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Current Opinion in Chemical Biology

The enzymatic conversion of phosphonates to phosphate by bacteria

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Phosphonates are ubiquitous organophosphorus compounds that contain a characteristic C–P bond which is chemically inert and hydrolytically stable. Bacteria have evolved pathways to metabolize these phosphonate compounds and utilize the products of these pathways as nutrient sources. This review aims to present all of the known bacterial enzymes capable of transforming phosphonates to phosphates. There are three major classes of enzymes known to date performing such transformations: phosphonatases, the C-P lyase complex and an oxidative pathway for C–P bond cleavage. A brief description of each class is presented.

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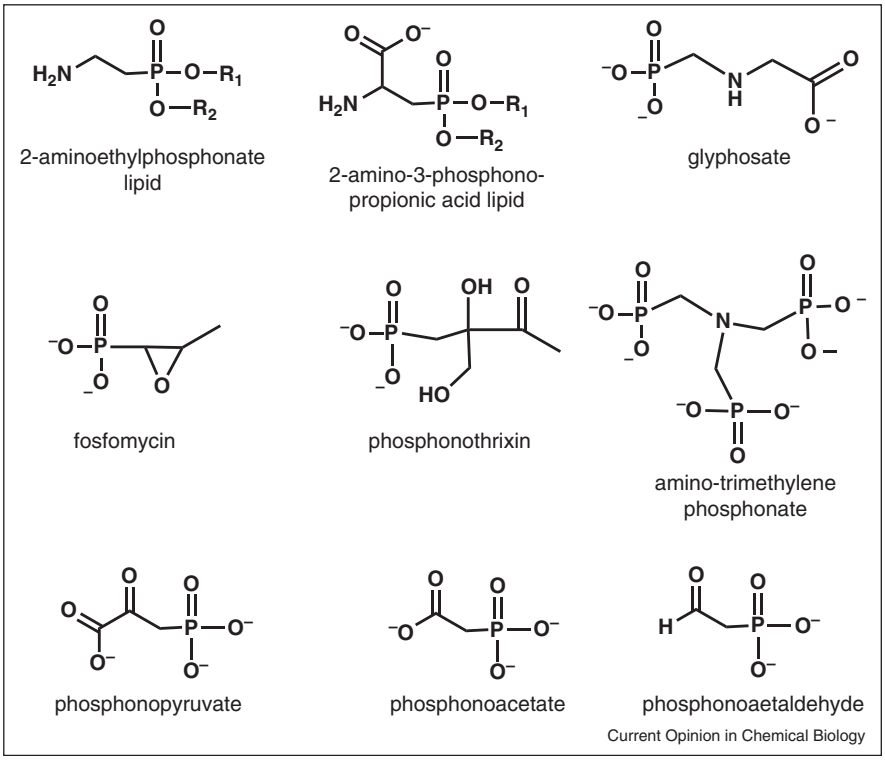
Phosphonates are organophosphorus compounds that contain a characteristic carbon–phosphorus (C–P) bond. This bond is chemically inert, hydrolytically stable and resistant to photolysis. Phosphonates are prevalent in all primitive life forms, where they can exist as integral components of membrane phosphonolipids, such as 2-aminoethylphosphonic acid and 2-amino-3-phosphonopropionic acid (Scheme 1). The presence of phosphonates in these lipid membranes confers rigidity to the membranes and protects the organisms against light and degradation from phosphatases [1–3]. Biologically relevant exopolysaccharides and glycoproteins also contain phosphonate moieties. Phosphonates are found extensively in antibiotics such as fosfomycin and phosphonothrixin, the herbicide glyphosate and the industrial detergent additive amino-trimethylene phosphonate (Scheme 1) [1–3]. It is estimated that in the US alone more than 20 000 tons of phosphonates are released annually into the environment in the form of herbicides and detergent wastes. With such large quantities of phosphonates being released into the environment, there is a significant interest in understanding the mechanisms by which phosphonates are degraded or metabolized by bacterial species [1]. The abundance and universal prevalence of phosphonates in the environment has led to the evolution of several bacterial species that are able to metabolize and utilize phosphonates as carbon and phosphorus sources [2–4]. There are three known classes of enzymes or enzymatic systems that have been mechanistically characterized which are capable of breaking the C–P bonds of phosphonate compounds. These include phosphonate hydrolases, the C-P lyase complex, and an oxidative pathway.

