

Insertion of heterometals into the NifEN-associated iron–molybdenum cofactor precursor

Janice M. Yoshizawa · Aaron W. Fay ·
Chi Chung Lee · Yilin Hu · Markus Walter Ribbe

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Abstract The cofactors of Mo-, V-, Fe-dependent nitrogenases are believed to be highly homologous in structure despite the different types of heterometals (Mo, V, and Fe) they contain. Previously, a precursor form of the FeMo cofactor (FeMoco) was captured on NifEN, a scaffold protein for FeMoco biosynthesis. This all-Fe precursor closely resembles the Fe/S core structure of the FeMoco and, therefore, could reasonably serve as a precursor for all nitrogenase cofactors. Here, we report the heterologous incorporation of V and Fe into the NifEN-associated FeMoco precursor. EPR and activity analyses indicate that V and Fe can be inserted at much reduced efficiencies compared with Mo, and incorporation of both V and Fe is enhanced in the presence of homocitrate. Further, native polyacrylamide gel electrophoresis experiments suggest that NifEN undergoes a significant conformational rearrangement upon metal insertion, which allows the subsequent NifEN–MoFe protein interactions and the transfer of the cofactor between the two proteins. The combined outcome of these in vitro studies leads to the proposal of a selective mechanism that is utilized in vivo to maintain the specificity of heterometals in nitrogenase cofactors, which is likely accomplished through the redox regulation of metal mobilization by different Fe proteins (encoded by *nifH*,

nifH, and *anfH*, respectively), as well as the differential interactions between these Fe proteins and their respective scaffold proteins (NifEN and VnfEN) in the Mo-, V-, and Fe-dependent nitrogenase systems.

Keywords Nitrogenase · Heterometal · NifEN · Iron–molybdenum cofactor · Assembly

Introduction

Nitrogenase is a complex metalloprotein that provides the biochemical machinery for the nucleotide-dependent reduction of dinitrogen (N₂) to ammonia (NH₃). Three homologous nitrogenases, which are distinguished by the heterometals (i.e., Mo, V, and Fe) at their respective cofactor centers, have been identified to date [1]. Among them, the most extensively studied member is the Mo-dependent nitrogenase, which consists of two redox-active proteins: the Fe protein and the MoFe protein. The Fe protein (encoded by *nifH*) is an α_2 -dimer of approximately 60 kDa, which has a [Fe₄S₄] cluster bound between the subunits and a nucleotide (i.e., MgATP) binding site located within each subunit; whereas the MoFe protein (encoded by *nifD* and *nifK*) is an $\alpha_2\beta_2$ -tetramer of approximately 230 kDa, which has a P-cluster ([Fe₈S₇]) bridged between each α/β subunit dimer and an FeMoco ([MoFe₇S₉X–homocitrate], where X is C, N, or O) buried within each α -subunit [2]. Catalysis by this nitrogenase involves repeated association and dissociation between the Fe protein and the MoFe protein and the ATP-dependent electron transfer within the Fe protein/MoFe protein complex from the [Fe₄S₄] cluster of the Fe protein, through the P-cluster, to the FeMoco of the MoFe protein, where substrate is reduced [3].

J. M. Yoshizawa · A. W. Fay · C. C. Lee · Y. Hu (✉) ·
M. W. Ribbe (✉)
Department of Molecular Biology and Biochemistry,
University of California,
2448 McLaugh Hall,
Irvine, CA 92697-3900, USA
e-mail: yilinh@uci.edu

M. W. Ribbe
e-mail: mribbe@uci.edu

The V- and Fe-dependent nitrogenases are also two-component systems comprising Fe protein (encoded by *vnfH*)/VFe protein (encoded by *vnfD*, *vnfG*, and *vnfK*), and Fe protein (encoded by *anfH*)/FeFe protein (encoded by *anfD*, *anfG*, and *anfK*), respectively [1]. The Fe proteins of V- and Fe-dependent nitrogenases share a significant degree of sequence homology with their counterpart in the Mo-dependent nitrogenase, all of which are α_2 -dimers containing a single $[\text{Fe}_4\text{S}_4]$ cluster [1]. Except for the presence of an additional small subunit (encoded by *vnfG* and *anfG*, respectively), the VFe and FeFe proteins are also homologous to the MoFe protein, both in the primary sequence and in the types of metal centers they contain (i.e., both have the P-cluster and the cofactor centers that are homologous to those found in the Mo-dependent nitrogenase). In particular, the cofactor centers of VFe protein (i.e., FeVco) and FeFe protein (i.e., FeFeco) are believed to be structurally homologous to FeMoco and, at least in the case of FeVco, it has been reported that its Fe/S core structure is very similar to that of the FeMoco [4]. The catalytic mechanisms of the V- and Fe-dependent nitrogenases likely resemble that of the Mo-dependent system, which requires the participation of both component proteins and the sequential electron transfer toward their respective cofactor sites. On the other hand, the substrate-reducing profiles of V- and Fe-dependent nitrogenases are similar to yet distinct from that of their Mo counterpart, and the different heterometal constituents in their cofactor centers likely contribute to their altered catalytic capacities.

