Structural Basis of the Selectivity of GenN, an Aminoglycoside N-Methyltransferase Involved in Gentamicin Biosynthesis

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ABSTRACT: Gentamics are heavily methylated, clinically valuable pseudotrisaccharide antibiotics produced by Micromonospora echinospora. GenN has been characterized as an S-adenosyl-l-methionine-dependent methyltransferase with low sequence similarity to other enzymes. It is responsible for the 3"-N-methylation of 3"-dehydro-3"-amino-gentamicin A2, an essential modification of ring III in the biosynthetic pathway to the gentamicin C complex. Purified recombinant GenN also efficiently catalyzes 3"-N-methylation of related aminoglycosides kanamycin B and tobramycin, which both contain an additional hydroxymethyl group at the C5" position in ring III. We have obtained eight cocrystal structures of GenN, at a resolution of 2.2 Å or better, including the binary complex of GenN and S-adenosyl-l-homocysteine (SAH) and the ternary complexes of GenN, SAH, and several aminoglycosides. The GenN structure reveals several features not observed in any other N-methyltransferase that fit it for its role in gentamicin biosynthesis. These include a novel N-terminal domain that might be involved in protein:protein interaction with upstream enzymes of the gentamicin X2 biosynthesis and two long loops that are involved in aminoglycoside substrate recognition. In addition, the analysis of structures of GenN in complex with different ligands, supported by the results of active site mutagenesis, has allowed us to propose a catalytic mechanism and has revealed the structural basis for the surprising ability of native GenN to act on these alternative substrates.

Methylation is an important reaction in the biosynthesis of specialized metabolites in both microorganisms and plants, where methylation on (principally) oxygen, nitrogen, or carbon contributes significantly to the chemical diversity and bioactivity of the products. Gentamincins are clinically valuable aminoglycoside antibiotics constructed from three modified sugar units, including the unusual aminocyclitol 2-deoxystreptamine (2-DOS). Gentamincins inhibit protein synthesis by interfering with initiation, codon fidelity, and translation. They are isolated from the filamentous bacterium Micromonospora echinospora as the gentamicin C complex, a mixture of five components differing in their methylation patterns (Figure 1A). Gentamicin C complex is effective against life-threatening sepsis caused by Gram-negative bacterial infections. Unfortunately, these vital drugs entail a serious risk of kidney damage and hearing loss, so there is great interest in generating novel gentamicin components with a potentially greater therapeutic window.

The later, diversity-generating steps in the biosynthetic pathway to gentamicins have been intensively studied in recent years, greatly aided by the sequencing of the gentamicin gene cluster. The pseudotrisaccharide intermediate 3"-dehydro-3"-amino-gentamicin A2 (DAA2) is first converted to gentamicin A by transfer of a methyl group to the amino group at position 3" in ring III11,12 (Figure 1A). The cobalamin-dependent radical SAM C-methyltransferase GenD1 then methylates the C4" in ring III of gentamicin A to produce gentamicin X2,13 the branch point in gentamicin biosynthesis. Further methylation at the C6" position in ring II of X2 catalyzed by GenK, a second cobalamin-dependent radical SAM C-methyltransferase,13-16 yields the intermediate G418, which undergoes amination and loss of ring II hydroxy groups to give gentamicins C2a and C2. Analogous amination and dihydroxylation of gentamicin X2 yields gentamicin C1a (Figure 1A). The final two components of the gentamicin C complex, C2b and C1, are formed by N-methyl transfer to the amino group at the 6"-position in, respectively, gentamicin C1a and gentamicin C2. The catalyst(s) for these terminal steps have not been identified. In contrast, GenN has been previously shown to catalyze.

