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In Vitro Phosphinate Methylation by PhpK from *Kitasatospora phosalacinea*

Williard J. Werner,[†] Kylie D. Allen,[†] Kaifeng Hu,[‡] Gregory L. Helms,[§] Brian S. Chen,[†] and Susan C. Wang*,[†]

[†]School of Molecular Biosciences, College of Veterinary Medicine, Washington State University, Pullman, Washington 99164, United States

[‡]National Magnetic Resonance Facility at Madison, University of Wisconsin, Madison, Wisconsin 53706, United States

[§]Center for Nuclear Magnetic Resonance Spectroscopy, Washington State University, Pullman, Washington 99164, United States

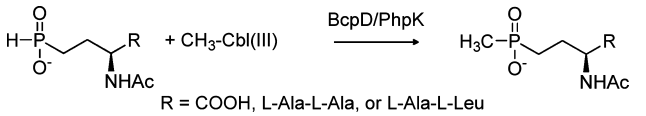
Supporting Information

ABSTRACT: Radical *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 has 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) to form 2-acetylamino-4-hydroxymethylphosphinylbutanoate (*N*-acetylphosphinothricin). 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 of the Supporting Information).[2] Because of 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 specifically investigated the phosalacine pathway.[2,9] Little is known about the formation of the unique C−P−C linkage 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 [$CH_3Cbl(III)$] and the methyl recipients were *N*-acetyldemethylphosphinothricin or 2-acetylamino-4-hydroxyphosphinylbutanoate (NAcDMPT) and the corresponding phosphinyl tripeptide, NAcDMPT-L-Ala-L-Ala (Scheme 1).[12] The resulting products are *N*-acetylphosphinothricin (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.

Scheme 1. Proposed *P*-Methyl Transfer Reactions



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 $CH_3Cbl(III)$ 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] Because none of the hypothesized substrates for this family of enzymes was nucleophilic, it appeared likely that these methyltransferases used a different mechanism for catalysis. Transfer of a methyl anion from $CH_3Cbl(III)$ 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 *S*-adenosyl-L-methionine (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

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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 $CH_3Cbl(III)$ 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 *K. phosalacinea* DSM 43860. Sequencing indicated that *phpK* from *K. phosalacinea* is more than 99% identical to the published *phpK* sequence from the bialaphos producer, *S. viridochromogenes*.[11,19] After overexpression in *Escherichia coli* and cell lysis, PhpK was not found in the soluble fraction and appeared to be expressed 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 of the Supporting Information).[21] Iron and sulfide analyses indicated approximately 5.9 mol of Fe and 4.4 mol of S per mole of PhpK.[22−24] Electron paramagnetic resonance (EPR) spectroscopy was used to verify the presence of the [4Fe-4S] cluster (Figure S3 of the Supporting Information). At 10 K, the EPR spectrum of a sample containing only PhpK and buffer showed a small g = 2.00 signal. 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 $[4Fe\text{-}4S]^+$ cluster. Addition of all components hypothesized to be required for the reaction (vide infra) resulted in the disappearance of the $[4Fe\text{-}4S]^+$ cluster.

Assaying PhpK for activity was a significant challenge because of 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 $[4Fe\text{-}4S]^+$. However, in practice, dithionite effected side reactions in the presence of SAM and $CH_3Cbl(III)$. Thus, if single-turnover conditions are assumed, the magnitude of the difference between the molecular masses of PhpK (~61 kDa) and NAcDMPT (209 Da) implied that large, milligram amounts of enzyme would be needed to observe comparatively small, microgram amounts of product.

