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The GenK-catalyzed C-6' Methylation in the Biosynthesis of Gentamicin: Isolation and Characterization of a Cobalamin-dependent Radical SAM Enzyme

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Abstract

The existence of cobalamin (Cbl)-dependent enzymes that are members of the radical *S*-adenosyl-L-methionine (SAM) superfamily was previously predicted based on bioinformatic analysis. A number of these are Cbl-dependent methyltransferases but the details surrounding their reaction mechanisms have remained unclear. In this report we demonstrate the *in vitro* activity of GenK, a Cbl-dependent radical SAM enzyme that methylates an unactivated *sp*³ carbon during the biosynthesis of gentamicin, an aminoglycoside antibiotic. Experiments to investigate the stoichiometry of the GenK reaction revealed that one equivalent each of 5'-deoxyadenosine and *S*-adenosyl-homocysteine are produced for each methylation reaction catalyzed by GenK. Furthermore, isotope-labeling experiments demonstrate that the *S*-methyl group from SAM is transferred to Cbl and the aminoglycoside product during the course of the reaction. Based on these results, one mechanistic possibility for the GenK reaction can be ruled out and further questions regarding the mechanisms of Cbl-dependent radical SAM methyltransferases, in general, are discussed.

The radical *S*-adenosyl-L-methionine (SAM, **1**) superfamily of enzymes^{1,2} comprise a diverse array of biological catalysts that share a common [4Fe-4S] cluster coordinated by three cysteines of a highly conserved CxxxCxxC motif in their active sites.³ The fourth iron of the cluster interacts with a bound SAM. Upon reduction to the +1 redox state, the [4Fe-4S]⁺ cluster donates an electron through inner-sphere *e*[−] transfer to trigger the reductive homolysis of the C5'-S bond of SAM (**1**) to produce methionine (**2**) and a 5'-deoxyadenosyl radical (5'-dAdo•, **3**). The latter can be used as a radical initiator in the subsequent chemical transformation catalyzed by the corresponding enzymes (Scheme 1).

A subset of radical SAM enzymes contains a cobalamin (Cbl) binding domain at the *N*-terminus. This expanding group of enzymes is found in a number of biosynthetic pathways,^{4,5} where the individual enzymes are implicated in a wide range of transformations including the methylation of unactivated C and P centers and the formation of the isocyclic ring systems of chlorophyll and bacteriochlorophyll.^{6–9} Thus far, the activities of only two Cbl-dependent radical SAM enzymes have been demonstrated *in vitro*. One is PhpK,¹⁰ which catalyzes methylation of the phosphinate group of 2-acetylamin-4-

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Supporting Information. Details regarding the cloning, expression, refolding, and reconstitution of GenK along with HPLC protocols and a list of abbreviations. This material is available free of charge via the Internet at <http://pubs.acs.org>.

hydroxyphosphinyl-butanoate in the biosynthesis of phosalacine in *Kitasatospora phosalacinea*. A more recent example is TsrM, which catalyzes the conversion of tryptophan to 2-methyl-tryptophan in the biosynthesis of thiostrepton A.¹¹ In both cases the detailed mechanism remains unknown.

Gentamicins are aminoglycoside antibiotics produced by *Micromonospora echinospora*.¹² Sequence analysis of the gentamicin C₁ (**6**) biosynthetic gene cluster suggested that the methylation step at C-6' of GenX₂ (**7**) to yield the clinically useful geneticin (G418, **8**),^{13,14} is likely catalyzed by a Cbl-dependent radical SAM enzyme, GenK (see Scheme 2).¹⁵ This assignment is supported by gene knockout experiments reported recently.^{16,17} An alignment of 13 putative Cbl-dependent radical SAM *sp*³ C-methyltransferases (including GenK, see Fig. S1) suggests that these enzymes harbor a single [4Fe-4S] cluster per monomer because only the cysteine residues comprising the CxxxCxxC motif are conserved. In order to shed light on the mechanisms of the reactions catalyzed by this fascinating set of enzymes, we undertook a study of the GenK-catalyzed reaction.

The *genK* gene was amplified from the genomic DNA of *M. echinospora* using PCR (polymerase chain reaction) and was cloned into a pET24 vector. The recombinant *genK* was heterologously expressed in *Escherichia coli*. Although GenK was quite well overproduced, the enzyme was only detected in insoluble inclusion bodies. Thus, it was subjected to a denaturation (with 5 M urea) and refolding protocol as described in the Supporting Information (SI). The refolded GenK was then incubated with FeCl₃ and Na₂S under anaerobic conditions for reconstitution of the [4Fe-4S] cluster. Unbound iron and sulfide was removed by gel filtration chromatography. The purity of refolded, reconstituted GenK was assessed by SDS-PAGE (Fig. S2). The UV-visible spectrum of reconstituted GenK and the bleaching of the characteristic absorbance shoulder at 420 nm in the presence of sodium dithionite are consistent with the presence of a bound Fe/S cluster (Fig. S2). Further analysis revealed that 3.5 ± 0.9 eq of iron and 3.8 ± 1.1 eq of sulfide are bound to each GenK monomer indicating that GenK contains a single [4Fe-4S] cluster as predicated by the amino acid sequence alignment (Fig. S1).

