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2,3-Bisphosphoglycerate-independent phosphoglycerate mutase is conserved among different phylogenic kingdoms

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We have previously demonstrated that maize (*Zea mays*) 2,3-bisphosphoglycerate-independent phosphoglycerate mutase (PGAM-i) is not related to 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase. With the aid of specific anti-maize PGAM-i antibodies, we demonstrate here the presence of a closely related PGAM-i in other plants. We also describe the isolation and sequencing of a cDNA-encoding almond (*Prunus amygdalus*) PGAM-i that further demonstrates this relationship among plant PGAM-i. A search of the major databases for related sequences allowed us to identify some novel PGAM-i from different sources: plants (*Arabidopsis thaliana, Oryza sativa* and *Antithamniom* sp.), monera (*Escherichia coli, Bacillus subtilis* and *Bacillus megaterium*) and animals (*Caenorhabditis elegans*). All of these amino acid sequences share a high degree of homology with plant PGAM-i. These observations suggest that the PGAM-i from several biological kingdoms constitute a family of protein different from other proteins with related enzymatic function and arose from a common ancestral gene that has diverged throughout its evolution.

Key words: Phosphoglycerate mutase; Almond; Sequence; Plants; Bacteria; Nematoda; Database search; Evolution.

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Introduction

Phosphoglycerate mutase (PGAM, EC5.4.2.1) is a glycolytic enzyme that catalyses the interconversion of 3-phosphoglycerate and 2-phosphoglycerate. The interconversion of these two monophosphoglycerates can be accomplished by a well-known reaction involving 2,3-bisphosphoglycerate (2,3-BPG) as a cofactor and catalysed by the 2,3-BPG-dependent phosphoglycerate mutase (PGAM-d) or by a not-so-well characterized reaction that does not require the presence of 2,3-BPG. This reaction is catalysed by 2,3-BPG-independent phosphoglycerate mutase (PGAM-i).

PGAM-d is present in all vertebrates, most invertebrates and some fungi and bacteria. It is found in monomeric, dimeric or tetrameric combinations of identical or similar subunits of ~30 kDa (see Fothergill-Gilmore and Watson, 1989, for a review). These subunits have been cloned from yeast (Fothergill-Gilmore and Watson, 1989), human (Shanske *et al.*,

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Abbreviations—PGAM, phosphoglycerate mutase; PGAM-d, 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase; PGAM-i, 2,3-bisphosphoglycerate-independent phosphoglycerate mutase; 2,3-BPG, 2,3-bisphosphoglycerate.

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1987; Sakoda *et al.*, 1988) and rat (Castellá-Escolá *et al.*, 1989; Uchida, 1991; Ureña *et al.*, 1992) tissues and show a high degree of amino acid conservation. PGAM-d also shows some homology with bisphosphoglycerate mutase, a multifunctional enzyme that primarily catalyses 2,3-BPG synthesis and breakdown but also possesses PGAM activity (Fothergill-Gilmore and Watson, 1989). Moreover, PGAM-d shows a striking similarity to the family of acid phosphatases and to Fru-2,6-P_2ase (Bazan *et al.*, 1989; Bazan and Fletterick, 1990), which indicates a common evolutionary origin for all these enzymes.

PGAM-i is present in all plants and some members of fungi, monera, protista and animal kingdoms (Carreras *et al.*, 1982). It is a monomeric enzyme with a relative molecular weight of 55–75 kDa (Fothergill-Gilmore and Watson, 1989; Graña *et al.*, 1989). We cloned a PGAM-i cDNA from maize (Graña *et al.*, 1992), the first PGAM-i to be cloned, and recently isolated its gene (Pérez de la Ossa *et al.*, 1994). Comparison between maize PGAM-i and PGAM-d sequences shows they are not evolutionarily related.

