The Purification and Characterization of Phosphonopyruvate Hydrolase, a Novel Carbon-Phosphorus Bond Cleavage Enzyme from *Variovorax* sp. Pal2*

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Phosphonopyruvate hydrolase, a novel bacterial carbon-phosphorus bond cleavage enzyme, was purified to homogeneity by a series of chromatographic steps from cell extracts of a newly isolated environmental strain of *Variovorax* sp. Pal2. The enzyme was inducible in the presence of phosphonoalanine or phosphonopyruvate; unusually, its expression was independent of the phosphate status of the cell. The enzyme had a molecular mass of 63 kDa with a subunit mass of 31.2 kDa. Activity of purified phosphonopyruvate hydrolase was Co²⁺-dependent and showed a pH optimum of 6.7–7.0. The enzyme had a $K_m$ of 0.53 mm for its sole substrate, phosphonopyruvate, and was inhibited by the analogues phosphonoformic acid, 3-phosphonopropionic acid, and hydroxymethylphosphonic acid. The nucleotide sequence of the phosphonopyruvate hydrolase structural gene indicated that it is a member of the phosphoenopyruvate phosphomutase/isocitrate lyase superfamily with 41% identity at the amino acid level to the carbon-to-phosphorus bond-forming enzyme phosphoenopyruvate phosphomutase from *Tetrahymena pyriformis*. Thus its apparently ancient evolutionary origins differ from those of each of the two carbon-phosphorus hydrolases that have been reported previously; phosphonoacetaldehyde hydrolase is a member of the haloacetate dehalogenase family, whereas phosphonoacetate hydrolase belongs to the alkaline phosphatase superfamily of zinc-dependent hydrolases. Phosphonopyruvate hydrolase is likely to be of considerable significance in global phosphorus cycling, because the phosphonopyruvate hydrolase is known to be a key intermediate in the formation of all naturally occurring compounds that contain the carbon-phosphorus bond.

Organophosphonates are a group of biogenic compounds characterized by the presence of a stable covalent carbon to phosphorus (C–P) bond. Although they are believed to have originated before the earth’s atmosphere became extensively oxygenated, C–P compounds have been isolated from a wide variety of extant life forms, and a significant percentage of biogenic phosphorus is believed to occur in the C–P linkage. Moreover a growing number of synthetic organophosphonates have found applications in industrial, agricultural, and domestic products and are ultimately disposed of to soils or natural waters. An understanding of C–P bond metabolism is thus a prerequisite to a fuller understanding of global biogeochemical phosphorus cycling.

Four distinct bacterial enzymes are known to carry out the C–P bond cleavage reaction that is central to organophosphonate mineralization. The C–P lyase complexes have a broad substrate specificity (1) and can act upon unsubstituted alkyl and aryl organophosphonates (general formula R–PO₃H₂). The two C–P hydrolases that act on phosphonoacetaldelyde (OHC–CH₂–PO₃H₂) (2) and phosphonoacetate (HOOC–CH₂–PO₃H₂) (3) are essentially specific to their respective substrates. A fourth C–P bond-metabolizing enzyme, phosphoenolpyruvate phosphomutase (PEP mutase), catalyzes the intramolecular rearrangement and interconversion of 3-phosphonopyruvate (COOH–CO–CH₂–PO₃H₂) and phosphoenolpyruvate (COOH–C(=CH₂)–O–PO₃H₂) (4). Phosphonopyruvate formation by this route is a key step in the biosynthesis of all known natural products that contain the C–P bond (5).

One of the most widely distributed of such biogenic C–P compounds is 2-amino-3-phosphonopropionic acid, commonly called phosphonoalanine (HOOC–CHNH₂–CH₂–PO₃H₂); it is formed through the transamination of phosphonopyruvate by many lower organisms, such as the sea anemone *Zoanthus sociatus* (6) and the protozan *Tetrahymena pyriformis* (7). Our earlier studies on its biodegradation showed that 47 of 100 randomly chosen environmental bacterial isolates had the ability to utilize phosphonoalanine as sole phosphorus source for growth (8). Subsequently Ternan et al. (9) reported the isolation of an environmental *Burkholderia cepacia* strain capable of growth on phosphonoalanine as sole source of carbon, nitrogen, and phosphorus. Cell extracts of the isolate contained a previously unknown P₂-independent activity designated phosphonopyruvate hydrolase (PHH) that catalyzed the hydrolytic cleavage of the C–P bond of phosphonopyruvate to yield pyruvate and P₂ (Fig. 1).