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methylation of the 3'-amino group in both kanamycin B (KanB) and tobramycin (Tob; Figure 1B), indicating the potential for this enzyme to generate novel aminoglycosides.\textsuperscript{11} GenN is an enzyme with low sequence similarity to other N-methyltransferases, with the exception of Sis30 from the biosynthetic pathway to sisomicin, an aminoglycoside closely related to gentamicin.\textsuperscript{17} There is increasing interest in using such class I S-adenosyl methionine- (SAM) dependent methyltransferases\textsuperscript{18} that act within biosynthetic pathways as tools for biocatalysis and biotransformation.\textsuperscript{19} Certain of these methyltransferases have been shown to accept a relatively broad range of substrates,\textsuperscript{19–21} while others have narrow substrate tolerance.\textsuperscript{3} Structural insights are therefore indispensable to future efforts to understand and engineer substrate preference.

Figure 1. (A) Methylation steps in gentamicin biosynthetic pathway. (B) GenN catalyzed 3'-N-methylation of kanamycin B and tobramycin.
We have determined the crystal structure at atomic resolution of GenN, in ternary complex with the reaction product S-adenosylhomocysteine (SAH) and each of several aminoglycoside ligands, and we have used site-directed mutagenesis to probe the roles of specific amino acid residues in binding and catalysis. This has provided insight into both the catalytic mechanism of GenN and the structural basis for its ability to accept these alternative substrates and underlined its promise as a tool for regiospecific methylation of further aminoglycoside templates.

**RESULTS AND DISCUSSION**

**Overall Structure.** The crystal structure of GenN was solved in cocomplex with S-adenosylhomocysteine (SAH) and several different substrates or products: the binary complex of GenN–SAH and the ternary complexes of GenN–SAH–DAA2, GenN–SAH–3′-N-methyltrobramycin (GenN–SAH–Tobm), GenN–SAH–KanB, GenN–SAH–3′-N-methylkanamycin B (GenN–SAH–KanBm), GenN–SAH–gentamicin X2 (GenN–SAH–X2), GenN–SAH–G418, and GenN–SAH–Tris(hydroxymethyl)aminomethane (GenN–SAH–Tris). GenN, in all these complexes, crystallized in the orthorhombic space group P2₁2₁2₁ with a single monomer in the asymmetric unit, and the crystals diffracted to a maximum resolution between 1.9 Å and 2.2 Å (Supporting Information Table 1). The protein exists in the crystal as a monomer, consistent with its behavior in solution during gel filtration. The crystal structure of the GenN–SAH–Tris was initially solved by single anomalous dispersion (SAD) after soaking with sodium iodide. Thirteen iodine atom peaks were identified which were used to calculate phases and to produce the initial electron density map. The other seven structures were solved by molecular replacement using the structure of the GenN–SAH–Tris complex as a search model.

The GenN structure (Figure 2A) is composed of two domains, a predominantly α-helical N-terminal domain (residues Met1–78) connected to a larger C-terminal SAM-binding domain (residues Pro79–321) containing the active site. The latter domain shares the general canonical fold of class I methyltransferases, closely related to the Rossmann nucleotide-binding fold, and consisting of a conserved eight-stranded β-sheet (1/β3, 1/β2, 1/β1, 1/β4, 1/β5, 1/β7, 1/β6, and 1/β8, Figure 2B) sandwiched between six α-helices and a β-hairpin (Figure 2A and C). As in other class I methyltransferases, the order of these eight strands is 3-2-1-4-5-7-6-8 with the seventh strand antiparallel to the others. However, GenN is distinctive in having an eighth strand of the β-sheet (β8) lying parallel to the sixth and appears to form a new topological subclass (class Io). The SAM and aminoglycoside binding sites are in the N-terminal portion of the β-sheet, between strands 1 and 2 (Figure 2C). GenN does not share extensive sequence similarity with other methyltransferases. A search using the program DALI identified the closest match to be the putative methyltransferase MJ0882 from *Methanococcus jannaschii* with a Z score of 17.1 and a RMSD of 3.0 Å (Figure 2D and Supporting Information Figure 1). The similarity between the GenN and MJ0882 sequences is 34%, extending over only 27% of the sequence. MJ0882 has a β-hairpin in the SAM binding domain as in GenN, but it has neither the extra eighth strand in the central β-sheet nor the N-terminal domain (Figure 2D). Other related topological...
structures revealed by DALI include 5'-methyluridine methyltransferase from *Thermus thermophilus* (Z score = 16.6), PrmC, a HemK methyltransferase involved in the methylation of RF1 during protein synthesis (Z score = 16.4), and the tRNA(m1G37) methyltransferase aTrm5 from *Methanococcus jannaschii* (Z score = 16.3). GenN does, however, match the classical SAM/SAH binding motifs identified for the class I methyltransferases, including the glycine-rich consensus “GXGXG” at the C-terminal portion of strand 1 of the central β-sheet, which interacts with the carboxypropyl portion of SAM/SAH, the acidic residue in motif II (Asp152 at the C-terminus of β2 in GenN) which hydrogen bonds to the ribose of SAM/SAH, and the acidic residue in motif III (Asp179 located between the β3 and β4) that interacts with the adenosyl group (Figure 2D and Supporting Information Figure 1).

