GenK-Catalyzed C-6′ Methylation in the Biosynthesis of Gentamicin: Isolation and Characterization of a Cobalamin-Dependent Radical SAM Enzyme

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S *Supporting Information*

ABSTRACT: The existence of cobalamin (Cbl)-dependent enzymes that are members of the radical *S*-adenosyl-L-methionine (SAM) superfamily was previously predicted on the basis of 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 1 equiv 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. On the basis of 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] comprises 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

Scheme 1. Initial Reaction Catalyzed by Radical SAM Enzymes
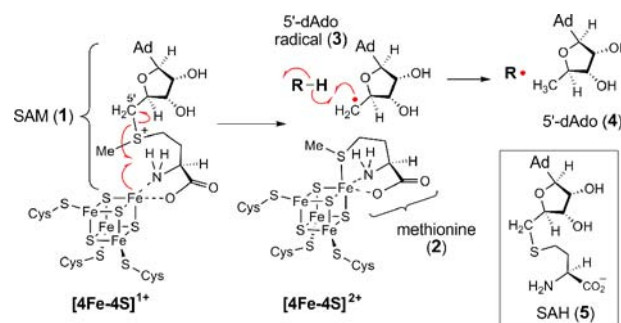
demonstrated *in vitro*. One is PhpK,[10] which catalyzes methylation of the phosphinate group of 2-acetylamino-4-hydroxyphosphinyl butanoate in the biosynthesis of phosalacine in *Kitasatospora phosalacinea*. A more recent example is TsrM from *Streptomyces laurentii*, which catalyzes the conversion of tryptophan to 2-methyltryptophan 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 C_1 (**6**) biosynthetic gene cluster suggested that the methylation step at C-6′ of GenX$_2$ (**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

Scheme 2. C-6′ Methylation Reaction Catalyzed by GenK in the Gentamicin Biosynthetic Pathway

gentamicin X$_2$ (**7**)   G418 (**8**)   gentamicin C$_1$ (**6**)
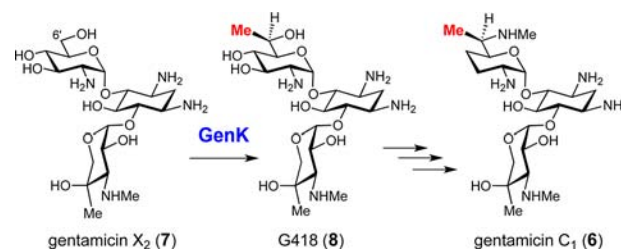
assignment is supported by gene knockout experiments reported recently.[16,17] An alignment of 13 putative Cbl-dependent radical SAM sp^3 C-methyltransferases (including GenK, see Figure S1) suggests that these enzymes harbor a single [4Fe-4S] cluster per monomer because only the cysteine residues comprising 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 $FeCl_3$ and Na_2S 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 (Figure 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 (Figure S2). Further analysis revealed that 3.5 ± 0.9 equiv of iron and 3.8 ± 1.1 equiv 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 (Figure S1).

The activity of GenK was assayed using 1 mM commercially available $GenX_2$ (**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 (Figure 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 (Figure 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 (Figure S4). The coordination of SO_2^- to the corrinoid cobalt[18] 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 the conversion of $GenX_2$ (**7**) to G418 (**8**). Both are described in the SI. The results shown in Figures 1 and 2 clearly indicate that GenK converts $GenX_2$ (**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 $GenX_2$, its production is coupled to G418 formation. Quantification of 5′-dAdo in traces b and c in Figure 1, and G418 in traces b and c in Figure 2, gives 0.49, 0.32, 0.49, and 0.40 mM, respectively, demonstrating that 5′-dAdo and
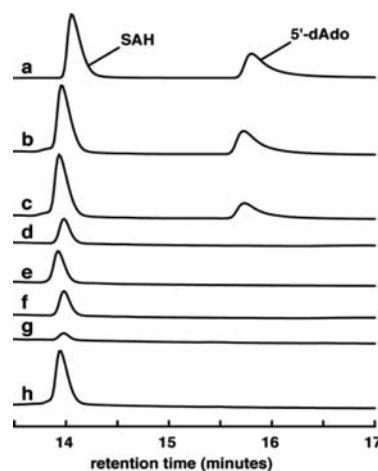

