

# PHOSPHONATES TO PHOSPHATE: A FUNCTIONAL ANNOTATION OF THE ESSENTIAL GENES OF THE *PHN* OPERON IN *ESCHERICHIA COLI*

SIDDHESH S. KAMAT, HOWARD J. WILLIAMS,  
AND FRANK M. RAUSHEL\*

Department of Chemistry, P.O. Box 30012, Texas A&M University,  
College Station, TX 77843, U.S.A.

E-MAIL: \*[raushel@tamu.edu](mailto:raushel@tamu.edu)

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## ABSTRACT

The reaction mechanism for the enzymatic conversion of methyl phosphonate to phosphate and methane in *Escherichia coli* has eluded researchers over the last three decades despite significant genetic and *in vivo* studies. The *phn* operon governs the C-P lyase activity in *E. coli*. The essential genes within the *phn* operon are *phnGHIJKLM*. The proteins encoded by *phnGHM* were over-expressed in *E. coli* and purified to homogeneity using standard protocols. The proteins encoded by *phnIJKL* were soluble only when expressed as N-terminal glutathione S-transferase (GST) fusion proteins. PhnI was shown to catalyse the formation of  $\alpha$ -D-ribose-1-methylphosphonate-5-triphosphate (RPnTP) from MgATP and methylphosphonate in the presence of PhnG, PhnH, and PhnL after *in situ* cleavage of the GST-tags. PhnI alone catalyses the hydrolytic cleavage of MgATP to adenine and D-ribose-5-triphosphate (RTP). PhnM catalyses the hydrolysis of  $\alpha$ -D-ribose-1-methylphosphonate-5-triphosphate (RPnTP) to  $\alpha$ -D-ribose-1-methylphosphonate-5-phosphate (PRPn) and pyrophosphate with attack of water on the  $\alpha$ -phosphoryl group of the triphosphate moiety of RPnTP. PhnJ was reconstituted with an iron-sulphur cluster through the anaerobic addition of FeSO<sub>4</sub>, Na<sub>2</sub>S and Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> under strictly anaerobic conditions. The [Fe<sub>4</sub>S<sub>4</sub>]-reconstituted PhnJ

GST-fusion protein catalyses the homolytic cleavage of the phosphorus-carbon bond of  $\alpha$ -D-ribose-1-methylphosphonate-5-phosphate (PRPn) to ultimately form  $\alpha$ -D-ribose-1,2-cyclic-phosphate-5-phosphate (PRcP) and methane in the presence of *S*-adenosyl-L-methionine (SAM) under strictly anaerobic conditions, after *in situ* cleavage of the GST-tag.

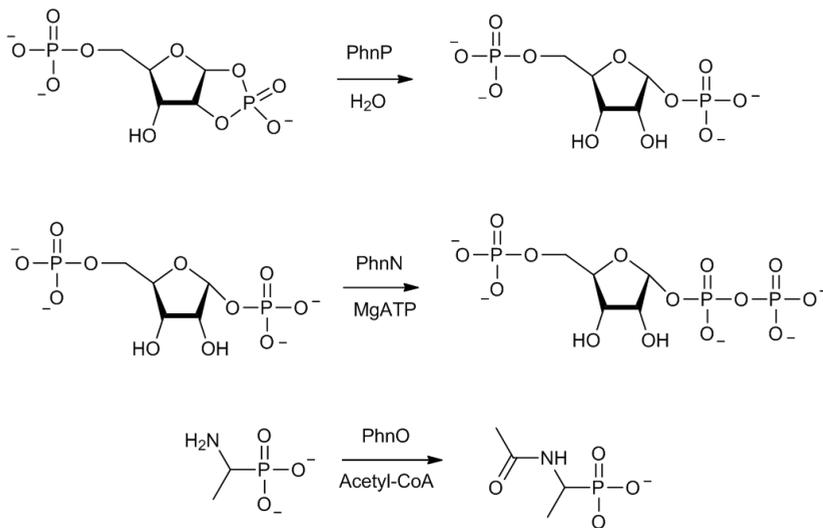
## INTRODUCTION

Carbon, nitrogen, oxygen, and phosphorus are essential elements required by all living organisms for survival. Of these elements, phosphorus is primarily incorporated as a derivative of phosphate where it is an integral component of nucleic acids, carbohydrates, and phospholipids. Phosphorus has many roles in many metabolic pathways and cell signalling. Most organisms have evolved to obtain phosphorus, as phosphate, directly from the environment. However, many Gram-negative bacteria, such as *Escherichia coli*, have the capability of utilizing organophosphonates as a nutritional source of phosphorus under conditions of phosphate starvation via the cryptic C-P lyase activity (1, 2). Phosphonates are organophosphorus compounds that contain one C-P bond. This bond is chemically inert and hydrolytically stable. Phosphonates are ubiquitous and some prominent examples include antibiotics (fosfomycin and phosphonothrixin), herbicides (glyphosate) and industrial detergent additives (amino-trimethylene phosphonate). It is estimated that more than 20,000 tons of phosphonates are released annually in the US alone from herbicide and detergent wastes (3, 4). The large quantities of phosphonates that are being released into the environment suggest that there needs to be a greater understanding of how these compounds are metabolized and degraded.

