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# Isolation and Characterization of the Carbon–Phosphorus Bond-forming Enzyme Phosphoenolpyruvate Mutase from the Mollusk *Mytilus edulis**

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**The enzyme phosphoenolpyruvate mutase was purified to homogeneity from the mollusk *Mytilus edulis*. The subunit size of the native homotetramer was determined to be 34,000 Da. The steady-state kinetic constants for catalysis of the conversion of phosphonopyruvate to phosphoenolpyruvate at pH 7.5 and 25 °C were measured at $k_{cat}$ = 34 $s^{-1}$, phosphonopyruvate $K_m$ = 3 $\mu$M, and $Mg^{2+}$ $K_m$ = 4 $\mu$M. The enzyme displayed a broad specificity for divalent metal ion activation; $Co^{2+}$, $Mn^{2+}$, $Zn^{2+}$, and $Ni^{2+}$ are activators, whereas $Ca^{2+}$ is not. Analysis of the pH dependence of the $Mg^{2+}$-activated mutase-catalyzed reaction of phosphonopyruvate revealed one residue that must be protonated (apparent p$K_a$ = 8.3) and a second residue that must be unprotonated (apparent p$K_a$ = 7.7) for maximal catalytic activity.**

Naturally occurring phosphonates are believed to be the legacy of reduced forms of phosphorus present on the earth prior to the formation of the present oxygen-based atmosphere (1). The direct linkage of the carbon unit to phosphorus in phosphonates distinguishes them from the more prevalent phosphate esters. The stability of the carbon–phosphorus bond to both solution and enzyme-catalyzed hydrolysis appears to be a key determinant in the diversity of biological activities displayed by natural and synthetic phosphonates (2). While a wide variety of phosphonates have been detected in bacteria, fungi, and animals (ranging from protozoans to man) (2), we are only beginning to learn about how they are acquired by the host organism (*i.e.* by synthesis or ingestion) and about the specific physiological roles that they play.

The phosphonate metabolites fosfomycin, bialaphos (Fig. 1), and K-26 (the tripeptide Ile-Tyr-(1-amino-2-(4-hydroxyphenyl) ethylphosphonic acid) secreted by certain strains of bacteria display biological activities (antibiotic, antifungal, and herbicidal) toxic to certain organisms, suggesting a possible function of the phosphonate in natural chemical warfare (2). The 2-aminoethyl phosphonate (AEP)[1] (Fig. 1) conjugates of glycan, lipid, and peptide macromolecules, found in a wide variety of parasitic organisms, seem to be connected with the ability of the parasite to infect the host organism and/or to avoid destruction by the host once it has been infected. For instance, both the AEP-lipopeptidophosphoglycan, which serves as the major cell-surface glycoconjugate of the epimastigote form of *Trypanosoma cruzi* (3, 27, 28), and the AEP-glycan antigen of the infectious capsule of *Bacteroides fragilis* (4) play probable roles in host recognition. On the other hand, the cellular membrane phosphonolipids of rumen Protozoa (5), the aquatic invertebrate parasite *Tetrahymena pyriformis* (a ciliated protozoan) (6), the bacteria parasite *Bdellovibrio bacteriovorus* (a bacterium) (7), and the plant pathogen *Pythium prolatum* (a fungus) (8) appear to enhance membrane stability in the phosphatase/lipase-rich host environment and thus promote parasite longevity.

In marine invertebrates (*e.g.* snail, sea hare, cuttlefish, clam, mussel, squid, and octopus), AEP-macromolecule conjugates are prevalent (2, 9). The novel forms of AEP-phosphonoglycosphingolipids found concentrated in the nerve tissues of these organisms (10, 29) suggest a possible neurological function that, in light of the discovery of AEP in human brain (11), may be far-reaching.

AEP and AEP conjugates are also found in certain insects (12, 13). For instance, AEP is the main phosphorous compound in locust hemolymph; in *Locusta migratoria*, it represents 60% of the total phosphorus (12). The function of "free AEP" and that of the two isoforms hemolymph apolipoprotein III, a glycoprotein containing AEP as a substituent on the carbohydrate unit (13), are yet unknown.

