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# *In vitro* Phosphinate Methylation by PhpK from *Kitasatospora Phosalacinea*

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## Abstract

Radical SAM (*S*-adenosyl-L-methionine), cobalamin-dependent methyltransferases have been proposed to catalyze the methylations of unreactive carbon or phosphorus atoms in antibiotic biosynthetic pathways. To date, none of these enzymes have been purified or shown to be active *in vitro*. Here we demonstrate the activity of the P-methyltransferase enzyme, PhpK, from the phosalacine producer *Kitasatospora phosalacinea*. PhpK catalyzes the transfer of a methyl group from methylcobalamin to 2-acetylamino-4-hydroxyphosphinylbutanoate (*N*-acetyldemethylphosphinothricin or NAcDMPT) to form 2-acetylamino-4-hydroxymethylphosphinylbutanoate (*N*-acetylphosphinothricin or NAcPT). This transformation gives rise to the only carbon-phosphorus-carbon linkage known to occur in Nature.

Compounds containing a carbon-phosphorus-carbon linkage are often used as neurotransmitter antagonists and enzyme inhibitors.[1] However, this bonding sequence is observed in only one naturally-occurring material and its derivatives: 2-amino-4-hydroxymethylphosphinylbutanoate, more commonly known as L-phosphinothricin (L-PT) or glufosinate (Chart S1).[2] Due to its structural similarity to L-glutamate, L-PT is a useful inhibitor of bacterial and plant glutamine synthetases.[3] L-PT is naturally produced by *Streptomyces hygroscopicus*, *Streptomyces viridochromogenes*, and *Kitasatospora phosalacinea*.[4–8] All three species normally produce L-PT as the first amino acid of an exported tripeptide (Chart S1). The *Streptomyces* tripeptide, L-phosphinothricylalanylalanine or bialaphos, is commercially used as an herbicide.[2,3] *K. phosalacinea* produces L-PT as part of an alternative tripeptide, L-phosphinothricylalanylleucine or phosalacine.

Multiple studies have examined the genetics and mechanistic enzymology of proteins in the two *Streptomyces* pathways, but no studies have been published specifically investigating the phosalacine pathway.[2,9] Little is known about the formation of the unique C-P-C linkage

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in any of the three organisms. The *bcpD* gene in *S. hygroscopicus* (*phpK* in *S. viridochromogenes*) was believed to encode the putative P-methyltransferase catalyzing the methylation of the electrophilic phosphinyl group, resulting in the formation of the C-P-C linkage.[7,10,11] Experiments with *Streptomyces* cell extracts indicated that the methyl donor was the vitamin $B_{12}$ derivative methylcobalamin ($CH_3$Cbl(III)) and the methyl recipient(s) were 2-acetylamino-4-hydroxyphosphinylbutanoate (NAcDMPT) and the corresponding phosphinyl tripeptide, NAcDMPT-L-Ala-L-Ala (Scheme 1).[12] The resulting product(s) are NAcPT and NAcPT-L-Ala-L-Ala, respectively. By extension, NAcDMPT and NAcDMPT-L-Ala-L-Leu are the likely substrates for the *K. phosalacinea* P-methyltransferase.

Sequencing indicated that the P-methyltransferases were related to a pair of putative methyltransferases from the fosfomycin (Fom3) and fortimicin (Fms7) biosynthetic pathways.[10,13] Fom3 and Fms7 have been hypothesized to catalyze the transfer of methyl groups from $CH_3$Cbl(III) to unreactive carbon, rather than phosphorus, atoms.[13,14] This posed an intriguing mechanistic problem; in nearly all cases studied, biological transfer of a methyl group occurs via an $S_N2$-type nucleophilic substitution reaction.[15] Since none of the hypothesized substrates for this family of enzymes were nucleophilic, it appeared likely that these methyltransferases used a different mechanism for catalysis. Transfer of a methyl anion from $CH_3$Cbl(III) was proposed, but such chemistry is unlikely to occur in aqueous solution[12].

