FeMo Cofactor Synthesis by a nif/H Mutant with Altered MgATP Reactivity*

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We have characterized a Nif" mutant of Azotobacter vinelandii, designated UW91 (Shah, V. K., Davis, L. C., Gordon, J. K., Orme-Johnson, W. H., and Brill, W. J. (1973) Biochim. Biophys. Acta 292, 246–255). The specific Fe protein mutation giving rise to the Nif" phenotype was shown by DNA sequencing and site-directed mutagenesis to be the substitution of a conserved alanine at position 157 by a serine. The UW91 Fe protein was purified and shown to have a normal [4Fe-4S] cluster and normal MgATP binding activity. The substitution of alanine 157 by serine, however, prevents the MgATP-induced conformational change that occurs for the wild-type Fe protein, prevents MgATP hydrolysis, and prevents productive electron transfer to the MoFe protein. The UW91 Fe protein does bind to the MoFe protein to give a normal cross-linking pattern; however, it does not compete very successfully with wild-type Fe protein in an activity assay. The UW91 MoFe protein was also purified and characterized and shown to be indistinguishable from the wild-type protein. Thus, the substitution of Fe protein residue alanine 157 by serine does not change the Fe protein's ability to function in FeMo cofactor biosynthesis or insertion. This demonstrates that these events do not require the MgATP-induced conformational change, MgATP hydrolysis, or productive electron transfer to the MoFe protein.

Molybdenum nitrogenase is composed of two separately purified proteins. The larger of the two, designated the molybdenum-iron protein (MoFe protein), is a M protein with tetramer composed of two α and two β subunits encoded by the nifD and nifK genes, respectively (1–4). The MoFe holoprotein has two distinct types of metal clusters. The P-clusters are believed to be [4Fe-4S]-type clusters with very unusual properties (2). The dominant hypothesis states that there are four P-clusters per MoFe protein, organized into two equivalent pairs (2, 3). Recent x-ray structural data further indicate that if that hypothesis is correct, then the P-clusters within each pair must be very close together (5). The other type of metal cluster in the MoFe protein is called the iron-molybdenum cofactor (FeMo cofactor) and is believed to be the site of substrate binding and reduction (1–3, 6).

The smaller of the two component proteins of nitrogenase, designated the iron protein (Fe protein), is composed of two identical subunits encoded by the nifH gene (4). These are bridged by a single [4Fe-4S] cluster (7) to form a M, ~60,000 holoprotein, which has two binding sites for MgATP (1–3). A preliminary x-ray structure of this protein, purified from Azotobacter vinelandii, has recently been reported (8). The Fe protein of nitrogenase has been reported to have three distinct functions. First, it is the physiological electron donor to the MoFe protein (1–3). To perform this well characterized function, the Fe protein must accept electrons from reduced flavodoxin or ferredoxin in vivo (or dichione in vitro), it must bind two molecules of MgATP, it must bind to the MoFe protein to form a complex, it must transfer one electron to the MoFe protein, and it must somehow couple this electron transfer reaction to MgATP hydrolysis (1–3). Thus, to serve as an electron donor, the Fe protein must have a normal [4Fe-4S]1+/2+ cluster, normal MoFe protein binding activity, and normal MgATP binding and hydrolytic activity.

A second function for the Fe protein concerns the initial biosynthesis of FeMo cofactor. FeMo cofactor is a M, ~100 metal cluster with stoichiometry of Mo:Fe:S:S:H:homocitrate (1–3, 6). Although the pathway for FeMo cofactor biosynthesis is not yet established, it has been known for some time that FeMo cofactor is synthesized separately from the FeMo protein polypeptides (9–11) and that its synthesis requires the combined action of the nifQ, B, N, E, and V genes (6, 12). In 1986, it was reported that mutants of Klebsiella pneumoniae and A. vinelandii that did not synthesize the nifH polypeptide also did not synthesize FeMo cofactor (10, 11). Based on these and other (13) experiments, it is now accepted that the nifH gene product is required for an early step in FeMo cofactor biosynthesis. However, it is not known what features of the Fe protein are required for this reaction.

