Purification and Characterization of a FeMo Cofactor-Deficient MoFe Protein†

Narasaiah Gavini,‡,§ Li Ma,‡ Gerald Watt,‖ and Barbara K. Burgess*,‡

Department of Molecular Biology and Biochemistry, University of California, Irvine, California 92717, and The Department of Chemistry, Brigham Young University, Provo, Utah 84602

Received February 3, 1994; Revised Manuscript Received July 25, 1994⊛

ABSTRACT: Previous studies have shown that the *nif*H gene product is required for FeMo cofactor biosynthesis and insertion and that a Δ*nif*H strain of *Azotobacter vinelandii* designated DJ54 accumulates a FeMo cofactor-deficient MoFe protein that is distinct from the FeMo cofactor-deficient protein synthesis by Nif B⁻, N⁻, or E⁻ strains [Tal, S., Chun, T., Gavini, N., & Burgess, B. K. (1991) *J. Biol. Chem. 266*, 10654–10657]. Here we report the purification and activation of the MoFe protein from DJ54. The purified protein is an $\alpha_2\beta_2$ tetramer that is indistinguishable from the wild-type MoFe protein by the criteria of SDS–polyacrylamide gel electrophoresis, native gel electrophoresis, and two-dimensional gel electrophoresis. It binds normally to its redox partner, the Fe protein, by the criterion of chemical cross-linking. It does not contain FeMo cofactor and does not catalyze significant C_2H_2 reduction or reductant-independent MgATP hydrolysis. It can, however, be activated with FeMo cofactor following the addition of the Fe protein and MgATP when an additional required component(s) is supplied by cell-free extracts from a Δ*nif*D strain of *A. vinelandii*. The purified DJ54 MoFe protein does contain P-clusters by the criteria of metal analysis, CD spectroscopy, cluster extrusion, and electrochemical reduction of the P_{OX} state. In the presence of dithionite it exhibits an axial $S = {}^1/_2$ EPR signal that integrates to 0.1–0.3 spin per $\alpha_2\beta_2$ tetramer. This signal has previously been observed for defective MoFe proteins from other organisms and for the VFe protein of *A. vinelandii* and may arise from a partially oxidized form of the P-clusters. The purified FeMoco-deficient MoFe protein can now be used to identify the spectral features specific for P-clusters in the MoFe and VFe proteins, for mechanistic studies, and as a starting material for *in vitro* assembly studies.

Molybdenum nitrogenase is composed of two separately purified proteins, whose complete structures have recently been solved by X-ray crystallography (Georgiadis et al., 1992; Kim & Rees, 1992a,b; Chan et al., 1993; Kim et al., 1993; Bolin et al., 1993). The larger of the two, the molybdenum–iron protein (MoFe protein), is an $\alpha_2\beta_2$ tetramer with the α and β subunits encoded by the *nif*D and *nif*K genes respectively. That protein has two types of metal centers, the iron molybdenum cofactors (FeMo cofactor) (two per tetramer), that have the composition Mo:Fe_7:S_9:homocitrate (Kim & Rees, 1992a; Smith & Eady, 1992; Burgess, 1990; Newton, 1992), and the P-clusters (two per tetramer) that each contain 8Fe and $8S^{2-}$ atoms in the form of bridged [4Fe-4S] clusters (Kim & Rees, 1992a). The FeMo cofactor can be isolated from purified MoFe protein and studied as a separate entity in the solvent *N*-methylformamide (Shah & Brill, 1977). The smaller of the two component proteins of nitrogenase, the iron protein (Fe protein), is a dimer of two identical subunits encoded by the *nif*H gene (Georgiadis et al., 1992; Dean & Jacobson, 1992). The two subunits are bridged by a single $[4Fe\text{-}4S]^{2+/+}$ cluster, and each has a single binding site for MgATP. For N_2 reduction to occur either *in vivo* or *in vitro*, electrons must be passed from the Fe protein to the MoFe protein in a reaction that is coupled to MgATP hydrolysis. Once in the MoFe protein, the electrons are transferred to the active FeMo cofactor center, where substrates are reduced (Smith & Eady, 1992; Burgess, 1990; Newton, 1992).

The assembly of a functional MoFe protein in a bacterial cell requires the participation of a large number of nitrogen fixation (*nif*) specific gene products (Dean & Jacobson, 1992). Gene products NifQ, B, N, E, V, and H are known to be required for the initial biosynthesis of the FeMo cofactor (Smith & Eady, 1992; Burgess, 1990; Newton, 1992) while others, NifH (Robinson et al., 1989) and NifY (White et al., 1992; Homer et al., 1993), appear to be required for the insertion of preformed FeMo cofactor into the MoFe protein polypeptides. Strains with mutations in the *nif*B (Shah & Brill, 1977), N, E (Shah & Brill, 1977; Tal et al., 1992), or H (Robinson et al., 1989; Tal et al., 1991; Robinson et al., 1987) genes accumulate FeMo cofactor-deficient MoFe proteins that can be activated *in vitro* by addition of isolated FeMo cofactor in *N*-methylformamide. Recent studies have established, however that the FeMo cofactor-deficient MoFe proteins synthesized by Nif B⁻, N⁻, and E⁻ strains are like each other but are unlike the protein synthesized by a Nif H⁻ strain (Tal et al., 1991).

The FeMo cofactor-deficient MoFe proteins from Nif B⁻ strains of *Klebsiella pneumoniae* (Hawkes & Smith, 1983) and *Azotobacter vinelandii* (Paustian et al., 1990) have previously been purified. Here we report the purification and characterization of a FeMo cofactor-deficient MoFe protein synthesized by a *nif*H deletion strain of *A. vinelandii*, designated DJ54 (Robinson et al., 1987).

MATERIALS AND METHODS

Reagents and Chemicals. DEAE-cellulose 52 was from Whatman, Q-Sepharose was from Pharmacia, and ACA34

† This work was supported by Grant R01 GM43144 from the National Institutes of Health.

* To whom correspondence should be addressed at the Department of Molecular Biology and Biochemistry, University of California, Irvine, CA 92717, (714) 856-4297 (phone) or (714) 856-8551 (FAX).

‡ University of California.

§ Present address: Department of Biological Sciences, Bowling Green State University, Bowling Green, OH 43403.

‖ Brigham Young University.

⊛ Abstract published in *Advance ACS Abstracts*, September 1, 1994.