All three nitrogenase cofactors (i.e., FeMoco, FeVco, and FeFeco) share the same apparatus at the early stages of biosynthesis. Assembly of all cofactors is presumably initiated by NifU and NifS (encoded by *nifU* and *nifS*), which mobilize Fe and S for the biosynthesis of small Fe/S fragments. These small Fe/S clusters are then transferred to NifB (encoded by *nifB*) and further processed into a large Fe/S core that possibly contains all Fe and S necessary for the generation of a mature cofactor. The biosynthetic pathway of nitrogenase cofactor is believed to branch at this point, which leads to the formation of FeMoco if the Fe/S core is further matured on NifEN (encoded by *nifE* and *nifN*), or the formation of FeVco and FeFeco if the Fe/S core is further matured on VnfEN (encoded by *vnfE* and *vnfN*) [5].

The role of NifEN as a scaffold protein for FeMoco maturation was initially proposed on the basis of a significant degree of sequence homology between NifEN and MoFe protein, which has led to the hypothesis that NifEN contains a “P-cluster site” that houses a P-cluster homolog and an “FeMoco site” that hosts the conversion of FeMoco precursor to a mature cofactor [5]. Recently, a Mo/homocitrate-free precursor, which closely resembles the Fe/S core of the mature FeMoco, was captured on NifEN of *Azotobacter vinelandii* [6, 7]. In an ATP-dependent

process, this NifEN-associated precursor can be converted to a mature FeMoco upon insertion of Mo and homocitrate by Fe protein. Following the maturation of the precursor, NifEN can serve as an FeMoco source and directly activate the FeMoco-deficient $\Delta nifB$ MoFe protein [8, 9]. Identification of such an all-Fe precursor implies that, instead of being assembled by the previously postulated mechanism that involves the coupling of $[\text{Fe}_4\text{S}_3]$ and $[\text{MoFe}_3\text{S}_3]$ subclusters, the FeMoco is assembled by having the complete Fe/S core structure in place before the insertion of Mo. Moreover, given the homology among the three nitrogenase cofactors, such an Fe/S core could reasonably act as a precursor for all cofactors. Transformation to FeVco could occur by insertion of V (instead of Mo) along with homocitrate into the precursor, whereas conversion to FeFeco could take place by insertion of Fe (instead of Mo) along with homocitrate into the precursor or by having the precursor proceed as is for homocitrate attachment.

Here, we report the heterologous incorporation of V and Fe into the NifEN-associated FeMoco precursor. EPR and activity analyses indicate that V and Fe can be inserted at much reduced efficiencies compared with Mo, and incorporation of both V and Fe is enhanced in the presence of homocitrate. Further, native polyacrylamide gel electrophoresis (PAGE) experiments suggest that NifEN undergoes a significant conformational rearrangement upon metal insertion, which allows the subsequent NifEN–MoFe protein interactions and the transfer of the cofactor between the two proteins. The combined outcome of these in vitro studies leads to the proposal of a selective mechanism that is utilized in vivo to maintain the specificity of heterometals in nitrogenase cofactors, which is likely accomplished through the redox regulation of metal mobilization by different Fe proteins (encoded by *nifH*, *vnfH*, and *anfH*, respectively), as well as the differential interactions between these Fe proteins and their respective scaffold proteins (NifEN and VnfEN).

Materials and methods

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

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, Edison, NJ, USA) in Burke’s minimal medium supplemented with 2 mM ammonium acetate. The growth rate was measured by the cell density at 436 nm using a Spectronic 20 Genesys (Spectronic Instruments, Westbury, NY, USA). After ammonium consumption, the cells were

derepressed for 3 h, followed by harvesting using a flow-through centrifugal harvester (Cepa, Lahr, Germany). The cell paste was washed with 50 mM tris(hydroxymethyl)aminomethane (Tris)-HCl (pH 8.0). Published methods were used for the purification of His-tagged NifEN, His-tagged *ΔnifB* MoFe protein, and nontagged Fe protein from *A. vinelandii* strains DJ1041, DJ1143, and AvOP, respectively [6, 10–12].

Maturation assays

The conversion of NifEN-associated precursor to “FeMoco”, “FeVco”, or “FeFeco” was performed in a 50-mL maturation assay containing 25 mM Tris-HCl (pH 8.0), 100 mg precursor-bound NifEN (designated NifEN^{Precursor}), 120 mg Fe protein, 0.4 mM homocitrate, 2.4 mM ATP, 4.8 mM MgCl₂, 30 mM creatine phosphate, 24 units/mL creatine phosphokinase, 20 mM dithionite (Na₂S₂O₄), and 0.4 mM Na₂MoO₄, NH₄VO₃, or FeCl₃, respectively. The conversion of NifEN-associated precursor to the homocitrate-free “FeMo”, “FeV”, or “FeFe” cluster was performed using the same procedure as described above, except that homocitrate was omitted from the maturation assay. In all cases, the maturation mixtures were stirred for 1 h at 30 °C and, subsequently, the various NifEN forms were reisolated (designated NifEN^{FeMoco}, NifEN^{FeVco}, NifEN^{FeFeco}, NifEN^{FeMo}, NifEN^{FeV}, and NifEN^{FeFe}, respectively) and subjected to EPR, reconstitution, and electrophoresis experiments.