In parallel with the ongoing investigation of phosphonate structure and function, the chemical pathways leading to phosphonate natural products have been actively pursued. The pathways leading to the commercially important antibiotic fosfomycin (16) and herbicide bialaphos (17) have been elucidated (14, 15, 30), as has the pathway leading to AEP (18) (Fig. 1). An important finding emerging from these studies is that the carbon–phosphorus bond-forming reaction of all three pathways is the rearrangement of phosphoenolpyruvate (PEP) to phosphonopyruvate (19, 31, 32) catalyzed by the enzyme PEP mutase. The bialaphos pathway also contains a second, very similar carbon–phosphorus bond-forming reaction, the rearrangement (and decarboxylation) of carboxyphosphoenolpyruvate to phosphinopyruvate (20) (Fig. 1). The two carbon–phosphorus bond-forming enzymes PEP mutase and carboxy-PEP mutase are structurally related (21).

Given that natural phosphonates share a common origin in the PEP to phosphonopyruvate step, it then follows that phosphonate producers may be identified on the basis of indigenous PEP mutase. Indeed, a PEP mutase screen has been successfully applied in the identification of phosphonate-producing soil bacteria (22). Our plan was to extend this idea further to higher

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[1] The abbreviations used are: AEP, 2-aminoethyl phosphonate; PEP, phosphoenolpyruvate; TEA, triethanolamine; Tricine, *N*-tris(hydroxymethyl)methylglycine; bis-Tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; CAPSO, 3-(cyclohexylamino)-2-hydroxyl-1-propanesulfonic acid.

