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Substrate-Tuned Catalysis of the Radical *S*-Adenosyl-L-Methionine Enzyme NosL Involved in Nosiheptide Biosynthesis**

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Abstract: *NosL is a radical S-adenosyl-L-methionine (SAM) enzyme that converts L-Trp to 3-methyl-2-indolic acid, a key intermediate in the biosynthesis of a thiopeptide antibiotic nosiheptide. In this work we investigated NosL catalysis by using a series of Trp analogues as the molecular probes. Using a benzofuran substrate 2-amino-3-(benzofuran-3-yl)propanoic acid (ABPA), we clearly demonstrated that the 5′-deoxyadenosyl (dAdo) radical-mediated hydrogen abstraction in NosL catalysis is not from the indole nitrogen but likely from the amino group of L-Trp. Unexpectedly, the major product of ABPA is a decarboxylated compound, indicating that NosL was transformed to a novel decarboxylase by an unnatural substrate. Furthermore, we showed that, for the first time to our knowledge, the dAdo radical-mediated hydrogen abstraction can occur from an alcohol hydroxy group. Our study demonstrates the intriguing promiscuity of NosL catalysis and highlights the potential of engineering radical SAM enzymes for novel activities.*

Nosiheptide is a clinically interesting and highly modified macrocyclic peptide natural product[1] belonging to the thiopeptide antibiotic family.[2] By targeting the bacterial 50S ribosomal subunit,[3] this compound exhibits highly potent activity against various contemporary bacterial pathogens, including methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococci* (VRE), and the hypervirulent BI/NAP1/027 strain of *Clostridium difficile*.[1a,4] Nosiheptide is generated from a ribosomally synthesized linear precursor peptide[5] and therefore belongs to the large class of ribosomally synthesized and posttranslationally modified peptides (RiPPs).[6] Nosiheptide also contains an indolic acid moiety that is not derived from the precursor peptide (Figure 1 A).[5a] A radical *S*-adenosyl-L-methionine (SAM) enzyme NosL catalyzes the conversion of L-Trp to 3-methyl-2-indolic acid (MIA), the precursor of the nosiheptide indolic acid moiety, and this reaction proceeds through an unusual carbon chain fragmentation–recombination process (Figure 1 B).[7] The same MIA synthase activity has also been demonstrated for NocL, which shares high sequence similarity with NosL and is involved in the biosynthesis of a nosiheptide structural analogue nocathiacin I.[8]

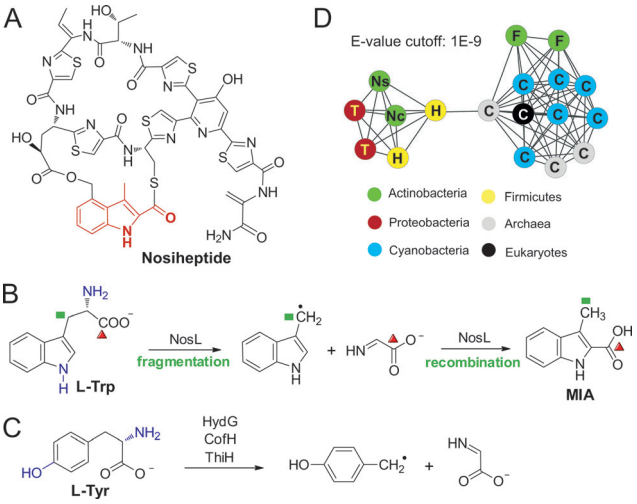

Figure 1. Nosiheptide and the reactions catalyzed by radical SAM aromatic amino acid lyases. A) Structure of nosiheptide. The indolic acid moiety derived from NosL-catalyzed reaction is shown in red. B) NosL-catalyzed MIA formation, which involves an unprecedented fragmentation–recombination process. The red triangles and green rectangles illustrate the previous ^{13}C-labeling studies of MIA biosynthesis.[7] C) HydG, CofH, and ThiH share a common step for cleaving the Cα–Cβ bond of L-Tyr. The possible hydrogen abstraction sites for NosL in (B) and for HydG, CofH, and ThiH in (C) are shown in blue. D) Protein similarity map of NosL-related enzymes. Each node in the network represents a protein sequence, and each edge represents sequences with BlastP E values below the indicated cutoff. Protein sequences are from the PDB and Swiss-Prot databases and are detailed in the SI. T, ThiH; H, HydG; Ns, NosL; Nc, NocL; C, CofH; F, FbiC, a fusion protein containing a discrete CofH domain.

