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Characterization of a Radical *S*-Adenosyl-L-methionine Epimerase, NeoN, in the Last Step of Neomycin B Biosynthesis

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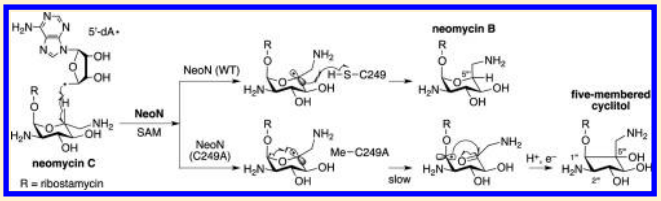
Supporting Information

ABSTRACT: The last step of neomycin biosynthesis is the epimerization at C-5‴ of neomycin C to give neomycin B. A candidate enzyme responsible for the epimerization was a putative radical *S*-adenosyl-L-methionine (SAM) enzyme, NeoN, which is uniquely encoded in the neomycin biosynthetic gene cluster and remained an unassigned protein in the neomycin biosynthesis. The reconstituted and reduced NeoN showed the expected epimerization activity in the presence of SAM. In the epimerization, 1 equiv of SAM was consumed to convert neomycin C into neomycin B. The site of neomycin C reactive toward epimerization was clearly confirmed to be C-5‴ by detecting the incorporation of a deuterium atom from the deuterium oxide-based buffer solution. Further, alanine scanning of the NeoN cysteine residues revealed that C249 is a critical amino acid residue that provides a hydrogen atom to complete the epimerization. Furthermore, electron paramagnetic resonance analysis of the C249A variant in the presence of SAM and neomycin C revealed that a radical intermediate is generated at the C-5‴ of neomycin C. Therefore, the present study clearly illustrates that the epimerization of neomycin C to neomycin B is catalyzed by a unique radical SAM epimerase NeoN with a radical reaction mechanism.



INTRODUCTION

Neomycin is a clinically important aminoglycoside antibiotic discovered by Waksman in 1949.[1] The core aminocyclitol of neomycin is 2-deoxystreptamine (2DOS), decorated with neosamine C (2,6-diamino-2,6-dideoxy-D-glucose) at C-4 and D-ribose at C-5 to give ribostamycin, which is further linked with neosamine C or neosamine B (2,6-diamino-2,6-dideoxy-L-idose) at C-3″ (C-3 of the ribose moiety) to yield neomycin C or neomycin B, respectively. The unique combination of these four units comprises the characteristic architecture of neomycin, which acts as an antibiotic by specifically interacting with bacterial ribosomal RNA (rRNA) to inhibit protein synthesis.[2] The crystal structure of the bacterial rRNA complex with neomycin B revealed that the configuration and conformation of the neosamine B moiety is important for these interactions with the RNA molecule.[3] In fact, neomycin B has a higher antibacterial activity than neomycin C, which is assumed to be a biosynthetic precursor of neomycin B.[4] Thus, the epimerization at C-5‴ of neomycin C is a critical biosynthetic reaction to acquire higher bioactivity.

Neomycin C is constructed from D-glucose-6-phospahte (G6P), two UDP-*N*-acetyl-D-glucosamines (UDP-GlcNAc), and 5-phosphoribose 1-diphosphate (PRPP) (Supporting Information (SI), Scheme S1).[5,6] First, G6P is converted to 2-deoxy-*scyllo*-inosose (2DOI) by the 2DOI synthase, NeoC.[7] Then, 2DOI is transaminated to 2-deoxy-*scyllo*-inosamine (2DOIA) by a PLP-dependent aminotransferase, NeoS. The C-1 hydroxyl group of 2DOIA is oxidized by a NAD-dependent dehydrogenase, NeoE, to give 3-amino-2,3-dideoxy-*scyllo*-inosose (amino-2DOI), which is transaminated to 2DOS by NeoS. 2DOS is then glycosylated with UDP-GlcNAc by a glycosyltransferase, NeoM, to give *N*-acetylparomamine, which is deacetylated by a deacetylase, NeoD, to afford paromamine.[8,9] The C-6′ position of paromamine is oxidized by a FAD-dependent dehydrogenase, NeoQ, followed by transamination with a PLP-dependent aminotransferase, NeoB, to give neamine.[10] Neamine is ribosylated with PRPP by a phosphoribosyltransferase, NeoL, and a phosphatase, NeoP, leading to ribostamycin. The enzymatic activities of a NeoL homologue, BtrL, and a NeoP homologue, BtrP, have been confirmed in vitro.[11] Ribostamycin is glycosylated with UDP-GlcNAc by a unique glycosyltrasnferase, NeoF, and the glycosylated compound is deacetylated by NeoD to give 6‴-deamino-6‴-hydroxyneomycin C (neomycin Y_2).[8] The C-6‴ position of neomycin Y_2 is oxidized by NeoQ, followed by transamination with NeoB to give neomycin C.[10] Enzymatic synthesis of neomycin C from ribostamycin has been successfully achieved.[6]

In the neomycin biosynthetic gene cluster, a unique hypothetical gene, *neoN*, encoding a putative radical *S*-adenosyl-L-methionine (SAM) protein remains to be characterized.[12] The *neoN* gene is conserved in structurally related aminoglycoside biosynthetic gene clusters such as lividomycin B[13] and paromomycin[14] that have neosamine B at C-3″ of the pseudotrisaccharide moieties (SI, Figure S1). Thereby, the

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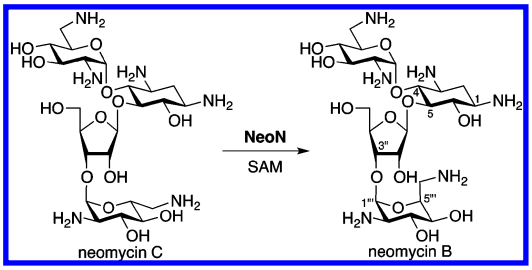
neoN gene was proposed to be involved in the epimerization of neomycin C to give neomycin B, presumably with radical SAM chemistry (Scheme 1).