0006-2960/94/0433-11842$04.50/0

was from Sepracor. ATP,[1] creatine phosphate, creatine phosphokinase, TES,[1] Tris,[1] Bis-Tris,[1] and CHES[1] were from Sigma, and $Na_2S_2O_4$ was from EM Science (Cherry Hill, NJ). Ampholines for two-dimensional gel electrophoresis were from LKB, Bromma, Sweden. Acrylamide, bis(acrylamide), ammonium persulfate, TEMED,[1] and SDS[1] were from Bio-Rad.

Cell Growth and Enzyme Assays. The wild-type *A. vinelandii* Fe and MoFe proteins were purified and analyzed as described elsewhere (Burgess et al., 1980). FeMo cofactor was isolated by the HCl/NaOH modification (Burgess et al., 1980) of the original isolation procedure (Shah & Brill, 1977). The construction of *A. vinelandii* strain DJ54, that contains a defined in-phase deletion in the *nif*H gene, is reported elsewhere (Robinson et al., 1987). For protein purification, DJ54 was grown under derepression conditions (Burgess et al., 1980) in a 200 L New Brunswick fermentor, with a mixing rate of 150 rpm and air addition rate of 50 L/min. The cells were harvested in mid-log phase, 3 h after ammonia was exhausted from the media, using a Sharples centrifuge. C_2H_2 reduction (Robinson et al., 1986), FeMo cofactor activation (Robinson et al., 1989), and phosphate assays (Fiske & Subbarow, 1952) were carried out using published procedures. All FeMo cofactor/DJ54 activation assays were carried out according to our published method (Robinson et al., 1989) except that purified DJ54 MoFe protein was added to cell-free extracts from a Δ*nif*D mutant of *A. vinelandii* designated DJ100 (Robinson et al., 1986).

Protein Characterization. Iron (Van DeBogart & Beinert, 1967) and molybdenum (Clark & Axley, 1955) determinations, one-dimensional and two-dimensional gel electrophoresis (Robinson et al., 1989), and Western analysis (Tal et al., 1991) were carried out as described elsewhere. Anaerobic native gel electrophoresis was performed by a modification of published procedures (Ornstein, 1964; Davis, 1964), whereby all buffers were degassed and the gel was prerun with 2 mM $Na_2S_2O_4$ under Ar for 15 min before loading the samples. The 6% acrylamide gels were run for 2.5 h at 100 V in a Vacuum Atmosphere's anaerobic box. EPR[1] spectra were obtained using a Bruker ESP 300E_z spectrophotometer, interfaced with an Oxford Instrument ESR-9002 liquid helium continuous-flow cryostat. CD[1] spectra were obtained using a Jasco J720 spectopolarimeter. UV/vis spectra were obtained with a Perkin Elmer Lambda UV/vis spectrometer. Extrusion experiments (Kurtz, 1982) and microcoulometric reduction measurements (Watt et al., 1993) were performed as described elsewhere.

RESULTS

Purification of the FeMo Cofactor-Deficient MoFe Protein from ΔnifH Strain DJ54. Because DJ54 is Nif$^-$, the cells were grown to mid-log phase on limiting ammonia and were then allowed to derepress for nitrogenase synthesis for 3 h before harvesting. When cells were grown in the 200 L fermentor, there were no obvious differences in cell growth behavior or cell yields when DJ54 was compared to the wild-type except, of course, that the wild-type cells started to grow again following derepression while the DJ54 cells did not.

[1] Abbreviations: ATP, adenosine triphosphate; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; TEMED, *N,N,N′,N′*-tetramethylethylenediamine; Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; HEPPS, *N*-(2-hydroxyethyl)piperazine-*N′*-3-propanesulfonic acid; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; SDS, sodium dodecyl sulfate; EPR, electron paramagnetic resonance; CD, circular dichroism.

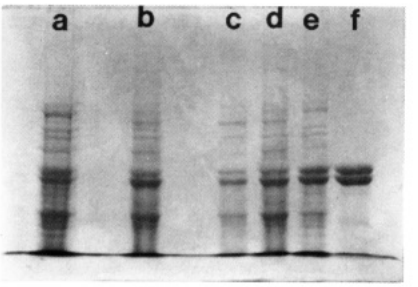
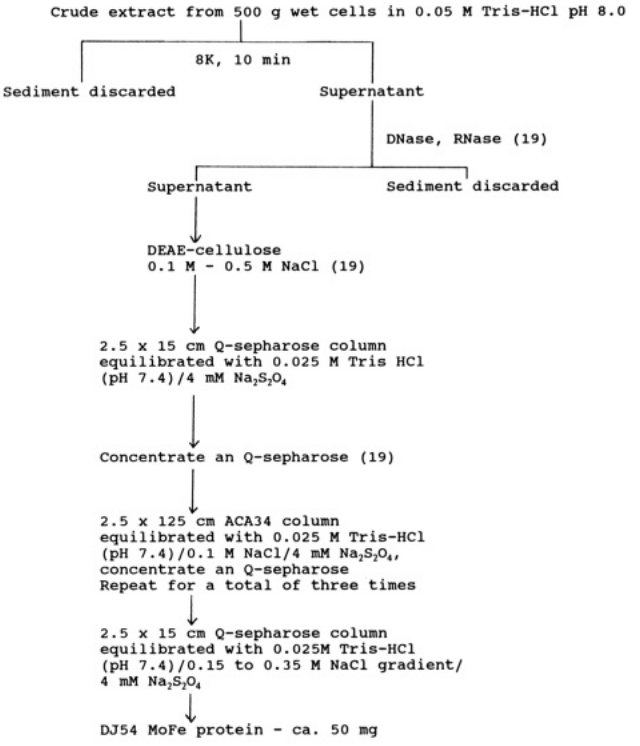


FIGURE 1: Coomassie-stained SDS–polyacrylamide (10%) gel electrophoresis separation of fractions obtained during the purification of the MoFe protein from DJ54. Lanes: (a) initial DEAE-cellulose (15 μg); (b) Q-Sepharose (15 μg); (c) first ACA54 (10 μg); (d) 2nd ACA54 (10 μg); (e) 3rd ACA54 (10 μg); and (f) final Q-Sepharose (10 μg). The steps are described in Scheme 1.

Scheme 1: Flow Diagram of the Purification of the DJ54 MoFe Protein



Wild-type *A. vinelandii* MoFe protein is purified anaerobically using a heat step followed by two DEAE-cellulose columns, followed by crystallization (Burgess et al., 1980). Because the DJ54 protein was completely denatured by the heat treatment at 56 °C for 5 min, it was necessary to design a new purification procedure. Scheme 1 is a flow diagram for purification of the DJ54 MoFe protein, and Figure 1 is a Coomassie-stained denaturing gel showing the protein at each stage of the purification. The final product appears to be at a similar level of purity as the wild-type MoFe protein purified using the crystallization method.