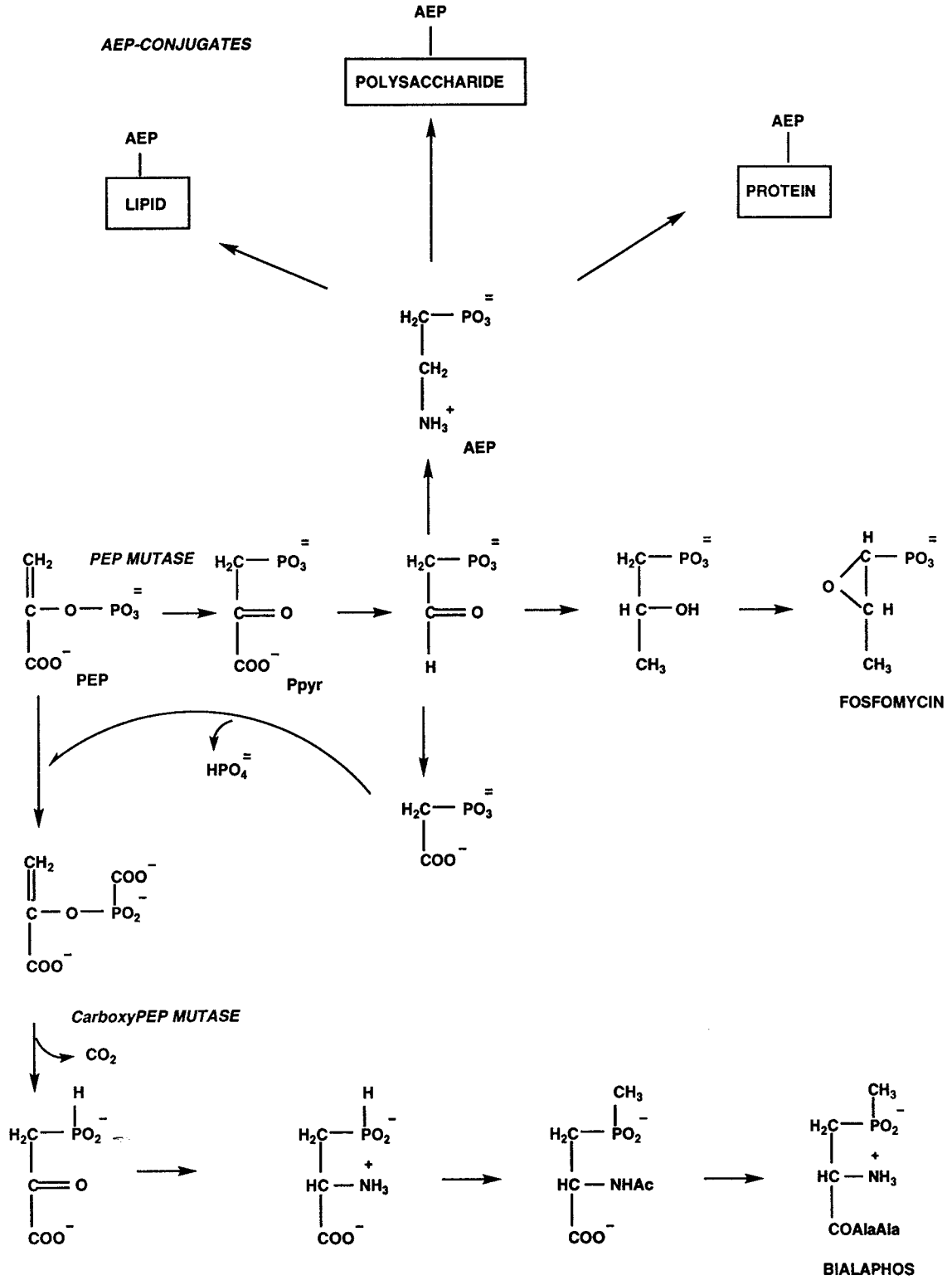


FIG. 1. **Known phosphonate biosynthetic pathways.** *Ppyr*, phosphopyruvate

organisms known to contain phosphonates for the purpose of distinguishing between ingestion, biosynthesis by faculative gut microbes, and active biosynthesis by the primary organism as possible modes of phosphonate acquisition. Saltwater mussels were reported to contain significant quantities of AEP (2), and so, prompted by their availability at local food stores, our search for PEP mutase in a "higher organism" started with the mussel *Mytilus edulis*. To ensure that the enzyme was indeed produced by the mussel and not by the microbes residing in its digestive track, we first demonstrated that the PEP mutase activity is highest (double) in the foot tissue. We then proceeded with the purification of the enzyme, which is reported in this paper along with the characterization of its physical and kinetic properties.

## EXPERIMENTAL PROCEDURES

*Purification of PEP Mutase from M. edulis*—Three-hundred grams of fresh tissue were blended (three 15-s intervals) in 100-g batches with

TABLE I
*Purification of PEP mutase from M. edulis*

| Step | Total protein[a] | Total activity[b] | Specific activity | Yield | Purification |
|---|---|---|---|---|---|
| | *mg* | *units* | *units/mg* | *%* | *-fold* |
| Supernatant of cell free extract (300 g tissue) | 13,700 | 164 | 0.012 | 100 | 1 |
| Ammonium sulfate (50–80%) | 1700 | 90 | 0.053 | 55 | 4 |
| Hydroxylapatite | 240 | 66 | 0.28 | 42 | 23 |
| Phenyl-Sepharose | 13 | 49 | 3.8 | 30 | 320 |
| DEAE-Sepharose | 1.2 | 30 | 25 | 18 | 2100 |
| Mono Q | 0.19 | 17 | 90 | 10 | 7500 |

[a] The protein concentration was monitored by the Bradford method (33) using bovine serum albumin as a standard.
[b] One unit of enzyme activity is defined as the amount of enzyme required for the production of 1 $\mu$mol of PEP from phosphonopyruvate/min (spectrophotometrically measured using the pyruvate kinase/lactate dehydrogenase coupling assay).