Analysis of the electrostatic surface of GenN reveals a large noncharged groove between the N- and C-terminal domains (Supporting Information Figure 2) indicating some potential for protein–protein interaction. The N-terminal subdomain of GenN is composed of a four α-helical bundle and a small β-hairpin (Figure 2A and B). A DALI search using this subdomain yielded the best, albeit low, Z scores (2.0–4.5), indicating a topology shared with several O-methyltransferases involved in the biosynthesis of natural products. The N-terminal domains in these O-methyltransferases are involved in homodimer formation. In contrast, GenN is monomeric in solution, although the results of kinetic experiments have suggested it might form a functional complex with neighboring enzymes GenD2 and GenS2 in the gentamicin biosynthetic pathway. Such an arrangement has been reported for coupled enzymes in the biosynthesis of 3,6-dideoxyhexose and of the aminoglycoside apramycin.

**The SAM/SAH Binding Site.** Although we initiated co-crystallization of GenN with SAM, either in the presence or in the absence of aminoglycoside, SAH rather than SAM was observed at the active site in all the binary and ternary complex structures, with or without 3’-N-methylation product. This is a very common observation in the crystallization of methyltransferases. The SAH molecule binds in a buried position between strands 1 and 2 of the central β-sheet (Figure 1B) and makes extensive hydrophobic and hydrogen bonding interactions with the protein (Figure 3A and Supporting Information Figure 3). These interactions include hydrogen bonding between the backbone amide oxygen of Gly131 and Cys132 in the glycine-rich motif I and the nitrogen of the carboxypropyl moiety of SAM/SAH. This nitrogen atom also interacts, via water molecules, with the carboxylate in the side chain of Asp129, the backbone amide nitrogens of Leu137 and Asn194, and the backbone amide oxygens of Met130 and Gly131. The Asp152 (motif II) interacts with the ribosyl hydroxyl groups of SAM/SAH, which also interact via water molecules with the backbone amide nitrogen of Asp107 and the backbone amide oxygen of Tyr105. The adenine moiety of SAM/SAH hydrogen-bonds to the carboxyl group of Asp179 (motif III) and to the backbone nitrogen of Met180. In other methyltransferases, the adenine moiety is located in a highly hydrophobic pocket, which in GenN is formed by the side chains of Met130, Val153, Met180, Pro196, Phe204, Leu207, Ala210, and Ile214 (Figure 3A, Supporting Information Figures 3 and 4).

Superimposition of the structures of all the solved GenN complexes shows that the mode of SAM/SAH binding, in an extended conformation typical of class I methyltransferases, is well conserved, with no major active site differences between SAH substrate and SAH product ternary complexes. The presence of 3’-N-methylated product caused a shift of only about 0.6 Å in the position of the sulfur when compared with the structure containing the corresponding nonmethylated ligand (Figure 2B). All attempts to crystallize uncomplexed GenN have failed, so it remains unclear whether there are significant conformational changes upon SAM/SAH binding.