In this work we studied the relationship of PGAM-i from different species by immunoneutralization and Western blot experiments. We also cloned a cDNA-encoding PGAM-i from a dicotyledoneus species, *Prunus amygdalus*. In addition, by means of database search we identified new PGAM-i sequences that had not previously been related to PGAM-i. The phylogenetic relationships among PGAM-i from different kingdoms were studied, and we conclude that the PGAM-i present in at least three kingdoms arose from a common progenitor and constitute a distinct family of protein sequences.

Materials and Methods

Enzymes and chemicals

Enzymes, substrates and cofactors were purchased from Boehringer-Mannheim (Mannheim, Germany) and Pharmacia (Uppsala, Sweden). ^{32}P-dCTP was from Amersham (Buckinghamshire, UK). Purified antibodies raised against maize seed PGAM were obtained in our laboratory as previously described (Graña *et al.*, 1989). Bradford reagent was from Bio-Rad (Richmond, CA, USA). Peroxidase-conjugated swine anti-rabbit IgG was from Dakopats (Glostrup, Denmark) and diaminobenzidine was from Sigma (St. Louis, MO, USA). *P. dulcis* cDNA library in lambda ZAP was from Stratagen (La Jolla, CA, USA). All other reagents were analytical grade.

Plant material

Plant seeds were kept at 30°C in a humid chamber in the dark for imbibition and germination (Graña *et al.*, 1993). Two- to 7-day-old seedlings or animal tissues were frozen in liquid nitrogen and powdered with a steel mortar. The frozen material was homogenized with a potter in 20 mM phosphate buffer, pH 7.5, for protein extractions and in 8M guanidinium hydrochloride, 20 mM 2-[N-Morpholino]ethanesulfonic acid (MES) and 20 mM EDTA, for total RNA isolation. Supernatants were removed after centrifugation, kept on ice and immediately processed. RNA was isolated and PGAM activity assayed as previously described (Graña *et al.*, 1989, 1993). Protein concentration was determined as described by Bradford (1976).

Immunological test and Northern blot analyses

Immunoneutralizations and Western blots of protein extracts were done essentially as previously described (Graña *et al.*, 1989). In all experiments, maize embryo protein extracts were used as internal control. Fifty micrograms of protein extract was loaded per lane. Northern blot experiments were conducted as previously described (Graña *et al.*, 1993) except that washes were less stringent (0.5–1 × SSC, 0.1% SDS).

cDNA library screening

A cDNA library made in lambda ZAP using mRNA from young almond roots was screened using cDNA restriction fragments derived from maize clone, which encodes almost the entire coding region for maize PGAM (Graña *et al.*, 1992). The cDNA insert was subcloned in pBluescript by *in vivo* excision and sequenced in an automatic DNA sequencer (ALF, Pharmacia).

Genebank and EMBL nucleotide databases were compared with maize PGAM-i amino acid sequence using the TFASTA (Madison, WI, USA) option of the GCG package.

Results

To ascertain the evolutionary relationship among PGAM-i of different species, including representatives of the plant, fungi, animal and monera kingdoms, immunoneutralization, Western blot and Northern blot analyses were performed. Figure 1 shows the ability of maize PGAM-i antibody to immunoneutralize PGAM-i activity from different sources. PGAM-i activity was immunoneutralized in extracts of all plants tested. In contrast, PGAM-i activity from other kingdoms, includ-

