Characterization of Nocl Involved in Thiopeptide Nocathiacin I Biosynthesis

A [4Fe-4S] CLUSTER AND THE CATALYSIS OF A RADICAL S-ADENOSYMETHIONINE ENZYME*

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The radical S-adenosymethionine (AdoMet) enzyme superfamily is remarkable at catalyzing chemically diverse and complex reactions. We have previously shown that NosL, which is involved in forming the indole side ring of the thiopeptide nosiheptide, is a radical AdoMet enzyme that processes L-Trp to afford 3-methyl-2-indolic acid (MIA) via an unusual fragmentation-recombination mechanism. We now report the expansion of the MIA synthase family by characterization of Nocl, which is involved in nocathiacin I biosynthesis. EPR and UV-visible absorbance spectroscopic analyses demonstrated the interaction between L-Trp and the [4Fe-4S] cluster of Nocl, leading to the assumption of nonspecific interaction of [4Fe-4S] cluster with other nucleophiles via the unique Fe site. This notion is supported by the finding of the heterogeneity in the [4Fe-4S] cluster of Nocl in the absence of AdoMet, which was revealed by the EPR study at very low temperature. Furthermore, a free radical was observed by EPR during the catalysis, which is in good agreement with the hypothesis of a glycyl radical intermediate. Combined with the mutational analysis, these studies provide new insights into the function of the [4Fe-4S] cluster of radical AdoMet enzymes as well as the mechanism of the radical-mediated complex carbon chain rearrangement catalyzed by MIA synthase.

Fe-S clusters are ubiquitous and ancient prosthetic groups that are essential for a wide range of biological processes (1–4). Besides their most prominent function of mediating electron transfer, another important role of Fe-S cluster is their direct interaction with the substrates in various enzymatically redox or nonredox reactions. The best characterized nonredox reaction of a Fe-S cluster is acutation, an enzyme in the citric acid cycle that utilizes a [4Fe-4S] cluster as a Lewis acid to activate the substrate (5). Enzymes catalyzing redox reactions, on the other hand, show remarkable versatility. These include the emerging large class of radical S-adenosymethionine (AdoMet)3 enzymes that perform the catalysis with AdoMet through a radical-associated mechanism (6–10). Since the discovery of the first member, lysine 2,3-aminomutase, in 1970 (11), the radical AdoMet superfamily is now believed to comprise of thousands of proteins that participate in numerous biochemical processes in animals, plants, and microorganisms. These enzymes contain a motif of three cysteine residues (usually as CXXXCXXC) that nucleate a [4Fe-4S] cluster for binding of AdoMet via a unique Fe (Feu) site uncoordinated to the cysteine residue. A common mechanism is shared for generation of a powerful reagent 5′-deoxyadenosyl radical, with the [4Fe-4S] cluster serving as an electron donor to reductively cleave the carbon-sulfur bond of AdoMet, thereby initiating highly diverse transformations. One fascinating aspect of radical AdoMet proteins is their potential to catalyze highly complex structural rearrangements, as exemplified by ThiC in thiamine biosynthesis (12) and MoaA in molybdenum cofactor biosynthesis (13, 14).

We recently demonstrated that NosL, a radical AdoMet enzyme involved in biosynthesis of the thioppeptide antibiotic nosiheptide (NOS) (Fig. 1A), catalyzes the rearrangement of L-Trp to afford 3-methyl-2-indolic acid (MIA) (15, 16). The shunt products, 3-methylindole and glyoxylate, were produced during the in vitro assay. In addition, glycine, assumedly originating from a glycyl radical intermediate, was detected by using a rapid-quench and derivatization method. These results strongly support an unprecedented fragmentation-recombination strategy that is utilized for the carbon chain reconstitution of L-Trp during the catalysis (Fig. 1B).

Notably, all of the NOS-like, e-series thiopeptides contain the MIA moiety (17), including nocathacin I (NOC-I, Fig. 1A) produced by Nocardia sp. ATCC200299 (18, 19), which represents the most pharmacologically promising members of the thiopeptide antibiotics numbering >80 entities reported to date. This indicates that NosL homologs might be involved in the biosynthetic pathways of all the e-series thiopeptides.

