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Discovery of a Cyclic Phosphodiesterase that Catalyzes the Sequential Hydrolysis of Both Ester Bonds to Phosphorus

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Abstract

The bacterial C-P lyase pathway is responsible for the metabolism of unactivated organophosphonates under conditions of phosphate starvation. The cleavage of the C-P bond within ribose-1-methylphosphonate-5-phosphate to form methane and 5-phosphoribose-1,2-cyclic phosphate (PRcP) is catalyzed by the radical SAM enzyme PhnJ. In *Escherichia coli* the cyclic phosphate product is hydrolyzed to ribose-1,5-bisphosphate by PhnP. In this study, we describe the discovery and characterization of an enzyme that can hydrolyze a cyclic phosphodiester directly to a vicinal diol and inorganic phosphate. With PRcP, this enzyme hydrolyzes the phosphate ester at carbon-1 of the ribose moiety to form ribose-2,5-bisphosphate, and then this intermediate is hydrolyzed to ribose-5-phosphate and inorganic phosphate. Ribose-1,5-bisphosphate is neither an intermediate nor substrate for this enzyme. Orthologs of this enzyme are found in the human pathogens *Clostridium difficile* and *Eggerthella lenta*. We propose that this enzyme be called cyclic phosphate dihydrolase (cPDH) and be designated as PhnPP.

In the absence of phosphate, many bacteria can utilize organophosphonates (Pn) as a source of phosphorus.[1] In *Escherichia coli*, the metabolic machinery needed to convert organophosphonates to phosphate is governed by the 14-cistron *phnCDEFGHIJKLMNOP* operon.[2] The enzyme complex (C-P lyase) that functions to catalytically cleave the hydrolytically stable carbon-phosphorus bond of organophosphate substrates is encoded by the genes *phnGHIJKLM*. The proteins encoded by *phnCDEF* are required for the transport of phosphonate substrates while the remaining genes, *phnNOP*, are needed for the expression of three accessory enzymes. Recently, the metabolic pathway for the conversion of methyl phosphonate to 5-phospho-D-ribose-1,2-cyclic phosphate (PRcP) and methane by the C-P lyase complex in *E. coli* has been elucidated.[3] The key enzyme in this transformation is PhnJ, which converts α-D-ribose-1-phosphonate-5-phosphate (PRPn) to 5-phospho-D-ribose-1,2-cyclic phosphate (PRcP) as illustrated in Scheme 1.[4] PRcP is subsequently hydrolyzed to D-ribose-1,5-bisphosphate (1,5-RbP) by PhnP. PhnP is a phosphodiesterase from cog1235, which is a subset of the metallo β-lactamase superfamily I enzymes.[5] The product of this reaction is then converted to 5-phosphoribosyl-1-pyrophosphate (PRPP) by the action of PhnN.[6] PhnO is an accessory enzyme, which has been shown to acetylate 1-aminoalkylphosphonic acids by acetyl CoA.[7]

In an effort to further elucidate the metabolic complexities of bacterial phosphonate metabolism, we discovered that while many bacteria have a set of genes similar to that

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Supporting Information
Cloning and purification details, description of experimental procedures and results, synthesis of substrates, and a list of predicted orthologs of the enzyme studied in this work. This material is available free of charge via the Internet at http://pubs.acs.org

found in *E. coli* for the utilization of organophosphonates, a small cluster of organisms lack the specific gene required for the hydrolysis of PRcP; *i.e.* a homolog to PhnP. Instead, some of these bacteria possess an enzyme of unknown function from cog0613 that belongs to the polymerase and histidinol phosphatase (PHP) family of proteins within the amidohydrolase superfamily (AHS). The structurally characterized members of the PHP family possess a distorted $(\beta/\alpha)_7$-barrel protein fold and contain a trinuclear metal center in the active site.[8,9] The genetic architecture for a subset of these organisms, relative to that found in *E. coli*, is highlighted in Figure 1. The mechanistically characterized enzymes from the PHP family hydrolyze phosphomonoesters by activating the nucleophilic water molecule with two divalent cations, while the third metal acts as a Lewis acid to the departing alcohol leaving group.[10] It was therefore anticipated that the uncharacterized enzyme from cog0613 would act to hydrolyze PRcP to 1,5-RbP via a reaction that was homologous to that catalyzed by PhnP.