We now describe the purification and properties of PHH from a soil isolate of a *Variovorax* sp. and the sequence analysis of

1 The abbreviations used are: C–P, covalent carbon to phosphorus bond; PEP mutase, phosphoenolpyruvate phosphomutase; PHH, phosphonopyruvate hydrolase; CAPS, 3-cyclohexylamino propanesulfonic acid.

*This work was supported by Biotechnology and Biological Sciences Research Council (UK) Grant 81/p11488 (1999–2002), a Strategic Research Infrastructure grant for Environmental Engineering and Biotechnology at The Queen’s University of Belfast, the Invest Northern Ireland RTD Centres of Excellence Programme, and by The Queen’s University Environmental Science and Technology Research Centre, Belfast BT9 7BL, Northern Ireland. Tel.: 44-0-28-90-272287; Fax: 44-0-28-90-835877; E-mail: j.quinn@qub.ac.uk.

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† The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY179862.

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the structural gene that encodes it. Intriguingly, the enzyme shares a common ancestry with PEP mutase, which is responsible for phosphonopyruvate biosynthesis (see above); the interaction of the two activities is thus likely to play an important regulatory role in global phosphorus cycling.

**EXPERIMENTAL PROCEDURES**

**Microorganism and Culture Conditions**—Samples from soils, natural water bodies, and waste treatment installations in Northern Ireland were incubated in mineral salts medium (8) with D,L-phosphonoalanine (5 mM) as sole carbon, nitrogen, and phosphorus source for 5–7 days. Cultures were plated on the same medium solidified with agar, single colonies were isolated, and those that grew most rapidly were chosen for further study.

Cells of Variovorax sp. Pal2 were routinely grown in batch culture at 30 °C on an orbital shaker at 100 rpm in mineral salts medium (8) supplemented with 3 g pyruvate/liter and 2 g gluconate/liter as carbon sources, 5 mM NH4Cl as nitrogen source, and 0.5 mM phosphonopantoic acid as phosphorus source. Cells were harvested in mid-log phase by centrifugation, washed twice in 20 mM HEPES-KOH buffer, pH 7.0, and stored at −20 °C.