The activity of GenK was assayed using 1 mM commercially available GenX₂ (**7**) as substrate in 50 mM Tris•HCl buffer (pH 8.0) containing 10 mM DTT, 4 mM SAM, and 1 mM methylcobalamin (MeCbl) or hydroxocobalamin (HOCbl). An experiment comparing GenK activity over a range of MeCbl concentrations showed that GenK activity is nearly maximized in the presence of 1 mM MeCbl (Fig S3). NADPH (4 mM) and methyl viologen (MV, 1 mM) were employed as the electron source and mediator, respectively, to reduce the GenK [4Fe-4S] cluster to the active +1 redox state. A combination of *E. coli* flavodoxin, flavodoxin reductase, and NADPH is also capable of activating GenK (Fig S4). Although dithionite is commonly used to activate radical SAM enzymes for *in vitro* studies, it is not effective for GenK. Neither NADPH and benzyl viologen (BV), dithionite and MV, nor dithionite and BV are capable of activating GenK (Fig S4). The coordination of SO₂⁻ to the corrinoid cobalt¹⁸ may compromise the ability of dithionite to support multiple turnovers with Cbl-dependent radical SAM enzymes.

The GenK reaction was monitored under two different reverse-phase HPLC conditions: one to detect the formation of 5'-dAdo (**4**) and SAH (**5**) and another to detect GenX₂ (**7**) to G418 (**8**). Both are described in the SI. The results shown in Figures 1 and 2 clearly indicate that GenK converts GenX₂ (**7**) to G418 (**8**) in the presence of either MeCbl or HOCbl (but not without Cbl), and that both 5'-dAdo (**4**) and SAH (**5**) are formed as co-products during turnover. Reconstitution of GenK with iron and sulfide is required to obtain activity. Since 5'-dAdo (**4**) is not generated in the absence of GenX₂, its production is coupled to G418 formation. Quantification of 5'-dAdo in trace b and c (Fig. 1) and G418 in traces b and c

(Fig. 2) gives 0.49, 0.32, 0.49, and 0.40 mM, respectively demonstrating that 5'-dAdo and G418 are produced at a ratio close to 1:1. The presence of SAH in assays without GenK indicates that a notable extent of Cbl methylation takes place non-enzymatically under the conditions employed even when MeCbl is used as substrate. This suggests that the C-Co bond of MeCbl is prone to non-enzymatic cleavage during the assay.

In order to confirm the role of cobalamin during methyl transfer from SAM to GenX₂, assays were conducted in the presence of either HOCbl or MeCbl and ¹³CD₃-methyl-SAM followed by mass spectroscopic analysis of G418 and Cbl. As expected, when ¹³CD₃-methyl-SAM was used as a substrate in a GenK reaction, the ¹³CD₃-label was detected in both G418 (Fig. 3) and Cbl (Fig. S5). These observations, together with the established Cbl-dependence of the GenK reaction, indicate that the S-methyl substituent in SAM is transferred to Cbl and, subsequently, to GenX₂ to give G418.

The quantity of 5'-dAdo and SAH produced by GenK was measured after an 8 hr incubation, as a function of [GenK] (2, 4, 6, 8, and 10 μM) revealing a linear relationship between the two (Fig S6). GenK (10 μM) was assayed over a timecourse to determine the stoichiometry of 5'-dAdo and SAH formation (depicted in Fig. 4). The results, obtained after subtraction of non-enzymatically produced SAH, clearly show that 5'-dAdo and SAH are produced in a 1:1 ratio. As mentioned above, Fig. 1 and 2 demonstrate that 5'-dAdo and G418 are also produced in a 1:1 ratio.

Several mechanisms can be envisioned for the GenK-catalyzed conversion of GenX₂ (**7**) to G418 (**8**) (Scheme 3). In the first mechanism (Scheme 3A), the 5'-dAdo• radical (**3**) generated via the reductive cleavage of SAM abstracts a hydrogen atom from C-6' of the substrate **7**. The resulting substrate radical (**9**) then accepts a methyl radical from Me-Cbl to generate Cbl(II) and the G418 product (**8**). A similar mechanism has been proposed for the putative C-methyl-transferase Fom3, which is a Cbl-dependent radical SAM enzyme catalyzing C-2 methylation of 2-hydroxyethylphosphonate in fosfomycin biosynthesis.^{19,20} In this mechanism, Me-Cbl could be regenerated after each turnover via reduction of Cbl(II) to Cbl(I), followed by methylation by SAM.