In *E. coli* the catalytic machinery for the “C-P lyase” activity has been localized to the *phn* gene cluster, which is induced only under conditions of limiting phosphate by the global *pho* regulation system. This operon consists of 14 genes denoted as *phnCDEFGHIJKLMN*OP. Genetic and biochemical experiments have demonstrated that the *phnGHIJKLM* genes encode proteins that are essential for the enzymatic conversion of phosphonates to phosphate. The genes *phnCDEF* encode proteins responsible for the binding and/or transport of phosphonates across cell membranes as well as regulation and modulation of the C-P lyase activity. The genes *phnNOP* encode proteins that have auxiliary functions and these three proteins have been functionally characterized (1, 5). Wanner, Hove-Jensen and co-workers have shown that PhnN catalyses the ATP-dependent phosphorylation of  $\alpha$ -D-ribose-1,5-bisphosphate (PRP) to  $\alpha$ -D-ribose-1-pyrophosphate-5-phosphate (PRPP) (6). Blanchard and co-workers have shown that PhnO catalyses the metal-dependent acetylation of aminoalkylphosphonic acids with acetyl CoA as the co-substrate (7). Zechel, Hove-Jensen and co-workers have shown that PhnP catalyses the metal-dependent hydrolysis of  $\alpha$ -D-ribose-1,2-cyclic phosphate-5-phosphate (PRcP) to PRP (8). These reactions are summarized in Scheme 1.

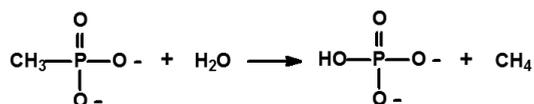
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## Phosphonates to Phosphate



**Scheme 1.** Reactions catalyzed by PhnN, PhnO, and PhnP.

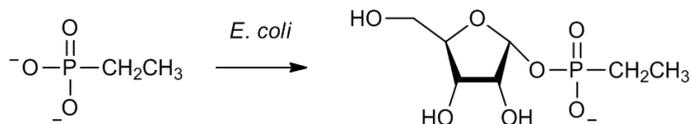
In *E. coli* methyl phosphonate is converted to phosphate and methane enzymatically as shown in Scheme 2 via the C-P lyase pathway. This transformation is the equivalent to the addition of water during the cleavage of the carbon-phosphorus bond, but an enzymatic mechanism for the conversion of inactivated phosphonates to phosphate is unknown.



**Scheme 2.** Conversion of methyl phosphonate to phosphate and methane.

At the start of our investigation there were no functional annotations for any of the seven proteins considered essential for C-P bond cleavage despite three decades of research on this pathway. The primary difficulty in elucidating the reactions catalysed by these enzymes has been the extremely poor solubility of the proteins encoded by the essential gene when removed from their cellular environment. The protein encoded by *phnH* was the only protein to have been reported as soluble. This protein has been crystallized and the three-dimensional structure (PDB id: 3fsu) has been solved (9). Despite computational and ligand binding studies, no clues were available that would have provided hints of the catalytic role of PhnH in the C-P lyase pathway. PhnM was predicted to be a hydrophobic protein and this has led to the speculation that the *phnGHIJKLM* gene products form a membrane-associated enzyme complex that functions collectively to catalyse C-P bond cleavage (1, 2). Zechel, Hove-Jensen and co-workers recently purified the gene products from the expression of the genes *phnGHIJK* as a soluble multi-protein complex (2, 10). Walsh, Floss and co-workers have shown that C-P bond cleavage occurs with racemization of stereochemistry at the

carbon centre. This result suggests that the C-P bond cleavage reaction proceeds via a radical-based mechanism (11). Frost and co-workers isolated  $\alpha$ -D-ribose-1-ethylphosphonate from the extracellular medium after providing radio-labelled ethylphosphonate to *E. coli* as the sole phosphorus source (12, 13). This transformation is illustrated in Scheme 3.



**Scheme 3.** Formation of  $\alpha$ -D-ribose-1-ethylphosphonate in *E. coli* from ethylphosphonate.

Based on the functional annotations to the auxiliary proteins (PhnNOP), and the previous literature available on the C-P lyase complex in *E. coli*, there were three conclusions that could be drawn at the start of this investigation: (a) there is a ribose donor/intermediate involved in the pathway for the metabolism of phosphonates (b) the chemistry on this pathway is initiated at the C-1 position of the ribose moiety and (c) the C-P bond breaks via a radical mechanism. Sequence analysis of PhnI indicated a weak similarity to purine and pyrimidine nucleosidases at very relaxed expectation values when BLASTED using the NCBI protein database. PhnJ has four conserved cysteine residues at the C-terminal end of the protein with a highly conserved spacing between the four cysteine residues. The conserved cluster of cysteine residues is suggestive of a metal binding site and a high probability of binding an iron-sulphur cluster (14). PhnM is a member of the amidohydrolase superfamily (AHS) and is found within COG3454 of the Cluster of Orthologous Groups (COG) of proteins. Enzymes within this superfamily of proteins catalyse hydrolysis reactions at carbon and phosphorus centres using a mono- or binuclear metal centre (15). PhnK and PhnL were predicted to be ATP-binding proteins of unknown function and perhaps function as permeases (16). Therefore, the preliminary bioinformatic and sequence analysis yielded hints for three probable reactions for the metabolism of organophosphonates: (a) a nucleosidase-type reaction catalysed by PhnI – presumably involving the synthesis of a phosphonate-ribose adduct; (b) a hydrolytic reaction catalysed by PhnM; and (c) a radical-based reaction for the cleavage of the C-P bond catalysed by PhnJ that would require an iron-sulphur cluster.