Members of the radical SAM enzyme superfamily utilize a [4Fe–4S] cluster to bind SAM and reductively cleave its carbon–sulfur bond to produce a highly reactive 5′-deoxyadenosyl (dAdo) radical, which initiates an amazingly diverse variety of reactions relevant to DNA repair, RNA and protein modification, and the biosynthesis of vitamins, coenzymes, and natural products.[9] For most radical SAM enzymes, the reaction is initiated by dAdo radical-mediated hydrogen abstraction from an sp^3 carbon, and such hydrogen is not exchangeable with solvent. In contrast, a NosL in vitro assay with fully deuterated Trp showed that the resulting 5′-deoxyadenosine (dAdoH) is not deuterium-labeled, suggesting that in NosL catalysis a dAdo radical likely initiates the reaction by abstracting a solvent-exchangeable hydrogen.[7] Two recent studies performed on hydrogenase-maturing

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enzyme HydG[10] and F_{420} biosynthesis protein CofH,[11] respectively, also showed that a dAdo radical abstracted the solvent-exchangeable hydrogen atoms in the reactions. HydG, CofH, and thiamine biosynthesis protein ThiH cleave the Cα–Cβ bond of L-Tyr (Figure 1C), and together with NosL and NocL that cleave the Cα–Cβ bond of L-Trp, these enzymes form a phylogenetically related group (Figure 1D). Likely because of the prevalence of tyrosyl and tryptophanyl radicals in biochemistry,[12] it was believed that the dAdo radical-mediated hydrogen abstraction was from the phenolic position of L-Tyr for HydG,[13] CofH,[14] and ThiH,[15] and from the indole nitrogen atom of L-Trp for NosL.[7] However, a recent structural study on NosL showed that the L-Trp amino group is placed spatially close to dAdoH in the enzyme active site, indicating the hydrogen abstraction may possibly be from the amino group instead of the indole nitrogen.[16]

To biochemically validate the site of the dAdo radical-mediated hydrogen abstraction in NosL catalysis, we synthesized 2-amino-3-(benzofuran-3-yl)propanoic acid (ABPA) (see the Supporting Information, SI) and used it as a mechanistic probe. ABPA is structurally very similar to Trp but lacks a solvent-exchangeable hydrogen atom on the aromatic ring (Figure 2A). This molecule can preclude the reaction if the hydrogen abstracted by the dAdo radical is from the indole nitrogen atom. Incubation of ABPA in the assay mixture containing reconstituted NosL, SAM, and dithionite resulted in a product with a protonated molecular ion at $m/z = 176.0553$ in LC-MS analysis, and this product was absent in the control assay in which the supernatant of boiled enzyme was used (Figure 2B). The suggested molecule formula of the product is $C_{10}H_8O_3$ ($[M+H]^+$ calc. 176.0552, 0.6 ppm error), which is consistent with an MIA analogue 3-methyl-2-benzofuranic acid (MBA). The identity of MBA was further confirmed by co-eluting with the synthetic standard in HPLC analysis (Figure 2B). The 3-methylindole (MI) analogue 3-methylbenzofuran (MB) was also observed in the reaction mixture (Figure 2C), and was confirmed by GC-MS analysis (Figure S1) and by co-eluting with the synthetic standard (Figure 2C). These results clearly demonstrated that the hydrogen abstracted by the Ado radical in MIA biosynthesis is not from the indole nitrogen but likely from the amino group of Trp, as suggested recently.[16] Because of the phylogenetically close relationship (Figure 1D) and similar catalytic activities of NosL-related enzymes, our results suggested that the Ado-radical-mediated hydrogen abstraction in the catalysis of ThiH, HydG, and CofH is not from the phenolic group but likely from the amino group of L-Tyr.