Scheme 1. Epimerization of Neomycin C to Neomycin B



In general, radical SAM enzymes contain a characteristic CxxxCxxC motif used to stabilize a 4Fe-4S cluster, whose reduced form $[4Fe\text{-}4S]^{+}$ reductively cleaves the C−S bond of SAM to generate L-methionine and a 5′-deoxyadenosyl radical that triggers various radical reactions.[15] Previously, many radical SAM enzymes, including the 2DOIA dehydrogenase BtrN involved in butirosin biosynthesis,[16] have been characterized in vitro. A presumed epimerization reaction in the transformation of neomycin C to neomycin B has not yet been characterized, although the involvement of radical SAM enzymes in the related epimerization has been suggested in the biosynthesis of polytheonamide[17] and avilamycin.[18] In the present paper, we describe the characterization of a radical SAM epimerase, NeoN, that catalyzes epimerization of neomycin C to neomycin B. Furthermore, we expand the radical SAM chemistry to generate unprecedented reaction products by introducing a single-point mutation into the catalytically critical cysteine residue.

RESULTS AND DISCUSSION

The *neoN* gene was expressed in *Escherichia coli* according to standard methods. To obtain enzymes that possess iron−sulfur clusters during cultivation in *E. coli*, we co-expressed the *neoN* gene with pRKSUF017, which was developed to express the *suf* gene cluster derived from *E. coli*.[19] The obtained cells were homogenated by sonication in a glovebox and purified by cobalt affinity chromatography using TALON resin. The NeoN protein was reconstituted by adding ferrous iron, ferric iron, and sodium sulfide, followed by reduction with sodium dithionite to obtain the presumably active form of the radical SAM enzyme (SI, Figure S2). The reconstituted and reduced NeoN was reacted with SAM and neomycin C. The NeoN reaction products were converted to 2,4-dinitrophenyl (DNP) derivatives for high-performance liquid chromatograpy (HPLC), ultraviolet−visible spectroscopy (UV/vis), and electrospray ionization mass spectrometry (ESI-MS) analyses. As shown in Figure 1, in the presence of SAM under anaerobic conditions, neomycin C was epimerized to neomycin B by NeoN. NeoN also converted neomycin Y_2 to neomycin Y_1, albeit with less efficiency than the neomycin C conversion (SI, Figures S3 and S4, Table S1, and Scheme S1). Thus, the 6‴-amino group seems to be partly involved in substrate recognition, but not necessarily. Neomycin Y_1 was not converted to neomycin B by a FAD-dependent dehydrogenase, NeoQ, and an aminotransferase, NeoB (Figure S3), indicating that these enzymes recognize the D-configuration of the hexose moiety of paromamine and neomycin Y_2. Consequently, the

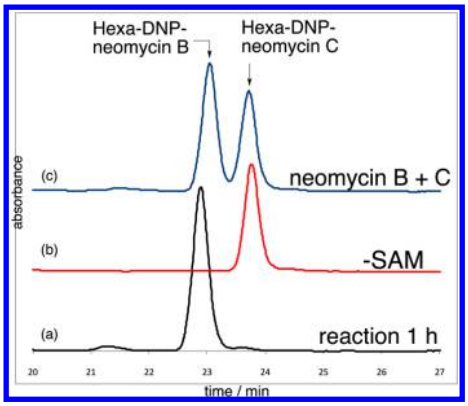


Figure 1. HPLC analysis (350 nm detection) of the NeoN reaction (1 h) with neomycin C and SAM: (a) NeoN (150 μM) reaction with SAM (2 mM), neomycin C (1 mM), and $Na_2S_2O_4$ (10 mM) in a glovebox, (b) without SAM, and (c) the hexa-DNP derivative of neomycin B and C.

characterized NeoN epimerization was found to be the last step of neomycin B biosynthesis.

During the epimerization reaction of neomycin C, the same amounts of 5′-deoxyadenosine (5′-dA) and neomycin B were generated, indicating that 1 equiv of SAM was consumed (SI, Figure S5). Thus, it appeared that SAM was not catalytic in the neomycin C epimerization. Presumably, the radical intermediate generated from neomycin C with 5′-deoxyadenosyl radical abstracts a hydrogen atom from a thiol or hydroxyl group of amino acid residues at the active site of the enzyme, but not from 5′-deoxyadenosine (SI, Scheme S2).

To verify this hypothesis, we carried out the NeoN reaction in a buffer prepared with deuterium oxide, and the product neomycin B was analyzed by LC-ESI-MS and NMR. Incorporation of a single deuterium atom into neomycin B was clearly confirmed by the ESI-MS by detecting m/z 1610 against m/z 1609 for the unlabeled neomycin B DNP derivative (SI, Figure S6). MS/MS analysis of the parent ions indicated that deuterium was incorporated into the neosamine B moiety. Furthermore, ^{1}H, ^{2}H, and ^{13}C NMR of the deuterium-labeled neomycin B clearly showed that the deuterium was introduced at the C-5‴ of neomycin B (SI, Figures S7 and S8). The ^{1}H signal at C-5‴ of neomycin B in ^{1}H NMR is significantly reduced (ca. one-fourth) in comparison with the other proton signals. This result indicates that a radical intermediate is generated at C-5‴ during epimerization through abstraction of the hydrogen atom at C-5‴ of neomycin C by 5′-deoxyadenosyl radical (Scheme S2). The radical intermediate then abstracts a hydrogen (deuterium in the D_2O buffer) atom from a thiol or hydroxyl group that is exchangeable with the water solvent molecules to complete the epimerization (Scheme 2). This reaction mechanism agrees well with previous feeding experiments with radioisotope-labeled glucose.[20] The tritium atom at C-5 of glucose is removed during biosynthesis, while the one at the C-4 of glucose is retained in neomycin B. Thus, the abstraction of the hydrogen atom at C-5‴ that disappears during the reaction seems to trigger epimerization at C-5‴.