## PROTEIN ISOLATION

The genes for the seven essential proteins encoded by the *phnGHIJKLM* gene cluster were cloned, expressed in *E. coli* and the proteins were purified to homogeneity (17). The genes for PhnG, PhnK, PhnL and PhnM were amplified and individually ligated into a pET30a(+) vector. Of these proteins only PhnG and PhnM were soluble after cell lysis. PhnG was purified using a standard polyhistidine-tag purification strategy and was stable in 150 mM NaCl at pH 8.5. PhnM was cloned without a polyhistidine-tag and was largely insoluble at

pH values below 8.5. This protein was isolated using gel filtration and anion exchange chromatography at pH 9.0. Since PhnM is a member of the AHS, the growth cultures and purification buffers were supplemented with 1 mM ZnCl<sub>2</sub>. The identities of PhnM and PhnG were validated by N-terminal amino acid analysis of the purified proteins. PhnK and PhnL were insoluble when expressed from a standard pET30a(+) vector. These genes were subsequently re-cloned with N-terminal glutathione S-transferase (GST) solubility tags using a pET42a(+) vector. These clones resulted in the expression of GST fused to the N-terminus of the target protein with a linker containing a Factor-Xa cleavage site. The PhnK and PhnL GST-fusion proteins were soluble and purified using a GST-affinity column (GE Healthcare). These two proteins required 1 mM dithiothreitol (DTT), 500 mM NaCl and 10% glycerol, pH 8.8, for stability (17).

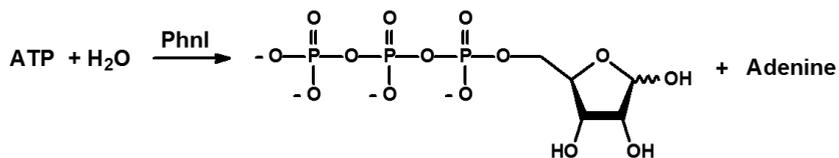
A pET28b(+) vector was used for the initial cloning of the genes for PhnH, PhnI and PhnJ. Of these proteins, only PhnH was soluble after cell lysis. This protein was purified as described previously (9). Since PhnI and PhnJ were insoluble, they were re-cloned into a pET42a(+) vector with a GST-tag using the same strategy as that for PhnK and PhnL. The purification of PhnI required 1 mM EDTA, 1 mM DTT, 500 mM NaCl and 10% glycerol at pH 8.5. PhnJ required 1 mM EDTA, 500 mM NaCl and 10% glycerol at pH 8.8. Of the seven proteins critical for cleavage of phosphorus-carbon bonds, PhnG, PhnH and PhnM were purified without any solubility tags. PhnI, PhnJ, PhnK and PhnL were purified as N-terminal GST-fusion proteins. The GST-fusion proteins precipitated within ~15 minutes after removal of the N-terminal GST-tag by Factor-Xa. All protein purifications contained 100 mM HEPES buffer at the specified pH (17).

## CATALYTIC FUNCTION OF PHN I

Prior investigations have hinted that a ribose intermediate is involved in the metabolism of phosphonate substrates prior to the conversion to phosphate (8, 12 – 13). Since PhnI showed a distant relationship to enzymes that are functionally validated as nucleosidases, we incubated this protein (10 μM) with a small and focused library of probable ribose donors including nucleosides, nucleotides and other ribose bearing compounds such as NAD<sup>+</sup>, NADP and ADP-ribose at concentrations of ~2 mM for up to 6 hours in the presence of 3 mM MgCl<sub>2</sub> at pH 8.5. The liberation of the free base was determined spectrophotometrically at 240–350 nm in the presence of coupling enzymes (1 μM) that are capable of deaminating adenine (18), guanine (19) or cytosine (20). Xanthine oxidase (50 Units) was added to all inosine containing compounds (21). The only active substrates found in these preliminary screening assays were the purine nucleoside di- and tri- phosphates i.e. ATP, ADP, GTP, GDP, ITP and IDP. The best substrates for PhnI were GTP and ATP with kinetic constants for the nucleosidase activity of ~10<sup>4</sup> M<sup>-1</sup>s<sup>-1</sup> for  $k_{cat}/K_m$ . The products of this reaction were D-ribose-5-triphosphate (RTP) and the free base (adenine or guanine when ATP or GTP were utilized, respectively). The reaction catalysed by PhnI with ATP is

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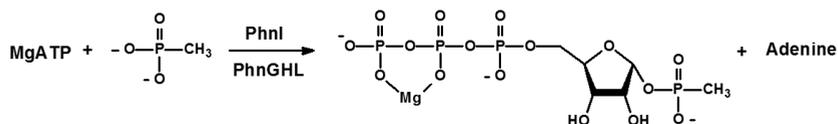
presented in Scheme 4. The RTP could be purified using a ResourceQ anion exchange column (GE Healthcare). The structure of RTP was initially identified by  $^{31}\text{P}$ -NMR spectroscopy and further confirmed by one- and two-dimensional NMR spectroscopy.