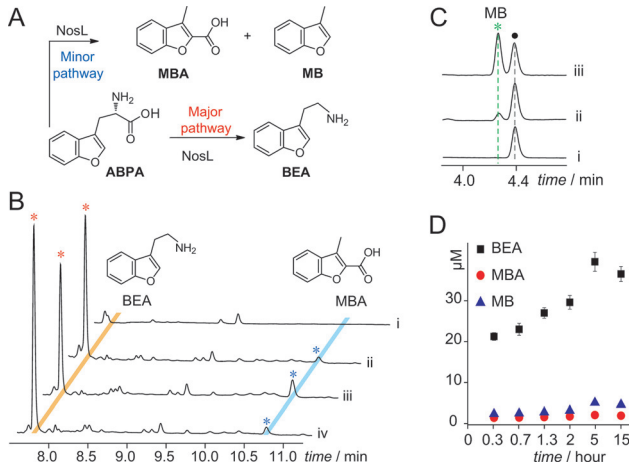

Figure 2. Tuning NosL chemistry by using an unnatural substrate ABPA. A) ABPA can be converted to MBA and 3-methybenzofuran (MB) by Cα–Cβ bond cleavage (minor pathway, ca. 15% conversion), or to BEA by decarboxylation (major pathway, ca. 85% conversion). B) HPLC analysis of the ABPA reaction mixture, using the supernatant of boiled NosL as a negative control (trace i), or using reconstituted NosL as the catalyst (trace ii). The NosL reaction mixture (trace ii) was also co-injected with either the synthetic MBA standard (trace iii) or BEA standard (trace iv) to confirm the production of these two compounds in the reaction. C) Production of MB in NosL-catalyzed reaction. Trace i and ii are the same from (B), whereas trace iii is the reaction mixture (trace ii) co-injected with the synthetic MB standard. HPLC analyses in (B) and (C) were performed using different eluting programs detailed in the SI. Red, blue, and green asterisks indicate BEA, MBA, and MB, respectively; the solid circle in (C) indicates an unknown compound that is independent of NosL-catalysis. D) Quantitative production of MBA, MB, and BEA in the NosL-catalyzed reaction. Assay was carried out by incubating 300 μM ABPA with ca 80 μM reconstituted protein, 500 μM SAM, and 2 mM of sodium dithionite in 50 mM MOPS buffer (pH 8.0). The time scale is not shown proportionally.

Surprisingly, we observed a major peak (shown by the red asterisks in Figure 2B) in HPLC analysis of the assay mixture, and this peak did not appear in the control reaction (Figure 2B). LC-MS analysis showed that the compound exhibited a protonated molecular ion at $m/z = 162.0918$, indicating the molecule formula $C_{10}H_{12}NO$ ($[M+H]^+$ calc. 162.0919, 0.6 ppm error). MS/MS analysis suggested that this compound may be 2-(benzofuran-3-yl)ethanamine (BEA), a decarboxylated product of ABPA (Figure S2). The identity of this compound was further confirmed by co-eluting with the authentic BEA standard in HPLC (Figure 2B). Production of BEA is time-dependent, and its yield is about 20-fold and 8-fold higher than that of MBA and MB, respectively (Figure 2D), suggesting that NosL was transformed to a non-oxidative decarboxylase by ABPA. To data, BlsE involved in the biosynthesis of the peptidyl nucleoside antibiotic Blasticidin S is the only known radical SAM enzyme that catalyzes a non-oxidative decarboxylation reaction.[17] The sequence similarity between BlsE and NosL is barely detectable, demonstrating the remarkable catalytic versatility of radical SAM superfamily enzymes. We failed to detect tryptamine in the NosL assay with Trp, indicating that the benzofuran ring may play a pivotal role in tuning enzyme conformation for the cryptic decarboxylase activity of NosL.

To study the dAdo radical-mediated hydrogen abstraction in APBA decarboxylation, we performed the assay in a buffer containing 67% D_2O. LC-MS analysis clearly showed incorporation of deuterium into dAdoH. Although the majority of labeled dAdoH is singly deuterated, doubly deuterated dAdoH is also observable (Figure 3A,B), demonstrating that the hydrogen abstracted by the dAdo radical in NosL catalysis is reversibly from the solvent-exchangeable site similar to the catalysis of HydG[10] and CofH.[11] In addition, we observed significant deuterium incorporation into BEA. This compound is singly deuterium-labeled and the doubly

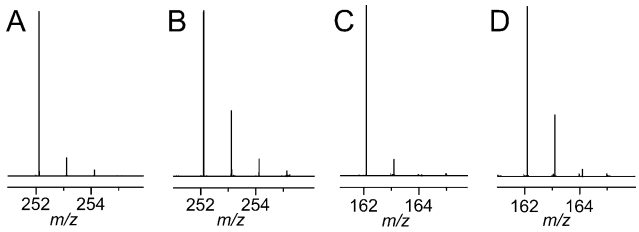

Figure 3. MS analysis of dAdoH and BEA from NosL-catalyzed reactions using ABPA as the substrate, showing the mass spectra of dAdoH produced in A) H_2O, and B) 67% D_2O, and the mass spectra of BEA produced in C) H_2O and D) 67% D_2O. The reaction conditions are detailed in the SI. MS/MS spectra of unlabeled BEA (m/z = 162. 1) from (C) and deuteriated BEA (m/z = 163.1) from (D) are shown in Figures S2 and S3 in the SI.

deuteriated BEA is insignificant, if not absent (Figure 3C,D). MS/MS analysis of deuterated BEA indicated that the deuterium is on the ethylamine part and not on the benzofuran ring (Figure S3).