To identify which amino acid residue donates the hydrogen atom to the presumable radical intermediate of neomycin C, we carried out alanine scanning of cysteine residues in NeoN. NeoN has eight cysteine residues, which were all mutated to alanine. Among the mutants, C226A, C247A, C271A, and C274A were self-cleaved during protein purification (SI, Figure

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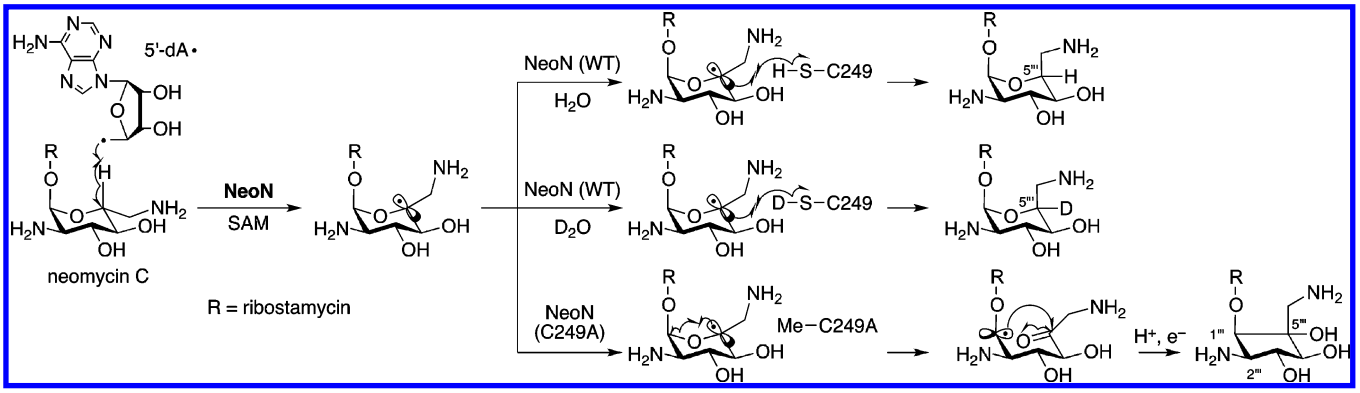
Scheme 2. NeoN and C249A Reaction with Neomycin C



S9), suggesting that these cysteine residues are important to maintain the conformation of NeoN. Conversely, a triple cysteine mutant for the radical SAM motif (C26/30/33A) was purified in the same manner as the wild-type (Figure S9) but did not display epimerization activity (SI, Figure S10). This result indicates that these cysteine residues are critical to construct the 4Fe-4S cluster in radical SAM enzymes. Interestingly, when C249A was reacted with neomycin C, a new enzymatic product, rather than neomycin B, was generated (Figure 2).

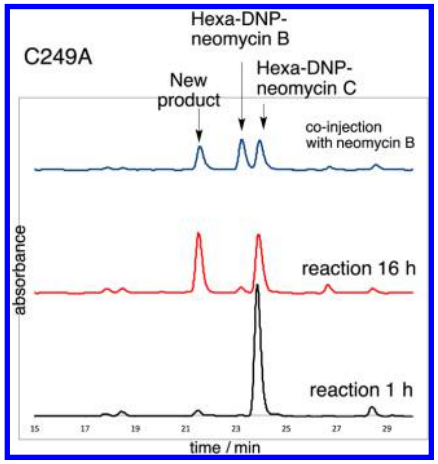


Figure 2. HPLC analysis (350 nm detection) of the C249A reaction with neomycin C and SAM: bottom, C249A reaction with SAM and neomycin C for 1 h; middle, for 16 h; top, co-injection of reaction mixture after 16 h with neomycin B.

The mass-to-charge ratio of the pseudomolecular ion of the new product determined by ESI-MS analysis was the same as those of neomycin B and C (SI, Figure S11). However, while ^{1}H NMR of the new product did show the existence of a ribostamycin structure with two anomeric protons, the anomeric proton corresponding to the neosamine B moiety disappeared (SI, Figure S12 and Table S2). ^{13}C NMR and DEPT135 analysis indicated that one carbon atom at 77.8 ppm is a quaternary carbon connected to an oxygen atom (SI, Figure S13 and Table S2). ^{1}H−^{1}H COSY, TOCSY, and HSQC analysis suggested the C-1‴ and C-4‴ species were continuously connected, and one aminomethyl group is isolated from this spin system (SI, Figures S14−S16). HMBC analysis indicated a correlation between the quaternary carbon atom with H-1‴, H4‴, and the aminomethyl group (SI, Figure S17). Consequently, a five-membered carbocyclic structure with an aminomethyl group was determined (Scheme 2). NOESY analysis showed the correlations between H-1‴ and H-2‴, H-2‴ and H-4‴, and H-3‴ and the aminomethyl group to establish the stereochemistry shown in Scheme 2 (SI, Figures S18 and S19). It is remarkable that this enzymatic reaction product was obtained by simply introducing a point mutation at the presumable catalytic residue.

Structurally related five-membered cyclitol-containing natural products such as bacteriohopanoid and pactamycin are isolated as metabolites of microorganisms (SI, Figure S20). Although no in vitro experimental evidence has yet been reported, putative radical SAM enzymes that are proposed to be involved in the formation of carbocyclic structures from carbohydrate origins are encoded in the biosynthetic gene clusters.[21,22] Thus, the C249A reaction mechanism with neomycin C in the present study sheds light on the mechanisms of such unprecedented carbocyclic formation from carbohydrate origins.

Further, this result suggests that the C249 thiol provides the hydrogen atom that is directly abstracted by a radical intermediate at C-5‴ to complete the epimerization of neomycin C (Scheme 2). This cysteine residue is conserved in NeoN homologues and seems to be a critical residue for epimerization (SI, Figure S21). A similar radical quenching mechanism by cysteine in the spore photoproduct lyase (SPL) reaction has been reported.[23] In the SPL reaction, a thymine radical intermediate generated from the spore photoproduct by 5′-deoxyadenosyl radical abstracts a hydrogen atom of C141 within DNA photodamage repair mechanisms.

Electron paramagnetic resonance (EPR) analysis of the as-isolated NeoN displayed a typical signal for a $[3Fe\text{-}4S]^{+}$ cluster (SI, Figure S22a). After reconstitution and reduction, however, an axial EPR signal with $g_{\|}$ = 2.03, $g_{\perp}$ = 1.92 at 10 K was observed, indicating the involvement of typical $[4Fe\text{-}4S]^{+}$ clusters in radical SAM proteins (Figure 3a). The EPR spectrum of the reduced NeoN in the presence of SAM revealed the coordination of SAM to the $[4Fe\text{-}4S]^{+}$ cluster (g = 2.00, 1.90, 1.86) (Figure S23b). However, addition of neomycin C affected the shape of the signal less (Figure S23c), which suggests that neomycin C might locate close to the $[4Fe\text{-}4S]^{+}$ cluster, but not with direct coordination as in the SAM case. In the presence of SAM and neomycin C, the signal for the $[4Fe\text{-}4S]^{+}$ cluster almost disappeared (Figure 3b), indicating that the $[4Fe\text{-}4S]^{+}$ cluster was oxidized to a $[4Fe\text{-}4S]^{2+}$ cluster by cleaving the SAM to initiate epimerization. However, no obvious EPR signal for the presumable organic radical