Phosphonate hydrolases

Phosphonate hydrolases have been generically referred to as 'phosphonatases'. The characteristic feature of the substrates for the phosphonatases is the presence of an electron withdrawing β-carbonyl group that facilitates bond delocalization and allows the heterolytic cleavage of the C–P bond. The phosphonate substrates hydrolyzed by this group of enzymes include phosphonopyruvate (PnPy), phosphonoacetate (PAA) and phosphonoacetaldehyde (Pald) (Scheme 1). Proteins belonging to the phosphonatase class of enzymes have evolved from different enzyme superfamilies, and have been characterized mechanistically and structurally.

The first reported phosphonopyruvate hydrolase (PPH) was identified from cell free extracts of an environmental isolate capable of utilizing phosphonoalanine as a carbon, nitrogen and phosphorus source, from *Burkholderia cepacia* Pal6 [5]. The PPH reaction is presented in Figure 1a, where phosphonopyruvate is converted to pyruvate and orthophosphate. On the basis of amino acid sequence identity, the gene for PPH has also been identified in *Variovorax* sp. Pal2, another environmental sample obtained from a soil isolate [6]. PPH belongs to the phospho(enol)pyruvate (PEP) mutase/isocitrate lyase superfamily of enzymes [7•]. PPH has a 40% amino acid sequence identity to PEP mutase and has a $(\beta/\alpha)_8$-barrel structural fold. The monomers associate as a tetramer and the 8th α-helix is swapped between two dimers [7•]. There are three available structures for PPH from *Variovorax* sp. Pal2: apo-enzyme (PDB id: 2HRW), PPH complexed with Mg^{2+} and phosphonopyruvate (PDB id: 2HJP), and PPH complexed with Mg^{2+} and oxalate (PDB id: 2DUA). The active site Mg^{2+} anchors the

Scheme 1



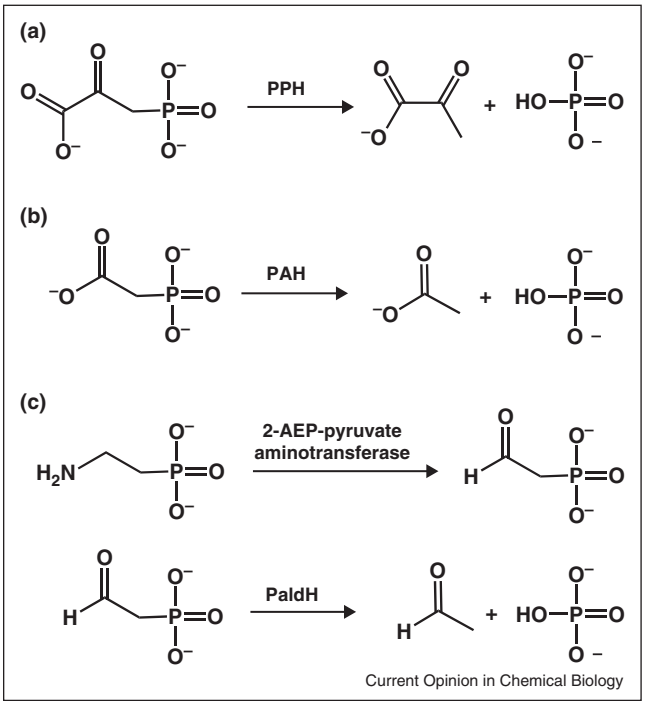
Structures of important phosphonates.

phosphonopyruvate substrate. On the basis of the available structures of PPH and mechanistic experiments, a consensus catalytic mechanism analogous to that of PEP mutase has been proposed (Figure 2) [5,6,7•,8]. PPH does not utilize any other phosphonate compounds besides phosphonopyruvate [6].