Throughout the purification, the protein was monitored by its absorbance at 405 nm. Just prior to the first ACA34 column the UV/vis spectrum of the partially purified protein (data not shown) revealed the presence of a cytochrome-like contaminant in the DJ54 protein. We have previously encountered large quantities of this contaminant while purifying other altered forms of the *A. vinelandii* MoFe protein (Li et al., 1990) but not while purifying the wild-type protein using similar purification steps. For the procedure shown in Scheme 1, the red cytochrome-like protein eluted just prior to the DJ54 MoFe protein on the ACA34 column. It was

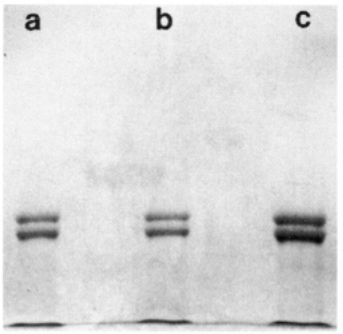


FIGURE 2: Denaturing gel analysis of mixtures of purified wild-type and DJ54 MoFe protein. One-dimensional SDS (6%) gel electrophoresis separation of (a) wild-type MoFe protein; (b) DJ54 MoFe protein; and (c) a 50:50 mixture of the two. 0.5 μg of protein was loaded per lane.

thus possible to remove it completely by going through the column several times (Scheme 1) and sacrificing yield at those steps. Because this contaminant runs on SDS gels in the same location as the β subunit of the MoFe protein (Li et al., 1990), the removal of this protein can be easily observed in Figure 1. Thus, early fractions have much darker β (lower) subunit bands while the pure fractions give equal intensity of the α and β subunit bands.

The purification procedure shown in Scheme 1 was carried out at room temperature under anaerobic conditions. If it was necessary to store a partially purified fraction overnight, it was pelleted in liquid nitrogen. It is important to note that at no time during the procedure were there any obvious differences in the behavior or stability of the protein when compared to that of the wild-type MoFe protein. The final yield at ca. 50 mg of DJ54 MoFe protein from 500 g of cells is lower than that of the wild-type protein using the crystallization method which involves fewer steps (Burgess et al., 1980), but was sufficient for the characterization described below.

Characterization by Gel Electrophoresis. The elution volumes obtained for the wild-type and DJ54 MoFe proteins on the ACA34 column used for the purification were the same, indicating that both proteins have similar native molecular weights. To determine if there were small differences in the subunit molecular weight, 6% SDS–polyacrylamide gels were run on mixtures of the two proteins. As shown in Figure 2, no differences were detected in subunit molecular weight and no bands other than the α and β subunit bands were observed. In addition, there were no differences in the reactivity of the denatured polypeptides with antibodies raised against the wild-type MoFe protein (data not shown). Figure 3 shows heavily loaded two-dimensional gel electrophoresis separations of wild-type MoFe protein, DJ54 MoFe protein, and mixtures of equal concentrations of the two prior to the final Q-Sepharose purification. These three samples are indistinguishable from one another, demonstrating that no change has occurred in the isoelectric point of either subunit as a result of the *nif*H deletion. Further, in agreement with the one-dimensional gels, these gels show that there are no other major protein bands observed for the DJ54 protein that are not observed for the wild-type. Taken together, these data demonstrate that, like the wild-type protein, the DJ54 protein is an $\alpha_2\beta_2$ tetramer.

The final confirmation that the FeMo cofactor-deficient MoFe protein purified from the DJ54 cells has a wild-type polypeptide organization is shown in Figure 4A, which is an anaerobic native gel electrophoresis separation of the purified wild-type and DJ54 MoFe proteins. The two proteins are indistinguishable on these gels, leading to the conclusion that

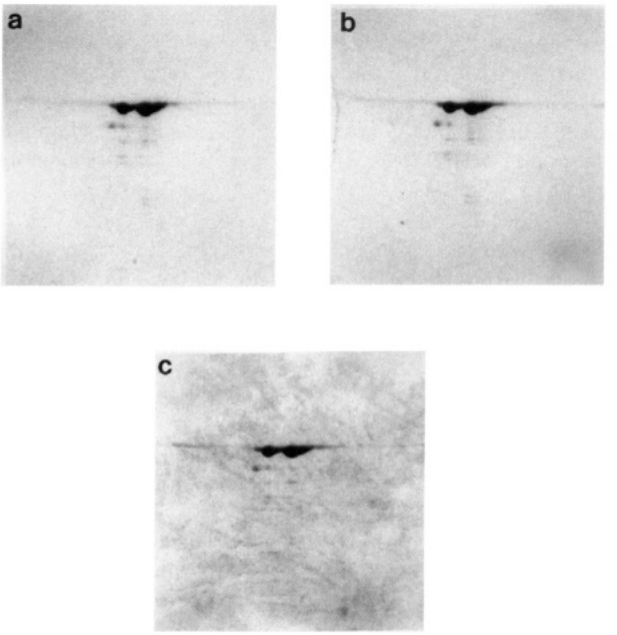


FIGURE 3: Two-dimensional gel electrophoresis separation of (a) wild-type MoFe protein; (b) DJ54 MoFe protein; and (c) a 50:50 mixture of the two. The partially purified protein was from the step just prior to the final Q-Sepharose column. 25 μg of protein was loaded in the first dimension.

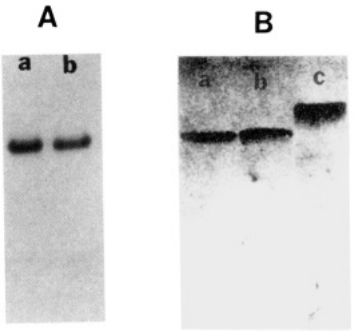


FIGURE 4: Native anaerobic polyacrylamide (6%) gel electrophoresis separations. Panel A compares (a) purified wild-type MoFe protein and (b) purified DJ54 MoFe protein, after staining with Coomassie. Panel B compares (a) purified wild-type MoFe protein, (b) wild-type cell-free extracts, and (c) DJ54 cell-free extracts after blotting and reacting with anti-MoFe protein antibody.

the migration behavior of the MoFe protein during native anaerobic gel electrophoresis **is not influenced by the presence or absence of the FeMo cofactor**. This result is not unexpected if the FeMoco site in the DJ54 protein is buried as it is in the wild-type protein (Kim & Rees, 1992a,b). As shown in Figure 4B and as previously discussed (Tal et al., 1991), the FeMo cofactor-deficient MoFe protein that accumulates in DJ54 cell-free extracts runs well above the position of the wild-type MoFe protein on native gels. The analysis of the purified DJ54 MoFe protein reported here now clearly establishes that this abnormal migration behavior does not result from a change in the molecular weight (Figure 2) or charge (Figure 3) of the α or β subunits, or from the simple absence of FeMo cofactor (Figure 4A). The simplest explanation is therefore that, in DJ54 cell-free extracts, the MoFe protein associates loosely with another protein that is lost during purification and that makes it run more slowly on native gels.