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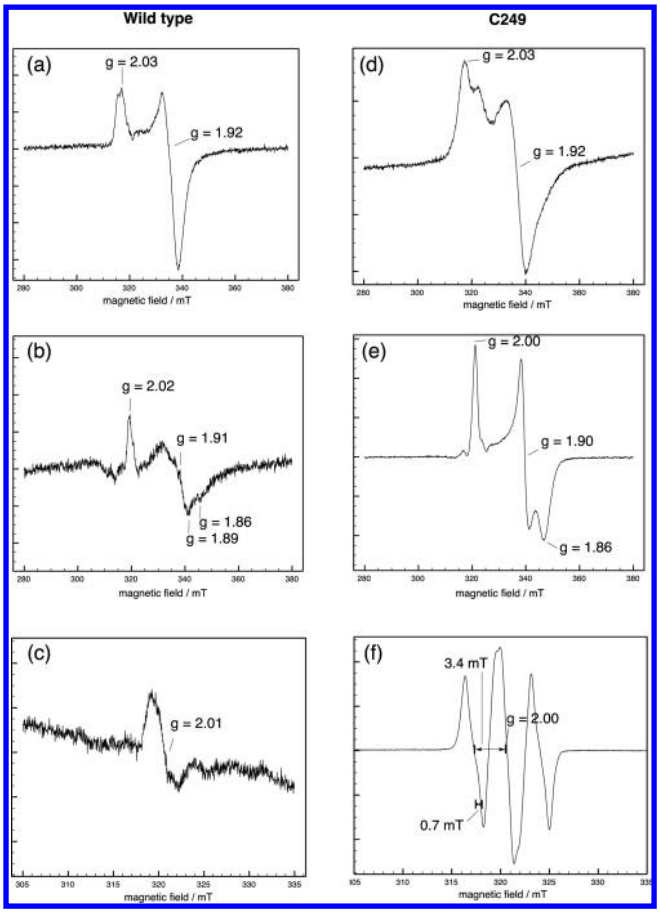

Figure 3. EPR analysis of the wild-type (WT) and C249A mutant NeoN. Spectra were recorded with modulation width, 0.6 mT; modulation frequency, 100 kHz; and microwave power, 1 mW. (a) WT (300 μM) + $Na_2S_2O_4$ (2 mM), 10 K; (b) WT (600 μM) + SAM (2 mM) + neomycin C (1 mM), 10 K; (c) same as in (b) but at 50 K; (d) C249A (500 μM) + $Na_2S_2O_4$ (2 mM), 10 K; (e) same as in (d) + SAM (2 mM) + neomycin C (1 mM); and (f) same as in (e) but at 50 K.

intermediate of neomycin C was observed, even at 50 K (Figure 3c), probably because the epimerization reaction proceeds rapidly.

EPR analysis of the reduced C249A variant at 10 K showed signals almost identical to those of the wild type, which indicates that C249A possesses the same $[4Fe\text{-}4S]^+$ cluster as a radical SAM enzyme (Figure 3d). Even in the presence of SAM and neomycin C, which was rapidly frozen after 5 min of reaction for EPR analysis, an EPR signal for the $[4Fe\text{-}4S]^+$ cluster was detected (Figure 3e), while the shape of the signal was significantly different from that of the wild type with substrates. Interestingly, a hyperfine-coupled EPR signal was clearly observed at 50 K, indicating that an organic radical of the NeoN reaction intermediate derived from neomycin C was trapped (Figure 3f). The hyperfine coupling values at $g = 2.00$ were estimated to be 3.4, 3.4, and 0.7 mT to indicate that the observed electron spin couples with two protons at C-6‴ and one at C-4‴. Thus, the EPR analysis of C249A in the presence of SAM and neomycin C revealed that an organic radical generated at C-5‴ of neomycin was detected (Scheme 2). In the C249A reaction with neomycin C, a presumable C-1‴ radical intermediate is generated via β-cleavage of the C-1‴-ring oxygen bond to give a five-membered cyclic product. This β-cleavage reaction thus seems to be a slow enough step to trap the radical intermediate, while the hydrogen abstraction at C-5‴ by 5′-deoxyadenosyl radical is fast.

The iron atom content in NeoN was estimated as 6.6 ± 0.9 per protein (SI, Figure S25), and the free sulfur atom content in NeoN was estimated as 5.8 ± 0.9 per protein (Figure S25). These values indicate that NeoN has another iron−sulfur cluster in addition to the 4Fe-4S cluster of the radical SAM motif. The UV spectrum of the C26/30/33A variant, which lacks the radical SAM motif, showed evidence of the characteristic absorption for the $[4Fe\text{-}4S]^{2+}$ cluster around 420 nm (Figure S9), indicating the existence of a second cluster. However, even after the reduction with dithionite, the C26/30/33A variant showed the characteristic absorption for $[4Fe\text{-}4S]^{2+}$ cluster, suggesting that the putative $[4Fe\text{-}4S]^{2+}$ cluster is not reduced under the examined experimental conditions with dithionite (Figure S9). EPR analysis of the C26/30/33A variant supports this property, since this putative second 4Fe-4S cluster is EPR silent (Figure S22d). A radical SAM 2DOIA dehydrogenase, BtrN, in the butirosin biosynthetic pathway is known to have similar enzymatic properties.[24] The second 4Fe-4S cluster in BtrN is proposed to accept one electron from the 2DOIA radical intermediate.

Sequence alignment of NeoN with BtrN[25] and anaerobic sulfatase maturating enzyme (anSME)[26] for formylglycine post-translational modification shows that C226, C247, C271, and C274 could be involved in a putative second 4Fe-4S cluster, which is part of the SPASM domain[26] (SI, Figure S26). A homology model of NeoN based on the anSME structure reveals that C249 is located close to the putative second 4Fe-4S cluster and might be able to interact with C247 (Figure S26). This homology modeling thus suggests that the putative second 4Fe-4S cluster is involved in the electron-transfer mechanism for the catalytic cycle. The oxidation state of the putative second 4Fe-4S cluster was divalent under the examined analytical conditions, judging from UV/vis and EPR analysis. Therefore, this divalent 4Fe-4S cluster could accept one electron through the disulfide bond formation from thiyl radical of C249. The reduced second 4Fe-4S cluster could then reduce the $[4Fe\text{-}4S]^{2+}$ cluster that is generated from the reductive cleavage of SAM in the radical SAM motif for the next catalytic cycle (Scheme 3). Since neomycin B was not epimerized to neomycin C at all under the examined reaction conditions (data not shown), this epimerization reaction is irreversible. Thus, the presumable disulfide formation seems to be the thermodynamic driving force for the irreversible epimerization.

