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Decoding the Nitrogenase Mechanism: The Homologue Approach

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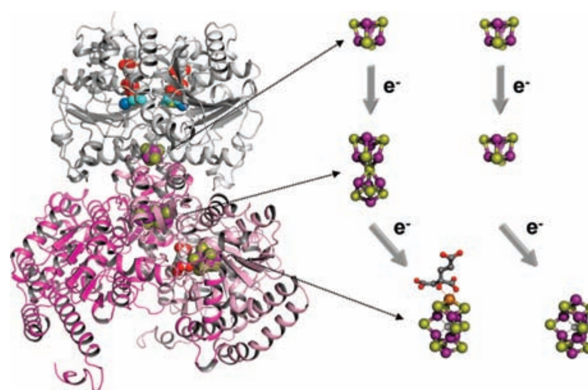
CONSPECTUS

The (Mo)-nitrogenase is a complex metalloenzyme that catalyzes the key step in the global nitrogen cycle, the reduction of atmospheric dinitrogen (N_2) to bioavailable ammonia (NH_3), at the iron−molybdenum cofactor (FeMoco) site of its molybdenum−iron (MoFe) protein component. Despite the fundamental significance of biological nitrogen fixation and extensive studies over the past decades, the catalytic mechanism of nitrogenase has not been deciphered.



One major challenge for the mechanistic study of nitrogenase is the redox versatility of its FeMoco center. The ability of FeMoco to shuttle between oxidation states in a rapid and unsynchronized manner results in a mixed oxidation state of the cofactor population during turnover. The substrate and the various intermediates can only interact with the FeMoco site in a transient manner, so it is extremely difficult to capture any substrate- or intermediate-bound form of nitrogenase for the direct examination of substrate−enzyme interactions during catalysis.

In this Account, we describe the approach of identifying a partially "defective" nitrogenase homologue, one with a slower turnover rate, as a means of overcoming this problem. The NifEN protein complex serves as an ideal candidate for this purpose. It is an $\alpha_2\beta_2$-heterotetramer that contains cluster-binding sites homologous to those found in the MoFe protein: the "P-cluster site" at the interface of the $\alpha\beta$-subunit dimer, which accommodates a $[Fe_4S_4]$-type cluster; and the "FeMoco site" within the α-subunit, which houses an all-iron homologue to the FeMoco. Moreover, NifEN mimics the MoFe protein in catalysis: it is capable of reducing acetylene (C_2H_2) and azide (N_3^-) in an ATP- and iron (Fe) protein-dependent manner. However, NifEN is unable to reduce proton (H^+) and N_2, and it is an inefficient enzyme with a restricted electron flux during the turnover.

The extremely slow turnover rate of NifEN and the possible "synchronization" of its FeMoco homologue at a certain oxidation level permit the observation of a new $S = {}^1/_2$ EPR signal upon turnover of C_2H_2 by NifEN, which is analogous to the signal reported for a MoFe protein variant upon turnover of the same substrate. This result is exciting, because it suggests the possibility of naturally enriching a C_2H_2-bound form of NifEN for the successful crystallization of the first intermediate-bound nitrogenase homologue.

On the other hand, the fact that NifEN represents a partially "defective" homologue of the MoFe protein makes it a promising mutational platform on which a functional MoFe protein equivalent may be reconstructed by introducing the missing features of MoFe protein step-by-step into NifEN. Such a strategy allows us to define the function of each feature and address questions such as the following: What is the function of P-cluster in catalysis? Are Mo and homocitrate the essential constituents of the cofactor in N_2 reduction? How does substrate accessibility affect the reactivity of the enzyme? This homologue approach could complement the mechanistic analysis of the nitrogenase MoFe protein, and information derived from both approaches will help achieve the ultimate goal of solving the riddle of biological nitrogen fixation.

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Introduction

Nitrogenase is a complex metalloenzyme that catalyzes the key step in the global nitrogen cycle: the nucleotide-dependent reduction of atmospheric dinitrogen (N_2) to bioavailable ammonia (NH_3).[1−3] Such a process, termed biological nitrogen fixation, is usually depicted as $N_2 + 8H^+ + 16MgATP + 8e^- \rightarrow 2NH_3 + H_2 + 16MgADP + 16P_i$. This reaction not only represents the major biological entry point of reduced nitrogen into the food chain but also embodies the formidable chemistry of breaking the triple bond of N_2 under ambient conditions. The fundamental significance and inherent complexity of biological nitrogen fixation has prompted extensive studies to obtain a molecular description of the nitrogenase. However, despite major efforts in the last decades,[1−6] the catalytic mechanism of nitrogenase has not been deciphered.

The complexity of the nitrogenase reaction originates, in part, from the intricate composition of this catalytic machinery. The best characterized molybdenum (Mo)-nitrogenase of *Azotobacter vinelandii* is a binary enzyme system comprising two redox-active metalloproteins.[2] One, designated the iron (Fe) protein, is an α_2-homodimer with one $[Fe_4S_4]$ cluster bridged between the two subunits and one ATP binding site located in each subunit;[7−9] the other, termed the molybdenum−iron (MoFe) protein, is an $\alpha_2\beta_2$-heterotetramer containing two unique metal centers: the P-cluster (or the "P" center), an $[Fe_8S_7]$ cluster ligated between each $\alpha\beta$-subunit dimer, and the iron−molybdenum cofactor (FeMoco, or the "M" center), a $[MoFe_7S_9X$-homocitrate$]$ cluster (X = C, N, or O) buried within each α-subunit.[10,11] Nitrogenase catalysis is a well-coordinated process that involves the participation of both component proteins and their associated metal centers. The Fe protein and the MoFe protein likely undergo repeated association/dissociation events in this process, where the Fe protein serves as an ATP-dependent reductase for the MoFe protein, transferring electrons from its $[Fe_4S_4]$ cluster through the P-cluster to the FeMoco of the MoFe protein, the site of substrate reduction.[2]

