A source of reductant is routinely added to the in vitro iron-molybdenum cofactor (FeMo-co) synthesis assay, although a requirement for reductant has not been established. This report demonstrates that the addition of reductant to the in vitro FeMo-co synthesis system is not required when Azotobacter vinelandii cell-free extract is prepared in buffer that lacks added reductant. The addition of reductant is required, however, if the A. vinelandii cell-free extract is chemically oxidized prior to addition to the assay. These results might suggest that extracts of A. vinelandii contain a physiological source of reductant that functions in the in vitro synthesis of FeMo-co. It is possible that the proteins required for FeMo-co biosynthesis (e.g. NIFNE and dinitrogenase reductase) are at the appropriate redox state to function in the assay. It is also possible that dinitrogen reductase and/or NIFNE (both Fe-S proteins required for FeMo-co synthesis) might catalyze the reductant-dependent reaction for FeMo-co synthesis. Dithionite, Ti(III) citrate, and NADH are able to serve as the source of reductant for in vitro FeMo-co biosynthesis.

Nitrogenase is a two-component metalloprotein that catalyzes the conversion of dinitrogen to ammonium (1). Dinitrogenase reductase is an α₂ dimer that contains a single Fe₅S₄ center. During substrate reduction, dinitrogenase reductase (also called NIFH, Fe protein, or component II) catalyzes the Mg²⁺-ATP-dependent transfer of electrons to dinitrogenase (2). Dinitrogenase (also called Mofe protein or component I) is an α₂β₂ tetramer that contains two pairs of unique metal clusters, known as the iron-molybdenum cofactor (FeMo-co), and the P-cluster (3–5). The electrons transferred to dinitrogenase are ultimately channeled to FeMo-co, the site of substrate reduction (5, 6).

In vitro system for the synthesis of FeMo-co has been described (7). The system includes ATP and an ATP-regenerating system, molybdate, homocitrate, NifB-co (an iron-sulfur cluster that is the apparent metabolic product of the nifB gene product), NIFNE (an α₂β₂ tetramer of the nifN and nifE gene products), and dinitrogenase reductase (8–11). In addition, a source of reductant (sodium dithionite) is routinely added to the in vitro FeMo-co synthesis assay.

Dithionite has served two roles in the in vitro investigation of nitrogenase. Dithionite was shown by Bulen et al. (12) to replace ferredoxin/flavodoxin as an electron donor to the enzyme. In addition, dithionite is an effective oxygen scavenger, thus protecting the oxygen-labile nitrogenase components. Because NIFNE, NifB-co, dinitrogenase reductase, and FeMo-co are all known to be extremely oxygen labile, dithionite has been included in the in vitro FeMo-co synthesis system. Here we test the requirement for dithionite to determine if it plays an active role in FeMo-co synthesis.

**EXPERIMENTAL PROCEDURES**

Materials—AG1-X8 and Sephadex G-25 were Pharmacia Biotech Inc. products. Sodium dithionite (Na₂S₂O₄; DTH) was purchased from Fluka. Na₅⁹MoO₄ was obtained from Nordion (Ontario, Canada). Indigo carmine and Ti(III) chloride were from Sigma. (NH₄)₂MoS₄ was a gift from D. Coucouvanis. Azotobacter vinelandii Strain and Growth Conditions—A. vinelandii strain UW45 (with a mutation within nifB gene (13)) was grown and derepressed, and extracts were prepared as described (14). The nifB gene product is required for the synthesis of cofactors for all three N₂ fixation systems (13). Where indicated, DTH was excluded from the N₃ x glycerol and the 25 mM Tris (pH 7.4) used in the osmotic-shock cell lysis procedure. Strain UW45 was grown and derepressed in molybdenum-free medium containing 1 mM Na₂WO₄ (7) for preparation of extracts used for ⁹⁹Mo experiments.