**Scheme 4.** Reaction catalyzed by PhnI in the presence of MgATP.

The formation of D-ribose-5-triphosphate from either ATP or GTP did not appear to be a productive route for the C-P lyase pathway since the phosphonate substrate was not connected to the ribose moiety. We therefore incubated a small library of phosphate and phosphonate derivatives (5 mM) with MgATP in the presence of PhnI to determine if any of these compounds (rather than water) could displace adenine. Surprisingly, phosphate, pyrophosphate, methylphosphate and sulfate inhibited the displacement of adenine from ATP but no inhibition was observed with methylphosphonate, ethylphosphonate, phosphonoacetic acid or 2-aminoethylphosphonate. The apparent inhibition constant for phosphate at an ATP concentration of 0.1 mM was  $\sim 0.30$  mM. RTP and adenine were the only products formed in the presence of either phosphate or methylphosphonate. There were no further changes in the reaction products whether we used the GST-tagged PhnI in the presence or absence of Factor Xa (22).

Since it was previously postulated that the C-P lyase reaction involved a multi-protein complex, all of the recombinantly purified proteins were added to the reaction mixture (10). No new products were detected other than RTP and adenine. Since PhnI, PhnJ, PhnK and PhnL were all GST-fusion proteins, Factor-Xa (50 Units) was added to the reaction mixture for *in situ* cleavage of the GST-tags. With the addition of Factor-Xa, a new compound was observed by  $^{31}\text{P}$  NMR spectroscopy at 17.8 ppm. Anion exchange chromatography was used to purify the newly formed compound. One- and two-dimensional NMR spectra were consistent with the formation of  $\alpha$ -D-ribose-1-methylphosphonate-5-triphosphate (RPnTP) as shown in Scheme 5. A small amount of the  $\beta$ -anomer was also detected (approximately 5–10% of the  $\alpha$ -anomer). In this transformation, all of the ATP was consumed but the overall yield of the phosphorylated product was approximately 30–35%; the rest of the ATP was converted to RTP.



**Scheme 5.** Formation of RPNTP by PhnI in the presence of PhnGHL after cleavage of the GST-tags.

To determine whether all of the proteins were required for this transformation, each protein was removed individually from the reaction mixture. The minimal set of proteins required for the nucleophilic attack of methylphosphonate on the anomeric carbon of MgATP to form adenine and form RPNTP were PhnI, PhnG, PhnH and PhnL. The kinetic constants for the reaction of methylphosphonate with ATP and GTP in presence of PhnI, PhnG, PhnH and PhnL were  $\sim 10^5 \text{ M}^{-1} \text{ s}^{-1}$  for  $k_{\text{cat}}/K_{\text{m}}$ . No reaction was observed with the fusion proteins and the GST-tags must be removed in situ for this activity. The partitioning of ATP to RTP and RPNTP likely results from partially cleaved GST-tagged protein and the precipitation of PhnI, PhnK, and PhnL after cleavage of these tags. The stoichiometry of the four proteins required for the formation of this complex and the individual functions of PhnG, PhnH and PhnL are currently uncertain.

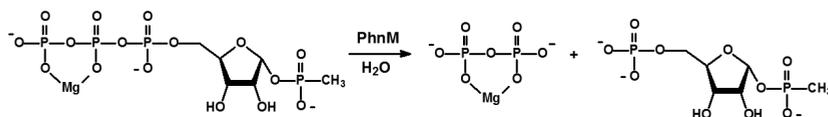
## CATALYTIC FUNCTION OF PHNM

PhnM is a member of the amidohydrolase superfamily (15). Enzymes within this superfamily use a mono- or binuclear metal centre to catalyse hydrolytic reactions at carbon and phosphorus centres. PhnM is grouped within COG3454. This COG consists of approximately 200 proteins of similar sequence from different bacteria. All proteins from the amidohydrolase superfamily have a  $(\beta/\alpha)_8$ -barrel structural fold. Sequence alignment analysis indicated that all of the enzymes in COG3454 have a highly conserved HxD motif at the end of  $\beta$ -strand 1, a histidine at the end of  $\beta$ -strand 5, a glutamate at the end of  $\beta$ -strand 6 and an aspartate at the end of  $\beta$ -strand 8. These protein sequences lack a clearly defined ligand from  $\beta$ -strand 4 that could serve to bridge two divalent cations and thus PhnM was predicted to bind a single divalent metal cation in the active site at the  $\alpha$ -metal binding site. PhnM was previously predicted to be a hydrophobic membrane bound protein (1, 2). Variation of the solution pH demonstrated that PhnM was soluble at pH values greater than 9.0. At this pH PhnM was soluble and could be purified without a solubility or affinity tag. Inductively coupled plasma mass spectrometry (ICP-MS) demonstrated that the purified PhnM contained  $\sim 1.2$  equivalents of  $\text{Zn}^{2+}$  per monomer when  $\text{Zn}^{2+}$  was supplemented in the growth media during protein expression.

Previous studies have suggested that  $\alpha$ -D-ribose-1-phosphonate-5-phosphate (PRPn) is a potential intermediate in the conversion of alkylphosphonates to phosphate by *E. coli* (8, 12). Hence it was rational to assume that one of the proteins in the *phn* operon would catalyse the hydrolysis of the  $\beta$ - and  $\gamma$ -phosphoryl groups from RPNTP that was isolated by

the action of PhnI, PhnG, PhnH and PhnL. PhnM was the prime candidate to perform this hydrolytic reaction. In addition to a set of highly conserved residues believed to coordinate the active site divalent cation, PhnM also possess five highly conserved arginine residues on the various loops that connect the eight  $\beta$ -strands and  $\alpha$ -helices. These loops are essential in determining substrate specificity. The positively charged arginine residues may participate in the coordination of the triphosphate moiety of the RPnTP substrate.