**The DAA2 Binding Site.** GenN-catalyzed methylation using SAM as the methyl donor converts DAA2 into gentamicin A (Figure 1). In the ternary complex of GenN–SAH–DAA2, DAA2 binds between strands 1 and 4 of the central β-sheet on the side opposite to the SAM/SAH binding site, with the 3’-amino group pointing toward the position of the methyl group of SAM (Figure 4A). The DAA2 binding cavity is almost exclusively defined by interactions with two long loops of the SAM-binding domain: L1 (residues 92–112), which connects the β-hairpin to the SAM binding domain, and L8 (residues 194–212), which connects strand β4 to helix α4 (Figure 4B). In all other class I methyltransferases, the position and size of the L1 and L8 loops are dramatically different, implicating L1 and L8 in determining the specificity of GenN for its aminoglycoside substrate. Primary sequence alignment of GenN with several N-methyltransferases whose structures have been solved also indicates that these regions are not conserved (Supporting Information Figure 1). Eight of the 21 residues of L1 and six of the 19 residues of L8 are negatively charged, to match the polycationic DAA2 (Figure 1). Glu98 in L1 hydrogen-bonds to the amino group of the glucosamine ring.
3'-N-methyl group and a 4''-C-methyl group, as well as a 6'-methyl group in the case of G418. These structures are essentially superimposable on the ternary complex with SAH and DAA2. Notably, the 4''-C-methyl group of X2 and G418 does not clash sterically with any active site residues (Supporting Information Figures 7A,B and 8), a hint that GenN might tolerate substrates bearing an equivalent modification at this position of ring II.

**Residues Potentially Involved in the Catalytic Mechanism of GenN.** Methyl transfer in SAM-dependent methyltransferases requires the close approach (to within about 3 Å) and precise orientation of the substrates to promote S_N2-like in-line nucleophilic attack by the acceptor on the methyl group of SAM. In the GenN–SAH–DAA2 ternary complex, the carbonyl of the amide side chain of Asn194 forms a hydrogen bond with the 3'-amino group of DAA2 (2.9 Å), helping to activate the amino group for nucleophilic attack on the electron-deficient methyl group of SAM. Two more hydrogen bonds, between the 3'-amine of the DAA2 and the main chain amide oxygen of Ser195 (3.1 Å) and with the carboxylate side chain of Asp107 (3.5 Å), reinforce the nucleophilicity of the 3'-nitrogen and would help stabilize the positive charge on this center arising during methyl transfer. At the same time, the amino group of the Asn194 carboxamide side chain closely (2.7 Å) interacts with the carboxyl group of SAM/SAH (Figure 5A), a potentially crucial interaction to bring the two substrates together for the methyl transfer to occur. The distance found between the 3'-amine of the DAA2 and the sulfonium moiety of SAH is 3.5 Å (Figure 4A), well within the expected range. A kink caused by Pro196 plays a key role in aligning the backbone amide NH group of Thr197 to make a hydrogen bond (2.9 Å) to the backbone carbonyl of Ser195, stabilizing the interaction between Ser195 and the 3'-amine. The role played by the "NSPT" (Asn194–Ser195–Pro196–Thr197) motif in GenN (Figure 5A and Supporting Information Figure 1) is similar to that of the conserved (D/N)PPY motif in the protein N(5)-glutamine methyltransferase PrmC/HemK, which positions the amide nitrogen of the glutamine residue. The same catalytic strategy has been reported for TaqI DNA methyltransferase. As shown in Figure 5B, a 100% conversion of DAA2 (10 nmoles) to gentamicin A by wild-type GenN (0.15 nmoles) was obtained after 10 min of incubation at 30 °C. A conservative Asp107 to Asn194 mutant of GenN (D107N) exhibited only 23% of the activity under the same assay conditions. Replacing the Asn194 with Asp (N194D), which presumably still retains the ability to deprotonate the 3'-amine but lost the interaction with the carboxyl group of SAM/SAH (Figure 5A), significantly decreased the activity to around 10% of the wild type enzyme. Therefore, none of these residues is uniquely essential for catalysis, consistent with a mechanism for catalysis in which numerous active site interactions combine to define the productive conformation of the substrates. The stability of the D107N and N104D GenN mutants was probed by circular dichroism, and characteristic curves for a protein containing both α helices and β sheets were obtained, indicating the integrity of the mutant proteins (Supporting Information Figure 9A–C).

