The FeMoco-deficient MoFe Protein Produced by a nifH Deletion Strain of Azotobacter vinelandii Shows Unusual P-cluster Features*

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The His-tag MoFe protein expressed by the nifH deletion strain Azotobacter vinelandii DJ1165 (ΔnifH MoFe protein) was purified in large quantity. The αβ2 tetrameric ΔnifH MoFe protein is FeMoco-deficient based on metal analysis and the absence of the S = 3/2 EPR signal, which arises from the FeMo cofactor center in wild-type MoFe protein. The ΔnifH MoFe protein contains 18.6 mol Fe/mol and, upon reduction with dithionite, exhibits an unusually strong S = 1/2 EPR signal in the g ~ 2 region. The indigo disulfonate-oxidized ΔnifH MoFe protein does not show features of the D2+ state of the P-cluster of the ΔnifB MoFe protein. The oxidized ΔnifH MoFe protein is able to form a specific complex with the Fe protein containing the [4Fe-4S]1+ cluster and facilitates the hydrolysis of MgATP within this complex. However, it is not able to accept electrons from the [4Fe-4S]1+ cluster of the Fe protein. Furthermore, the dithionite-reduced ΔnifH MoFe can be further reduced by Ti(III) citrate, which is quite unexpected. These unusual catalytic and spectroscopic properties might indicate the presence of a P-cluster precursor or a P-cluster trapped in an unusual conformation or oxidation state.

The metalloenzyme nitrogenase complex catalyzes the biological reduction of dinitrogen to ammonia (for recent reviews, see Refs. 1–6). The enzyme is composed of two separately purifiable proteins, the iron (Fe) protein and the molybdenum-iron (MoFe)† protein. The Fe Protein is a 60-kDa dimer of two identical subunits encoded by the nifH gene. The two subunits are bridged by a [4Fe-4S] cluster, and each subunit has a binding site for MgATP. The more complicated MoFe protein is a 230-kDa αβ2 tetramer with the α and β subunits encoded by the nifD and nifK genes, respectively. The MoFe protein contains two different types of metal clusters, the [8Fe-7S] cluster (P-cluster) bridged between each αβ subunit pair and the [MoFe-9S-homocitrate] cluster (FeMoco) located within each α subunits. Substrate reduction by the enzyme requires both component proteins, with the Fe protein serving as a specific reductant of the MoFe protein, which in turn provides the site of substrate reduction. To carry out the catalytic function of nitrogenase, the reduced Fe protein first binds two molecules of MgATP and undergoes a conformational change before forming a complex with the MoFe protein. Then, coupled with MgATP hydrolysis, electrons are transferred from the Fe protein to the P-clusters of the MoFe protein within the complex. This process is followed by the dissociation and re-reduction of the oxidized Fe protein and the dissociation of MgADP from the MoFe protein. Finally, the electrons are believed to be transferred from the P-cluster to the FeMoco, where substrate reduction occurs.

FeMoco-deficient, but P-cluster containing MoFe proteins have proved to be useful for the study of two major aspects of the nitrogenase research, the maturation of MoFe protein (7–18) and the features of the P-cluster (10, 19–21). Two types of 100% FeMoco-deficient MoFe proteins, presumably different catalytically and structurally, have been isolated and characterized. One was expressed by a nifB deletion strain (12, 21–22), and the other was expressed by a nifH deletion strain (10). The nifB gene product (NiFb) is involved in the synthesis of the FeMoco (23), a process independent of the production of the MoFe protein polypeptides (24–26). NiFb produces an iron- and sulfur-containing FeMoco precursor, FeMoco-co, presumably the starting point of the FeMoco synthesis (23). In addition, FeMoco synthesis in Azotobacter vinelandii also requires the Fe protein, reductant, MgATP, a protein designated γ (15), and the combined action of at least five more nif gene products, including nifN, nifE, nifX, nifQ, and nifV (for reviews, see Refs. 27–34 and 35). The Fe protein is involved not only in the biosynthesis of FeMoco but also in the insertion of preformed FeMoco into a FeMoco-deficient form of the MoFe protein during the final maturation of the holo-MoFe protein (27–34). This maturation process most likely occurs in a series of steps. Initially the FeMoco site is inaccessible to FeMoco insertion (7–9). The conversion to another form with the FeMoco site accessible for FeMoco insertion involves at least the Fe protein, GroEL, MgATP, and γ (9, 11–15, 18). All required components for this conversion are available in vivo in nifB deletion strains. Therefore, this step has already occurred and the FeMoco-deficient MoFe protein that accumulates in this strain (ΔnifB MoFe protein) has the FeMoco site in an open conformation and can be directly activated with isolated FeMoco (9). In contrast, the conversion to an accessible FeMoco binding site cannot occur in the absence of Fe protein, which is the case in the nifH deletion strain. Consequently, MoFe proteins produced by nifH deletion strains (ΔnifH MoFe protein) of A. vinelandii, such as DJ54, have the FeMoco site in a closed conformation (7–10).