EPR spectroscopy

All EPR spectroscopy samples were prepared in a Vacuum Atmospheres dry box (Vacuum Atmospheres, Hawthorne, CA, USA) at an oxygen level of less than 4 ppm. The dithionite-reduced samples contained 15 mg/mL protein, 10% glycerol, 2 mM Na₂S₂O₄, and 25 mM Tris-HCl (pH 8.0). The indigo disulfonate (IDS)-oxidized samples were prepared by incubating proteins with excess IDS for 30 min and subsequently removing IDS using an anion-exchange column. Spectra were collected in perpendicular mode using a Bruker ESP 300 E_z spectrophotometer (Bruker, Billerica, MA, USA) interfaced with an Oxford Instruments ESR-9002 liquid-helium continuous-flow cryostat (Oxford Instruments, Oxford, UK). All spectra were recorded using a gain of 5×10^4 , a modulation frequency of 100 kHz, a modulation amplitude of 5 G, and a microwave frequency of 9.62 GHz.

Reconstitution analysis

The reconstitution of the FeMoco-deficient *ΔnifB* MoFe protein was performed in a 0.8-mL assay containing

25 mM Tris-HCl (pH 8.0), 20 mM Na₂S₂O₄, and 0.5 mg *ΔnifB* MoFe protein. Insertion of various cofactors/clusters was initiated by the addition of 2 mg cofactor/cluster-bound NifEN (see “Maturation assays”) to the assay, and the reaction mixture was then incubated for 30 min at 30 °C before being terminated and examined for enzymatic activities as described earlier [13].

Anaerobic native PAGE

Native PAGE of equimolar mixtures of *ΔnifB* MoFe protein (230 kDa) and various NifEN forms (210 kDa) containing different cofactor/cluster sources was performed in a Vacuum Atmospheres dry box at an oxygen level of less than 4 ppm. A precast 7.5% Tris-HCl gel (Bio-Rad) was prerun at 80 V for 1 h in an anaerobic running buffer containing 2 mM dithionite before the protein samples were loaded in the sample wells of the gel. Each sample contained 25 mM Tris-HCl (pH 8.0), 80% glycerol, 7 μg *ΔnifB* MoFe protein and 8 μg of one of the various NifEN forms (i.e., NifEN^{Precursor}, NifEN^{FeMoco}, NifEN^{FeMo}, NifEN^{FeVco}, NifEN^{FeV}, NifEN^{FeFeco}, and NifEN^{FeFe}) so that the molar ratio between *ΔnifB* MoFe protein and NifEN was approximately 1:1 in all cases. The gel was stained by Coomassie blue upon the completion of electrophoresis.

Results

Previously, an “FeMoco”-bound form of NifEN was generated by incubating NifEN^{Precursor} with Fe protein, MgATP, dithionite, MoO₄²⁻, and homocitrate and reisolating NifEN (i.e., NifEN^{FeMoco}) after such a treatment [14]. Recently, a “FeMo”-cluster-bound form of NifEN (i.e., NifEN^{FeMo}) was obtained by the same procedure, except that homocitrate was omitted from the incubation mixture (unpublished data). Such an “FeMo” cluster is a homocitrate-free yet Mo-containing equivalent of NifEN-associated “FeMoco”, and the difference between the two cluster species reflects the impact of homocitrate on Mo incorporation (unpublished data). To investigate whether V and Fe can be heterologously incorporated into the NifEN-associated FeMoco precursor, four NifEN forms—NifEN^{FeVco}, NifEN^{FeV}, NifEN^{FeFeco}, and NifEN^{FeFe}, respectively—were prepared using the same procedures as those used for the generation of NifEN^{FeMoco} and NifEN^{FeMo}, except that Mo(VI) (supplied in the form of MoO₄²⁻) in the incubation mixture was replaced by V(V) (supplied in the form of VO₃⁻) and Fe(III) (supplied in the form of FeCl₃), respectively. These V- and Fe-treated NifEN forms were subsequently compared with the Mo-bound forms of NifEN for the assessment of heterometal insertion into the NifEN-bound

precursors (see “Materials and methods” for the preparation of the various NifEN forms in this work).

