Refining the pathway of carbide insertion into the nitrogenase M-cluster

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Carbide insertion plays a pivotal role in the biosynthesis of M-cluster, the cofactor of nitrogenase. Previously, we proposed a carbide insertion pathway involving methyltransfer from SAM to a FeS precursor and hydrogen abstraction from this methyl group that initiates the radical-based precursor maturation. Here we demonstrate that the methyl group is transferred to a precursor-associated sulfur before hydrogen abstraction, thereby refining the initial steps of the carbide insertion pathway.
Results
Defining the initial point of methyl attachment. To pinpoint the entry point of the methyl group into the pathway of carbide insertion, NiFeN-B was first incubated with SAM to allow methyltransfer to occur and subsequently treated by acid and analysed for methyl-bound species. Consistent with mobilization of the methyl group of SAM and abstraction of hydrogen from this methyl group by a 5-dA• radical that is generated upon homolytic cleavage of a second equivalent of SAM. (I). The process continues with further deprotonation/dehydrogenation of the resultant methylene radical into a carbide ion concomitant with the incorporation of the 9th sulfur and the radical-based rearrangement and coupling of the two [FeS4]-like modules of the K-cluster into an [Fe8S9C] L-cluster. The L-cluster is then matured into an M-cluster on NiFeN following NiFeH-mediated insertion of Mo and homocitrate and transferred to its target binding site in NiFeDK, completing the assembly of M-cluster. While attractive, certain details of this model, such as where the methyl group is initially attached on transfer and whether methyltransfer occurs before hydrogen abstraction, have remained unclear.

Here we show that the methyl group of SAM is transferred to a K-cluster-associated sulfur before hydrogen abstraction from this group takes place. This observation establishes the sequence of events during the initial phase of carbide insertion and reveals an interesting SN2-type reaction that involves direct methyltransfer to a metallosulfur cluster.

Figure 1 | Proposed carbide insertion pathway. It begins with the transfer of a methyl group from one equivalent of SAM to the K-cluster (I) and abstraction of a hydrogen atom from the methyl group by a 5-dA• radical that is generated upon homolytic cleavage of a second equivalent of SAM (II). The process continues with further dehydrogenation/deprotonation of the methylene radical concomitant with its incorporation into the L-cluster as an interstitial carbide atom (III). The L-cluster is further matured into an M-cluster on insertion of Mo and homocitrate (IV). The Nif proteins hosting events I–IV during the assembly of M-cluster are indicated in the figure. Atoms of the clusters are coloured as follows: Fe, orange; S, yellow; C, gray; Mo, cyan. HC, homocitrate.
Figure 2 | HPLC analyses of cleavage products of SAM/analogs and GC/GC-MS analyses of acid-quenched products upon incubation of NifEN-B/ NifEN-BFeSe with SAM/analouges. (a) HPLC elution profiles of (1) SAM, SAH and 5′-dAH standards; (2) SAM alone; (3) SAM and (4) [methyl-d3] SAM in the presence of reduced NifEN-B. (b,c) GC-MS (inset) and GC analyses of acid-quenched incubation mixtures containing SAM (b) or [methyl-d3] SAM (c), and NifEN-B in the reduced (black), oxidized (red) or re-reduced (blue) state. The acid-quenched products were identified as methanethiol (b, inset) and methane-d3-thiol (c, inset) based on their GC retention times and respective m/z ratios of 47 and 50. (d) HPLC elution profiles of (1) SAM, SAH and 5′-dAH standards; (2) SAM alone; and (3) SAM in the presence of reduced NifEN-BFeSe. (e) GC-MS full scan (inset) and SIM (m/z = 96) analyses of acid-quenched incubation mixtures containing reduced NifEN-BFeSe in the presence (black) and absence (red) of SAM. The acid-quenched product was identified as methylselenol based on its GC–MS retention time and fragmentation pattern. (f) HPLC elution profiles of (1) SAH and 5′-dAH standards; (2) allyl SAM alone; and (3) allyl SAM in the presence of reduced NifEN-B. (g) GC-MS full scan (inset) and SIM (m/z = 74) analyses of acid-quenched incubation mixtures containing reduced NifEN-B in the presence (black) and absence (red) of allyl SAM. The acid-quenched product was identified as allylthiol based on its GC–MS retention time and fragmentation pattern.
Likewise, when the incubation mixture comprising [methyl-\textsuperscript{d3}] SAM, Ni\textit{f}EN-B and reductant was quenched by acid, a volatile product with identical retention time to that of methanethiol could be detected on the same GC column (Fig. 2c, black). GC–MS analysis identified this product as methane-\textsubscript{d3} (CD\textsubscript{3}SH) based on an \textit{m/z} ratio of 50 (Fig. 2c, inset), confirming that the methyl group in methanethiol originated from the methyl group of SAM.

