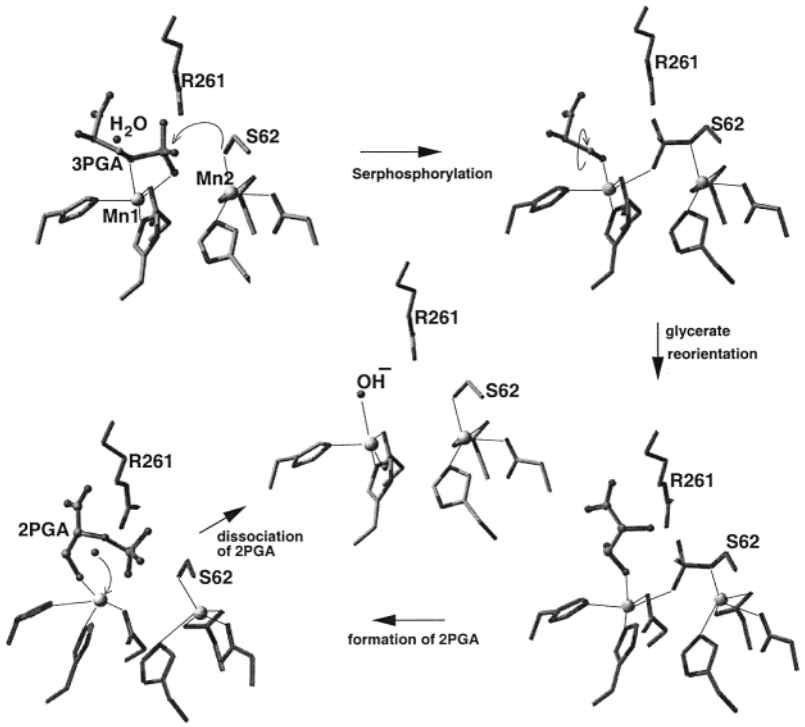

Figure 7. Proposed catalytic mechanism of *B. stearothermophilus* iPGM. The catalytic mechanism is as described by Jedrzejas et al.[3,4]

ing the metal ion−phosphate bridge. (d) The E−phosphate complex then dissociates into enzyme and a free inorganic phosphate. This latter process is very slow, 35 s^{-1}, and is the rate-limiting step of the AP reaction. For the transferase activity of AP, in step c the phosphate group is transferred to the hydroxyl moiety of an alcohol group.

E. Catalytic Mechanism for iPGM and Its Comparison to AP Catalysis

The mechanism of phosphate group transfer from 3PGA to 2PGA or vice versa by iPGM is quite similar to the phosphotransferase reaction catalyzed by AP. The major intermediates of the proposed catalytic mechanism for iPGM are as follows (Figure 7):[3,4] (a) 3PGA binds in the active site of iPGM during which the ester oxygen O1P and O3P coordinate to Mn1. The remaining two phosphate oxygen atoms coordinate with Arg261 in a bidentate fashion (Figure 5A). Ser62 also coordinates with Mn2 and an O2P oxygen atom of the phosphate group; the phosphorus atom is within interacting distance of Ser62. Both Mn1 and Mn2 are in a distorted square planar coordination geometry (Figure 7, Table 2). (b) Ser62 moves to occupy the fifth coordination site of the phosphorus atom and forms a phosphoserine intermediate which is followed by breaking the bond between the phosphate and the D-glyceric acid part of the substrate. During this event the phosphate group maintains its bidentate interaction with Arg261, and Ser62 continues to coordinate to Mn2. Mn1 maintains its distorted square pyramidal coordination geometry as does Mn2. During this step the two Mn^{2+} ions are bridged by the phosphate group. (c) The newly formed O3 end of the glyceric acid (previously O1P) coordinates with Mn1, and this is likely followed by extraction of a proton from the O2 oxygen atom of the D-glycerate by Asp154 (Figure 4). The glycerate then undergoes repositioning to bring the O2 oxygen atom close to the phosphorus atom. (d) The O2 oxygen replaces the previous ester oxygen of the substrate and interacts with the phosphorus of the phosphoserine intermediate; this leads to the breaking of the phosphoserine ester bond and the transfer of the phosphate group to the D-glyceric acid. The newly formed 2PGA moves away from the serine residue, which maintains its coordination with Mn2. (e) The 2PGA complex dissociates into the enzyme and free 2PGA as a water molecule, Wat17, moves to coordinate with the Mn1 ion by hydrolyzing the Mn1−O3 bond and providing an extra hydrogen for the leaving O3 end of the new substrate. Mn1 coordinates to the resultant OH^- group. The next substrate molecule binding in the active site of iPGM replaces this OH^-, which likely picks up a H^+ from the water microenvironment. There is no evidence that the redox properties of Mn^{2+} ions play any role in catalysis, as the Mn^{2+} ions retain their 2+ oxidation state throughout the course of the reaction.