The third Fe protein function involves the insertion of preformed FeMo cofactor into an inactive, FeMo cofactor-deficient MoFe protein. This step in the assembly of an active FeMo holoprotein requires not only the nifH polypeptide but also MgATP (14). However, again it is not known what features of the Fe protein are required for FeMo cofactor insertion.

In order to determine which features of the Fe protein are required for each of these three functions, we have begun to characterize nif/H mutants. Here, we discuss the characterization of an A. vinelandii nif/H mutant, designated UW91 (15).

EXPERIMENTAL PROCEDURES

Materials—The A. vinelandii Fe and MoFe proteins were purified and analyzed as described elsewhere (16) (using QAE-Sepharose and not DEAE-cellulose for the final Fe protein column) to give specific activities of ~1,900 and 2,900 nmol of H₂ evolved · min⁻¹ · mg⁻¹, respectively. FeMo cofactor was isolated by the HCl/NaOH modification (16, 17) of the original isolation procedure (18). A. vinelandii strain UW91 was kindly provided by Prof. Dennis Dean, from the Anaerobic Laboratories at Virginia Polytechnic Institute and State University. Ampholines for two-dimensional gel electrophoreses were

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from LKB, Bromma, Sweden. Acrylamide, bisacrylamide, ammonium persulfate, TEMED1 and SDS were from Bio-Rad. Whatman DEAE-cellulose 52 was obtained from Baxter, Irvine, CA. ATP, phosphocreatine, creatine phosphokinase, TES, Tris, Bis-Tris, HEPPS, CHES, Sephadex G-25, and EDC were from Sigma. Sodium dithionite was from EM Science, Cherry Hill, NJ. QAE-Sepharose was from Pharmacia LKB Biotechnology Inc. Ammonium tetrathiomolybdate was purchased from Aldrich. Bathophenanthrolinedisulfonate was from GFS Chemicals, Columbus, OH. All radiochemicals were purchased either from Amersham Corp. or from Du Pont-New England Nuclear. All restriction endonucleases and T, DNA ligase were from Boehringer-Mannheim.

Cloning and Sequencing the AuUW91 nifH Gene—Total chromosomal DNA was isolated from A. vinelandii UW1 using standard methods (19). Since the nifH gene does not have any sites for the restriction enzyme PstI, the chromosomal DNA of AuUW91 was digested with PstI and was ligated into the PstI-cleaved plasmid pUC18 (20). Escherichia coli strain TG1 was transformed with this ligation mixture, and colonies that were resistant to ampicillin (50 μg/ml) and colorless on Luria broth agar plates containing X-gal and isopropyl-β-D-thiogalactoside were selected as recombinants. These colonies were subjected to colony hybridization analysis using the nifH gene, and one type A. vinelandii strain was the probe. To confirm this, a BglII-EcoRI fragment harboring the nifH gene was labeled with 32P using a random primer labeling kit obtained from Amersham Corp. The hybridization was carried out as described elsewhere (21). Plasmid DNA was isolated from the colonies that hybridized with the nifH gene, and the presence of a DNA fragment corresponding to the nifH gene was identified by restriction enzyme mapping. A PstI fragment carrying the nifH gene of AuUW91 was then subcloned into the PstI site of M13 mp18 (20), and the nucleotide sequence was determined on both strands by the dideoxy chain termination method (22) using nifH primers.

Site-directed Mutagenesis—The oligonucleotide-directed mutagenesis was carried out using the Oligonucleotide-directed in vitro Mutagenesis System purchased from Amersham Corp. and used according to the manufacturer’s instructions. A chemically synthesized oligonucleotide primer corresponding to the DNA region of interest and an oligonucleotide complementary to the sequence with the appropriate base change, was hybridized to the single-stranded M13 recombinant plasmid DNA. The second strand was synthesized using the DNA polymerase I Klonef fragment and T, DNA ligase, and heteroduplexes were generated. The newly synthesized strand was phosphorylated by replacing dCTP with dCTPαS during the synthesis. This reaction was then used to generate nicked DNA that was capable of phosophorhodiolated genomic DNA. E. coli strain TG1 was transfected with this mutagenized DNA (21) and the mutants grown, derepressed for nitrogenase synthesis, and stored at -80 °C (13). To screen the clones, 5 μg of genomic DNA was electrophoresed on 1.0% agarose gels and transferred to nitrocellulose filters. The filters were hybridized with the nifH gene, and the presence of a DNA fragment hybridizing with the nifH gene was detected by autoradiography after digestion with restriction enzymes.

