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## REVIEW

# PhnJ - A novel radical SAM enzyme from the C-P lyase complex <sup>☆</sup>

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Phosphonate meta-  
bolism;  
C-P bond cleavage;  
Hydrogen transfer**Abstract**

PhnJ from the C-P lyase complex catalyzes the cleavage of the carbon-phosphorus bond in ribose-1-phosphonate-5-phosphate (PRPn) to produce methane and ribose-1,2-cyclic-phosphate-5-phosphate (PRcP). This protein is a novel radical SAM enzyme that uses glycyl and thiyl radicals as reactive intermediates in the proposed reaction mechanism. The overall reaction is initiated with the reductive cleavage of S-adenosylmethionine (SAM) by a reduced [4Fe-4S]<sup>1+</sup>-cluster to form an Ado-CH<sub>2</sub>• radical intermediate. This intermediate abstracts the *proR* hydrogen from Gly-32 of PhnJ to form Ado-CH<sub>3</sub> and a glycyl radical. In the next step, there is hydrogen atom transfer from Cys-272 to the Gly-32 radical to generate a thiyl radical. The thiyl radical attacks the phosphorus center 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 *proS* hydrogen of Gly-32 to produce a thiophosphate intermediate, methane, and a radical intermediate at Gly-32. The final product, PRcP, is formed by nucleophilic attack of the C2-hydroxyl on the transient 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 molecule without the use of another molecule of SAM or involvement from the [4Fe-4S]-cluster again. © 2015 The Authors. Published by Elsevier GmbH. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

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## Introduction

Many bacteria have the ability to grow on organophosphonates as a source of phosphorus when the phosphate concentration is very low (White and Metcalf, 2007). In *Escherichia coli* the metabolism of phosphonates is governed by the set of 14 genes localized with the *phn* operon (Metcalf and Wanner, 1993). We have recently determined the metabolic pathway for the conversion of methylphosphonate (MPn) to ribose-1,2-cyclic-phosphate-5-phosphate (PRcP) and methane (Kamat et al., 2011). This pathway is initiated by the displacement of adenine from ATP to form ribose-1-phosphonate-5-triphosphate (RPnTP) in a reaction catalyzed by PhnI in the presence of PhnG, PhnH, and PhnL. In the next step PhnM, a member of the amidohydrolase superfamily, catalyzes the cleavage of pyrophosphate from RPnTP to generate ribose-1-phosphonate-5-phosphate (PRPn). In the final step PhnJ catalyzes the cleavage of the P-C bond in PRPn to form ribose-1,2-cyclic-phosphate-5-phosphate (PRcP) and methane (Kamat et al., 2011). The overall transformation is summarized in Scheme 1.

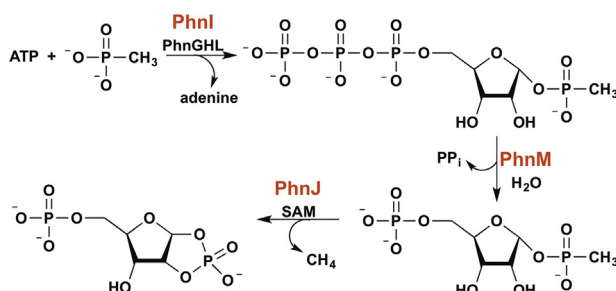
Chemically, the most interesting reaction catalyzed by enzymes in this pathway is the one initiated by PhnJ where the chemically inert and hydrolytically stable C-P bond of the phosphonate intermediate is cleaved. The reaction catalyzed by PhnJ requires S-adenosylmethionine (SAM) and a reduced [4Fe-4S]-cluster in addition to the substrate and thus this enzyme is similar to a superfamily of enzymes that are collectively known as radical SAM enzymes. In preliminary experiments it was demonstrated that at the end of the reaction that one enzyme-equivalent of methionine and 5'-deoxyadenosine were formed from one molecule of SAM. These results are consistent with the reductive cleavage of the SAM cofactor by the [4Fe-4S]-cluster to transiently generate a 5'-deoxyadenosyl radical and methionine. The 5'-deoxyadenosyl radical would likely function as a radical initiator for the ultimate cleavage of C-P bond in the

substrate (Booker and Grove, 2010). However, the mechanism for the actual cleavage of the C-P bond is not clear. The experiments described in this presentation will outline how the chemical mechanism for the reaction catalyzed by PhnJ was elucidated.