Interaction of the DJ54 MoFe Protein with the Wild-Type Fe Protein. The native gel analysis just described suggests that the global surface structure of the DJ54 MoFe protein is likely to be similar to that of the wild-type. Experiments

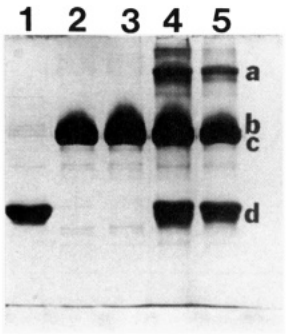


FIGURE 5: Analysis of the cross-linked product generated by the reaction of wild-type Fe protein with either the wild-type MoFe protein or the DJ54 MoFe protein. Conditions were as described elsewhere (Kurtz, 1982). Fractions of the samples from each reaction were analyzed by SDS–polyacrylamide gel electrophoresis on 10% gels, and the protein bands were visualized after staining with Coomassie Blue. Lane 1, wild-type Fe protein; lane 2, wild-type MoFe protein; lane 3, DJ54 MoFe protein; lane 4, mixture of wild-type Fe protein and wild-type MoFe protein cross-linked using EDC; and lane 5, mixture of wild-type Fe protein and DJ54 MoFe protein cross-linked using EDC. Band a, cross-linked product composed of Fe protein and β subunit of MoFe protein (Willing & Howard, 1990); band b, α subunit of MoFe protein; band c, β subunit of MoFe protein; and band d, Fe protein.

Table 1: Catalytic Activities

		MoFe protein [nmol of product/(min·mg)]	
atmosphere	product	wild-type	DJ54
C_2H_2	C_2H_4	1864 ± 178	9.1 ± 1.1
Ar	PO_4^{2-}	3972 ± 31	40.2 ± 2.6

were run to test whether or not this protein could bind to the wild-type Fe protein using the approach taken by Willing et al. (1989). They have previously shown that glutamate residue 112 of the wild-type Fe protein could be chemically cross-linked to lysine residue 399 of the β-subunit of the wild-type MoFe protein by 1-ethyl-3-[3′-(dimethylamino)propyl]carbodiimide (Willing & Howard, 1990). Figure 5 shows that the cross-linking behaviors of the DJ54 MoFe protein and wild-type protein are identical. Thus, at least insofar as this cross-linking reaction is concerned, the DJ54 MoFe protein does bind to the Fe protein in a manner analogous to that of the wild-type MoFe protein/Fe protein complex.

Catalytic Activity of the DJ54 MoFe Protein. Because the Fe protein is required for FeMo cofactor biosynthesis and insertion (Smith & Eady, 1992; Burgess, 1990; Robinson et al., 1989), the purified DJ54 MoFe protein does not contain FeMo cofactor. Table 1 shows that the protein also has barely detectable levels of C_2H_2 reduction activity. Although MgATP binds to the Fe protein alone, MgATP hydrolysis only occurs within the Fe protein/MoFe protein complex. It is important to note that while some MoFe protein mutant/wild-type Fe protein complexes still carry out reductant-independent MgATP hydrolysis, the DJ54 MoFe protein supports only about 1% of the wild-type levels of MgATP hydrolysis (Table 1).

In earlier studies we have shown that in cell-free extracts of Δ*nif*H strain DJ54 an inactive form of the MoFe protein is accumulated that runs more slowly than the wild-type protein on native gels (Figure 4B) (Tal et al., 1991). That form cannot be activated simply be adding FeMo cofactor in NMF; however, it can be fully activated to wild-type levels of activity by adding FeMo cofactor in the presence of the Fe protein and MgATP (Robinson et al., 1989; Tal et al., 1991). In early purification attempts we used the latter assay to monitor

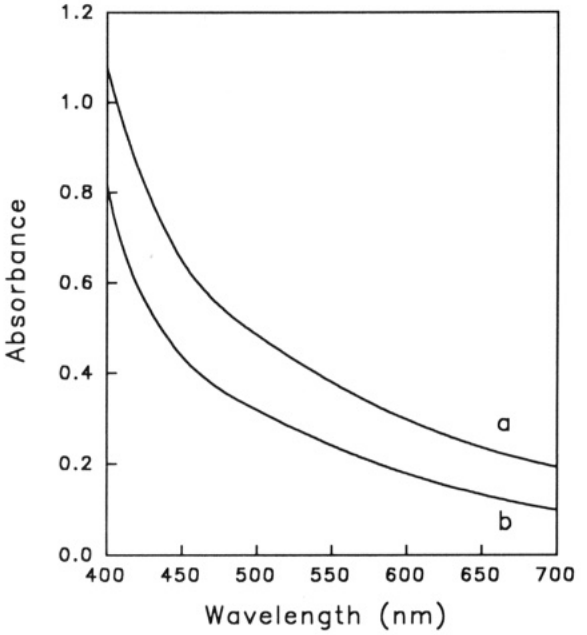


FIGURE 6: Absorbance spectra of purified, dithionite-reduced (a) wild-type and (b) DJ54 MoFe proteins. Protein concentrations were 4 mg/mL.

the purification of the DJ54 protein. For the wild-type MoFe protein, initial DEAE-cellulose column fractions (Scheme 1) yielded activity of ca. 400 nmol of C_2H_2 reduced/(min·mg of MoFe protein). For the DJ54 MoFe protein following activation with FeMo cofactor, highly variable results were obtained for the initial DEAE-cellulose fraction, ranging from ca. 12 to ca. 350 nmol of C_2H_2 reduced/(min·mg of protein). If the DJ54 protein was put directly onto the ACA34 column following the first DEAE column, highly variable results were again obtained. Further investigation of the above phenomenon revealed an inverse correlation between the purity of the DJ54 protein and our ability to activate it with isolated FeMo cofactor in the presence of the Fe protein and MgATP. The more pure the fraction, the lower the activity, until the purified DJ54 MoFe protein described here could not be activated at all. Again, something that is present is cell-free extracts, that is needed for activation, is being removed during purification.