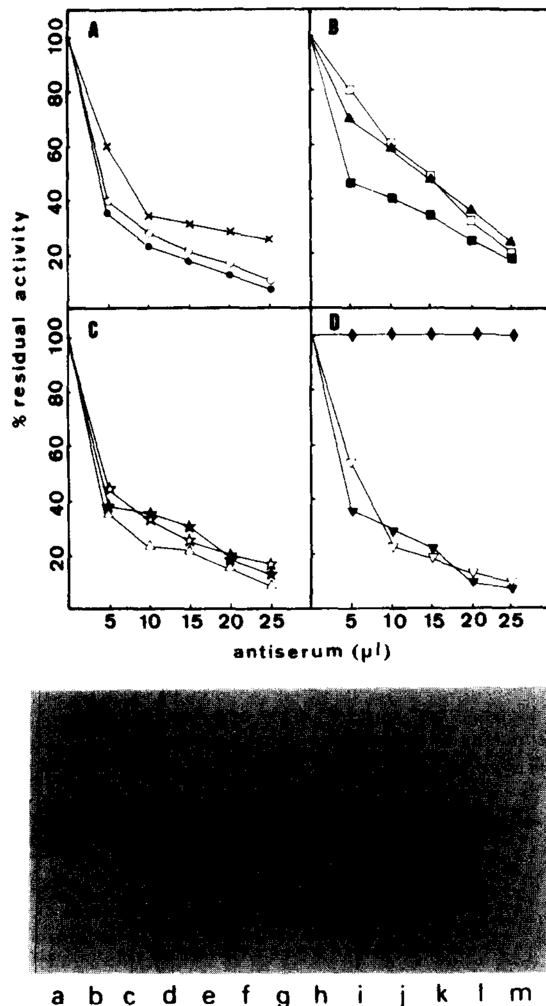

Fig. 1. Immunoneutralization and Western blot of PGAM-i from different sources. (Top). Immunoneutralization. (A) Monocotyledonae: ×, onion; ○, Black Mexican Sweet; ●, maize. (B and C) Dicotyledoneae: □, chickpea; ▲, lentil; ■, haricot bean; ☆, tobacco; ★, almond; △, tomato. (D) ▽, carrot; ▼, pine; ◆, starfish, mushroom and spider. (Bottom) Western blot. a, almond; b, Black Mexican Sweet; c, maize embryo; d, maize root; e, maize coleoptile; f, wheat germ root; g, wheat germ coleoptile; h, rice root; i, rice coleoptile; j, haricot bean; k, onion; l, iris; m, fern.

ing members of the Arthropoda, Equinoderma and Nematoda classes, a gram-positive bacteria (*Bacillus subtilis*) and a fungus (mushroom, *Agaricus bisporus*), which also have the co-factor-independent enzyme, was not immunoneutralized.

Western blot experiments show a similar spectrum of cross-reactions (Fig. 1). In addition, a faint band with relative molecular mass similar to that of plant PGAM was observed in starfish protein extracts. In contrast, no specific PGAM-i bands were observed in members of three different kingdoms: *B. subtilis, A. bisporus* and *Caenorhabditis elegans* (data not shown).

Northern blot experiments were performed using maize PGAM-i cDNA as a probe, and, as expected, the results were even more specific. At high stringency, a band of ~2.1 kb was observed in all maize tissues tested, including Black Mexican Sweet variety. At low stringency we found a weak band of similar mobility in several plant RNA preparations, but in these conditions the background was high and specificity difficult to assess (not shown).

The immunological studies clearly demonstrate a significant degree of PGAM-i similarity among plants. To further analyze the relationship between the PGAM-i of different plant species, the cDNA corresponding to almond PGAM, a dicotyledonous plant belonging to the Rosaceae family, was cloned using the maize clone as a probe. The isolated almond cDNA was 1.5 kb long (not shown) and contained an open reading frame coding for 487 amino acids. Comparison of the deduced amino acid sequences of almond and maize PGAM indicates that the 1.5-kb almond cDNA contained ~90% of the entire coding region. Furthermore, Western blot experiments predicted a protein molecular mass similar to that of maize PGAM (Fig. 1, bottom). Comparison of the nucleotide sequence of almond and maize showed only 76% identity, which explains the poor cross-hybridization observed in plant Northern blots using maize PGAM cDNA as a probe. Amino acid homology was greater than at cDNA level because many base changes take place in the third position of codons and in many cases imply no changes in the amino acid sequence. Thus, the predicted almond amino acid sequence is 84% identical and 91% similar to that of maize (Fig. 2). These data, together with the immunological data presented above, indicate a high degree of similarity among PGAM from different plants.