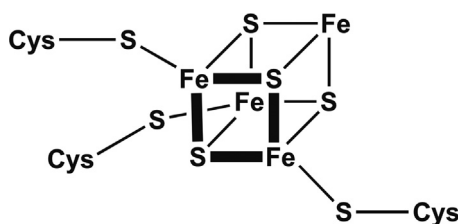
## Three initial questions

At the start of this investigation we set out to address three questions that would help elucidate the chemical mechanism for the reaction catalyzed by PhnJ. The first of these questions is this identity of the three cysteine residues that are required for the formation of the [4Fe-4S]-cluster. In PhnI there are four cysteine residues near the C-terminal end of the protein that are absolutely conserved in all of the organisms that possess this gene. These cysteine residues include Cys-241, Cys-244, Cys-266, and Cys-272 for PhnJ from *E. coli* with the invariant spacing of C<sub>X2</sub>C<sub>X21</sub>C<sub>X5</sub>C (3). However, in most of the radical SAM enzymes identified to date, the spacing of these cysteine residues that form the iron-sulfur cluster is C<sub>X3</sub>C<sub>X2</sub>C (Frey et al., 2008). Shown in Scheme 2 is a cartoon of a typical [4Fe-4S]-cluster found in all radical SAM enzymes. In these complexes three of the four irons are coordinated to the protein via the side chain thiolates of three cysteine residues. The fourth iron is ultimately coordinated by the SAM cofactor.

The second question to be addressed is the origin of the hydrogen formed during the formation of the 5'-deoxyadenosine product. As illustrated in Scheme 3 when SAM is reductively cleaved by the iron-sulfur center, the carbon-sulfur bond is replaced with a carbon-hydrogen bond and the origin of this hydrogen is unknown in the PhnJ catalyzed reaction. Since the 5'-deoxyadenosyl radical intermediate is thought to function as a radical initiator of the reaction, the origin of this hydrogen would help to illuminate the overall reaction mechanism.



Scheme 1 Metabolic pathway for the conversion of methyl phosphonate to phosphate by the C-P lyase complex of enzymes.



**Scheme 2** Cartoon of a [4Fe-4S]-cluster in radical SAM enzymes.

The third question seeks to uncover the origin of the new hydrogen that is formed in the methane product after cleavage of carbon-phosphorus bond in the substrate PRPn as illustrated in [Scheme 4](#). It was assumed at the initiation of this project that the C-P bond would be cleaved in a radical process and the origin of the hydrogen in the methane product would help to elucidate the structure of the putative radical intermediates ([Frost et al., 1987](#)).

### Mutation of four conserved cysteine residues

The four conserved cysteine residues of PhnJ from *E. coli* (C241, C244, C266, and C272) were individually mutated to alanine. The mutant enzymes were purified to homogeneity and characterized as to whether these mutant proteins could assemble an iron-sulfur cluster. The only protein that could form an iron-sulfur cluster was the C272A mutant. The UV-visible spectrum and the EPR spectrum of the C272A mutant were identical to those obtained for the wild-type enzyme (data not shown) ([Kamat et al., 2013](#)). These results indicated that the [4Fe-4S]-cluster in PhnJ was coordinated to the protein via C241, C244, and C266 having the spacing of C<sub>X2</sub>C<sub>X21</sub>C. This spacing of cysteine residues has not been observed in any other radical SAM enzyme making PhnJ unique among the known radical SAM enzymes. Although the C272A mutant was able to form an iron-sulfur cluster this enzyme was catalytically inactive, suggesting that C272 was important for catalytic activity of PhnJ.