The gene for Elen0235 (gi|257790010) was PCR-amplified by standard methods from the genomic DNA of *Eggerthella lenta* strain 1899B (ATCC 25559). The gene was subsequently subcloned into a high-copy plasmid pET30(a) to express the appropriate protein with 6 x His-tag at the C-terminus in *E. coli*. The enzyme (denoted here as Elen0235) was purified to >95% homogeneity using Ni-NTA chromatography at 4 °C, and excess imidazole was removed by dialysis. Similarly, the recombinant enzyme for PhnP (gi|16131918) from *E. coli* was obtained and purified to homogeneity. The putative substrate, PRcP, was chemically synthesized from PRPP using a modification of a published procedure.[6] The reactions catalyzed by Elen0235 and PhnP were determined by incubating the purified enzymes with PRcP and the products of the reaction characterized by ^{31}P-NMR.

The ^{31}P-NMR spectrum of PRcP is presented in Figure 2A. The phosphate attached to the hydroxyl group at C5 resonates at 4.57 ppm, while the 1,2-cyclic phosphate resonates at 19.31 ppm. In the proton-coupled ^{31}P-NMR spectrum the cyclic phosphate appears as a doublet of doublets while the phosphate at C5 is a triplet. The ^{31}P NMR spectrum of the product of the reaction catalyzed by PhnP (D-ribose 1,5-bisphosphate) is presented in Figure 2B. The phosphate attached to the hydroxyl group at C5 resonates at 4.57 ppm, whereas the phosphate at C1 resonates at 3.02 ppm. In the proton-coupled spectrum the phosphate at C1 appears as a doublet and the phosphate at C5 appears as a triplet. All of the substrate has been consumed. The ^{31}P-NMR spectrum of the products from the hydrolysis of PRcP catalyzed by Elen0235 is presented in Figure 2C. The resonance that appears at 3.24 ppm is phosphate (a singlet in both the proton-coupled and decoupled spectrum. The resonance at 4.56 ppm is from the phosphate at C5 of D-ribose-5-phosphate. Clearly, both enzymes consume PRcP. However, the product of the reaction catalyzed by PhnP is D-ribose-1,5-bisphosphate (1,5-RbP) but the products of the reaction catalyzed by Elen0235 are D-ribose-5-phosphate and phosphate.

Quite remarkably, the reaction catalyzed by Elen0235 with the cyclic phosphate substrate must involve two consecutive hydrolytic reactions. This observation thus requires that either D-ribose-1,5-bisphosphate (1,5-RbP), D-ribose-2,5-bisphosphate (2,5-RbP), or both compounds function as reaction intermediates. Ribose-1,5-bisphosphate was isolated as the product from the enzymatic hydrolysis of PRcP by PhnP. The other potential intermediate, ribose-2,5-bisphosphate, was chemically synthesized from PRcP by acid hydrolysis and then purified by anion exchange chromatography. [11] At alkaline and neutral pH, the resonances corresponding to the 2- and 5-phosphates of 2,5-RbP largely overlap with one other (Figure 3B). Therefore, the pH was adjusted to 5.8 to enable the observation of two distinct phosphorus resonances. At this pH, 2,5-RbP shows four resonances (Figure 3A). The corresponding ^{1}H-^{31}P coupled NMR spectrum exhibits two pairs of doublets and triplets corresponding to the phosphate groups at C2 and C5 of the ribose ring, respectively. The

relative amounts of the two species, based on the integral values from the ^{31}P-NMR spectrum, are 32% : 68%. These species represent the two anomers of 2,5-RbP as the percentages correspond reasonably well with the relative amounts of the α- and β-anomers of D-ribose-5-phosphate in solution (36% : 64%) determined using ^{13}CNMR at pH 4.5.[12] When 1,5-RbP and 2,5-RbP were incubated with Elen0235 in separate reactions, there was no change in the ^{31}P-NMR spectrum of 1,5-RbP (Figure S2), while the reaction mixture with 2,5-RbP showed the formation of two new signals (Figure 3C). These two resonances coincided with the products of enzymatic hydrolysis of PRcP by Elen0235, and were confirmed to be D-ribose-5-phosphate and inorganic phosphate.