The unique structure of the P-cluster has led to the hypothesis that this cluster could serve as an effective "capacitor" that mediates the delivery of different numbers of electrons to FeMoco,[2] thereby allowing the FeMoco to adopt various oxidation states for the reduction of a wide range of substrates. Indeed, apart from its physiological substrate (i.e., N_2), nitrogenase is capable of reducing a variety of alternative substrates, such as acetylene (C_2H_2), azide (N_3^-), cyanide (CN^-), and hydrazine (N_2H_4), each requiring the accumulation of a different number of electrons at the FeMoco site for substrate binding and reduction.[2] On the other hand, the redox-versatility of FeMoco presents a major challenge for the mechanistic studies of nitrogenase. The ability of FeMoco to shuttle between the different oxidation states in a rapid and unsynchronized manner results in a mixed oxidation state of the FeMoco population during turnover, with which the substrates and the various intermediates can only interact in a transient manner. Consequently, it is extremely difficult to capture any substrate- or intermediate-bound form of nitrogenase for the direct examination of substrate−enzyme interactions during catalysis. Recently, a combined genetic and spectroscopic strategy was employed to overcome this problem. By altering the substrate accessibility and limiting the electron flux, a number of substrates or intermediates were successfully trapped on the MoFe protein.[5,6] These studies, together with the identification of a central atom of FeMoco that may be involved in nitrogenase turnover,[10] could prove instrumental in elucidating the mechanistic details of nitrogenase. Meanwhile, the search for alternative approaches continues, with the ultimate goal to solve the riddle of biological nitrogen fixation.

The Homologue Approach: Compare and Conquer

One approach that should be fully exploited is the identification and characterization of enzymatic systems that are homologous to nitrogenase. Given the major problems encountered in the mechanistic analysis of nitrogenase, it would be highly desirable if a homologous enzyme could be identified, one that has a slow electron flux through the system during the substrate turnover so that a specific oxidation state(s) of the cofactor can be naturally populated for the binding of a certain substrate(s) or intermediate(s). Furthermore, by carefully comparing such a partially "defective" homologous enzyme with the fully competent nitrogenase and deducing the origins of the differences in the catalytic capacities of the two systems, the key elements for the nitrogenase reaction could be systematically established.

NifEN serves as an ideal candidate for this purpose. Better known as a scaffold protein for FeMoco assembly, NifEN is an $\alpha_2\beta_2$-heterotetramer that shares considerable sequence homology with the MoFe protein.[12,13] It contains cluster-binding sites that are homologous to those found in the MoFe protein: a "P" center at the interface of each $\alpha\beta$-subunit dimer, which houses a P-cluster homologue, and an "M" center within each α-subunit, which hosts the conversion of a FeMoco precursor to a mature cluster prior to its transfer to the MoFe protein. While the P-cluster homologue in NifEN was identified

earlier as an [Fe_4S_4]-type cluster,[14] the FeMoco precursor was captured on NifEN only recently, when a His-tagged form of NifEN was isolated from an *A. vinelandii* strain containing deletions of *nifHDK* genes in the genomic background. Such a strain allows the capture of an FeMoco precursor on NifEN because of (i) the absence of the Fe protein (encoded by *nifH*), which prevents the final step of FeMoco maturation on NifEN (i.e., the incorporation of Mo and homocitrate into the FeMoco) from occurring, thereby trapping the FeMoco in a precursor state on NifEN, and (ii) the absence of the MoFe protein (encoded by *nifDK*), which interrupts the transfer of FeMoco from NifEN to the MoFe protein upon the completion of FeMoco assembly, thereby allowing the accumulation of the FeMoco precursor on NifEN.[15] This NifEN-bound FeMoco precursor was subsequently identified as an iron-only homologue closely resembling the Fe/S core structure of the FeMoco in the MoFe protein,[16] which can be converted, *in vitro*, to a fully matured FeMoco upon incubation with molybdate (MoO_4^{2-}), homocitrate, Fe protein, MgATP, and dithionite.[17]

With an [Fe_4S_4] cluster at the "P" center and an Fe-only FeMoco homologue (or FeMoco precursor) at the "M" center, NifEN seems to have the equivalents for both clusters that are involved in the electron transfer within the MoFe protein (Figure 1). One question naturally follows: can NifEN substitute for the MoFe protein as the redox partner of the Fe protein and catalyze the reduction of at least some substrates of the MoFe protein? Indeed, when coupled with the Fe protein, NifEN is capable of reducing C_2H_2 and N_3^- to C_2H_4 and NH_3, respectively, the same products generated by the MoFe protein in the same reactions (Figure 2).[18] Furthermore, like the MoFe protein, NifEN requires the Fe protein to function in the capacity of an ATP-dependent reductase during catalysis, because neither C_2H_2 nor N_3^- is reduced by NifEN if ATP is absent, if ATP is replaced by ADP or nonhydrolyzable ATP analogues, or if the Fe protein is replaced by its A157S variant that is specifically defective in ATP hydrolysis.[18] Finally, in the NifEN-catalyzed reactions, CO is a potent inhibitor for the reduction of both C_2H_2 and N_3^-, whereas C_2H_2 and N_3^- can serve as inhibitors for each other.[18] Such a pattern of CO inhibition, as well as the mutual inhibition between C_2H_2 and N_3^-, has also been observed in the MoFe protein-catalyzed reactions.[2] Together, these observations suggest that NifEN is a catalytically competent homologue of the MoFe protein.

NifEN: "Mission: Slow-Down"

Having established that NifEN is a homologous enzyme to the nitrogenase MoFe protein, the next question is: does NifEN fit the bill of a partially "defective" nitrogenase homologue that could facilitate the mechanistic analysis of nitrogenase?