In Vitro FeMo-co Synthesis Assay—9 ml serum vials were flushed with purified argon and rinsed with anoxic 25 mM Tris-HCl (pH 7.4) that contained 1.7 mM DTH. This buffer will hereafter be referred to as Tris-HCl/DTH. The anoxic buffer was removed, and the vials were then rinsed with DTH-free 25 mM Tris-HCl that had been sparged with nitrogen and degassed on a gassing manifold to remove any traces of DTH from the vials. A complete FeMo-co synthesis reaction mixture was prepared by combining the following: 100 μl of the appropriate A. vinelandii cell-free extract and 5 μl of purified NifB-co (9) were added to the reaction mixtures, 5 μl of the purified NifB-co contained approximately 8.5 nmoles of DTH, which is not sufficient to support significant FeMo-co synthesis in assays that contain indigo carmine-oxidized cell-free extract (described below, see Table III). The total volume of the reaction mixture was 553 μl. The vials were incubated in a rotary water bath shaker at 30 °C for 30 min. This phase of the assay is referred to as the preincubation phase. During this phase, FeMo-co is synthesized and inserted into apodinitrogenase (apodinitrogenase refers to the dinitrogenase protein that lacks FeMo-co this form of the protein contains the P-clusters). After the 30 min of incubation, the indicated amount of (NH₄)₂MoS₄ was added to the assays to prevent further insertion of FeMo-co into apodinitrogenase (15). The (NH₄)₂MoS₄ stock solution was prepared in N-methylformamide that had been degassed on a gassing manifold. The vials were incubated at room temperature for 5 min, and the activity of the newly formed dinitrogenase was measured using the acetylene reduction assay as described previously (7). To monitor the incorporation of ⁹⁹Mo into FeMo-co, in vitro FeMo-co synthesis reactions were carried out as described above with the fol-
lowing exceptions. The concentration of nonradioactive molybdate was lowered 100-fold (10 μM of 10 μM Na2MoO4 were added to each assay), and Na2MoO4 (0.5 μCi/assay, carrier free) was added to each reaction. In addition, extracts of strain UW45 that was grown and derepressed on tungsten-containing medium were used in these assays. At the end of the preincubation phase of the assay, 100 μM of glycerol (that had been degassed in a gas manifold) were added to all reactions. The reactions contained 100 μM of this mixture were then applied to anoxic, native polycrylamide gels as described previously (16). Species with associated Mo were visualized using a PhosphorImager as described previously with the exception that the dried gels were exposed to the phosphor screen for 1–2 h (16).

Treatment of Extract with Indigo Carmine—5 g of strain UW45 (nifB) cell-free extract was treated with chromic acid shock as described previously (14) with the exception that DTH was excluded from all solutions. 15–20 ml of the extract were chemically oxidized by incubation with 3 mM oxidized indigo carmine (E° = −125 mV) for 15 min. The indigo carmine was removed from the extract using a column consisting of a 1 × 10-cm AG1-X8 layer on top of a 1 × 7-cm Sephadex G-25 layer. The column was equilibrated with 50 mM Tris-HCl (pH 7.4) and was operated in a Vacuum Atmospheres Company glove box containing less than 3 ppm O2.

Preparation of Reductant-free FeMo-co.—A source of reductant-free FeMo-co was needed to investigate the requirement of reductant for in vitro activation of apodinitrogenase by FeMo-co. Acid-treated dinigro- genase, which is a source of FeMo-co, can be readily prepared in a form that is free of reductant. All procedures were carried out under an argon atmosphere at 0–4 °C in the absence of dithionite. 15 mg of crystallized dinigro- genase from A. vinelandii were added to 2 ml of anoxic distilled H2O containing 3–4 glass beads (3-mm diameter) in a screw-capped centrifuge tube modified (9) so that the contents could be degassed on a gassing manifold. The contents were acidified with 125 μl of 0.4 M citric acid. After 2–3 min on ice, 250 μl of 0.4 M Na2HPO4 were added, and the contents were further incubated for 15 min before centrifugation at 500 × g for 2 min. The supernatant was discarded, and the pellet was resuspended in 2 ml of 25 mM Tris-HCl (pH 7.4) that had been sparged with N2 and degassed on a gassing manifold. This suspension, which is free of added reductant, was used as a source of FeMo-co.