EPR analyses

The NifEN-associated precursor undergoes a significant transformation upon the attachment of Mo, as both NifEN^{“FeMoco”} and NifEN^{“FeMo”} display EPR features that are different from those of NifEN^{“Precursor”} (Fig. 1). The insertion of Mo introduces similar $S = 3/2$ signals (which center at $g = 3.96$) into the EPR spectra of NifEN^{“FeMoco”} and NifEN^{“FeMo”}, both in the dithionite-reduced (Fig. 1a) and in the IDS-oxidized (Fig. 1b) states. Additionally, in the dithionite-reduced state, a $g = 2.03$ feature appears in the spectra of both NifEN^{“FeMoco”} and NifEN^{“FeMo”} (Fig. 1a, red and blue traces). Concurrent with the appearance of these Mo-associated EPR features, the $S = 1/2$ signals of dithionite-reduced NifEN^{“FeMoco”} and NifEN^{“FeMo”} decrease in magnitude by approximately 50% upon Mo insertion (Fig. 1a), whereas the $g = 1.92$ feature (Fig. 1b, black trace) disappears completely in the spectra of IDS-oxidized NifEN^{“FeMoco”} and NifEN^{“FeMo”} (Fig. 1b). As the $g = 1.92$ feature and approximately 50% of the $S = 1/2$ signal have been previously assigned to the NifEN-associated precursor,¹ the disappearance of these signals along with the appearance of the Mo-associated signals represent the conversion of the precursor to a Mo-containing cluster species [14]. Therefore, these parameters can be used to monitor the insertion of other heterometals into the NifEN-associated precursor.

Upon treatment with V or Fe, the NifEN-associated precursor undergoes changes that are similar to yet distinct from those associated with the insertion of Mo. Like NifEN^{“FeMoco”} and NifEN^{“FeMo”}, in the dithionite-reduced state, NifEN^{“FeVco”}, NifEN^{“FeV”}, NifEN^{“FeFeco”}, and

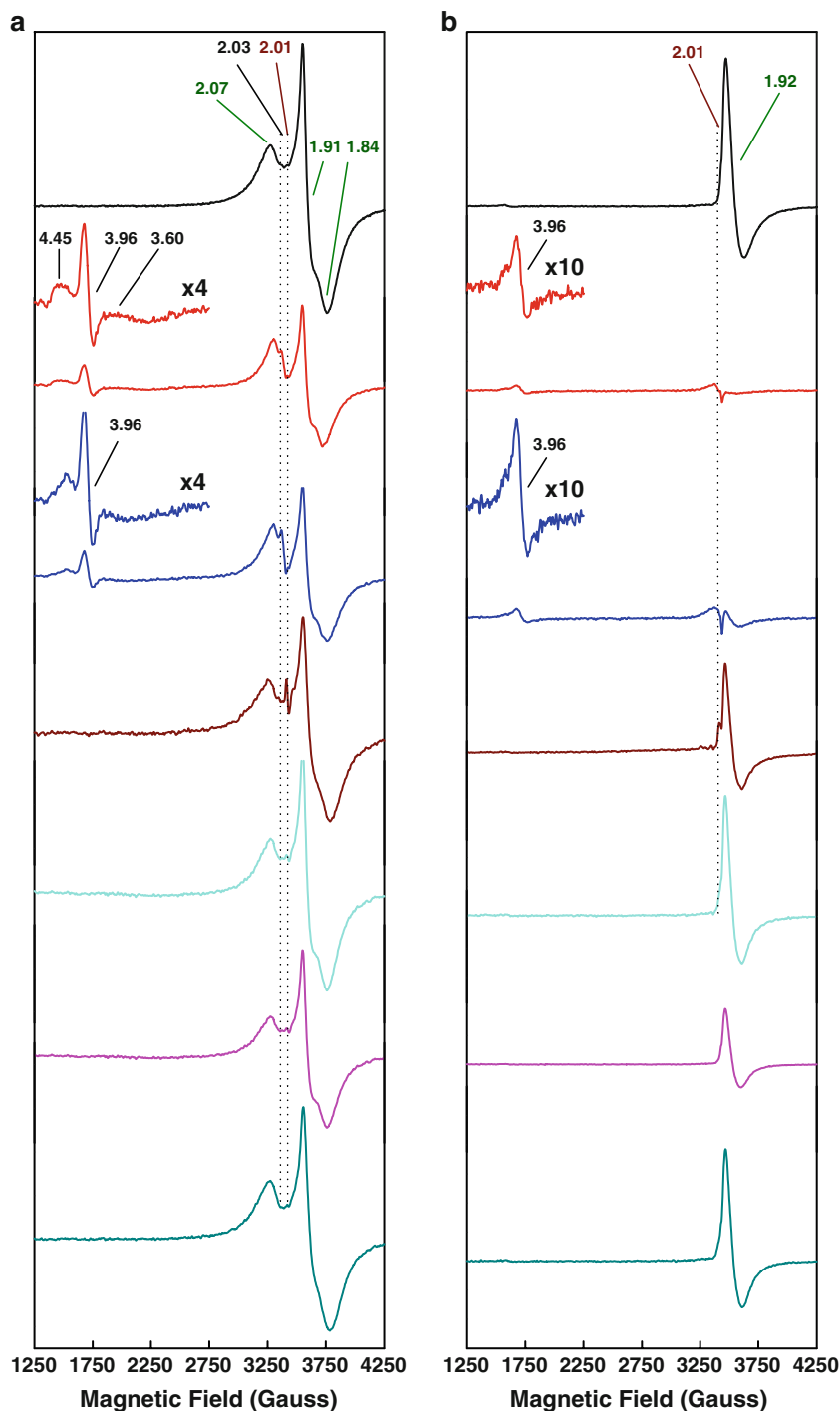
NifEN^{“FeFe”} (Fig. 1a) all display $S = 1/2$ signals of reduced intensity relative to that displayed by NifEN^{“Precursor”} (Fig. 1a). However, in these cases, the levels of signal reduction are much lower than those in the cases of NifEN^{“FeMoco”} and NifEN^{“FeMo”}. Similarly, the $g = 1.92$ feature of the precursor is reduced in size in the spectra of IDS-oxidized NifEN^{“FeVco”}, NifEN^{“FeV”}, NifEN^{“FeFeco”}, and NifEN^{“FeFe”} (Fig. 1b); however, in contrast to what is observed in the cases of NifEN^{“FeMoco”} and NifEN^{“FeMo”}, this feature is far from being obliterated from the spectra of these NifEN forms. Perhaps the biggest difference between the Mo-bound and the V- or Fe-treated cluster species is the absence of an $S = 3/2$ signal in the latter cases, although a new feature appears at $g = 2.01$, which can be best visualized in the spectra of both dithionite-reduced and IDS-oxidized NifEN^{“FeVco”} (Fig. 1). Together, these observations suggest that V and Fe can be incorporated into the NifEN-bound precursor. However, the lesser degree of decrease in the sizes of the precursor-associated signals upon V and Fe treatment, particularly in the absence of homocitrate, points to a lesser degree of metal incorporation into the V- and Fe-bound NifEN forms. Moreover, the absence of a distinctive $S = 3/2$ signal from the spectra of V- and Fe-bound NifEN forms suggests that, compared with the Mo-bound clusters, these V- and Fe-containing clusters possess different electronic properties (which may originate from the different spectroscopic properties of V and Fe) and/or they are present in different protein environments [which could indicate a different conformational rearrangement of NifEN (or the lack thereof) upon the insertion of V or Fe (see “Native PAGE” for further discussion)].