### Origin of new hydrogen in 5'-deoxyadenosine

When the carbon-sulfur bond is reductively cleaved by the iron-sulfur center in radical SAM enzymes a new carbon-hydrogen bond is ultimately formed in the 5'-deoxyadenosine product ([Scheme 3](#)). Our initial proposal for this transformation was that the putative 5'-deoxyadenosyl radical would act as a radical initiator to initiate hydrogen atom transfer from Cys-272 to form a thiyl radical. To test this proposal we conducted the reaction catalyzed by PhnJ in D<sub>2</sub>O. If there was hydrogen atom transfer from Cys-272, then the 5'-deoxyadenosine would be labeled with a single deuterium when the reaction was conducted in D<sub>2</sub>O. However, when the PhnJ reaction is conducted in D<sub>2</sub>O no deuterium was found in the 5'-deoxyadenosine product. This result demonstrates that the putative 5'-deoxyadenosyl radical does not initiate hydrogen atom transfer from cysteine or any other solvent exchangeable site from either the substrate or enzyme.

A limited number of radical SAM enzymes are known to catalyze the formation of glycy radicals as intermediates ([Eklund and Fontecave, 1999](#)). To test whether PhnJ catalyzed the formation of a glycy radical we expressed PhnJ in minimal media that was supplemented with di-deuterated glycine. The protein was purified and then subjected to hydrolysis by trypsin. The peptide fragments were then analyzed by mass spectrometry to determine the extent of labeling of the glycine residues with deuterium. The mass spectra demonstrated that 66% of the glycine residues were di-deuterated, 15% were mono-deuterated, and 19% were unlabeled with deuterium. This protein was designated as PhnJ-glycine-d<sub>2</sub>. When this protein was used in a typical PhnJ reaction, the 5'-deoxyadenosine that was isolated at the end of the reaction was labeled with a single atom of deuterium ( $m/z=253.1$  for the [M+H<sup>+</sup>]). These results demonstrated quite clearly that the 5'-deoxyadenosyl radical initiated the hydrogen atom transfer from a glycine residue on PhnJ to form 5'-deoxyadenosine and a glycy radical.

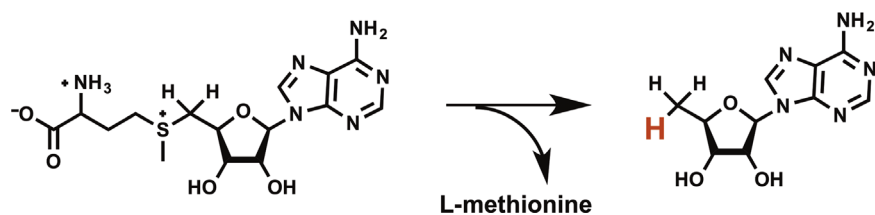
### Origin of new hydrogen in methane

After the carbon-phosphorus bond in PRPn is cleaved in the reaction catalyzed by PhnJ, a new carbon-hydrogen bond is formed during the formation of methane ([Scheme 4](#)). When the PhnJ catalyzed reaction was conducted in D<sub>2</sub>O under conditions of multiple turnovers, the methane that was isolated contained a single atom of deuterium ( $m/z=17$ ). This result demonstrated that the new hydrogen originated from a site that could ultimately exchange with solvent. When this same enzyme sample was used to catalyze the PhnJ reaction in D<sub>2</sub>O under conditions of a single turnover by limiting the amount of substrate, the methane that was recovered at the end of the reaction did not contain deuterium ( $m/z=16$ ). This result demonstrated that under limiting turnover conditions that hydrogen atom transfer to the putative methyl radical did not come from a source that readily exchanged with solvent.

