Construction of a Form of the MoFe Protein of Nitrogenase That Accepts Electrons from the Fe Protein but Does Not Reduce Substrate*

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The direction of electron flow through nitrogenase is generally believed to be from the Fe protein to the P-clusters to the FeMo cofactor and then to substrate. In order to examine oxidation states of the P-clusters that might be involved in this pathway, we have constructed a form of the MoFe protein that contains a species called the MoFe cluster (Gavini, N., Ma, L., Watt, G., and Burgess, B. K. (1995) Biochemistry 33, 11842-11849) in place of FeMo cofactor. This MoFe cluster-containing protein was purified, and the presence of the cluster was confirmed by reisolation of the MoFe cluster followed by EPR spectroscopy. The protein does not reduce protons or acetylene, however, upon the addition of the Fe protein and MgATP, MgATP hydrolysis occurs at a rate 28% of the wild-type protein. As isolated in the presence of excess dithionite the MoFe cluster-containing protein is EPR silent. Upon addition of the Fe protein and MgATP a g = 1.94 EPR signal develops that integrates to about 1 spin per P-cluster. This signal only develops when both the Fe protein and MgATP are added and it arises from the P-clusters.

Molybdenum nitrogenase is composed of two separate proteins whose complete structures have recently been determined by x-ray crystallography (1-7). The smaller of the two, designated the iron protein (Fe protein), is a dimer of two identical subunits encoded by the nifH gene (1, 8). It contains two binding sites for MgATP and has a single (4Fe-4S)3+/4+ cluster. The larger of the two component proteins is designated the molybdenum-iron protein (MoFe protein) and is an a2b2 tetramer with the a and b subunits encoded by the nifD and nifK genes, respectively. It has two types of metal centers, the P-clusters (two per tetramer) that each contain 8Fe and 8S2- atoms in the form of bridged (4Fe-4S) clusters and, the iron molybdenum cofactors (FeMo cofactor) (two per tetramer) that have the composition MoFe3S9 homocitrate (2, 9-11). The FeMo cofactor is responsible for the S = 3/2 EPR signal exhibited by the MoFe protein in its dithionite reduced state (9-11).

For substrate reduction to occur the MoFe protein must first receive electrons, one at a time, from the Fe protein in a reaction that is coupled to MgATP hydrolysis (12-17). Because all products that leave nitrogenase (e.g. N2, H2) have been reduced by multiples of two electrons, the MoFe protein must be able to accumulate several electrons before products are released (12). At present, it is not clear if those electrons are stored in the P-clusters, in the MoFe cofactor or if, when they enter the MoFe protein one at a time, they are immediately used to produce enzyme-bound substrate reduction intermediates. One major obstacle to obtaining this type of information is that one substrate, H+, is always available. Thus, once the Fe protein, MgATP, and the MoFe protein are put together in the presence of reductant, electron flow is continuous with electrons coming into the MoFe protein from the Fe protein and leaving via the substrate reduction product H2. It is therefore not possible to stop the nitrogenase reaction halfway through in order to examine the oxidation state(s) of the P-clusters or FeMo cofactor centers. We wanted to construct a form of the MoFe protein that could accept electrons from the Fe protein-MgATP complex but that could not reduce H+. We have previously reported the purification and characterization of a FeMo cofactor-deficient form of the MoFe protein that contains P-clusters (18). In a separate study we also reported the large scale isolation and characterization of a species designated the MoFe cluster that contains the metal portion of FeMo cofactor that gives rise to the S = 3/2 EPR signal but which appears to be missing homocitrate and does not support any substrate reduction (19). Here we report the purification and characterization of a form of the MoFe protein that is constructed in vitro by inserting MoFe cluster into the FeMo cofactor-deficient protein.

