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Radical SAM-dependent Carbon Insertion into the Nitrogenase M-cluster

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Abstract

The active site of nitrogenase, M-cluster, is a metal-sulfur cluster containing a carbide at its core. Using radiolabeling experiments, we show that this carbide originates from the methyl group of *S*-adenosylmethionine (SAM) and that it is inserted into the M-cluster by the assembly protein NifB. Our SAM cleavage and deuterium substitution analyses suggest similarity between the mechanism of carbon insertion by NifB and the proposed mechanism of RNA methylation by the radical SAM enzymes RlmN and Cfr, which involves methyl transfer from one SAM equivalent, followed by hydrogen atom abstraction from the methyl group by a 5'-deoxyadenosyl radical generated from a second SAM equivalent. This work is an initial step toward unraveling the significance of the interstitial carbide and providing insights into the nitrogenase mechanism.

The M-cluster serves as the active site of Mo-nitrogenase, where inert nitrogen is converted to bioavailable ammonia (1). Arguably one of the most complex protein-bound metallocusters, the M-cluster has a core composition of 1Mo:7Fe:9S and can be viewed as [MoFe₃S₃]- and [Fe₄S₃]-subclusters bridged by three sulfide atoms (Fig. 1). In addition, an endogenous homocitrate moiety is attached to the Mo and a μ_6 -light atom is coordinated at the center of the metal-sulfur core (Fig. 1). The initial discovery of the interstitial atom was exciting because of its potential relevance to the nitrogenase mechanism (2). Recent studies identified this light atom as carbide (3, 4), prompting questions as to where this carbide originates and how it is inserted into the M-cluster.

The M-cluster is synthesized sequentially on two assembly proteins, NifB and NifEN, before it is delivered to NifDK, the catalytic component of nitrogenase (5). Functional analyses of NifB provided initial insights into the carbon insertion process. NifB has a CXXXCXXC signature motif that is characteristic of a family of radical *S*-adenosylmethionine (SAM or AdoMet) enzymes (6, 7). Additionally, it contains sufficient ligands to potentially accommodate the entire complement of iron atoms in M-cluster (5). These observations led to the proposal that NifB uses a SAM-dependent mechanism to generate the Fe-S core of the M-cluster, which is then processed on NifEN into a mature M-cluster and delivered to its target location in NifDK (5). Acquiring proof for this hypothesis has proven to be challenging. Initial attempts to characterize NifB from *Azotobacter vinelandii* were hampered by the instability of this protein in aqueous solutions. Recently, mimicking the natural *nifN*(3)-(5) *nifB* fusion in *Clostridium pasteurianum*, this problem was circumvented by fusing the *nifB* and *nifN* genes of *A. vinelandii*, resulting in the expression of a NifEN-B fusion protein that consists of two components that act sequentially in M-cluster biosynthesis (8).

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Subsequent metal, activity, UV/vis and EPR analyses established the presence of two transient clusters, designated L- and K-cluster, respectively, on the NifEN-B fusion protein (8, 9). The K-cluster consists of a pair of $[\text{Fe}_4\text{S}_4]$ clusters; whereas the L-cluster is a $[\text{Fe}_8\text{S}_9]$ cluster that closely resembles the Fe-S core of the M-cluster (8, 10-12; Fig. 1). Located near the $[\text{Fe}_4\text{S}_4]$ cluster at the SAM domain of NifB, the two 4Fe units of K-cluster can be coupled into an 8Fe L-cluster in the presence of SAM (8; Fig. 1). Subsequently, the L-cluster is transferred to and matured on NifEN upon insertion of Mo and homocitrate by NifH, an ATP-dependent reductase that also serves as the obligate electron donor for NifDK during catalysis (8, 13-15; Fig. 1). The resultant M-cluster is then transferred from NifEN to NifDK via direct protein-protein interactions (8, 13, 14; Fig. 1). The sequential conversion of K- to L- to M-cluster suggests that the K- and L-clusters represent consecutive snapshots of M-cluster biosynthesis. Moreover, the SAM-dependence of the conversion of K- to L-cluster points to a synthetic route to bridged metalloclusters that relies on radical chemistry at the SAM domain of NifB. Given the ability of SAM to serve as a carbon donor (16-19), it is possible that the interstitial carbide is derived from SAM and that it is incorporated during the formation of the L-cluster on NifB.