The reaction in the reverse direction is essentially the same as in the forward direction, with the only difference being that the roles of the O2 and O3 oxygen atoms of the D-glycerate part of the substrate switch around.[4] In this direction O2 coordinates with Mn1 and O3 performs a nucleophilic attack on the phosphorus atom of the phosphoserine intermediate. Coordination of the ester oxygen of either of the substrates (3PGA or 2PGA) with Mn1 determines that the product of the reaction will be the other substrate/product form because only the oxygen atom not coordinating with Mn1 will be available for chemical modification. In essence, the iPGM catalyzes a reaction analogous to the phosphotransferase

reaction catalyzed by AP, with the D-glycerate assuming a role analogous to that of an alcohol in the transferase reaction catalyzed by AP. In addition to the mechanistic and functional similarities discussed above, the sequence conservation of the metal binding residues suggests that both APs and iPGMs evolved from a common ancestral enzyme. Indeed, proteins with significant sequence homologies to both APs and iPGMs have been identified among archaebacterial proteins.[6]

F. Discussion

Zn^{2+} and Mn^{2+} often take part in the catalytic mechanisms of metalloenzymes that are activated by OH^- bound to the metal ion.[19] Such metalloenzymes often adopt α/β folds (as do AP and iPGM) in which the metal binding and active sites are located at the edge of the β-sheet core of these enzymes. The direct interactions and coordination of protein residues and substrate to the metal ions aid in catalysis by maintaining these moieties in the proper orientation for catalysis. Such interactions in proteins are often affected by second-order interactions, defined as interactions which are some distance away from the active site. These interactions of more distant residues with residues in the active site or with secondary structure elements affecting the orientation of the active site are essential for the proper orientation of the active site residues, the metal ions, and the hydroxide group for catalysis.[3]

For many enzymes that utilize Zn^{2+} or Mn^{2+} in catalysis, the affinity for these metal ions is so high that the ions do not readily dissociate from the protein, thus making demonstration of a metal ion requirement somewhat difficult. In addition, with this extremely tight binding of metal ions, changes in pH between 6 and 8 may not affect metal binding enough to alter enzyme activity significantly. This latter situation may describe the case for iPGMs of plant species and some bacteria, for which no divalent cation requirement has been definitively established; indeed, the activity of at least one plant iPGM is extremely insensitive to pH between values of 6 and 8.[59] However, the identification of Mn^{2+} in the *E. coli* iPGM and the conservation of the residues involved in Mn^{2+} coordination in *B. stearothermophilus* iPGM in all known iPGMs strongly suggest that these enzymes contain two tightly bound metal ions, although it is certainly possible that these ions could be Zn^{2+} and not Mn^{2+}.