Activation of Apodinitrogenase by FeMo-co (FeMo-co Insertion Assay)—The FeMo-co insertion assays were performed as described previously (5) except that the extract of A. vinelandii strain UW45 was free of DTH and had been oxidized with indigo carmine. In addition, reductant-free FeMo-co (described above) was added to the reactions as the source of FeMo-co.

Preparation of DTH, Ti(III) Citrate, NADH, and Dithiothreitol—Ti(III) citrate—Ti(III) citrate was prepared as described previously (17). Species with associated Ti(III) were visualized using a PhosphorImager as described previously with the exception that the dried gels were exposed to the phosphor screen for 1–2 h (16).

TABLE I

| Extracta | DTH present during FeMo-co synthesisb | Activityc |
|------------------|-------------------------------|---------|
| a. Plus DTH | + | 3.84 |
| b. Plus DTH | − | 2.91 |
| c. Minus DTH | + | 3.69 |
| d. Minus DTH | − | 2.72 |
| e. Oxidized UW45 | + | 2.94 |
| f. Oxidized UW45 | − | 0.56 |

*a UW45 extracts were prepared by osmotic shock as previously described. DTH was added to or excluded from the 4 M glycerol and the 0.025 M Tris-HCl (pH 7.4) buffer as indicated.

**In vitro FeMo-co synthesis assays were performed as described under "Experimental Procedures." DTH was added to or excluded from the reaction mixtures as indicated. At the end of the 30-min incubation, 40 nmoles of (NH4)2MoS4 were added to prevent subsequent insertion of FeMo-co into apodinitrogenase. At this time, 0.8 ml of ATP-regenerating mixture and 10 μl of purified dinigro- genase reductase (0.1 mg of protein) were added. The vials were brought to atmospheric pressure, and the C2H2 reduction phase of the assay was initiated by adding 0.5 ml of C2H2.

*Expressed as nmol C2H4 formed per min/mg protein. Values are the averages of duplicate assays that have variations of 10% or less.

**Extracts of strain UW45 were prepared by the osmotic shock procedure using 4 M glycerol and 0.025 M Tris-HCl (pH 7.4), from which DTH was excluded. The extract was oxidized with indigo carmine as described under "Experimental Procedures."
in vitro the lack of insertion of FeMo-co into apodinitrogenase. is due to the lack of FeMo-co synthesis (Table I) as opposed to and thus suggest that the lack of dinitrogenase activity when indigocarmine-oxidized extract contains a sufficient amount of The results presented here, however, demonstrate that the chemical oxidants (as was done in this study) does not result in genase previously revealed that mixing dinitrogenase with the redox properties of the P-clusters of more amenable to FeMo-co activation. A thorough analysis of the apodinitrogenase population that is activable by FeMo-co. The order and time of component addition to these assays clearly reveals that this difference in activability is not due to any instability of the apodinitrogenase or the presence of reductant, apodinitrogenase is more amenable to FeMo-co synthesis reaction from which DTH was excluded during the preincubation phase. Comparison of the activities of the assays reported in Table II (line a), to which DTH was added as a component of the ATP-regenerating mixture after the initial 30-min preincubation phase, and Table II (line b), to which (NH₄)₂MoS₄ was added to prevent subsequent insertion of FeMo-co into apodinitrogenase prior to the addition of DTH to the assay, suggests that in the absence of reductant, apodinitrogenase is more amenable to activation by FeMo-co. The order and time of component addition to these assays clearly reveals that this difference in activity is not due to any instability of the apodinitrogenase or the presence of reductant. One explanation for this observation is that there is a mixed population of apodinitrogenase in the oxidized extract (most likely apodinitrogenases at various oxidation states) and that addition of reductant during the activation assay significantly increases the percentage of the apodinitrogenase population that is activable by FeMo-co. The DTH presumably reduces the P-clusters of apodinitrogenase and the reduced form(s) of apodinitrogenase appears to be more amenable to FeMo-co activation. A thorough analysis of the redox properties of the P-clusters of A. vinelandii dinitrogenase previously revealed that mixing dinitrogenase with chemical oxidants (as was done in this study) does not result in the P-clusters being in a single well defined redox state (21). The results presented here, however, demonstrate that the indigo carmine-oxidized extract contains a sufficient amount of apodinitrogenase that is activable in the absence of reductant and thus suggest that the lack of dinitrogenase activity when reductant is excluded from the preincubation phase of the in vitro FeMo-co synthesis reaction (that utilizes oxidized extract) is due to the lack of FeMo-co synthesis (Table I) as opposed to the lack of insertion of FeMo-co into apodinitrogenase.