RESULTS AND DISCUSSION

The A. vinelandii strain UW91 (AuvUW91) was originally isolated in 1973 by NTG mutagenesis (15). Because this strain was unable to fix nitrogen in vivo, experiments were carried out using whole cells and cell-free extracts to identify the cause for the Ni2+ phenotype. These early experiments revealed that AuUW91 synthesized a MoFe protein that gave approximately wild-type levels of C2H2 reduction activity and exhibited the wild-type S = 3/2 EPR signal (15) that is now known to arise from protein bound FeMo cofactor (1, 2, 6). Thus, the MoFe protein in this strain appeared to be normal and was not believed to be responsible for the Ni2+ phenotype. This same series of experiments showed that although the nifH polypeptide was synthesized by AuUW91, the UW91 Fe protein had no activity in the in vitro C2H2 reduction assay (15). Thus, mutation of the Fe protein was proposed to be responsible for the Ni2+ phenotype. Because it is now known that the Fe protein has multiple functions, these early experiments suggest that the Fe protein synthesized by UW91 cannot function as the electron donor to the MoFe protein but can probably function in both MoFe cofactor biosynthesis and insertion.

Confirmation That the UW91 Fe Protein Functions...
mally in Both FeMo Cofactor Biosynthesis and Insertion—To confirm that the MoFe protein synthesized by UW91 is normal, the protein was purified and characterized. The successful purification procedure was identical with that used for the wild-type MoFe protein (16). Each protein is stable to the heat step used in that procedure, and both proteins crystallize, which is the final purification step. The migration behavior of the purified UW91 and wild-type MoFe proteins on one-dimensional SDS and on two-dimensional gels is also identical (data not shown). Table I compares the specific activities of both MoFe proteins for \( \text{C}_2\text{H}_2 \) reduction, \( \text{H}_2 \) evolution, and \( \text{N}_2 \) reduction. Again, the two MoFe proteins are indistinguishable in these assays.

To determine whether or not the FeMo cofactor site of the UW91 MoFe protein is normal, the protein was examined using EPR spectroscopy. As reported initially for cell-free extracts (15), the \( S = 3/2 \) EPR signal exhibited by the FeMo cofactor center of the purified UW91 MoFe protein is identical with the signal exhibited by the purified wild-type MoFe protein (data not shown). To further confirm that the two FeMo cofactor sites are identical, FeMo cofactor was extracted from the UW91 MoFe protein into N-methylformamide (17, 18). The behavior of the UW91 FeMo cofactor during this isolation procedure and, as shown in Fig. 1, its subsequent ability to activate the inactive FeMo cofactor-deficient MoFe protein, were both indistinguishable from the behavior of the wild-type FeMo cofactor.

The above data confirm that the UW91 Fe protein can function in vivo in both FeMo cofactor biosynthesis and in FeMo cofactor insertion into the MoFe protein polypeptides. To test whether the UW91 Fe protein can function in the insertion reaction in vitro, the experiment shown in Fig. 2 was performed. Panel A of this figure illustrates that the FeMo cofactor-deficient MoFe protein found in cell-free extracts from the \( \Delta \text{nifH} \) strain DJ54 does not exhibit any \( \text{C}_2\text{H}_2 \) reduction activity until FeMo cofactor is added, when a small amount of active MoFe holoprotein is assembled (13). As shown in Fig. 2, this activity is not enhanced by adding MgATP with the FeMo cofactor or by adding the wild-type Fe protein with the FeMo cofactor. However, because both MgATP and the Fe protein are required to optimize the insertion of preformed FeMo cofactor into the FeMo cofactor-deficient MoFe protein (14), the activity is dramatically enhanced by adding wild-type Fe protein, together with MgATP (Fig. 2). The data in Fig. 2B further demonstrate that the UW91 Fe protein can function as well as the wild-type Fe protein in this in vitro FeMo cofactor insertion assay.