Earlier studies suggested that the ΔnifB and ΔnifH MoFe proteins are different in not only the conformations of their FeMoco binding sites but also their P-cluster features (10, 21).
The exact function and redox properties of the P-cluster have been the focus of discussion for a long time (2). Which oxidation states of the P-cluster are required in the process of electron transfer from the Fe protein to the FeMoco of the MoFe protein still remain controversial, and several possibilities have been suggested. Some favor the theory that involves two-electron oxidized P\(^{2+}\) states or at least states more oxidized than P\(^{3+}\), the dithionite-reduced state of the P-cluster (36, 37), where others favor redox states more reduced than P\(^{2+}\) (2, 19). A comparative study of the \(\Delta nif/H\) and \(\Delta nif/H\) MoFe proteins can be used to address this problem as well as many other remaining questions about the function and assembly of the P-cluster. However, the instability of these FeMoco-deficient MoFe proteins and the loss of protein activity during the time-consuming purification procedure prevented reasonable protein yields for subsequent experiments. Recently, an efficient one step purification of a His-tag version of the \(\Delta nif/B\) MoFe protein has been reported that allowed the isolation of a sufficient amount of protein for further studies (21). Here we report a detailed catalytic and biophysical characterization of \(\Delta nif/H\) MoFe protein using the same expression and purification strategies for this mutant protein.

**EXPERIMENTAL PROCEDURES**

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

**Construction of the Variant A. vinelandii Strain, Cell Growth, and Protein Purification—**A. vinelandii \(\Delta nif/H\) mutant strain DJ1165, expressing a FeMoco-deficient His-tag MoFe protein (\(\Delta nif/H\) MoFe protein), was constructed by transforming competent cells of A. vinelandii DJ1141 with plasmid pDB115, which contained the \(nifH\) gene with a deletion within its coding sequence. The successful transformation of A. vinelandii strain DJ1141 by pDB115 was confirmed by the inability of the transformed cells to grow without a source for nitrogen fixation. The result of A. vinelandii DJ1165 contains an in-frame deletion within the \(nifH\) gene, extending from codon 158 to 200. The construction of plasmid pDB115 (38) and A. vinelandii strain DJ1141 (21) has been described elsewhere. The FeMoco-deficient His-tag MoFe protein expressed by A. vinelandi DJ1143 is designated as \(\Delta nif/B\) MoFe protein, and the construction of the A. vinelandii DJ1145 has been reported in detail previously (21).

A. vinelandii mutant strains DJ1165, DJ1143, CA12 (\(\Delta nif/HDK\)), and wild type were grown in 180-liter batches in a 200-liter New Brunswick fermentor on 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 (Spectronic Instruments, Rochester, NY). After the consumption of the ammonia, the cells were de-repressed for 3 h followed by harvesting using a flow-through centrifugal harvester (Cepa). The cell paste was washed with 50 mM Tris-HCl (pH 8.0) and kept on dry ice until needed.

Published methods were used for the purification of wild-type Fe protein (39), wild-type MoFe protein (40), \(\Delta nif/B\) MoFe protein (21), and the preparation of crude extract of A. vinelandii strain CA12 (18). \(\Delta nif/H\) MoFe protein was purified as described for the \(\Delta nif/B\) MoFe protein (21) using a slight modification. All buffers contained 10% glycerol.