The novel decarboxylase activity of NosL suggests the conformational diversity of the enzyme. Several examples have shown that one protein can adopt different conformational states by binding different ligands and exert different activities, which are referred to as catalytic promiscuities.[18] The intriguing promiscuity of NosL prompted us to further probe its catalysis by using Trp analogues. We next tested NosL activity with 2-hydroxy-3-(indol-3-yl)propanoic acid (HIPA), an alcohol analogue of Trp (Figure 4A). Incubation of HIPA in the presence of reconstituted NosL and other

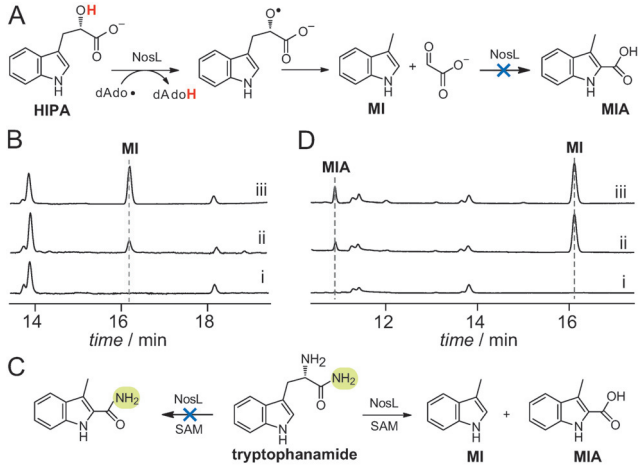

Figure 4. Probing NosL catalysis by using HIPA and tryptophanamide. A) NosL catalyzes the Cα–Cβ bond cleavage of HIPA but did not synthesize MIA from HIPA. B) HPLC analysis of the HIPA reaction mixture, using the supernatant of boiled NosL as a negative control (trace i), and using reconstituted NosL as the catalyst (trace ii); the reaction mixture (trace ii) was co-injected with the authentic standard (trace iii) to confirm the production of 3-methylindole (MI). C) NosL converted tryptophanamide to MI and MIA but not to 3-methyl-2-indolic amide. D) HPLC analysis of the tryptophanamide reaction mixture, using the supernatant of boiled NosL as a negative control (trace i), and reconstituted NosL as the catalyst (trace ii); the reaction mixture (trace ii) was co-injected with the authentic standard (trace iii) to confirm the identity of MIA.

required components led to the production of MI in the assay (Figure 4B), suggesting that the dAdo radical-mediated hydrogen abstraction can occur not only from an amino group but also from an alcohol hydroxy group (Figure 4A). Such a type of hydrogen abstraction is, to the best of our knowledge, unprecedented in radical SAM chemistry. It is noteworthy that the bond dissociation energy (BDE) of the alcohol O–H is higher than that of the dAdoH C–H bond (e.g., 440 kJ and 425 kJ for methanol O–H and dAdoH C–H bonds, respectively).[19] The fact that the yield of MI produced from HIPA is significantly lower than that produced from Trp (Figure S4) is likely because of the thermodynamically unfavorable hydrogen abstraction. We did not observe MIA production in the reaction; a possible reason is that dehydroglycine, not glyoxylate, is a necessary intermediate in MIA biosynthesis. We found that MIA was not produced when MI and glyoxylate were used in the NosL assay, suggesting that at least one of MI and glyoxylate is a shunt product rather than intermediate in MIA biosynthesis (Figure 4A).

We further probed the NosL activity by using tryptophanamide, an amide analogue of Trp (Figure 4C), questioning whether the carboxamide group can be transferred to indole C2 just like the carboxyl group of Trp. Analysis of the resulting assay mixture showed that 3-methyl-2-indolic amide was not produced in the reaction (Figure 4C). On the contrary, MIA was produced, and the identity of this compound was confirmed by co-eluting with the authentic MIA in HPLC (Figure 4D). The yield of MIA produced from tryptophanamide is slightly decreased but comparable to that produced from Trp (Figure S4), indicating that tryptophanamide is a kinetically competent substrate for MIA biosynthesis.

In summary, this investigation provides direct biochemical evidence supporting that the dAdo radical-mediated hydrogen abstraction in NosL catalysis is not from the indole nitrogen but from the amino group of L-Trp. Such a hydrogen abstraction mechanism very likely applies to radical SAM Tyr lyases, including ThiH, HydG, and CofH/FbiC. Our study also demonstrates the remarkable catalytic versatility of NosL and provides new insights into the mechanism of its catalysis. More importantly, we have revealed a cryptic non-oxidative decarboxylase activity of NosL and showed that different enzyme activities can be tuned by perturbation of the substrate structure. The intriguing catalytic promiscuity of NosL could thus inspire future efforts to investigate the mechanisms of radical SAM enzymes in modulating the reactivity of radical species and tuning the catalytic outcomes, and to engineer these enzymes for novel and/or improved activities.

Keywords: biosynthesis · catalytic promiscuity · radical reactions · *S*-adenosylmethionine · thiopeptides

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