NifEN-B complex of *Azotobacter vinelandii* is fully functional in nitrogenase FeMo cofactor assembly

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Assembly of nitrogenase FeMoco is one of the key processes in bioinorganic chemistry. NifB and NifEN are two essential elements immediately adjacent to each other along the biosynthetic pathway of FeMoco. Previously, an 8Fe-precursor of FeMoco was identified on NifEN; however, the identity of the biosynthetic intermediate on NifB has remained elusive to date. Here, we present a combined biochemical and spectroscopic investigation of a His-tagged NifEN-B fusion protein of *Azotobacter vinelandii*. Our data from the EPR and activity analyses confirm the presence of the 8Fe-precursor in the NifEN entity of NifEN-B; whereas those from the metal, EPR, and UV/Vis experiments reveal the presence of additional [Fe₄S₄]-type cluster species in the NifB entity of NifEN-B. EPR-, UV/Vis- and metal-based quantitative analyses suggest that the newly identified cluster species in NifEN-B consist of both SAM-motif (CXXXCXXC)- and non-SAM-motif-bound [Fe₄S₄]-type clusters. Moreover, EPR and activity experiments indicate that the non-SAM-motif [Fe₄S₄] cluster is a NifB-bound intermediate of FeMoco assembly, which could be converted to the 8Fe-precursor in a SAM-dependent mechanism. Combined outcome of this work provides the initial insights into the biosynthetic events of FeMoco on NifB. More importantly, the full capacity of NifEN-B in FeMoco biosynthesis demonstrates the potential of this fusion protein as an excellent platform for further investigations of the role of NifB and its interaction with NifEN during the process of FeMoco assembly.

Nitrogenase plays an essential role in biological nitrogen fixation, a process that converts the atmospheric dinitrogen (N₂) to the bioavailable ammonia (NH₃) (1). The “conventional” molybdenum (Mo)-nitrogenase consists of two components: an ATP-dependent reductase (Fe protein) and a catalytic component (MoFe protein). Both proteins contain metal centers: The homodimeric Fe protein holds a [Fe₄S₄] cluster between the two subunits; whereas the $\alpha_2\beta_2$ -tetrameric MoFe protein houses a P-cluster ([Fe₈S₇]) at each α/β -subunit interface and a FeMo cofactor, or FeMoco ([MoFe₇S₉X-homocitrate], where X = C, N or O), within each α -subunit (2, 3). During catalysis, these metal centers presumably align with one another upon the ATP-dependent association of the two component proteins, mediating the transfer of electrons from the [Fe₄S₄] center, via the P-cluster, to the FeMoco, where substrate reduction occurs (4). The properties of these clusters, therefore, are crucial for the reactivity of nitrogenase; in particular, the distinctive structural/chemical features of FeMoco—the active center of nitrogenase—underlie the unique reaction mechanism of this enzyme.

The biosynthesis of FeMoco (Fig. S1) is arguably one of the most complicated processes in bioinorganic chemistry (5). Analogous to the chemical synthetic approach of FeMoco topologies (6, 7), the biosynthetic pathway of FeMoco begins with the generation of small Fe-S building blocks; however, the latter process involves the stepwise assembly of small Fe-S fragments into a high-nuclearity cluster through the action of various *nif*-encoded proteins. It has been proposed that NifUS mobilizes Fe and S for the sequential formation of [Fe₂S₂] and [Fe₄S₄] clusters, which are transferred to NifB and assembled into a large Fe-S core that possibly contains all Fe and S for the generation of a mature FeMoco. Subsequently, this Fe-S core is transferred to NifEN

and further processed into a fully complemented FeMoco before the latter is delivered to its destined location within the MoFe protein (5).