The observation of attachment of this SAM-derived methyl group to an acid-labile sulfur atom is exciting, as it provides the first indication that the methyl group of SAM is transferred to a sulfur atom of a FeS cluster, such as one of the [\textit{Fe\textsubscript{2}S\textsubscript{2}}] modules of the K-cluster in our proposed model (see Fig. 1). To further investigate the source of sulfur in the methanethiol product, Ni\textit{f}EN-B was first treated with an iron chelator to remove its endogenous FeS clusters and then reconstituted with FeCl\textsubscript{3}/Na\textsubscript{2}Se or FeCl\textsubscript{3}/Na\textsubscript{2}S. When SAM was incubated with the Fe/Se-reconstituted Ni\textit{f}EN-B protein (designated Ni\textit{f}EN-B\textsubscript{FeSe}) in the presence of dithionite, it was cleaved into SAH and 5'-dA\textsubscript{•}H (Fig. 2d, trace c)—the same products generated from SAM cleavage by the as-isolated Ni\textit{f}EN-B or the Fe/S-reconstituted Ni\textit{f}EN-B protein (designated Ni\textit{f}EN-B\textsubscript{FeS})—although the efficiency of SAM cleavage by Ni\textit{f}EN-B\textsubscript{FeSe} was lower than that by Ni\textit{f}EN-B or Ni\textit{f}EN-B\textsubscript{FeS}. When this incubation mixture was quenched by acid, a volatile product was detected by GC analysis and identified as methylselenol (CH\textsubscript{2}SeH) by GC–MS analysis based on a \textit{m/z} ratio of 96, as well as a characteristic fragmentation pattern of this compound (Fig. 2e, inset). This result firmly establishes the S atom of a FeS cluster as the point of methyl group attachment and points to the possibility that this methyltransfer step occurs via an SN2-type reaction before the abstraction of a hydrogen atom from the methyl group.

**‘Uncoupling’ methyltransfer and hydrogen abstraction.** To investigate the sequence of events between methyltransfer and hydrogen abstraction, the two events were ‘uncoupled’ through substitution of allyl SAM, an analogue containing an allyl group. Hydrogen abstraction, the two events were ‘uncoupled’ through substitution of allyl SAM, an analogue containing an allyl group.

(\textit{CH\textsubscript{2}=CH\textsubscript{CH}} \textsubscript{2}=CH\textsubscript{3}) based on an \textit{m/z} ratio of 50 (Fig. 2c, inset), confirming that the methyl group in methanethiol originated from the methyl group of SAM.

\textit{Discussion}

While the post-hydrogen-abstraction events along the carbide insertion pathway are yet to be explored, the current work has established the first two steps in this process as an SN2-type methyltransfer from SAM to the sulfur atom of the K-cluster, followed by hydrogen abstraction of this SAM-derived methyl group by a 5'-dA\textsubscript{•} radical. Given that the endogenous FeS clusters could be removed from Ni\textit{f}EN-B and readily reconstituted by Fe/S or Fe/Se (see Fig. 2d,f) through a method specific for FeS cluster formation, the 9\textsuperscript{th} sulfur that is required along with the carbide for the coupling and rearrangement of the two [\textit{Fe\textsubscript{5}S\textsubscript{5}] like cluster modules of K-cluster into an [\textit{Fe\textsubscript{8}S\textsubscript{9}}] L-cluster seems to be associated with the FeS cluster instead of the protein environment. One appealing scenario is that the 9\textsuperscript{th} sulfur is a 'dangling' sulfur attached externally to one Fe atom of the K-cluster, which serves as the initial point of methyl attachment. Such ‘dangling’ sulfurs have been observed in the cases of other radical SAM-dependent enzymes such as RimO, MiaB and HydG\textsubscript{12,13}, as well as the radical enzyme (R)-2-hydroxyisocaproyl-CoA dehydratase\textsuperscript{14}, which enable a variety of important FeS chemistry in biological systems. Strategic labelling of this sulfur by Se may shed light on the insertion of this atom that is intertwined with the insertion of carbide, which could facilitate further refinement of the assembly pathway of M-cluster.