**Protein Characterization and Spectroscopy**—All spectroscopic samples were prepared in a vacuum atmosphere dry box with an oxygen level of less than 4 ppm. Unless noted otherwise, all samples were in 25 mM Tris-HCl (pH 8.0), 10% glycerol, and 2 mM Na\(_2\)S\(_2\)O\(_4\). MoFe protein samples were oxidized by the addition of excess of indigo disulfonate and incubated for 30 min. Subsequently, indigo disulfonate was used to determine the insertion mixture was subsequently determined as described previously (40). The product of each assay was then analyzed as published elsewhere (44).

**RESULTS AND DISCUSSION**

The His-tag \(\Delta nif/H\) MoFe Protein Is an \(\alpha_2\beta_2\) Tetramer—Using the previously described method (21), up to 200 mg of His-tag \(\Delta nif/H\) MoFe protein/200 g of cells of A. vinelandii DJ1165 was purified after one step. This is a 10-fold yield increase compared with that of the conventional purification procedure (10), which was essential for subsequent extensive catalytic and biophysical characterization of this protein.

Fig. 1 shows that the purified His-tag \(\Delta nif/H\) MoFe is an \(\alpha_2\beta_2\) tetramer:
tetramer, as was described of the non-His-tag version of the ΔnifH MoFe protein from the A. vinelandii strain DJ54 (10). It has been reported that FeMoco-deficient MoFe protein in DJ54 crude extracts ran well above the position of wild-type MoFe protein on native gels. This was interpreted as a result of loose association of this mutant protein with another protein that was subsequently lost during the long purification procedure (9, 10). However, even with our fast purification procedure, we were not able to detect any protein, which was associated with the purified His-tag ΔnifH MoFe protein (Fig. 1, lane 6). It is worth mentioning that there is a significant difference between the subunit composition of the His-tag and non-His-tag versions of the ΔnifB MoFe proteins of A. vinelandii. In contrast to the αβγδ hexamer of nifB MoFe protein of A. vinelandii UW45 (16, 21), the αβγδ tetrameric His-tag ΔnifB MoFe protein of A. vinelandii DJ1143 does not contain an additional γ subunit (Ref. 21 and Fig. 1, lane 4). Interestingly, although γ is not regulated by nif genes, is believed to mediate the insertion of the FeMoco into the FeMoco-deficient MoFe protein (15), the His-tag ΔnifB MoFe protein was found to be almost fully reconstituted after the addition of isolated FeMoco in N-methylformamide and was therefore considered catalytically active (21). The ΔnifB MoFe protein produced by Klebsiella pneumoniae also exists as an αβγδ hexamer (12–14), and the additional γ subunit of this organism has been identified as the nifY gene product (13, 14). The protein NifY is also present in A. vinelandii, although it was known to be different from γ. Both NifY and γ are expressed at very low levels in A. vinelandii (21). A large quantity purification and characterization of NifY and γ will be required to address the remaining questions about the exact function of γ and protein interactions involving γ during nitrogenase biosynthesis.

The P-cluster Features of ΔnifH MoFe Protein Are Different from Those of ΔnifB MoFe Protein—Table I shows that isolated His-tag ΔnifH MoFe protein contains 18.6 mol of Fe/mol of protein and no detectable molybdenum. The analysis of isolated His-tag ΔnifB MoFe protein reveals a metal content almost identical to that of His-tag ΔnifH MoFe. The iron content of both mutant proteins, which is around 60% that of wild-type MoFe protein, and the absence of molybdenum in both matches previously described results of presumably P-cluster-containing but FeMoco-deficient MoFe proteins (10, 16, 21). The FeMoco deficiency of His-tag ΔnifH and ΔnifB MoFe protein is confirmed by the absence of the well characterized S = 3/2 EPR signal, which arises from the FeMoco center of wild-type MoFe protein (Fig. 2, trace 1).