The second experiment that investigated whether FeMo-co is synthesized in the absence of reductant but is not inserted into the apodinitrogenase present in the oxidized extract employed in vitro FeMo-co synthesis reactions that contained Na₂¹⁹⁹MoO₄. Following the preincubation phase of the in vitro FeMo-co synthesis reaction, the proteins were separated by electrophoresis on anoxic native gels, and ¹⁹⁹Mo-labeled FeMo-co associated with proteins in the reaction mixture was detected. Previous studies have demonstrated that in the absence of apodinitrogenase, FeMo-co synthesized in vitro is associated with the gamma protein (16). The gamma protein becomes associated with the α2β2 form of apodinitrogenase to generate the α2β2 form, which can be activated by FeMo-co (22, 23). If FeMo-co insertion into apodinitrogenase but not FeMo-co synthesis requires that reductant be added to the oxidized extract, ¹⁹⁹Mo-labeled FeMo-co might be expected to be associated with gamma in a reaction system that uses oxidized extract and lacks DTH. The results presented in Fig. 1 clearly show that when the oxidized extract is used in the in vitro FeMo-co synthesis assay, no ¹⁹⁹Mo-labeled FeMo-co is associated with gamma (or any other protein other than the molybdenum storage protein, a non-nif protein that has been observed to accumulate ¹⁹⁹Mo both in vivo and in vitro (Refs. 24 and 25; Fig. 1, lane 2). When DTH is added to an identical assay, however, ¹⁹⁹Mo-labeled FeMo-co is observed to be associated with both dinitrogenase and gamma (in addition to the molybdenum storage protein (Fig. 1, lane 1)). The lack of ¹⁹⁹MoFeMo-co associated with gamma protein in the assay employing the oxidized extract provides further evidence that reductant is required for in vitro FeMo-co biosynthesis.

In agreement with the results presented in Table I, ¹⁹⁹Mo-labeled FeMo-co is associated with dinitrogenase in the assay that lacked DTH during the preincubation phase and contained extract of strain UW45 prepared in the absence of DTH (but not oxidized; Fig. 1, lane 3). These data provide further evidence that the A. vinelandii extract contains a physiological reductant(s) that functions in the in vitro synthesis of FeMo-co. From the results presented here, however, it is not clear if a stoichiometric amount of reductant is required for each molecule of FeMo-co synthesized or if the reductant is merely required to reduce the proteins involved in FeMo-co biosynthesis (e.g. NIFNE and dinitrogenase reductase) to the redox state in which they are active in the biosynthesis of FeMo-co.