In contrast to the situation described above, Mn^{2+} can readily be removed from iPGMs of *Bacillus* species.[32] The relatively weak binding of Mn^{2+} to these latter enzymes, as well as the coordination of these catalytically important Mn^{2+} ions seem likely to be significant components of the pH sensitivity of these iPGMs, as protons should compete efficiently with Mn^{2+} ions for association with coordinating histidine residues. As expected based on the latter argument, the binding of Mn^{2+} to *B. stearothermophilus* iPGM is remarkably pH sensitive, with binding of only one Mn^{2+} detected at pH 6 with a K_d of $\sim$50 μM while at pH 8 there are two Mn^{2+} bound, one with a K_d of $\leq$ 4 μM and the other with a K_d of $\leq$50 μM (M. Chander and P. Setlow, unpublished results). As expected, the loss of one ion from the binuclear metal ion cluster in the iPGM's active site on going from pH 8 to 6 is accompanied by a significant decrease in enzyme activity (M. Chander and P. Setlow, unpublished results).

Presumably the selective advantage of accumulating a 3PGA reserve in the dormant spore was the driving force for the regulation of the activity of these PGMs during sporulation and spore germination, with the pH changes during these latter processes being a convenient way to modulate Mn^{2+} binding to PGM and thus enzyme activity. In addition, one might expect direct effects of pH on the activity of these enzymes, since even slight displacements of Mn^{2+} ions from their ideal positions would be expected to alter their coordination to substrates, products, and intermediates in catalysis. Indeed, the maximum activity of the *B. stearothermophilus* iPGM (i.e., at very high Mn^{2+} concentration) decreases 5- to 10-fold between pH 8 and 6 (M. Chander and P. Setlow, unpublished results).

G. Why Two Metal Ions in Active Centers of Enzymes?

It is interesting that both iPGM and AP use a binuclear metal complex for their catalysis. Some metalloenzymes utilize a single metal site, and some employ binuclear or even multinuclear metal binding sites. Metals in bimetallic centers usually exhibit correlated physicochemical properties, especially when these metal ions are coordinated through a monatomic bridging ligand like a hydroxyl group or water molecule; this appears to be particularly true when the metal ions possess uncompensated net charges. In the case where there is a lack of bridging, bridging through larger polyatomic groups (e.g., carboxylate or imidazole), or a significant distance between metal ions, the correlation of their properties is limited. Often in this latter situation the two metals are required for distinct functions related to enzyme catalysis or for catalysis of different steps in a sequence of catalytic events. The advantages of binuclear metal centers are as follows: (i) charge delocalization, (ii) smaller activation barriers due to delocalized charge, (iii) the ability to bind larger substrates, (iv) easier electrostatic activation of substrate or ionization of a water molecule, and (v) decreased transition-state energy for reactions.[60] Binuclear electrostatic effects are estimated to decrease the pK_a for proton dissociation from a water molecule by as much as 3−4 units. The features listed above make binuclear metalloenzymes uniquely suited to accomplish some very specialized reactions. The coupled function of two metal ion complexes specifically facilitates the following processes: (i) activation of substrates, (ii) ionization of a water molecule, (iii) stabilization of transition state, and (iv) selectivity and molecular recognition of substrate.

Carboxylate groups are thought to play an additional role by providing structural bridges. They spatially separate and screen the two metal ions to minimize intermetallic interference. In the cases of

AP and iPGM, such bridging is facilitated through phosphate-group oxygen atoms. A phosphate can bridge the two metals, Zn^{2+} or Mn^{2+}, through three atoms (two oxygens and one phosphorus), and this may contribute to an even more pronounced electronic effect relative to a carboxylate group.