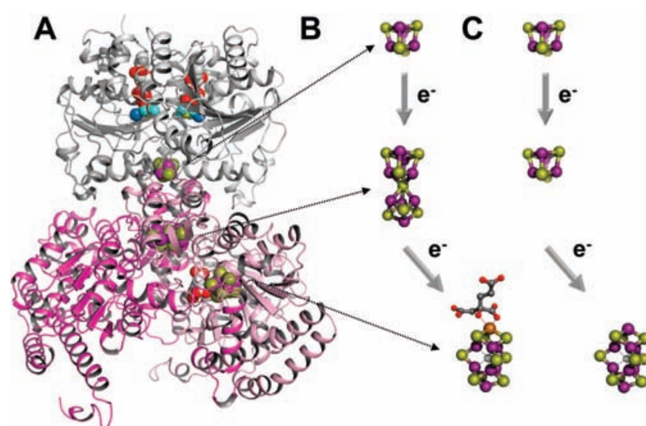

FIGURE 1. Components of the electron transfer chains in the Fe protein/MoFe protein system (nitrogenase) and the Fe protein/NifEN system (nitrogenase homologue). (A) The crystal structure of the MgADP·AlF_4^--stabilized complex between the Fe protein and one $\alpha\beta$-dimer of the MoFe protein. (B) The electron transfer pathway between the Fe protein and the MoFe protein. (C) The hypothetical electron transfer pathway between the Fe protein and NifEN. It has been proposed that, during nitrogenase catalysis, electrons flow from the [Fe_4S_4] cluster of the Fe protein to the P-cluster and then the FeMoco of the MoFe protein. Likewise, electrons could flow from the [Fe_4S_4] cluster of the Fe protein to the [Fe_4S_4] cluster and then the FeMoco homologue of NifEN. The two subunits of the Fe protein are colored dark and light gray, and the α- and β-subunits of the MoFe protein are colored light purple and magenta. The atoms of the metal centers are color as follows: Mo, orange; Fe, purple; S, yellow; O, red; C, dark gray; X (C, N, or O), light gray. These presentations are generated in PYMOL using 1N2C and 1M1N PDB coordinates.[8,10]

The answer to this question is a definite yes. NifEN has a much narrower substrate profile than the MoFe protein (Figure 2). Reduction of C_2H_2 by NifEN, like that by the MoFe protein, is a two-electron process, because C_2H_4 is the only detected product.[18] Reduction of N_3^- by NifEN, however, differs from that by the MoFe protein in the number of electrons involved. While the MoFe protein can reduce N_3^-, stepwise, by two, six, and eight electrons to $N_2 + NH_3$, $N_2H_4 + NH_3$, and NH_3, respectively, the NifEN-catalyzed reaction is "stuck" at the first step, because (i) NifEN cannot reduce N_2, (ii) no N_2H_4 production is detected; and (iii) N_2H_4 is not a substrate of NifEN.[18] Apparently, NifEN is only capable of reducing substrates with a limited amount of electrons, because the reactions of both C_2H_2- and N_3^--reduction involve no more than two electrons. This argument is further substantiated by the inability of NifEN to reduce CN^- and N_2: the former involves four or six electrons and the latter eight (i.e., six electrons for the reduction of N_2 and two for the concomitant reduction of H^+).[18] Interestingly, the reduction of H^+ to H_2 in the absence of N_2, another two-electron reaction, is missing from the catalytic

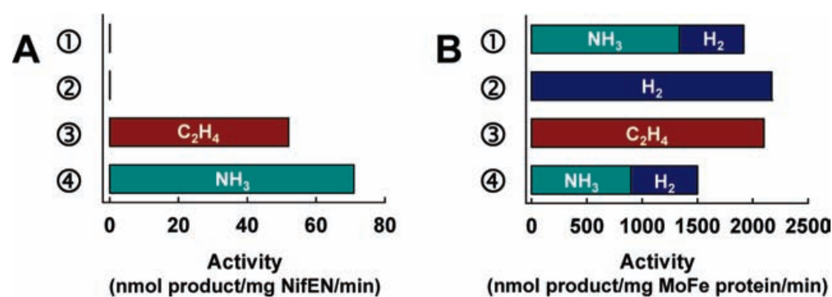

FIGURE 2. Substrate reducing activities of NifEN and MoFe protein. Shown are the activities of N_2 (①), H^+ (②), C_2H_2 (③), and N_3^- (④) reduction by NifEN (A) and MoFe protein (B) of *A. vinelandii*. NifEN is unable to reduce N_2 and H^+, but it is capable of catalyzing the two-electron reduction of C_2H_2 and N_3^- (A). The MoFe protein, on the other hand, is able to catalyze the concomitant reduction of N_2 and H^+, the reduction of H^+ that is independent of N_2, and the reduction of C_2H_2 and N_3^-. In the MoFe protein-catalyzed reaction, N_3^- can be reduced by two, six, and eight electrons to $N_2 + NH_3$, $N_2H_4 + NH_3$, and NH_3, respectively.

repertoire of NifEN.[18] This observation suggests that the H_2 evolution utilizes a different reaction mechanism or a different reaction site than the two-electron reduction of C_2H_2 and N_3^-.[18] Apart from having a limited substrate range, NifEN is much less active than the MoFe protein (Figure 2) and requires a lower solution potential (as suggested by the concentration of dithionite) and a higher excess of reductase (as indicated by the molar ratio of Fe protein to NifEN) to achieve the maximum activities.[18] The optimal reduction of C_2H_2 or N_3^- by NifEN occurs at a dithionite concentration of 0.4 mM (calculated solution potential of ca. −490 mV[19]) and an Fe protein/NifEN ratio of approximately 70.[18] In comparison, the MoFe protein requires a dithionite concentration of 20 mM (calculated solution potential of ca −440 mV[19]) and an Fe protein/MoFe protein ratio of 30 for the maximum activities.[18] The combined outcome of these studies points to a restricted electron flux through NifEN, rendering it an inefficient enzyme with a very slow substrate turnover rate.