When PhnM was incubated with purified RPnTP in the presence of 1 mM ZnCl<sub>2</sub> and 5 mM MgCl<sub>2</sub>, <sup>31</sup>P-NMR identified 2 new reaction products, consistent with  $\alpha$ -D-ribose-1-methylphosphonate-5-phosphate (PRPn) and pyrophosphate (17). This observation was consistent with the hydrolytic cleavage of  $\alpha$ - and  $\beta$ - phosphoryl groups of the triphosphate moiety of RPnTP. This reaction is shown in Scheme 6.



**Scheme 6.** Reaction catalyzed by PhnM.

To determine the exact position of the hydrolytic attack, the reaction was performed in a 1:1 mixture of H<sub>2</sub>O and H<sub>2</sub>O<sup>18</sup>. The O<sup>18</sup> label was found exclusively in the PRPn product. This result was indicative of an attack of the activated hydroxide on the  $\alpha$ -phosphorus of the triphosphate moiety of the substrate. All of the kinetic measurements were performed on this reaction using the Pi Colorlock (Gold) phosphate detection kit from Innova Biosciences according to the manufacturer's instruction at pH 8.5. The pyrophosphate produced in this reaction was converted to inorganic phosphate by the addition of inorganic pyrophosphatase from Baker's yeast (Sigma). The kinetic constants obtained for the hydrolysis of RPnTP by PhnM were  $k_{\text{cat}} = 6.4 \text{ s}^{-1}$ ,  $K_m = 56 \mu\text{M}$  and  $k_{\text{cat}}/K_m = 1.1 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ . Zn<sup>2+</sup> and Mg<sup>2+</sup> supplementation were essential for optimal activity of PhnM.

To determine the substrate profile for PhnM, D-ribose-5-triphosphate (RTP) and D-ribose-5-diphosphate (RDP) were tried as substrates. Both RTP and RDP were synthesized by the action of PhnI on ATP and ADP, respectively, and purified using anion exchange chromatography (17). Both RTP and RDP were substrates for PhnM. In both cases D-ribose-5-phosphate was one product. The other product was pyrophosphate for RTP and inorganic phosphate for RDP. The kinetic constants for the hydrolysis of RTP by PhnM were  $k_{\text{cat}} = 6.0 \text{ s}^{-1}$ ,  $K_m = 98 \mu\text{M}$  and  $k_{\text{cat}}/K_m = 6.1 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ . For RDP the kinetic constants were  $k_{\text{cat}} = 0.08 \text{ s}^{-1}$ ,  $K_m = 200 \mu\text{M}$  and  $k_{\text{cat}}/K_m = 3.8 \times 10^2 \text{ M}^{-1}\text{s}^{-1}$  (17). Thus, the substrate profiles showed that the RPnTP was the best substrate and that PhnM hydrolyses the triphosphate moiety to form the PRPn and pyrophosphate. Enzyme activity with PRPn is 300-fold more active than with RDP. The kinetic constants did not change for the hydrolysis

of RPNTP when PhnG, PhnH, PhnI, PhnK, PhnL and Factor-Xa were added to the assay mixture. Therefore there is no evidence that PhnM forms a complex with these other proteins.

## CATALYTIC FUNCTION OF PHNJ

The deletion of *phnJ* from *E. coli* led to the identification of  $\alpha$ -D-ribose-1-methylphosphonate (RPN) in the growth medium and this has led to the prediction that  $\alpha$ -D-ribose-1-methylphosphonate-5-phosphate (PRPN) is the ultimate substrate for the actual C-P lyase reaction (8). This expectation was consistent with our experimental results since this compound was synthesized from ATP and methylphosphonate by the combined actions of PhnI, PhnG, PhnH, PhnL and PhnM. Of the remaining two enzymes in the *phn* operon, the most likely protein to catalyse the actual carbon-phosphorus bond cleavage was PhnJ. PhnJ has four conserved cysteine residues with a spacing of  $Cx_2Cx_{21}Cx_5C$  near the C-terminal end of the protein. These four conserved cysteine residues could form a metal-thiolate cluster or more likely, an iron-sulphur cluster. The cleavage of PRPN to PRcP (or PRP) was assumed to require a radical mechanism and such reactions could be catalysed by radical SAM enzymes.