Phosphonoacetate is biogenically available to bacteria from the degradation of 2-aminoethylphosphonate (2AEP) [9•]. The first phosphonoacetate hydrolase (PAH) activity was found in *Pseudomonas fluorescens* 23F, a bacterial isolate from the sludge of a laundry waste treatment plant in Ireland [10]. PAH catalyzes the hydrolysis of phosphonoacetate to yield acetate and orthophosphate (Figure 1b). PAH belongs to the alkaline phosphatase superfamily [11]. Members of this superfamily have an active site consisting of a binuclear metal center with a highly conserved serine or threonine residue that is utilized to form a covalent phospho-enzyme adduct as an integral part of the catalytic mechanism [12]. There are two PAH superfamily enzymes that have been extensively characterized: PAH from *P. fluorescens* 23F [9•] and *Sinorhizobium meliloti* 1021 (phnA) [13•]. Both of these enzymes have high specificity for zinc, but the PAH from *S. meliloti* can be activated with Mn^{2+} or Fe^{2+}. The structure of PAH from *P. fluorescens* has been determined in a complex with phosphonoformate (PDB id: 1EI6). The structure of PAH from *S. meliloti* has been determined for the apo-enzyme (PDB id: 3SZY), complexed with phosphonoacetate (PDB id: 3T02), acetate (PDB id: 3SZZ), vanadate (PDB id: 3T00) and phosphonoformate (PDB id: 3T01). On the basis of these crystal structures, PAH possess a catalytic core of the alkaline phosphatase superfamily and uses a conserved threonine residue to form the phospho-enzyme adduct in the catalytic cycle. However, this enzyme possesses a unique capping domain analogous to the nucleoside pyrophosphatase/phosphodiesterase enzyme family that is critical for shielding the substrate from solvent during turnover. A detailed reaction mechanism for the PAH has been proposed (Figure 3) [9•,13•].

Phosphonoacetaldehyde (Pald) is formed biologically by the action of 2AEP-pyruvate transaminase, which uses 2AEP and pyruvate as substrates and yields Pald and L-alanine as products. Pald is subsequently hydrolyzed by phosphonoacetaldehyde hydrolase (PaldH) to form acetaldehyde and orthophosphate (Figure 1c). PaldH belongs to the haloalkanoic acid dehydrogenase (HAD) superfamily of enzymes [14,15]. Members of the HAD superfamily perform diverse set of transformations that require the Mg^{2+} dependent formation of a covalent intermediate to an active site aspartate residue [16]. PaldH from *Bacillus cereus* is the most extensively

Figure 1



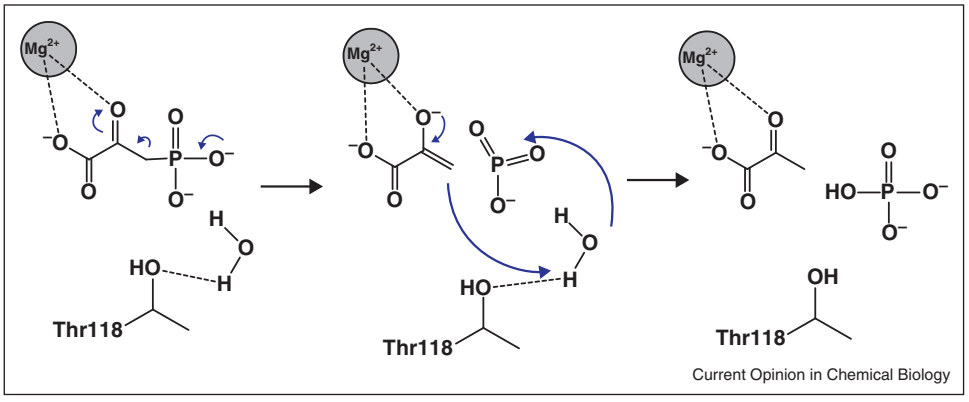
Reactions catalyzed by phosphonate hydrolases. These include reactions catalyzed by phosphonopyruvate hydrolase (PPH), phosphonoacetate hydrolase (PAH) and phosphonoacetaldehyde hydrolase (PaldH).

studied phosphonatase [17,18,19•]. Several X-ray structures are available for this enzyme including the wild-type PaldH complexed with Mg^{2+} (PDB id: 1RQN), tungstate (PDB id: 1FEZ), vinyl sulfonate (PDB id: 1RQL), and a covalently trapped intermediate (PDB id: 2IOF). On the basis of the X-ray structures and mechanistic studies, a triad of catalytic residues consisting of lysine, histidine, and aspartate has been identified. The lysine residue forms a Schiff's base with the substrate to activate Pald. The histidine residue activates a water molecule for hydrolytic attack and the aspartate residue forms the phospho-enzyme complex for cleavage of the C–P bond. A comprehensive mechanism has been proposed for PaldH based on the mechanistic characterization and the available X-ray structures (Figure 4) [17,18,19•].