The function of NifEN as a scaffold protein for FeMoco assembly is suggested by a considerable degree of sequence homology between NifEN and MoFe protein, which in turn leads to the hypothesis that NifEN contains a “P-cluster site” that houses a P-cluster homolog and a “FeMoco site” that hosts the conversion of a FeMoco precursor to a mature FeMoco (5). This hypothesis has been confirmed by combined biochemical, spectroscopic and structural studies of a precursor-bound form of NifEN (8–11). Crystallographic analysis shows that this NifEN species is structurally homologous to the MoFe protein (11); furthermore, it reveals the presence of a [Fe₄S₄] cluster at the P-cluster site and an all-Fe FeMoco precursor at the FeMoco site in this protein. The all-Fe precursor is compatible in geometry with the EXAFS-derived 8Fe model (i.e., an [Fe₈S₉] cluster), which closely resembles the Fe-S core of FeMoco (9, 11); moreover, it is nearly surface exposed, poised to be transformed into a mature FeMoco following the Fe protein-mediated insertion of Mo and homocitrate (10, 11).

The fact that the FeMoco is assembled by having the complete Fe-S core structure in place prior to the insertion of Mo and homocitrate suggests a significant role of NifB, the biosynthetic apparatus immediately upstream of NifEN, in FeMoco assembly. Indeed, deletion of *nifB* results in the generation of a cofactor-deficient MoFe protein, suggesting that NifB is an essential factor for the biosynthesis of FeMoco (12). The exact function of NifB in this process, however, remains largely unknown. Sequence analysis shows that NifB has a CXXXCXXC signature motif (designated SAM-motif from here on) at the N terminus, which is characteristic of a family of radical S-adenosylmethionine (SAM)-dependent enzymes. Additionally, there are plenty of potential ligands in the sequence of NifB (Fig. S2) that could accommodate the complete complement of Fe atoms in FeMoco. The biogenesis of the Fe-S core on NifB, therefore, may signify a previously undescribed synthetic route to bridged metal centers that relies on the radical chemistry of SAM (13). Unfortunately, the intermediate of FeMoco biosynthesis is yet to be identified on NifB, mostly due to the difficulty of obtaining a precursor-bound form of NifB.[†]

Here, we present a combined biochemical and spectroscopic investigation of a His-tagged NifEN-B fusion protein of *Azotobacter vinelandii*. Such an “integrated” assembly unit exists naturally in *Clostridium pasteurianum* (15) and is successfully engineered here by fusing NifN and NifB of *A. vinelandii* together. Our results indicate the presence of a [Fe₄S₄]-type cluster

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[†]A cofactorless NifB species has been studied previously (14).

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species in the NifB entity of NifEN-B along with the $[\text{Fe}_4\text{S}_4]$ clusters associated with the SAM-motif of NifB. Moreover, the simultaneous spectroscopic response of these two types of $[\text{Fe}_4\text{S}_4]$ clusters to SAM is accompanied by an increased formation of the previously identified 8Fe-precursor on NifEN-B, suggesting that the non-SAM-motif $[\text{Fe}_4\text{S}_4]$ cluster is a NifB-bound intermediate of FeMoco that could be converted to the 8Fe-precursor in a SAM-dependent mechanism.

Results

His-tagged NifEN-B is homologically expressed in *A. vinelandii* and purified at a yield of approximately 350 mg/kg cell paste. The successful fusion between NifN and NifB of *A. vinelandii* is confirmed by SDS/PAGE analysis, showing an approximately 100 kDa band that corresponds to the fused NifN-B subunit and an approximately 50 kDa band that corresponds to the His-tagged NifE subunit (Fig. 1A). The NifEN-B complex has a molecular mass of approximately 300 kDa, consistent with a subunit composition of $(\text{NifE})_2(\text{NifN-B})_2$ (Fig. 1B).