Enzymes from the PHP family have been shown previously to catalyze the hydrolysis of phosphomonoesters by nucleophilic attack of hydroxide at the phosphorus center.[10] With regard to the hydrolysis of 2,5-RbP by Elen0235, the reaction is expected to occur similarly. It is unclear, however, whether the initial phosphodiesterase activity with PRcP occurs by the attack of hydroxide on phosphorus or the anomeric carbon of the D-ribose ring. This issue was addressed by incubating PRcP with Elen0235 in the presence of 56% H_2 ^{18}O. It is known that there is a small chemical shift difference in the ^{31}P-NMR signal of phosphate esters when ^{18}O is substituted for ^{16}O.[13] For phosphate, the chemical shift difference is 0.021 ppm upfield for each ^{18}O substitution, and is additive.[14] If the hydroxide attacks at the phosphorus center in both hydrolytic steps of this reaction, then three separate resonances are expected for the inorganic phosphate product. The results are presented in Figure 4 and the percentage of the phosphate species containing 0, 1, and 2 atoms of oxygen-18 are 22% : 49% : 29%. The expected percentages, based on the attack of two water molecules directly with the phosphorus center, are 19% : 49% : 31%. The results clearly demonstrate that both water molecules used in the overall reaction react directly with the phosphorus center.

The kinetic parameters for the hydrolysis of PRcP and 2,5-RbP were determined by monitoring the rate of formation of inorganic phosphate. The apparent kinetic constants for the hydrolysis of PRcP at pH 8.0 and 25 °C are as follows: $k_{cat} = 2.0 \pm 0.2\ s^{-1}$; $K_m = 14 \pm 3$ μM; and $k_{cat}/K_m = 1.4 \pm 0.1 \times 10^5\ M^{-1}\ s^{-1}$. The kinetic constants for hydrolysis of 2,5-RbP by Elen0235 are as follows: $k_{cat} = 7.4 \pm 0.3\ s^{-1}$; $K_m = 23 \pm 2$ μM; and $k_{cat}/K_m = 3.3 \pm 0.2 \times 10^5\ M^{-1}\ s^{-1}$. The rate constants for the enzymatic hydrolysis of PRcP and 2,5-RbP by Elen0235 are thus very similar to one another. To determine whether or not Elen0235 catalyzed the hydrolysis of PRcP processively, 4.0 mM PRcP was incubated with 0.5 μM Elen0235 at pH 8.0 until the reaction proceeded to ~50% completion. The reaction was quenched by adding 0.5 mM EDTA, and the reaction mixture was passed through a 10 kDa molecular weight cut-off centrifugal filter to remove the enzyme. The pH of the filtrate was adjusted to 5.8 and the products of the reaction were analyzed by ^{31}P-NMR spectroscopy to determine whether or not the intermediate, 2,5-RbP, was released into solution (Figure 5).

The reaction products clearly show the formation of a significant amount of 2,5-RbP as an intermediate. The approximate ratios of PRcP, 2,5-RbP and R5P, calculated from the integration of the phosphorus resonances, are 22% : 39% : 39%. This observation demonstrates that the reaction mechanism is not processive and confirms that 2,5-RbP is a kinetically competent intermediate that is released from the enzyme site as the initial product of PRcP hydrolysis.