**Methods**

Unless noted otherwise, all chemicals and reagents were obtained from Fisher Scientific or Sigma-Aldrich. All proteins were handled under argon in an anaerobic chamber containing <4 p.p.m. O\textsubscript{2}.

**Cell growth and protein purification.** Azotobacter vinelandii was grown at 30 °C in 180-l batches in a 200-l fermenter (New Brunswick Scientific) in Burke’s minimal medium supplemented with 2 mM ammonium acetate. Growth rates were monitored via cell density at 436 nm using a Spectronic 20 Genesys spectrometer (Spectronic Instruments). On ammonia depletion, cells were allowed to de-repress for 3 h before being harvested via a flow-through centrifugal harvester (Cepa). The cell paste was washed with 50 mM Tris–HCl (pH 8.0) buffer before it was frozen and stored at –80 °C. Published methods were adapted to the purification of His-tagged Ni\textit{f}EN-B\textsuperscript{B}.

**Radiolabel assays with [methyl-\textsuperscript{14}C] S-adenosyl-L-methionine.** Five different reactions were assembled, each containing, in a total volume of 32 μl, 100 mM Tris–HCl (pH 8.0), 250 μM [methyl-\textsuperscript{14}C] SAM (American Radiolabeled Chemicals, Inc.) and Ni\textit{f}EN-B (j) in the as-isolated state, where an excess (10 mM) Na\textsubscript{2}S\textsubscript{2}O\textsubscript{4} was supplied; (ii) in a reduced state, where excess Na\textsubscript{2}S\textsubscript{2}O\textsubscript{4} was removed from the as-isolated Ni\textit{f}EN-B (see i) via gel filtration with Sephadex G-25 fine resin. 

**Figure 3 | Capture of Ni\textit{f}EN-B proteins in different redox states on affinity (IMAC) resin after incubation with [methyl-\textsuperscript{14}C] SAM.** Ni\textit{f}EN-B samples were as following: (1) reduced by excess dithionite; (2) reduced by dithionite, followed by removal of excess dithionite; (3) oxidized by IDS; (4) oxidized by IDS and then re-reduced by dithiothreitol; and (5) oxidized by IDS and then re-reduced by dithionite.
(GE Healthcare); (iii) in an oxidized state, where Na$_2$SO$_4$-free NiFEN-B was oxidized with excess I$_2$, followed by the removal of excess I$_2$ via AG1-X8 resin (Bio-Rad); (iv) in a weakly reduced state, in which oxidized NiFEN-B was re-reduced by the addition of 5 mM DTT; and (v) in a re-reduced state, where the Na$_2$SO$_4$-free NiFEN-B (see ii) was first oxidized by I$_2$ and then re-reduced by the addition of 10 mM Na$_2$SO$_4$. All reactions were incubated for 30 min at 25°C with intermittent shaking before being passed over Immobilized-metal affinity chromatography (IMAC) IMAC sepharose resin (10% Na$_2$S$_2$O$_4$) that was equilibrated with a buffer containing 25 mM Tris–HCl (pH 8.0), 10% glycerol and 500 mM NaCl. The IMAC sepharose resin was washed three times with 700 µl buffer containing 25 mM Tris–HCl (pH 8.0), 10% glycerol, 500 mM NaCl and 40 mM imidazole. The IMAC sepharose samples were then re-suspended in the wash buffer and applied directly onto a piece of Whatman 3-MM chromatography paper. The blots were dried and exposed to a GE Healthcare Storage Phosphor Screen GP (20 × 25 cm) for 48 h before imaging on a GE Healthcare Typhoon Trio⁺ variable mode imager.