**Synthesis of 3-Phosphonopyruvate**—The trillithium salt of phosphonopyruvate was prepared according to the method of Sparkes et al. (10) by chemical synthesis services, Belfast, Northern Ireland via chemical trifugation, washed twice in 20 mM HEPES-KOH buffer, pH 7.0, and stored at −20 °C. For preparation of crude extract, the washed cells (~15 g wet weight from 5 liters of the culture) were suspended in 65 ml of 20 mM HEPES-KOH buffer, pH 7.0, and disrupted by sonication on ice for 6 min (30-s sonication followed by 2-min cooling) at 16 KHz. The cellular debris was removed by centrifugation (10,600 × g for 30 min at 4 °C), and the supernatant was immediately used in the next step. For ammonium sulfate fractionation, the cell-free supernatant was fractionated in two steps by addition of solid ammonium sulfate at 20 °C and stirring for 1 h. The precipitate obtained between 2.1 and 3.1 M was collected by centrifugation (10,600 × g for 30 min at 4 °C) and dissolved in 20 mM HEPES-KOH buffer, pH 7.0. For phosphonopyruvate hydrolysis, the protein fraction obtained by salt precipitation was applied to a phenyl-Sepharose HP column (5.0 × 4.0 cm) (Amersham Biosciences) equilibrated with 50 mM HEPES-KOH buffer, pH 7.0, containing 1 M (NH4)2SO4. The column was washed with 3 bed volumes of 0.5 M (NH4)2SO4 in 50 mM HEPES-KOH buffer, pH 7.0. The proteins were eluted at 8 ml/min first with 0.1 M (NH4)2SO4 in 50 mM HEPES-KOH buffer, pH 7.0 (200 ml), and then the enzyme with the buffer alone (160 ml). Fractions containing active enzyme were pooled, concentrated, and desalted with 20 mM HEPES-KOH buffer, pH 7.0, by ultrafiltration through a Vivaspin 15 concentrator (10,000 molecular weight cut-off) (Sartorius). For anion-exchange chromatography (column 1), the enzyme solution was applied to a Q-Sepharose HP anion-exchange column (1.6 × 4.8 cm) (Amersham Biosciences) equilibrated with 20 mM HEPES-KOH buffer, pH 7.0. Proteins were eluted at 2.5 ml/min, applying an initial isocratic step in the equilibration buffer (27 ml) followed by a linear gradient of 0–0.2 M NaCl in the same buffer (80 ml). Fractions containing the enzyme were pooled and dialyzed against 25 mM HEPES-KOH buffer, pH 7.0, containing 0.5 M NaCl. For immobilized metal ion affinity chromatography, the enzyme solution (1.1 ml) was applied to a chelating-Sepharose column (1.0 × 7.5 cm) (Amersham Biosciences) charged with Cu2+ and equilibrated with 25 mM HEPES-KOH buffer, pH 7.0, containing 0.5 M NaCl. The column was washed with the same buffer (20 ml), and the bound proteins were eluted at 2 ml/min first with a linear gradient of 0–0.5 M NH4Cl in 25 mM HEPES-KOH buffer, pH 7.0, containing 0.5 M NaCl. The active fractions were pooled, concentrated, and desalted with 20 mM HEPES-KOH buffer, pH 7.8, by ultrafiltration as before. For anion-exchange chromatography (column 2), the sample (1.1 ml) was applied to a Q-Sepharose HP column (1.0 × 4.5 cm) equilibrated with 20 mM HEPES-KOH buffer, pH 7.8. The column was washed with the same buffer (10 ml), and the bound proteins were removed with a linear gradient of 0–0.2 M NaCl in the same buffer at a flow rate of 1 ml/min. PPH was eluted in a single major peak at about 0.19 M NaCl. The active fractions were pooled, dialyzed, and concentrated by ultrafiltration as before and stored in aliquots with 25% glycerol at −20 °C.

**Gel Electrophoresis**—SDS-PAGE used precast 8–16% Tris/glycine or NuPAGE BisTris gels in an X cell 11 mini-cell with Mark 12 molecular mass standards (Invitrogen). Gels were stained with EZBlue gel staining reagent (Sigma) or the SilverQuest kit (Invitrogen). Electrophoresis under non-denaturing conditions used precast 8–16% Tris/glycine polyacrylamide gels (Invitrogen). Enzyme activity was located by immersion of the gel for 15 min at 37 °C in the phosphonopyruvate hydrolysis assay mixture and then detecting the phosphate released with the Fiske and Subbarrow reagent (see above); the enzyme’s activity was manifested by a blue band. Protein was detected using the EZBlue reagent (Sigma).

**Size Exclusion Chromatography**—This was performed using an Ultrahydrogel SEC 3,000 column (Beckman Coulter) equilibrated with 20 mM sodium phosphate buffer, pH 7.2, containing 0.25 M NaCl, at a flow rate of 1 ml/min.

**Gel Electrofocusing**—Isoelectric focusing gels at pH 5–7 (Invitrogen) were used with pI standards (WVR International).