Figure 1. HPLC traces showing the production of both 5′-dAdo (**4**) and SAH (**5**) during the GenK reaction. Trace a is a standard composed of authentic 5′-dAdo (**4**) and SAH (**5**). The remaining traces result from reaction mixtures prepared as described in the text containing (b) MeCbl, (c) HOCbl, (d) MeCbl but no GenK, (e) HOCbl but no GenK, (f) MeCbl but no $GenX_2$, (g) no Cbl, (h) MeCbl and non-reconstituted GenK. Detector was set at 260 nm.
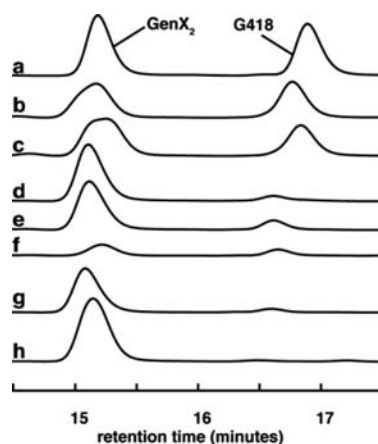
Figure 2. HPLC traces showing conversion of $GenX_2$ (**7**) to G418 (**8**) in the presence of GenK. Prior to HPLC analysis, the assay mixture was subjected to the reaction with 1-fluoro-2,4-dinitrobenzene (DNFB) (see SI for details). Absorbance was monitored at 340 nm to detect DNFB-derivatized aminoglycosides. Trace a is a standard composed of derivatized, authentic $GenX_2$ (**7**) and G418 (**8**). Traces b−h are as described in the legend of Figure 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.

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 $GenX_2$, assays were conducted in the presence of either HOCbl or MeCbl and $^{13}CD_3$-*methyl*-SAM followed by mass spectroscopic analysis of G418 and Cbl. As expected, when $^{13}CD_3$-*methyl*-SAM was used as a substrate in the GenK reaction, the $^{13}CD_3$-label was detected in both G418 (Figure 3) and Cbl (Figure S5). These observations, together with the established Cbl-dependence of the GenK reaction,
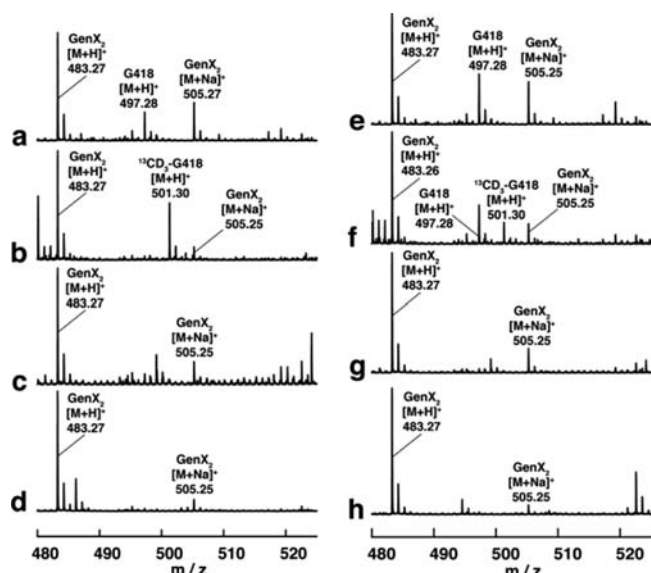
Figure 3. Mass spectra of aminoglycoside substrate and product displaying incorporation of $^{13}CD_3$ from $^{13}CD_3$-*methyl*-SAM into product. The spectra correspond to assays with (a) HOCbl + unlabeled SAM, (b) HOCbl + $^{13}CD_3$-*methyl*-SAM, (c) HOCbl + unlabeled SAM without GenK, (d) HOCbl + $^{13}CD_3$-*methyl*-SAM without GenK, (e) MeCbl + unlabeled SAM, (f) MeCbl + $^{13}CD_3$-*methyl*-SAM, (g) MeCbl + unlabeled SAM without GenK, and (h) MeCbl + $^{13}CD_3$-*methyl*-SAM without GenK.

indicate that the *S*-methyl substituent in SAM is transferred to Cbl and, subsequently, to GenX$_2$ to give G418.