In the last few years, several projects have been started to identify new genes in an organism such as *C. elegans* (Waterston *et al.*, 1992), among others. Different strategies have been used to accomplish this, but basically cDNA libraries are constructed such that all different cDNAs are represented with similar probabilities. Several clones are randomly isolated and partially sequenced. As a result, partial nucleotide sequences corresponding to several unknown gene products are available in the major databases. In view of the high degree of similarity between maize and almond cDNAs, we searched these databases for PGAM-i-like sequences derived from species distant in evolution. In this way we identified several nucleotide sequences coding for

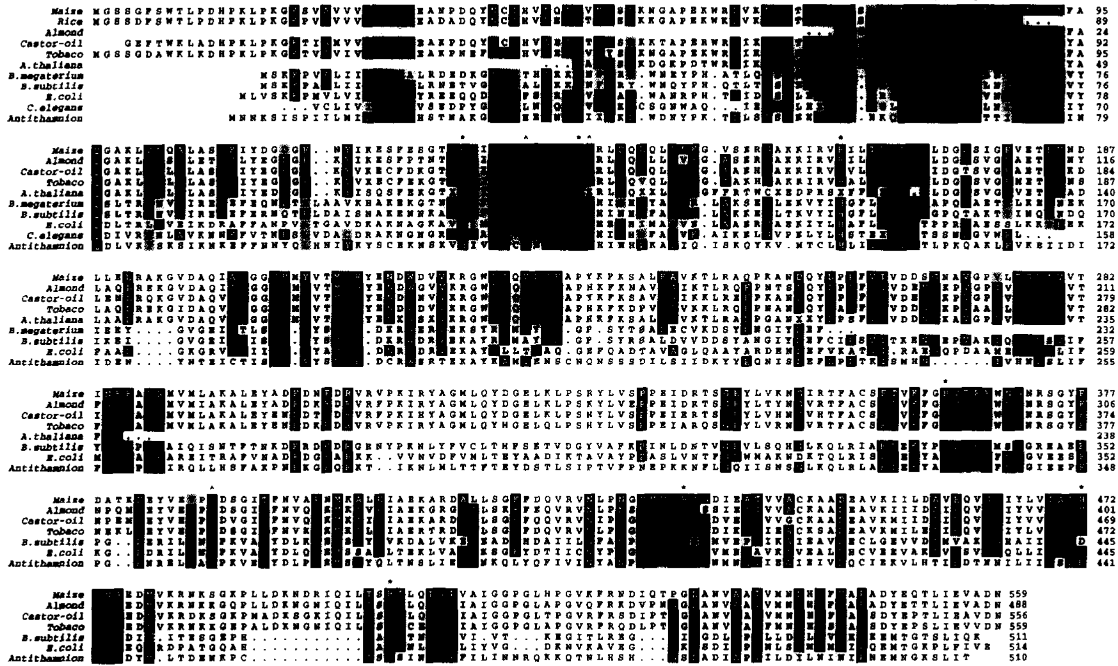

Fig. 2. Predicted sequence of almond PGAM-i and alignment with maize PGAM-i and other known PGAM-i-like sequences. Amino acid in black are identical in all sequences, whereas amino acids in grey are similar. The conserved His and Ser are shown by * and ^, respectively.

gene products, which showed a notable degree of similarity with maize PGAM-i (Fig. 2). Three plant sequences with high similarity to maize PGAM-i were found: (1) the 5′fragment, 267 bp long, from rice (*Oryza sativa*); (2) the central fragment, 717 bp long, from *A. thaliana* and (3) the full-length PGAM-i cDNA sequence from *Antithamniom* sp. We also found three PGAM-i-related sequences from the monera kingdom: (1) the full-length PGAM-i cDNA sequence from *Escherichia coli,* (2) a 1533 bp long fragment, 90% full-length sequence from *B. subtilis* and (3) a 696 bp long fragment from *B. megaterium* (Schläpfer and Zuber, 1992). Moreover, we detected a further sequence, a 474 bp long fragment from the Nematoda *C. elegans*. The deduced protein sequences are 29–91% identical and 55–97% similar to those from the first described maize PGAM-i sequence (Figs. 2 and 3). The identity and similarity data shown in Fig. 3 were obtained by comparing the amino acid sequence available for each species. Thus, some percentages may differ to a certain extent from those derived from comparison of the full-length amino acid sequences.