CP lyase complex

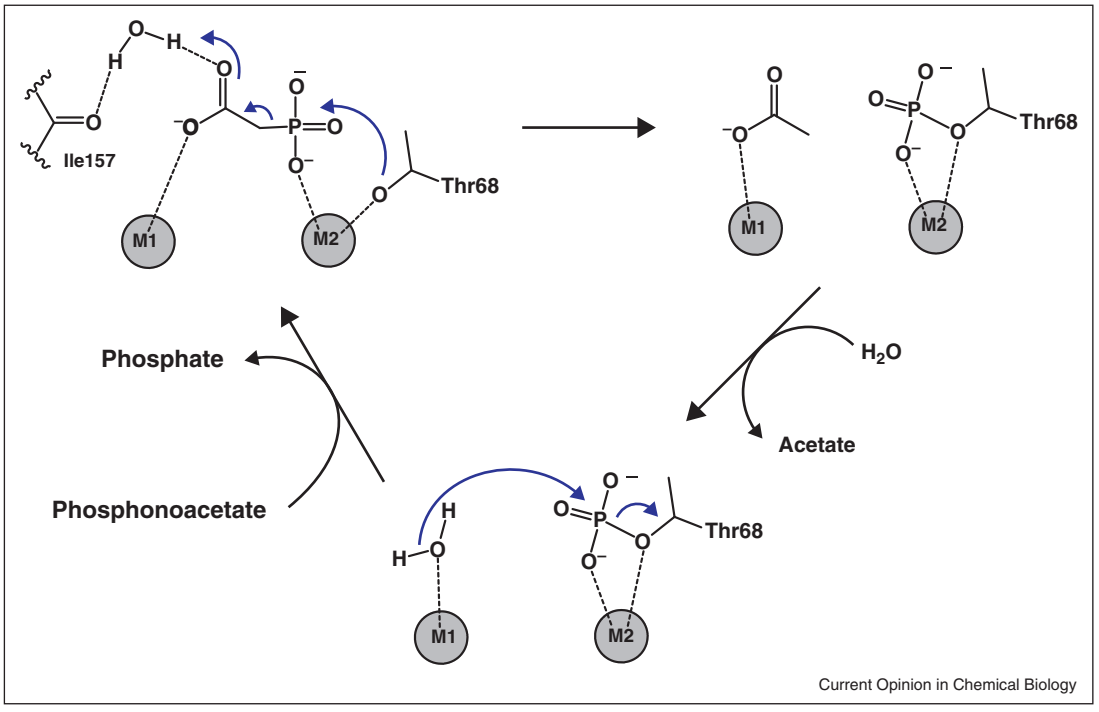
The C-P lyase complex has been studied for over four decades. The C-P lyase activity is very promiscuous and was first studied in cell free extracts of several bacterial strains capable of metabolizing a broad spectrum of phosphonates including unactivated substrates such as methylphosphonate (MPn) and ethylphosphonate (EtPn) [20,21]. The C-P lyase activity in bacteria is governed by the 14-cistron *phnCDEFGHIJKLMNOP* operon. It is upregulated under conditions of phosphate starvation, and is regulated by the PhoR/PhoB two component signaling system [22,23]. The C-P lyase activity was predicted to arise from the gene products of the *phn* operon forming a membrane-localized multienzyme complex in bacteria [22,23]. The gene products PhnC, PhnD and PhnE are phosphonate transporters, based on sequence analysis and phenotypes obtained from transposon-based mutants grown on different phosphonates [22,23]. The proteins PhnG, PhnH, PhnI, PhnJ, PhnK, PhnL and PhnM are absolutely critical for C-P lyase activity and form the minimal catalytic set for C-P lyase activity. Deletion, or mutation to any one of these genes results in complete abolishment of the C-P lyase activity [22,23]. PhnN, PhnO and PhnP perform accessory functions and were predicted to be involved in the metabolism of the intermediates and products formed on this pathway [22,23]. Several studies have demonstrated that the C-P lyase activity involves a radical-based homolytic cleavage of the C–P bond of organophosphonates [24,25]. Feeding studies involving ^{32}P-ethylphosphonate as the sole phosphorus source for *Escherichia coli* led to the identification of α-D-ribose-1-ethylphosphonate as a