Characterization of the UW91 Genotype—To confirm that the UW91 mutation is located in the \( \text{nifH} \) gene, and not in another gene that affects Fe protein activity (e.g. \( \text{nifM} \) (4)), the chromosomal \( \text{nifH} \) gene in UW91 was replaced with the wild-type \( \text{nifH} \) gene as described under “Experimental Procedures.” Fig. 3 (a and b) shows that the wild-type \( \text{nifH} \) is able to replace the defective UW91 \( \text{nifH} \) to restore the wild-type phenotype. To identify the specific mutation in UW91, its \( \text{nifH} \) gene was then cloned and sequenced as described under “Experimental Procedures.” Two differences were found between the published wild-type sequence (31) and the \( \Delta \text{nifH} \) sequence determined here. One difference was a change of codon 159 from GCC to TCC, resulting in the substitution of serine for alanine at position 157 of the Nif H protein sequence. This G to T mutation is the type commonly caused by NTG mutagenesis. The second difference was the deletion of the entire codon GAA at \( \text{nifH} \) position 266, resulting in the deletion of a glutamate residue at position 265 of the Nif H sequence. This deletion is surprising because it is
addition of an excess of purified wild-type Fe protein.

2.182 A157S strains. As expected, the MoFe protein is active in all strains, whereas only the wild-type and ΔE265 strains show significant Fe protein activity. Surprisingly, the UW91 strain consistently shows higher crude extract activities for the MoFe protein than does the wild-type strain. Purification results indicate that these higher levels of crude extract activities are due to larger quantities of the MoFe protein being present in the UW91 strain. Although the cause for this apparent overexpression of the MoFe protein in strain UW91 has not been determined, it is not due to the mutation in the nifH gene. Thus, both site-directed mutants, ΔE265 and A157S, show wild-type, as opposed to overexpressed, levels of MoFe protein activity (Table II).

Structural Locations of the A157S and ΔGlu265 Mutations—Normand and Bousquet (32) have recently compiled 22 different sequences for Nif H, Vnf H, and Anf H, and Fig. 4A compares these sequences in the region of E265. This residue is neither conserved nor located in a highly conserved region, which is again consistent with the mutagenesis data showing that the residue is not required for Fe protein activity. Fig. 4B compares these sequences in the region of A157. This residue, an adjacent methionine, and several other residues in this region are completely conserved in all Nif H, Vnf H, and Anf H sequences. These data are consistent with the mutagenesis data showing that the A157S mutation results in an inactive Fe protein.

Previous studies have identified three regions of the A. vinelandii Nif H polypeptide that are believed to be important for Fe protein function. These include 1) the [4Fe-4S] cluster ligands, C265 and C266 (37, 38), 2) a putative MgATP-binding region, G11 through S312 (35), and 3) a putative MoFe protein interaction site near E265 identified by cross-linking experiments (27, 36). The region identified here, around A157, has not previously been shown to be important for Fe protein function. Also, it is not close in sequence to either the [4Fe-4S] cluster ligands or to the likely MgATP-binding site.

Physical Properties of the UW91 Fe Protein—The UW91 Fe protein was purified to homogeneity using the same procedure normally used for the wild-type protein (16). During this purification, both proteins ran at the same rate on gel filtration columns, indicating that the wild-type and the UW91 proteins are both dimers having the same native molecular weight. As shown in Fig. 5A, the two proteins also migrate at the same rate on SDS-gel electrophoresis, illustrating that the apparent subunit molecular weight of the protein has not been affected by either mutation. Fig. 5B is a two-dimensional gel electrophoresis separation of a mixture of the purified UW91 and wild-type Fe proteins. The proteins run in slightly different positions, and by loading varying quantities of one type or the other, it can be seen that the wild-type protein runs to the right, on the more acidic side of the gel. This is consistent with the change in the Fe protein’s isoelectric point caused by the deletion of E265 in the UW91 mutant protein.