**The Structural Basis for Kanamycin B and Tobramycin As Alternative Substrates for GenN.** We have previously shown that GenN is able to methylate both KanB and Tob to produce, respectively, 3'-N-methylkanamycin B (KanBm) and 3'-N-methyltobramycin (Tobm). Both kanamycin and tobra-
mycin (Figure 1) are analogues of DAA2 that differ in having an extra hydroxymethyl group at the C4″ position, and tobramycin also lacks the 3′-hydroxy group (Figure 1B). Crystals of ternary complexes of GenN–SAH–KanB, GenN–SAH–KanBm, and GenN–SAH–Tobm (Supporting Information Figure 7C–E) were obtained by cocrystallization with the alternative substrates in the presence of SAM. The presence of KanBm in the GenN active site reveals that methyl transfer occurred to some extent during the process of crystallization. The structures of all these complexes were very similar to the structures of the binary complex GenN–SAH and the ternary complexes GenN–SAH–DAA2 and GenN–SAH–Tris, with near-identical orientation of the ligands within the active site. This suggests a relatively rigid structure of GenN unaffected by the binding of substrates/products, in contrast with certain other methyltransferases where loops poorly defined in the structure of the binary enzyme–SAH complex become well-ordered in ternary complexes.22,37,38

A comparison of the conformation of active site residues in the several ternary complexes, and their likely hydrogen-bonding interactions with the aminoglycoside substrate reveal a plausible basis for the initially surprising ability of GenN to methylate kanamycin B and tobramycin. Although the hydrogen bond observed between the nucleophilic 3′-amino group of DAA2 and the side chain of Asp107 is missing in the structures of GenN–SAH–KanB, GenN–SAH–KanBm, and GenN–Tobm–SAH, this is compensated by a new hydrogen bond donated from the 4″-hydroxymethyl group of KanB or Tob to the side chain carboxamide of Asn198 (Figure 6). As a result, the 3″-amino group in these complexes remains at almost the same position as in GenN–SAH–DAA2 and is still the most chemically reactive and best positioned nucleophile in the vicinity of the methyl group of SAM (Figure 6). A total of 89% and 54% of KanB and Tob, respectively, were methylated by wild-type GenN after incubation at 30 °C for 10 min (Figure 5B). The weaker activity of GenN on KanB and Tob than on

Figure 5. Putative catalytic residues of GenN and the effect of their mutation on the activity of GenN. (A) NSPT motif and proposed catalytic mechanism of GenN. (B) 3″-N-methylation of DAA2, kanamycin B, and tobramycin catalyzed by wild-type GenN and its mutants (N194D, D107N, N198D, and N198L) after 10 min incubation at 30 °C. The error bars stand for standard deviation of duplicate data points.

Figure 6. Binding of kanamycin B, 3″-N-methylkanamycin B, and SAH in GenN–SAH–KanB (green) and GenN–SAH–KanBm (light blue) complexes. The distance between the 3″-N of kanamycin B and the interacting NSPT motif is indicated.
DAA2 could be explained by the fact that only two residues (the side chain of Asn194 and the backbone carbonyl of Ser195), instead of three as with DAA2, are carrying out the deprotonation of the target 3'-amine. It is therefore not surprising that the N194D mutation was more detrimental to the activity of GenN toward KanB and Tob (Figure 5B). N198L mutation did not affect the activity of GenN on DAA2. The same mutant, however, was significantly less active on KanB and Tob. Conservative mutant N198D, which presumably is still able to hydrogen bond with the 4'-hydroxymethyl group of KanB or Tob, showed similar activity to the wild-type enzyme (Figure 5B). These observations support the notion that the interactions between the Asp198 side chain and the 4'-hydroxymethyl group of KanB or Tob plays a role in stabilizing the binding of these substrate analogs in the enzyme.