Overall, we propose the NeoN reaction mechanism as follows (Scheme 3). First, SAM is coordinated on the $[4Fe\text{-}4S]^+$ cluster, and neomycin C is placed close to the SAM binding site. SAM is then reductively cleaved by the $[4Fe\text{-}4S]^+$ cluster to give L-methionine and 5′-deoxyadenosyl radical, which abstracts the hydrogen atom at C-5‴ of neomycin C to form a radical intermediate and 5′-deoxyadenosine. The radical intermediate abstracts the C249 thiol hydrogen atom from the opposite site of 5′-deoxyadenosine to give neomycin B and a thiyl radical at C249. The orientation of SAM, neomycin, and C249 seems to be critical to determine the stereochemistry of the dead-end product neomycin B. The thiyl radical of C249 forms a disulfide bond with C247 on the putative second iron−sulfur cluster, which is thus reduced to $[4Fe\text{-}4S]^+$. For the next catalytic cycle, one-electron transfer from the reduced second iron−sulfur cluster to the oxidized iron−sulfur cluster in the

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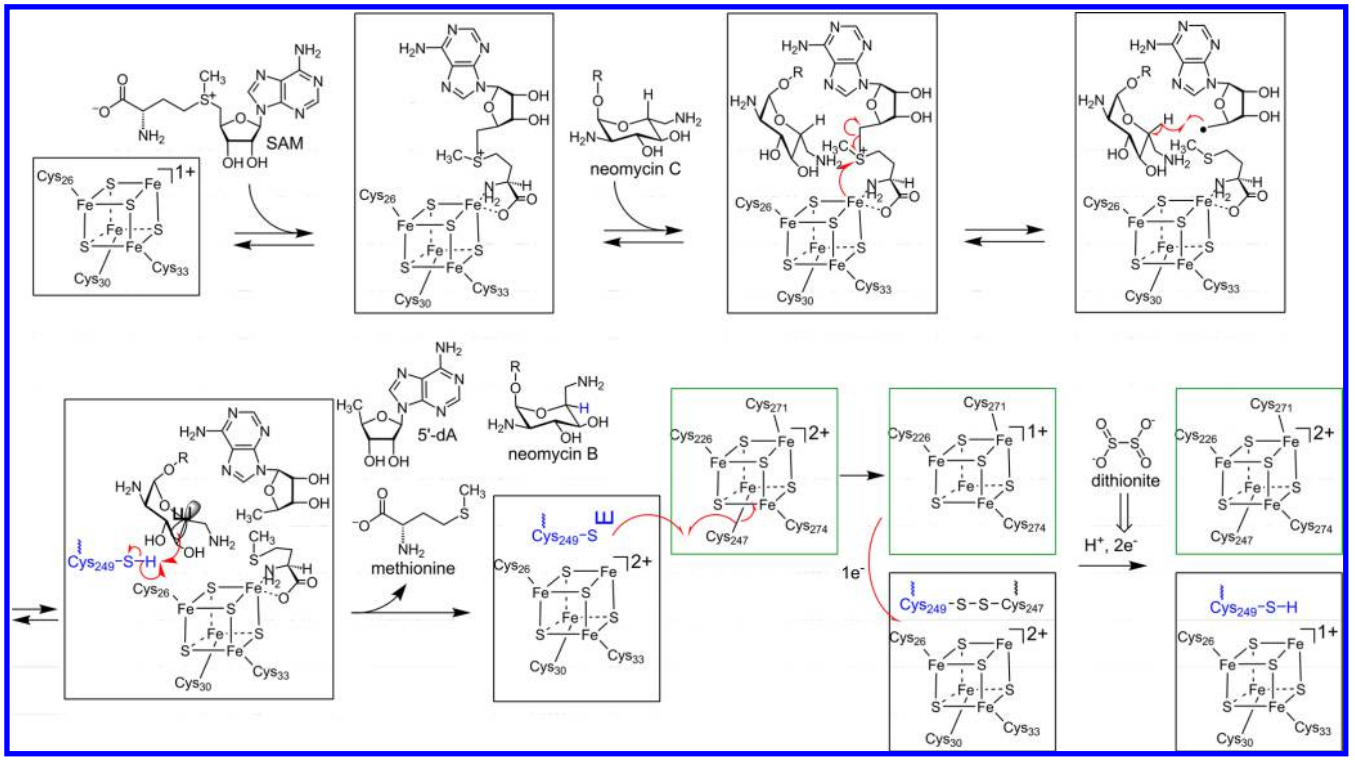
Scheme 3. Proposed NeoN Reaction Mechanism



radical SAM motif occurs. Although the mechanism for the reduction of the oxidized cysteine is unclear, under the experimental reaction conditions, an excess amount of dithionite could reduce it. Under the physiological conditions, thioredoxin reductase-type enzyme could be involved in the critical reduction step. Because we observed only a weak EPR signal in the wild-type NeoN reaction in the presence of SAM and neomycin C (Figure 3c,d), the existence of a thiyl radical of C249 and the reduced form of the putative second iron−sulfur cluster is unclear at the moment. More detailed biochemical analysis is necessary to understand the electron-transfer mechanism. Crystal structural analysis of NeoN could help to understand the function of the putative second iron−sulfur cluster in the SPASM domain.

CONCLUSIONS

In conclusion, the function of a unique radical SAM epimerase, NeoN, was clearly elucidated. Thus, the last piece of the neomycin biosynthesis is finally understood. In addition, this finding expands the mechanistic knowledge about radical SAM enzymes that catalyze epimerization at chemically inactive positions of molecules with radical chemistry.