The absorbance at 405 nm for equal concentrations of the wild-type and UW91 Fe proteins is identical, indicating that both proteins contain the same amount of iron. This is confirmed in Fig. 6, which compares the EPR signals exhibited by equal concentrations of the wild-type and UW91 Fe proteins in their dithionite-reduced states. The spectra for the two proteins are indistinguishable. Thus, the defect in the UW91 Fe protein that keeps it from serving as the physiological electron donor to the MoFe protein is not an altered [4Fe-4S] cluster. It is important to note that the UW91 Fe protein also cannot serve as an electron donor to the MoFe protein in vitro, where dithionite is used as an artificial electron donor. Since the data in Fig. 6 clearly show that the UW91 [4Fe-4S] cluster can be reduced by dithionite, the defect does not appear to involve the electron transfer to the Fe protein but rather some essential step in electron transfer from the Fe protein to the MoFe protein. This indicates that the defect is unlikely
to involve the interaction of the Fe protein with its physiological electron donor, flavodoxin.

**Interaction of the UW91 Fe Protein with MgATP**—The wild-type Fe protein is known to have two binding sites for MgATP (37). To test whether or not the UW91 Fe protein also binds MgATP, a gel filtration experiment was performed whereby the Fe protein was loaded onto a Sephadex G-25 column equilibrated with [14C]ATP. As shown in Fig. 7, as with the wild-type Fe protein, the UW91 Fe protein co-eluted with the [14C]ATP. Quantitation of these data show that the results for the wild-type and UW91 Fe proteins are indistinguishable and that both proteins have two binding sites for MgATP.

The binding of MgATP to the wild-type Fe protein is known to cause 1) the reduction potential to be lowered by 50–100 mV (37, 38, 39), 2) exposure of SH groups and the [4Fe-4S] cluster to the solvent (30, 40), 3) a dramatic change in the circular dichroism spectrum, especially for the oxidized protein (3), and 4) a change in the electron paramagnetic resonance signal (38). All of these changes are believed to be caused by a MgATP-induced conformational change in the Fe protein. To test whether or not a similar conformational change occurs for the UW91 Fe protein, we preformed the experiment shown in Fig. 8. **Panel A** shows that when bathophenanthrolinedisulfonate is added to the wild-type Fe protein, only a small amount of iron is chelated immediately and an absorbance base line at 535 nm is quickly established. When MgATP is then added to the solution, all the iron is chelated to form an orange Fe2+ phenanthrol ine complex that is observed as a dramatic increase in the absorbance at 535 nm.

Experiments of this type have been interpreted as suggesting that the [4Fe-4S] cluster is normally somewhat buried within the Fe protein but that MgATP binding exposes the [4Fe-4S] cluster to the chelator (30, 38). Fig. 8 shows that the addition of MgATP to the UW91 Fe protein in the presence of bathophenanthrolinedisulfonate does not cause exposure of the [4Fe-4S] cluster. Thus, binding of MgATP to the UW91 Fe protein does not cause the protein conformational change observed for the wild-type protein.

Although MgATP binds to the wild-type Fe protein alone, MgATP hydrolysis occurs only when the MoFe protein is added (41). When this MgATP hydrolysis is productive, it is coupled to electron transfer from the Fe protein to the MoFe protein (41). The stoichiometry of this productive reaction is 2 MgATPs hydrolyzed per electron transferred. Under some circumstances (e.g. in the presence of the MoFe protein in-
hbitor cyanide (42)), unproductive MgATP hydrolysis can also occur, leading to ATP/2e− ratios much greater than the typical 1:1. Because the UW91 Fe protein cannot support substrate reduction by the MoFe protein, by definition, it cannot carry out productive MgATP hydrolysis. Table III shows that it also does not carry out unproductive MgATP hydrolysis.