150 ml of lysis buffer (50 $\mu$g/ml trypsin inhibitor, 1 mM benzamide HCl, 2.5 mM EDTA, 5 mM dithiothreitol, 1 mM $MgSO_4$, and 200 mM thiamine pyrophosphate in 50 mM Hepes, pH 7.5, at 0 °C) in a Waring blender. The blended tissue was then homogenized using a Con-Torque motor (Eberbach Corp.)-driven homogenizer (Potter-Elvehjem Tissue Grinder). Following centrifugation (17,000 × *g*, 30 min), the supernatant was made 50% saturated in ammonium sulfate with gentle stirring at 0 °C. The protein precipitate was removed by centrifugation, and then the ammonium sulfate in the supernatant was increased to 80%. The protein was collected by centrifugation, dissolved in 5 volumes of TEA buffer (50 mM triethanolamine, pH 7.5, 5 mM $MgCl_2$, and 0.5 mM dithiothreitol), and chromatographed at 4 °C on a 4.8 × 40-cm hydroxylapatite column pre-equilibrated with TEA buffer, pH 7.5. The column was eluted with 500 ml of 0.05 M $NaH_2PO_4$ in TEA buffer, pH 7.5, followed by a 2-liter linear gradient of $NaH_2PO_4$ (0.05–0.50 M) in TEA buffer, pH 7.5. The PEP mutase-containing fractions, identified (using the spectrophotometric coupled assay solution containing 0.1 mM phosphonopyruvate, 5 mM $MgCl_2$, 1 mM ADP, 0.2 mM NADH, 0.5 mM dithiothreitol, 40 units/ml pyruvate kinase, and 20 units/ml lactate dehydrogenase in 50 mM Hepes, pH 7.5) at ~0.4 M phosphate, were pooled and made 20% saturated with ammonium sulfate (gentle stirring at 0 °C). The resulting solution was chromatographed on a 3.7 × 40-cm phenyl-Sepharose column pre-equilibrated with 10% ammonium sulfate in TEA buffer, pH 7.5. The column was eluted with 250 ml of the equilibration buffer followed by a 2-liter linear gradient of 10 to 0% ammonium sulfate in TEA buffer, pH 7.5. The PEP mutase-containing fractions (eluted at ~0% ammonium sulfate) were pooled; concentrated to 20 ml with an Amicon ultrafiltration apparatus; diluted 10-fold with TEA buffer, pH 7.5; and then concentrated once again. Following two more cycles of dilution and concentration, the 3-ml enzyme sample was chromatographed on a 1 × 50-cm DEAE-Sepharose Fast Flow column. The column was eluted with 200 ml of TEA buffer, pH 7.5, followed by a 400-ml linear gradient of KCl (0–0.2 M) in the same buffer. The PEP mutase-containing fractions (eluting at ~0.1 M KCl) were pooled and then concentrated to 10 ml with an Amicon ultrafiltration apparatus. The sample was diluted 20-fold with TEA buffer, pH 7.5, and then concentrated again. This cycle was repeated twice more before concentrating to a final volume of 2 ml for chromatography on a 0.5 × 10-cm Mono Q HR 5/5 prepacked fast protein liquid chromatography column. The column was eluted with a 24-ml linear gradient of 0.02–0.2 M KCl in TEA buffer, pH 7.5. The PEP mutase-containing fractions (eluting at ~0.1 M KCl) were stored at −80 °C.

*Molecular Mass Determination*—The mussel PEP mutase subunit size was determined by SDS-polyacrylamide gel electrophoresis analysis (12% separating gel and 3% stacking gel) using the following molecular mass standards: phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). A linear semilog plot of log molecular mass *versus* distance traveled on the gel was constructed. The subunit size of mussel PEP mutase was determined from the measured distance traveled on the gel by extrapolation from the plot. The molecular size of native mussel PEP mutase was determined using a 1.5 × 81-cm Sephacryl S-200 column that had been equilibrated with TEA buffer, pH 7.5, and calibrated using the Pharmacia gel filtration calibration kit (ribonuclease A (13.7 kDa), chymotrypsinogen A (25 kDa), ovalbumin (43 kDa), aldolase (158 kDa), and catalase (232 kDa)) according to the manufacturer's instructions. Chromatographies were carried out at 4 °C using TEA buffer, pH 7.5, as eluant and a peristaltic pump to maintain a constant flow rate of 0.3 ml/min. The elution volume was measured for mussel PEP mutase in two independent trials, and the molecular mass of the mutase was extrapolated from the linear semilog plot of protein molecular mass *versus* observed elution volume.

*N-terminal Sequence Analysis of Mussel PEP Mutase Holoenzyme and Protease V8-cleaved Enzyme*—Purified PEP mutase was chromatographed on an SDS-polyacrylamide gel and then transfer-blotted to a polyvinylidene difluoride membrane using a Hoefer Semiphor transfer blotter. Following membrane destaining and drying, the protein was excised and sequenced using Edman degradation techniques (Applied Biosystems Model 470 gas-phase sequenator). *Staphylococcus aureus* strain protease V8 (0.03 mg) digestion was carried out with 0.15 mg of PEP mutase preincubated in 30 $\mu$l of $H_2O$, 8 $\mu$l of mercaptoethanol, and 16 $\mu$l of 10% SDS at 20 °C for 5 min. The reaction was terminated (after 2–80 min of incubation) by mixing a 6-$\mu$l aliquot with 4 $\mu$l of SDS loading buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.025% (w/v) bromphenol blue, and 0.5% mercaptoethanol). Following heating at 100 °C for 4 min, the sample was chromatographed on a precast 16% Tricine gel (Novex). The peptide fragments were blotted onto a polyvinylidene difluoride membrane. One major peptide, identified by Coomassie Blue staining, was sequenced as described above.