Activity assays

Recently, it was reported that NifEN^{“Precursor”} is capable of catalyzing the reduction of some substrates of MoFe protein [i.e., acetylene (C_2H_2) and azide (HN_3)], albeit at lower specific activities [15]. Compared with NifEN^{“Precursor”}, both NifEN^{“FeMoco”} and NifEN^{“FeMo”} display nearly no C_2H_2 - and HN_3 -reducing activities on their own, particularly in the case of NifEN^{“FeMoco”} (Table 1). On the other hand, both NifEN^{“FeMoco”} and NifEN^{“FeMo”} can activate (or reconstitute) the FeMoco-deficient $\Delta nifB$ MoFe protein (Table 1), and the level of $\Delta nifB$ MoFe protein reconstitution by NifEN^{“FeMoco”} is comparable to that by isolated FeMoco [14]. Interestingly, NifEN^{“Precursor”} shows its highest activities in C_2H_2 and HN_3 reduction at 0.4 mM dithionite, whereas these activities of NifEN^{“Precursor”} are practically eliminated at 20 mM dithionite [15]. The optimal Mo incorporation into NifEN^{“Precursor”}, on the other hand, occurs at 20 mM dithionite, as NifEN^{“FeMoco”} prepared at 20 mM dithionite displays approximately threefold higher activity

¹ NifEN contains two types of clusters: an $[Fe_4S_4]$ -type cluster at the $\alpha\beta$ -dimer interface and an FeMoco precursor (containing seven or eight Fe atoms) at the cofactor site in the α -subunit [5, 6]. Previous EPR analyses revealed that the $S = 1/2$ signal in the EPR spectrum of dithionite-reduced NifEN is a mixture of signals originating from both the $[Fe_4S_4]$ cluster and the precursor, which behave differently upon variations of temperature and power [6]. On the basis of these power- and temperature-dependent EPR experiments, approximately half of the $S = 1/2$ signal was assigned to the precursor, and the other half was assigned to the permanent $[Fe_4S_4]$ cluster [6]. Upon Mo insertion, the portion of the $S = 1/2$ signal that originates from the precursor (approximately 50%) disappears, whereas the portion of the $S = 1/2$ signal that originates from the $[Fe_4S_4]$ cluster (approximately 50%) remains [8]. The $g = 1.92$ feature has been assigned to the precursor on the basis of the observation of this signal in the spectrum of the IDS-oxidized NifEN (which contains both the precursor and the $[Fe_4S_4]$ cluster) but not in the spectrum of the IDS-oxidized $\Delta nifB$ NifEN (which contains only the $[Fe_4S_4]$ cluster as a result of *nifB* deletion) [6].