Interaction of the UW91 Fe Protein with the MoFe Protein—Kinetic studies of wild-type nitrogenase, involving the measurement of substrate reduction activity, have shown that there are two productive Fe protein-binding sites per MoFe protein and that the two proteins associate only transiently (41). Because these proteins must dissociate following each electron transfer, tight complexes are expected to be inactive. In fact, a tight heterologous two-to-one complex involving the

![Fig. 7. Analysis of MgATP binding to the wild-type Fe protein (A) and the UW91 Fe protein (B).](image)

![Fig. 8. Reaction of the purified wild-type Fe protein (A) and the purified UW91 (B) Fe protein with bathophenanthroline disulfonate following the addition of MgATP. The 1-mL anaerobic solutions contained 0.8 mg of purified Fe protein and 1.5 mM bathophenanthroline disulfonate. MgATP was added at the indicated times to a final concentration of 2.5 mM ATP. Spectra were recorded on a Varian 634 model spectrophotometer. If the UW91 Fe protein (or the wild-type Fe protein) was exposed to MgATP following the addition of the bathophenanthroline disulfonate, all of the iron was released from the Fe protein.](image)

### Table III

| Product measure | Fe protein present |
|----------------|-------------------|
|                | UW91              |
| H₂             | 2085 ± 76         | 0.8 ± 0          |
| Pₐ             | 3942 ± 42         | 18.0 ± 2         |

*This experiment was carried out at a 5-fold molar excess of Fe protein to MoFe protein.*

![Fig. 9. Acetylene reduction activity of the purified MoFe protein at a molar ratio of five wild-type Fe protein to one MoFe protein (0.6 mg of Fe, 0.4 mg of MoFe) following the addition of increasing amounts of either the wild-type Fe protein (O) or the UW91 Fe protein (E). To avoid problems with salt inhibition, both Fe proteins were desalted on Sephadex G-25 prior to their addition. The lowest level of UW91 Fe protein added represents a 50%;50% mixture of UW91 to wild-type Fe protein. The highest level added represents a 7.5-fold excess of UW91 Fe protein over wild-type Fe protein. Clostridium pasteurianum Fe protein and the A. vinelandii MoFe protein have been isolated, and it is inactive (43, 44). Thus, the inability of the UW91 Fe protein to transfer an electron to the MoFe protein could be explained by either too tight or too weak association of the two proteins. Too tight binding seems unlikely because the original report on UW91 (15) and the data in Table II show that when wild-type Fe protein is added to UW91 cell-free extracts, it can bind to the MoFe protein in those extracts to give active enzyme.*

To further test the strength of the interaction between the UW91 Fe protein and the MoFe protein, the competition experiment shown in Fig. 9 was performed. In this experiment, increasing amounts of the UW91 Fe protein were added to a molar ratio of 1:5 wild-type Fe protein to MoFe protein. For the control, increasing amounts of the wild-type Fe protein were added. As illustrated in Fig. 9, this control gave the expected result (41), showing the specific activity of the MoFe protein increases as more and more wild-type Fe protein is added. In contrast, when increasing amounts of the UW91 Fe protein were added, productive interaction of the wild-type Fe protein with the MoFe protein was inhibited (Fig. 9), indicating that the UW91 Fe protein does bind to the MoFe protein. However, the extent of inhibition is much less than expected if the wild-type and UW91 Fe proteins are equally competitive. For example, a 50%;50% mixture of the wild-type and UW91 proteins gave only 9.2% inhibition, and the highest inhibition shown in Fig.
9, 22%, is only obtained with a 7.5-fold excess of U991 Fe protein over wild-type Fe protein. These data could indicate that the MoFe protein has a strong preference for the MgATP-induced conformation of the wild-type Fe protein, a conformation that does not exist for the U921 protein. Alternately, in the absence of MgATP, the conformations of the U921 and the wild-type Fe proteins could be different, with the MoFe protein having a preference for the wild-type conformation.