*Determination of Steady-state Kinetic Constants for Mussel PEP Mutase*—The $k_{cat}$ and $K_m$ values were determined from initial velocity data. The rate of PEP formation in reactions containing ~2 milliunits/ml PEP mutase, 5 mM $MgCl_2$, 1–100 $\mu$M phosphonopyruvate, 50 mM Hepes, 0.5 mM dithiothreitol, pH 7.5 (at 25 °C), and the coupling system (1 mM ADP, 0.2 mM NADH, 40 units/ml pyruvate kinase, and 20 units/ml lactate dehydrogenase) was monitored by measuring the decrease in solution absorbance at 340 nm ($\Delta\epsilon$ = 6.2 $mM^{-1}$ $cm^{-1}$). The initial velocity data were analyzed using Equation 1 and the Fortran HYPERL program of Cleland (23),

$$v_o = V_m[E][S]/K_m + [S] \quad \text{(Eq. 1)}$$

where $v_o$ is the initial velocity, $V_m$ is the maximum velocity, [E] is the total enzyme concentration, [S] is the substrate concentration, and $K_m$ is the Michaelis constant for the substrate. The $k_{cat}$ was calculated by dividing the $V_m$ value by the concentration of PEP mutase (based on protein determination using the Bradford assay (33)) used to catalyze the reactions.

*Determination of pH Rate Profiles for Mussel PEP Mutase*—The $V_m$ and $V_m/K_m$ values were determined from initial velocity data measured (as described above) as a function of reaction pH. The reaction solutions were buffered at the following pH ranges: pH 6.0–6.5 with 50 mM bis-Tris, pH 7.0–7.5 with 50 mM Hepes, pH 8.0–8.5 with 50 mM Tricine, and pH 9.0–10.0 with 50 mM CAPSO. The data were analyzed using Equations 2 and 3 and the Fortran BELL and HABELL programs of Cleland (23),

$$\log Y = \log(c/(1 + [H]/K_1 + K_2/[H])) \quad \text{(Eq. 2)}$$

$$\log Y = \log(c/(1 + [H]/K_1)) \quad \text{(Eq. 3)}$$

where $Y = V_m$ or $V_m/K_m$, $c$ is the pH-independent value of $Y$, [H] is the hydrogen ion concentration, and $K_1$ and $K_2$ are dissociation constants of groups that ionize.

*Examination of Mussel PEP Mutase Metal Ion Cofactor Specificity*—The velocity of PEP formation in reaction solutions containing ~2 milliunits/ml PEP mutase, 100 $\mu$M phosphonopyruvate, and varying amounts of $MgCl_2$, $MnCl_2$, $CoCl_2$, $ZnCl_2$, $NiCl_2$, or $CaCl_2$ in 50 mM Hepes, pH 7.5 (at 25 °C), was monitored at 233 nm ($\Delta\epsilon$ = 1.5 $mM^{-1}$ $cm^{-1}$). The initial velocity data were analyzed using Equation 1 and the Fortran HYPERL program of Cleland (23).

RESULTS

*Purification of PEP Mutase from a Saltwater Mollusk (M. edulis)*—PEP mutase was purified from homogenized mussel tissue by initial ammonium sulfate fractionation of the cellular extract followed by hydroxylapatite, phenyl-Sepharose, DEAE-Sepharose, and Mono Q column chromatography. The results of the purification steps are summarized in Table I. The homogeneous enzyme (analyzed by SDS-polyacrylamide gel electrophoresis), having a specific activity of 90 units/mg, was obtained in a yield of 0.2 mg/300 g of tissue. The enzyme is relatively stable as indicated by minimal loss of activity upon storage in TEA buffer and 10% glycerol, pH 7.5, at 4 °C for at least 2 weeks and at −80 °C for at least 4 weeks.

*Physical and Kinetic Properties of Mussel PEP Mutase*—The molecular mass of denatured PEP mutase was determined by SDS-polyacrylamide gel electrophoresis analysis to be ~34 kDa. The molecular mass of the native protein, a homotetramer, was determined by using gel filtration techniques to be ~144 kDa. The N-terminal sequence of the protein and the sequence of a high pressure liquid chromatography-purified V8 proteolytic peptide are shown in Fig. 2 and are aligned with the corresponding regions of the *Streptomyces hygroscopicus* (24), *Streptomyces wedmorensis* (14), and *T. pyriformis* (21) PEP mutase sequences. The turnover rate for $Mg^{2+}$-activated PEP mutase was measured in the thermodynamically favored direction of the reaction, phosphonopyruvate to PEP. At 25 °C and pH 7.5, $k_{cat}$ = 34 $s^{-1}$, and phosphonopyruvate $K_m$ = 3.3 ± 0.3 μM.