In 2001, these four proteins were identified as members of the radical SAM superfamily.[16] Radical SAM proteins contain three cysteines, typically within a conserved CXXXCXXC motif, which bind a [4Fe-4S] cluster. The reduced +1 state of the cluster donates an electron to SAM, resulting in homolytic cleavage of the carbon-sulfur bond to form the 5′-deoxyadenosyl radical required for catalysis.[16] The revelation that these enzymes were members of the radical SAM superfamily suggested another possible mechanism for catalysis: transfer of the methyl group as a radical.[9,17] The existence of a chemical precedent for organic radical methylation by $CH_3$Cbl(III) in aqueous solution indicated that the methyl radical mechanism was feasible.[18]

To gain insight into this group of possible radical SAM methyltransferases, we chose to study the P-methyltransferase from *Kitasatospora phosalacinea* DSM 43860. Sequencing indicated that *phpK* from *K. phosalacinea* is over 99% identical to the published *phpK* sequence from the bialaphos producer, *S. viridochromogenes.* [11,19] After overexpression in *E. coli* and cell lysis, PhpK was not found in the soluble fraction and appeared to express solely in inclusion bodies. To purify the enzyme, we modified a literature procedure for the solubilization and refolding of an iron-sulfur/corrinoid protein.[20] All steps were performed in an anaerobic chamber (Coy Laboratory Products). After refolding, PhpK was further purified by anionic exchange chromatography, and the hypothesized [4Fe-4S] clusters were anaerobically reconstituted.

PhpK was dark brown and displayed an ultraviolet-visible spectrum consistent with that of other [4Fe-4S] proteins with a local maximum at 420 nm (Figure S2).[21] Iron and sulfide analyses indicated approximately 5.9 moles Fe and 4.4 moles S per mole of PhpK.[22–24] EPR spectroscopy was used to verify the presence of the [4Fe-4S] cluster (Figure S3). At 10 K, the EPR spectrum of a sample containing only PhpK and buffer showed a small signal at $g$ = 2.00. Addition of sodium dithionite, a strong chemical reductant, resulted in a new, broad spectral feature at $g$ = 1.93 consistent with an EPR-active, reduced $[4Fe\text{-}4S]^{+1}$ cluster. Addition of all components hypothesized to be required for the reaction (*vide infra*) resulted in the disappearance of the $[4Fe\text{-}4S]^{+1}$ cluster.

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Assaying PhpK for activity was a significant challenge due to the lack of an appropriate ultraviolet or visible handle and significant polarity and/or structural similarities leading to low chromatographic separation. Perhaps most importantly, proposed radical SAM mechanisms can lead to suicide inactivation of PhpK *in vitro*; use of SAM as a substrate theoretically traps the [4Fe-4S] cluster of the enzyme in an inactive +2 state.[9,17] In theory, excess sodium dithionite could reactivate the enzyme by reducing the cluster back to $[4Fe\text{-}4S]^{+1}$. However, in practice, dithionite effected side reactions in the presence of SAM and $CH_3$Cbl(III). Thus, assuming single turnover conditions, the magnitude of the difference between the molecular weights of PhpK (~61 kD) and NAcDMPT (209 Da) implied that large amounts (mg) of enzyme would be needed to observe comparatively small (μg) amounts of product.

We turned to NMR spectroscopy to overcome these experimental limitations. PhpK was incubated with SAM and sodium dithionite, then NAcDMPT (Asischem), $CH_3$Cbl(III), and the enzyme MTAN (to relieve possible product inhibition) were added and anaerobically incubated at ~20 °C.[25,26] The reaction mixtures were denatured, and PhpK was removed by ultrafiltration. After partial purification and concentration, the resulting material was dissolved in $D_2O$ (Cambridge Isotope Labs). Two-dimensional $^{1}H$-$^{31}P$ gHSQC spectra were collected using a coupling constant of 15 Hz. Peak positions varied slightly due to differences in final pH and/or spectrometers. The methylene protons at C-4 of NAcDMPT displayed a strong crosspeak at 1.38 ppm ($^{1}H$) and 30.0 ppm ($^{31}P$) due to coupling with $^{31}P$ (Figures S4, S5, S6, and S7). In the presence of all reaction components above, PhpK catalyzed partial conversion of NAcDMPT to NAcPT as demonstrated by the appearance of a new crosspeak at (1.07, 44.0) ppm corresponding to the methyl group of NAcPT (Figures S5 and S6). Spiking the sample with chemically-synthesized NAcPT increased the intensity of this crosspeak. The amount of NAcPT in the final NMR sample was estimated to be [~100 μM], which corresponds to [~15–20 μM] in the original reaction. Given the initial protein concentration of [~30 μM], under these specific conditions PhpK catalyzes single turnover. In a control lacking dithionite, hypothesized to be required for reduction of the [4Fe-4S] cluster, the NAcPT-associated gHSQC crosspeak is not observed (Figure S4). Thus, our results suggest that PhpK catalyzes the methylation of NAcDMPT in a potentially radical SAM-dependent fashion.