To test whether the purified DJ54 protein could be activated if the missing component(s) were supplied, we used cell-free extracts from a Δ*nif*D strain of *A. vinelandii* as a source of the missing component(s). Because this strain does not synthesize the structural α subunit MoFe protein polypeptide, any MoFe protein activity observed must arise from the added, purified DJ54 MoFe protein. Thus, when the DJ100 extracts were activated as described in Materials and Methods by addition of FeMo cofactor, Fe protein, and MgATP, there was no detectable activity. In another control, where purified DJ54 MoFe protein was added to the DJ100 extracts with Fe protein and MgATP but without FeMo cofactor, there was also no detectable activity. However, when DJ100 extracts were incubated with Fe protein, MgATP, purified DJ54 MoFe protein, and FeMoco, the DJ54 protein was activated to give 105 ± 4 nmol of C_2H_2 reduced/(min·mg of protein). Thus, the purified DJ54 protein can be activated with FeMo cofactor, the Fe protein, and MgATP if the additional factor(s) is supplied by the DJ100 extracts.

Evidence for the Presence of P-Clusters. For the wild-type MoFe protein 53% of the total iron content is associated with the P-clusters and the rest with the FeMo cofactor centers. Figure 6 compares the visible spectra of equal concentrations

Table 2: Metal Analysis

	MoFe protein (nmol/mg of protein)		
metal	wild-type	DJ54	%[a]
Fe	98	53	54
Mo	7.0	0.8	11

[a] Percent of metal present in DJ54 relative to wild-type.

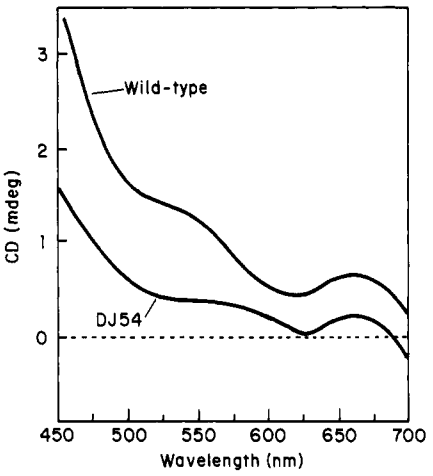


FIGURE 7: CD spectra of purified, dithionite-reduced wild-type and DJ54 MoFe proteins. Protein concentrations were 6.3 mg/mL.

of the wild-type and DJ54 MoFe proteins in their dithionite-reduced states. The general shape of the spectra are similar for both proteins, showing a broad decrease in absorbance in the 400–750 nm region and no obvious features. It is also apparent from Figure 6 that the DJ54 protein has much less brown color (i.e., iron) than the wild-type protein. This is quantitated in Table 2 which shows that the DJ54 MoFe protein has ca. 54% of the iron present in the wild-type MoFe protein. Like the wild-type absorbance spectra shown in Figure 6, the visible CD spectra of the wild-type MoFe protein should reflect the environments of both the P-clusters and the FeMo cofactor centers. Figure 7 compares the CD spectra of the dithionite-reduced wild-type and DJ54 MoFe proteins. These data clearly show that a major feature present at ca. 660 nm is extremely similar quantitatively and qualitatively when DJ54 is compared to the wild-type. In contrast, the feature at ca. 520 nm is much smaller in the DJ54 protein. Although the feature at ca. 520 nm could arise from FeMo cofactor that is missing in the DJ54 MoFe protein, previous studies have shown that initial oxidation of the P-clusters in the wild-type MoFe protein also causes this feature to diminish (Stephens, 1985). Thus, it is possible that the CD data indicate that the P-clusters in the DJ54 MoFe protein are more oxidized than those in the wild-type protein (see EPR discussion below).

When the wild-type MoFe protein is denatured using organic solvents in the presence of thiolate ligands, the iron associated with the 8Fe-containing P-clusters can be removed from the protein in the form of 2[4Fe-4S] clusters with each Fe atom ligated by the added thiolate (Kurtz et al., 1979). Figure 8 shows that the addition of an analogous denaturing mixture, containing benezenethiolate as a potential ligand (Kurtz, 1982), to the purified DJ54 MoFe, yields a species whose absorption spectrum is indistinguishable from that of synthetic $[Fe_4S_4(SPh)_4](Et_4N)_2$ in the same solvent. Thus, like the P-clusters in the native protein (Kurtz et al., 1979), the P-clusters of the DJ54 protein can be extruded as [4Fe-4S] clusters.

In wild-type MoFe protein the 8Fe-containing P-clusters (two per tetramer) are diamagnetic in the dithionite-reduced

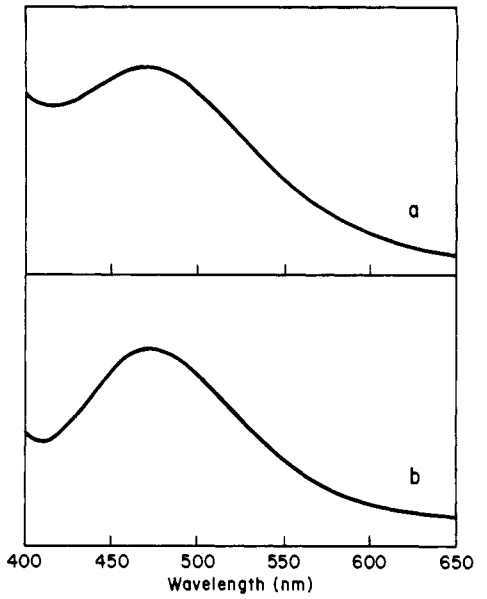


FIGURE 8: Extrusion of [4Fe-4S] centers from the purified DJ54 MoFe protein. The clusters were extruded as described elsewhere (Kurtz, 1982). Shown is (a) the synthetic complex $[Fe_4S_4(SPh)_4](Et_4N)_2$ compared to (b) the difference absorbance spectra of the DJ54 MoFe protein (after addition of the extrusion mixture minus before the addition of the extrusion mixture).