**Protein Sequencing**—Approximately 50 μg of dried enzyme was desiccated (in 15 μl) and water (15 μl), and a small crystal of CNBr was added. After incubation at room temperature for 24 h the sample was lyophilized and dissolved in 10 μl of SDS-PAGE sample buffer. Pure enzyme and its CNBr fragments were separated in NuPAGE BisTris gels (Invitrogen) and blotted by the semi-dry method onto polyvinylidene difluoride membranes (ProBlot; Applied Biosystems). Using 10 mM CAPS buffer, pH 11.0, containing 0.1% Tween 20, Protein bands were visualized by EZBlue (Sigma) staining and excised. Automated Edman sequencing was performed by the Microchemical Facility, The Babraham Institute (Cambridge, United Kingdom).

**Mass Spectrometry**—This was carried out with a Voyager-DE matrix-
Characterization of Phosphonopyruvate Hydrolase

### Table I

| Procedure                        | Volume | Total units | Protein | Specific activity | Yield | Purification |
|----------------------------------|--------|-------------|---------|------------------|-------|--------------|
| Crude extract                    | 60.5   | 271.0       | 5.2     | 0.88             | 100   | 1            |
| (NH₄)₂SO₄                       | 35.0   | 243.0       | 4.1     | 1.7              | 90    | 2            |
| Phenyl-Sepharose                 | 15.0   | 170.8       | 1.63    | 7.0              | 70    | 8            |
| Q-Sepharose, pH 7.0              | 4.0    | 148.0       | 0.9     | 41.0             | 61    | 48           |
| Chelating Sepharose (Cu²⁺)       | 1.1²   | 93.9        | 1.2     | 70.0             | 35    | 81           |
| Q-Sepharose, pH 7.8              | 1.1²   | 51.9        | 0.32    | 163.0            | 19    | 189          |

* Volume after concentration by ultrafiltration.

Enzyme Stability—The effect of temperature on the stability of PPH was determined by exposure of the reaction mixture without substrate at temperatures from 30 to 75 °C for 10 min prior to performing the assay. The activity in the dialyzed preparation was 61 units/mg, and after EDTA treatment this fell to 30 units/mg; all activities were compared with the latter value.

Effect of pH—The pH optimum was determined using the standard assay with different sets of 100 mM buffers as follows: sodium succinate/NaOH, pH 3.5–5.5, MOPS/NaOH, pH 7.0–8.0, HEPES/KOH, pH 6.4–7.4, and H₂BO₃/NaOH, pH 8.0–10.0. The effect of temperature—PPH activity was assayed at different temperatures over the range from 0 to 60 °C. The following temperature profile was used: initial denaturing at 95 °C for 1 min at 0 °C to the reaction mixture and then incubating different metal ions (5 mM final concentration) for 5 min at 37 °C prior to measuring activity. The activity in the dialyzed preparation was 61 units/mg, and after EDTA treatment this fell to 30 units/mg; all activities were compared with the latter value.

Determination of Kinetic Parameters—In the presence of phosphonopyruvate by using concentrations ranging from 0.05 to 25.0 mM. Activity was measured at 37 °C as described above.

Cloning and Analysis of the pphA Gene—General DNA isolation and manipulation techniques were carried out following published protocols (12). Restriction endonuclease digestions and ligations with T4 DNA ligase were performed as recommended by the enzymes’ suppliers (Amersham Biosciences and Sigma). Plasmid pGEM-T Easy (Promega) was used for cloning of the PCR fragments. Plasmids pUC18 and pUC19 containing inserts from the pphA gene region and PCR fragments were used as templates for the CEQ DTCS Dye Terminator Cycle sequencing kit (Beckman Coulter). DNA sequences were obtained using an automatic DNA sequencing kit (CEQ2000; Beckman Coulter). The nucleotide sequences of both strands were determined. Initial computer analysis of the sequences was performed using the ClustalW (13) with parameters set at default values.