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3 The abbreviations used are: AdoMet, S-adenosymethionine; Feu, unique Fe; MIA, 3-methyl-2-indolic acid; NOC-I, nocathacin I; NOS, nosiheptide.
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thiopeptides for the MIA moiety formation. Indeed, noCL, which encodes a protein with 78% identity to NosL, was identified within the biosynthetic gene cluster of NOC-I (20). Heterologous expression of noCL in the nosL deletion mutant readily restored NOS production, confirming that they are functionally interchangeable.

We now report the characterization of NocL as a radical AdoMet MIA synthase by in vivo and in vitro studies. EPR and UV-visible absorbance analysis indicate that besides interacting with AdoMet, a fraction of [4Fe-4S] cluster of the enzyme also interacts with L-Trp. Comparative analysis of EPR spectra at different temperatures revealed that the [4Fe-4S] cluster is heterogeneous in the absence of AdoMet, supporting the non-specific interaction of the [4Fe-4S] cluster with other nucleophiles. During the steady state of catalysis, a free radical was observed by EPR, which is consistent with the presence of a glycyl radical as proposed previously in NosL-catalyzed reaction (16). Combined with the mutational analysis, these studies provided new insights into the function of [4Fe-4S] cluster in radical AdoMet enzymes, as well as the mechanism of the radical-mediated carbon chain reconstitution of L-Trp catalyzed by MIA synthase.

EXPERIMENTAL PROCEDURES

Materials—Biochemicals and media were purchased from Sinopharm Chemical Reagent Co. or Oxoid. Restriction enzymes were from TaKaRa Biotechnology. Chemicals were from Sigma-Aldrich except 1-[3H]Trp and 1-[13C]Trp, which were purchased from Cambridge Isotope Laboratory.

DNA Isolation, Manipulation, and Sequencing—DNA isolation and manipulation in Escherichia coli were carried out according to standard methods (21). PCR amplifications were carried out on an Authorized Thermal Cycler (Eppendorf AG 22331, Hamburg, Germany) using either Taq DNA polymerase or PfuUltra™ High Fidelity DNA polymerase. Primer synthesis and DNA sequencing were performed at the Shanghai Invitrogen Biotech Co. and Chinese National Human Genome Center.

Sequence Analysis—Protein comparison was carried out by available BLAST methods. Amino acid sequence alignment was performed by the ClustalW method, and the DRAWTREE and DRAWGRAM methods, respectively, from BIOLOGYWORKBENCH 3.2 software.

Production and Activity Assay of NocL—A 1.2-kb PCR product containing noCL was amplified by PCR using the primer pairs 5’-A CAT ATG GCG GAA TAC CCC GG-3’ (Ndel site underlined) and 5’-A GAA TTC TCA GCC GAT CGG GAT GAC G-3’ (EcoRI site underlined) from pSL5001, a pOJ446-based Nocardia sp. ATCC202099 genomic library cosmid that contains the NOC-I biosynthetic gene cluster (20), and then cloned into pMD18-T vector (TaKaRa Biotechnology) to yield pSL4150. After sequencing to confirm the fidelity, the 1.2-kb Ndel/EcoRI fragment was recovered from pSL4150 and ligated into the same site of pET28a, making the recombinant construct pSL4151 for expressing noCL to give the N-terminal His6-tagged protein. Expression, purification, reconstitution, and in vivo and in vitro activity assay of NocL were all performed as described previously for NosL (16). The quantification of Fe and labile S per molecule of the protein was performed in triplicate according to the methods described previously (22, 23).
Preparation of EPR Samples for Analyzing the Fe-S Cluster—Four aliquots of a 250-μl solution containing 100 mM NaCl, 10 mM DTT, and 100 μM reconstituted NosL in 50 mM MOPS buffer (pH 8.0) were placed in the microtubes. Reagents were added to each tube in the order shown to give the following final concentrations: MOPS buffer (pH 8.0), control, sodium dithionite (2 mM), sodium dithionite (2 mM), and AdoMet (1 mM), or sodium dithionite (2 mM) and L-Trp (1 mM). After mixing by pipette, each sample was loaded into a quartz EPR tube, frozen in liquid nitrogen, and then stored on dry ice before recording the spectra.