EXPERIMENTAL PROCEDURES

Materials—The wild-type Azotobacter vinelandii Fe and MoFe proteins (20), the MoFe cluster (19), and the FeMo cofactor-deficient MoFe protein from nifH strain DJ 54 (18) were purified and analyzed as described elsewhere. DEAE-cel lulose 52 was from Whatman, Q-Sepharose was from Pharmacia, and AcA34 was from Bio Selgra. ATP, creatine phosphate, creatine phosphokinase, TES1, Tris, Bis-Tris, CHES, and (NH4)2SO4 were from Sigma and Na2S2O4 was from EM Science (Cherry Hall, NJ). NMF was from Aldrich and methyl ethyl ketone was from Fisher and both were pretreated as described elsewhere (19).

Cell Growth, Activation, and Protein Purification, and Characterization—The construction of A. vinelandii strain DJ 54, that contains a defined in-phase deletion in the nifH gene, is reported elsewhere (21). For protein purification cells were grown as described previously (18). Large scale insertion of MoFe cluster into the FeMo cofactor-deficient MoFe protein and subsequent purification of the protein product are described in the text. CH4 reduction (1, 22), H2 evolution (22), phosphate assays (23), iron determinations (24), molybdenum determinations (25), and gel electrophoresis (26) were carried out as previously reported. EPR spectra were obtained using a Bruker ESP 300E spectrophotometer, interfaced with an Oxford Instrument ESR-9002 liquid helium continuous flow crystal. Spin quantitations were obtained under nonsaturating conditions using a double integration method.

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1 The abbreviations used are: TES, N-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid; Bis-Tris, 2-(bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; CHES, 2-(N-cylohexylamino)ethanesulfonic acid; NMF, N-methyl formamide; HEPPS, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
MoFe Protein/Electron Transfer

RESULTS AND DISCUSSION

A ΔnifH strain of A. vinelandii designated DJ 54 accumulates an inactive FeMo cofactor-deficient but P-cluster containing MoFe protein that can be fully activated in vitro in a reaction that requires the addition of isolated FeMo cofactor in NMF, the Fe protein, MgATP, and at least one additional protein (18, 21, 26–30). Recently we reported the large scale isolation and purification of MoFe cluster-containing MoFe protein from wild type A. vinelandii (32). That protein appeared to be a hexamer with two additional subunits of unknown origin. To construct this protein we developed a large scale modification in place of FeMo cofactor (19).

Figure 1 shows that following the addition of FeMo cofactor, the Fe protein, and MgATP to the DJ54 extracts the MoFe protein peak is restored. Thus, the elution profile from the DEAE-cellulose column provided the first indication that the MoFe cluster insertion reaction had been successful.

The MoFe protein fraction from the DEAE-cellulose column was further purified according to Scheme I. Typical yields are 200 mg of protein from 500 g of cells. As shown in Fig. 2, the final product is nearly homogeneous by the criterion of Coomassie-stained SDS-polyacrylamide gel electrophoresis. Fig. 2 also shows that like the wild-type MoFe protein, the MoFe cluster-containing protein has only two types of subunits corresponding to the α and β subunits of the MoFe protein. This is in contrast to a form of the MoFe protein that was constructed in vitro by inserting FeMo cofactor into a purified FeMo cofactor-deficient MoFe protein synthesized by a NifB strain of A. vinelandii (20). That protein appeared to be a hexamer with two additional subunits of unknown origin.

Evidence for the Presence of MoFe Cluster—Several criteria were used to establish that the purified protein contained the MoFe cluster. First, as shown in Table I, the protein has >90% of the Fe and Mo content of the wild-type protein. Second, as shown in Fig. 3, the intensity of the visible spectrum is now very similar to that obtained for the wild-type MoFe protein whereas the FeMo cofactor-deficient MoFe protein purified from DJ 54 has greatly reduced absorbance (18). Third, the circular dichroism spectra of the wild-type protein and the MoFe cluster-containing protein show two features at 530 and 660 nm (Fig. 4), whereas the FeMo cofactor-deficient protein shows only the 660-nm P-cluster feature and is missing the 520-nm FeMo cofactor feature (18). The data in Fig. 4 further
show that the shape of the 520-nm feature of the MoFe cluster-containing protein differs substantially from that of the wild-type protein. This is consistent with the known structural differences between MoFe cluster and FeMo cofactor which include the probable absence of the chiral ligand homocitrate (19). Fourth, as shown in Fig. 5, the MoFe cluster can be reisolated from the purified protein to yield a species whose EPR spectrum is indistinguishable from the MoFe cluster originally added to the DJ54 cell-free extracts. Taken together these data demonstrate that we have constructed and purified a form of the MoFe protein that differs from wild-type by the substitution of MoFe cluster for FeMo cofactor.