On the basis of the N-terminal amino acid sequence of the purified enzyme, a forward oligonucleotide primer was designed: 5’-TTCATGNGCATGCGGGCNGCAAC3’ (JQ102). A reverse primer was designed using the sequence of the internal 17.4-kDa polypeptide: 5’-TTGGAAGCTTTCCTCGCTTTA3’ (JQ111). A 300-bp fragment of the pphA gene was amplified by PCR, using total Variovorax sp. Pal2 genomic DNA and primers JQ102 and JQ111. The reaction was carried out in a volume of 25 μl with concentrations of deoxynucleotide triphosphates at 200 μM and the JQ102 and JQ111 primers at 0.15 μM each. The following temperature profile was used: initial denaturing at 95 °C for 1 min and then 30 cycles of 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 1 min. The stability of PPH—The enzyme had a molecular mass of 31.187 kDa determined by mass spectrometry and 63 kDa by size exclusion chromatography under non-denaturing conditions. This indicates that PPH is a dimer. The pl of the purified protein was 5.35. The N-terminal sequences of the complete polypeptide and two internal fragments generated by CNBr (20.4 and 17.4 kDa) were determined (Table II).

### RESULTS

Isolation of Variovorax sp. Strain Pal2—A program of enrichment culture on D,L-phosphonoalanine was carried out using samples from local soils, natural water bodies, and waste treatment installations. Bacterial strains capable of metabolizing the compound as sole carbon, nitrogen, and phosphorus source were obtained from all six sites sampled; the isolates were found to utilize only the L-enantiomer of phosphoala

16 S rRNA Gene Sequencing Analysis—An almost complete 16 S rRNA gene from isolate Pal2 was amplified by PCR with the universal primers described by Pascual et al. (14). Sequencing of the 16 S rRNA gene and its analysis were carried out as described previously (15).
Characterization of Phosphonopyruvate Hydrolase

FIG. 2. SDS-PAGE analysis of PPH fractions. a, lanes 1 and 9, Mark 12 molecular mass standards (Invitrogen); lane 2, fraction after ammonium sulfate precipitation (2.1–3.1 M); lane 3, PPH fraction eluted from a hydrophobic interaction chromatography column; lane 4, PPH fraction eluted from Q-Sepharose HP, pH 7.0, column; lane 5, active fraction eluted from an immobilized metal ion affinity chromatography column; lanes 6–8, purified PPH fraction (lane 6, protein loaded 0.8 μg) eluted from Q-Sepharose HP, pH 7.8. Numbers at the left are molecular masses in kilodaltons. Proteins on the gel were visualized by silver staining, b, lane 1, purified PPH fraction (loaded 1.5 μg), visualized by Coomassie Blue staining; lanes 2 and 3, purified PPH fraction (loaded with 0.03 and 0.3 μg, respectively), visualized by activity staining.

TABLE II

Sequences of the amino terminus and CNBr-derived peptides

| Sample | Amino acid sequence |
|--------|--------------------|
| Complete polypeptide | MTKNQALRAALDSGRLFTAMAAHNLPLVA* |
| 20.4 kDa fragment | MRAIASTVISPLAIDDF |
| 17.4 kDa fragment | MIEDKTPFPQDSLRTDGQELVRIFEGG |

(M), methionine was presumed to be the amino acid residue preceding the CNBr cleavage sites.

Segments used for the design of oligonucleotides for PCR were underlined.

The effect of temperature was also determined, and 37 °C was selected for routine use.

As the hydrolases described for other phosphonate compounds were shown to be metalloproteins (2, 3), the effect of EDTA treatment and metal ion addition to the dialyzed enzyme were investigated (Table III). Exposure to EDTA reduced activity by about 50%. Co²⁺ and, to some extent, Ni²⁺ and Mg²⁺, was found to restore PPH activity. Ca²⁺ and Cu²⁺ had no effect, whereas Mn²⁺ and Cu²⁺ reduced the activity by 41 and 45%, respectively. These results suggest the enzyme is dependent on Co²⁺, and this ion was included in the assay mixtures.

**Kinetics and Specificity**—The stoichiometry of the catalyzed reaction was confirmed by the equimolar release of pyruvate and phosphate from phosphonopyruvate with the purified enzyme. The ability of the enzyme to catalyze the intramolecular rearrangement of phosphonopyruvate to phosphoenolpyruvate was examined using the coupled ADP-pyruvate kinase-NADH-lactate dehydrogenase system of Nakashita et al. (17); this revealed a rate of phosphoenolpyruvate formation some 1000-fold lower than that of pyruvate and P₇. Phosphonopyruvate did not itself serve as a substrate for phosphonopyruvate hydratase. In addition, some small phosphatase substrates (glycerophosphate, phospho-L-serine, and phosphoglycolic acid) were examined as possible PPH substrates; none were hydrolyzed.

