LETTER

doi:10.1038/nature12061

The catalytic mechanism for aerobic formation of methane by bacteria

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Methane is a potent greenhouse gas that is produced in significant quantities by aerobic marine organisms[1]. These bacteria apparently catalyse the formation of methane through the cleavage of the highly unreactive carbon–phosphorus bond in methyl phosphonate (MPn), but the biological or terrestrial source of this compound is unclear[2]. However, the ocean-dwelling bacterium *Nitrosopumilus maritimus* catalyses the biosynthesis of MPn from 2-hydroxyethyl phosphonate[3] and the bacterial C–P lyase complex is known to convert MPn to methane[4–7]. In addition to MPn, the bacterial C–P lyase complex catalyses C–P bond cleavage of many alkyl phosphonates when the environmental concentration of phosphate is low[4–7]. PhnJ from the C–P lyase complex catalyses an unprecedented C–P bond cleavage reaction of ribose-1-phosphonate-5-phosphate to methane and ribose-1,2-cyclic-phosphate-5-phosphate. This reaction requires a redox-active [4Fe–4S]-cluster and *S*-adenosyl-L-methionine, which is reductively cleaved to L-methionine and 5′-deoxyadenosine[8]. Here we show that PhnJ is a novel radical *S*-adenosyl-L-methionine enzyme that catalyses C–P bond cleavage through the initial formation of a 5′-deoxyadenosyl radical and two protein-based radicals localized at Gly 32 and Cys 272. During this transformation, the *pro-R* hydrogen from Gly 32 is transferred to the 5′-deoxyadenosyl radical to form 5′-deoxyadenosine and the *pro-S* hydrogen is transferred to the radical intermediate that ultimately generates methane. A comprehensive reaction mechanism is proposed for cleavage of the C–P bond by the C–P lyase complex that uses a covalent thiophosphate intermediate for methane and phosphate formation.

The glutathione *S*-transferase (GST) fusion protein of PhnJ from *Escherichia coli* was purified under anaerobic conditions[8]. The isolated protein was dark brown in colour, had an absorbance maximum at a wavelength of 410 nm and was EPR silent (produced no electron paramagnetic resonance signal) (Fig. 1a). Addition of dithionite to the isolated protein resulted in the loss of absorbance at 410 nm and yielded an EPR-active species (Fig. 1a). At a temperature of 12 K the EPR signal was strongest, and at 48 K the signal was significantly weaker (Supplementary Fig. 1). These results are consistent with the initial isolation of an intact $[4Fe–4S]^{2+}$-cluster that can be further reduced by dithionite to the $[4Fe–4S]^{1+}$ oxidation state[9–12].

Iron–sulphur cluster formation in most radical *S*-adenosyl-L-methionine (SAM) enzymes requires coordination to three cysteine residues in a CX_3CX_2C motif[13–15] (X, any amino acid). PhnJ lacks the signature radical SAM enzyme motif but has four cysteine residues with a $CX_2CX_{21}CX_5C$ spacing near the carboxy-terminal end of the protein. To determine which three of the four cysteine residues of PhnJ are required for assembly of the [4Fe–4S]-cluster, we mutated Cys 241, Cys 244, Cys 266 and Cys 272 to Ala. All of the mutant enzymes were inactive for the production of methane, and the Cys241Ala, Cys244Ala and Cys266Ala mutants were unable to assemble a [4Fe–4S]-cluster (Supplementary Fig. 2). The Cys272Ala mutant protein was dark brown in colour and the ultraviolet–visible spectrum was identical to that of wild-type PhnJ (Fig. 1b). After reduction of the Cys272Ala mutant protein with dithionite, the absorbance maximum at 410 nm was lost and an EPR-active species was formed that is identical to that of wild-type PhnJ (Fig. 1b). Cys 241, Cys 244 and Cys 266 are therefore required for the formation of the [4Fe–4S]-cluster in PhnJ, and Cys 272 is critical for catalytic activity.