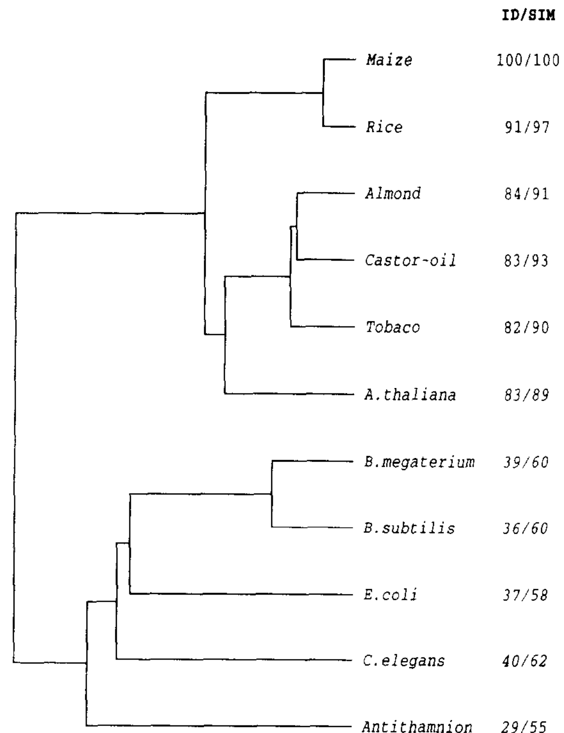

Fig. 3. Evolutionary tree of PGAM-i-like enzymes. Identity (ID) and similarity (SIM) of the enzymes with respect to maize PGAM-i are indicated on the right.

Discussion

Glycolytic enzymes are ancient widely distributed proteins that have evolved slowly and are particularly suitable for the study of the

evolution of enzymes. In particular, PGAM is found in all organisms, with the only exception being some thermoacidophile bacteria (Fothergill-Gilmore and Watson, 1989). Knowledge of PGAM-d and PGAM-i, the two groups of this enzyme, differs greatly. PGAM-d is a well-known enzyme that has been studied in depth from different organisms. In contrast, knowledge of PGAM-i is more limited. Fortunately, understanding of PGAM-i has rapidly increased since it was first purified (Graña *et al.*, 1989), its cDNA cloned (Graña *et al.*, 1992) and its gene isolated and sequenced (Pérez de la Ossa *et al.*, 1994) from maize. In this article we present an evolutionary study of PGAM-i that includes identification of several sequences, not previously identified as PGAM-i, obtained from the databases.

Comparison of maize and almond sequences shows a high degree of identity. This identity among plant PGAM-i is enhanced by immunological and database studies. Moreover, although the work here presented was being carried out, two additional plant PGAM-i from tobacco (*Nicotiana tabacum*) and castor oil (*Ricinus communis*) were cloned (Huang *et al.*, 1993). Taken together, all of these studies clearly demonstrate a high degree of conservation of these enzymes in the plant kingdom and suggest a close evolutionary relationship. A singular case is that of *Antithamniom* sp., a plant whose PGAM amino acid sequence, obtained from the database, had not been identified as PGAM-i to date. This sequence possessed only 29% identity and 55% similarity with maize, the pattern used in this work. Although *Antithamniom* is a rhodophyta plant, it is one of the least differentiated, and some authors include it in the monera kingdom (Margulis and Schwartz, 1982). Moreover, the amino acid sequence of *Antithamniom* sp. has been deduced from a plastid DNA sequence. Thus, its low degree of identity with other plants is not so surprising.