For iPGM, the presence of two metal ions in its active site likely facilitates most if not all of the processes described above, and both Mn^{2+} ions also have specialized functions in catalysis. Mn1 interacts principally with the glycerate part of the 2/3PGA substrate and takes part primarily in the phosphotransferase part of reaction through facilitation of reorientation of the glycerate moiety. Mn2 interacts principally with the phosphate group of the substrate and primarily facilitates the formation and cleavage of the phosphoryl−enzyme intermediate. Both of these processes are essential for iPGM catalysis, and the enzyme would not be expected to be functional without either of the Mn^{2+} ions. Both Mn^{2+} ions are also relatively close together, and they likely influence one another's behavior during the various steps of the catalytic process. AP behaves very similarly to iPGM in its catalysis utilizing two Zn^{2+} ions instead of two Mn^{2+} ions. One Zn^{2+} ion is involved mainly in the phosphatase reaction (creation and hydrolysis of the phosphoenzyme intermediate) and the other in the phosphotransferase reaction in addition to the precise positioning of the substrate and catalytic residues for catalysis. However, depending on the type of the substrate utilized, AP does not always carry out a phosphotransferase reaction.

IV. Other Binuclear Metalloenzymes

There are several other enzymes with known three-dimensional structures that contain a binuclear metal ion complex in their active sites. These two metal ions sometimes share a ligand in the catalytic site, as in the case for iPGM and AP. Three of these enzymes which in addition perform functions related to that of iPGM are described and analyzed below and compared functionally and structurally to *B. stearothermophilus* iPGM. There is, however, no significant structural similarity between these three enzymes and iPGM in terms of either the active site structure or the overall three-dimensional structure. This reinforces the importance and functional and evolutionary implications related to the similarity of the structures of the active sites of *E. coli* AP and *B. stearothermophilus* iPGM.

A. Enolase

Enolase, like PGM, is another enzyme in the glycolytic pathway and catalyzes the reversible dehydration of 2PGA to form phosphoenolpyruvate (PEP). PEP has a highly activated phosphate group that is utilized for ATP synthesis in the final step of glycolysis.[60] Different views of enolase catalysis have been proposed based on independent structural studies of this enzyme from *Saccharomyces cerevisiae*[8,61−62] or lobster[63] in different laboratories. The number of metal ions detected and the positioning of these metals differ between these studies. One of these

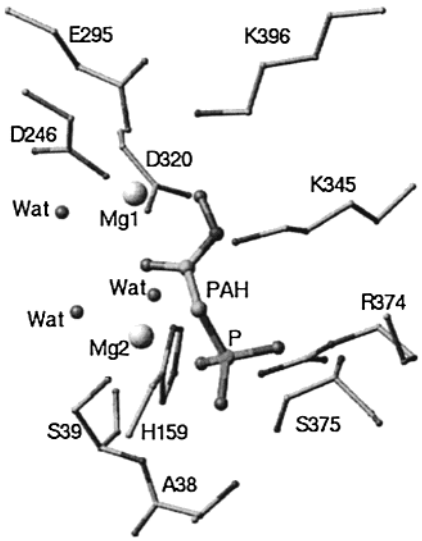

Figure 8. Active site residues of *S. cerevisiae* enolase. The interactions of both Mg^{2+} ions as well as a reaction intermediate analogue, the inhibitor phosphonoacetohydroxamate (PAH), with the protein are shown as determined by Wedekind et al. (PDB: 1EGB).[8]

structures is that of the dimeric *S. cerevisiae* enolase containing a bimetal complex with Mg^{2+} (Figure 8).[8] According to recent mechanistic studies involving structures of complexes of this enzyme with two Mg^{2+} ions and with both substrate and product molecules, enolase, similarly to iPGM and AP, contains a bimetallic active site with either Mg^{2+} or Mn^{2+} ions.[9] The two metal sites differ, however, because the second site is occupied only in the presence of 2PGA (catalytic site). The first site is called a structural site and contains a tightly bound metal ion. Unlike the situation with Mn^{2+} in iPGM, the coordination geometry of both Mg^{2+} ions, Mg1 and Mg2, located in the enolase active site is octahedral and involves protein residue side chains, a substrate/product, and water molecules; both metals are also μ-bridged through a carbonyl oxygen of the substrate/product. The binding of 2PGA to enolase is also different than that in iPGM. For example, in enolase both metal ions interact with the carboxylate group of the 2PGA, while in iPGM there are no interactions between this carboxylate group and Mn^{2+}. Binding of the inhibitor phosphonoacetohydroxamate, a reaction intermediate analogue, in the yeast enolase active site is shown in Figure 8. Although a bimetal structure/mechanism has been reported for the *S. cerevisiae* enolase based on the crystallographic studies of Larsen et al.[9] and the NMR and EPR studies of Poyner et al.,[64] there are other data which indicate that enolase may not contain a bimetallic center.[63]