It was shown previously that 5′-deoxyadenosine (Ado-CH_3) and L-methionine are formed from the utilization of SAM during the reaction catalysed by PhnJ and that approximately one enzyme equivalent of SAM is consumed under single or multiple turnovers[8]. The reductive cleavage of SAM by the $[4Fe–4S]^{1+}$-cluster requires the transient formation of a 5′-deoxyadenosyl radical (Ado-CH_2•) that subsequently abstracts a hydrogen atom from either the enzyme or the substrate as

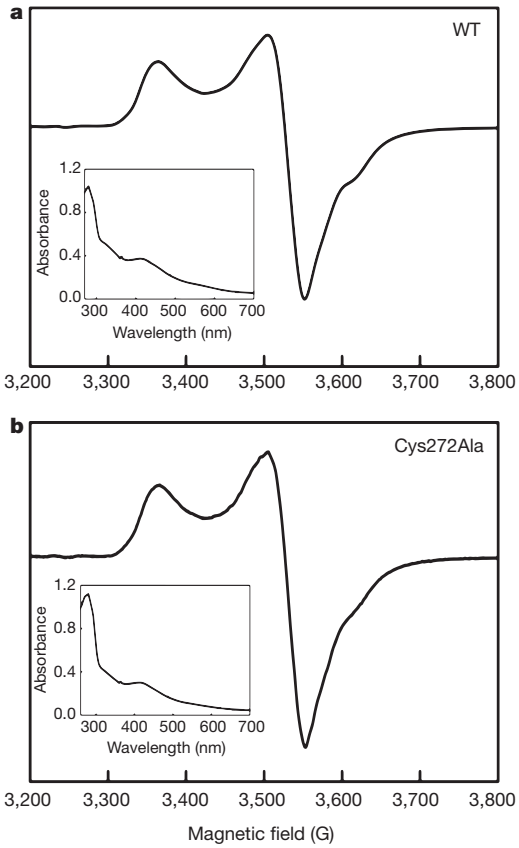

Figure 1 | EPR spectra of wild-type PhnJ and the Cys272Ala mutant. a, EPR spectrum of wild-type (WT) PhnJ (180 μM) after reduction with dithionite at 12 K. The EPR spectrum is characteristic of a reduced $[4Fe–4S]^{1+}$-cluster with *g* values of 2.01, 1.92 and 1.87. Inset, ultraviolet–visible spectrum of as-isolated wild-type PhnJ (18 μM). **b**, EPR spectrum of PhnJ Cys272Ala mutant (158 μM) after reduction with dithionite at 12 K. Inset, ultraviolet–visible spectrum of as-isolated PhnJ Cys272Ala mutant (17 μM). The EPR spectra were obtained under these instrument settings: 9.46-GHz microwave frequency, 0.2-mW microwave power, and 10-G modulation amplitude.

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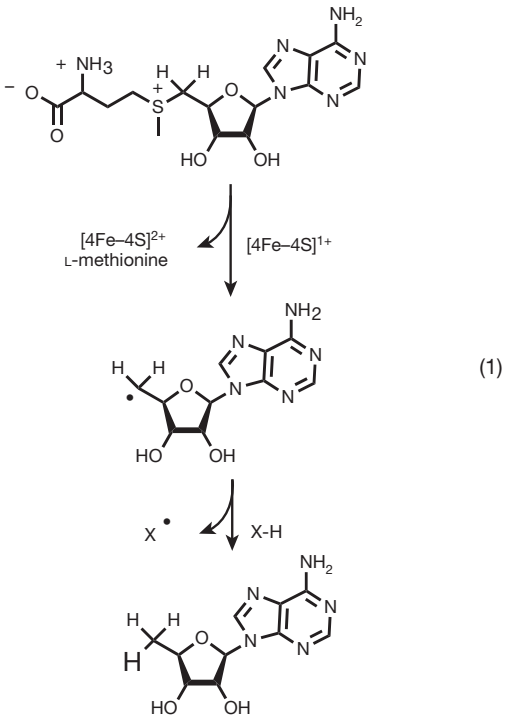
illustrated in equation (1)[13–15] (where X-H denotes the enzyme or substrate and H denotes the abstracted hydrogen atom). To determine whether or not hydrogen atom abstraction occurs from a solvent exchangeable site, the reaction catalysed by PhnJ was conducted in H_2O and then in 90% D_2O. The product, Ado-CH_3, was isolated and the isotopic composition determined by mass spectrometry. For the reaction conducted in H_2O, the mass of the $(M+H)^+$ ion of the isolated Ado-CH_3 was 252.1 Da. (Fig. 2a). When the reaction was performed in 90% D_2O, the $(M+H)^+$ ion mass was also 252.1 Da (Fig. 2b). These results demonstrate that the hydrogen atom that is transferred to the Ado-CH_2• radical does not originate in a solvent exchangeable site in either the protein or the substrate.