Fig. 1 EPR properties of various NifEN forms in (a) dithionite-reduced and (b) IDS-oxidized states. *Black* NifEN^{Precursor}, *red* NifEN^{“FeMoco”}, *blue* NifEN^{“FeMo”}, *brown* NifEN^{“FeVco”}, *cyan* NifEN^{“FeV”}, *pink* NifEN^{“FeFeCo”}, *green* NifEN^{“FeFe”}. The dithionite-reduced samples were measured at 6 K, whereas the IDS-oxidized samples were measured at 15 K. The $S = 3/2$ signals of NifEN^{“FeMoco”} and NifEN^{“FeMo”} are enlarged, and the g values are indicated. The g values of the features associated with the precursor ($g = 2.07$, 1.91, and 1.84), the Mo-bound clusters ($g = 4.45$, 3.96, and 3.60), and the V- and Fe-bound clusters ($g = 2.01$) are labeled in *green*, *black*, and *brown*, respectively



in reconstituting the $\Delta nifB$ MoFe protein than NifEN^{“FeMoco”} prepared at 0.4 mM [14]. Apparently, there is a redox-dependent “switch” between the catalytic and biosynthetic capacities of NifEN, as the calculated solution potentials of 0.4 and 20 mM dithionite are approximately -490 and -440 mV, respectively [16]. More importantly, the disappearance of the substrate-reducing activities of

NifEN^{“FeMoco”} and NifEN^{“FeMo”}—which indicates the disappearance of the catalytically competent precursor upon insertion of metal—correlates with the disappearance of the precursor-specific features in their EPR spectra (Fig. 1). Thus, the decrease in the substrate-reducing activities of NifEN^{“FeMoco”} and NifEN^{“FeMo”}, along with the increase in their capacities to reconstitute $\Delta nifB$ MoFe

Table 1 The substrate-reducing and reconstitution activities of various NifEN forms

Assay type	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	NH ₃ formation from HN ₃
Substrate-reducing activities ^a					
NifEN ^{Precursor}	52 ± 7	0	0	0	73 ± 10
NifEN ^{FeMoco}	3 ± 1 (94)	0	0	0	5 ± 1 (93)
NifEN ^{FeMo}	7 ± 5 (87)	0	0	0	8 ± 1 (89)
NifEN ^{FeVco}	24 ± 7 (54)	0	0	0	43 ± 2 (41)
NifEN ^{FeV}	34 ± 2 (35)	0	0	0	55 ± 5 (25)
NifEN ^{FeFeco}	12 ± 4 (77)	0	0	0	12 ± 4 (84)
NifEN ^{FeFe}	37 ± 4 (29)	0	0	0	35 ± 2 (52)
Reconstitution activities ^b					
NifEN ^{Precursor}	0	0	0	0	ND
NifEN ^{FeMoco}	1,065 ± 88	1,100 ± 19	334 ± 40	323 ± 23	ND
NifEN ^{FeMo}	55 ± 9	62 ± 5	50 ± 16	64 ± 7	ND
NifEN ^{FeVco}	12 ± 1	12 ± 1	0	7 ± 2	ND
NifEN ^{FeV}	<1	0	0	2 ± 1	ND
NifEN ^{FeFeco}	15 ± 1	22 ± 2	0	0	ND
NifEN ^{FeFe}	3 ± 1	0	0	<1	ND

Percentage decreases in the substrate-reducing activities of various NifEN forms relative to those of NifEN^{Precursor} are given in *parentheses*. The lower detection limits are 0.01, 0.02, 0.001, and 0.02 nmol/min/mg of protein for C₂H₄ formation under C₂H₂/Ar, H₂ formation under Ar, NH₃ formation under N₂, and H₂ formation under N₂, respectively

ND not determined

^a Assays were performed in the presence of 0.4 mM dithionite as described in “Materials and methods.” The substrate-reducing activities of various NifEN forms were measured by replacing MoFe protein with NifEN in the nitrogenase activity assays. Data are expressed as nanomoles per minute per milligram of NifEN

^b Assays were performed in the presence of 20 mM dithionite as described in “Materials and methods.” The reconstitution activities of various NifEN forms were measured by combining NifEN (the source of the cofactor) with the cofactor-deficient *ΔnifB* MoFe protein (the acceptor of the cofactor) in the nitrogenase reconstitution assays. NifEN^{Precursor} does not show any substrate-reducing activity under these conditions [15]. Therefore, all activities detected in these assays originate from the MoFe protein upon reconstitution by the NifEN forms that carry matured cofactors/clusters. Data are expressed as nanomoles per minute per milligram of *ΔnifB* MoFe protein

protein, can be used to analyze the extent to which V and Fe are heterologously inserted into the NifEN-associated precursor.

Like the Mo-treated NifEN (i.e., NifEN^{FeMoco} and NifEN^{FeMo}), V-treated NifEN (i.e., NifEN^{FeVco} and NifEN^{FeV}) and Fe-treated NifEN (i.e., NifEN^{FeFeco} and NifEN^{FeFe}) exhibit decreased C₂H₂ and HN₃ reducing activities relative to NifEN^{Precursor} (Table 1). However, the V- and Fe-treated NifEN forms show a lesser degree of decrease in activity than the Mo-treated NifEN forms, especially in the absence of homocitrate (Table 1). The V- and Fe-treated forms of NifEN can also activate *ΔnifB* MoFe protein (Table 1); yet, compared with the Mo-bound forms of NifEN, the levels of activation of *ΔnifB* MoFe protein by these NifEN forms are greatly diminished and, in particular, the capacities of the homocitrate-deficient V- and Fe-bound NifEN (i.e., NifEN^{FeV} and NifEN^{FeFe}) to activate the *ΔnifB* MoFe protein are nearly abolished (Table 1).