Despite the presumably identical cofactor content, ΔnifH MoFe and ΔnifB MoFe protein are clearly distinguishable by color. Isolated ΔnifH MoFe protein has a light brown color, in contrast to the previously described reddish brown color of the ΔnifB MoFe protein (21). The apparent differences in color to the eye are consistent with observed differences in the visible range absorption spectra (Fig. 3). The spectrum of ΔnifB MoFe protein shows a broad shoulder in the ~475–575-nm range, in contrast to that of the ΔnifH MoFe protein, which is essentially featureless in the entire visible region. The difference of the colors and absorption spectra of these two proteins indicates a possible difference between their P-clusters in terms of structure or oxidation state. As shown in Fig. 2 (trace 2), isolated His-tag ΔnifB MoFe protein exhibits a S = 1/2 EPR signal that is recognized in the g ~ 2 region and integrated to 0.22 spin/MoFe protein. This signal has been described previously (10,
Unusual P-cluster Features of ΔnifH MoFe Protein

The IDS-oxidized ΔnifH MoFe Protein Does Not Show Features of the P2+ State of the P-cluster—The P\textsuperscript{N} state of the P-cluster of wild-type and His-tag Δnif/H MoFe proteins can be two-electron-oxidized to the P\textsuperscript{2+} state by IDS (21). This state can be recognized by a g = 11.8 signal observed in the parallel mode EPR (21, 50, 51). Mössbauer data indicate that the “P-cluster” of the isolated Δnif/H MoFe protein is in a more oxidized state than that of the Δnif/H MoFe protein (a detailed analysis will follow in a later report). If the Δnif/H MoFe protein contains a more oxidized P-cluster than that of the Δnif/H MoFe protein, it should show the P\textsuperscript{2+} EPR signal upon IDS oxidation.

12, 19, 21, 49) and was interpreted as a very minor species of the P-cluster population (21). The origin of this signal could be explained by the presence of either a P-cluster precursor that is not fully processed (21, 49) or a P-cluster in a state that is more oxidized or reduced than the dithionite-reduced P\textsuperscript{N} state (10, 19). The identical S = 1/2 EPR signal in the g ~ 2 region is also present in the His-tag Δnif/H MoFe protein sample (Fig. 2, trace 3). However, in this case the signal is much stronger and integrated to 0.7 spin/MoFe protein. This is the highest value that has ever been described for this signal and may be the result of the improved protein purification method that helps to prevent protein degradation or destruction of the metal clusters. If the S = 1/2 EPR signal in the g ~ 2 region arises from a P-cluster precursor, then it should be a major component of the His-tag Δnif/H MoFe protein.

Reduced Fe Protein Cannot Transfer Electrons to the IDS-oxidized Δnif/H MoFe Protein—The IDS-oxidized Δnif/H MoFe protein in its P\textsuperscript{2+} state can be reduced to the P\textsuperscript{1+} state through a MgATP-dependent one-electron transfer from the [4Fe-4S]\textsuperscript{1+} state of the Fe protein (21). The P\textsuperscript{1+} state of the P-cluster can be recognized by a rhombic signal in the perpendicular mode EPR with g values of 2.05, 1.94, and 1.81 at pH 7.4 (21, 52).Fig. 4A (traces 1 and 2) shows the appearance of the expected g = 11.8 parallel mode EPR signal, and Fig. 4B (traces 1 and 2) shows the concurrent disappearance of the S = 1/2 perpendicular mode EPR signal in the g ~ 2 region after the oxidation of the P-cluster of the Δnif/H MoFe protein from the P\textsuperscript{N} to the P\textsuperscript{2+} state by IDS. The g = 11.8 parallel mode EPR signal cannot be observed in the case of the IDS-oxidized Δnif/H MoFe protein (Fig. 4A, trace 4). However, the disappearance of the S = 1/2 perpendicular mode EPR signal (Fig. 4B, traces 1 and 2) and the simultaneous increase of the absorbance in the visible region around 420 nm (Fig. 5B) are evidences for the oxidation of a cluster. An identical absorbance increase at 420 nm was observed in the case of the IDS-oxidized Δnif/H MoFe protein (Fig. 5A). These results show that the Δnif/H MoFe protein, like the Δnif/H MoFe protein, can be oxidized by IDS. However, IDS-oxidized Δnif/H MoFe protein does not show the expected features of the P\textsuperscript{2+} state, indicating the presence of a possible structurally different cluster from the known P-cluster structure.