(1)

Because hydrogen atom transfer to the Ado-CH_2• radical intermediate does not occur from a solvent exchangeable site, the next most probable source of this hydrogen atom was postulated to be a Gly residue[16–19]. PhnJ was therefore expressed and purified from an M9-minimal medium supplemented with [2,2-$^2H_2$]-Gly. The mass spectrum (Supplementary Fig. 3) of a typical tryptic peptide (^{164}FGHIATTY AYPVK^{176}) demonstrated that the average deuterium content of the Gly residues was as follows: PhnJ-Gly-h_2, 19%; PhnJ-Gly-hd, 15%; PhnJ-Gly-d_2, 66%. When the Gly-labelled protein was used to catalyse the C–P lyase reaction in H_2O, the Ado-CH_3 had a predominant $(M+H)^+$ ion mass of 253.1 Da and the deuterium content of the newly formed Ado-CH_3 was ~74% (Fig. 2c). Therefore, hydrogen atom transfer within PhnJ must occur from one of the eight conserved Gly residues to the transient Ado-CH_2• radical intermediate and consequently forms a glycyl radical.

During cleavage of the C–P bond of ribose-1-phosphonate-5-phosphate (PRPn) to ultimately form ribose-1,2-cyclic-phosphate-5-phosphate (PRcP), a new C–H bond is formed in the methane product, but the origin of the hydrogen atom is unknown (equation (2)). To determine the direct source of this hydrogen atom, the reaction catalysed by PhnJ was conducted in H_2O and D_2O under conditions of single and multiple turnovers of substrate using wild-type PhnJ, and PhnJ that was uniformly labelled with deuterated Gly. The methane produced in these reactions was trapped and subjected to mass spectrometric analysis to determine the ratio of unlabelled (CH_4) and deuterated (CH_3D) methane. When unlabelled PhnJ was incubated with less than one enzyme equivalent of substrate and the reaction

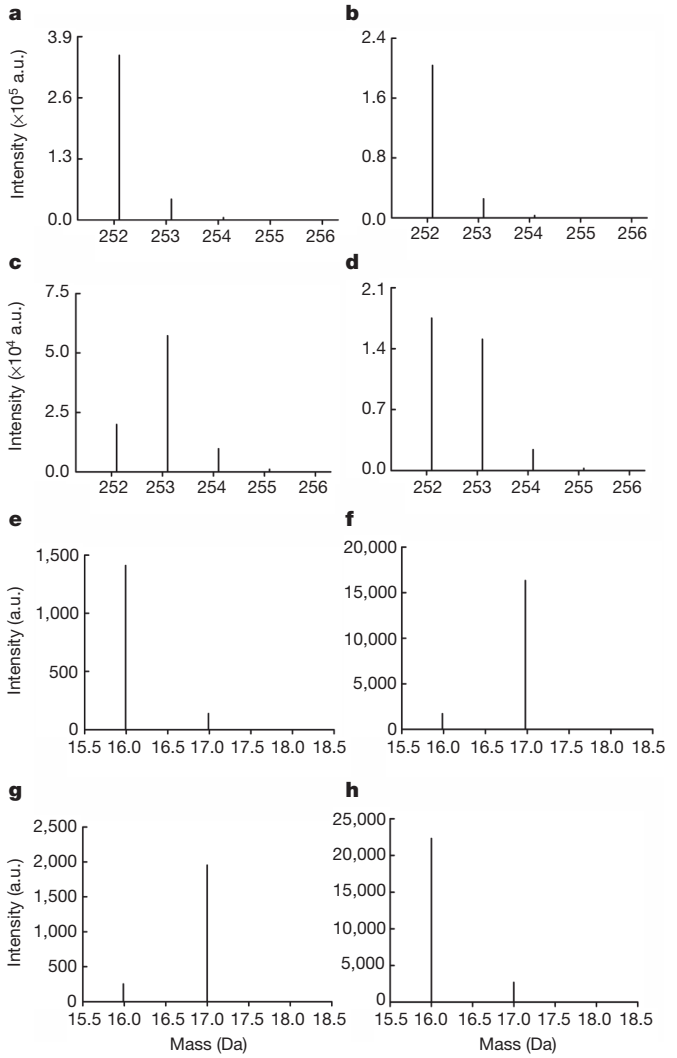
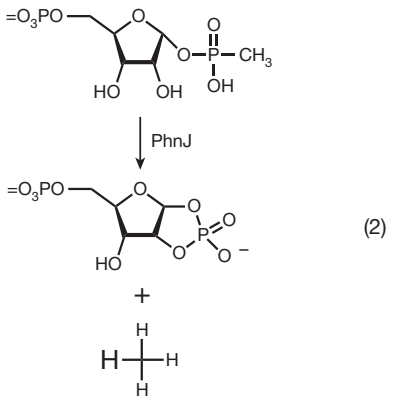