We turned to nuclear magnetic resonance (NMR) spectroscopy to overcome these experimental limitations. PhpK was incubated with SAM and sodium dithionite, and then NAcDMPT (Asischem), $CH_3Cbl(III)$, and the enzyme 5′-methylthioadenosine nucleosidase (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 Laboratories). Two-dimensional $^1H-^{31}P$ gradient heteronuclear single-quantum correlation (gHSQC) spectra were recorded using a coupling constant of 15 Hz. Peak positions varied slightly because of differences in final pH and/or spectrometers. The methylene protons at C-4 of NAcDMPT displayed a strong cross-peak at 1.38 ppm (^1H) and 30.0 ppm (^{31}P) due to coupling with ^{31}P (Figures S4−S7 of the Supporting Information). In the presence of all reaction components described above, PhpK catalyzed partial conversion of NAcDMPT to NAcPT as demonstrated by the appearance of a new cross-peak at 1.07 and 44.0 ppm corresponding to the methyl group of NAcPT (Figures S5 and S6 of the Supporting Information). Spiking the sample with chemically synthesized NAcPT increased the intensity of this cross-peak. The concentration of NAcPT in the final NMR sample was estimated to be ~100 μM, which corresponds to ~15−20 μM in the original reaction mixture. Given the initial protein concentration of ~30 μM, under these specific conditions PhpK catalyzes a single turnover. In a control lacking dithionite, hypothesized to be required for reduction of the [4Fe-4S] cluster, the NAcPT-associated gHSQC cross-peak is not observed (Figure S4 of the Supporting Information). 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 $^{13}CH_3Cbl(III)$ and assayed PhpK with either a combination of $^{12}CH_3Cbl(III)$ and $^{13}CH_3Cbl(III)$ or $^{13}CH_3Cbl(III)$ alone.[27] The $^1H-^{31}P$ gHSQC spectrum for the mixed-isotope reaction displayed the cross-peak previously observed for the methyl protons of NAcPT at 1.06 and 43.8 ppm (Figure S7 of the Supporting Information). Two new cross-peaks appeared at 0.95 and 43.7 ppm and at 1.16 and 44.1 ppm. In the reaction mixture containing only $^{13}CH_3Cbl(III)$, a very clear exclusive correlation spectroscopy pattern (indicated by the asterisks) of the H−P cross-peaks (centered at 1.15 and 43.1 ppm and indicated by the x) is observed because of the passive couplings of ^1H and ^{31}P to the attached ^{13}C nuclei (Figure 1). This pattern matches the additional cross-peaks

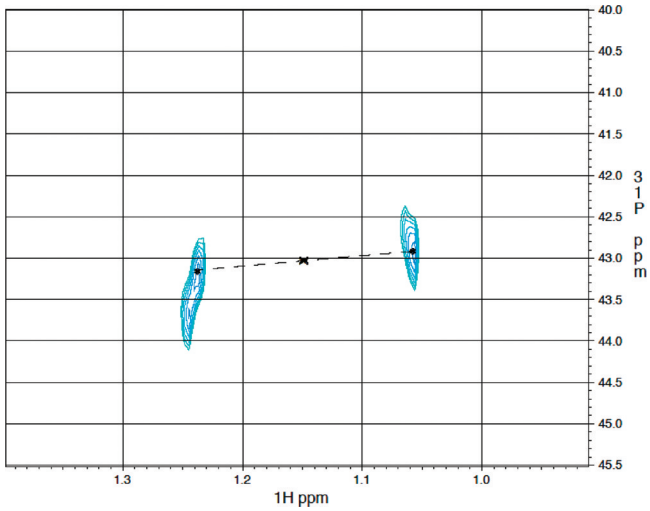

Figure 1. H−P gHSQC spectrum of the partially purified $^{13}CH_3$−Cbl(III) PhpK reaction mixture. The $^{13}CH_3$ H−P cross-peaks are centered at 1.15 and 43.1 ppm (designated by the x).

observed in the mixed-isotope experiment. The one-bond J coupling ($^1J_{CH}$) is ~127 Hz, which matches the predicted value. Under these conditions, $CH_3Cbl(III)$ 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

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$CH_3Cbl(III)$ as a direct methyl donor for the final L-PT antibiotic.[12]

In summary, we have established an active, in vitro system for studying 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 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 $CH_3Cbl(III)$, and 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 Cbl-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.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures and EPR and NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Telephone: (509) 335-7714. Fax: (509) 335-4159. E-mail: susan_wang@wsu.edu.

Author Contributions

W.J.W. and K.D.A. contributed equally to this work.

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