Like NifEN, NifEN-B is fully active as a FeMoco donor for the FeMoco-deficient ΔnifB MoFe protein upon incubation with dithionite, Fe protein, MgATP, MoO_4^{2-} and homocitrate (Table 1). Thus, NifEN-B should contain the previously identified, 8Fe-precursor in its NifEN entity, which can be converted to a mature FeMoco following such a treatment (8). This argument is further supported by the ability of NifEN-B to catalyze the reduction of acetylene (C_2H_2) and azide (N_3^-) in combination with Fe protein, MgATP, and dithionite (Table 2), a characteristic feature of NifEN that is bound with an 8Fe-precursor (16). The definitive proof for the presence of 8Fe-precursor in NifEN-B comes from the comparative EPR analysis of NifEN and NifEN-B in the indigodisulfonate (IDS)-oxidized state, where both proteins display a $g = 1.94$ signal (Fig. 2A, black and brown traces) that is solely attributed to the NifEN-associated 8Fe-precursor (8).[‡] Compared to NifEN, NifEN-B is 26% and 21% more active in assembly (Table 1) and catalysis (Table 2), respectively, which can be correlated further with a 15% increase in the signal intensity of the oxidized NifEN-B relative to that of the oxidized NifEN (Fig. 2A, black and brown traces). This observation points to a somewhat elevated accumulation of 8Fe-precursor on NifEN upon the fusion of NifB.

More excitingly, the Fe content of NifEN-B is considerably higher (by 1.85-fold) than that of NifEN, which is strongly indicative of the presence of additional cluster species in NifEN-B (Table 3). Indeed, NifEN-B (Fig. 2B, brown trace) displays a much more complex signal than NifEN (Fig. 2B, black trace) in the dithionite-reduced state, with additional features appearing at $g = 2.02$, 1.95, and 1.94 that overlap with the $S = 1/2$ signal of NifEN.[‡] Interestingly, these extra EPR features respond dramatically to SAM, showing a decrease in magnitude that eventually leads to their disappearance upon the addition of increasing amounts of SAM (Fig. 2B, blue and green traces). The complete disappearance of these features in the spectrum of NifEN-B at 40 mM SAM (Fig. 2B, green trace) results in a "restoration" of the $S = 1/2$ signal typically exhibited by dithionite-reduced NifEN (Fig. 2B, black trace), illustrating the irresponsiveness of the NifEN-associated clusters to SAM in the dithionite-reduced state. These results facilitate the identification of additional cluster species on NifEN-B, which are clearly distinguishable from the NifEN-associated clusters based on their response to SAM. Given the presence of a SAM-motif in NifB, it is logical

Fig. 1. Molecular mass of NifEN-B. (A) Coomassie blue-stained 10% SDS/PAGE of NifEN and NifEN-B. Lane 1, 15 μg of protein standard; lane 2, 1.5 μg of purified NifEN; lane 3, 1.5 μg of purified NifEN-B. The NifE and NifN-B bands are approximately 50 kDa and approximately 100 kDa, respectively. (B) Determination of the native molecular mass of NifEN-B by gel filtration. V_0 , void volume; V_e , elution volume. Protein standards (open circle) are: conalbumin (75 kDa), aldolase (158 kDa), ferritin (440 kDa) and thyroglobulin (669 kDa). The apparent native molecular mass of NifEN-B (red triangle) is approximately 300 kDa.

to assign these SAM-responsive cluster species to the NifB entity of NifEN-B.

The spectral contribution of the additional cluster species on NifEN-B is subsequently derived from the difference spectrum between NifEN-B (Fig. 2B, brown trace) and NifEN-B plus 40 mM SAM (Fig. 2B, green trace) in the dithionite-reduced state. Such a spectrum consists of a distinctive $S = 1/2$ signal with g values of 2.02, 1.95, and 1.94, suggesting that the newly identified clusters on NifEN-B are of $[\text{Fe}_4\text{S}_4]$ nature (Fig. 3A). Furthermore, this $S = 1/2$ signal integrates to 2.7 spins/mol protein, suggesting that it could originate from more than two equivalents of $[\text{Fe}_4\text{S}_4]^{1+}$ clusters. This assumption is substantiated by the comparative UV/Vis analysis of NifEN and NifEN-B, which reveals a significantly increased shoulder at 420 nm of the absorption spectrum of IDS-oxidized NifEN-B (Fig. 3B) that corresponds to an increased amount of $[\text{Fe}_4\text{S}_4]$ -type clusters in this protein. Quantitative analysis based on the ΔA_{420} [§] indicates that NifEN-B contains 3.4 mol more $[\text{Fe}_4\text{S}_4]$ cluster equivalents than NifEN per mol protein (Table 4). As NifB contains a signature SAM-motif that is capable of binding a $[\text{Fe}_4\text{S}_4]$ cluster, two of the three $[\text{Fe}_4\text{S}_4]$ cluster equivalents could be assigned to the two SAM motifs in the two NifB entities of NifEN-B (assuming a full occupancy of these sites); whereas the remaining cluster content could be attributed to another cluster species of $[\text{Fe}_4\text{S}_4]$ characteristics that is not directly bound to the SAM-motif. This assignment is further corroborated by a considerable difference between the Fe contents of NifEN-B (29.8 ± 2.2 mol Fe/mol protein) and NifEN (16.1 ± 3.3 mol Fe/mol protein), which could easily account for the presence of another $[\text{Fe}_4\text{S}_4]$ -type cluster species in addition to the two SAM-motif-bound $[\text{Fe}_4\text{S}_4]$ clusters in NifEN-B (Table 3).