Preparation of EPR Samples for Analyzing the Free Radical—Sample were prepared containing 100 mM NaCl, 10 mM DTT, and 100 μM reduced NosL in 50 mM MOPS buffer (pH 8.0) in a total volume of 900 μl, which was divided into three aliquots. L-Trp, L-[2H8]Trp, or L-[1-13C]Trp was added to each solution to a final concentration of 1 mM. The samples were transferred to EPR tubes, and after 3 min of incubation at room temperature the solutions were quickly frozen in liquid nitrogen and then stored on dry ice before recording the spectra.

EPR Spectroscopy—EPR spectra were obtained at X-band using a Bruker EMX plus 10/12 spectrometer system, equipped with an Oxford ESR910 liquid helium continuous flow cryostat using a Bruker EMX plus 10/12 spectrometer system, equipped with an Oxford ESR910 liquid helium continuous flow cryostat with an Oxford ESR910 liquid helium continuous flow cryostat

UV-Visible Absorbance Analysis—Samples were prepared as described above for the EPR analysis of the Fe-S cluster. The samples were diluted 5-fold with MOPS buffer (pH 8.0) before transfer into a cuvette sealed with rubber septa for UV-visible analysis. The protein concentration was kept the same in all samples. To obviate the possible effect of cuvette difference, baseline corrections were performed before recording each spectrum.

Site-specific Mutagenesis for Expressing Mutant NosL—The recombinant plasmid pSL4150 (pMD18-T derivative) that contains a 1.2-kb fragment encoding NosL served as the template. PCR amplifications were carried out, by using the primer pair 5'-GAC TCC GAG TGC GAT CCA GAA CCC GGT GTC CTT GAC ATC GCA CTC GGA GTC GCA G-3' and 5'-CTT GGC CAT CGA GTA ATC TCT GCA CTC GGA GTC GCA G-3' for the M96Y mutation; the primer pair 5'-GGC TGC TTC GCA ACC AAC CAC TGC GAC-3' and 5'-GTT GGT GTT GTG GTA GAG CGC GAC GAA CGT GTG CAG CCG GGG CG-3' for the P83A mutation; the primer pair 5'-GGC TTC TTC ACC GCC GGC TAC GAG AAC AAT TAC ACC C-3' and 5'-GTA CTT GTC CTC GTA CGC GCC GGT GAG GAA GCC GAC G-3' for the G243A mutation; the primer pair 5'-C GTC AAC CCC GGT GTC GCC ATC GGA CTG CAC CTG G-3' and 5'-CAG TGT CAG TCC GCC GAT GCC GAC ACC GGG GTT GAC G-3' for the L245A mutation; and the primer pair 5'-CCC GGT GTC CTC ATC GCC CAC CTG CTC GTG GTG GCC G-3' and 5'-CAG ATC CAG TCC GCC GAT GCC GAC ACC GGG GTT GAC G-3' for the G247A mutation. Exchanged nucleotides are underlined. After sequencing to confirm the fidelity, the mutant DNA fragments were cloned individually into pET28a, yielding the recombinant constructs for producing NosL mutants (i.e. pSL4153 for P83A, pSL4155 for M96Y, pSL4157 for E138A, pSL4159 for G243A, pSL4161 for G245A, and pSL4163 for G247A). The recombinant strain SL4151, which produces the native NosL, was utilized as the positive control in the parallel analytic process.

RESULTS AND DISCUSSION

Identification of NosL as a Radical AdoMet MIA Synthase—nosL was cloned and ligated in pET28a followed by introduction into E. coli BL21 (DE3), yielding the recombinant strain SL4151 that produces NosL in an N-terminally His6-tagged form. HPLC-MS analysis of the culture broth of SL4151 revealed the production of MIA, with a yield (44 ± 4 mg/liter) similar to that of the recombinant strain SL4101 for expressing NosL (42 ± 5 mg/liter) (Fig. 2A, i and ii). MIA production was not detected in the control strain SL4100 carrying the pET28a vector alone (Fig. 2A, iii), indicating that NosL is a MIA synthase.