Fig. 4A shows that the parallel mode P\textsuperscript{2+} EPR signals of the sample with the oxidized Δnif/H MoFe protein alone (Fig. 6A,
which arises from the $P^{1+}$ state of the P-cluster with $g$ values of 2.05, 1.95, and 1.81 (Fig. 6B, trace 1 + 3). In contrast, the $S = 1/2$ EPR signals of the MgADP-bound $[4Fe-4S]^{1+}$ Fe protein alone (Fig. 6B, trace 2) or this conformation of Fe protein incubated with oxidized $\Delta nifB$ MoFe protein (Fig. 6B, trace 2 + 3) are identical. Therefore, electron transfer from the Fe protein in the MgADP-bound state to the $\Delta nifB$ MoFe protein does not occur.

Qualitatively and quantitatively indistinguishable spectra are observed in samples of IDS-oxidized $\Delta nifH$ MoFe protein incubated with $[4Fe-4S]^{1+}$ Fe protein and either MgADP or MgATP (Fig. 6B, traces 1 + 4 and 2 + 4). Both spectra are identical to the $[4Fe-4S]^{1+}$ Fe protein in its MgADP-bound conformation in terms of the $S = 1/2$ EPR signal (Fig. 6B, trace 2). These data indicate that both MgADP and MgATP are unable to facilitate electron transfer from the $[4Fe-4S]^{1+}$ cluster of the Fe protein to the IDS-oxidized $\Delta nifH$ MoFe protein. However, the shape of the $S = 1/2$ EPR signal of the $[4Fe-4S]^{1+}$ Fe protein clearly shows that MgATP is hydrolyzed to MgADP during the incubation (Fig. 6B, trace 1 + 4). This observation is consistent with the observation of a MgATP hydrolysis activity promoted by purified His-tag $\Delta nifH$ MoFe protein of 5915 ± 113 nmol MgATP hydrolysis/min/mg of protein, which is ~55% of the activity of wild-type MoFe protein (Table II). Purified His-tag $\Delta nifB$ MoFe protein shows a similar activity (Table II) and was previously considered to be catalytically active (21). It has been reported that $\Delta nifH$ MoFe protein of A. vinelandii DJ54 was not able to facilitate MgATP hydrolysis by complex formation with the Fe protein to a significant extent (10). However, damages of the fragile mutant protein during the described laborious and time-consuming purification method might be the cause of the loss of the activity of the protein. Despite their abilities to hydrolyze MgATP, the FeMoco-deficient His-tag $\Delta nifH$ and $\Delta nifB$ MoFe proteins are not able to demonstrate any substrate-reducing activities, which can be measured by $H_2$ evolution, $C_2H_2$ reduction, and the $N_2$ fixation assays (Table II).

The Fe Protein Forms a Normal Complex with the $\Delta nifH$ MoFe Protein—The chelation assay is another way to study interaction between the Fe protein and the MoFe protein. The binding of MgATP to the $[4Fe-4S]^{1+}$ Fe protein induces a conformational change that involves the contraction of the protein (44, 55–57). Binding of two molecules of MgADP to the state of the Fe protein causes a different, less dramatic conformational change that does not involve a global change in the protein radius of gyration (57). Fig. 7 shows that this reaction can be easily monitored using a chelation assay based on the fact that in the absence of MgATP (or the presence of MgADP) the $[4Fe-4S]^{1+}$ Fe protein is resistant to chelation by bathophenanthroline disulfonate, whereas in the presence of MgATP, iron is rapidly removed from the protein (58, 59). Once the $[4Fe-4S]^{1+}$ Fe protein is in its MgATP conformation, it is able to form a very specific complex with the MoFe protein. In this complex, the MoFe protein sequesters the $[4Fe-4S]^{1+}$ cluster of the Fe protein, thereby protecting it from chelation (55, 58). Fig. 7, A–C, show that there are no observed differences between wild-type, $\Delta nifH$, and $\Delta nifB$ MoFe protein in this chelation protection assay, indicating normal complex formation between the MoFe protein and the Fe protein in all cases.

In summary, the oxidized $\Delta nifH$ MoFe protein is able to form a specific complex with the $[4Fe-4S]^{1+}$ Fe protein and facilitate the hydrolysis of MgATP within this complex. These data indicate that the $\Delta nifH$ MoFe protein in this study is present in the correct structural conformation and active in promoting MgATP hydrolysis. However, in contrast to the case of the $\Delta nifB$ MoFe protein, no concurrent oxidation of the $[4Fe-4S]^{1+}$
cluster of the Fe protein or electron transfer to the oxidized
\( \text{nifH} \) MoFe protein is observed. Possible explanations for
missing the electron transfer step in the case of this altered
protein are either that the P-cluster in this protein exists in a
precursor form or the P-cluster is trapped in an unusual con-
formation or oxidation state that is unable to accept electrons.