These findings raise several mechanistic questions. It is clear that this enzyme treats PRcP and 2,5-RbP as two distinct substrates of comparable affinity and efficiency. Moreover, the enzyme is able to distinguish between the P-O bonds at C1 of PRcP and 1,5-RbP. The former is rapidly hydrolyzed while hydrolysis of the latter could not be detected. This result implies that the enzyme has sufficient specificity, but is surprisingly promiscuous in its ability to catalyze the hydrolysis of a phosphodiester and a phosphomonoester. There is no

evidence to suggest that the net reaction is accomplished via separate active sites. The overall hydrolytic reaction from PRcP to 2,5-RbP to R-5-P and P_i is presented in Scheme 2.

Purple acid phosphatase (PAP) from pig liver and red kidney bean can catalyze the hydrolysis of methyl *p*-nitrophenyl phosphate (MpNPP) to *p*-nitrophenol (pNP), inorganic phosphate, and methanol.[15] PAP cannot hydrolyze bis-*p*-nitrophenyl phosphate (bis-pNPP), and the authors of this study state that the diesterase activity has no biological relevance. PAP has an Fe^{3+}-M^{2+} (M = Fe, Zn or Mn) cluster in the active site that apparently hydrolyzes the MpNPP diester in a processive manner. It has also been reported that two enzymes from *Myxococcus xanthus* can hydrolyze cAMP to adenosine and orthophosphate.[16]

Elen0235 has all of the metal-binding residues that are present in other members of the PHP family and this enzyme should possess a trinuclear active site. The mechanism of hydrolysis can be envisaged to be similar to that proposed for L-histidinol phosphate phosphatase, another PHP family enzyme from cog1387. The α- and β-metal ions activate the nucleophilic hydroxide that bridges these two metal ions. The third metal ion (denoted as the γ-metal ion) serves as a Lewis acid by interacting with the oxygen of the leaving group alcohol.

A protein sequence BLAST analysis and genomic context study of Elen0235 revealed that a limited number of organisms possess a homolog of this enzyme, as opposed to a majority of the organisms possessing the C-P lyase operon which have PhnP. A list of organisms that contain an enzyme that is predicted to hydrolyze PRcP to R-5-P and P_i is provided in Table S1. This observation raises the question why these organisms, which include the highly virulent and multi-drug resistant human gut pathogen *Clostridium difficile* 630, have ribose-5-phosphate and inorganic phosphate as the terminal products of the C-P lyase pathway instead of PRPP. The enzyme PRPP synthetase converts R-5-P to PRPP by pyrophosphoryl transfer from ATP, and all the organisms that possess an ortholog to Elen0235 possess a copy of this enzyme.

In summary, we have discovered an enzyme that specifically hydrolyzes a cyclic phosphodiester to a vicinal diol and inorganic phosphate in a non-processive manner (Scheme 2). we propose that this enzyme be called cyclic phosphate dihydrolase (cPDH) and be designated as PhnPP.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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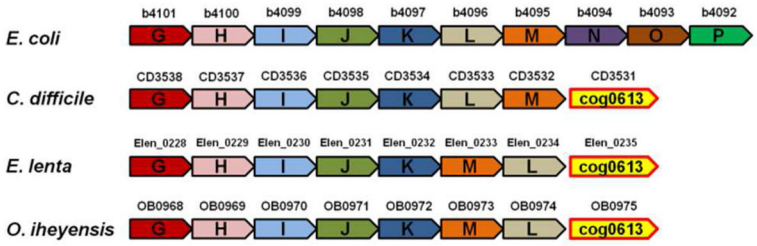


Figure 1.
Schematic representation of organization of open reading frames encoding enzymes that constitute the C-P lyase pathway in select organisms.