Recently, Willing and Howard have taken a different approach to study the interaction of the Fe and MoFe proteins (36). They have shown that glutamine residue 112 of the wild-type Fe protein could be chemically cross-linked to lysine protein; and wild-type MoFe protein cross-linked using EDC; Imw protein.

A concentration of were carried out in 0.5-ml volumes containing 50 mM TES-KOH, analyzed by SDS-polyacrylamide gel electrophoresis on 10% gels, and then were terminated by adding SDS sample buffer as described elsewhere (27). Fractions of the samples from each reaction were analyzed by SDS-polyacrylamide gel electrophoresis on 10% gels, and the protein bands were visualized after staining with Coomassie Blue. Lane 1, wild-type Fe protein; lane 2, mixture of wild-type Fe protein and wild-type MoFe protein; lane 3, mixture of wild-type Fe protein and wild-type MoFe protein cross-linked using EDC; lane 4, U921 Fe protein; lane 5, mixture of U921 Fe protein and wild-type MoFe protein; lane 6, mixture of U921 Fe protein and wild-type MoFe protein cross-linked using EDC. Band a, cross-linked product composed of Fe protein and β subunit of the MoFe protein (27); band b α subunit of the MoFe protein; band c, β subunit of the MoFe protein; band d, Fe protein.

In earlier studies, we have shown that an A. vinelandii strain DJ54 that has a defined deletion in the nifH gene does not synthesize FeMo cofactor (13). That organism does synthesize an inactive FeMo cofactor-deficient MoFe protein (10, 14). As illustrated in Fig. 2, that protein can be fully activated by isolated FeMo cofactor in vitro, if both the Fe protein and MgATP are also added. Because the wild-type Fe protein binds to both MgATP and the MoFe protein, one interpretation of these results is that the Fe protein and MgATP are working together to make the FeMo cofactor site of the MoFe protein accessible for FeMo cofactor insertion (14). Another possibility is that MgATP does not exert its effect by interacting directly with the Fe protein but rather by interacting with the FeMo cofactor-deficient MoFe protein, or FeMo cofactor, or some other, as yet unrecognized, component of the system. If that is the case, then FeMo cofactor insertion is likely to be a sequential process whereby the Fe protein step must occur prior to the step that requires MgATP. Otherwise, it is difficult to understand the need to add MgATP to the insertion assay in vitro (Fig. 2) when MgATP was obviously already present in vivo.

The data presented here show that the substitution of a conserved alanine by a serine at position 157 of the Nif H sequence results in a Fe protein that can still function in FeMo cofactor biosynthesis and insertion. That protein has a normal [4Fe-4S] cluster (6) and normal MgATP binding activity (Fig. 7), and therefore, these findings might be required for FeMo cofactor biosynthesis and/or insertion. This protein, however, does not undergo the MgATP-induced conformational change, does not hydrolyze MgATP, and does not carry out productive electron transfer to the MoFe protein. Thus, we conclude that these reactions are not required for either the initial synthesis of FeMo cofactor or for its subsequent insertion into the MoFe protein.

**Implication for FeMo Cofactor Biosynthesis and Insertion**—During a normal nitrogenase reaction, the Fe protein's [4Fe-4S] cluster must be reduced, the Fe protein must bind MgATP, a MgATP-induced conformational change must occur, the Fe protein must bind to the MoFe protein, and it must undergo production electron transfer coupled to MgATP hydrolysis. The results presented here show that the substitution of A157 by serine does not affect the [4Fe-4S] cluster structure or reduction and does not affect MgATP binding but does prevent the MgATP-induced conformational change. These data strongly suggest that this highly conserved region of the protein, which is not near the [4Fe-4S] cluster ligands (7, 33) or the putative MgATP-binding site (34, 35), is directly involved in the MgATP-induced conformational changes. This A157S variant of the Fe protein also appears to be altered in its ability to bind productively to the MoFe protein and blocked in its ability to hydrolyze MgATP and transfer electrons to the MoFe protein. These results, therefore, suggest that the MgATP-induced conformational change may be a prerequisite for these subsequent reactions.

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