| PEP Mutase | N-terminal | Internal Sequence |
|---|---|---|
| *Mytilus edulis* | 1 STKVKKTTQLKQMLN | DKLFPKTNSLHD?RA |
| *T. pyriformis* | 10 SS-TRKTTQLKNMIQ | 123 DKIFPKRNSLLDDGR |
| *S. hygroscopicus* | 7 ANGDRGTTRSAGGR | 127 DKVFPKMNSFFGDGH |
| *S. wedmorensis* | 136 VKEVGTTPNVRLSR | 262 DKEGLKRNSLFGTDV |

FIG. 2. **The N-terminal sequence of *M. edulis* PEP mutase holoenzyme and the N-terminal sequence of an *M. edulis* PEP mutase protease V8 fragment (prepared as described under "Experimental Procedures" and labeled *Internal Sequence*) aligned with matching regions occurring in the sequences of the PEP mutases from *T. pyriformis* (21), *S. hygroscopicus* (24), and *S. wedmorensis* (14).**

*Metal Ion Specificity of Mussel PEP Mutase*—The steady-state kinetic constants for metal ion activation of PEP mutase were determined at a fixed saturating concentration of phosphonopyruvate and varying metal ion concentrations. The $V_m$ and apparent $K_m$ values, measured for the $Mg^{2+}$-, $Mn^{2+}$-, $Co^{2+}$-, $Zn^{2+}$-, $Ni^{2+}$-, or $Ca^{2+}$-activated enzyme at pH 7.5 and 25 °C, are reported in Table II.

*pH Dependence of Mussel PEP Mutase Catalysis*—The $V_m$ and $V_m/K_m$ values were determined (at varying phosphonopyruvate concentrations and a fixed saturating $Mg^{2+}$ concentration) as a function of the reaction solution pH. The results are presented in the pH rate profiles of Fig. 3. Analysis of the $V_m/K_m$ data with Equation 2 gave an apparent p$K_1$ of 7.7 ± 0.2 and an apparent p$K_2$ of 8.3 ± 0.2. Analysis of the $V_m$ data with Equation 3 gave an apparent p$K_1$ of 6.5 ± 0.1.

TABLE II

*Michaelis constant ($K_m$) for the metal ion activator and maximum velocity ($V_m$; standardized to $V_m$ for $Mg^{2+}$ activation) measured for the M. edulis PEP mutase-catalyzed conversion of phosphonopyruvate to PEP*

See "Experimental Procedures" for details. Kinetic values previously measured for the *T. pyriformis* (26) and *P. gladioli* (25) PEP mutases are given in parentheses.

| Metal ion | $M^{2+}$ $V_m$/$Mg^{2+}$ $V_m$ | $K_m$ |
|---|---|---|
| | | μM |
| $Mg^{2+}$ | 1.0 (1.0,[a] 1.0[b]) | 4 ± 1 (4,[a] 5[b]) |
| $Co^{2+}$ | 0.7 (0.9,[a] 0.5[b]) | 0.3 ± 0.1 (1,[a] 2,[b]) |
| $Mn^{2+}$ | 0.3 (0.5,[a] 0.3[b]) | 0.11 ± 0.02 (0.02,[a] 7[b]) |
| $Zn^{2+}$ | 0.5 (2.3,[a] 0.4[b]) | 0.18 ± 0.04 (7[b]) |
| $Ni^{2+}$ | 0.6 | 2.2 ± 0.2 |
| $Ca^{2+}$ | No activity | |

[a] *P. gladioli* PEP mutase.
[b] *T. pyriformis* PEP mutase.