The fact that the presumed SAM-motif- and non-SAM-motif-clusters collectively respond to SAM (Fig. 2B, blue and green traces) suggests that the events that occur on the SAM-motif-cluster(s) are likely coupled to those on the non-SAM-motif-cluster(s). Moreover, concomitant with the response of these two types of $[\text{Fe}_4\text{S}_4]$ clusters to SAM, the 8Fe-precursor-specific, $g = 1.94$ signal of NifEN-B is further enlarged by 17% and 29%, respectively, in the presence of 10 mM and 40 mM SAM (Fig. 2A, blue and green traces). Accompanying these spectroscopic changes, the maturation activity (Table 1) and catalytic capacity (Table 2) of NifEN-B are further increased by 54%

[‡]NifEN contains two types of clusters: the permanent $[\text{Fe}_4\text{S}_4]$ cluster, which is located between the α - and β -subunits; and the 8Fe-precursor ($[\text{Fe}_8\text{S}_7]$), which is situated at the α -subunit (8, 11). In the IDS-oxidized state, the 8Fe-precursor displays a characteristic $g = 1.94$ feature; whereas the permanent $[\text{Fe}_4\text{S}_4]$ clusters are EPR-silent (8). In the dithionite-reduced state, however, these clusters jointly give rise to an $S = 1/2$ signal (8).

[§] ΔA_{420} refers to the difference in the absorption at 420 nm between the oxidized and reduced forms of the same protein. See *Materials and Methods* for the quantification of $[\text{Fe}_4\text{S}_4]$ clusters based on ΔA_{420} (17).

Table 1. Maturation activities of NifEN and NifEN-B

Assay condition	Activities*				
	C ₂ H ₄ formation under C ₂ H ₂ /Ar	H ₂ formation under Ar	NH ₃ formation under N ₂	H ₂ formation under N ₂	Average percentage
NifEN	825 ± 16 (100)	876 ± 9 (100)	407 ± 22 (100)	258 ± 13 (100)	(100)
NifEN-B	1057 ± 21 (128)	1069 ± 3 (122)	508 ± 15 (125)	335 ± 14 (130)	(126)
NifEN-B + SAM†	1611 ± 1 (195)	1474 ± 31 (168)	759 ± 13 (186)	444 ± 22 (172)	(180)
MoFe protein	1990 ± 22 (241)	2048 ± 63 (234)	910 ± 27 (224)	531 ± 22 (206)	(226)

Data are shown as nmol per min per mg MoFe protein. The maturation activities of NifEN and NifEN-B (±SAM) refer to their abilities to reconstitute the cofactor-deficient MoFe protein once their associated precursors are converted to mature cofactors upon incubation with Fe protein, MgATP, homocitrate, MoO₄²⁻, and dithionite (8, 10, 11). The maturation activities of NifEN are set as 100%, and the percentages of activities of other proteins are expressed accordingly. All percentages are given in parentheses.

*The lower detection limits are 0.01, 0.02, 0.001, and 0.02 nmol per min per mg protein for C₂H₄ formation under C₂H₂/Ar, H₂ formation under Ar, NH₃ formation under N₂, and H₂ formation under N₂, respectively.