**Catalytic Activity of the MoFe Cluster-containing Protein**—In recent years a number of altered forms of the MoFe protein have been constructed in vitro and a few have been purified. Although many forms that contain alterations of the polypeptide in the FeMo cofactor pocket and those that have alterations in the homocitrate portion of FeMo cofactor have little or no N2 fixation activity all proteins purified to date are able to reduce H+ to H2 (12, 33). As described in the Introduction, our goal was to construct a form that was similar enough to the wild-type MoFe protein that it could accept electrons from the Fe protein but that was not able to transfer those electrons to any substrate, including H+. Table II shows that, as expected, the MoFe cluster-containing protein does not have detectable levels of either C2H2 reduction or H2 evolution activity.

**TABLE I**

| Metal            | MoFe protein | Wild type | MoFe cluster | %* |
|------------------|--------------|-----------|--------------|----|
|                  | nmol/mg of protein |          |              |    |
| Fe               | 96 (±10)     | 98 (±10)  | 102          |    |
| Mo               | 6.9 (±0.5)   | 6.2 (±0.5)| 90           |    |

*Percentage of metal present in MoFe cluster-containing protein relative to wild type.

**FIG. 2.** Coomassie-stained one-dimensional SDS-polyacrylamide (10%) gel electrophoresis separation of: (a) wild-type MoFe protein and (b) MoFe cluster-containing protein.

**FIG. 3.** Absorption spectra of: (a) wild-type MoFe protein at 5 mg/ml, (b) the MoFe cluster-containing protein at 5.5 mg/ml, and (c) the FeMo cofactor-deficient MoFe protein purified from DJ54 at 5 mg/ml. All samples were in the dithionite reduced state.

**FIG. 4.** CD spectra of purified (a) wild-type MoFe protein and (b) MoFe cluster-containing protein. Both samples were in their dithionite reduced states. Protein concentrations were 10 mg/ml.

**FIG. 5.** EPR spectra of isolated MoFe cluster in methyl ethyl ketone at 4 K. A, MoFe cluster isolated in the presence of excess dithionite. B, MoFe cluster isolated in the absence of excess dithionite. a, isolated from MoFe cluster-containing protein. b, isolation from wild-type MoFe protein. The isolation method is described elsewhere (19). Microwave power, microwave frequencies, and modulation amplitude were: 5 mW, 9.43 GHz, and 5 Gauss, respectively. It should be noted that the second of the two g = 2 resonances observed for isolated MoFe cluster is present in control solutions of NMF with dithionite but not cluster present (19).
proteins, the MgATP concentration and other biochemical conditions like pH, temperature, and ionic strength. This MgATP hydrolysis is absolutely required for electron transfer between the Fe protein and the MoFe protein and under ideal conditions occurs with a stoichiometry of 2MgATPs hydrolyzed per electron transferred. Under non-ideal conditions, in the presence of certain inhibitors like cyanide (12, 33), or for some mutant variants of the MoFe protein (e.g. Ref. 34), electron transfer and MgATP hydrolysis become uncoupled to give MgATP/electron ratios much greater than two. In a previous study we showed that the FeMo cofactor-deficient MoFe protein purified from DJ 54 catalyzed only about 1% of the wild-type levels of MgATP hydrolysis (18). Table II shows that the MoFe cluster-containing protein does hydrolyze MgATP at significant rates of ~28% of wild-type even though no electrons are being transferred to substrate. Thus like the wild-type MoFe protein, this new form of MoFe protein must have a structure that can induce the necessary conformational change in the Fe protein that allows MgATP hydrolysis to occur.