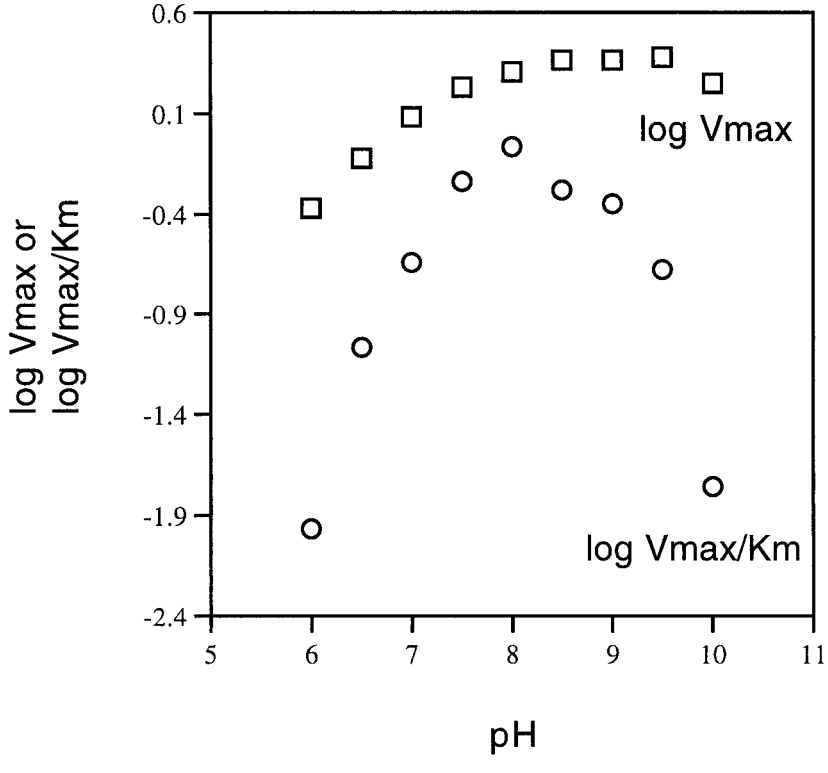


FIG. 3. **Plot of log $V_m$ and log $V_m/K_m$ (measured for the *M. edulis* PEP mutase/$Mg^{2+}$-catalyzed conversion of phosphonopyruvate to PEP at 25 °C) *versus* reaction solution pH.** The data were analyzed using Equations 2 and 3, respectively. See "Experimental Procedures" for details.

TABLE III
*Summary of the properties of known PEP mutases*

| Source (Ref.) | Pathway | Subunit | Native | $k_{cat}$ | $K_m$ |
|---|---|---|---|---|---|
| | | *kDa* | | $s^{-1}$ | $\mu M$ |
| *M. edulis* | AEP | 34[a] | Tetramer | 34 (25 °C) | 3 |
| *T. pyriformis* (21, 26) | AEP | 32.8[b] | Dimer | 77 (25 °C) | 4 |
| *S. hygroscopicus* (24) | Bialophos | 33.7[b] | ? | | |
| *P. gladioli* (25) | Fosfomycin | 61[a] | Tetramer | 2600 (30 °C) | 20 |
| *S. wedmorensis* (15) | Fosfomycin | 48.3[b] | ? | | |

[a] Determined by SDS-polyacrylamide gel electrophoresis analysis of the denatured protein.
[b] Determined by gene sequencing.

## DISCUSSION

Thus far, PEP mutase has been shown to function in three different biosynthetic pathways: one leading to fosfomycin, another to bialaphos, and the third to AEP (Fig. 1). The fosfomycin pathway PEP mutase-encoding gene from *S. wedmorensis* (14) has been sequenced, and the enzyme has been purified/characterized from a probable fosfomycin-producing strain, *Pseudomonas gladioli* (25). The bialaphos pathway PEP mutase-encoding gene from *S. hygroscopicus* (24) has been sequenced, but because of its instability, the *S. hygroscopicus* enzyme has only been partially purified (19). Finally, PEP mutase has been purified/characterized from the AEP producers *T. pyriformis* (26) and *M. edulis*, and the encoding gene from *T. pyriformis* (21) has been sequenced. Here, we compare the physical and kinetic properties of mussel PEP mutase with those reported for the microbial enzymes.

The comparison made between the two stretches of mussel PEP mutase sequence (obtained by automated amino acid sequencing) and matching regions found in the full sequences of the bialaphos pathway PEP mutase from *S. hygroscopicus,* the fosfomycin pathway PEP mutase from *S. wedmorensis*, and the AEP pathway PEP mutase from *T. pyriformis* (Fig. 2) indicates that the match may be closest with the *T. pyriformis* enzyme. The sequence identity existing between (full sequence) pairs of the microbial mutases ranges from 32 to 34%, whereas the sequence identity between the stretches of mussel and *T. pyriformis* PEP mutases falls in the 50–60% range.