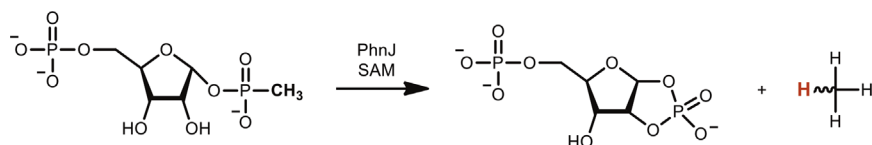
At this point the most likely source of the new hydrogen on methane was proposed to be the glycine residue that participated in hydrogen atom transfer to the 5'-deoxyadenosyl radical. To test this conjecture the PhnJ-catalyzed reaction was conducted with the protein that was uniformly labeled with deuterium (PhnJ-glycine-d<sub>2</sub>). When PhnJ-glycine-d<sub>2</sub> was used as the catalyst in H<sub>2</sub>O under single turnover conditions, the methane that was isolated contained a single atom of deuterium ( $m/z=17$ ). When the labeled PhnJ-glycine-d<sub>2</sub> was used as a catalyst in H<sub>2</sub>O under conditions of multiple turnovers, the methane was unlabeled ( $m/z=16$ ). These results demonstrated quite clearly that the source of the new hydrogen in methane derives directly from one of the hydrogen atoms of a glycine residue contained within PhnJ.

### New questions

The previous experiments demonstrated that a glycine residue within the active site of PhnJ was involved in hydrogen atom transfer to the putative 5'-deoxyadenosyl radical to form a glycy radical. This glycine residue was also



**Scheme 3** The formation of 5'-deoxyadenosine from the reductive cleavage of the carbon-sulfur bond in SAM. The red hydrogen illustrates the new C-H bond that is formed during the course of the reaction catalyzed by PhnJ.



**Scheme 4** Formation of the products PRcP and methane in the reaction catalyzed by PhnJ. The red hydrogen in methane illustrates the new C-H bond that is formed after the cleavage of the C-P bond in the substrate.

involved in hydrogen atom transfer to the putative methyl radical during the formation of the methane product. These experiments also demonstrated that during the course of the reaction catalyzed by PhnJ, that the glycine residue would exchange its hydrogen atoms with those of solvent. The most likely source of this solvent exchange reaction would be via hydrogen atom transfer with Cys-272. This process is illustrated in [Scheme 5](#). These results raised two additional questions about the mechanism of the reaction catalyzed by PhnJ. The first of these questions is the specific glycine residue that functions in these hydrogen atom transfers. In principle the specific hydrogen that is labeled with deuterium could be determined by conducting the reaction in  $D_2O$  under multiple turnover conditions. This would initiate the exchange of the hydrogen atoms of a single glycine residue with deuterium. The identity of this glycine residue could then be determined by mass spectrometry after cleavage of PhnJ with trypsin. There are eight conserved glycine residues in PhnJ.

The second question concerns the stereochemistry of the hydrogen atom transfer. Glycine is achiral but the two hydrogen atoms can be differentially labeled with deuterium at the *proR* and *proS* positions. With the labeled enzyme, PhnJ-glycine- $d_2$ , one of the deuterium atoms is transferred to the 5'-deoxyadenosyl radical and the other is transferred to the putative methyl radical. To determine the stereospecific origin of these hydrogen atom transfers PhnJ can be labeled with glycine residues that are stereospecifically labeled with deuterium at either the *proR* or *proS* position.