The quantity of 5′-dAdo and SAH produced by GenK was measured after an 8 h incubation, as a function of [GenK] (2, 4, 6, 8, and 10 μM), revealing a linear relationship between the two (Figure S6). GenK (10 μM) was assayed over a time course to determine the stoichiometry of 5′-dAdo and SAH formation (depicted in Figure 4). The results, obtained after
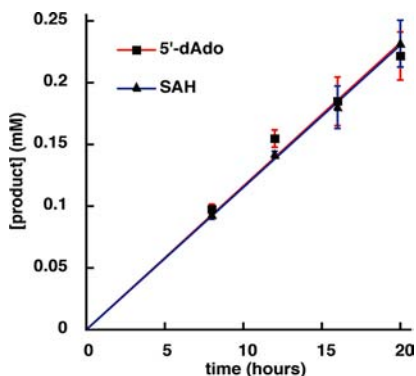

Figure 4. Stoichiometry of 5′-dAdo and SAH production during the GenK reaction.

subtraction of non-enzymatically produced SAH, clearly show that 5′-dAdo and SAH are produced in a 1:1 ratio. As mentioned above, Figures 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 GenX$_2$ (**7**) to G418 (**8**) (Scheme 3). In the first mechanism (Scheme 3A), the 5-dAdo• (**3**) generated via the reductive cleavage of SAM abstracts a hydrogen atom from C-6′ of the substrate **7**. The resulting

Scheme 3. Possible GenK Reaction Mechanisms^a
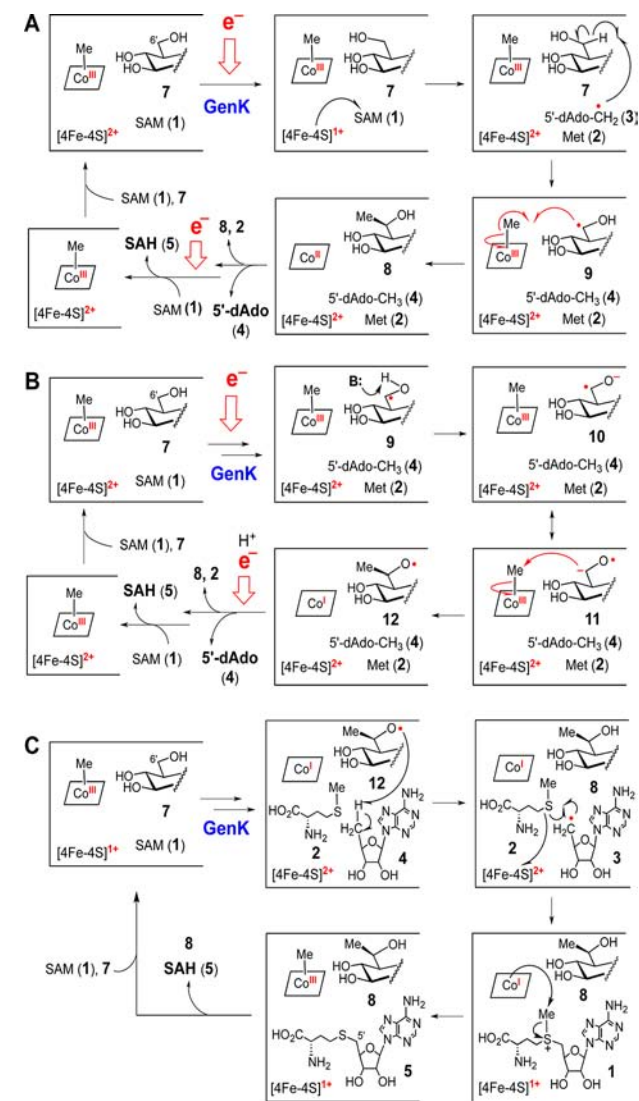
^{a}(A) GenX$_2$ radical (**9**) is quenched by the transfer of methyl radical from Me-Cbl(III) to give Cbl(II), which is reduced to Cbl(I) in order to accept a new methyl group from SAM. (B) Transfer of methyl cation to GenX$_2$ ketyl radical (**11**) followed by reduction and protonation of product 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 CH$_3^+$.

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 (Scheme 3B,C). 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 DesII[21−23] 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 alkoxy 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 on the basis of 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 GenX$_2$ (**7**) to give 5′-dAdo (**4**) and the GenX$_2$ radical (**9**). The second SAM is used to re-methylate 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.

■ ASSOCIATED CONTENT

Ⓢ 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.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

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