The cleavage pattern of SAM upon interaction with NifEN-B provides evidence that SAM is the source of the interstitial carbide (20). HPLC analysis shows that SAM remains intact in the absence of NifEN-B (Fig. 2A, 1), but is cleaved into two products in the presence of NifEN-B and reductant (Fig. 2A, 2). The two products display HPLC retention times characteristic of *S*-adenosyl-homocysteine (SAH or AdoHcy) and 5'-deoxyadenosine (5'-dAH) (Fig. 2A, 3 and 4). Liquid chromatography-mass spectrometry (LC-MS) analysis further confirms this identification based on mass-to-charge (m/z) ratios and fragmentation patterns of SAH and 5'-dAH (Fig. S1). The same pattern of SAM cleavage was documented for RlmN and Cfr, two radical SAM methyltransferases (16-18). One mechanism proposed for the RlmN- and Cfr-catalyzed reactions (Fig. S2) involves transfer of the methyl group from one equivalent of SAM to a polypeptide cysteine residue via an $\text{S}_\text{N}2$ mechanism, followed by generation of a 5'-deoxyadenosyl radical (5'-dA $\bullet$) via the reductive cleavage of a second equivalent of SAM. Subsequently, 5'-dA $\bullet$ abstracts a hydrogen atom from the methylcysteine, generating 5'-dAH and a presumed methylene radical on cysteine that initiates the radical-based methylation of RNA (18, 19, 21).

Deuterium substitution experiments illustrate that the reaction catalyzed by NifEN-B also involves hydrogen atom abstraction from the methyl group of SAM. As observed previously for RlmN and Cfr (10), incubation of NifEN-B with [methyl- d_3]-SAM, where the three hydrogen atoms of the methyl group are labeled with deuterium, results in a mixture of deuterium-enriched 5'-dAD and unlabeled 5'-dAH (Fig. 2B, right). The formation of 5'-dAD reflects the abstraction of deuterium from the labeled methyl group by 5'-dA $\bullet$; whereas the detection of 5'-dAH suggests the occurrence of (1) abortive cleavage of SAM, in which the 5'-dA $\bullet$ abstracts a solvent-derived hydrogen atom (17, 18); (2) hydrogen atom abstraction by 5'-dA $\bullet$ from an unlabeled methyl group, which is generated on NifEN-B through the action of unlabeled SAM during cell growth and remains associated with NifEN-B after purification; or (3) a combination of both events. These experiments firmly establish the initial hydrogen removal from the methyl group as a net transfer of a hydrogen atom. Continued removal of hydrogen atoms from this group, which is required for the generation of a carbide, likely occurs through proton abstraction via acid/base chemistry (22, 23); alternatively, it could involve the transfer of a hydride to Fe^{3+} and the subsequent removal of protons by the metal cluster in NifEN-B, a mechanism analogous to the conversion of H_2 to H^+ 2 by NiFe hydrogenase (24) and a single Fe model complex (25).

The similarity between NifB- and RlmN/Cfr-catalyzed reactions in using SAM-derived methyl group as a carbon source raises the possibility of directly visualizing the entry of

carbon into NifEN-B and the flow of carbon from NifEN-B onward through carbon-14 (^{14}C) labeling experiments. Such experiments can be carried out by incubating [methyl- ^{14}C]-SAM (1) with NifEN-B alone; or (2) with NifEN-B, NifH and apo NifDK in a maturation assay that also contains dithionite, ATP, molybdate, and homocitrate (13). The former allows the SAM-dependent conversion of K-cluster to L-cluster on NifEN-B, which can be used to assess whether carbon is incorporated in the L-cluster; whereas the latter allows the assembly process to proceed with the maturation of L-cluster to M-cluster by NifH and the transfer of M-cluster to apo NifDK, which can be used to determine whether carbon is transferred together with the cluster species between these proteins (see Fig. 1). Alone, the His-tagged NifEN-B can be captured on affinity (IMAC) resin after incubation with excess [methyl- ^{14}C]-SAM; in a maturation assay, it can be strategically combined with His- or non-tagged NifH and apo NifDK, followed by the capture of His- and non-tagged proteins on affinity (IMAC) and anion exchange (DEAE) resins, respectively (Fig. 3A; Fig. S3).