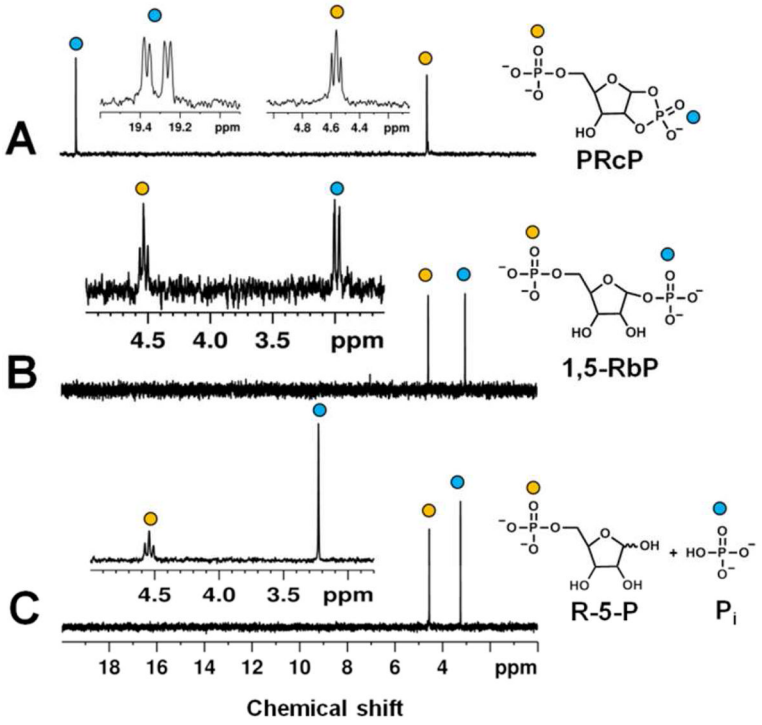


Figure 2.

^{31}P-NMR spectra of PRcP and products of the reactions catalyzed by PhnP and Elen0235 at pH 8.5. (A) 4 mM 5-phosphoribose-1,2-cyclic phosphate (PRcP). (B) Product of the enzymatic hydrolysis of 1 mM PRcP by PhnP from *E. coli*. (C) Products of the enzymatic hydrolysis of 2 mM PRcP by Elen0235. The insets show the respective ^{1}H-^{31}P coupled NMR spectra.

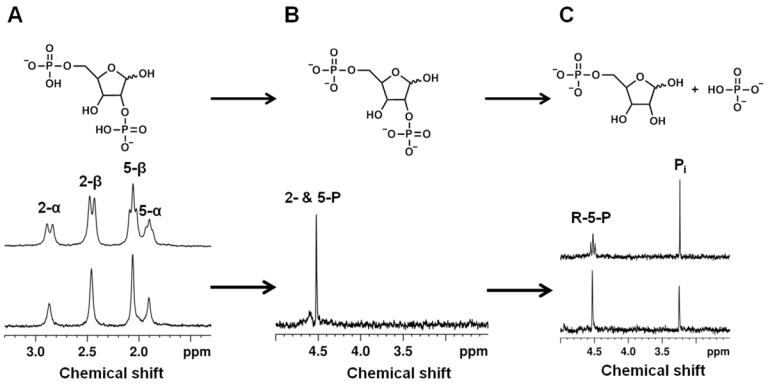


Figure 3.

^{31}P-NMR spectra of synthesized ribose-2,5-bisphosphate (2,5-RbP) and the products of its hydrolysis by Elen0235. (A) 4 mM 2,5-RbP at pH 5.8. (B) 2 mM 2,5-RbP at pH 8.5. (C) Products of hydrolysis of 2 mM 2,5-RbP by Elen0235 at pH 8.5. In panels A and C, ^{1}H-^{31}P coupled spectra are shown above ^{1}H-^{31}P decoupled spectra.