### Identification of the special glycine residue

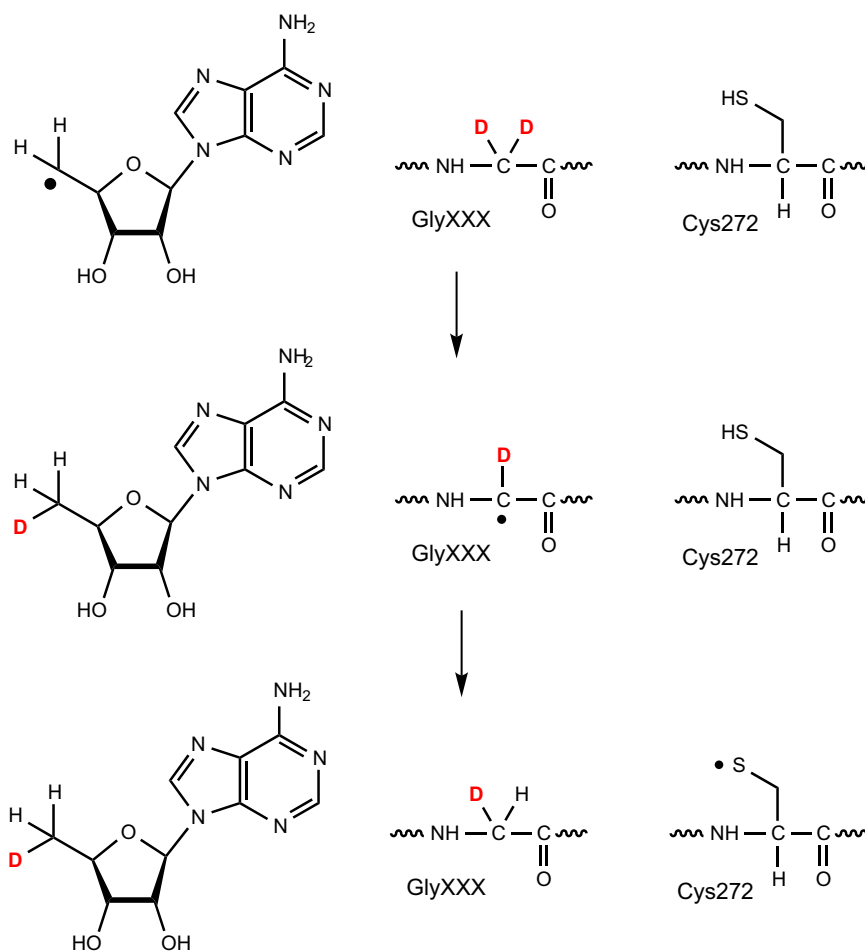
To determine which of the eight conserved glycine residues within PhnJ was responsible for the hydrogen atom transfers to both 5'-deoxyadenosine and Cys-272, the reaction catalyzed by PhnJ was conducted in  $D_2O$  under multiple turnover conditions. Under these conditions a single glycine residue is expected to incorporate deuterium as a function of time as deuterium atoms from the solvent are transferred from Cys-

272 to this glycine residue. Aliquots of the reaction were removed as a function of time, PhnJ was digested with trypsin and the peptide fragments were subjected to mass spectrometric analysis. Only a single peptide became deuterated during the course of the incubation. The sequence of this peptide is as follows: 26-AVAIPGYQVPFGGR-40. There are three glycine residues in this peptide but only one of these glycine residues, Gly-32, is conserved across all homologs of PhnJ that have been sequenced to date ([Kamat et al., 2013](#)).

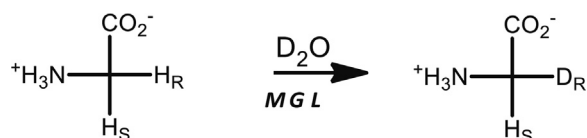
To confirm that Gly-32 is the glycine residue which is critical for hydrogen atom transfers in the reaction catalyzed by PhnJ, this residue was mutated to an alanine residue. As expected, this mutant, G32A, was inactive toward the formation of methane and PRcP from PRPr. We therefore concluded that Gly-32 was the residue that was responsible for hydrogen atom transfers in the reaction catalyzed by PhnJ.

### Stereospecific hydrogen atom transfers

The  $\alpha$ -carbon of glycine is prochiral. From the experiments described previously, we determined that one of the hydrogen atoms of Gly-32 is ultimately found in 5'-deoxyadenosine and the other hydrogen is found in methane during the first turnover of the reaction catalyzed by PhnJ. To determine the stereospecificity of these transfers, we expressed PhnJ in minimal media that was supplemented with glycine that was specifically deuterated at the *proR* position. The monodeuterated glycine was made by incubating glycine with the enzyme methionine- $\gamma$ -lyase (MGL) in the presence of  $D_2O$  ([Koulikova et al., 2011](#)). This reaction is illustrated in [Scheme 6](#). The protein was designated as PhnJ-glycine- $d_R$ . When this protein was utilized as a catalyst under single turnover conditions, the 5'-deoxyadenosine that was isolated from the reaction mixture was labeled with deuterium but the methane was not. These results are consistent with the transfer of the *proR* hydrogen to the 5'-deoxyadenosyl intermediate during the formation of 5'-deoxyadenosine and the transfer of the *proS* hydrogen to the putative methyl



**Scheme 5** Proposed hydrogen atom transfer from a glycine residue in the active site of PhnJ to the putative 5'-deoxyadenosyl radical and the subsequent hydrogen atom transfer from Cys-272.