EPR of the Isolated MoFe Cluster-containing Protein—As shown in Fig. 6 and discussed in detail elsewhere (19), isolated MoFe cluster in the presence of dithionite exhibits an S = 3/2 EPR signal very similar to the one exhibited by the FeMo cofactor site of the wild-type MoFe protein. As discussed above this same EPR signal can be obtained by reisolation of the MoFe cluster from the MoFe cluster-containing MoFe protein (Fig. 5). As shown in Fig. 6, the FeMo cofactor-deficient MoFe protein purified from DJ 54 is EPR silent in the g = 3–5 region of the spectrum but exhibits a g = 1.94 signal. That signal, which is discussed in detail elsewhere, arises from the P-clusters (18). It is not observed for wild-type MoFe protein but has been observed for FeMo cofactor-deficient proteins from three bacterial species and is very similar to a signal exhibited by the VFe protein from A. vinelandii (35–38). In all cases where it has been observed, however, the g = 1.94 signal integrates to only about 0.1 to 0.3 spin per molecule. Fig. 6 shows that as isolated in the presence of excess dithionite the MoFe cluster-containing protein is EPR silent in the g = 3–5 region. Whether or not the protein as isolated exhibits the g = 1.94 signal varies from preparation to preparation, but when present, it is always a very minor component that integrates to <0.1 spin per molecule.

EPR in the g = 3–5 Region—The first question that arises concerning the data in Fig. 6 is: why doesn’t the MoFe cluster-containing protein exhibit the S = 3/2 EPR signal when the protein is in the presence of excess dithionite? One possibility is that on binding to the protein the structure of the cluster is changed such that either the ground state spin state is altered or the signal is broadened so that it cannot be observed. A second possibility is that the MoFe cluster is no longer in the S = 3/2 state. For wild-type MoFe protein the S = 3/2 state of the FeMo cofactor can undergo reversible oxidation and reduction to yield states that are EPR silent as shown in Equation 1.

\[
\text{Substrate reduction} \quad S = \text{integer} \quad \Rightarrow \quad S = 3/2 \quad \Rightarrow \quad S = 0 \quad (\text{Eq. 1})
\]

\[
\text{Fe protein/MgATP} \quad \Rightarrow \quad S = 3/2
\]

It is therefore possible that the MoFe cluster center is EPR silent because it is either more oxidized or more reduced than the S = 3/2 state. More oxidized seems unlikely because the isolated MoFe cluster can be easily reduced by excess dithionite and because reduction of the protein with methyl viologen also failed to produce the S = 3/2 signal. Another possibility is that the MoFe cluster center might be stuck in the fully-reduced S = integer state because the MoFe cluster insertion reaction requires the addition of the Fe protein and MgATP and the MoFe cluster cannot reduce substrate.

We have previously identified three oxidation states of isolated MoFe cluster in methyl ethyl ketone as shown in Equation 2 (19).

\[
S = 3/2 \quad \Rightarrow \quad S = 0 \quad \Rightarrow \quad S = 1/2 \quad \text{(Eq. 2)}
\]

When the MoFe cluster is isolated from wild-type MoFe protein in the presence of excess dithionite it remains in the S = 3/2 state, while when it is isolated in the absence of dithionite the cluster is oxidized and ends up in the S = 1/2 or S = 0 states (Ref. 19 and Fig. 5B). In contrast, when the MoFe cluster is isolated from the MoFe cluster-containing protein in the presence (Fig. 5A) or absence (Fig. 5B) of dithionite it always ends up in the reduced S = 3/2 state. This result is consistent with the MoFe cluster center of the protein starting out in a more reduced state than the FeMo cofactor center of the dithionite-reduced MoFe protein. This result would be inconsistent with the MoFe cluster site being present initially in the S = 0 oxidized state. At present, however, we cannot distinguish between a redox state change and structural causes for the absence of the S = 3/2 signal.