One explanation for the "defects" of NifEN in substrate reduction is the presence of an $[Fe_4S_4]$ cluster, instead of an $[Fe_8S_7]$ P-cluster, at the "P" center of this protein (Figure 2). It is possible that the $[Fe_4S_4]$ cluster in NifEN does not mediate the electron transfer as effectively and flexibly as the P-cluster in the MoFe protein, resulting in (i) a much reduced electron flow through NifEN and (ii) a more oxidized state of the FeMoco homologue, to which only select substrates, such as C_2H_2 and N_3^-, can bind. The extremely slow turnover rate of NifEN (as shown by the activity of NifEN in C_2H_2- and N_3^--reduction) and the possible "synchronization" of its FeMoco homologue at a certain oxidation level (as suggested by the ability of NifEN to catalyze only the two-electron reduction of C_2H_2 and N_3^-) are precisely the features desired for an effective mechanistic analysis of nitrogenase, because the combined effect of both may permit a direct observation of the enzyme−substrate interactions in the NifEN-catalyzed reac-

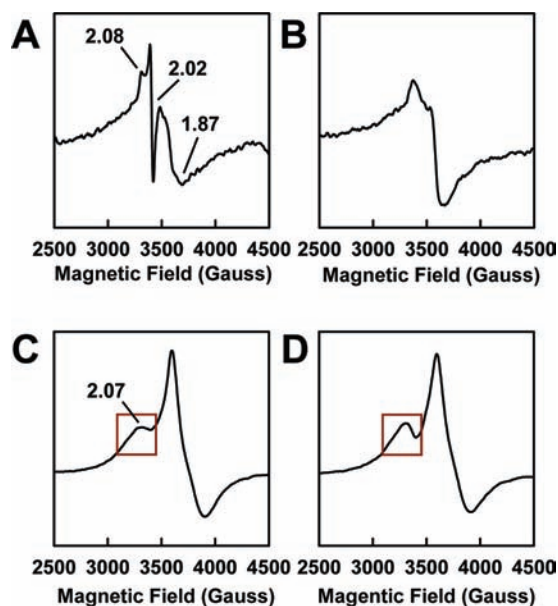

FIGURE 3. EPR properties of NifEN. Shown are the EPR spectra of NifEN under turnover (A) and nonturnover (B) conditions of C_2H_2 and under turnover (C) and nonturnover (D) conditions of N_3^-. The turnover samples (A, C) contain ATP, which is absent from the nonturnover samples (B, D). The EPR spectra in the presence of C_2H_2 and N_3^- were recorded at 30 and 6 K, respectively.

tions. Indeed, upon C_2H_2 turnover, a new $S = {}^1/_2$ signal appears at $g = 2.02$ of the EPR spectrum of NifEN (Figure 3A).[18] This signal, which is best visualized at 30 K, is absent from the spectrum of NifEN under nonturnover conditions (Figure 3B).[18] In the case of N_3^-, although no new EPR features can be observed upon turnover, the $g = 2.07$ feature of the NifEN-associated signal decreases in magnitude, which is most pronounced at 6 K (Figure 3C,D).[18] These observations are exciting, because one of the major stumbling blocks for the spectroscopic investigations of the nitrogenase mechanism is that no additional EPR features have been directly observed for the wild-type MoFe protein under normal turnover conditions, likely due to a diversely mixed oxidation state of the cofactor center that "dilutes" out any signal that may origi-

nate from a particular substrate- or intermediate-bound state of the enzyme.[6] Furthermore, an analogous $S = {}^1/_2$ signal has been reported for a variant form of the MoFe protein upon C_2H_2 turnover.[20] Thus, catalysis by NifEN likely mimics that by the MoFe protein and the active centers in NifEN and MoFe protein may undergo similar redox changes during the turnover of the same substrate (C_2H_2 in this case). The comparable yet considerably slower turnover of NifEN could prove advantageous in naturally enriching a C_2H_2-bound form of NifEN and eventually allowing the successful crystallization of the first intermediate-bound nitrogenase homologue, a feat yet to be accomplished for this complex enzyme system.

NifEN: "Project: Restoration"

The fact that NifEN represents a partially "defective" homologue of the MoFe protein makes it particularly interesting for comparisons to be made between the two proteins, which may reveal features missing in the former that are responsible for the catalytic capacity of the latter. The sequence alignment between NifEN and MoFe protein reveals that NifEN does not have the complete complement of residues of the MoFe protein in three key regions: the P-cluster site (or the "P" center), the cofactor site (or the "M" center), and the "proton gating" residue (Figure 4). Based on this comparison, a "restoration" strategy can be developed to introduce these missing features step-by-step back into NifEN, which may allow us to define the function of each feature and address the following questions that are of significant relevance to the mechanistic studies of nitrogenase.

To "P" or Not To "P"? The P-cluster is ligated at the α/β subunit interface of the MoFe protein by six cysteine residues, three from the α-subunit ($Cys^{\alpha62}$, $Cys^{\alpha88}$, and $Cys^{\alpha154}$) and three from the β-subunit ($Cys^{\beta70}$, $Cys^{\beta95}$, and $Cys^{\beta153}$) (Figure 5). In the presence of excess dithionite, the P-cluster is present in the P^N state, where all of the Fe atoms in the cluster are believed to be ferrous. Upon indigo disulfonate (IDS) oxidation, the P-cluster can be converted to the P^{OX} (or P^{2+}) state by a two-electron oxidation process, during which process one-half of this $[Fe_8S_7]$ cluster is "opened" up concomitantly with the added coordination from the backbone amide nitrogen of $Cys^{\alpha88}$ to one Fe atom and the O_γ of $Ser^{\beta188}$ to another Fe atom of the cluster (Figure 5).[11] Although the mechanistic relevance of the P^{OX} state is as yet unclear, the observation of a structural rearrangement of the P-cluster upon a change in its oxidation state suggests that the P-cluster may indeed undergo concurrent oxidative/structural changes during catalysis. Thus, the presence of all six cysteine residues along with $Ser^{\beta188}$ is likely the prerequisite both for the formation of the P-cluster (where a sufficient amount of coordination is required to secure the cluster in place) and for the functionality of the P-cluster in catalysis (where a proper manner of reorientation is needed to accommodate the redox-associated structural changes of the cluster).