The radical SAM superfamily was first identified in 2001 by Sofia and co-workers, based on the structural and mechanistic similarities of lysine aminomutase (LAM), biotin synthase (BioB), lipoic acid synthase (LipA) and pyruvate formate lyase activating enzyme (PflA). Each of these enzymes uses a structurally conserved  $(\beta/\alpha)_8$ -TIM barrel motif to produce a 5'-deoxyadenosyl radical from S-adenosyl-L-methionine (SAM) using an unusual  $[Fe_4S_4]$ -cluster which is redox active in the reduced  $[Fe_4S_4]^{1+}$  and the oxidized  $[Fe_4S_4]^{2+}$  oxidation states (23). A recent comprehensive review by Frey *et al.* (24), has suggested that more than 2800 proteins belong to this superfamily. A signature feature for the members of this superfamily is a  $Cx_3Cx_2C$  motif near the N-terminal end of the protein. These three cysteine residues nucleate the  $[Fe_4S_4]$ -cluster. This cluster binds SAM to the unique Fe that is not ligated to any three cysteine residues. The 5'-deoxyadenosyl radical generated from the reductive cleavage of SAM, abstracts a hydrogen atom from the substrate or the protein (to generate a protein radical) initiating the reaction. With the successful reconstitutions of ThiC (25) ( $Cx_2Cx_4C$ ), HmdA (26) ( $Cx_5Cx_2C$ ), and Dph2 (27), a more diverse combination of conserved cysteine residues can function for the assembly of the  $[Fe_4S_4]$ -cluster and perform similar radical reactions. Another comprehensive review by Booker *et al.* (28), lists more examples of members of the radical SAM superfamily lacking the canonical  $Cx_3Cx_2C$  motif as well as enzymes possessing multiple iron-sulphur clusters. Elp3, a component of the factor required for transcriptional elongation ( $Cx_4Cx_9Cx_2C$ ) is one such example. Another example is PqqE, which is one of the six proteins required for the biosynthesis of pyrrolo-quinoline quinone. This protein contains a non-canonical  $Cx_2Cx_{27}C$  motif binding a  $[Fe_4S_4]$ -cluster capable of producing a 5'-deoxyadenosyl radical upon reduction along with another cluster of the canonical  $Cx_3Cx_2C$  motif.

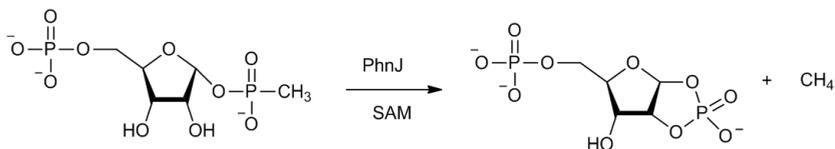
Literature precedents have hinted at the formation of some sort of metal-thiolate cluster on PhnJ (14), based on the highly invariant  $Cx_2Cx_{21}Cx_5C$  motif at the C-terminal of the protein. PhnJ purified aerobically with a GST-tag, had a black colour. This was presumably due the non-specific binding of iron sulphide from the degradation of an iron-sulphur cluster upon exposure to air. Metal analysis using ICP-MS showed the presence of  $\sim 2.2$  equivalents of Fe per monomer of aerobically purified PhnJ. Thus PhnJ appeared to be capable of binding iron and sulphide. The metal ions bound to the aerobically purified PhnJ were removed with the addition of EDTA to the purification buffers. The apo-PhnJ was made anaerobic by passing argon through the protein solution and then an excess of sodium dithionite was added (5 mM). PhnJ was incubated in the glove box for four hours. Throughout the reconstitution experiments the oxygen concentration was maintained below 4 ppm. Reconstitution of the  $[Fe_4S_4]$ -cluster was initiated by slow dropwise addition of a 50-fold enzyme excess of  $FeSO_4$ . After equilibrating anaerobically for three hours, a 50-fold excess of  $Na_2S$  was added and the mixture incubated for an additional three hours. The excess iron and sulphide that formed insoluble iron sulphide (black solid) was removed by centrifugation, followed by ultrafiltration through a 10 kDa membrane. The protein with the reconstituted iron-sulphur cluster had a reddish-brown colour. The UV-visible absorption spectrum of PhnJ, reconstituted with iron and sulphide, had a broad absorption band centred at 403 nm that is indicative of a  $[Fe_4S_4]$ -cluster (23, 24, 28). The absorption band disappears upon addition of 1 mM dithionite, suggesting the reduction of the  $[Fe_4S_4]^{2+}$  cluster to the  $[Fe_4S_4]^{1+}$  species, which is the active form for most radical SAM enzymes (17). The discoloration is also indicative of the redox activity of the proteins within the radical SAM superfamily (24, 28). Quantitation of the UV-visible spectrum of PhnJ suggested that the chemical reconstitution yield was approximately 40%.

Even though there were prior suggestions that PRPn was the ultimate substrate for PhnJ, the putative C-P bond cleaving enzyme from the gene knockout studies carried out by Zechel, Hove-Jensen and co-workers (8, 10), there were still two compounds that could have been potential substrates for cleavage by PhnJ of the C-P bond: PRPn and RPnTP. Both of these compounds were incubated with 125  $\mu M$  PhnJ that was chemically reconstituted with a  $[Fe_4S_4]$ -cluster, 2 mM SAM and 1mM dithionite, under anaerobic conditions at pH 6.8. The reactions were analysed by  $^{31}P$  NMR spectroscopy but there was no change in the NMR spectrum for either substrate. Since PhnJ was expressed and purified as an N-terminal GST-fusion protein, the reaction was supplemented with Factor-Xa to cleave the GST-tag. No change in the  $^{31}P$  NMR spectrum was observed with RPnTP, but a new resonance appeared at 16.2 ppm using PRPn as a substrate (17). The increase in the resonance at 16.2 ppm correlated with a decrease in the phosphonate resonance of PRPn at 16.6 ppm. The new resonance splits into a triplet in the  $^1H$ -coupled  $^{31}P$  spectrum (17) demonstrating that the product is no longer a methyl phosphonate. The new resonance was consistent with a cyclic phosphate and the proton coupling constant of 21 Hz indicates that the phosphate moiety of the product was esterified to the hydroxyls attached to C1 and C2 of the ribose. This result was consistent with the findings of Zechel, Hove-Jensen and co-

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## Phosphonates to Phosphate

workers for the substrate of PhnP (8, 17). Thus, PhnJ requires a reduced  $[\text{Fe}_4\text{S}_4]$ -cluster and SAM to catalyse the formation of  $\alpha$ -D-ribose-1,2-cyclic phosphate-5-phosphate from PRPn. The overall reaction is illustrated in Scheme 7.