Figure 2 | Mass spectra of 5′-deoxyadenosine and methane from reactions catalysed by PhnJ. a–d, Mass spectra of 5′-deoxyadenosine for PhnJ in H_2O (**a**), PhnJ in 90% D_2O (**b**), PhnJ-Gly-d_2 in H_2O (**c**) and PhnJ-Gly-d_R in H_2O (**d**).Typical reaction compositions were 120 μM PhnJ, 1 mM PRPn (60 μM in **d**), 2 mM SAM, 1 mM dithionite, 50 units Factor Xa, 150 mM HEPES (pH 8.5), 0.5 M NaCl and 10% (w/v) glycerol. Typical reaction volume was 200 μl. **e–h**, Mass spectra of methane for wild-type PhnJ in 90% D_2O for a single-turnover experiment (**e**), wild-type PhnJ in 90% D_2O for multiple turnovers (**f**), PhnJ-Gly-d_2 in H_2O for a single-turnover experiment (**g**) and PhnJ-Gly-d_2 in H_2O for multiple turnovers (**h**). Typical reaction compositions were 150 μM PhnJ, 75 μM PRPn for single-turnover experiments, 1.5 mM PRPn for multiple-turnover experiments, 2 mM SAM, 1 mM dithionite, 50 units Factor Xa, 150 mM HEPES (pH 8.5), 0.5 M NaCl and 10% (w/v) glycerol. Typical reaction volume was 1.0 ml; typical headspace volume was 500 μl. a.u., arbitrary units.

conducted in 90% D_2O, the methane product was unlabelled with a mass-to-charge ratio (m/z) of 16 (Fig. 2e). When the reaction was initiated with ten enzyme equivalents of substrate in 90% D_2O, the methane product was predominantly labelled with deuterium with m/z 17 (Fig. 2f). When PhnJ-Gly-d_2 was used to initiate the reaction in H_2O with less than one enzyme equivalent of substrate, the isolated methane product predominantly contained a single deuterium label with m/z 17 (Fig. 2g). Finally, when PhnJ-Gly-d_2 was used to initiate the reaction in H_2O under multiple-turnover conditions, the methane product was unlabelled with m/z 16 (Fig. 2h). Under single-turnover conditions, the origin of the new hydrogen in the methane product derives exclusively from one of the Gly residues of PhnJ. Under multiple-turnover conditions, the origin of the new hydrogen in the methane product is determined from whether the reaction was conducted in

H_2O or D_2O. Therefore, during the course of the reaction catalysed by PhnJ, the active-site Gly residue directly participates in hydrogen atom transfer to the intermediate that forms methane. During multiple turnovers, the original hydrogen atoms contained within this Gly residue are ultimately replaced with those from bulk solvent.



(2)

According to the deuterium-labelling studies of PhnJ, one of the two prochiral hydrogen atoms from a PhnJ Gly residue is initially transferred to the transient Ado-CH_2• radical and the other hydrogen is transferred to the methyl group of the substrate during the course of the reaction. To determine the stereochemical origin of each of these hydrogen atom transfers, PhnJ was expressed in a medium containing Gly labelled with deuterium in the *pro-R* position and hydrogen in the *pro-S* position. The Gly used for this experiment was 68% *R*-[2-^{2}H]-Gly and 32% unlabelled Gly (Supplementary Fig. 4). Wild-type PhnJ was expressed in M9-minimal medium supplemented with 20 mM *R*-[2-^{2}H]-Gly. Mass spectrometric analysis of a tryptic peptide fragment, ^{27}AVAIPGYQVPFGGR^{40}, demonstrated that the average deuterium content at the *pro-R* position of the Gly residues in the isolated PhnJ (PhnJ-Gly-d_R) was ~52% (Supplementary Fig. 5). PhnJ-Gly-d_R was used to catalyse the C–P lyase reaction under conditions where the initial substrate concentration of PRPn was less than one equivalent of PhnJ. Under these single-turnover conditions, the Ado-CH_3 was shown by mass spectrometry to be 44% labelled with deuterium (Fig. 2d). No deuterium was found in the methane product (Supplementary Fig. 6). Therefore, the *pro-R* hydrogen of an unknown Gly from PhnJ is transferred to the Ado-CH_2• radical intermediate during the course of the C–P lyase reaction and the *pro-S* hydrogen is used in the formation of methane.