B. Pyruvate Kinase

Pyruvate kinase is yet another enzyme of the glycolytic pathway and uses the PEP produced by enolase to phosphorylate ADP generating ATP and pyruvate. This enzyme also binds multiple metal ions, including the divalent metal ions Mg^{2+}, Co^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+}.[10] The structure of the rabbit muscle enzyme shows the enzyme's tetrameric nature and the details of the bimetallic active site (Figure

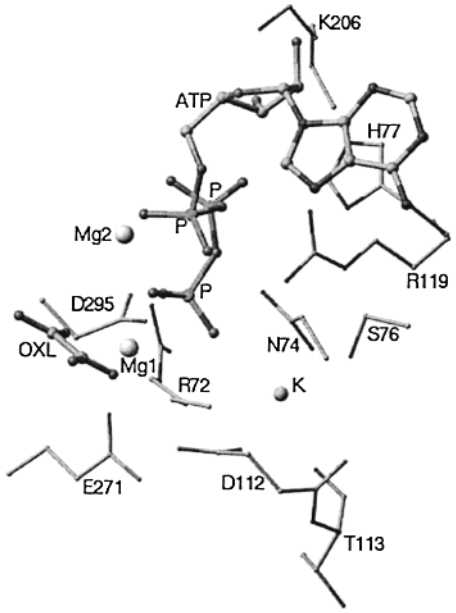

Figure 9. Active site residues of rabbit muscle pyruvate kinase. The interactions of Mg1 (Mn1), Mg2 (Mn2), and K^+ with the protein are shown as described by Larsen et al. (PDB: 1A49).[10]

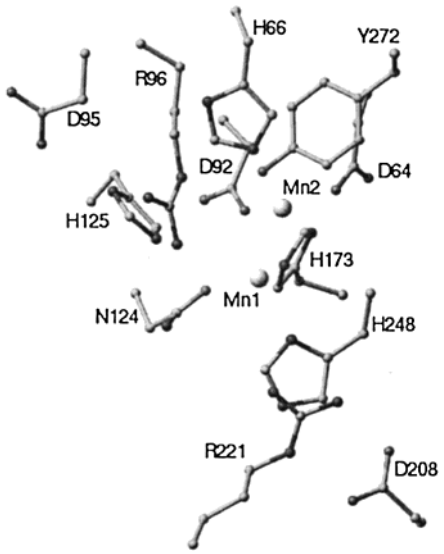

Figure 10. Active site residues of rabbit muscle PP1. The interaction between both metal ions, Mn1 and Mn2, and their interaction with the protein are shown as described by Goldberg et al.[11] and Egloff et al.[12] (PDB: 1FJM).

9).[10,65−68] One of the Mn^{2+} ions, Mn1 (Mg1), in the active site has six ligands, six oxygen atoms of either carboxylate groups or solvent molecules. The second metal in the structure[10] is 5.7 Å away from the Mn1 and is a K^+ which is tetracoordinated with oxygen atoms of protein residues. Another binding site for Mn^{2+} ions, Mn2 (Mg2), requires the presence of the nucleotide substrate for metal binding.[10,68] In the more recent structure of Larsen et al.,[10] both Mn^{2+} ions were replaced by Mg^{2+} ions (Figure 9). Again, the geometry of the metal ions is not square pyramidal or tetrahedral as in iPGM and AP.