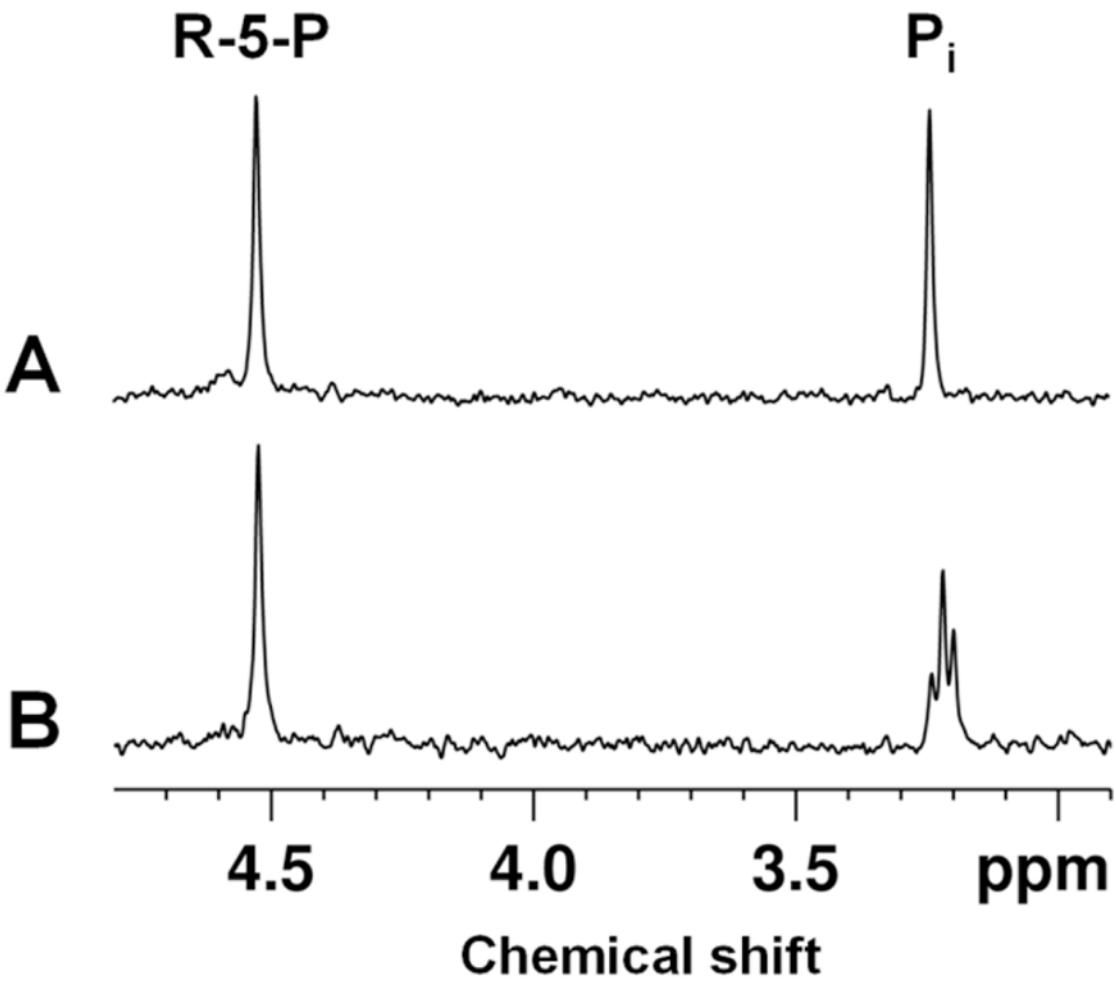


Figure 4.
^{31}P-NMR (^{1}H-decoupled) spectra of the reaction products from PRcP catalyzed by Elen0235 when the reaction is carried out in 100% H_2 ^{16}O (panel A) and 56% H_2 ^{18}O (panel B).