Figure 2



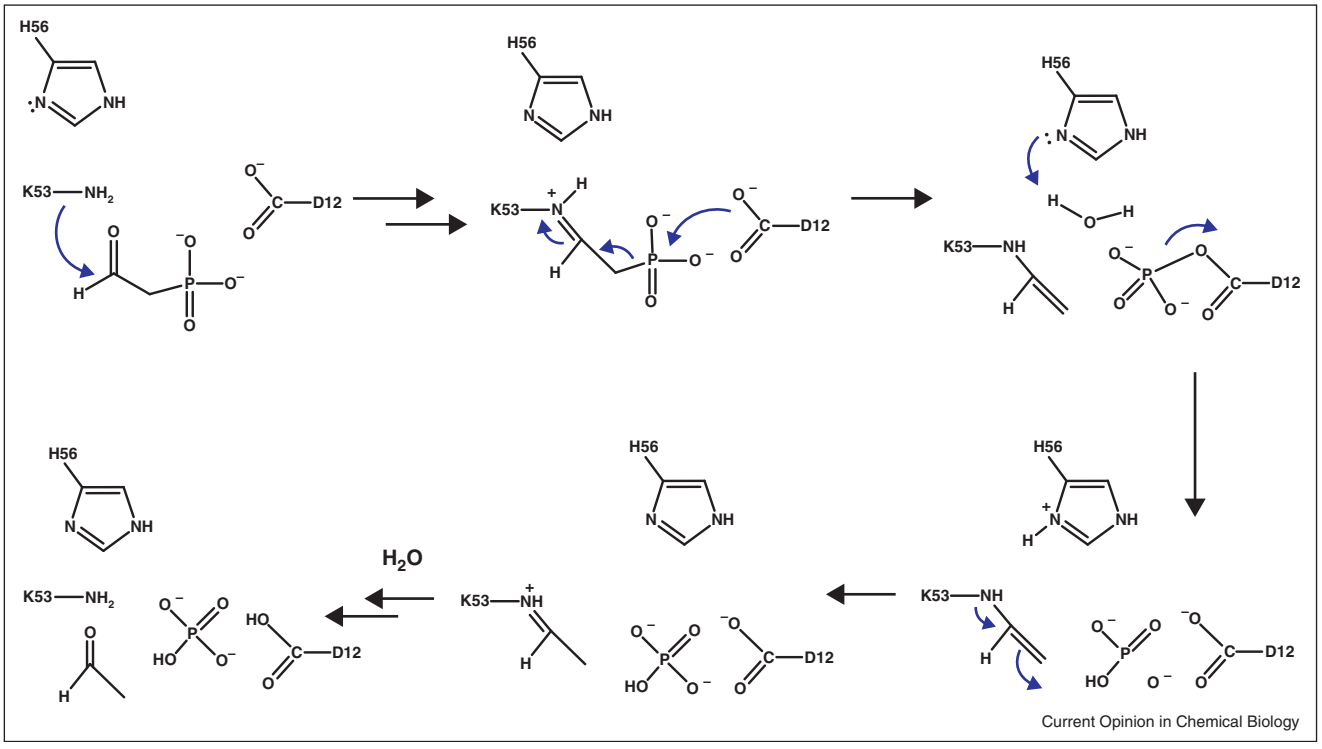
Mechanism of the reaction catalyzed by phosphonopyruvate hydrolase (PPH) from *Variovorax* sp. Pal2.

Figure 3



Mechanism of the reaction catalyzed by phosphonoacetate hydrolase (PAH). [Amino acid numbering follows that of PhnA from *Sinorhizobium meliloti* 1021. However the PAH from *Pseudomonas fluorescens* 23F invokes an identical mechanism, with the exception that the Ile is replaced by two lysine residues. M1 and M2 represent metals 1 and 2].

