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## **The B-12-Radical SAM enzyme PoyC catalyzes valine C-beta-Methylation during Polytheonamide Biosynthesis**

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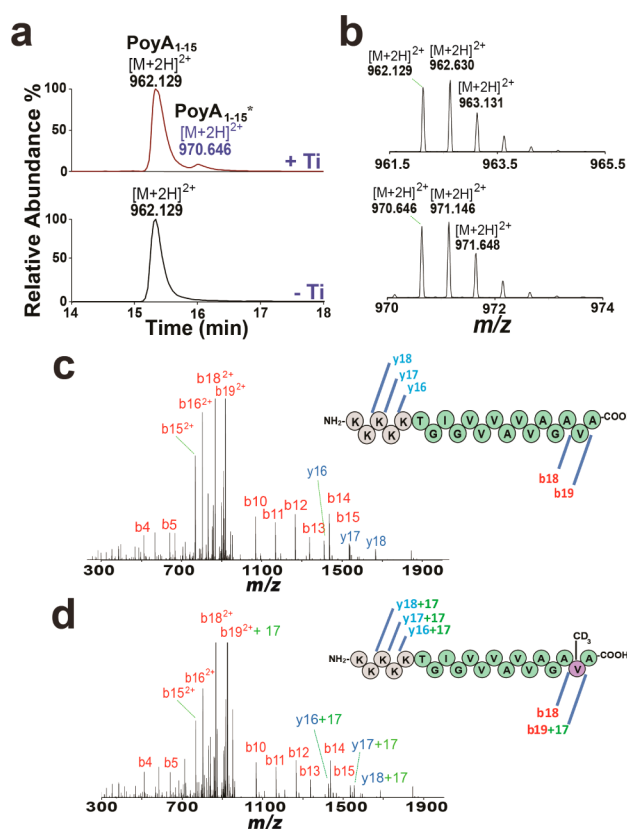
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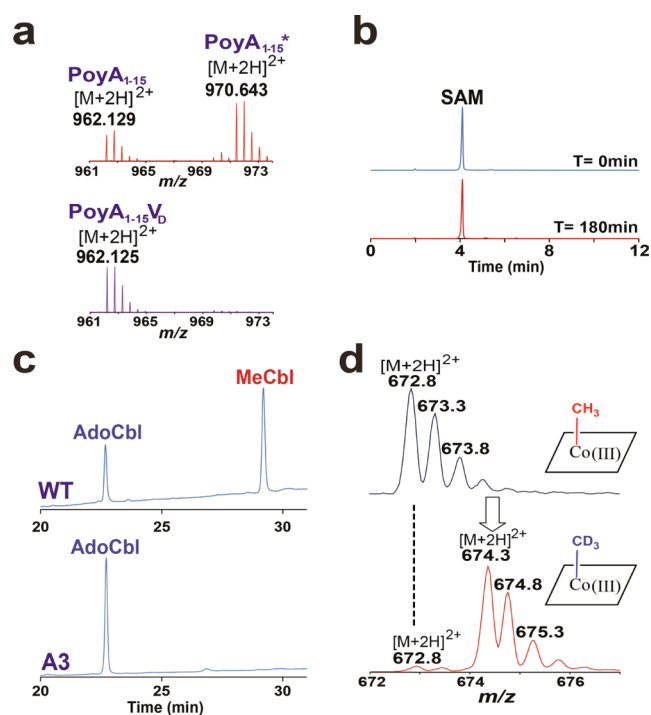




**Figure 3.** (a) LC–MS analysis of PoyA<sub>1–15</sub> incubated with PoyC and CD<sub>3</sub>-SAM under anaerobic conditions in the presence (+Ti) or absence (–Ti) of titanium citrate. See the SI for the experimental conditions. (b) High-resolution LC–MS analyses of PoyA<sub>1–15</sub> (upper trace) and PoyA<sub>1–15</sub>\* (lower trace). (c, d) High-resolution LC–MS/MS analyses of (c) PoyA<sub>1–15</sub> and (d) PoyA<sub>1–15</sub>\*. See Supplementary Figure 2 for the full spectra.

LC–MS/MS analysis of the modified PoyA<sub>1–15</sub>\* peptide revealed the positioning of the methyl group on the penultimate amino acid, L-Val<sub>14</sub> (Figure 3c,d, Supplementary Figure 2, and Supplementary Tables 1 and 2). Interestingly, Val<sub>14</sub> is one of the few C-methylated and epimerized residues found in native polytheonamide A (Figure 1b). Previous studies have shown that *in vivo*, heterologous coexpression of PoyA with PoyD<sup>6</sup> leads to epimerization of amino acid residues, suggesting that epimerization is the first step in polytheonamide A biosynthesis. However, since *E. coli* is unable to produce *de novo* cobalamin, coexpressions of PoyA with PoyB or PoyC were inconclusive. To test whether PoyC acts prior to or after epimerization, we synthesized a peptide containing one D-Val at position 14 (PoyA<sub>1–15</sub>V<sub>D</sub>). As shown, introduction of one D-Val led to methyl transfer inhibition (Figure 4a). This result further supports the hypothesis that PoyC catalyzes methyl transfer only on L-amino acids. Consistent with this hypothesis, most of the C-methylated residues are L-configured in polytheonamide A (Figure 1b).