EXPERIMENTAL PROCEDURES

Expression and Purification of NeoN. The *neoN* gene (originally deposited as the *neoH* gene) was amplified by PCR with primers neoN-F (5′-GACACcatATGACCACCGACATC-3′) and neoN-R (5′-GCGTGCCGGACCAGCtCGAgCCGC-3′) using the cosmid cfra10 that contains the neomycin biosynthesis gene cluster derived from *Streptomyces fradiae* NBRC 12773[7] as a template DNA. PCR reaction with PrimeSTAR HS DNA polymerase (Takara) was performed under the following conditions: 30 cycles of 98 °C for 10 s, 55 °C for 5 s, and 72 °C for 1 min 20 s for extension of DNA. The manufacturer's protocol was used to amplify the target DNA. PCR product was treated with Takara Ex Taq DNA polymerase reaction and then sub-cloned into the pT7Blue T-vector (Novagen) to obtain pT7Blue-neoN using *Escherichia coli* DH5α. After confirmation of the sequence (Fasmac), the *Nde*I-*Xho*I fragment of pT7Blue-neoN was inserted into pET28a (Novagen) vector to obtain pET28-neoN. The pET28-neoN plasmid and pRKSUF017[19] were introduced into *E. coli* C41(DE3) (Lucigen) in the presence of 30 μg/mL of kanamycin and 5 μg/mL of tetracycline.

E. coli C41(DE3) carrying pET28-neoN and pRKSUF017 was grown in 500 mL baffled flasks of LB medium containing 30 μg/mL of kanamycin and 5 μg/mL of tetracycline at 37 °C until OD_{600} 0.4−0.6 with 200 rpm agitation. The expression was induced by addition of 0.2 mM isopropyl β-D-thiogalactopyranoside (IPTG), 200 μM $FeSO_4(NH_4)_2SO_4$, and 200 μM L-cysteine. The cultivation was continued at 28 °C for 18−21 h with 100 rpm agitation. The cells were harvested by centrifugation at 7000 rpm for 10 min, washed with 20 mM HEPES−NaOH (pH 7.5), and stored at −30 °C until use. The wet cells were placed in a glovebox (UNICO) and suspended in buffer A (20 mM HEPES−NaOH, pH 7.5, 200 mM KCl, 10% glycerol), which was degassed with a freeze−thaw procedure under reduced pressure, followed by bubbling with a mixture of argon and hydrogen gas purified through a reduced Cu catalyst. The suspension of cells on an aluminum beads bath that was kept at cooled temperature was disrupted by sonication (QSONICA) for 10 s with a 10 s interval (total 5 min) in the glovebox. Cell debris was removed by centrifugation at 12 000 rpm for 20 min at 4 °C. The supernatant was loaded onto a TALON resin (Clontech) column (1 × 3 cm), which had been previously equilibrated with buffer A. The column was washed with 15 mL of buffer A plus 20 mM imidazole to remove unbound proteins. NeoN was eluted using buffer A plus 200 mM imidazole. The brownish protein solution was collected and desalted with a PD-10 desalting column (GE Healthcare) equilibrated with buffer A.

The purified NeoN was treated with 5 mM dithiothreitol (DTT) at room temperature for 15 min. $FeSO_4(NH_4)_2SO_4$ (0.25 mM), $FeCl_3$ (0.25 mM), and Na_2S (0.5 mM) were then added, and the mixture was incubated at room temperature for 30 min. The NeoN sample was then desalted by passing through a PD-10 desalting column equilibrated with buffer A. The obtained reconstituted NeoN was concentrated with an Amicon Ultra-0.5 mL device (Ultracel-10K, Millipore, 10 000 MWCO) at 10 000 rpm at 10 °C and then reduced

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by sodium dithionite (10 mM, Kanto Chemical) for 1 h at room temperature. Protein concentrations were determined with the Bradford assay (Bio-Rad) with BSA as a standard or Nano Drop Lite (Thermo Scientific) to estimate a concentration of NeoN (1 unit at A_{280} as 1 mg/mL). UV/vis absorption spectra of NeoN were determined by using a Shimadzu UV-2450 spectrophotometer. Enzymes were freshly prepared for each set of experiments.

Enzymatic Reaction of NeoN. The reduced NeoN (150 μM) was incubated with 1 mM neomycin C (prepared enzymatically from ribostamycin with NeoFDQB[6]) and 2 mM SAM (Sigma-Aldrich) in buffer A in the presence of 10 mM sodium dithionite at room temperature for 1 h. The reaction was quenched by addition of the same volume of ethanol. The quenched reaction solution (20 μL) was treated with 20 μL of 5% 1-fluoro-2,4-dinitrobenzene (Kanto Chemical) in methanol, 10 μL of DMSO, and 2 μL of 2 M NaOH at 60 °C for 1.5 h. The DNP derivatives of aminoglycosides were extracted with 500 μL of ethyl acetate, and the organic layers were evaporated. The residue was dissolved in 25 μL of CH_3CN, and an aliquot (5 μL) of the solution was injected into a HPLC (Hitachi L7100 pump, L-7405-UV detector, L-7300 column oven or L-6250 pump, L-4000 UV detector, L-7300 column oven) equipped with a TSKgel ODS-100Z column (5 μm, 4.6 × 250 mm, TOSOH). Elution was performed with 60% CH_3CN for 10 min and then a linear gradient to 75% CH_3CN for 20 min at a flow rate of 0.9 mL/min at 40 °C, monitored at 350 nm. The standard curves were generated with the authentic neomycin B and C [a mixture of 85:15 (Nacalai Tesque); 0.05, 0.1, 0.2, 0.5, 1.0, and 2.0 mM] after DNP derivatiztion so as to correlate peak area for the amount of neomycin B and C.

The formed 5′-deoxyadenosine was also detected by HPLC. An aliquot (5 μL) of the quenched enzymatic solution was injected into a HPLC (same instrument mentioned above) equipped with a TSKgel ODS-100Z column (5 μm, 4.6 × 250 mm, TOSOH). Elution was performed with 10% CH_3CN for 20 min at a flow rate 0.9 mL/min at 40 °C, monitored at 254 nm. The standard curves were generated with the authentic 5′-deoxyadenosine (Sigma-Aldrich, 0.05, 0.1, 0.2, 0.5, 1.0, and 2.0 mM) so as to correlate peak area with the amount of 5′-deoxyadenosine.

For the quantitative analysis of neomycin and 5′-deoxyadenosine formations, NeoN (40 μM) was incubated with 0.5 mM neomycin C, 1.0 mM SAM in buffer A in the presence of 10 mM sodium dithionite at room temperature for 10, 30, 60, 120, and 180 min and 15 h.