Indeed, when His-tagged NifEN-B is captured on IMAC resin after incubation with [methyl- ^{14}C]-SAM, it retains radioactivity after extensive wash with buffer, suggesting a transfer of ^{14}C label from [methyl- ^{14}C]-SAM to NifEN-B (Fig. 3A, 1). Following the incubation of His-tagged NifEN-B with non-tagged NifH and His-tagged NifDK in a maturation assay, the ^{14}C label can be detected in the IMAC fraction (*i.e.*, NifEN-B and NifDK), but not in the DEAE fraction (*i.e.*, NifH), indicating that the ^{14}C label is not harbored on NifH (Fig. 3A, 2). When His-tagged NifDK is replaced by non-tagged NifDK in the same maturation assay, however, the ^{14}C label appears in the DEAE fraction (*i.e.*, NifH and NifDK), suggesting a transfer of ^{14}C label from NifEN-B to NifDK (Fig. 3A, 3). A further substitution of His-tagged NifH for non-tagged NifH in this assay clearly shows the presence of radioactivity in the DEAE fraction (*i.e.*, NifDK), providing definitive proof for the transfer of ^{14}C label to NifDK upon cluster maturation (Fig. 3A, 4). When [methyl- ^{14}C]-SAM is replaced by [carboxyl- ^{14}C]-SAM in the same set of experiments, radioactivity is not detected in any of the protein fractions (Fig. 3B). Thus, the radioactive labeling of NifEN-B and NifDK does not originate from the carboxyl group or from the non-specific binding of SAM to these proteins; rather, it originates from the ^{14}C -labeled methyl group of SAM.

The ^{14}C label can be further traced to the cluster species extracted from NifEN-B and NifDK. The amount of extractable ^{14}C -labeled cluster species on NifEN-B (Fig. 3C, 1, left) is significantly decreased after the maturation of L-cluster and transfer of M-cluster from NifEN-B to apo NifDK (Fig. 3C, 2, left), and such a decrease is accompanied by an appreciable accumulation of extractable ^{14}C -labeled cluster species on NifDK following the incorporation of M-cluster (Fig. 3C, 2, middle). Activity analyses confirm that the clusters extracted from NifEN-B and NifDK are L- and M-cluster, respectively, as the former needs to be matured further on NifEN before it can be inserted into apo NifDK (Fig. 3D, 4 and 5), while the latter can be used directly to reconstitute apo NifDK (Fig. 3D, 6). SDS-PAGE analysis further demonstrates the strict association of ^{14}C label with the cluster species, as no radioactivity is detected in the polypeptides of [methyl- ^{14}C] SAM-treated NifEN-B or NifDK (Fig. S4). The absence of ^{14}C label from NifEN-B polypeptides also implies a direct transfer of methyl-derived carbon species to the K-cluster. This is supported by data from a post-translational modification (PTM) analysis, which reveal the absence of methyl or methylene group from NifEN-B polypeptides after treatment with SAM (26).

Based on these observations, we propose two plausible mechanisms for the carbon insertion by NifB, which are clearly distinct in the requirement for reductant in the initial step of methyl transfer (Fig. 4). The first mechanism (Fig. 4A) begins with the transfer of a methyl group from SAM to a sulfide of the K-cluster via a reductant-independent $\text{S}_\text{N}2$ mechanism, generating SAH and a cluster that at least transiently contains a methanethiol ligand. Then,

reductive cleavage of a second equivalent of SAM gives rise to a 5 -dA•, which abstracts a hydrogen atom from the methyl group, generating 5 -dAH and a methylene thiol radical. This scenario is consistent with the fact that Fe²⁺ in the K-cluster is not sufficiently nucleophilic for the initial methyl group transfer to generate an Fe-C bond (27). Consequently, it must involve a switch of the ligation of the C intermediate to Fe atom(s), as well as the removal of the other two hydrogen atoms of this intermediate, which eventually leads to the formation of an interstitial carbide atom that is coordinated by six Fe atoms. The second mechanism (Fig. 4B) starts with the reductive cleavage of a methyl group from SAM, which generates a transient methyl radical that could be captured on Fe²⁺ of the K-cluster to directly form an Fe-C bond. This scenario is analogous to the capture of C radicals by Co²⁺ in vitamin B12 radical chemistry (28, 29), and it eliminates the need for ligand exchange of the C intermediate. The subsequent hydrogen transfer could proceed in a manner identical to that in the first mechanism, which involves the initial hydrogen atom abstraction by 5 -dA• and the subsequent proton abstraction that leads to the formation of carbide.