PoyC possesses a predicted unusual rSAM motif: Cx7Cx2C. To probe its function, we generated the corresponding triple variant Ax7Ax2A (Supplementary Figure 3). As expected, this mutant was unable to produce 5'-dA and to transfer a methyl group to the substrate (Figure 4b). However, contrary to TsrM,<sup>1,2</sup> this mutant was also impaired for SAH production, suggesting different functions for the [4Fe–4S] center in these two B<sub>12</sub>-rSAM enzymes.



**Figure 4.** (a) LC–MS analyses of PoyA<sub>1–15</sub> (upper trace) and PoyA<sub>1–15</sub>V<sub>D</sub> (lower trace) after incubation in the presence of PoyC. See the SI for the experimental conditions. (b) SAM cleavage assay performed with the PoyC Ax7Ax2A mutant (UV detection at 257 nm). Samples were assayed at  $T = 0$  min (upper trace) and after 180 min of incubation with the mutant enzyme (lower trace). See the SI for the experimental conditions. (c) HPLC analysis of the cobalamin derivatives present in PoyC (WT) and the Ax7Ax2A mutant (A3) (UV detection at 278 nm). (d) LC–MS analyses of MeCbl present in PoyC at  $T = 0$  h (upper trace) and after 1 h of incubation with its peptide substrate, Ti (10 mM) and CD<sub>3</sub>-SAM (2 mM) (lower trace).

Because PoyC is the first B<sub>12</sub>-rSAM enzyme isolated natively loaded with a B<sub>12</sub> cofactor (i.e., without addition of cobalamin derivatives during enzyme refolding, purification or reaction), we aimed to characterize its B<sub>12</sub> content. PoyC, carefully handled in the dark, was found to contain essentially two B<sub>12</sub> derivatives identified as AdoCbl and MeCbl by their HPLC profiles (Figure 4c and Supplementary Figure 4), their UV–vis spectra and MS analyses (Supplementary Figure 5). PoyC proved to contain  $0.27 \pm 0.08$  mol of MeCbl and  $0.07 \pm 0.02$  mol of AdoCbl per polypeptide chain.

The apparent nonselectivity of PoyC for the upper axial ligand of cobalamin was surprising since the methionine synthase from *E. coli* was purified as a mixture of forms containing hydroxo- or methylcobalamin<sup>27</sup> while the “corrinoid iron–sulfur protein” was isolated as a cob(II)alamin complex.<sup>28,29</sup> It is well-known that when supplied with cobalamin derivatives, *E. coli* produces large amounts of AdoCbl,<sup>30</sup> which was likely incorporated during the heterologous expression of PoyC. Interestingly, the Ax7Ax2A mutant also contained AdoCbl, but we did not detect MeCbl (Figure 4c). This result suggests a critical role of the [4Fe–4S] center in cobalamin methylation. It is also consistent with the fact that the Ax7Ax2A mutant is unable to produce SAH and thus to transfer the methyl group from SAM to cobalamin.

As shown, the AdoCbl/MeCbl content of the enzyme did not change during the reaction (Supplementary Figure 6). However, MeCbl was almost entirely converted into CD<sub>3</sub>-Cbl during the

reaction (Figure 4d), establishing that the enzyme-bound cobalamin is recycled from SAM during catalysis.

Collectively, our results demonstrate that PoyC is a B<sub>12</sub>-rSAM enzyme with an unusual Cx7Cx2C radical SAM motif identified only in a putative rSAM enzyme<sup>31</sup> and the predicted bottromycin methyltransferases.<sup>7</sup> We purified PoyC with two bound alkylcobalamins: AdoCbl and MeCbl. On the basis of the UV–vis spectrum, it appears that these cobalamins have a base-off coordination, similar to what has recently been proposed for TsrM.<sup>20</sup> Using Ti as the reducing system, we have also demonstrated that PoyC is a peptide C-methyltransferase catalyzing the reductive cleavage of SAM. Ti has been extensively used for the reactivation of methionine synthase<sup>26</sup> and recently with a B<sub>12</sub>-dependent P-methyltransferase.<sup>24</sup> However, in this latter case, no 5'-dA or SAH was reported.

In agreement with our results, it is reasonable to assume that PoyC catalyzes the reductive cleavage of SAM into the 5'-dA radical. This radical species likely abstracts a C<sub>β</sub>-H atom,<sup>32</sup> generating a carbon-centered radical intermediate. Currently, how the methyl group is attached and what are the physiological oxidation states of cobalamin remain open questions. The homolytic cleavage of MeCbl and the formation of a methyl radical intermediate, as elegantly reported in methyl-coenzyme M reductase,<sup>33</sup> would provide a reasonable solution to attach the methyl group to the carbon-centered radical. Cob(II)alamin, after reduction to cob(I)alamin, could react with SAM to regenerate MeCbl. We have previously shown that TsrM catalyzes methyl transfer to sp<sup>2</sup> carbon atoms<sup>2</sup> and rigorously demonstrated that TsrM, contrary to PoyC, does not catalyze the reductive cleavage of SAM or abstracts a substrate H atom.<sup>1,2</sup> It thus appears that, depending on the hybridization of the carbon atom (sp<sup>2</sup> vs sp<sup>3</sup>), B<sub>12</sub>-rSAM enzymes have evolved different mechanisms requiring or not requiring the formation of 5'-dA radical.

Finally, our data show that PoyC catalyzes methyl transfer only on L-amino acid residues. This result indicates that methylation occurs prior epimerization and suggests that PoyB is likely responsible for the synthesis of the N-terminal *tert*-butyl group in polytheonamide A, although other roles cannot be ruled out yet.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b06697.

Experimental procedures and supplementary figures (PDF)

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### Notes

The authors declare no competing financial interest.

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