†The form of SAM used in the maturation assays is S-(5'-adenosyl)-L-methionine chloride.

and 47%, respectively, upon the addition of 10 mM SAM, both of which are accountable by the formation of more 8Fe-precursors on NifEN-B. Together, these observations strongly suggest that the non-SAM-motif [Fe₄S₄] cluster is a NifB-bound intermediate that could be converted to an 8Fe-precursor of FeMoco in a SAM-dependent mechanism.

Discussion

Consistent with the sequence-based prediction of a high degree of hydrophobicity of *A. vinelandii* NifB (18), our earlier attempts at characterizing a His-tagged form of NifB from this organism have been hampered by the extreme instability of NifB in aqueous solutions. In this work, this problem is circumvented by creating a fusion between NifB and NifN of *A. vinelandii*, which may protect certain hydrophobic areas of NifB through the interactions between the NifB and NifEN entities of NifEN-B. A large quantity of NifEN-B can be obtained this way, likely facilitated by the well-expressed, high-affinity His-tagged NifEN entity that “pulls” the NifB entity alongside during the purification process, resulting in a high yield of NifB normally not achieved by the purification of NifB alone. Moreover, the fact that the NifEN-B complex is fully active as a FeMoco donor for the cofactor-deficient MoFe protein not only precludes the absolute requirement for carrier protein(s) that deliver the cluster intermediate(s) from NifB to NifEN but also suggests a high capacity of NifB to interact with NifEN as a monomer during the assembly process of FeMoco.

The slightly increased accumulation of 8Fe-precursor on NifEN-B could result from a more efficient transfer of cluster species from NifB to NifEN, or a more protected/stabilized precursor-binding site near the surface of NifEN upon the “locking” of NifB on NifEN. More importantly, the biosynthetic intermediate that occurs prior to the 8Fe-precursor is “backed up” on NifB

through the fusion of NifB and NifEN, the two key assembly machineries immediately adjacent to each other along the biosynthetic pathway of FeMoco. The [Fe₄S₄] nature of this cluster intermediate is consistent with the widely accepted hypothesis that NifB receives the [Fe₄S₄] clusters from NifUS for the subsequent formation of a large Fe-S core (5, 13); whereas the formation of an 8Fe-precursor from these 4Fe-intermediates is reasonable from the standpoint of synthetic chemistry (6, 7). What's more, the simultaneous responses of this [Fe₄S₄]-type intermediate and the SAM-motif [Fe₄S₄] cluster to SAM and the concomitant increase in the amount of the 8Fe-precursor on NifEN-B point to a significant role of radical SAM chemistry in the NifB-based assembly events.

Fig. 2. EPR properties of NifEN-B. (A) EPR spectra of IDS-oxidized NifEN (black), NifEN-B (brown), NifEN-B plus 10 mM SAM (blue), and NifEN-B plus 40 mM SAM (green). The $g = 1.94$ signal, which is specific to the NifEN-associated 8Fe-precursor, is indicated. Spectra were collected in perpendicular-mode at 50 mW and 20 K. The $g = 1.94$ signals of NifEN and NifEN-B (in the absence of SAM) are saturated in intensity at 20 K. (B) EPR spectra of dithionite-reduced NifEN (black), NifEN-B (brown), NifEN-B plus 10 mM SAM (blue), and NifEN-B plus 40 mM SAM (green). All g values are indicated. The spectrum of NifEN-B in the presence of 40 mM SAM (green) is nearly identical in line shape to that of NifEN (black), with a slight shift in the g values. Spectra were collected in perpendicular mode at 50 mW and 10 K. The $S = 1/2$ signals of NifEN and NifEN-B (in the absence of SAM) are saturated in intensity at 10 K.