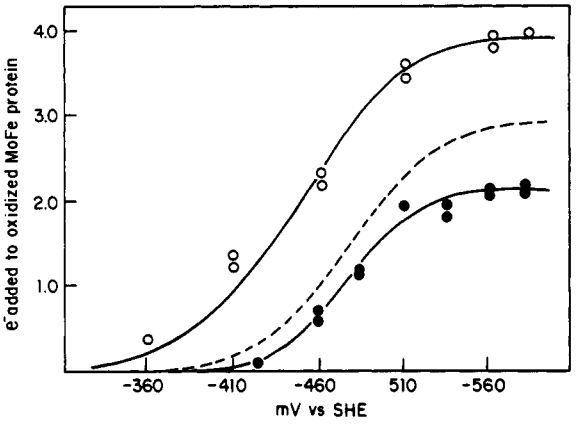


FIGURE 9: Microcoulometric reduction of indigodisulfonate-oxidized DJ54 MoFe protein. Upper curve (O—O) is reduction of DJ54 oxidized by excess indigodisulfonate. The solid line is a Nernst fit computed with $n = 1$ and $E_{1/2} = -455$ mV. The lower curve (●—●) is dithionite-free DJ54 partially oxidized by controlled addition of indigodisulfonate. The solid line is computed with $n = 1$ and $E_{1/2} = -470$ mV. The dotted line is the reduction of indigodisulfonate-oxidized wild-type MoFe protein and is computed with $n = 1$ and $E_{1/2} = -470$ mV. The minor differences in $E^{0'}$ are within the day-to-day variation of the method and are not believed to be significant.

state, which is designated P_N (Zimmermann et al., 1978). Extensive spectroscopic and redox titration experiments have established that two electrons can be removed from each P-cluster to yield an S = integer state designated P_{OX} (Surerus et al., 1992; Pierik et al., 1993). It has further been established that the P_{OX} to P_N transition exhibits $n = 1$ behavior with an $E^{0'}$ of ca. –470 mV versus SHE[1] (Watt et al., 1993). Thus, the $\alpha_2\beta_2$ protein that contains two P-clusters in the P_{OX} state should accept four electrons. Figure 9 shows the methyl viologen-mediated electrochemical reduction of the P_{OX} state of the DJ54 MoFe protein prepared by oxidation with indigodisulfonate. Separate experiments gave the same results with methylene blue oxidation. As shown in Figure 9, as expected for normal P-clusters, each molecule of oxidized DJ54 MoFe protein can accept four electrons with an $n = 1$ value and an $E^{0'} \sim -460$ mV versus SHE. Only one redox

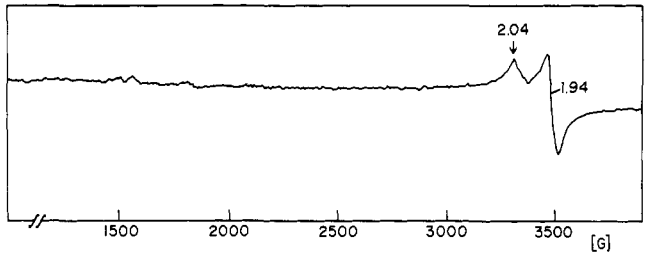


FIGURE 10: EPR spectrum of purified DJ54 MoFe protein at 4 K in the presence of dithionite. Protein concentration was 6.3 mg/mL. Microwave power, microwave frequency, modulation amplitude, and gain were 5 mW, 9.43 GHz, 2.886 G, and 5×10^4, respectively.

region is observed, confirming that no FeMo cofactor centers are present in the DJ54 protein. Selective oxidation of the DJ54 protein by about two electrons gives a reduction curve with parameters almost identical to the four electron oxidized species (Figure 9). Thus, all P-cluster redox states in the DJ54 protein are behaving identically. This same type of experiment has been performed previously on the native MoFe protein (Watt et al., 1993). Native MoFe protein P-clusters were also shown to be oxidized by a total of four electrons, but with one center having a slightly more positive reduction potential than the remaining three. Figure 9 shows that the four redox centers present in DJ54 have almost identical properties to the three centers in the native MoFe protein.

EPR Properties. For the wild-type MoFe protein in the presence of excess dithionite, the P-clusters are in the P_N state, which is believed to contain all Fe^{2+} iron atoms (Zimmermann et al., 1978). This state is diamagnetic. In contrast, as isolated in the presence of excess $Na_2S_2O_4$, the purified DJ54 MoFe protein exhibits the g = 1.94 EPR signal shown in Figure 10 which is not observed above 30 K. This signal integrates to ca. 0.1–0.3 spin per $\alpha_2\beta_2$ tetramer.

Confirmation That the DJ54 MoFe Protein Does Not Contain FeMo Cofactor. A number of lines of evidence demonstrate that, as expected, the DJ54 MoFe protein does not contain FeMo cofactor. First, the data in Table 2 show that the DJ54 MoFe protein does not contain sufficient Fe or Mo to account for the presence of FeMo cofactor. Second, when redox titration experiments of the type shown in Figure 9 are performed on the wild-type MoFe protein, two redox processes are evident, one for the P-clusters and one for the FeMo cofactor (Watt et al., 1993). In contrast, the experiment with the purified DJ54 MoFe protein shows only one redox region corresponding to the P-clusters (Figure 9). Finally, the EPR data for the DJ54 MoFe protein (Figure 10) do not show the $S = {}^3/_2$ EPR signal that is exhibited by the FeMo cofactor site of the MoFe protein. Thus, the purified DJ54 MoFe protein does not contain FeMo cofactor.

DISCUSSION

When bound within the MoFe protein, the FeMo cofactor is the site of substrate binding and reduction by nitrogenase (Smith & Eady, 1992; Burgess, 1990; Newton, 1992). *In vivo*, FeMo cofactor is known to first be assembled apart from the MoFe protein polypeptides and to later be inserted into a FeMo cofactor-deficient MoFe protein (Robinson et al., 1986; Ugalde et al., 1984; Filler et al., 1986). Although the details of FeMo cofactor biosynthesis are still unclear, it has been known for some time that the reactions require the participation of, at least, the *nif*Q, -B, -V, -N, and -E gene products (Smith & Eady, 1992; Burgess, 1990; Newton, 1992). Strains with mutations in *nif*B, -N, or -E therefore do not synthesize FeMo cofactor. However, they do synthesize inactive, FeMo cofactor-deficient MoFe proteins that can be activated *in vitro* by addition of isolated FeMo cofactor in *N*-methylformamide (Shah & Brill, 1977).