**Scheme 6** Synthesis of monodeuterated glycine using methionine- $\gamma$ -lyase in D<sub>2</sub>O.

radical during the formation of methane in the reaction catalyzed by PhnJ.

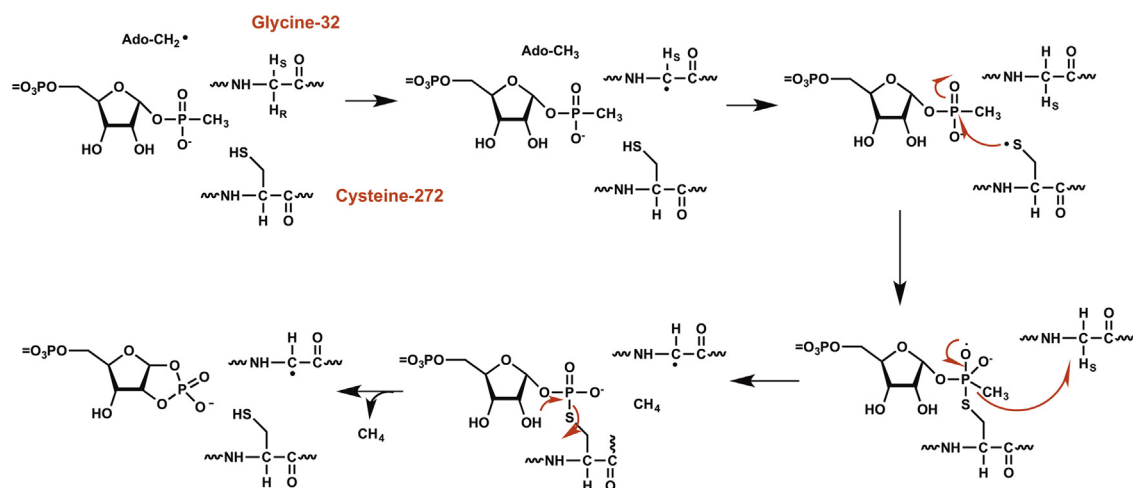
### Proposed reaction mechanism

The experiments conducted with PhnJ were used to formulate a working model for the reaction mechanism for the cleavage of the carbon-phosphorus bond in PRPn. The proposed mechanism is presented in [Scheme 7](#). In this mechanism the reaction starts with the reductive cleavage of the carbon-sulfur bond of SAM by the reduced iron-sulfur center. This generates methionine and a 5'-deoxyadenosyl radical intermediate. In the next step there is hydrogen atom transfer of the *proR* hydrogen of Gly-32 to form 5'-

deoxyadenosine and a glycy radical. This step is followed by hydrogen atom transfer from Cys-272 to the glycy radical, leading to the formation of a transient thiyl radical. The thiyl radical attacks the phosphorus center of the phosphate substrate to generate an unstable pentavalent substrate-based radical intermediate. This intermediate collapses via homolytic cleavage of the carbon-phosphorus bond to make a covalent thiophosphate intermediate and methane. The methane is formed via the transfer of the *proS* hydrogen of Gly-32. In the last step the hydroxyl at C2 of the substrate makes a nucleophilic attack on the phosphorus center of the thiophosphate intermediate. This step regenerates Cys-272 and forms the ultimate product PRcP. The next turnover occurs after hydrogen atom transfer from Cys-272 to the transient glycy radical ([Kamat et al., 2013](#)). Ongoing experiments are directed at the detection of the proposed radicals by EPR spectroscopy and isolation of the putative thiophosphate intermediate.

### Conflict of interest

The authors declare that there is no conflict of interest.



Scheme 7 Proposed reaction mechanism for carbon-phosphorus bond cleavage by PhnJ.

## Acknowledgments

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