Table 2. Catalytic activities of NifEN and NifEN-B

Enzyme	Activities*		
	C ₂ H ₄ formation under C ₂ H ₂ /Ar	NH ₃ formation from N ₃ ⁻ under Ar	Average percentage
NifEN	49 ± 1 (100)	53 ± 2 (100)	(100)
NifEN-B	57 ± 2 (116)	66 ± 7 (125)	(121)
NifEN-B + SAM†	87 ± 5 (178)	83 ± 6 (157)	(168)

Data are shown as nmol per min per mg NifEN entity. The catalytic activities of NifEN and NifEN-B (±SAM) refer to their abilities to reduce certain nitrogenase substrates in combination with Fe protein, MgATP, and dithionite (15). The catalytic activities of NifEN are set as 100%, and the percentages of activities of other proteins are expressed accordingly. All percentages are given in parentheses.

*The lower detection limits are 0.01 and 0.001 nmol per min per mg protein for C₂H₄ formation under C₂H₂/Ar, and NH₃ formation from N₃⁻ under Ar, respectively.

†The form of SAM used in the maturation assays is S-(5'-adenosyl)-L-methionine chloride.

Table 3. Iron and acid-labile sulfide contents of NifEN and NifEN-B

	Fe	S ²⁻
NifEN	16.1 ± 3.3 (100)	19.0 ± 1.8 (100)
NifEN-B	29.8 ± 2.2 (185)	33.4 ± 4.4 (176)

Data are shown as mol Fe or S²⁻ per mol protein. The Fe and S²⁻ contents of NifEN are set as 100%, and percentages of the Fe and S²⁻ contents of NifEN-B are expressed accordingly. All percentages are given in parentheses.

A SAM-induced decrease of the magnitude of the SAM-motif-associated $S = 1/2$ signal has been documented earlier (19) and could be explained by the oxidation of the unique SAM-motif [Fe_4S_4] cluster from the +1 state to the EPR-silent, +2 state upon the binding of SAM, which subsequently supplies electrons for the reductive cleavage of SAM (20). The simultaneous decrease in the signal intensity of the [Fe_4S_4]-type intermediate, therefore, reflects an immediate impact of the redox events at the SAM-motif on the electronic properties of the [Fe_4S_4]-intermediate—perhaps through an initial redox modification of the [Fe_4S_4] cluster, followed by a more complicated rearrangement of two [Fe_4S_4] modules into an 8Fe-cluster—both of which capable of rendering the cluster species in an EPR-silent state. While it is unclear how spectroscopic changes of the [Fe_4S_4]-type intermediate are exactly coupled to those of the SAM-motif [Fe_4S_4] clusters, the concomitant disappearance of their EPR signals implies a close proximity of their locations in NifB that permits an instant action of one on the other.

An analogous pair of adjacent [Fe_4S_4] clusters has been identified in the molybdenum cofactor (Moco) biosynthesis protein MoaA (21, 22), which happens to be the closest relative to NifB in primary sequence. Crystallographic analysis reveals the presence of a SAM-motif [Fe_4S_4] cluster and a GTP-binding [Fe_4S_4] cluster that may work in concert with each other, generating a 5'-dA[•] radical upon the binding of SAM, which is then delivered to 5'-GTP for the formation of precursor Z (22). Compared to MoaA, NifB is considerably larger in size and does not contain a specific GTP-binding motif in its sequence; however, in addition to the three Cys ligands that belong to the SAM-motif at the N terminus, it has at least six highly conserved Cys residues across the entire length of the sequence. Thus, a similar cluster arrangement could be made for NifB, which places its SAM-motif-specific [Fe_4S_4] cluster strategically next to one (or two) assembly-related [Fe_4S_4] cluster(s) for the SAM-dependent assembly of these clusters into an 8Fe-cluster. It should be noted, however, that the radical SAM-dependent enzymes are highly diverse in their actions (20). Therefore, NifB may act in a completely different manner than any known member of this enzyme family; particularly, the incorporation of an extra S atom and, possibly, an interstitial X atom, by NifB into the coupled/rearranged [Fe_4S_4] modules requires a highly specialized mechanism that has never been defined before.