Oxidation/Reduction of the P-cluster—When the wild-type MoFe protein is in the presence of excess dithionite the P-clusters are in an S = 0 state designated P_N. This state is believed to be best represented as two bridged [4Fe-4S]_P clusters (4, 17). As shown in Fig. 7, the P-clusters in the dithionite-reduced MoFe cluster-containing protein are also primarily present in an EPR silent state but there is sometimes a small population of the state that exhibits the g = 1.94 EPR signal.

Table II

| Atmosphere | Wild type | MoFe cluster containing | Product | % of wild type |
|------------|-----------|-------------------------|---------|---------------|
| C_2H_2     | 1810 (±108) | ND*                     | C_2H_6  |
| Ar         | 1890 (±140)  | ND*                     | H_2    |
| Ar         | 3600 (±180)  | 1000 (±50)              | PO_4^- 28 |

* ND, not detectable above background.
or a form that is either more oxidized or more reduced by one could arise from a structurally different form of the P-clusters protein-MgATP complex. The result in a structure that can now accept electrons from the Fe signal. In fact, the signal shown in Fig. 7 this led to a dramatic increase in the size of the g = 1.94 signal, which integrates to 1 spin per P-cluster. This reaction absolutely requires not only the addition of the Fe protein but also MgATP which is hydrolyzed during the course of the reaction (Table II). In addition, this reaction does not occur for the FeMo cofactor-deficient MoFe protein purified from DJ 54 (Fig. 7, c and d). Thus the insertion of the FeMo cluster into the FeMo cofactor-deficient protein appears to result in a structure that can now accept electrons from the Fe protein-MgATP complex. The g = 1.94 signal shown in Fig. 7 could arise from a structurally different form of the P-clusters or a form that is either more oxidized or more reduced by one electron than the dithionite reduced P₅₇ state, because the addition or subtraction of one electron from an S = 0 cluster would be expected to yield an S = 1/2 state.

Electron Transfer Through the MoFe Protein—For wild-type nitrogenase following electron transfer from the Fe protein, the S = 3/2 FeMo cofactor-center of the MoFe protein is reduced to an EPR silent, but paramagnetic state (14–16). The disappearance of this EPR signal occurs following the addition of one electron per FeMo cofactor center, indicating that FeMo cofactor is the final location for the first electron that enters the MoFe protein from the Fe protein (17). Although it seems likely that the electrons are transferred from the Fe protein to the P-clusters and then to FeMo cofactor, there is currently no compelling experimental evidence to suggest that it is the case. An insight into this problem may be given by the variant of the MoFe protein reported here. As described above this protein cannot reduce proteins or acetylene, but is capable of supporting MgATP hydrolysis in the presence of Fe protein and dithionite, when this occurs a g = 1.94 EPR signal develops which is given by the P-clusters and which integrates to 1 spin per P-cluster. This could mean that the P-clusters are being reduced below their normal level after transfer of electrons from Fe protein, or that they are becoming oxidized after reducing the MoFe cluster and cannot be reduced by Fe protein. In the future, Mössbauer experiments on isotopic hybrids of this protein should be able to distinguish these possibilities and provide a useful probe of electron transfer mechanisms within the MoFe protein.

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**FIG. 7.** EPR spectra recorded at 8 K for: (a) MoFe cluster-containing protein in the presence of excess dithionite, (b) the sample in a after incubation for 2 min at room temperature following the addition of purified Fe protein (to give a 4:1 MoFe:Fe protein ratio) and 2 mM MgATP and an ATP generation system, (c) the FeMo cofactor-deficient MoFe protein purified from DJ 54 in the presence of excess dithionite, and (d) the sample in c after treatment with the Fe protein and MgATP as described in b. The protein concentrations for both the MoFe cluster containing protein and the DJ 54 protein were 4.0 mg/mL. The gains of all EPR spectra are 5 × 10⁻⁴.
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