**Conclusion.** Aminoglycosides are clinically valuable natural products, and gentamicin is one of the most functionalized molecules of this group. Gentamicin is of particular interest due to its effectiveness against sepsis caused by Gram-negative bacteria and the potential of related metabolites in the treatment of human inherited diseases that are associated with premature termination codons. The biosynthesis of gentamicin has been intensively studied over the past few years, and several methyltransferases have been characterized in vivo and in vitro, including the class I SAM-dependent N-methyltransferase GenN, which has low sequence similarity to any other N-methyltransferase. The fact that GenN is active against alternate aminoglycoside substrates opens the possibility of exploring its use to generate novel methylated aminoglycoside derivatives. In the class I SAM-dependent methyltransferases, a highly conserved SAM-binding fold has evolved to accommodate a multiplicity of catalytic mechanisms, including metal-dependent catalysis, involvement of a specific general base, electrostatic interactions, and deployment of numerous hydrogen-bond interactions to stabilize the transition state. The structure of GenN described herein has provided the first insight into the structure, function, and mechanism of N-methyltransferases handling highly basic intermediates in aminoglycoside biosynthesis. The GenN structure has several special features, not observed in other N-methyltransferases, which confer its ability to bind polycationic molecules. In addition, a GenN mechanism of catalysis is proposed for which support has been gained by analysis of the activity of specific mutants. Finally, this work has also allowed the rationalization of the previous finding of excellent GenN activity against KanB and Tob and provides the starting point for future efforts to harness the enzyme for production of novel aminoglycosides.

**METHODS**

**Cloning, Expression, and Purification of GenN.** The genN gene from *Micrococcus chinosorus* ATCC15835 was cloned into plasmid pET28a (+), expressed in *E. coli* BL21(DE3) cells (Novagen), and purified to apparent homogeneity by nickel-NTA affinity chromatography and gel filtration as previously described. The N-terminal His-tag was retained in subsequent experiments. Briefly, *E. coli* BL21(DE3) cells harboring the GenN expression plasmid were grown at 37 °C to an A600 of 0.6−0.8. The overexpression of GenN was induced by the addition of isopropyl β-D-thiogalactopyranoside (0.2 mM) and culturing at 18 °C for 20 h. Cells were harvested by centrifugation (4000g, 4 °C, 30 min) and resuspended in lysis buffer (50 mM Tris-HCl (pH 7.8), 150 mM NaCl). GenN was eluted using a linear gradient of imidazole (up to 500 mM) in a buffer of 50 mM Tris-HCl (pH 7.8) and 150 mM NaCl on an Äkta Purifier (GE Healthcare). Fractions containing GenN were concentrated to 15−20 mg mL$^{-1}$, and the enzyme was stored at −80 °C until use.

**Crystallization and Diffraction.** Crystallization trials were carried out using the sitting-drop method. GenN at 10 mg mL$^{-1}$ was incubated in the presence of 5 mM SAM and submitted to crystallization screening using Crystal Screens I and II and SaltRx (Hampton Research), Wizard I and II and Precipitant Synergy (Emerald Biosystem), and JCSG+ and PACT (Qiagen). The initial trials were performed using a Honeybee robot at the crystallization facility of LNBlu-Campinas-Brazil using the sitting drop method in 96 well plates. The drops had a total volume of 0.4 μL, comprising equal volumes of protein solution and well solution. The plates were stored in an automated imaging system (Roche Image 1000, Formulatrix) at 20 °C. Crystals of GenN appeared after 3−4 days, and the best conditions were optimized manually by hanging drop vapor diffusion using 24 well Linbro plates. The best and most reproducible crystallization conditions for GenN were (1) 0.1 M MgCl$_2$, 0.1 M Tris-HCl (pH 8.5), 20% (v/v) PEG 400, 20% (w/v), and PEG 8000 and (2) 0.2 M CaCl$_2$, 0.1 M HEPES (pH 7.0), and 20% (w/v) PEG 6000. The crystals were allowed to grow for approximately 10 days. To obtain the structure of GenN in ternary complex with different aminoglycosides and SAH, the protein was incubated for 10 min in the presence of 5 mM SAM and 10 mM of the aminoglycoside ligands, KanB, X2, Tob, G418, and DAA2. DAA2 was isolated and purified from a fermentation culture of the ΔgenN mutant of ATCC15835. The identity and the purity of DAA2 was confirmed by LC-ESI-HRMS (Supporting Information, Method 1 and Figure 10). All other aminoglycosides used are commercially available products (KanB and X2 from TOKU-E and Tob and G418 from Sigma). All the ternary complexes with SAH and various aminoglycoside ligands were crystallized under condition 1, while the crystals of the binary complex with SAH were obtained under condition 2.