Although the details of the NifB-hosted biosynthetic events are yet to be explored, the outcome of this work has provided an important framework for further investigations of the role

Fig. 3. Spectroscopic features of the newly identified cluster species on NifEN-B. (A) Difference EPR spectrum (solid red) between NifEN-B (dashed dark gray) and NifEN-B plus 40 mM SAM (dashed light gray) in the dithionite-reduced state. (B) Difference UV/Vis absorption spectrum (solid red) between NifEN-B (solid dark gray) and NifEN-B plus 40 mM SAM (solid light gray) in the IDS-oxidized state; and difference UV/Vis absorption spectrum (dashed red) between IDS-oxidized NifEN-B (dashed dark gray) and NifEN-B plus 40 mM SAM (dashed light gray) in the dithionite-reduced state. Shown are the visible ranges of the absorption spectra between 375 nm and 775 nm.

of NifB and its interaction with NifEN through the characterization of the fully functional NifEN-B fusion protein. Future work will focus on addressing some of the key questions of FeMoco assembly—such as the exact mechanism of Fe-S core formation through radical-SAM-dependent chemistry, and the identity and incorporation of the μ_6 -interstitial X atom—in hopes of further elucidating the structural-functional relationship of the unique FeMoco of nitrogenase.

Materials and Methods

Unless noted otherwise, all chemicals and reagents were obtained from Fisher Scientific or Sigma-Aldrich.

Construction of *A. vinelandii* Strains. *A. vinelandii* strain YM65A was constructed as follows. First, two plasmids were generated: One, designated plasmid #1, contained a kanamycin-resistance cassette flanked by the chromosomal sequences upstream of the *nifB* gene and downstream of the *fdx* gene; the other, designated plasmid #2, contained a 3'-end fragment of *nifN* gene (with the stop codon removed) that was immediately followed by *nifB* and *fdx* genes and a 5'-end fragment of *nifX* gene. Then, using a previously described method (23, 24), plasmids #1 and #2 were sequentially transformed into DJ1041, an *A. vinelandii* strain expressing a His-tagged form of NifEN in the *nifHDKTY*-deletion background. These procedures led to the successful construction of YM65A, an *A. vinelandii* strain expressing a His-tagged form of the NifEN-B fusion protein, as well as the small Fdx protein, behind the *nifH* promoter in a *nifHDKTYB/fdx*-deletion background.

Cell Growth and Protein Purification. All *A. vinelandii* strains were grown in 180 L batches in a 200 L New Brunswick fermentor (New Brunswick Scientific) in Burke's minimal medium supplemented with 2 mM ammonium acetate. The growth rate was measured by cell density at 436 nm using a Spectronic 20 Genesys spectrophotometer (Spectronic Instruments). Following ammonia depletion, cells were de-repressed for 3 hr and then harvested immediately using a flow-through centrifugal harvester (Cepa). The cell paste was washed

Table 4. Cluster content based on UV/Vis data

	A ₄₂₀ (Oxidized)*	A ₄₂₀ (Reduced) [†]	ΔA ₄₂₀ (Oxidized-Reduced) [‡]	Cluster Content (mol/mol protein) [§]
ΔnifB NifEN [¶]	0.193	0.099	0.094	2.0
NifEN	0.365	0.221	0.144	3.2
NifEN-B	0.889	0.584	0.305	6.6

*Absorption of a protein oxidized by excess IDS at 420 nm.

[†]Absorption of a protein reduced by dithionite at 420 nm.

[‡]Difference in absorption between the oxidized and reduced forms of the same protein at 420 nm.

[§]The cluster content is calculated based on ΔA₄₂₀ and an ε₄₂₀ (absorption coefficient at 420 nm) of 6,000 M⁻¹ cm⁻¹, which was determined previously for the [Fe_4S_4] cluster of nitrogenase Fe protein (16).

[¶]ΔnifB NifEN represents a precursor-free form of NifEN, which contains two permanent [Fe_4S_4] clusters—one at each α/β-subunit interface of the protein (8).

with a 50 mM Tris-HCl (pH 8.0) buffer. Published methods were adapted to the purification procedures of His-tagged NifEN-B, NifEN, $\Delta nifB$ NifEN, MoFe protein and $\Delta nifB$ MoFe protein (8, 25), as well as the nontagged Fe protein (26).