Contrary to the MoFe protein, NifEN has only four cysteine ligands, three from the α-subunit ($Cys^{\alpha37}$, $Cys^{\alpha62}$, and $Cys^{\alpha124}$, which correspond to $Cys^{\alpha62}$, $Cys^{\alpha88}$, and $Cys^{\alpha154}$ of the MoFe protein, respectively) and one from the β-subunit ($Cys^{\beta81}$, which corresponds to $Cys^{\beta95}$ of the MoFe protein) (Figure 5). However, the other two cysteine residues of the MoFe protein, namely, $Cys^{\beta70}$ and $Cys^{\beta153}$, are replaced by $Ser^{\beta56}$ and $Gly^{\beta139}$, respectively, in NifEN. Furthermore, $Ser^{\beta188}$ of the MoFe protein is replaced by $Asp^{\beta174}$ in NifEN (Figure 5). Such a ligand composition accommodates, instead of an $[Fe_8S_7]$ cluster, an $[Fe_4S_4]$ cluster at each α/β subunit interface of NifEN, which could account for the low electron flux through NifEN and the limited amount of substrates that can be catalyzed by NifEN. In particular, it may explain the inability of NifEN to catalyze the reduction of H^+ to H_2, either as an obligate coproduct of N_2 reduction or as a sole product in the absence of N_2 (Figure 2). While the absence of the coevolution of H_2 could be interpreted as an indirect effect of the missing P-cluster, which renders the FeMoco homologue in a more oxidized state that is prohibitive for the concomitant binding/reduction of H^+/N_2 at the cofactor site; the absence of the sole evolution of H_2 could be explained by a direct involvement of the P-cluster as the site for the independent H_2 evolution, which would be consistent with the impact of the missing P-cluster on the H^+ reduction, but not on the C_2H_2 and N_3^- reduction (the other two-electron reactions that likely occur at the cofactor site). Indeed, although there is no direct evidence for the involvement of the P-cluster in H_2 evolution, the possibility that H_2 could be evolved from the P-cluster has been discussed.[2] Furthermore, it has been shown that one redox couple of the P-cluster (P^{2+}/P^{1+}) can undergo coupled electron and proton transfer.[21] Thus, it is reasonable to speculate that some H_2 could be evolved from the P-cluster site if the flow of proton/electron is diverted from the usual route of "destination: cofactor", particularly in the absence of N_2.

The function of the P-cluster can be analyzed by restoring the missing cysteine and serine ligands and duplicating a P-cluster site in each α/β subunit half of NifEN. An overall increase in the enzymatic activities and an expansion in the substrate profile could be expected upon such a transformation. NifEN may be able to reduce at least some substrates that require more than two electrons for binding/reduction (e.g., CN^-) or catalyze the N_3^- reduction beyond the two-electron