The identity of the specific Gly residue within PhnJ that is involved in two distinct hydrogen atom transfers during the course of the C–P lyase reaction was determined by two complementary experiments. In the first experiment, the reaction catalysed by PhnJ was conducted in 75% D_2O under multiple-turnover conditions. Under these reaction conditions, one of the Gly residues in PhnJ must exchange the *pro-R* and *pro-S* hydrogen atoms with deuterium from solvent. The reactions were quenched at various times and PhnJ was isolated. After proteolytic digestion with trypsin, the peptide fragments were analysed by mass spectrometry to identify those peptides that incorporated deuterium. The only peptide found labelled with deuterium during the course of this experiment was ^{27}VAIPGYQVPFGGR^{40} (Fig. 3a). After 12 min, the total deuterium content of a single Gly residue was ~36%. This peptide contains three Gly residues but only Gly 32 is absolutely conserved in PhnJ.

To confirm that Gly 32 is directly involved in hydrogen atom transfers to the transient Ado-CH_2• radical, we mutated this residue to alanine. The purified PhnJ Gly32Ala mutant was brown and it had the same absorbance maximum at 410 nm as wild-type PhnJ (Supplementary Fig. 3). After reduction with dithionite, the absorbance at 410 nm was lost, consistent with a redox-active [4Fe–4S]-cluster. When PhnJ Gly32Ala was incubated with all of the ingredients required for the C–P lyase reaction, the formation of PRcP and methane was not detected. However, Ado-CH_3 was detected and, thus, an Ado-CH_2• radical was formed in the active site of this mutant. To assess whether a hydrogen atom was abstracted by the Ado-CH_2• radical from Ala-32, we expressed the PhnJ Gly32Ala mutant in a minimal medium supplemented with L-[2,3,3,3-$^2H_4$]-Ala. The PhnJ Gly32Ala-Ala-d_4 mutant protein was incubated with the same ingredients described above for the unlabelled mutant and the Ado-CH_3 was isolated and subjected to mass spectrometric analysis; deuterated Ado-CH_3 (66%) was the major product (Fig. 3b). These results confirm that there is hydrogen atom transfer specifically from Gly 32 in PhnJ to the Ado-CH_2• radical during the reaction cycle.

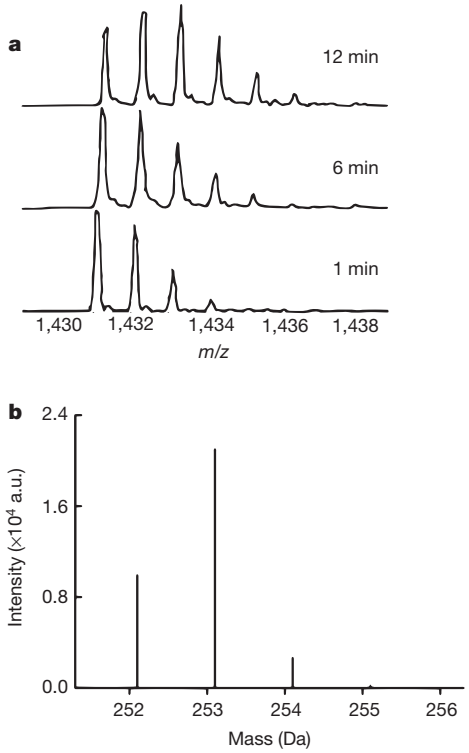


Figure 3 | Identification of Gly 32 as the site of the glycyl radical. **a**, Time course for the incorporation of deuterium into the peptide ^{27}VAIPGYQVPFGGR^{40} when the PhnJ reaction was conducted in D_2O. The reaction volume was 200 µl and contained 25 µM PhnJ, 300 µM PRPn, 1 mM SAM, 500 µM dithionite, 25 units Factor Xa, 150 mM HEPES (pH 8.5), 0.5 M NaCl and 10% (w/v) glycerol, in 75% D_2O. During the reaction course, 50 µl of reaction volume was loaded onto an SDS polyacrylamide gel and analysed by mass spectrometry after in-gel trypsin digestion. **b**, Mass spectrum of 5′-deoxyadenosine isolated from the PhnJ Gly32Ala mutant labelled with [2,3,3,3-$^2H_4$]-Ala. The reaction mixture contained 100 µM PhnJ-Gly32Ala-Ala-d_4, 1 mM PRPn, 2 mM SAM, 1 mM dithionite, 50 units Factor Xa, 150 mM HEPES (pH 8.5), 0.5 M NaCl and 10% (w/v) glycerol in a volume of 50 µl.