Figure 4



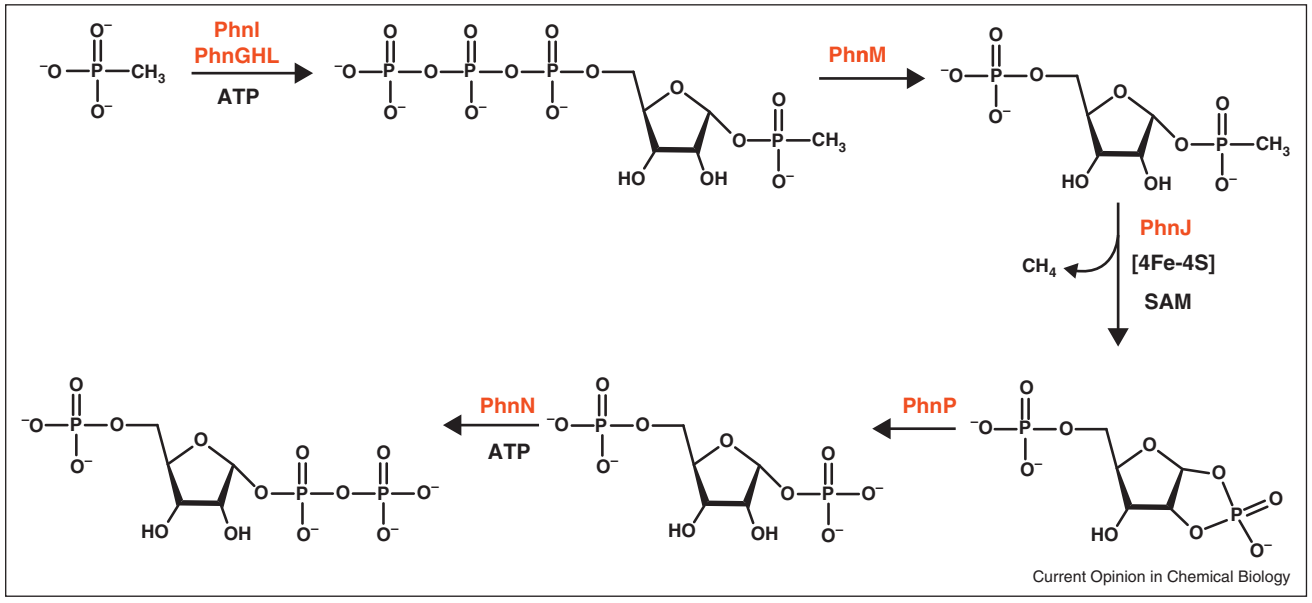
Mechanism of the reaction catalyzed by phosphonoacetaldehyde hydrolase (PaldH) from *Bacillus cereus*.

probable intermediate on this pathway and the involvement of a ribose moiety in this pathway [26]. Over the last few years, the functions of most gene products involved in catalysis of the C-P lyase activity have been annotated and the C-P lyase pathway has been elucidated [27].

PhnO, an accessory protein, was shown to be involved in the catabolism of 1-aminoalkylphosphonic acids, where the *phnO* gene product *N*-acetylates 1-aminoalkylphosphonic acids for further metabolism in the C-P lyase pathway [28,29]. PhnN is annotated as ribose-1,5-bisphosphokinase, where PhnN catalyzes the phosphorylation of ribose-1,5-bisphosphate (RbP) to 5-phosphoribosyl-1-pyrophosphate (PRPP), a central biological metabolite [30]. Deletion studies of the *phn*P gene product led to the cellular accumulation and identification of ribose-5-phosphate-1,2-cyclic phosphate (PRcP) as the potential substrate of PhnP [31•]. PhnP belongs to the metallo-β-lactamase superfamily, and bears sequence and structural homology to tRNase Z phosphodiesterases and catalyzes the hydrolysis of PRcP to yield RbP. There are two available X-ray structures of PhnP in the PDB from *E. coli* K-12: the wild-type enzyme (PDB id: 3G1P), and the wild-type enzyme complexed with vanadate, an inhibitor of PhnP (PDB id: 3P2U). On the basis of the X-ray structures and mechanistic studies, a detailed mechanism for PhnP is now available [32]. These two reactions form the back-end of the C-P lyase pathway (Figure 5). The front-end of the pathway starts with the biosynthesis of the ribose-1-phosphonate adduct as shown using methylphosphonate as the initial substrate. This reaction is initiated by the action of PhnI, a novel nucleosidase with the assistance of PhnG, PhnH and PhnL. The products obtained from ATP and methylphosphonate are adenine and α-D-ribose-1-methylphosphonate-5-triphosphate (RPnTP) [33•]. Of the genes involved in this transformation, PhnH is the only one that has an X-ray structure (PDB id: 2FSU) but the structure of this protein has yielded no insights into the role that this protein plays in this transformation [34]. RPnTP is hydrolyzed by PhnM to yield α-D-ribose-1-methylphosphonate-5-phosphate (PRPn) and pyrophosphate. PhnM is a member of the amidohydrolase superfamily (AHS) of enzymes [33•]. Members of the AHS superfamily utilize a mono or binuclear metal center to catalyze hydrolytic reactions of esters, amides, and amine functional groups at carbon and phosphorus centers [35]. PRPn is the substrate for the cleavage of the C–P bond via the C-P lyase pathway by PhnJ, a member of the radical SAM superfamily of enzymes [36,37]. Most members of the radical SAM superfamily utilize a redox active [4Fe–4S]-cluster and *S*-adenosyl-L-methionine to generate a transient 5′-deoxyadenosyl radical that initiates complex transformations by abstracting a hydrogen atom at unactivated carbon centers [36,37]. PhnJ catalyzes the transformation of PRPn to PRcP and methane (when methylphosphonate is the substrate) [33•]. The detailed outline of the entire pathway is summarized in Figure 5. Recent efforts have yielded isolation of PhnGHIJK as a multiprotein complex; however this complex was found to be catalytically incompetent [38].