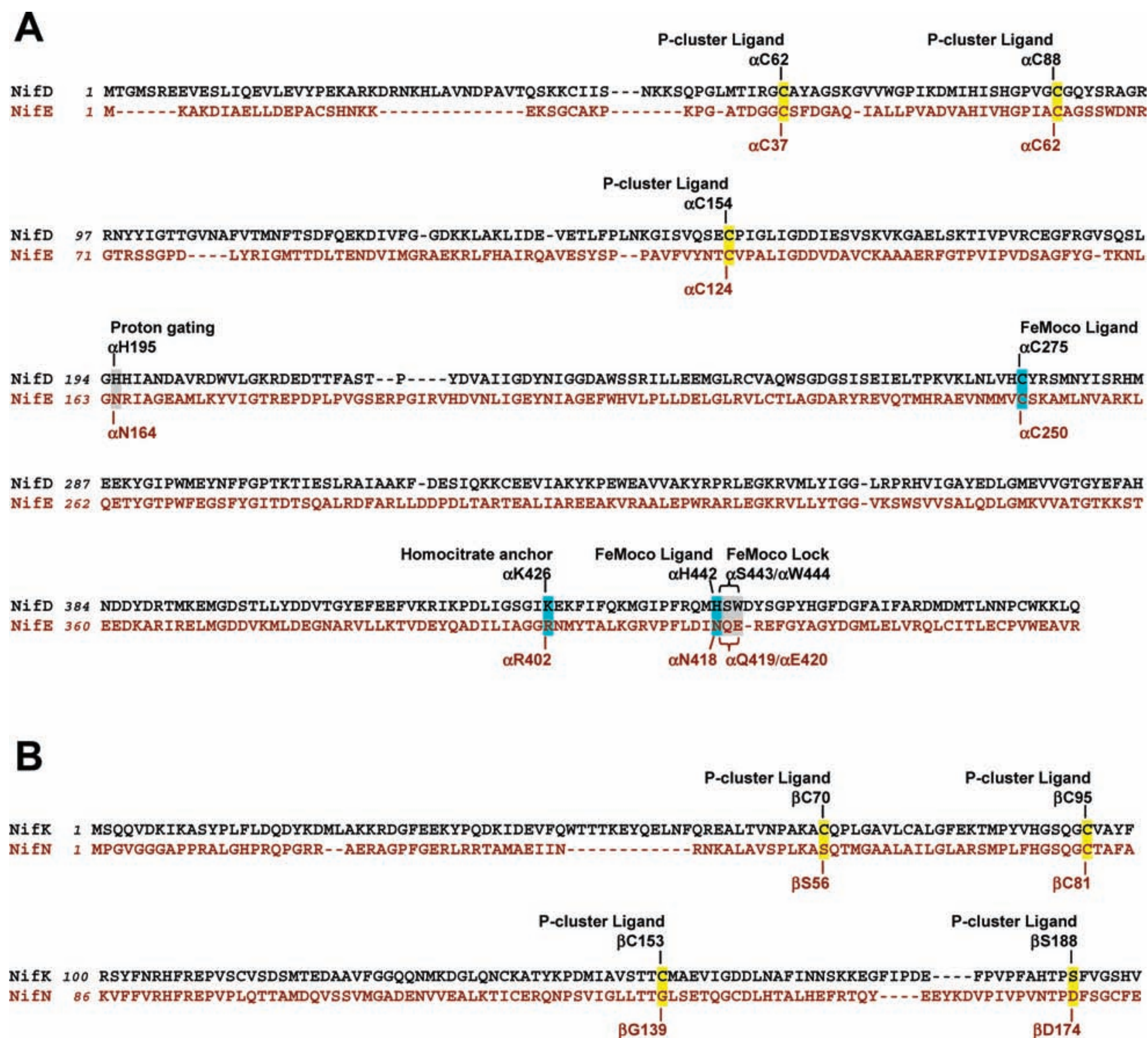

FIGURE 4. Comparison between the primary sequences of NifEN and MoFe protein. (A) Partial sequence alignment of the α-subunits of MoFe protein (NifD) and NifEN (NifE) from *A. vinelandii*. (B) Partial sequence alignment of the β-subunits of MoFe protein (NifK) and NifEN (NifN) from *A. vinelandii*. In NifEN, one of the FeMoco ligands (cysteine) is conserved; whereas the other (histidine) is replaced by an asparagine. The homocitrate anchor (lysine) is replaced by an arginine. Additionally, the FeMoco "lock" (tryptophan) is replaced by a glutamate. The P-cluster ligands (coordinating the P^N state) are also partially preserved in NifEN, with four of the six cysteine ligands conserved and the other two replaced by serine and glycine. The additional serine ligand for the P-cluster (coordinating the P^{OX} state) is replaced by an aspartate. The proton gating residue ($His^{\alpha195}$) is replaced by an asparagine in NifEN.

step. Additionally, it may be able to catalyze independent H_2 evolution, if the P-cluster is indeed the site for this reaction. On the other hand, NifEN may still lack the ability to reduce N_2 due to the absence of Mo and homocitrate from the iron-only FeMoco homologue (see below) or the lack of a certain residue that is crucial for proton gating during catalysis (see below).

Variation on a Theme of "M"? The FeMoco homologue in NifEN closely resembles the Fe/S core structure of the FeMoco in the MoFe protein; however, it is free of Mo and homocitrate. While this all-iron FeMoco homologue is catalytically active, its reactivity is rather limited (Figure 2). This finding coincides with the fact that the natural variants of FeMoco, namely, the iron–vanadium cofactor (FeVco) of the V-nitrogenase (where Mo is replaced by V) and the iron–iron cofactor (FeFeco) of the Fe-only nitrogenase (where Mo is completely absent), are less active than the FeMoco, especially in the case of the FeFeco (which, like the FeMoco homologue on NifEN, is of all-iron composition).[1] Together, these observations suggest a key role for Mo in nitrogenase reactivity. The absence of the homocitrate from the FeMoco homologue likely adds to the impact of the missing Mo on the

FIGURE 5. Comparison between the "P" centers in NifEN and MoFe protein. Consistent with the presence of four cysteine ligands (Cys$^{\alpha37}$, Cys$^{\alpha62}$, Cys$^{\alpha81}$, and Cys$^{\alpha124}$), the "P" center in NifEN is an [Fe_4S_4]-type cluster (A). In contrast, the "P" center in the MoFe protein is an [Fe_8S_7] cluster (B). In the reduced state (P^N), it is bridged by six cysteine ligands, three from the α-subunit (Cys$^{\alpha62}$, Cys$^{\alpha88}$, and Cys$^{\alpha154}$) and three from the β-subunit (Cys$^{\beta70}$, Cys$^{\beta95}$, and Cys$^{\beta153}$) (B). Upon a two-electron oxidation, the P-cluster can be converted to the oxidized state (P^{OX}), in which half of the cluster is "opened" up concomitantly with the additional coordination from Cys$^{\alpha88}$ and Ser$^{\beta188}$ (B).

catalytic capacity of NifEN, particularly where N_2 is the intended substrate. It has been proposed that the homocitrate switches from a bidentate ligation to a monodentate ligation to the Mo atom of the FeMoco during catalysis, which frees up a binding site for N_2 on Mo.[22] The absence of both homocitrate and Mo from the FeMoco precursor, therefore, may explain the inability of NifEN to employ such a mechanism for N_2 reduction.