Of 20 phosphonate compounds tested activity was detected only with phosphonopyruvate. The possible inhibitory effects of the other 18 compounds on the purified enzyme were also studied (Table IV). It was found that only 3-phosphononionic acid (17%), hydroxymethylphosphonic acid (25%), and phosphonoformic acid (73%) caused significant inhibition (at 5 mM). As in the case of the phosphonoacetate hydratase (3), P₇ was found to hydrolyze PPH activity by about 50%. Co²⁺ and, to some extent, Ni²⁺ and Mg²⁺, was found to restore PPH activity. Ca²⁺ and Cu²⁺ had no effect, whereas Mn²⁺ and Cu²⁺ reduced the activity by 41 and 45%, respectively. These results suggest the enzyme is dependent on Co²⁺, and this ion was included in the assay mixtures.

**Nucleotide Sequence Analysis of the pphA Gene**—N-terminal amino acid sequences of PPH and its fragments produced by CNBr fragmentation were used to design primers for analysis of the PPH (pphA) locus (Table II). PCR reactions with JQ102 and JQ111 primers yielded a fragment of 300 bp, which was cloned into pGEM-T Easy vector and analyzed. This fragment was subsequently used as a hybridization probe for detection of pphA fragments and clones.

Two clones containing the pphA gene region were identified. These were pPPH5 (which contained a 3.4-kb SmaI fragment) and pUPH11 (containing a 3.0-kb SmaI fragment). The nucleotide sequence of the pphA gene was determined. This gene encodes a putative PPH protein of 290 amino acids with an estimated molecular mass of 31,177 Da. This is in good correspondence with the molecular mass obtained for the purified PPH by mass spectrometry. The N-terminal sequences of the PPH protein and the two of its CNBr fragments corresponded to translated amino acid sequences of pphA. The start codon of the pphA gene was unequivocally identified by comparison with the PPH N-terminal amino acid sequence. To further

TABLE III

Effect of metal ions on phosphonopyruvate hydrolase activity

| Metal (5.0 mM) | PPH activity % |
|----------------|----------------|
| None           | 100            |
| Cobalt         | 227            |
| Nickel         | 154            |
| Magnesium      | 143            |
| Cesium         | 96             |
| Calcium        | 95             |
| Manganese      | 59             |
| Copper         | 55             |
Characterization of Phosphonopyruvate Hydrolase

TABLE IV
Effect of addition of non-substrate phosphonate compounds on phosphonopyruvate hydrolase activity

| Phosphonate (5 mM) | Relative activitya | % |
|-------------------|--------------------|---|
| None              | 100                | 100 |
| 2-Phosphonopropionic acid | 100              | 100 |
| 2-Phosphobutyrinic acid | 100              | 100 |
| 2-Phosphonoacetaldehyde | 104              | 104 |
| Phosphonoalanine | 102                | 102 |
| 1-Aminobutylphosphonic acid | 96               | 96 |
| 1-Aminoethoxyphosphonic acid | 100        | 100 |
| 2-Aminoethoxyphosphonic acid | 95            | 95 |
| 3-Aminopropylphosphonic acid | 100         | 100 |
| 4-Aminophosphonic acid | 100                | 100 |
| Methylphosphonic acid | 99                 | 99 |
| Ethylphosphonic acid | 100                | 100 |
| Phenylphosphonic acid | 100                | 100 |
| Hydroxymethylphosphonic acid | 75               | 75 |
| 3-Phosphonopropionic acid | 83                 | 83 |
| Phosphonoacetic acid | 93                 | 93 |
| Phosphonoformic acid | 24                 | 24 |
| Diethyphosphonic acid | 98                 | 98 |
| Isopropylphosphonic acid | 100                | 100 |

a 100% activity was 119 units/mg of protein. Activity values are the means of duplicate determinations.

confirm that the cloned gene encodes phosphonopyruvate hydrolase, cell extracts from cultures of *Escherichia coli* JM109 containing plasmids pPPH5, pUPH11, and pUC18 (negative control) were prepared and analyzed for PPH activity. Specific activities of 0.65 units/mg and 0.2 units/mg of protein were found in cells that contained pPPH5 and pUPH11, respectively. No PPH activity was found in the negative control.