C. Serine/Threonine Protein Phosphatase-1

Serine/threonine protein phosphatase-1 (PP1), a metallophosphoesterase, catalyzes the hydrolytic removal of phosphate from phosphoserine or phosphothreonine residues in proteins, a reaction that with protein kinases plays a crucial role in regulation of many biochemical pathways including glycolysis. The structure and mechanism of action of the rabbit muscle PP1 have been reported by Goldberg et al.,[11] and Egloff et al.[12] (Figure 10), and this enzyme seems to require Mn^{2+} for activity.[11] PP1 contains a binuclear cluster of $Mn^{2+}-Fe^{2+}$ or $Mn^{2+}-Mn^{2+}$ pairs;[11,12] Zn^{2+}, however, seems to substitute for Mn^{2+} in vivo, and the data suggest that PP1 may have a mixed metal cluster. At least two mechanisms of action of PP1 have been suggested that involve both metal ions; in these mechanisms the phosphate group coordinates to both metals through its oxygen atoms (not shown), a water molecule, and a carboxylate oxygen of an active site aspartic acid residue (Figure 10). The coordination geometry for metal1, Mn1, is square pyramidal, whereas for metal2, Mn2, it is distorted trigonal bipyramidal. The average distance between these two metals is 3.3 Å due to a double bridge between these ions through a carboxylate oxygen atom of Asp92 as well as a water molecule. The remaining ligands are protein residues or solvent molecules. For Mn1, this coordination is different from that with Mn^{2+} in iPGM, but the coordination of Mn2 is similar to that in iPGM. However, the metal coordination in PP1 is different than the tetrahedral coordination of the Zn^{2+} ions in AP.

V. Conclusions

Some glycolytic enzymes discussed in this review such as iPGM, pyruvate kinase, and possibly enolase utilize binuclear metal center with divalent cations such as Mn^{2+}, Zn^{2+}, and Mg^{2+} to produce ATP, an essential source of energy for multiple biochemical processes. Some of these enzymes are very specific with respect to the metal ions utilized for catalysis, in particular iPGM which has a very strict requirement for Mn^{2+}. Others, such as enolase, can function with a variety of different metals including Mg^{2+} or Mn^{2+}. Binuclear metalloenzymes are found not only in the glycolytic pathway, and an example of such an enzyme is serine/threonine phosphatase-1. Like enolase, serine/threonine phosphatase-1 retains catalytic activity with more than one type of divalent cation. The properties of many other binuclear metalloenzymes have also been covered in recent reviews by Lipscomb and Strater,[70] Dismukes,[60] Christianson,[19] Christianson and Cox,[20] and Ash et al.[71] The discussion of all of the metalloenzymes discussed in these reviews has not been repeated here, and the reader can find more information in these reviews.

The two enzymes which are the focus for this review are *B. stearothermophilus* iPGM and *E. coli* AP. Both of these enzymes can catalyze a phosphatase and a phosphotransferase reaction as a part of their overall catalytic cycle. The phosphotransferase reaction is an inherent part of iPGM catalysis but is an alternative to transfer of phosphate to water for AP. The details of the classification of iPGM as a binuclear Mn^{2+} metalloenzyme have just recently been revealed through the determination of the

crystal structure of this enzyme from *B. stearothermophilus*. The availability of three-dimensional structural information for this enzyme then revealed major similarities between this enzyme's active site, its utilization of two divalent metal ions, and its catalytic mechanism with those of the well characterized AP from *E. coli*. These similarities strongly imply that iPGMs and APs evolved from a common ancestral enzyme and unite both types of enzymes in a single class.[72]

VI. Abbreviations

AP	alkaline phosphatase
ATP	adenosine 5′-triphosphate
ADP	adenosine diphosphate
DPG	diphosphoglyceric acid
PAH	phosphonoacetohydroxamate
PEP	phosphoenolpyruvate
2PGA	2-phosphoglyceric acid
3PGA	3-phosphoglyceric acid
PGM	phosphoglycerate mutase
dPGM	DPG-dependent PGM
iPGM	DPG-independent PGM
PP1	serine/threonine protein phosphatase-1

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