In 1986 Filler et al. published the additional observation that strains of *K. pneumoniae* that had mutations in the *nif*H gene did not synthesize FeMo cofactor (Filler et al., 1986). This led them to the surprising conclusion that the *nif*H gene, that encodes the Fe protein polypeptide, was somehow required for the biosynthesis or stability of the FeMo cofactor. The same conclusion was reached independently at the same time by Robinson et al. (1986). They observed that strains of *A. vinelandii* that were missing one, or the other, or both of the MoFe protein polypeptides still synthesized FeMo cofactor, while strains that were additionally missing the Fe protein polypeptide did not. The latter observation subsequently led to the construction of a strain of *A. vinelandii* designated DJ54 that had a defined deletion in the *nif*H gene (Robinson et al., 1987). DJ54 is Nif$^-$, does not synthesize the Fe protein, and does not synthesize FeMo cofactor, but does accumulate an inactive, FeMo cofactor-deficient form of the MoFe protein whose purification and characterization are described herein.

The Sequence of Events in MoFe Protein Assembly. Although metalloproteins play a variety of essential roles in biology, very little is known about the biosynthesis of metal centers and their incorporation into proteins. For the MoFe protein, the first assembly process that occurs is the biosynthesis of the FeMo cofactor on a scaffold apart from the MoFe protein polypeptides (Robinson et al., 1986; Ugalde et al., 1984; Filler et al., 1986). The second process that occurs may involve several steps, but appears to result in the synthesis of a FeMo cofactor-deficient MoFe protein like the one we have now purified from DJ54. The data described above establish that this protein is an $\alpha_2\beta_2$ tetramer (Figures 2 and 3) and that it does not contain FeMoco, but that it does contain P-clusters by the criteria of metal analysis (Table 2, Figure 6), CD spectroscopy (Figure 7), extrusion experiments (Figure 8), and electrochemical reduction of the P_{OX} state (Figure 9). The *nif*H gene product is therefore only required for FeMo cofactor biosynthesis and not for P-cluster biosynthesis.

Previously published data show that, in DJ54 cell-free extracts, the Fe protein and MgATP are required to convert the DJ54 MoFe protein to a form of the MoFe protein with the FeMoco factor site open (Robinson et al., 1989; Tal et al., 1991). That open form of the MoFe protein accumulates in mutant strains of *A. vinelandii* or *K. pneumoniae* that have the Fe protein, but that do not synthesize FeMo cofactor because of defects in the *nif*B gene. It is now known that the open (NifB$^-$) form is not an $\alpha_2\beta_2$ tetramer but rather a stable $\alpha_2\beta_2\gamma_2$ hexamer and that the γ subunit is required for FeMo cofactor insertion (Hawkes & Smith, 1983; Paustian et al., 1990; White et al., 1992; Homer et al., 1993; Allen et al., 1993). Because DJ54 extracts contain this additional protein, they can be fully activated with FeMo cofactor as long as the Fe protein and MgATP are also added (Robinson et al., 1989; Figure 4B). Because the purified DJ54 MoFe protein does not contain this additional protein (Figures 2, 3, and 4A), it cannot be similarly activated unless the protein is supplied, which we have now accomplished by addition of cell-free extracts from Δ*nif*D strain DJ100. The additional subunit has not as yet been purified and characterized but appears to be *nif*Y in *K. pneumoniae* (White et al., 1993; Homer et al., 1993) and something else in *A. vinelandii* (Homer et al., 1993; Allen et al., 1993). The DJ54 MoFe protein purified here, and the activation assay reported here using DJ100 extracts,

will now be used as a starting point for the purification of the required protein(s).

Mechanistic Considerations. In spite of the fact that the MoFe protein has been extensively characterized by X-ray crystallography and biochemical and biophysical methods, the role of the P-clusters in nitrogenase turnover has not been established (Smith & Eady, 1992). One reason for the lack of information is that the MoFe protein contains two types of metal clusters and it is not always possible to unambiguously assign one spectral feature to the P-clusters and another to the FeMo cofactor. Because the purified DJ54 protein contains only P-clusters, it can now be used to deconvolute the more complex spectrum obtained for the MoFe protein. For example, the data in Figure 7 unambiguously assign the CD feature at ca. 660 nm to the P-clusters, and this feature can now be monitored in turnover experiments with the MoFe holoprotein. A second complication with studies of the MoFe holoprotein is that protons from water are a substrate for this enzyme. Consequently, it is not possible to stop the nitrogenase reaction part way through and look at the early steps in electron transfer that are likely to occur between the Fe protein and the P-clusters. The FeMo cofactor-deficient MoFe protein described here is an $\alpha_2\beta_2$ tetramer that runs like the wild-type MoFe protein on native gels (Figure 4A) and that binds normally to the Fe protein by the criteria of chemical cross-linking (Figure 5). Thus, it appears to have a global organization similar to that of the wild-type protein. Levels of MgATP hydrolysis by the DJ54 MoFe protein/wild-type Fe protein complex are extremely low but are reproducibly above background and could easily account for a single turnover. Future studies will therefore be aimed at examining in more detail the interaction of the Fe protein/MgATP complex with the P-clusters of this FeMo cofactor-deficient MoFe protein.

Significance of the g = 1.94 EPR Signal. The EPR data illustrated in Figure 10 show that the P-cluster environment in the DJ54 protein is not identical to that in the MoFe holoprotein. Interestingly, the EPR signal shown in Figure 10 is extremely similar to one published by Zumft and Mortenson in 1973 which they demonstrated arose from an inactive Mo-deficient form of the *Clostridium pasteurianum* MoFe protein (Zumft & Mortenson, 1973). They suggested that this demolybdo MoFe protein might be a biosynthetic precursor to the MoFe holoprotein, a conclusion that is fully consistent with this study of DJ54. Indeed, the reported properties of their protein and ours are so similar that it appears very likely that they are in fact the same form of the protein isolated from two different organisms. Further, a similar signal that integrated to only ca. 0.1 spin per molecule has also been observed for an inactive FeMo cofactor-deficient MoFe protein synthesized by a Nif B$^-$ strain of *K. pneumoniae* (Hawkes & Smith, 1983). Thus, this protein appears common among a diverse group of diazotrophs.

Finally, it is important to note that an EPR signal with very similar properties to the one shown in Figure 10 has also been observed for the VFe protein for *A. vinelandii* (Hales et al., 1989; Morningston & Hales, 1987). For the VFe protein it was not known if this signal arises from the P-clusters or from the FeMo cofactor centers. The data presented here unambiguously assign that signal to the P-clusters. Those data also suggest either that the currently available VFe protein preparations are mixtures of wild-type VFe protein and FeV cofactor-deficient VFe protein or that the P-clusters in the VFe protein are in a slightly different environment from the P-clusters in the MoFe holoprotein, an environment that stabilizes the state giving rise to the EPR signal shown in Figure 10.