Active NosL was then obtained by purification and reconstitution under the strictly anaerobic conditions where O2 concentration was less than 5 parts/million, yielding a sample in a dark brown color characteristic of Fe-S-cluster-containing proteins. Quantitative analysis (22, 23) revealed that each mol of the protein contained 4.8 ± 0.4 mol of Fe and 4.4 ± 0.3 mol of labile S, indicating that the enzyme contains an active [4Fe-4S] cluster. Indeed, NosL was shown to produce MIA and 3-methylindole (a shunt product arising from reduction of the 3-methylenindole intermediate) in vitro after addition of the reductant sodium dithionite and the substrates AdoMet and L-Trp. Each assay (20 μM NosL, 0.5 mM AdoMet, and 0.5 mM L-Trp) produced 36 ± 3 μM MIA, with the ratio of MIA to 3-methylindole typically around 1:3 when 500 μM dithionite was used (Fig. 2A, iv). This ratio dramatically decreased to about 1:8 when the dithionite concentration was increased to 4 mM (Fig. 2A, v). The reaction does not proceed in the absence of AdoMet (Fig. 2A, vi), indicating its AdoMet dependence. 5′-Deoxyadenosine, the trademark of radical AdoMet protein, was also detected in the reaction. Although 5′-deoxyadenosine was produced in the absence of L-Trp, the yield was substantially enhanced by the addition of L-Trp to the reaction mixture (Fig. 2B). Together, these results firmly established that NosL is a radical AdoMet protein that catalyzes the conversion of L-Trp to MIA in a fashion similar to that of NosL.

Interaction of the [4Fe-4S] Cluster of NosL with L-Trp—EPR analysis of the reconstituted NosL resulted in a silent spectrum (Fig. 3i), in accordance with a diamagnetic [4Fe-4S]2+ cluster. When NosL was reduced by sodium dithionite, an axial spectrum with g values of 2.02 and 1.91 was observed, closely resembling those of NosL and other radical AdoMet proteins (Fig. 3j).
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3ii). Addition of AdoMet to the reduced protein resulted in a rhombic spectrum with g tensors of 2.01, 1.89, and 1.80 (Fig. 3ii). The conversion of axial to rhombic symmetry is quite common in the radical AdoMet superfamily (24, 25), a likely explanation of this may be the loss of near C3 symmetry of the common in the radical AdoMet superfamily (24, 25), a likely nonspecific coordinations (discussed in more detail below) has an approximately C3 symmetry. This usually leads to near axial EPR spectra of radical AdoMet proteins despite a few exceptions, such as the pyruvate formate-lyase-activating enzymes that exhibit the rhombic signals (27, 28). The significant shift in the spectrum and the emergence of a rhombic signal by addition of L-Trp to NocL strongly support the interaction of L-Trp with the [4Fe-4S] cluster, which possibly arises from binding of the nucleophilic group of L-Trp with the [4Fe-4S] cluster further, UV-visible absorbance analysis was performed. The reconstituted NocL showed a spectrum typical of radical AdoMet enzymes, exhibiting an obvious absorption between 350 nm and 450 nm (Fig. 5A). After dithionite reduction, new absorptions emerged around 400 nm and 420 nm, and a sharply increased absorbance at 380 nm was observed (Fig. 5B). Addition of AdoMet to the reduced protein led to a weakened absorbance in the 420 nm region but no apparent change in the 350 nm region (Fig. 5C), corresponding to the interaction of AdoMet with the [4Fe-4S] cluster. Addition of L-Trp to the protein, however, also led to a change in the spectrum, with the absorption decreased slightly in both the 420 and 600 regions (Fig. 5D). Given that the protein concentration was maintained at the same level in all assays, this UV-visible analysis was consistent with the EPR study described above and indicated that certain interaction of L-Trp with the [4Fe-4S] indeed had taken place, which changed the structure as well as the spectra of the [4Fe-4S] cluster.