On the basis of the experiments described in this report, we propose the following reaction mechanism for PhnJ during cleavage of the C–P bond in PRPn to form PRcP and methane (Fig. 4). PhnJ is a novel radical SAM enzyme that uses Gly 32 and Cys 272 during the cleavage of C–P bonds. In the proposed mechanism, the reaction starts with the reductive cleavage of SAM by the reduced $[4Fe–4S]^{1+}$-cluster to form the Ado-CH_2• radical intermediate. In the second step, the Ado-CH_2• intermediate abstracts the *pro-R* hydrogen from Gly 32 to generate Ado-CH_3 and a glycyl radical. In the third step, there is stereospecific hydrogen atom transfer from Cys 272 to the Gly 32 radical to make a thiyl radical on the side chain of Cys 272, and the Gly residue is regenerated. However, we note that there is no direct evidence for the formation of a thiyl radical in this study. In the fourth step, the thiyl radical attacks the phosphonate moiety of the substrate, PRPn, to create a transient thiophosphonate radical intermediate. Collapse of this intermediate, by

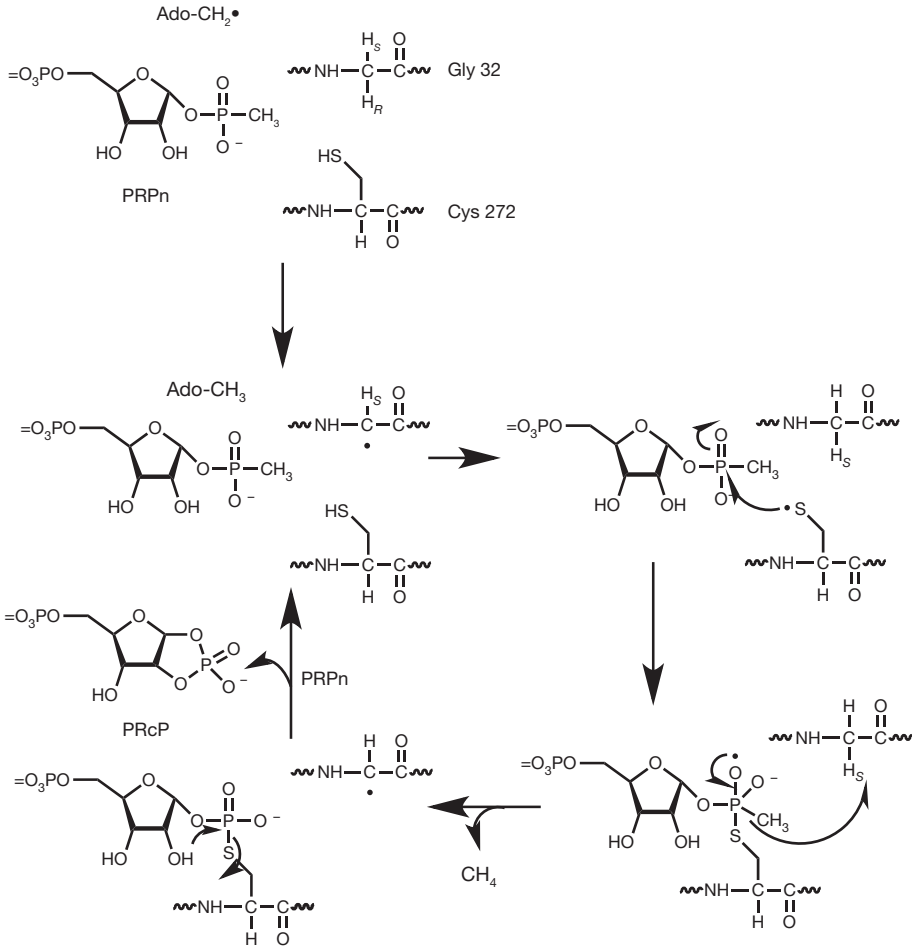
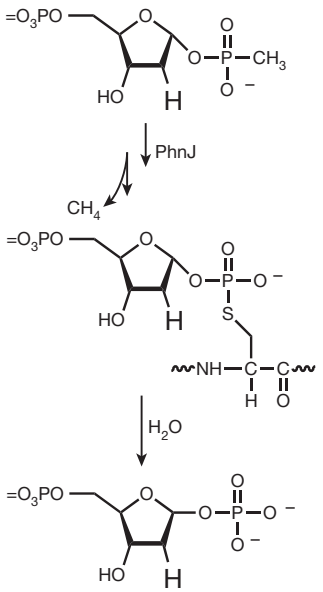