The \( \text{nifH} \) MoFe and \( \text{nifB} \) MoFe Proteins Can Be Reduced
by Ti(III) Citrate—All iron atoms of the P\textsubscript{N} state of the MoFe
protein, prepared by sodium dithionite reduction, are believed
to be in the Fe\textsubscript{2} state (21, 60). There is no indication that the
P-cluster can be reduced further to a lower oxidation state.
Recently it was described that the [4Fe-4S] cluster of the Fe
protein could exist not only in the [4Fe-4S]\textsuperscript{1} state, generated
by dithionite reduction, but also in the all-ferrous [4Fe-4S]\textsuperscript{0}
couple, generated by Ti(III) citrate (41, 61). It has been proposed
that the [4Fe-4S]\textsuperscript{2} couple, rather than the well established
[4Fe-4S]\textsuperscript{2/1} couple, is utilized \textit{in vivo} during nitrogenase
turnover (42, 62). This finding raised the question as to
whether it is also possible to reduce the metal centers of the
\( \text{nifH} \) MoFe and \( \text{nifB} \) MoFe protein beyond the dithionite-
reduced state as was isolated. The perpendicular mode EPR
signals in the \( g_2 \) region that arise from the dithionite-
reduced states of the \( \text{nifB} \) MoFe (Fig. 8, \textit{trace} 1) and \( \text{nifH} \)
MoFe protein (Fig. 8, \textit{trace} 3) are greatly diminished upon the
reduction with Ti(III) citrate (Fig. 8, \textit{traces} 2 and 4). The

### Table II

Comparison of the activities of purified MoFe proteins

| Atmosphere          | Product         | \( \text{MoFe protein} \) Wild type | \( \text{ΔnifB} \) | \( \text{ΔnifH} \) |
|---------------------|-----------------|-----------------------------------|-----------------|-----------------|
|                     |                 | nmol/min/mg protein | %    | nmol/min/mg protein | %    | nmol/min/mg protein | %    |
| 10% C\textsubscript{2}H\textsubscript{2}/90% Ar | C\textsubscript{2}H\textsubscript{4} | 1860 ± 87 | 100 | 3 ± 1 | >1 | 9 ± 1 | >1 |
| 100% N\textsubscript{2}  | NH\textsubscript{3} | 1002 ± 148 | 100 | 0 ± 0 | 0 | 0 ± 0 | 0 |
| 100% N\textsubscript{2}  | H\textsubscript{2} | 542 ± 47 | 100 | 0 ± 0 | 0 | 0 ± 0 | 0 |
| 100% Ar              | H\textsubscript{2} | 2417 ± 161 | 100 | 0 ± 0 | 0 | 0 ± 0 | 0 |
| 100% Ar              | P\textsubscript{4} | 10962 ± 585 | 100 | 4750 ± 50 | 45 | 5915 ± 113 | 54 |

Fig. 7. Protection of the iron chelation of the [4Fe-4S]\textsuperscript{1} cluster in the Fe protein by wild type (A), \( \text{nifB} \) (B), and \( \text{nifH} \) (C) MoFe protein. The formation of the complex between the iron chelator bathophenanthroline disulfonate and the iron from the [4Fe-4S]\textsuperscript{1} clusters of the Fe proteins was measured at 535 nm in the presence of either MgATP, MgATP and MoFe protein or MgADP. A final concentration of 0.2 mM ADP or ATP and 0.4 mM MgCl\textsubscript{2} was used. The MoFe and Fe protein concentrations were 0.13 and 0.08 mg/ml, respectively. Curves obtained in the presence of MgATP and MoFe protein were fitted to single exponential equations over a period of 100 s, giving the observed rate constants of 0.070, 0.068, and 0.069 s\textsuperscript{-1} for the wild-type, \( \text{nifB} \), and \( \text{nifH} \) MoFe protein, respectively.