Heterogeneity of the [4Fe-4S]+ cluster in NocL—The findings showed that the [4Fe-4S] cluster of NocL interacted with L-Trp inspired us to probe the nature of the cluster further. We proposed that the Fe site in the [4Fe-4S] cluster is coordination-deficient. As a result, when in the absence of the natural substrate AdoMet,
the Fe\textsubscript{u} site could act toward other nucleophiles, including bulky solvent, small molecules in the milieu, or even the residues from the protein itself (Fig. 4). In addition to the identification of interaction between the \([4Fe-4S]\) cluster of NocL and \(\text{L-Trp}\) here, the finding of a DTT-coordinated \([4Fe-4S]\) cluster in the MoaA crystal structure is also consistent with this proposal (29). The nonspecific interactions of the \([4Fe-4S]\) cluster of NocL with unidentified ligands may cause the heterogeneity of the cluster. Unlike that of \(\text{L-Trp}\), however, these interactions may be very weak, and consequently, the resulting heterogeneity of the \([4Fe-4S]\) cluster might not be discerned by EPR spectroscopy under the routine conditions. To support this point experimentally, we performed comparative EPR analysis of NocL by lowering the temperature, similar to a method previously used to ascertain the heterogeneity of \([4Fe-4S]\) cluster in coproporphyrinogen III oxidase HemN (30). We reasoned that different species in a heterogeneous system could have different characteristics such as the relaxation time, and this might lead to the change in spectrum at the lowered temperature.

To achieve a clear comparison, we decreased the EPR temperature from 13 K to a very low temperature of 2 K. The EPR spectra of reduced NocL at this temperature shifted dramatically, with a significantly broadened line width and a changed line shape, in part due to the power saturation effect. Notably, a new signal at \(g = 2.12\) was observed (Fig. 6A), supporting the heterogeneity of the \([4Fe-4S]\) cluster in NocL. A similar observation is also found in the spectra of NocL in the presence of \(\text{L-Trp}\) (Fig. 6B). Intriguingly, in the presence of \(\text{AdoMet}\) the spectrum at 2 K is quite similar to that at 13 K, although line width broadening resulting from saturation still occurred (Fig. 6C). The \(g = 2.12\) signal is apparently absent in the spectrum, ruling out an origin in signal dispersion or irrelevant background and demonstrating that the nucleophiles interacting nonspecifically with the \([4Fe-4S]\) cluster are replaced by \(\text{AdoMet}\) in this case. These results provided the EPR data of the \([4Fe-4S]\) cluster at very low temperature and indicated that the \([4Fe-4S]\) cluster of NocL is heterogeneous in the absence of \(\text{AdoMet}\), probably resulting from the nonspecific interaction of \([4Fe-4S]\) cluster with other nucleophiles at the Fe\textsubscript{u} site. Although the detailed nature seems obscure, the finding of these nonspecific interactions of the \([4Fe-4S]\) cluster gave insights into our understanding of radical \(\text{AdoMet}\) enzymes and may facilitate the design of molecular probes or inhibitors to study Fe-S cluster-containing enzymes.

**Identification of a Putative Glycyl Radical**—Glycyl radicals are common in biochemistry and are involved in a variety of biotransformations (31). However, the radicals identified so far usually reside on protein scaffolds whereas the free, unbound glycyl radical is rarely seen and to our knowledge, has not been characterized by EPR spectroscopy. We previously identified a trace amount of glycine in the NosL-catalyzed reaction by applying a rapid-quench assay (16), which was proposed to come from the glycyl radical intermediate during the catalysis.
Density function theory calculations supported this proposal because homolysis of the $\alpha$-$C\beta$ bond of Trp radical leading to a glycyl radical is energetically favored over heterolysis leading to dehydrogycine. The EPR spectra of glycol radicals characterized so far are characteristic of a 2-fold splitting caused by the nuclear hyperfine coupling of the hydrogen with the unpaired electron on Ca (32, 33). The spectra of Trp radicals, on the other hand, are anisotropic and variable in different cases (34, 35). We examined the glycol radical and/or possibly the Trp radical during the steady state of Nocl catalysis by EPR spectroscopy under conditions that focus on and enhance the free radical signal. The reduced Nocl exhibited a very weak signal at $g = 2.001$, which serves as the background (Fig. 7A). When frozen under the steady state of catalysis, the signal intensity showed 4–5-fold enhancement, indicating the production of new radical(s) during the catalysis. Interestingly, by subtracting the background, a splitting signal centered at $g = 2.0015$ was found (Fig. 7B), which correlated well with a free glycyl radical. The $g$ value of this signal is slightly smaller than the well characterized protein-based glycyl radical usually found at $g = 2.002$–2.004 (33), possibly reflecting the difference of the radicals in the free and protein-based forms. However, as the spectrum is background-subtracted, the complex non-Gaussian line shape might make $g$ value determination inaccurate. Use of $\mathrm{L}^3\mathrm{Trp}$ in place of $\mathrm{L}^2\mathrm{Trp}$ led to the collapse of the signal into a singlet (Fig. 7C), which may arise from the 6-fold smaller splitting constant for deuterium relative to hydrogen. Substitution of $\mathrm{L}^2\mathrm{Trp}$ by $\mathrm{L}^3\mathrm{Trp}$ showed significant broadening and alteration of the signal (Fig. 7D), confirming that the radical is centered on $\mathrm{Ca}$ and further supporting it to be a glycyl radical. The broadened line width and the complex line shape of the spectra implied that other free radicals such as a Trp radical might also exist in the assay in addition to glycyl radical.