**Selenium labelling experiment of NiFEN-B.** To generate selenium (Se)-labelled clusters on NiFEN-B, the protein was first treated with an iron chelator that removed the endogenous iron–sulfur (FeS) cluster species from the protein, followed by re-isolation of the apo NiFEN-B from the mixture. Specifically, 18 µl of NiFEN-B was incubated with 20 mM bathophenanthroline disulfonate in the presence of 2 mM Na$_2$SO$_4$, 25 mM Tris–HCl (pH 8.0) and 10% glycerol for 30 min with gentle stirring. Subsequently, bathophenanthroline disulfonate was removed from the protein by passing the incubation mixture via a 10 µl packed Q-Sepharose Fast Flow column (GE Healthcare). The resultant apo NiFEN-B was nearly colourless and exhibited no signal in electron paramagnetic resonance spectroscopy, and displayed no capability to serve as a precursor source in the L-cluster maturation assay, suggesting that its endogenous FeS clusters have been removed.

The apo NiFEN-B protein was chemically reconstituted with [Fe$_4$S$_4$] or [Fe$_4$Se$_4$] clusters following the procedures described in the Methods section. Na$_2$S$_2$O$_4$-free NiFEN-B (see i) was first oxidized by Na$_2$S$_2$O$_4$ and then re-reduced by the addition of 5 mM DTT; and (v) in a re-reduced state, where the mass spectrometry transfer line and ion source were maintained at 25°C. The inset indicates that the mass spectrometry transfer line and ion source were maintained at 25°C. The yield of allylthiol was detected in these experiments.

**Acid-quenching experiments.** A published method was adapted for the NiFEN-B-dependent production of methanethiol, methylselenol or allylthiol$^{22}$. First, excess Na$_2$S$_2$O$_4$ was removed from NiFEN-B via gel filtration with Sephadex G-25 fine resin that was equilibrated with an oxygen-free buffer containing 25 mM Tris–HCl (pH 8.0). Immediately following the removal of excess reductant, 4 nmol of NiFEN-B or NiFEN-B$^{\text{FeSe}}$ was added to a sealed 300-µl glass vial, which contained 200 nmol SAM, [methyl-d$_5$]-SAM or allyl SAM in a total volume of 100 µl, as well as 100% argon at 2 psi in the headspace. The 100 µl reactions were then incubated for 30 min at 25°C before being quenched by 25 µl of 1 M HCl.

To observe the formation of the volatile methanethiol, methane-d$_3$-thiol, or methylselenol, the acid-quenched samples were incubated at 60°C for 15 min and equilibrated to room temperature for 10 min before the entire headspace was injected by a gas-tight syringe onto a GC with flame photometric detection (SRI Instruments 8610C system). The reaction was subjected to a linear gradient of 2–15% buffer B over 10 min, a constant flow of 80% buffer B for 3 min. The GC inlet and oven temperatures were maintained at 30°C (for methanethiol or methane-d$_3$-thiol analysis) or 50°C (for methylselenol analysis), while the mass spectrometry transfer line and ion source were maintained at 250°C. The mass spectral data were acquired under SIM conditions and compared to those reported in the National Institute of Standards and Technology database. Approximately 50% of the maximum possible yield of methanethiol or methane-d$_3$-thiol was detected in these experiments.

To observe the formation of allylthiol, the acid-quenched samples were incubated at 25°C for 10 min before allylthiol was extracted into 100 µl of dichloromethane and inner-sampled in the ESI source of the mass spectrometer. The mass spectral data were acquired under SIM conditions and compared to those reported in the National Institute of Standards and Technology database. Approximately 30% of the maximum possible yield of allylthiol was detected in these experiments.

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**Author contributions**

J.A.W. performed the experiments, J.A.W., Y.H. and M.W.R. designed the research and analysed the data, Y.H. and M.W.R. supervised the research and wrote the manuscript.

**Additional information**

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