The other amino acid sequences deduced from DNA sequences from database search belong to bacteria and Nematode. They present 36–40% identity and 58–62% similarity with maize. The overall identity is of the same order (50%) as that present among yeast and human PGAM-d and bisphosphoglycerate mutase. These identities are similar to those found in other glycolytic enzymes such as enolase, which, from *B. subtilis*, is 41% similar to that from tomato (Van Der Straeten *et al.*, 1991). These data, compiled from individuals from three of the five kingdoms, again confirm the conservation of glycolytic enzymes throughout evolution.

Among bacteria, *E. coli* stands out. It has been proposed that it belongs to the PGAM-d group (D'Alessio and Josse, 1971). This relationship with PGAM-d is enhanced by several data: (1) it binds to Cibacron Blue-Sepharose and is eluted by 2,3-BPG (Price and Stevens, 1983); (2) it is inhibited by vanadate (data not shown), a reagent that does not inactivate PGAM-i; and (3) it is not immunoneutralized by anti-PGAM-i antibodies (Graña *et al.*, 1989). However, its amino acid sequence presents 37% identity and 58% similarity with maize, and its relation with PGAM-i is very clear (Fig. 2). The amino acid sequence and properties of *E. coli* indicate that the pattern of PGAM evolution is very complex. It is clear that all vertebrates possess PGAM-d and all plants PGAM-i, which shows that both groups derived from two different ancestral genes. All other considerations are ambiguous because cofactor dependence of some PGAM, such as *E. coli*, could be altered during evolution. It is clear that more sequences must be determined to ascertain the evolutive pattern of PGAM. Comparison of all of these sequences permits us to propose an evolutionary tree of PGAM-i enzymes (Fig. 3), which has been determined considering only the amino acid sequences known to date. Many of these sequences are complete, but some only partially so. Thus, some changes due to their unknown amino acid sequences may not be ruled out.

PGAM-d and PGAM-i differ in their mechanisms. PGAM-d acts through an intramolecular mechanism that involves the formation and hydrolysis of a phosphoenzyme intermediate with the participation of two histidine residues (Fothergill-Gilmore and Watson, 1989). PGAM-i acts through an intramolecular mechanism, and the phosphoenzyme intermediate has not been detected. However, studies with ^{18}O-labeled substrate indicate that the phosphoenzyme is involved (Britton *et al.*, 1971; Breathnach and Knowles, 1977; Blatter and Knowles, 1980). The amino acid residues involved as phosphoacceptor in PGAM-i are unknown, and although it is possible that, like PGAM-d, a histidine may be involved, the participation of a serine may play this role (Graña *et al.*, 1992).

It is also noteworthy that comparison of all PGAM-i sequences shows the presence of some conserved protein domains that contain four serines and eight histidines, amino acids that can act as phosphoacceptors. Thus, some of these clusters may participate in the maintenance of enzyme conformation and/or include part of the active site. PGAM-i from *Bacillus* sp. is Mn^{2+}-dependent (Shing and Setlow, 1978; Watabe and Freese, 1979), which sug-