**Data Collection and Processing and Structure Determination.** Data collection of GenN crystals was carried out at different Synchrotron facilities as indicated in Table S1. The data sets were processed using the program XDS$^{40}$ and scaled using AIMLESS$^{41}$ from the CCP4i suite. The structure of GenH-SAH-Tris was determined by single wavelength anomalous (SAD) diffraction using the incorporation of iodine atoms. For this, sodium iodide was added (to 100 mM) to a well solution containing native crystals of GenN, and the mixture was incubated for 5 min. The anomalous signal was checked by the program XDS,$^{42}$ and the structure determination was performed using the program Phaser and AutoSol$^{43}$ from the Phenix suite.$^{44}$ The structure was refined using Phenix.refine$^{45}$ and REFMAC.$^{46}$ Manual building, visual inspection, and analysis were carried out using COOT,$^{47}$ and further analysis and figure preparation was performed using the PyMOL Molecular Graphics System, Version 1.8 (Schrödinger, LLC). The quality of the structure was checked using the program Molprobity (Supporting Information Table 1).$^{48}$

**GenN Mutagenesis and Assay of Native and Mutant Enzymes.** Site-directed mutants of GenN were generated using the QuickChange method (Strategene; see Supporting Information Table 2 for sequences of PCR primers) using the wild-type genN gene inserted in a pLEX-A2 vector (Eurofins) between the Ndel and BamHI sites as the template. PCR amplifications were carried out using HF Phusion DNA polymerase (NEB) with 30 cycles of denaturation at 98 °C for 10 s, annealing at 60 °C for 30 s, and extension at 72 °C for 2 min followed by a final extension at 72 °C for 10 min. The resulting PCR products were digested with DpnI at 37 °C for 1 h to remove the template before being introduced into *E. coli* Novabla...
(Novagen). The Ndel/BamHI fragments of the plasmids bearing the desired mutation were purified and inserted into plasmid pET28a (+), and the recombinant plasmids were verified by DNA sequencing. The mutant enzymes were expressed and purified from E. coli as for the wild-type GenN. Circular dichroism measurements were performed to assess the stability of the GenN mutants using a J810 spectropolarimeter (JASCO) with a Peltier temperature controller and a quartz cuvette of 0.04 cm optical path. The wild type and mutant GenN proteins were measured at a concentration of 0.166 mg mL⁻¹ in a buffer constituted of 10 mM potassium phosphate and 150 mM NaCl, at pH 7.5. Spectra were obtained between the wavelengths of 184 and 260 nm with a scanning speed of 50 nm min⁻¹. Origin 2016 was used to plot the curves. End-point assays of GenN activity were carried out using 2 mM SAM as a methyl donor, 0.4 mM substrate, and 10 μM enzyme in 50 mM Tris-HCl, at pH 7.5 at 30 °C for 10 min. Product formation was monitored by LC-ESI-MS (Supporting Information Method 2).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.7b00466.

Figures S1–S10, Tables S1 and S2, and supplementary Methods (PDF)

Accession Codes

Atomic coordinates and structure factors have been deposited in the PDB under accession codes PDB: SUAT, SU0N, STYQ, SU18, SU1E, SU07, SU19, SU11.

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Author Contributions

P.d.S.B. performed the expression, purification, structure determination, and analysis of all GenN structure complexes. F.H. performed the cloning, mutagenesis experiments, and activity assays of wild type and mutant enzymes. S.L. and Y.S. purified and checked the quality of DAA2. M.V.B.D., P.F.L., F.H., and P.d.S.B. wrote the manuscript, and M.V.B.D. led the project.

Notes

The authors declare no competing financial interest.

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