Enzymatic Reaction of NeoN with Neomycin Y_2 (6‴-Deamino-6‴-hydroxyneomycin C). The reduced NeoN (150 μM) was incubated with 1 mM neomycin Y_2 (13 mg) and 2 mM SAM (22.3 mg) in 10 mL of buffer A in the presence of 10 mM sodium dithionite at room temperature for 12 h in a glovebox. The formed neomycin Y_1 (6‴-deamino-6‴-hydroxyneomycin C, 7.4 mg) was purified according to the purification method for the deuterium-labeled neomycin B mentioned below. ^{1}H NMR (500 MHz, D_2O): δ 1.65 (q, J = 12.5 Hz, 1H, H-2-ax), 2.24 (dt, J = 4.2, 12.7 Hz, 1H, H-2-eq), 3.12−3.25 (m, 3H, H-4′, H-1, H-3), 3.29 (dd, J = 3.9, 10.8 Hz, 1H, H-6′), 3.34−3.41 (m, 2H, H-2′, H-6′), 3.46 (m, 1H, H-2‴), 3.58 (t, J = 8.9 Hz, 1H, H-6), 3.70 (m, 1H, H-4‴), 3.71−3.92 (m, 7H, H-5″, H-6‴, H-6‴, H-4, H-5, H-5″, H-3′), 3.96 (ddd, J = 3.4, 8.5, 10.1 Hz, 1H, H-5′), 4.00 (m, 1H, H-5‴), 4.12−4.16 (m, 1H, H-3‴, H-4″), 4.32 (dd, J = 2.3, 4.9 Hz, 1H, H-2″), 4.41 (dd, J = 5.1 6.7 Hz, 1H, H-3″), 5.14 (d, J = 1.6 Hz, 1H, H-1‴), 5.33 (d, J = 2.1 Hz, 1H, H-1″), 5.88 (d, J = 3.8 Hz, 1H, H-1′). ^{13}C NMR (125 MHz, D_2O): δ 30.4, 40.3, 48.8, 50.3, 51.3, 53.9, 60.4, 61.2, 66.7, 67.9, 68.7, 69.0, 71.0, 73.1, 73.7, 75.0, 75.6, 81.5, 81.5, 85.1, 95.4, 95.9, 109.9.

Enzymatic Reaction of NeoN in D_2O-Containing Buffer. The buffer of the reconstituted NeoN solution was exchanged with buffer B [20 mM HEPES−NaOH, pD 7.8, 200 mM KCl, 10% glycerol prepared with D_2O (Acros Organics, 99.8 at.% enriched)] three times during the ultracentrifugation with an Amicon Ultra-0.5 mL device. The percentage of D_2O in the buffer was estimated to be ca. 90%. The obtained solution was reduced by sodium dithionite (10 mM) for 1 h at room temperature and then used for the reaction. The other reaction conditions were the same as mentioned above. An aliquot (2 μL) of DNP-derivativzed enzymatic solution was injected into an LC-ESI-MS instrument [Shimadzu LCMS-2010EV mass spectrometer equipped with LC-20AD pump, CTO-20A column oven, and SPD-M20AUV detector, or LCQ (Finnigan) mass spectrometer equipped with Shiseido semi-micro pump SI-1/2001, UV detector SI-1/2002, and SSC-2300 column oven (Senshu)]. A TSKgel ODS-100Z column (3 μm, 2.0 × 150 mm, TOSOH) was used for separation. Elution was performed with 60% CH_3CN for 10 min and then a linear gradient to 75% CH_3CN for 20 min at a flow rate of 0.1 mL/min at 40 °C, monitored at 350 nm. The negative mode was selected to detect DNP derivatives of aminoglycosides.

To isolate deuterium-labeled neomycin B for NMR analysis, the reduced NeoN (100 μM) was incubated with 1 mM neomycin C (4.9 mg) and 2 mM SAM (8.5 mg) in 10 mL of buffer B in the presence of 10 mM sodium dithionite at room temperature for 12 h in a glovebox. The reaction was quenched by addition of the same volume of ethanol. After centrifugation to remove the precipitate, the solution was loaded on a DOWEX AG1-X8 column (OH^- form, 2 × 6 cm) to obtain an unbound fraction that contains cationic compounds. The solution was then loaded on an Amberlite CG-50 column (NH_4^+ form, 2 × 6 cm), washed with 0.1 M of aqueous ammonia, and then eluted with 1 M of aqueous ammonia to obtain a free form of neomycin B. After removal of the solvent, the residue was dissolved in water and loaded on a DOWEX AG1-X8 column (SO_4^{2-} form, 1 × 6 cm) to obtain the sulfate salt of neomycin B as a white solid (4.9 mg). ^{1}H, ^{2}H, and ^{13}C NMR spectra were recorded with a Bruker DRX-500 spectrometer. Deuterium oxide (Acros Organics, 99.8 at.% enriched) was used as an NMR solvent. Chemical shifts are reported in δ values based on the solvent signal (δ_H 4.65) as a reference. ^{1}H NMR (500 MHz, D_2O): δ 1.96 (q, J = 12.6 Hz, 1H, H-2-ax), 2.42 (dt, J = 4.3, 12.7 Hz, 1H, H-2-eq), 3.20 (dd, J = 5.9, 13.6 Hz, 1H, H-6′), 3.32 (d, J = 13.5 Hz, 1H, H-6‴), 3.38 (d, J = 13.8 Hz, 1H, H-6‴), 3.40−3.50 (m, 4H, H-1, H-2′, H-6′, H-3), 3.56 (t, J = 1.5 Hz 1H, H-2‴), 3.68−3.74(m, 2H, H-6, H-5″), 3.79 (d, J = 2.2 Hz, 1H, H-4‴), 3.88 (dd, J = 2.8, 12.4 Hz, 1H, H-5″), 3.91−3.96 (m, 2H, H-5, H-5′), 4.02 (t, J = 9.3 Hz, 1H, H-3′), 4.13 (t, J = 9.6 Hz, 1H, H-4), 4.18−4.22 (m, 2H, H-4″, H-3‴), 4.30 (m, 1H, H-5‴), 4.42 (dd, J = 1.6, 4.7 Hz, 1H, H-2″), 4.51 (dd, J = 4.9, 7.0 Hz, 1H, H-3″), 5.26 (d, J = 1.5 Hz, 1H, H-1‴), 5.40 (d, J = 1.8 Hz, 1H, H-1″), 6.03 (d, J = 3.9 Hz, 1H, H-1′). ^{13}C NMR (125 MHz, D_2O): δ 28.4, 40.3, 40.4, 48.6, 50.0, 50.9, 53.7, 60.2, 67.1, 67.7, 68.1, 69.4, 71.0, 72.5, 73.3, 74.8, 75.4, 81.2, 84.9, 95.1, 95.1, 110.2.