ACKNOWLEDGMENT

We wish to thank Mr. Daryl Wong for running the two-dimensional gels and Mrs. Linda Silver for running the native gels. We thank Professor Brian Hales for helpful discussions concerning the $S = {}^1/_2$ EPR signal observed for the DJ54 MoFe protein.

REFERENCES

Allen, R. M., Homer, M. J., Chatterjee, R., Ludden, P. W., Roberts, G. P., & Shah, V. K. (1993) *J. Biol. Chem. 268*, 23670–23674.

Bolin, J. T., Campobasso, N., Muchmore, S. W., Morgan, T. V., & Mortenson, L. E. (1993) in *Molybdenum Enzymes, Cofactors and Model Systems* (Stiefel, E. I., Coucouvanis, D., & Newton, W. E., Eds.) ACS Symposium Series 535, pp 169–192, American Chemical Society, Washington, DC.

Burgess, B. K. (1990) *Chem. Rev. 90*, 1377–1406.

Burgess, B. K., Jacobs, D. B., & Stiefel, E. I. (1980) *Biochim. Biophys. Acta 614*, 196–209.

Chan, M. K., Kim, J., & Rees, D. C. (1993) *Science 260*, 792–794.

Clark, L. J., & Axley, J. H. (1955) *Anal. Chem. 27*, 2000–2003.

Davis, B. J. (1964) *Ann. N.Y. Acad. Sci. 121*, 407–427.

Dean, D. R., & Jacobson, M. R. (1992) in *Biological Nitrogen Fixation* (Stacey, G., Burris, R. H., & Evans, H. J., Eds.) pp 763–834, Chapman and Hall, New York.

Filler, W. A., Kemp, R. M., Ng, J. C., Hawkes, T. R., Dixon, R. A., & Smith, B. E. (1986) *Eur. J. Biochem. 160*, 371–377.

Fiske, C. H., & Subbarow, Y. (1952) *J. Biol. Chem. 66*, 325–400.

Georgiadis, M. M., Komiya, H., Chakrabarti, P., Woo, D., Kornuc, J. J., & Rees, D. C. (1992) *Science 257*, 1653–1659.

Hales, B. J., Ture, A. E., & Hoffman, B. M. (1989) *J. Am. Chem. Soc. 111*, 8519–8520.

Hawkes, T. R., & Smith, B. E. (1983) *Biochem. J. 209*, 43–50.

Homer, M. J., Paustian, T. D., Shah, V. K., & Roberts, G. P. (1993) *J. Bacteriol. 175*, 4907–4910.

Kim, J., & Rees, D. C. (1992a) *Science 257*, 1677–1682.

Kim, J., & Rees, D. C. (1992b) *Nature 360*, 553–560.

Kim, J., Woo, D., & Rees, D. C. (1993) *Biochemistry 32*, 7104–7115.

Kurtz, D. M., Jr. (1982) *Biochem. Biophys. Res. Commun. 104*, 437–442.

Kurtz, D. M., Jr., McMillian, R. S., Burgess, B. K., Mortenson, L. E., & Holm, R. H. (1979) *Proc. Natl. Acad. Sci. U.S.A. 96*, 4986–4989.

Li, J.-G., Tal, S., Robinson, A. C., Dang, V., & Burgess, B. K. (1990) *J. Bacteriol. 172*, 5884–5891.

Morningston, J. E., & Hales, B. J. (1987) *J. Am. Chem. Soc. 109*, 6854–6855.

Newton, W. E. (1992) in *Biological Nitrogen Fixation* (Stacey, G., Burris, R. H., & Evans, H. J., Eds.) pp 877–930, Chapman & Hall, New York.

Ornstein, L. (1964) *Ann. N.Y. Acad. Sci. 121*, 321–349.

Paustian, T. D., Shah, V. K., & Roberts, G. P. (1990) *Biochemistry 29*, 3515–3522.

Pierik, A. J., Wassink, H., Haaker, H., & Hagen, W. R. (1993) *Eur. J. Biochem. 212*, 51–61.

Robinson, A. C., Burgess, B. K., & Dean, D. R. (1986) *J. Bacteriol. 166*, 180–186.

Robinson, A. C., Dean, D. R., & Burgess, B. K. (1987) *J. Biol. Chem. 262*, 14327–14332.

Robinson, A. C., Chun, T. W., Li, J.-G., & Burgess, B. K. (1989) *J. Biol. Chem. 264*, 10088–10095.

Shah, V. K., & Brill, W. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3249–3253.

Smith, B. E., & Eady, R. R. (1992) *Eur. J. Biochem. 205*, 1–15.

Stephens, P. J. (1985) in *Molybdenum Enzymes* (Spiro, T. G., Ed.) pp 117–160, John Wiley & Sons, New York.

Surerus, K. K., Hendrich, M. P., Christie, P. D., Rottgardt, P., Orme-Johnson, W. H., & Münck, E. (1992) *J. Am. Chem. Soc. 114*, 8579–8590.

Tal, S., Chun, T., Gavini, N., & Burgess, B. K. (1991) *J. Biol. Chem. 266*, 10654–10657.

Ugalde, R. A., Imperial, J., Shah, V. K., & Brill, W. J. (1984) *J. Bacteriol. 159*, 888–893.

Van DeBogart, M., & Beinert, H. (1967) *Anal. Biochem. 20*, 325–334.

Watt, G. D., Huang, H., & Redding, K. R. N. (1993) in *Molybdenum Enzymes, Cofactors and Model Systems* (Stiefel, E. I., Coucouvanis, D., & Newton, W. E., Eds.) ACS Symposium Series 535, pp 243–256, American Chemical Society, Washington, DC.

White, T. C., Harris, G. S., & Orme-Johnson, W. H. (1992) *J. Biol. Chem. 267*, 24007–24016.

Willing, A. H., & Howard, J. B. (1990) *J. Biol. Chem. 265*, 6596–6599.

Willing, A. H., Georgiadis, M. M., Rees, D. C., & Howard, J. B. (1989) *J. Biol. Chem. 264*, 8499–8503.

Zimmermann, R., Münck, E., Brill, W. J., Shah, V. K., Hanzl, M. T., Rawlings, J., & Orme-Johnson, W. H. (1978) *Biochim. Biophys. Acta 537*, 185–207.

Zumft, W. G., & Mortenson, L. E. (1973) *Eur. J. Biochem. 35*, 401–409.