Figure 4 | Proposed mechanism for the reaction catalysed by PhnJ. The reaction cascade is initiated by formation of an Ado-CH2• radical. This intermediate abstracts the *pro-R* hydrogen from Gly 32 to form a glycyl radical. Hydrogen atom transfer from Cys 272 to the Gly 32 radical generates a thiyl radical on the side chain of Cys 272. This radical attacks the phosphonate moiety of the substrate to create a thiophosphonate radical intermediate. Homolytic C–P bond cleavage and hydrogen atom transfer from the original *pro-S* hydrogen of Gly 32 produces a thiophosphate intermediate, methane, and regenerates the radical intermediate at Gly 32. The ultimate product, PRcP, is formed by nucleophilic attack of the C2 hydroxyl on the covalent thiophosphate intermediate.

means of homolytic C–P bond cleavage and hydrogen atom transfer from the original *pro-S* hydrogen of Gly 32, produces a thiophosphate intermediate, methane, and regenerates the radical intermediate at Gly 32. The ultimate product, PRcP, is formed by nucleophilic attack of the C2 hydroxyl on the covalent 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 can be repeated with another substrate without the use of another molecule of SAM or any further involvement of the [4Fe–4S]-cluster.

The proposed reaction mechanism postulates the existence of a thiophosphate intermediate. To provide experimental support for this reaction intermediate, we synthesized the 2-deoxy substrate analogue, 2-dPRPn, in the anticipation that the lack of the C2 hydroxyl group of the substrate would trap the covalent intermediate as illustrated in equation (3)[8]. When PhnJ was incubated with ten enzyme equivalents of 2-dPRPn, and the other required ingredients of the C–P lyase reaction, ~2.3 enzyme equivalents of methane were detected by gas chromatography and approximately ~1.6 enzyme equivalents of 2-deoxyribose-1,5-bisphosphate were identified by ^{31}P-NMR spectroscopy (Supplementary Fig. 7). To assess whether any of the initial substrate was covalently attached to PhnJ, the enzyme was first treated with EDTA and then filtered through a 3 kDa membrane to remove the iron and all other small molecular weight molecules associated with the protein sample. The enzyme was digested with trypsin and the tryptic peptides analysed by ^{31}P-NMR to search for peptide fragments containing a phosphorylated enzyme-adduct. The ^{31}P-NMR spectrum revealed the appearance of two major resonances, one at a chemical shift of 4.5 p.p.m. and the other at a chemical shift of 25.0 p.p.m. (Fig. 5). The resonance at 4.5 p.p.m. correlates with the phosphate moiety at C5 of the proposed intermediate, and the resonance at 25.0 p.p.m. is consistent with the thiophosphate moiety at C1[20]. However, other types of phosphorus-containing adducts can resonate in this region of the ^{31}P-NMR spectrum and attempts to identify a modified tryptic peptide by mass spectrometry failed.



(3)

Pyruvate formate lyase and the anaerobic ribonucleotide reductase are two well-characterized proteins from the glycyl radical enzyme (GRE) family that utilize two subunits to catalyse their respective reactions[16–19]. Both of these systems have a smaller subunit, comprising ~250 amino acids, that functions as an activase (pyruvate formate lyase activase and ribonucleotide reductase activase). These proteins