The changes in the catalytic activities and the spectroscopic features of NifEN upon heterometal insertion can be quantitatively expressed by setting the percentage decreases in the activities of substrate reduction (i.e., C₂H₂ and HN₃ reduction) and those in the sizes of the *S* = 1/2 signal (dithionite-reduced) and the *g* = 1.92 feature (IDS-oxidized) of NifEN^{FeMoco} (as compared with NifEN^{Precursor}) to represent the complete conversion of the precursor, and calculating the percentage decreases in these three parameters of the other NifEN forms relative to those of NifEN^{FeMoco} (Fig. 2). Clearly, there is a strong correlation between these three parameters, which align well with one another in each case (Fig. 2). Moreover, a tendency of increased metal incorporation in the presence of homocitrate can be observed in all cases (Fig. 2, NifEN^{FeMoco} vs. NifEN^{FeMo}, NifEN^{FeVco} vs. NifEN^{FeV}, NifEN^{FeFeco} vs. NifEN^{FeFe}). Finally, the level of metal incorporation under these experimental conditions can be assigned in the order Mo > Fe > V, as this is the order of the

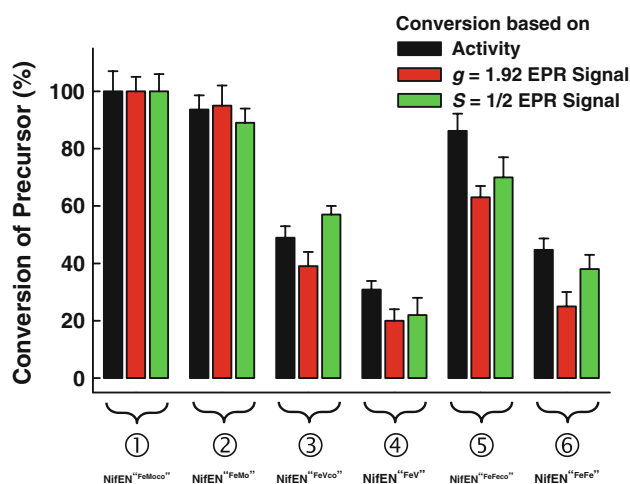


Fig. 2 Conversion of NifEN-associated precursor to various cofactors/clusters. Shown are the percentages of cluster conversion in NifEN^{FeMoco} (1), NifEN^{FeMo} (2), NifEN^{FeVco} (3), NifEN^{FeV} (4), NifEN^{FeFeco} (5), and NifEN^{FeFe} (6). The percentage of cluster conversion in NifEN^{FeMoco} was set as 100% (1), where the actual percentage decreases in the substrate-reducing activity (1, black), in the intensity of the $g = 1.92$ EPR feature (1, red), and in the intensity of the $S = 1/2$ signal (1, green) of NifEN^{FeMoco} are 94, 100, and 50%, respectively, relative to those of NifEN^{Precursor}. The levels of cluster conversion in other NifEN forms were calculated on the basis of the percentage decreases in the substrate-reducing activity (2–6, black), in the intensity of the $g = 1.92$ EPR feature (2–6, red), and in the intensity of the $S = 1/2$ signal (2–6, green) relative to those of NifEN^{FeMoco} (1, set at 100%)

percentage decreases in the three parameters of the Mo-treated (Fig. 2, NifEN^{FeMoco} and NifEN^{FeMo}), Fe-treated (Fig. 2, NifEN^{FeFeco} and NifEN^{FeFe}), and V-treated (Fig. 2, NifEN^{FeVco} and NifEN^{FeV}) NifEN forms.²

Native PAGE

The insertion of heterometal can be further monitored by complex formation between NifEN (the cofactor donor) and $\Delta nifB$ MoFe protein (the cofactor acceptor) at an approximate molar ratio of 1:1 in the anaerobic native PAGE. When NifEN^{Precursor} is mixed with $\Delta nifB$ MoFe protein, two separate bands can be visualized in the native gel, suggesting that these two proteins cannot form a strong complex (Fig. 3, lane 1). NifEN^{FeMoco} (Fig. 3, lane 2) or



Fig. 3 Anaerobic native polyacrylamide gel electrophoresis of equimolar mixtures of $\Delta nifB$ MoFe protein and various NifEN forms. Lane 1 $\Delta nifB$ MoFe protein plus NifEN^{Precursor}, lane 2 $\Delta nifB$ MoFe protein plus NifEN^{FeMoco}, lane 3 $\Delta nifB$ MoFe protein plus NifEN^{FeMo}, lane 4 $\Delta nifB$ MoFe protein plus NifEN^{FeVco}, lane 5 $\Delta nifB$ MoFe protein plus NifEN^{FeV}, lane 6 $\Delta nifB$ MoFe protein plus NifEN^{FeFeco}, and lane 7 $\Delta nifB$ MoFe protein plus NifEN^{FeFe}