Site-Directed Mutagenesis. The plasmid pET28-neoN was used as a template DNA for PCR-based site-directed mutagenesis via the QuikChange site-directed mutagenesis method. The oligonucleotides listed in SI, Table S3 were used as primers. After confirmation of the point mutation by DNA sequence analysis (Fasmac), the respective resultant plasmids were introduced into *E. coli* C41(DE3) with pRKSUF017. The recombinant mutant proteins of NeoN were purified almost to homogeneity according to the above-mentioned method for the wild type.

Enzymatic Reaction of C249A with Neomycin C. The reduced C249A (150 μM) was incubated with 1 mM neomycin C and 2 mM SAM in 10 mL of buffer A in the presence of 10 mM sodium dithionite at room temperature for 30 h in a glovebox. The formed compound (5.4 mg) was purified according to the purification method for the deuterium-labeled neomycin B mentioned above. ^{1}H NMR (500 MHz, D_2O): δ 1.53 (q, J = 12.7 Hz, 1H, H-2-ax), 2.16 (dt, J = 4.2, 12.8 Hz, 1H, H-2-eq), 3.05 (m, 1H, H-3), 3.12 (d, J = 13.3 Hz, 1H, H-6‴), 3.12−3.18 (m, 2H, H-6′, H-1), 3.22 (dd, J = 3.7, 10.7 Hz, 1H, H-2′), 3.32 (d, J = 13.7 Hz, 1H, H-6‴), 3.33−3.40 (m, 2H, H-4′, H-6′), 3.51 (dd, J = 6.4, 9.0 Hz, 1H, H-2‴), 3.56 (t, J = 9.5 Hz, 1H, H-6), 3.60−3.68 (m, 2H, H-4, H-5″), 3.70−3.85 (m, 4H, H-5, H-4‴, H-5″, H-3′), 3.93 (dd, J = 7.2, 9.2 Hz, 1H, H-3‴), 3.96 (m, 1H, H-5′), 4.04 (d, J = 6.1 Hz, 1H, H-1‴), 4.08−4.15 (m, 1H, H-4″, H-3″), 4.29 (dd, J = 2.5 4.1 Hz, 1H, H-2″), 5.33 (d, J = 2.3 Hz, 1H, H-1″), 5.79 (d, J = 4.3 Hz, 1H, H-1′). ^{13}C NMR (125 MHz, D_2O): δ 31.3, 40.3, 41.2, 48.9, 50.4, 54.0, 54.5, 60.9, 68.2, 69.0, 69.4, 71.0, 73.4, 73.4, 77.8, 78.9, 79.2, 79.4, 80.7, 81.1, 83.4, 85.1, 95.8, 109.8.

EPR Analysis. The reconstituted NeoN and C249A was reduced with sodium dithionite (10 mM or 2 mM) at room temperature for 5−30 min. Solutions of the reduced NeoN (300 μL, 300−600 μM) were

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transferred to EPR tubes and flash frozen in liquid nitrogen in a glovebox. To prepare the NeoN−SAM complex, the reduced NeoN with 2 mM sodium dithionite was reacted with SAM (2 mM) at room temperature for 5 min and then freeze-quenched in liquid nitrogen. To prepare the NeoN−SAM−neomycin C complex, the reduced NeoN with 2 mM sodium dithionite was reacted with SAM (2 mM) and then 1 mM neomycin C at room temperature for 1 or 5 min and then freeze-quenched in liquid nitrogen. The prepared sample tubes were kept in liquid nitrogen until the EPR analysis. EPR spectra were measured using an X-band ESR spectrometer (JES-RE1X, JEOL) and an X-band microwave output unit (JEOL). To control low temperatures, a digital temperature controller (Science Instrument) was utilized. EPR was measured by the CW-EPR (10 GHz, X-band) method. Spectra were recorded with modulation width of 0.6 mT, modulation frequency of 100 kHz, and microwave power of 1 mW. EPR for the iron−sulfur cluster was measured at 10 K and for the organic radical at 50 K.

Determination of the Iron Contents. Quantitation of the iron content was conducted using the reported procedure.[27] Excess amount of iron was removed from the reconstituted NeoN solution by passage through a PD-10 desalting column equilibrated with buffer A. The reconstituted NeoN solution (100 μL) was then treated with 100 μL of 1 M HCl at 100 °C for 5 min. To the solution were added 100 μL of 7.5% (w/v) ammonium acetate, 500 μL of 4% ascorbate, 100 μL of 2.5% sodium lauryl sulfate, and 1.5% ferene to form the Fe(II)−ferene complex to analyze the absorption at 593 nm. The iron concentrations of the samples were determined according to the standard curve generated by 50, 100, 200, 500, 1, and 1000 μM $FeSO_4(NH_4)_2SO_4$.

Determination of the Sulfur Contents. Quantitation of the sulfur content was conducted using the reported procedure.[28] Excess amount of sulfur was removed from the reconstituted NeoN solution by passing through a PD-10 desalting column equilibrated with buffer A. The NeoN solution (200 μL) was then treated with 600 μL of 1% zinc(II) acetate and 50 μL of 1 M NaOH at room temperature for 15 min. To the solution were added 100 μL of 0.2% (w/v) *N*,*N*-dimethyl-*p*-phenylenediamine and 150 μL of 10 mM $FeCl_3$, and the mixture reacted at room temperature for 20 min to form methylene blue, which shows a characteristic absorption at 670 nm. The sulfur concentrations in the samples were determined according to the standard curve generated by 50, 100, 200, 500, 1, and 1000 μM Na_2S.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Characterization data of enzymatic reaction products, UV/vis spectra of proteins, additional EPR spectra of proteins, sequence alignment, and other data. This material is available free of charge via the Internet at http://pubs.acs.org.

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The authors declare no competing financial interest.

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