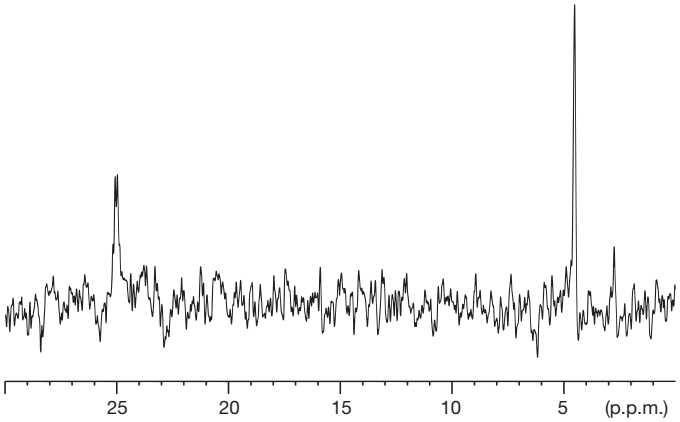


Figure 5 | ^{31}P-NMR spectrum of tryptic fragments of PhnJ after reaction with 2-dPRPn. In this experiment, 1 mM 2′-dPRPn was used as the substrate with 120 μM PhnJ. The reaction was quenched by the addition of EDTA and the PhnJ was isolated by ultrafiltration. PhnJ was digested with trypsin at pH 7.0. The resonance at 4.5 p.p.m. corresponds to the 5-phosphate of the ribose moiety of the covalent intermediate proposed in the mechanism depicted in equation (3). The resonance at 25 p.p.m. is consistent with the thiophosphate moiety of the proposed intermediate. The NMR spectrum was collected at pH 7.0 and 10 °C.

contain a radical SAM Cys motif, bind a redox-active [4Fe–4S]-cluster and generate a transient Ado-CH_2• intermediate from SAM. The Ado-CH_2• radical initiates the formation of a glycyl radical by abstracting the *pro-S* hydrogen of a highly conserved Gly residue on the larger subunit, of ~760 amino acids, which subsequently generates a catalytically competent thiyl radical at a conserved Cys residue of the larger subunit[21,22].

The polypeptide sequence of PhnJ consists of only 290 amino acids and is thus much smaller than other GREs (Supplementary Fig. 8). The hallmark of the proposed PhnJ reaction mechanism is the participation of a redox-active [4Fe–4S]-cluster, the transient formation of Ado-CH_2• from SAM, and the presence of two protein-based radicals from Gly 32 and Cys 272 that act in tandem for the cleavage of the C–P bond in phosphonate substrates. On the basis of labelling studies, the *pro-R* hydrogen of Gly 32 is abstracted by Ado-CH_2•, whereas the *pro-S* hydrogen is abstracted in all other GREs[21]. Both hydrogen atoms from Gly 32 are eventually transferred during the course of the C–P lyase reaction catalysed by PhnJ, which is unprecedented in any other GRE. The mechanistic characterization of the PhnJ reaction mechanism expands the repertoire of glycyl radical SAM enzymes and establishes a novel C–P bond cleaving reaction.

METHODS SUMMARY

Protein expression and purification. The gene for the expression of PhnJ was amplified and cloned into a pET42a(+) vector as an amino-terminal GST fusion protein, as described earlier[8]. For preparing PhnJ with deuterated Gly or Ala, the cells were grown in M9-minimal medium. The purification of wild-type PhnJ and mutant proteins was performed anaerobically in an MBraun LabMaster SP glove box, with oxygen levels of less than 4 p.p.m. The soluble protein fraction was applied to a GSTrap column (GE Healthcare, 5 ml) and eluted with reduced glutathione.

PhnJ-catalysed reactions. All PhnJ reactions were performed anaerobically (oxygen concentration less than 4 p.p.m. at all times) in an MBraun LabMaster SP glove box. A typical reaction contained 120–140 μM PhnJ, 2 mM SAM, 1 mM sodium dithionite, ×1 Factor Xa buffer, variable concentrations of PRPn, 150 mM HEPES buffer (pH 8.5), 0.5 M NaCl and 10% (w/v) glycerol. All reaction ingredients were incubated and the reaction initiated by the *in situ* cleavage of the GST tag by addition of 50 units of Factor Xa. Typical reaction volumes were 150–200 μl.

Received 15 November 2012; accepted 8 March 2013.
Published online 24 April 2013.

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Supplementary Information is available in the online version of the paper.

Acknowledgements We thank D. Barondeau for use of the anaerobic chamber, R. Stipanovic for use of the gas chromatography mass spectrometer, A. Mehta and T. Begley for use of the liquid chromatography mass spectrometer, and C. Hilty for use of the ^{31}P-NMR spectrometer. We thank P. A. Lindahl for help with the EPR measurements (GM084266). This work was supported by the Robert A. Welch Foundation (A-840).

Author Contributions S.S.K., H.J.W. and F.M.R. designed the experiments. S.S.K. did the cloning and purification, performed the reactions and made all samples for analysis. S.S.K. and H.J.W. did the NMR, gas chromatography and gas chromatography mass spectrometry experiments. M.C. collected and analysed the EPR data. S.S.K. and L.J.D. did the trypsin digestion and peptide analysis. The manuscript was written by S.S.K. and F.M.R.

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