NifEN^{FeMo} (Fig. 3, lane 3), on the other hand, forms a tight band in the native gel upon incubation with $\Delta nifB$ MoFe protein, suggesting that both can form complexes with the $\Delta nifB$ MoFe protein. There is a certain level of complex formation between $\Delta nifB$ MoFe protein and NifEN^{FeVco} (Fig. 3, lane 4), NifEN^{FeV} (Fig. 3, lane 5), NifEN^{FeFeco} (Fig. 3, lane 6), or NifEN^{FeFe} (Fig. 3, lane 7), as the lower NifEN band is reduced in intensity in all cases. However, consistent with the much reduced levels of V and Fe incorporation (Fig. 2), the NifEN band is still clearly visible, which either represents the unconverted portion of NifEN (i.e., NifEN^{Precursor}) that is unable to complex with the $\Delta nifB$ MoFe protein or suggests an “incorrect” conformational change of NifEN upon the heterologous incorporation of V or Fe that renders it incapable of complex formation with the $\Delta nifB$ MoFe protein (Fig. 3, lanes 4–7). The latter account would be consistent with our proposal that the missing $S = 3/2$ signal in the cases of V- and Fe-treated NifEN forms may reflect the differences in the protein environments where the V-, Fe-, and Mo-containing clusters reside (see “EPR analyses”). Together, these results suggest a “productive” conformational rearrangement of NifEN upon the effective insertion of the “correct” heterometal into the precursor.

Discussion

Our observation that V and Fe can be incorporated into the NifEN-associated precursor suggests the ability of Fe protein to mobilize metals other than Mo. Indeed, it was reported earlier that V and Mn can bind to the Fe protein in the presence of nucleotide [17, 18]. Additionally, in the case of V, it has been demonstrated that V(V) (supplied in the form of VO_4^{3-}) is reduced to V(IV) by the Fe protein

² The metal contents of the various NifEN forms have been analyzed by colorimetric methods and inductively coupled plasma mass spectrometry analyses. The degrees of precursor conversion in NifEN^{FeMo}, NifEN^{FeVco}, and NifEN^{FeV} are 90, 60, and 42%, respectively, by taking the Mo content in NifEN^{FeMoco} as a result of complete cluster conversion and comparing the Mo or V content of NifEN^{FeMo}, NifEN^{FeVco}, and NifEN^{FeV} with the Mo content of NifEN^{FeMoco} (data not shown). These numbers are consistent with the percentages of precursor conversion derived from the EPR and activity analyses (Fig. 2).

before it can bind to the nucleotide binding site of the latter [17]. This finding is consistent with the outcome of our previous X-ray absorption spectroscopy/extended X-ray absorption fine structure analysis, which indicates a decrease in the effective oxidation state of Mo (as compared with the supplied form of MoO_4^{2-}) upon its binding to the Fe protein in an ATP-dependent process, as well as a further reduction of Mo when homocitrate is attached to it [9]. Thus, the Fe protein/ATP-mediated heterometal mobilization may involve the proper reduction of any given metal (in association with the homocitrate) by the Fe protein, which in turn governs the efficiency at which the metal is “loaded” onto the Fe protein. This argument is particularly valid in light of the redox-dependent nature of the metal incorporation into the NifEN-associated precursor (see “Results”). The poor efficiencies of V and Fe insertion under the optimal conditions for Mo insertion, therefore, can be explained by the “incorrect” redox potential that is achieved by the *in vitro* dithionite/Fe protein (encoded by *nifH*) system. Under the *in vivo* conditions, the “correct” redox potentials for V and Fe insertion are likely accomplished by the *vnfH*- and *anfH*-encoded Fe proteins, which differ from that attained by the *nifH*-encoded Fe protein. It has been reported that in *Azotobacter chroococcum* the *vnfH*-encoded Fe protein has a midpoint potential different from that of the *nifH*-encoded Fe protein in the MgADP-bound state [19]. Thus, it is possible that the selectivity of heterometals for the cofactor centers of the Mo-, V-, and Fe-dependent nitrogenases is determined by the different redox potentials of the Fe protein components of these nitrogenases.

Concomitant with the incorporation of Mo into the NifEN-associated precursor, NifEN apparently undergoes a conformational change that allows the subsequent delivery of FeMoco from NifEN to MoFe protein upon direct protein–protein interaction (see “Results”). As NifEN is highly homologous to the MoFe protein, such a conformational change of NifEN likely occurs during the Fe-protein-mediated metal insertion process, where Fe protein interacts with NifEN in a manner that is analogous to that of its interaction with the MoFe protein during catalysis. Thus, the “correct” interaction between the Fe protein and NifEN is crucial for the proper transfer of the matured cofactor from its assembly site in NifEN to its final binding site in MoFe protein. Given the existence of two scaffold proteins for the assembly of nitrogenase cofactors (i.e., NifEN and VnfEN), as well as the presence of three homologous yet distinct Fe proteins (i.e., *nifH*-, *vnfH*-, and *anfH*-encoded Fe proteins), it is possible that the

interaction between a particular Fe protein and its designated scaffold protein during the process of metal insertion is unique to each of the three homologous nitrogenase systems. These differential scaffold protein–Fe protein interactions, along with the redox regulation of metal mobilization by Fe proteins, may provide the structural–chemical basis for the metal specificity of nitrogenase cofactor biosynthesis.

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