**Scheme 7.** Reaction catalyzed by PhnJ in the presence of SAM.

Gas chromatography (GC) and GC-mass spectrometry (MS) were used to confirm the formation of methane. All GC and GC-MS samples were prepared anaerobically. The reconstituted PhnJ was incubated with 2 mM SAM, 1 mM dithionite, 5 mM PRPn, and Factor-Xa in a sealed tube for 5 hours. 50  $\mu\text{L}$  of the headspace (500  $\mu\text{L}$ ) was removed using a gas-tight Hamilton syringe and injected into the GC instrument (Hewlett Packard HP 6890 GC system with FID detector and manual injection) equipped with a 30 m X 0.32 mm I.D. SGE Solgel 1 column used in splitless injection mode, at 20 psig He carrier pressure, with a temperature program of 50  $^{\circ}\text{C}/1$  min, to 100  $^{\circ}\text{C}$  at 10  $^{\circ}\text{C}/\text{min}$ . The same reaction was conducted for the GC-MS experiment using a Thermo Electron Corporation DSQ quadrupole GC-MS instrument with Finnigan Trace GC Ultra chromatograph at 70 E.V. EI ionization. The temperature program and injection mode were the same. The column was a 30 m x 0.25 mm I.D. SGE BP1 column operated at 1 mL/min constant flow of He. 100  $\mu\text{L}$  of the headspace was injected into the GC-MS instrument. Gas chromatographic analysis of the headspace above the liquid showed a single peak that co-eluted with a methane standard. The formation of methane was confirmed by coupling the output of the GC to a mass spectrometer and detection of a mass of 16. Thus, the two products formed from the action of PhnJ on PRPn are methane and PRcP. To determine whether there were any byproducts of this reaction, an analysis was conducted to check for the formation of methanol and formaldehyde. Alcohol dehydrogenase from yeast was used to test the formation of methanol using  $\text{NAD}^+$ . The reaction showed no formation of methanol. For the detection of formaldehyde, purpald was used. A standard curve was made and the reaction mixture showed no formation of formaldehyde. The addition of SAM, PhnJ, a reductant (dithionite) and Factor-Xa were required for the formation of PRcP and methane from the  $[\text{Fe}_4\text{S}_4]^{1+}$  reconstituted PhnJ under strictly anaerobic conditions.

To determine the fate of SAM during the reaction, HPLC and amino acid analysis were employed. Amino acid analysis of the reaction products from SAM in the reaction catalysed by PhnJ was performed by the Protein Chemistry Laboratory, Texas A&M University. The derivatized amino acids were separated by reverse phase HPLC and detected by UV absorbance with a diode array detector or by fluorescence using an in-line fluorescence detector. For the reactions involving PhnJ, the concentrations of the components were as follows:

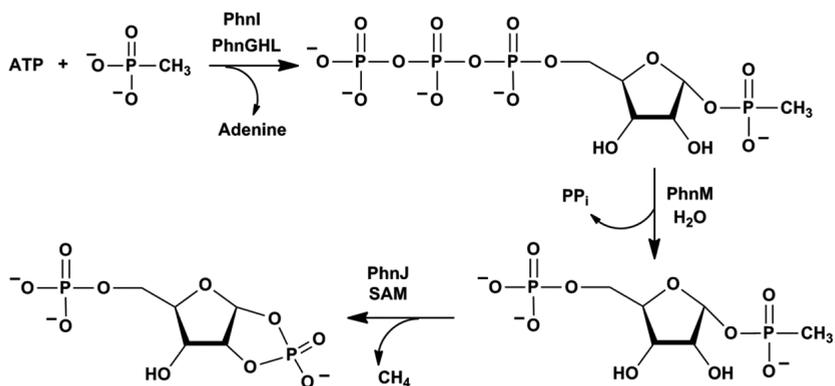
PhnJ = 70  $\mu$ M, SAM = 2.0 mM, dithionite = 1.0 mM, PRPn = 1.0 mM in 150 mM HEPES, containing 250 mM NaCl, 10% v/v glycerol and 1X Factor Xa reaction buffer at pH 8.5 in a volume of 1.0 mL. The reaction was initiated by adding 50 units of Factor Xa. As a control, PhnJ was eliminated from the reaction mixture. All of the reactions were kept anaerobic for three hours after which the precipitated enzyme was removed by centrifugation. The reaction was filtered through a 3 kDa ultra-filtration membrane and the flow-through was collected. The control reaction showed less than 0.01  $\mu$ M L-methionine. In the reaction mixture with PhnJ, 150  $\mu$ M of L-methionine was detected. The HPLC analysis for determining the reaction products of SAM with PhnJ was performed with an AKTA Purifier FLPC/HPLC system with a C 18 column (Cosmosil 5C 18-AR-II 4.6  $\times$  150 mm Nacalai, USA). The C 18-column was pre-calibrated with SAM, 5'-deoxyadenosine (5DA) and 5'-deoxy-5'-methylthioadenosine (MTA) for the elution profiles of the standards. For the reactions involving PhnJ, the concentrations of the components were as follows: PhnJ = 60  $\mu$ M, SAM = 0.5 mM, dithionite = 1 mM,  $\alpha$ -D-ribose-1-methylphosphonate-5-phosphate = 2 mM in 150 mM HEPES, containing 250 mM NaCl, 10% v/v glycerol and Factor-Xa reaction buffer at pH 8.5 in a volume of 1.0 mL. The reaction was initiated by adding 50 units of Factor-Xa to the reaction. As a control, dithionite was eliminated in one reaction and PhnJ was eliminated in another. All of the reactions were kept anaerobic for 3 hours after which the precipitated enzyme was removed by centrifugation. The reaction was filtered through a 3 kDa ultra-filtration membrane and the flow-through was collected. 250  $\mu$ L of this material was loaded onto a C 18-column and the HPLC traces were collected. HPLC analysis showed the formation of 5'-deoxyadenosine and amino acid analysis confirmed the formation of methionine. These products were only formed when PhnJ (with a reconstituted iron-sulphur centre), Factor-Xa, SAM, dithionite and PRPn were added to the reaction mixture under strictly anaerobic conditions. The omission any one of the above components resulted in no formation of 5'-deoxyadenosine. The concentration of PRcP, 5'-deoxyadenosine, methionine, and methane formed in the presence of PhnJ showed that 1–10 turnovers of product were formed per PhnJ. The small number of turnovers per enzyme may reflect the poor solubility of PhnJ after proteolytic cleavage of the N-terminal GST-fusion tag.