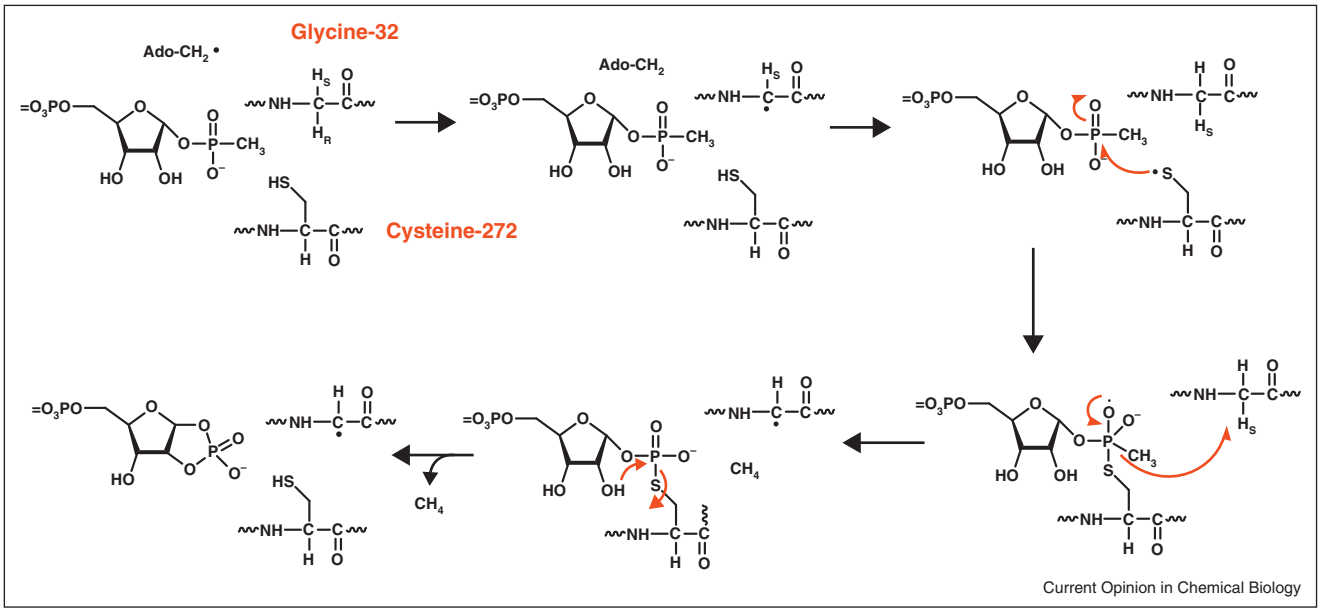
The chemical reaction mechanism for cleavage of the C–P bond in PRPn has been elucidated [39••]. The proposed reaction mechanism starts with the reductive cleavage of SAM by a reduced iron-sulfur cluster to form

Figure 5



The C-P lyase pathway from *Escherichia coli*.

Figure 6



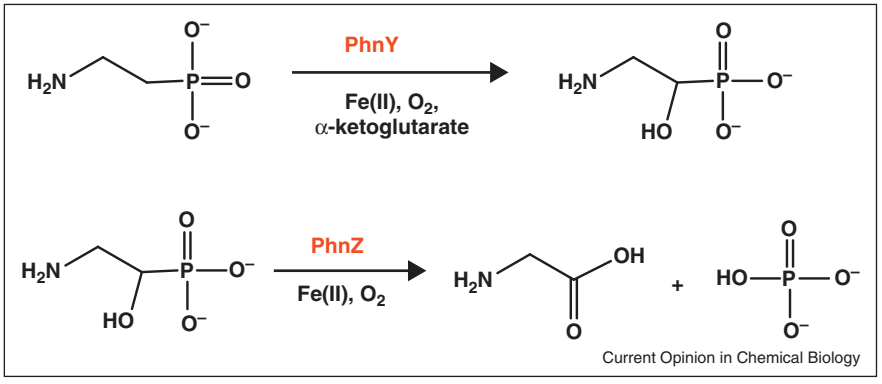
Proposed mechanism for the reaction catalyzed by PhnJ.