Other than having some elements missing from its composition, the FeMoco homologue is present in a protein environment that is similar to, yet distinct from, that of the FeMoco in the MoFe protein. While Cys$^{\alpha275}$ (the ligand for the Fe-end of the FeMoco) is preserved as Cys$^{\alpha250}$ in NifEN, His$^{\alpha442}$ (the ligand for the opposite Mo-end of the FeMoco) is replaced by Asn$^{\alpha418}$ (Figure 6). In addition, Lys$^{\alpha426}$ (the homocitrate anchor at the Mo-end of the FeMoco) is replaced by Arg$^{\alpha402}$ in NifEN (Figure 6). Together, these alterations could contribute to a somewhat different orientation of the cofactor in NifEN. This theory was confirmed by our recent XAS/EXAFS analysis, which reveals that the Mo atom in the NifEN-bound FeMoco has an asymmetrical ligation to the three first-shell Fe atoms.[23] Apart from the differences in the cofactor ligands, several residues that presumably "pack" the FeMoco tightly in the polypeptide matrices of the MoFe protein are missing from NifEN; most notably, the Trp$^{\alpha444}$ residue that "locks" the FeMoco in place by its bulky side chain,[13] as well as a conserved Ser$^{\alpha443}$ right next to it, are replaced by Glu$^{\alpha420}$ and Gln$^{\alpha419}$, respectively, in NifEN (Figure 6). The absence of such a "lock" mechanism, along with the presence of a different set of ligands at the "M" center of NifEN, may result in a different orientation of the FeMoco homologue and an altered substrate reducing profile of the protein.

The functions of these key elements at the cofactor site can be analyzed in combination with the restoration of the P-cluster site. A dramatic increase in enzymatic activity and a further expansion in the substrate profile of NifEN could be expected upon the simultaneous restoration of both the P-cluster and the FeMoco sites. It would be most interesting to see whether N_2 can be reduced by NifEN following these transformations, because Mo and homocitrate are now incorporated into the FeMoco and oriented correctly at the FeMoco site. Both elements have been associated with N_2 reduction: Mo is the key constituent of the only known inorganic metal complex that is capable of N_2 reduction;[24] whereas the homocitrate has also been implicated in N_2 reduction.[22] However, the ability of NifEN to reduce N_2 could still be impaired by the absence of a residue that is crucial for delivering protons to the reaction of N_2 reduction (see below). Consequently,

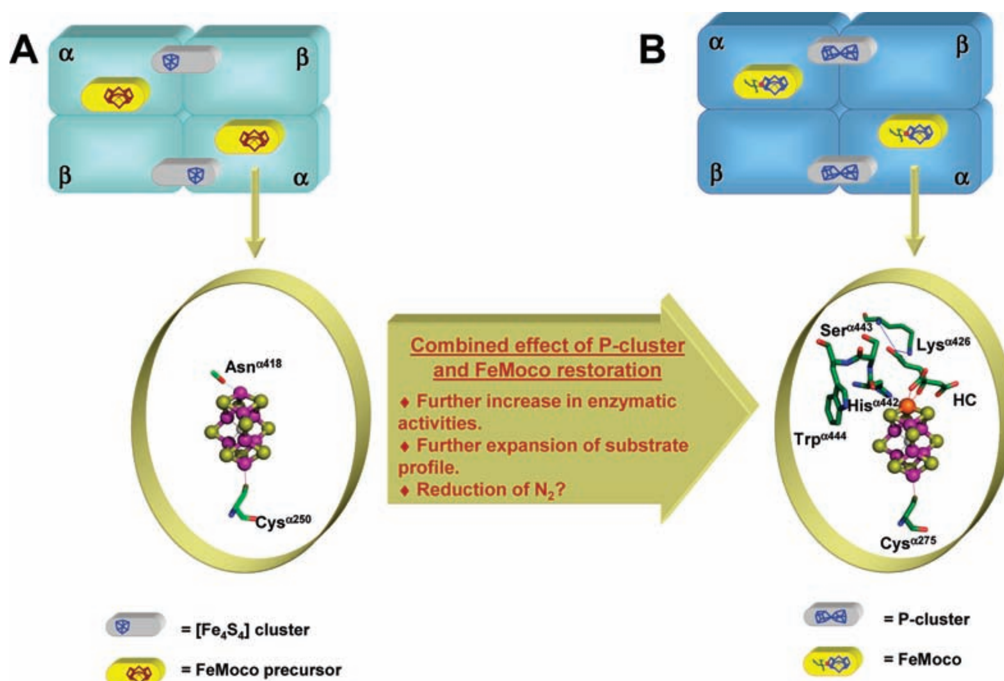

FIGURE 6. Comparison between the "M" centers in NifEN and MoFe protein. The "M" center in NifEN is a Mo- and homocitrate-free homologue of the FeMoco, and it is likely ligated by Asn$^{\alpha 418}$ and Cys$^{\alpha 250}$, respectively, at the opposite ends of the cluster (A). In contrast, the "M" center in the MoFe protein is a fully complemented FeMoco, and it is ligated by His$^{\alpha 442}$ and Cys$^{\alpha 275}$, respectively, at the Mo-end and the opposite Fe-end of the cluster (B). The homocitrate entity at the Mo-end of the cluster is further coordinated by Lys$^{\alpha 426}$ (B). Additionally, two highly conserved residues, Trp$^{\alpha 444}$ and Ser$^{\alpha 443}$, have been proposed to be part of a "lock" mechanism that secures the FeMoco in place (B).

NifEN may still be defective in N_2 reduction even when both the P-cluster and the FeMoco sites are restored in the protein.

Asparagine versus Histidine: Open, Sesame? Several residues in the MoFe protein have been implicated in controlling the access of substrates to the FeMoco site. Among them, the most interesting is His$^{\alpha 195}$. This residue is likely part of a putative proton (H^+) delivery chain to the active cofactor site, because the ability of the αH195N variant of the MoFe protein to evolve H_2 is significantly impaired.[25,26] Moreover, the decreased ability of the αH195N MoFe protein variant to evolve H_2 is associated with its inability to reduce N_2, although its ability to bind N_2 remains largely unaffected.[25,26] The function of His$^{\alpha 195}$ is particularly interesting from the mechanistic perspective and, therefore, has served as a topic of intense research in the recent years.[6,25,26] One possible account of the role of His$^{\alpha 195}$ in nitrogenase catalysis is that this residue is specifically involved in supplying H^+ to the N_2 reduction reaction, both for the formation of NH_3 and for the obligate H_2 evolution that accompanies the N_2 reduction, but it is not associated with the reduction of H^+ that is independent of the N_2 reduction. This argument is corroborated by the observation that the crude extract of the αH195N MoFe protein variant shows the same extent of decrease in its ability to evolve H_2 under Ar (where H^+ is the sole substrate) and N_2 (where both N_2 and H^+ are the intended substrates).[25] What is more, it implies that the H_2 evolution by nitrogenase may utilize two different mechanisms (i.e., one is N_2-dependent and the other is N_2-independent) or occur at different locations [e.g., the P-cluster being one potential site for the N_2-independent H_2 evolution (see above)].