## RECONSTITUTION OF THE C-P LYASE PATHWAY

The mechanism for the conversion of alkyl phosphonates to phosphate in *E. coli* was elucidated. PhnI (a novel nucleosidase), in the presence of PhnG, PhnH, and PhnL, catalyses the formation of RPnTP from MgATP and methylphosphonate. PhnM (an amidohydrolase) catalyses the hydrolysis of RPnTP to form pyrophosphate and PRPn. PhnJ can be chemically reconstituted with a [Fe<sub>4</sub>S<sub>4</sub>]-cluster with ferrous sulfate, sodium sulphide and sodium dithionite under strictly anaerobic conditions. The reconstituted PhnJ catalyses the homolytic cleavage of the C-P bond of PRPn to form PRcP and methane in the presence of SAM. These reactions are summarized in Scheme 8.

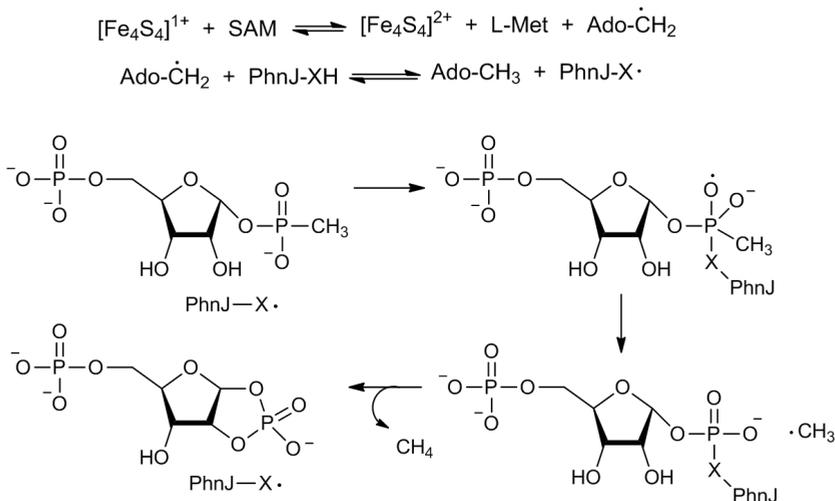
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## Phosphonates to Phosphate



**Scheme 8.** Reaction pathway for the conversion of methyl phosphonate to methane and PRcP.

PhnJ is a putative radical SAM enzyme and the working model for the cleavage of the C-P bond of the phosphonate moiety is presented in Scheme 9 (17). In this mechanism the reduced iron-sulphur cluster transfers an electron to SAM and forms an oxidized iron-sulphur cluster, methionine, and 5'-deoxyadenosyl radical. The 5'-deoxyadenosyl radical abstracts a hydrogen atom from the protein generating a protein based radical and to generate 5'-deoxyadenosine. This protein based radical attacks the phosphorus of the phosphonate forming a protein-substrate adduct. This results in the homolytic cleavage of the C-P bond, coupled with formation of a transient methyl radical. Methane is formed presumably from the abstraction of a hydrogen atom from either the protein residue to regenerate the protein radical or from 5'-deoxyadenosine to regenerate the 5'-deoxyadenosyl radical. Attack of the C2 hydroxyl generates the cyclic diester phosphate product PRcP. The formation of PRcP by PhnJ is consistent with the known function of PhnP that catalyses the hydrolysis of PRcP to PRP (8).



**Scheme 9.** Working model for the reaction mechanism catalyzed by PhnJ.

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