It should be noted that the radical SAM-based carbide insertion is an integral part of a synthetic strategy to couple the two 4Fe units of K-cluster into an 8Fe L-cluster (Fig. 4). It is accompanied by the incorporation of a cluster-bridging sulfide, as well as the structural rearrangements of Fe-S clusters through the facile ligand replacement of their tetrahedral Fe²⁺/Fe³⁺ atoms via an addition/elimination mechanism (30, 31). Details of this process, such as whether the initial methyl transfer requires a reductant and how the starting methyl group is processed into a carbide atom, are yet to be defined. Nevertheless, this work is a step toward unraveling the structure-function relationship of the interstitial carbide, which could provide insights into the mechanism of nitrogenase.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. (A) Biosynthesis of M-cluster on NifEN-B fusion protein, which involves SAM-dependent conversion of K-cluster to L-cluster on NifB, transfer of L-cluster to NifEN, maturation of L-cluster upon NifH-mediated insertion of Mo and homocitrate, and transfer of the resultant M-cluster to NifDK. (B) Coupling of the 4Fe units of K-cluster into an 8Fe L-cluster via carbon- and sulfur-insertion, and conversion of the L-cluster to a mature M-cluster upon insertion of Mo and HC (homocitrate). The L-cluster represents an all-iron homolog of the M-cluster.

Fig. 2. (A) HPLC elution profiles of SAM in the (1) absence and (2) presence of NifEN-B and dithionite, compared with those of (3) SAH and (4) 5'-dAH standards. (B) LC-MS analysis of 5'-dAH recovered after incubation with unlabeled SAM (left) or [methyl- d_3] SAM (right). The ratio of 5'-dAH to SAH produced in the presence of NifEN-B was 1.8 ± 0.1 ($N=5$). In a maturation assay, the yields of SAH and M-cluster were 2.0 ± 0.2 and 1.7 ± 0.1 μM , respectively, in the presence of 10 μM SAM and 10 μM NifEN-B ($N=5$).

Fig. 3.

(A) Protein fractions captured on affinity (IMAC) and anion-exchange (DEAE) resins after incubation of [methyl-¹⁴C]-SAM with (1) His-tagged NifEN-B alone; (2) His-tagged NifEN-B, non-tagged NifH, and His-tagged NifDK; (3) His-tagged NifEN-B, non-tagged NifH, and non-tagged NifDK; and (4) His-tagged NifEN-B, His-tagged NifH, and non-tagged NifDK. Assays 2-4 also contained dithionite, ATP, molybdate, and homocitrate. (B) Experiments (B, 1-4) were identical to those in (A, 1-4), except that [carboxyl-¹⁴C]-SAM was used instead of [methyl-¹⁴C]-SAM. Experiment (A, 4) was included as a positive control here (B, 5). (C) Clusters extracted from (1) the IMAC fraction in (A, 1); and (2) the IMAC fraction (left) and DEAE (right) fraction in (A, 4). (D) Activities of C₂H₂ reduction by extracted clusters alone (1-3), after maturation and transfer of clusters to apo NifDK (4, 5), or upon direct transfer of cluster to apo NifDK (6). Clusters used for activity analyses were from C1, left (1, 4); C2, left (2, 5); and C2, middle (3, 6), respectively. Activities of clusters upon maturation and/or incorporation into apo NifDK (4-6) are indicated by *.

Fig. 4.

Proposed mechanisms of carbide insertion involve (A) transfer of a methyl group from SAM to the 4Fe K-cluster via an SN2 mechanism, followed by reductive cleavage of a second equivalent of SAM to generate 5-dA•, which abstracts a hydrogen atom from the methyl group on the K-cluster; or (B) reductive cleavage of a methyl group from SAM, followed by transfer of the resultant methyl radical to the 4Fe K-cluster and abstraction of a hydrogen atom of this group by 5-dA•. Continued deprotonation of the C intermediate in A or B must occur concomitant with its incorporation into the 8Fe L-cluster as an interstitial carbide atom. Atoms of the clusters are colored as follows: Fe, orange; S, yellow; C, grey.