Molecular Mass Determination. The native molecular mass of His-tagged NifEN-B was determined by gel filtration on an Ultrogel ACA 34 column (Pall Life Science; ID: 2.5 cm, Length: 1.4 m) at a flow rate of 0.5 mL/min. Conalbumin (75 kDa), aldolase (158 kDa), ferritin (440 kDa) and thyroglobulin (669 kDa) were used as protein standards (GE Healthcare Biosciences).

FeMoco Maturation Assay. The FeMoco maturation assay contained, in a 1.5 mL total volume, 25 mM Tris-HCl (pH 8.0), 20 mM dithionite ($\text{Na}_2\text{S}_2\text{O}_4$), 0.45 mg $\Delta nifB$ MoFe protein, 1.4 mg Fe protein, 0.3 mM homocitrate, 0.3 mM sodium molybdate (Na_2MoO_4), 0.8 mM ATP, 1.6 mM MgCl_2 , 10 mM creatine phosphate, 8 units creatine phosphokinase and, in some cases, 10 mM SAM. The FeMoco maturation was initiated with the addition of 2 mg NifEN or 3 mg NifEN-B to this mixture, which was then incubated at 30 °C for 30 min before it was examined for enzymatic activities (see below). The amounts of NifEN and NifEN-B in the assays were determined by titration experiments as described previously (8), which represented the amounts of protein required for the maximum possible maturation of FeMoco.

Metal, Sulfur, and Activity Determination. Iron and acid-labile sulfide were determined by published methods (27–29). The maturation activities were determined as described previously (30). The products H_2 and C_2H_4 were analyzed as published elsewhere (31); NH_3 was determined by a high performance liquid chromatography fluorescence method (32).

Electron Paramagnetic Resonance Spectroscopy. All samples for EPR analysis were prepared in a Vacuum Atmospheres dry box (Vacuum Atmospheres) with an oxygen level of less than 4 ppm. The dithionite-reduced NifEN and NifEN-B protein samples were in a pH 8.0 buffer containing 10% glycerol,

2 mM $\text{Na}_2\text{S}_2\text{O}_4$ and, in some cases, 10 mM or 40 mM SAM. The indigodisulfonate (IDS)-oxidized NifEN and NifEN-B samples were prepared by incubating samples with excess IDS for 5 min (33). A final concentration of 10 mM or 40 mM SAM was then added to some of these samples. All EPR spectra were recorded by a Bruker ESP 300 E_z spectrophotometer (Bruker) interfaced with an Oxford Instruments ESR-9002 liquid-helium continuous-flow cryostat (Oxford Instruments), using a microwave power of 50 mW, a gain of 5×10^4 , a modulation frequency of 100 kHz and a modulation amplitude of 5 G. A microwave frequency of 9.62 GHz was used to accumulate 5 scans of perpendicular-mode EPR spectra at 10 K (reduced samples) or 20 K (oxidized samples). Spin quantification of EPR signals was performed as described earlier (24).

UV/Vis Absorption Spectroscopy. Dithionite was removed from the reduced $\Delta nifB$ NifEN, NifEN, and NifEN-B protein samples by passage through a Sephadex G-25 Fine column (GE Healthcare Biosciences) in a Vacuum Atmospheres dry box with an oxygen level of less than 4 ppm. The protein samples were then oxidized by incubation with IDS for 5 min, followed by removal of excess IDS via passage through an anion exchange column (33). Aliquots of each oxidized sample were then diluted to 100 μL and placed anaerobically in sealed quartz cuvettes (1 cm light path), and UV/Vis spectra of these samples were recorded with a Hewlett Packard 8453A spectrophotometer (Agilent Technologies) against a buffer blank. Subsequently, these oxidized samples were re-reduced by supplementation of dithionite to a final concentration of 2 mM, followed by the recording of their UV/Vis spectra as described above. The $[\text{Fe}_4\text{S}_4]$ cluster content was estimated for each protein based on the difference in absorption at 420 nm (ΔA_{420}) between the oxidized and the reduced protein samples and an ϵ_{420} of 6,000 $\text{M}^{-1}\text{cm}^{-1}$ (17).

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