Interestingly, the histidine to asparagine mutation is "duplicated" in the native sequence of NifEN, because NifEN has an aparagine residue (Asn$^{\alpha 164}$) in a position that corresponds to His$^{\alpha 195}$ in the MoFe protein (Figure 7). The presence of an asparagine residue instead of a histidine residue in NifEN, therefore, could abolish the ability of NifEN to catalyze the concomitant formation of H_2 and NH_3. Should this be the case, the restoration of both the P-cluster and the FeMoco sites in NifEN (above) could restore the ability of the protein to evolve H_2 independently of the N_2 reduction, yet it may still leave the protein "handicapped" in its ability to reduce N_2; whereas an additional mutation of Asn$^{\alpha 164}$ to histidine may further increase the activity of NifEN in H_2 evolution and restore the capacity of NifEN in N_2 reduction. In the latter scenario, NifEN may also be able to catalyze the reduction of all MoFe protein substrates with the same efficiency as the MoFe protein, because this protein likely has all the key elements that are crucial for the catalytic ability of the MoFe protein in place.

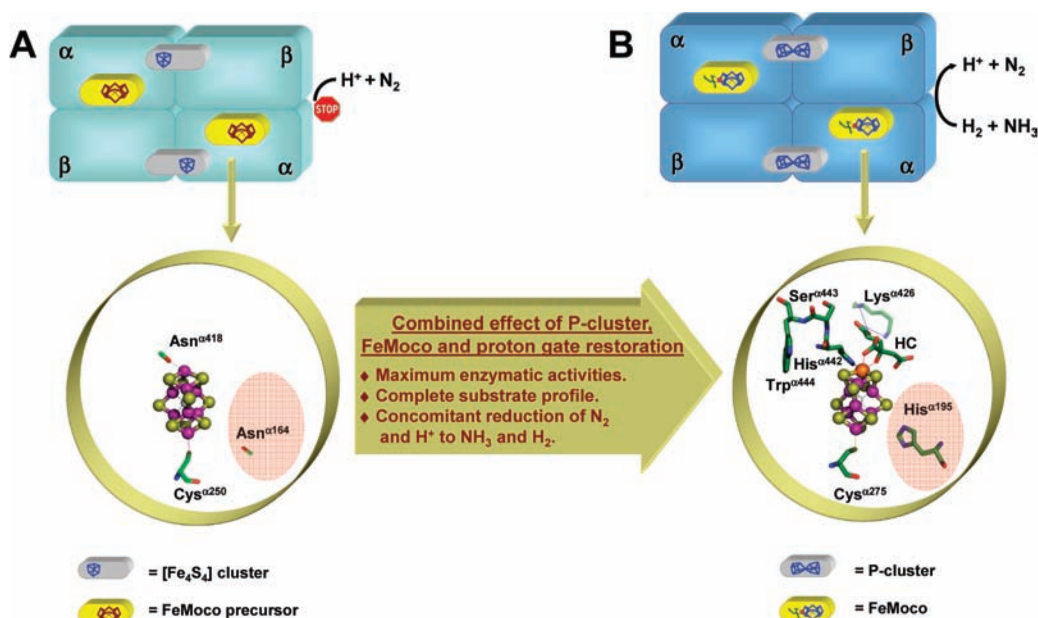

FIGURE 7. Comparison between NifEN and MoFe protein in the "proton gating" mechanism. In NifEN, an essential "proton gating" residue is replaced by another residue ($Asn^{\alpha164}$) near the "M" center (A). In the MoFe protein, this "proton gating" residue ($His^{\alpha195}$) is likely involved in the delivery of H^+ to the active "M" center for the concomitant reduction of H^+ and N_2 (B).

Future Outlook

As an excellent candidate in the application of a homologue approach to the mechanistic analysis of nitrogenase, NifEN can be used as-is for the capture of alternative substrates (e.g., C_2H_2), which facilitates a direct examination of the enzyme−substrate interactions during catalysis, or it can be used as a mutational platform on which a functional MoFe protein equivalent may be reconstructed by restoring the features of MoFe protein step-by-step in NifEN, which permits a detailed assessment of the functions of these feature in catalysis.

With a better understanding of the catalytic machinery of nitrogenase, the key features of the MoFe protein can then be introduced into NifEN in a "mix-and-match" manner in hopes of generating a low-flux nitrogen-fixing system that allows the capture of N_2 or nitrogenous intermediates. For example, the missing P-cluster ligands can be added in the NifEN sequence one or two at a time to create a P-cluster variant with a sufficient yet lower capacity to deliver electrons to the cofactor; alternatively, the FeMoco ligands can be restored one by one, and Mo can be incorporated into the iron-only FeMoco homologue either alone or in combination with the homocitrate to alter the reactivity of the active cofactor center; finally, the partial restoration of the P-cluster site or the FeMoco site can be combined with the mutations of the residues controlling substrate accessibility to further regulate the substrate profile. While it is too early to predict the outcome of these studies, we are optimistic that they could provide useful insights into the catalytic "black box" of nitrogenase. Information derived from this homologue approach could complement that gained through the mechanistic analysis of the nitrogenase MoFe protein and, together, they could help us work toward our ultimate goal of decoding the nitrogenase mechanism. Our future outlook in this regard is bright.

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FOOTNOTES

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