Data base searches of GenBank™ and EMBL with the BLAST program (18) and homology analyses showed that the PPH protein has similarities with a number of members of the PEP mutase superfamily. The highest scores were with PEP mutases from *T. pyriformis* (19) (41% amino acid sequence identity) and *Mytilus edulis* (20, 21) (40% identity). A slightly lower level of identity (38%) was found with the bacterial PEP mutase from *Streptomyces viridochromogenes*. Even less pronounced similarities were found between PPH and 2-methylisocitrate lyase from *Salmonella typhimurium* (32% identity) and isocitrate lyase of *E. coli* (20% identity) (Fig. 3). Alignment of the PPF sequence with several proteins belonging to the PEP mutase superfamily is presented in Fig. 3. The conserved amino acid residues between PPH and PEP mutases in several superfamily proteins, there is a good conservation of regions (Fig. 3, positions 72–99, 152–165, 188–198, 230–240, 278–295, and 355–360). It is particularly notable that most of the amino acid residues that comprise the active site of *M. edulis* PEP mutase (20) are conserved in PPH from *Variovorax* sp. Pal2.

DISCUSSION

Phosphonopyruvate is produced biogenically through the rearrangement of the high energy phosphate ester phosphonoenolpyruvate by PEP mutase, the major C–P bond-forming enzyme identified to date. Thus the compound is central to the formation of all natural phosphonates. In some organisms phosphonoenolpyruvate may subsequently be aminated to phosphonoalanine (22); however, the initial step in all of the major biosynthetic pathways of phosphate natural products is believed to be its decarboxylation to phosphonoacetaldehyde by phosphonopyruvate decarboxylase (22). Because the equilib-

![FIG. 3. Amino acid alignment of PPH with proteins of the PEP mutase superfamily. The sequences aligned are as follows: PPH from *Variovorax* sp. Pal2 (accession number AY179862), PEP mutase from *T. pyriformis* (PEP_TETPY; accession number P33182), PEP mutase from *M. edulis* (PEPM_MYTED; accession number P56839), PEP mutase from *S. viridochromogenes* (PEP_STRVR; accession number O86937), PEP mutase from *S. typhimurium* (PRPB_ST; accession number P05313), PEP mutase from *S. hygroscopicus* (SSHYG_THRIC; accession number P075313), and phosphonopyruvate decarboxylase from *Phormidium* (PPD_DEC; accession number Q56062). The residues conserved among the known PPF mutase sequences (20) are indicated in the figure by shaded bold letters. The *M. edulis* PEP mutase active site residues (20) are underlined. Residues conserved in all aligned sequences are indicated by asterisks.](image-url)
PH is the third substrate-specific bacterial phosphonohydrolase capable of heterolytic C–P bond cleavage to be described to date. In its requirement for a divalent metal ion it has similarities to many phosphoryl transferring enzymes including the C–P hydrolases specific to phosphonoacetate (3) and phosphonoacetaldehyde (25); the former is zinc-dependent whereas in the latter magnesium is a cofactor in a mechanism that involves both an active site nucleophile and formation of a Schiff-base intermediate (25). PPH is also similar to the other two hydrolases in its homodimeric structure. However, the subunit sizes are different; the phosphonoacetate hydrolase is 30 kDa whereas the phosphonoacetaldehyde hydrolase is 40 kDa.