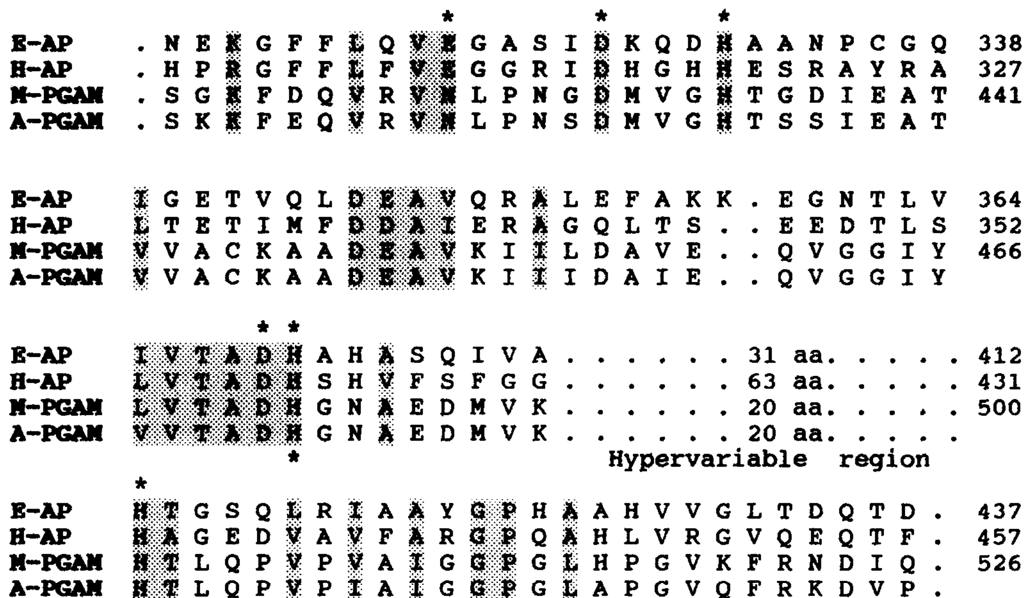

Fig. 4. Comparison of the amino acid sequences of maize (M) and almond (A) PGAM-i with *E. coli* (E-AP) and human (H-AP) alkaline phosphatases. Shaded sequences represent similar regions between alkaline phosphatases and plant PGAM-i, and asterisks represent the metal binding sites of alkaline phosphatases. The histidine conserved in both PGAM-d and PGAM-i is indicated by an additional asterisk below the amino acid sequence.

gests that it may be a metalloprotein, although it possesses an amino acid composition similar to that of maize PGAM-i. Therefore, if all PGAM-i have evolved from a common ancestor, it may be that some PGAM-i have conserved a metal binding site regulation during evolution, whereas others have not.

The similarity of sequences among PGAM-i, especially the conserved protein domains containing Ser and His, underlines the need for mutagenesis experiments to elucidate the amino acid residues involved in the active site of PGAM-i.

A possible relationship between maize PGAM and the alkaline phosphatase family was previously proposed based on the observation that some clusters of amino acids involved in alkaline phosphatase metal binding properties are present in the maize PGAM-i amino acid sequence (Graña *et al.*, 1992). The corresponding residues are also present in the deduced amino acid sequence of plant PGAM-i (Fig. 4). In this figure only maize and almond PGAM-i sequences are represented, but the clusters are also present in all other plant PGAM-i known. This increases the likelihood of a common origin for alkaline phosphatase and PGAM-i. However, the suggested putative phosphorylation site triads (Asp^{266}-Ser-GLy^{268}) (Graña *et al.*, 1992) are not conserved, but other serines followed by glycine are also present in plant PGAM-i. The PGAM-i primary sequences present the amino acid residues Ala^{470}-Asp-His-Gly-Asn^{474} that, with the exception of the substitution of Asp for Ala, are identical to human and rat PGAM-d sequences. This His is involved in the phosphotransfer reaction of all PGAM-d and is also conserved in the alkaline phosphatase primary sequence (Fig. 4).

In conclusion, our data suggest that PGAM-i form a homogeneous branch in which all of their members may have evolved from the same ancestral gene. Thus, according to their phylogenic origin, PGAMs can be classified in two distinct groups: PGAM-d, including bisphosphoglycerate mutase, which has been shown to be evolutionarily related to a family of acid phosphatases (Bazan *et al.*, 1989; Bazan and Fletterick, 1990), and PGAM-i, including Mn^{2+}-dependent *Bacillus* sp. PGAM, which has been related to alkaline phosphatases (Graña *et al.*, 1992). These enzymes form a family of proteins distributed among plant, animal and bacterial species.

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