**Mutational Analysis of Nocl**—Nocl and Nosl both belong to the ThiH subfamily of radical AdoMet enzymes that cleave the $\alpha$-$C\beta$ bond of aromatic amino acids (26, 36, 37). Similar to other radical AdoMet proteins, this protein subfamily possesses a conserved CXXXXCXC motif for binding of the [4Fe-4S] cluster, a conserved “GE” motif proposed for binding of the methionine part of AdoMet, and a relatively conserved “GXLXG” motif for binding of the adenine part of AdoMet (Fig. 8) (38). For Nosl we previously replaced each cysteine residues in the CXXXXCXC motif and the glycine residue in the GE motif of Nosl to alanine. All mutants lost the ability to synthesize MIA, indicating the importance of these residues to the biological activity of the MIA synthase (16).
AdoMet binding. Most of the radical AdoMet proteins possess the motif CXXXCX\Phi C, where \( \Phi \) represents aromatic amino acids (30). This is true for ThiHs and HydG, but for Nocl or NosL, the aromatic acid is replaced by methionine. When replacing the methionine in the CXXXCXMC motif of Nocl to tyrosine, the mutant showed a 2-fold decreased production of MIA compared with the native enzyme, indicating that this methionine residue in Nocl may not play a specific role. The decreased activity of the mutant may arise from the conformational change caused by the large \( p \)-hydroxyphenyl group of tyrosine.

We also probed the effect of conformational change to the catalytic activity. The entire protein subfamily contains a conserved proline in the middle of the first of N-terminal \( \beta \)-sheet (38), which may be important for protein conformation. Replacement of this residue by alanine resulted in only a 1.5-fold decrease of MIA production, indicating that the residues outside the protein active site may not be essential for the protein catalysis.

CONCLUSION

We have expanded the MIA synthase family by \textit{in vivo} and \textit{in vitro} characterization of Nocl, which is involved in biosynthesis of the thiopeptide antibiotic NOC-I. The catalytic efficiency of Nocl is similar to that of NosL, confirming the functional complementarity of these proteins. EPR and UV-visible spectrometries revealed the interaction of the [4Fe-4S] cluster with \( \lambda \)-Trp, leading to the notion of non-specific interaction of the [4Fe-4S] cluster with other nucleophiles via the Fe\( \lambda \) site. Comparative EPR analysis at different temperatures showed the heterogeneity of [4Fe-4S] cluster in the absence of AdoMet and supported the notion of non-specific interactions. Identification of these interactions will certainly contribute to future studies, not only for radical AdoMet proteins but also for other Fe-S-containing enzymes.

The most prominent feature of MIA synthase family is the fragmentation-recombination process, in which a putative glycol radical serves as the key intermediate during the catalysis. The EPR study here revealed the presence of this radical, supporting our previous mechanistic hypothesis (16) and paving the way for investigation into the radical chemistry of the catalytic process. Mutational analysis further demonstrated the indispensability of the conserved motif for [4Fe-4S] and AdoMet binding. Complete understanding of the intriguing chemistry of the radical AdoMet MIA synthase is awaiting future studies, such as the structural elucidation of the enzymes.

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