Analysis of the corresponding sequences of the three enzymes suggests, however, that they have very different ancestries. Thus whereas phosphonoacetaldehyde hydrolase belongs to the haloacid dehalogenase family that includes several phosphoryl hydrolases (26), and phosphonoacetate hydrolase is a member of the alkaline phosphatase superfamily of zinc-dependent phosphatases. Thus, whereas phosphonoacetaldehyde hydrolase belongs to the haloacid dehalogenase family that includes several phosphoryl hydrolases (26), and phosphonoacetate hydrolase is a member of the alkaline phosphatase superfamily of zinc-dependent phosphatases (27), PPH shows strong homology (Fig. 3) to the members of a recently identified superfamily (28) that contains isocitrate lyase and PEP mutase.

Unsurprisingly, in view of their common substrate, PPH showed highest levels of amino acid sequence identity with the PEP mutases; in particular the active site residues identified in show the highest levels of amino acid sequence identity with the PEP mutase of the marine mussel (28) are highly conserved in PPH, as they are in other members of the superfamily (20). These amino acids include the Lys-120 and Asp-58 conserved in PPH, as they are in other members of the superfamily (20) that contains isocitrate lyase and PEP mutase.

Characterization of Phosphonopyruvate Hydrolase

Acknowledgments—We greatly appreciate the help of G. J. Allen with various techniques. We are grateful to J. Lawson for performing the size exclusion chromatography and Dr. Amanda Cross for carrying out the mass spectrometry. We also thank Professor D. Dunaway-Mariano for advice and encouragement in this study.

REFERENCES

1. Wanner, B. L. (1994) Biochim. Biophys. Acta 1212, 332–350
2. La Nauze, J. M., Rosenberg, H., and Shaw, D. C. (1970) Biochim. Biophys. Acta 212, 332–350
3. McGrath, J. W., Wisdom, G. B., McMullan, G., Larkin, M. J., and Quinn, J. P. (1997) J. Microbiol. Biotechnol. 14, 4673–4680
4. McGrath, J. W., Ternan, N. G., and Quinn, J. P. (1997) Lett. Appl. Microbiol. 24, 69–73
5. Nakashita, H., Shimazu, A., Hida, T., and Seto, H. (1992) J. Bacteriol. 174, 6857–6861
6. Pearson, W. R., and Lipman, D. J. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 2444–2448
7. Nakashita, H., Kozuka, K., and Seto, H. (2000) Biochim. Biophys. Acta 1490, 159–162
8. Jia, Y., Lu, Z., Huang, K., Herzberg, O., and Dunaway-Mariano, D. (1999) Biochemistry 38, 14165–14173
9. Nakashita, H., Watanabe, A., and Seto, H. (1992) J. Biol. Chem. 267, 383–389
10. McGrath, J. W., Ternan, N. G., and Quinn, J. P. (1997) Lett. Appl. Microbiol. 24, 69–73
11. Fiske, C. H., and Subbarow, Y. (1925) J. Biol. Chem. 66, 375–400
12. Sambrook, J., and Russell, D. W. (2001) Molecular Cloning: A Laboratory Manual, 3rd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
13. Thompson, J. D., Higgins, D. G., and Gibson T. J. (1994) Nucleic Acids Res. 22, 4673–4680
14. Pascual, C., Lawson, P. A., Farrow, J. A. E., Gimenez, M. N., and Collins M. D. (1995) Int. J. Syst. Bacteriol. 45, 724–728
15. Kulakov, L. A., McAlister, M. B., Ogden, K. L., Larkin, M. J., and O’Hanlon, J. F. (2002) Appl. Envir. Microbiol. 68, 1548–1555
16. Deleted in proof
17. Nakashita, H., Shimazu, A., Hida, T., and Seto, H. (1992) Biochim. Biophys. Acta 1175, 145–147
18. Schwartz, D., Recktenwald, J., Pelzer, S., and Wohlleben, W. (1998) FEBS Lett. 468, 149–157
19. Hanlon, J. F. (2002) Biophys. Acta 1548, 131–140
20. Jia, Y., Lu, Z., Huang, K., Herzberg, O., and Dunaway-Mariano, D. (1999) Biochemistry 38, 14165–14173