a 5′-deoxyadenosyl radical intermediate (Ado-$CH_2^{\bullet}$). This intermediate then abstracts the *proR* hydrogen from Gly-32 to produce 5′-deoxyadenosine (Ado-CH_3) and a glycyl radical. The glycyl radical subsequently abstracts a hydrogen atom from Cys-272 to make a thiyl radical. The thiyl radical then attacks the phosphonate moiety of the substrate, PRPn, to form a transient thiophosphonate radical intermediate. This intermediate collapses via homolytic C–P bond cleavage and hydrogen atom transfer from the original *proS* hydrogen of Gly-32, to produce a covalent thiophosphate intermediate and methane. The final product, PRcP, is formed by nucleophilic attack of the C2-hydroxyl on the covalent thiophosphate intermediate. This reaction regenerates the free thiol group of Cys-272. After hydrogen atom transfer from Cys-272 to the Gly-32 radical, the entire process is repeated with another substrate without the use of another molecule of SAM or involvement from the iron-sulfur cluster again. The proposed reaction mechanism is presented in Figure 6.

Oxidative pathway

Recently, a new bacterial pathway has been discovered for the metabolism of 2-aminoethylphosphonic acid (2AEP) from marine-based metagenomic DNA screens capable of using 2AEP as a sole phosphorus source for Δphn mutants in *E. coli*. A new pair of genes *phnY* and *phnZ* was discovered from this screen [39••]. Sequence analysis of PhnY indicates that it belongs to the non-heme Fe(II)/α-ketoglutarate-dependent dioxygenase family

Figure 7



The oxidative pathway for the metabolism of 2-aminoethylphosphonate (2AEP) from marine-based metagenomic screens.

and PhnZ is annotated as a metal-ion dependent phosphohydrolase of the HD-family of enzymes [40•]. PhnZ in often found closely associated with the *phn* operon encoding the C-P lyase activity, and forms a large class of uncharacterized proteins associated with and complementing this operon [40•]. PhnY and PhnZ catalyze sequential reactions to metabolize 2AEP (Figure 7). PhnY was shown to catalyze the hydroxylation of 2AEP to form 2-amino-1-hydroxyl-ethylphoshonic acid (2A1HEP). PhnY utilizes iron in the ferrous oxidation state, α-ketoglutarate, and a reducing agent such as ascorbate to convert 2AEP to 2A1HEP using molecular oxygen as the source of oxygen for the hydroxylation reaction. The mechanism of hydroxylation of 2AEP by PhnY is predicted to be analogous to that of TauD, another non-heme Fe(II)/α-ketoglutarate dependent hydroxylase involved in the catabolism of taurine [41]. PhnY was found to be very specific for 2AEP, and did not accept any other phosphonates such as methylphosphonate, ethylphosphonate as substrates [40•]. PhnZ possess the invariant 'HD' motif that is characteristic of the HD-hydrolase enzyme family, and shares similarities to myo-inositol oxygenase (MIOX), another member of this family [42]. PhnZ is shown to utilize the product of PhnY, 2A1HEP, as a substrate to yield glycine and orthophosphate as products. Molecular oxygen is used by PhnZ to oxidatively cleave the C–P bond of 2A1HEP. PhnZ is predicted to utilize a binuclear iron center in the active site, where Fe(II) reduces molecular oxygen to generate a Fe(III)-superoxo species, which oxidizes the substrate. A mechanism for the cleavage of the C–P bond of 2A1HEP has been proposed that is analogous to that proposed previous for MIOX [40•,41,42].

Currently there are three broad classes of enzymatic systems capable of metabolizing phosphonates. These include the phosphonatases, C-P lyases and an oxidative pathway. We have attempted to provide a brief description and summarize the recent progress made in the area. Extensive mechanistic and structural studies are available for all of these phosphonatases. With the recent functional discoveries of the genes involved in the C-P lyase pathway and the oxidative pathway, mechanistic and structural studies on these systems would become available in the very near future. With the advent of genome sequencing technologies, and the availability of genomes from various environmental sources, it is only a matter of time before other novel reactions and biological pathways capable of metabolizing phosphonates will emerge.

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