To verify the donor of the methyl group in this reaction, we synthesized $^{13}CH_3$Cbl(III) and assayed PhpK with either a combination of $^{12}CH_3$Cbl(III) and $^{13}CH_3$Cbl(III) or $^{13}CH_3$Cbl(III) alone.[27] The $^{1}H$-$^{31}P$ gHSQC spectrum for the mixed-isotope reaction displayed the crosspeak previously observed for the methyl protons of NAcPT at (1.06, 43.8) ppm (Figure S7). Two new crosspeaks appeared at (0.95, 43.7) ppm and (1.16, 44.1) ppm. In the reaction containing only $^{13}CH_3$Cbl(III), a very clear E.COSY pattern (indicated by the asterisks) of the H-P crosspeaks (centered at (1.15, 43.1) ppm and indicated by the "x") is observed due to the passive couplings of $^{1}H$ and $^{31}P$ to the attached $^{13}C$ nuclei (Figure 1). This pattern matches the additional crosspeaks observed in the mixed-isotope experiment. The one-bond $J$ coupling of $^{1}J_{CH}$ is ~127 Hz, which matches the predicted value. Under these conditions, $CH_3$Cbl(III) apparently serves as the sole source for the methyl group of NAcPT. Our data agree with a previous report in which cell-free extracts used only $CH_3$Cbl(III) as a direct methyl donor for the final L-PT antibiotic.[12]

In summary, we have established an active, *in vitro* system to study a P-methyltransferase, PhpK, that is broadly applicable to related enzymes including Fom3 and Fms7. We are now investigating the hypothesis that PhpK uses a radical SAM mechanism for catalysis.[9,17] Isolation of 5′-deoxyadenosine and/or other SAM cleavage products will provide strong evidence supporting a radical mechanism, as would the trapping of Cbl(II). Is NAcDMPT or the tripeptide NAcDMPT-L-Ala-L-Leu the physiological substrate? Another question is

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whether $CH_3Cbl(III)$ is truly the methyl group donor or, as is the case in other $CH_3Cbl(III)$- and methylcorrinoid-dependent enzymes, whether Cbl functions as a coenzyme and $CH_3Cbl(III)$ is formed as a methylated intermediate. Previous work in *S. hygroscopicus* showed that the methyl group of NAcPT originates from $CD_3$-methionine, conserving all three deuterons.[28] Taken together, these data suggest that the methyl group is derived from methionine, is transferred to $CH_3Cbl(III)$, then is added to NAcDMPT by PhpK. This contrasts with recent reports on the Cfr- and RlmN-catalyzed radical SAM methylation reactions in which one of the three methyl protons is derived from a separate source.[29,30] Although methionine is not a methyl donor, it can be converted to the methyl donor SAM by SAM synthetase. PhpK may undergo reductive reactivation with SAM, forming $CH_3Cbl(III)$ in a manner similar to that observed in cobalamin-dependent methionine synthase.[17] The work presented here sets the stage for the deeper mechanistic investigation of these and other intriguing questions surrounding this fascinating family of enzymes.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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## ABBREVIATIONS

| | |
|---|---|
| **SAM** | *S*-adenosyl-L-methionine |
| **NAcDMPT** | *N*-acetyldemethylphosphinothricin or 2-acetylamino-4-hydroxyphosphinylbutanoate |
| **NAcPT** | *N*-acetylphosphinothricin |
| **PT** | phosphinothricin |
| **$CH_3Cbl(III)$** | methylcobalamin |
| **[4Fe-4S]** | four-iron, four-sulfur |
| **EPR** | electron paramagnetic resonance |
| **NMR** | nuclear magnetic resonance |
| **MTAN** | 5′-methylthioadenosine nucleosidase |
| **gHSQC** | gradient heteronuclear single quantum correlation |
| **ppm** | parts per million |

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**E.COSY** exclusive correlation spectroscopy

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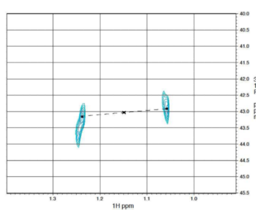


**Figure 1.**
H-P gHSQC spectrum of the partially purified $^{13}CH_3$-Cbl(III) PhpK reaction. The $^{13}CH_3$ H-P crosspeaks are centered at (1.15, 43.1) ppm (designated by the "x").

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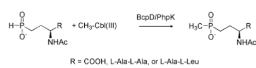


**Scheme 1.**
Proposed P-methyl transfer reactions

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