![Fig. 7](image-url)

Fig. 8. EPR spectra of dithionite (1 and 3) and Ti(III) citrate (2 and 4) reduced \( \text{nifB} \) (1 and 2) and \( \text{nifH} \) (3 and 4) MoFe protein. All samples (10 mg/ml protein) were prepared and measured as described under “Experimental Procedures.”
reversibility of this effect (data not shown) in combination with an increase of the absorbance at 420 nm upon Ti(III) citrate addition (data not shown) indicates a reduction of the metal clusters of both FeMoco-deficient MoFe proteins. This behavior could be ascribed to one of the following explanations; (a) the P-cluster is reduced to a lower oxidation state than that of P^N or (b) a P-cluster precursor that is not fully processed is present in an amount corresponding to the intensity of the EPR signal in the g ~ 2 region and is the reason for the described spectroscopic changes.

**Activation of ΔnifH MoFe Protein**—Previous studies show that FeMoco-deficient MoFe proteins expressed by *A. vinelandii* strains with deletion or mutation of the *nifH* gene cannot be activated simply by adding FeMoco in N-methylformamide (8–10, 18). Several components are required for the insertion of FeMoco into these FeMoco-deficient MoFe proteins, such as the Fe protein, MgATP, γ, and GroEL (9, 11–15, 18). In addition, one, or most likely, several more unidentified components are required for this process, which makes the reconstitution of the proteins difficult. Cell-free extracts of *A. vinelandii* mutant stains like DJ100 (Δ*nifD*) or CA12 (Δ*nifHK*) are usually supplied as a source of the missing component(s) in activation assays (10, 18). However, it was reported that the maximal activation of purified ΔnifH MoFe protein of *A. vinelandii* DJ54 was only around 5% of that of the wild-type MoFe protein (10). Our attempt to activate the His-tag ΔnifH MoFe protein yielded an activity of 70 nmol of C₂H₄ reduced/min/mg of protein, which also is ~5% wild-type activity. The fact that the full activity of the protein could not be restored can be explained by the failure of FeMoco insertion into the MoFe protein due to the un-optimal assay conditions. However, the presence of a P-cluster precursor or a P-cluster in an unusual conformation or oxidation state that is unable to carry out substrate reduction could also lead to the low activity of the reconstitution assays despite a successful FeMoco insertion. EPR samples of the reconstitution attempts do not show the S = 3/2 EPR signal, which arises from the FeMoco center of the dithionite-reduced wild-type MoFe protein (data not shown). This indicates the possibility of a failed FeMoco insertion, although it does not exclude the possibility that the presence of an unusual P-cluster species has perturbed the reconstituted FeMoco center.

**Conclusion**—In the current work, an improved method was used to isolate the fragile FeMoco-deficient His-tag ΔnifH MoFe protein, which allowed the purification of large amounts of this protein for further studies. In contrast to the results of earlier studies of the non-His-tag version of the ΔnifH MoFe protein (10), this protein showed MgATP hydrolysis activity as well as the capability of oxidation and reduction based on data obtained from EPR and UV-visible spectroscopy. The failure in detection of these properties of the non-His-tag version of the ΔnifH MoFe protein in previous studies could be explained by destruction of the fragile protein during the time-consuming purification procedure. The unusually strong S = 1/2 EPR signal in the g ~ 2 region exhibited by the His-tag ΔnifH MoFe protein could be ascribed to the presence of a P-cluster precursor or a P-cluster trapped in an unusual conformation or oxidation state in the ΔnifH MoFe protein. The presence of a P-cluster species in this mutant protein, which is unable to carry out substrate reduction, would explain the inability of this MoFe protein to restore full activity. It has been proposed previously that during the P-cluster biosynthesis, [4Fe-4S] clusters may be formed separately on the α and β subunit and that they are later combined to form the P-cluster (48). The absence of an intact P-cluster in the protein, which links the α and β subunit of the ΔnifH MoFe protein, would explain its high instability and unusual features. The presence of a P-cluster precursor in ΔnifH MoFe protein would imply a function of the Fe protein in the P-cluster assembly that has not been described so far. Extended x-ray absorption fine structure and crystallographic investigations will be used to determine the structures of the P-clusters in ΔnifH MoFe protein and ΔnifH MoFe protein and reveal the origin of the observed characteristics of the ΔnifH MoFe protein.

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