Given that the transfer of a methyl radical is heretofore unprecedented among the Cbl-dependent methyltransferases, mechanisms involving the transfer of a methyl cation from Me-Cbl were also considered (Schemes 3B and 3C). The early steps of these alternative mechanisms are identical to those of Scheme 3A. However, after hydrogen atom abstraction (**7** → **9**), the radical intermediate **9** is deprotonated to form the ketyl radical **10/11**, similar to those observed in the reactions catalyzed by DesII²¹⁻²³ and (*R*)-2-hydroxyacyl-CoA dehydratase.^{24,25} Nucleophilic attack by **11** at Me-Cbl leads to the transfer of an electrophilic methyl cation (**11** → **12**), yielding a Cbl(I) intermediate (as in traditional Me-Cbl chemistry)^{26,27} and an alkoxide product radical (**12**). The radical **12** could then be quenched by an external electron and proton (Scheme 3B), or via hydrogen atom transfer from 5'-dAdo (**4**) (Scheme 3C). In this latter mechanism, SAM (**1**) is regenerated while restoring the [4Fe-4S] cluster to the +1 redox state. The resultant SAM could then methylate Cbl(I), releasing SAH (**5**) as the sole adenosylated product. Thus, in this mechanism, the input of exogenous electrons is not required and only a single equivalent of SAM is consumed during each catalytic cycle. This is in contrast to mechanisms A and B, in which two equivalents of SAM (**1**) (in addition to two reducing equivalents) are consumed for each methyl transfer event catalyzed by GenK.

The observation that 5'-dAdo (**4**), SAH (**5**), and G418 (**8**) are each produced in equivalent amounts (Figure 4) is consistent with mechanisms A and B, but not C. Thus, mechanism C can be ruled out based on our current data. The catalytic cycle of GenK, and likely other

Cbl-dependent radical SAM methyltransferases, consumes two SAM molecules and two reducing equivalents. The first molecule of SAM acts as the source of 5'-dAdo• (3), which abstracts a hydrogen atom from C-6' of GenX2 (7) to give 5'-dAdo (4) and the GenX₂ radical (9). The second SAM is used to re-methylated Cbl(I) during turnover to regenerate Me-Cbl for a subsequent round of catalysis. Efforts are currently underway to further elucidate the chemical mechanism of this intriguing enzyme. Although it is a technical challenge to study Cbl-dependent radical SAM enzymes, this emerging class of biocatalysts offers considerable potential for the discovery of new and unprecedented enzyme chemistry.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. HPLC traces showing the production of both 5'-dAdo (**4**) and SAH (**5**) during the GenK reaction. Trace (a) is a standard composed of authentic 5'-dAdo (**4**) and SAH (**5**). The remaining traces result from reaction mixtures prepared as described in the text containing (b) MeCbl, (c) HOCbl, (d) MeCbl but no GenK, (e) HOCbl but no GenK, (f) MeCbl but no GenX₂, (g) no Cbl, (h) MeCbl and non-reconstituted GenK. Detector was set at 260 nm.

Figure 2.

HPLC traces showing conversion of GenX₂ (**7**) to G418 (**8**) in the presence of GenK. Prior to HPLC analysis, the assay mixture was subjected to the reaction with 1-fluoro-2,4-dinitrobenzene (DNFB) (see SI for details). Absorbance was monitored at 340 nm to detect FDNB-derivatized aminoglycosides. Trace (a) is a standard composed of derivatized, authentic GenX₂ (**7**) and G418 (**8**). Traces b–h are as described in the legend of Fig. 1. The small peaks at ~16.6 min in traces d–g and ~15.4 min in trace f are unrelated to product and substrate.

Figure 3.

Mass spectra of aminoglycoside substrate and product displaying incorporation of $^{13}\text{CD}_3$ from $^{13}\text{CD}_3$ -methyl-SAM into product. The spectra correspond to assays with (a) HOCbl + unlabeled SAM, (b) HOCbl + $^{13}\text{CD}_3$ -methyl-SAM, (c) HOCbl + unlabeled SAM without GenK, (d) HOCbl + $^{13}\text{CD}_3$ -methyl-SAM without GenK, (e) MeCbl + unlabeled SAM, (f) MeCbl + $^{13}\text{CD}_3$ -methyl-SAM, (g) MeCbl + unlabeled SAM without GenK, (h) MeCbl + $^{13}\text{CD}_3$ -methyl-SAM without GenK.

Figure 4.
Stoichiometry of 5'-dAdo and SAH production during the GenK reaction.

Scheme 1.
Initial reaction catalyzed by radical SAM enzymes.

Scheme 2.

The C-6' methylation reaction catalyzed by GenK in the gentamicin biosynthetic pathway.

Scheme 3.

Possible GenK reaction mechanisms. (A) GenX₂ radical (**9**) is quenched by the transfer of methyl radical from Me-Cbl(III) to give Cbl(II), which is reduced to Cbl(I) in order to accept a new methyl group from SAM. (B) Transfer of methyl cation to GenX₂ ketyl radical (**11**) followed by reduction and protonation of product radical (**12**). (C) Product radical (**12**) is quenched by a hydrogen from 5'-dAdo (**4**) to give 5'-dAdo• (**3**), which can be re-incorporated into SAM. In this mechanism, a single equivalent of SAM can serve as a source of both 5'-dAdo• (**3**) and CH₃⁺.