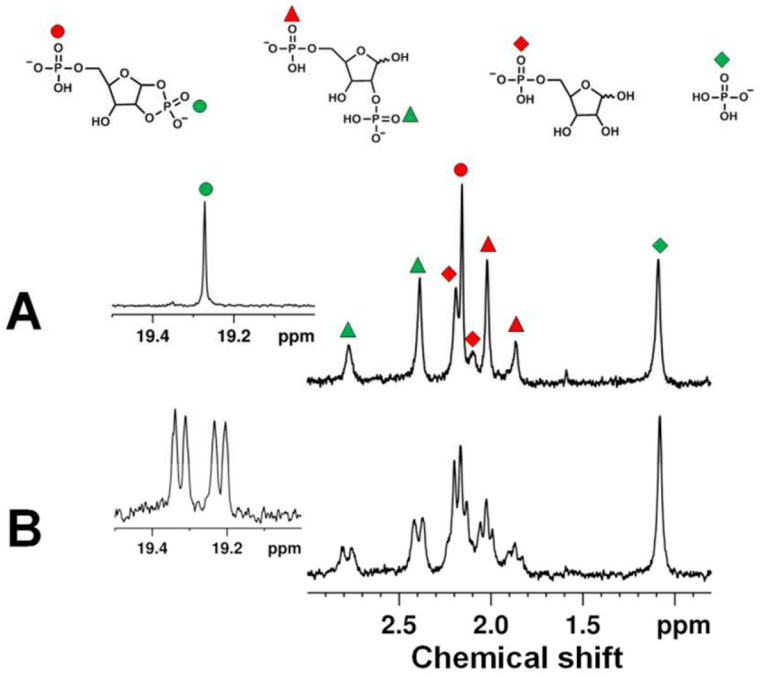
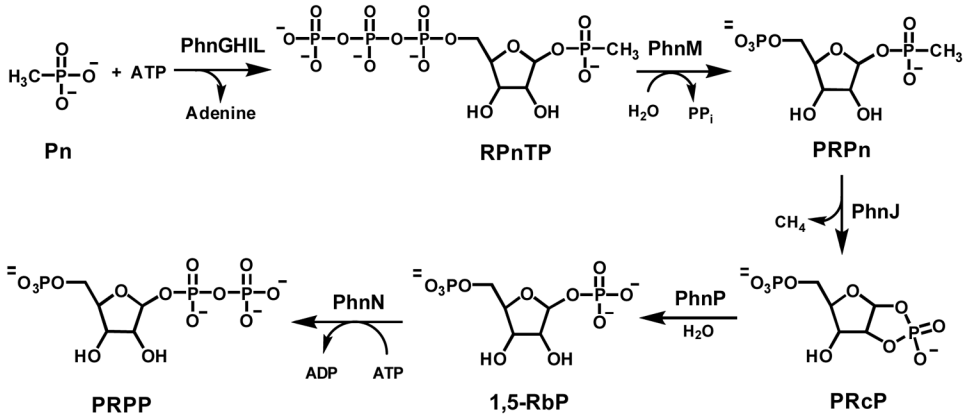
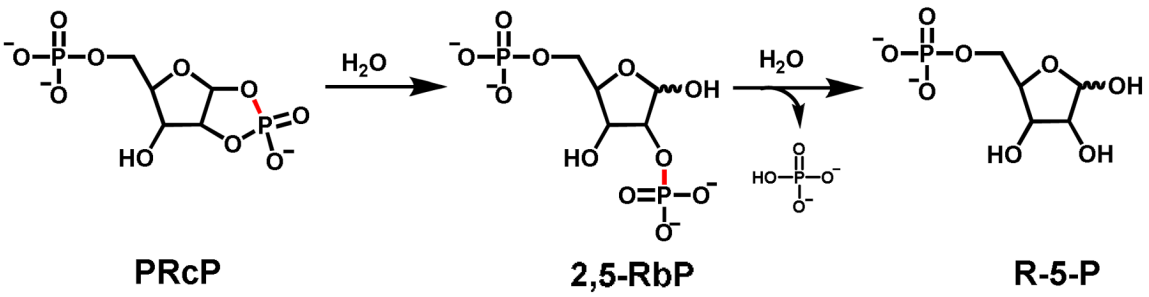


Figure 5.
^{31}P-NMR spectrum for the partial hydrolysis of 4 mM PRcP by 0.5 μM Elen0235. (A) ^{1}H-^{31}P decoupled spectrum. (B) ^{1}H-^{31}P coupled spectrum. The inset shows the resonance for the 1,2-cyclic phosphate group of the remaining substrate, PRcP.



Scheme 1.
C-P lyase pathway in *E. coli*.



Scheme 2.
Overall reaction catalyzed by Elen0235.