The subunit sizes of the PEP mutases and their kinetic constants are summarized in Table III. The two fosfomycin pathway PEP mutases, at 48 and 61 kDa, are considerably larger than the 33–34-kDa AEP and bialaphos pathway mutases. The quarternary structure of the PEP mutase is dimeric in the case of the *T. pyriformis* enzyme and tetrameric in the cases of the *P. gladioli* and *M. edulis* PEP mutases.

Overall, the steady-state kinetic constants measured for the mutases (Table III) are reasonably close. The turnover rates of the *T. pyriformis* and *M. edulis* enzymes are equal, as are the $K_m$ values. The $k_{cat}$ of the *P. gladioli* PEP mutase appears comparatively higher (~9-fold) and the $K_m$ value lower (~5-fold); however, these constants were measured under conditions different from those used with the other two enzymes (including a 5 °C higher temperature), which may significantly affect the values measured.

In previous studies of the *T. pyriformis* PEP mutase (26), it was shown that substrate and metal cofactor binding is rapid equilibrium-ordered, with metal ion binding following substrate binding. We do not yet know what role the metal ion plays in catalysis; however, preliminary results from EPR studies suggest that it binds to phosphate bridge oxygen and carboxylate oxygen of the PEP substrate ligand bound in the central complex. For all three PEP mutases examined (Table II), the $V_m$ values (measured under conditions of saturating phosphonopyruvate and metal ion) are remarkably close for $Mg^{2+}$, $Mn^{2+}$, $Co^{2+}$, and $Zn^{2+}$ serving in the capacity of activator. The metal ion $K_m$ value, which is equivalent to the dissociation constant for metal binding to the enzyme-pyruvate-metal complex, shows distinctly more variation with metal cofactor used in the case of the *M. edulis* and *P. gladioli* PEP mutases than it does with the *T. pyriformis* enzyme (Table II). Whereas the $K_m$ values measured for $Mg^{2+}$, $Mn^{2+}$, $Co^{2+}$, and $Zn^{2+}$ are all in the 2–7 $\mu$M range in the case of the *T. pyriformis* mutase (26), in the case of the *M. edulis* and *P. gladioli* mutases (25), the softer metal ions bind at least an order of magnitude tighter than does $Mg^{2+}$.

The $V_m$ and $V_m/K_m$ pH profiles reported previously for the *T. pyriformis* mutase (26) can now be compared with those measured for the *M. edulis* enzyme (Fig. 3). The $V_m$ profile for the *T. pyriformis* PEP mutase was found to be flat over the pH range of 6–10, thus providing no information about catalytic groups in the enzyme-pyruvate-$Mg^{2+}$ complex. The $V_m/K_m$ profile, on the other hand, was found to be bell-shaped, defining apparent ionization constants (for the free enzyme) of $pK_1 = 6.2 \pm 0.1$ and $pK_2 = 8.4 \pm 0.1$. These ionizations may have been missed in the $V_m$ pH profile because of shifted $pK_a$ values or because they are important only for substrate binding. Both the $V_m$ and $V_m/K_m$ pH profiles measured for the *M. edulis* PEP mutase reveal ionizations of essential groups. The $V_m$ pH profile shows a "break" at pH 9.5 (Fig. 3), indicating loss of catalytic activity at higher pH. Because of an insufficient number of data points beyond pH 9.5, we did not attempt to fit the curve to define the apparent $pK_a$ of the group ionizing at high pH. Instead, the data between pH 6 and 9 were fit to define the ionization of a group within the enzyme-pyruvate-$Mg^{2+}$ complex having an apparent $pK_a$ of $6.5 \pm 0.1$. Protonation of this group results in loss of catalytic activity. The $V_m/K_m$ pH profile measured for the *M. edulis* PEP mutase resembles that of the *T. pyriformis* enzyme and defines ionizations of catalytic groups in the free enzyme with $pK_1 = 7.7 \pm 0.2$ and $pK_2 = 8.3 \pm 0.2$.

In conclusion, the isolation of PEP mutase from *M. edulis* demonstrates that active phosphonate synthesis takes place in higher organisms. The primary phosphonate of mollusks is AEP, and therefore, it is highly likely that the PEP mutase isolated serves as the carbon–phosphorus bond-forming enzyme of the *M. edulis* AEP biosynthetic pathway. Comparison of the known properties of the *M. edulis* and *T. pyriformis* PEP mutases indicates very homologous structures with similar but not identical catalytic properties. The bacterial (fosfomycin and bialaphos pathway) PEP mutases are also related to the mussel enzyme, but perhaps not as closely.

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