Stability of the Components Involved in the in Vitro Synthesis of FeMo-co—To more definitively demonstrate that the lack of FeMo-co synthesis observed in the absence of DTH (when using the oxidized extract) is due to the requirement of reductant for FeMo-co synthesis and is not the result of the instability of the components required for FeMo-co biosynthesis in the absence of DTH, the following assays were performed. The components required for FeMo-co synthesis (molybdate, homocitrate, MgATP, oxidized extract of strain UW45, and NifB-co) were incubated in the absence of DTH for the standard 30-min incubation time. At this time, DTH was added to the assays and the reactions were incubated for an additional 30 min to allow FeMo-co synthesis and insertion into apodinitrogenase to occur. Comparison of the activities of the assays presented in Table II, line d, to which DTH was added after the 30 min

| Table II | Effect of DTH on in vitro reactions |
|----------|-----------------------------------|
| Précipitation system | Addition following preincubation | Second addition following preincubation | Activity |
| a. Complete minus DTH | FeMo-co | | 6.10 |
| b. Complete minus DTH | FeMo-co (NH₄)₂MoS₄ | 1.72 |
| c. Complete minus DTH | (NH₄)₂MoS₄ | FeMo-co | 0.06 |
| d. Complete minus DTH | DTH | (NH₄)₂MoS₄ | 1.53 |
| e. Complete | | | 1.44 |

a. The preincubation phase of in vitro FeMo-co synthesis reactions was performed as described in Table I. Where indicated, 1,300 nmol DTH, 30 nmol (NH₄)₂MoS₄, or reductant-free FeMo-co was added, and the reactions were incubated for at least an additional 15 min. Following this incubation, 30 nmol (NH₄)₂MoS₄ or reductant-free FeMo-co were added to the appropriate assays, and the reactions were again incubated for 15 min. The C₂H₄ reduction phase of the assay was then initiated by adding 0.8 ml of ATP-regenerating mixture (that contained DTH as described under “Experimental Procedures”) and 10 μl of purified dinitrogenase reductase (0.1 mg of protein). The vials were brought to atmospheric pressure, and 0.5 ml of C₂H₂ were added.

b. Activity is expressed as nmol C₂H₄ formed per min/mg protein.

c. The complete system contained 100 μl of 25 mM Tris-DTH (pH 7.4), 10 μl of 1 mM Na₂MoO₄, 20 μl of 5 mM homocitrate, 200 μl of an ATP-regenerating mixture (containing 3.6 mM ATP, 6.3 mM MgCl₂, 51 mM phosphocreatine, 20 units/ml creatine phosphokinase, and 6.3 mM DTH), 200 μl of oxidized extract of strain UW45, and 5 μl of purified NifB-co. DTH was excluded from the 0.025 ml Tris-HCl (pH 7.4) and the ATP-regenerating mixture in “minus DTH” reactions. 23). If FeMo-co insertion into apodinitrogenase but not FeMo-co synthesis requires that reductant be added to the oxidized extract, ¹⁹⁹Mo-labeled FeMo-co might be expected to be associated with gamma in a reaction system that uses oxidized extract and lacks DTH. The results presented in Fig. 1 clearly show that when the oxidized extract is used in the in vitro FeMo-co synthesis assay, no ¹⁹⁹Mo-labeled FeMo-co is associated with gamma (or any other protein other than the molybdenum storage protein, a non-nif protein that has been observed to accumulate ¹⁹⁹Mo both in vivo and in vitro (Refs. 24 and 25; Fig. 1, lane 2). When DTH is added to an identical assay, however, ¹⁹⁹Mo-labeled FeMo-co is observed to be associated with both dinitrogenase and gamma (in addition to the molybdenum storage protein (Fig. 1, lane 1)). The lack of ¹⁹⁹MoFeMo-co associated with gamma protein in the assay employing the oxidized extract provides further evidence that reductant is required for in vitro FeMo-co biosynthesis.

In agreement with the results presented in Table I, ¹⁹⁹Mo-labeled FeMo-co is associated with dinitrogenase in the assay that lacked DTH during the preincubation phase and contained extract of strain UW45 prepared in the absence of DTH (but not oxidized; Fig. 1, lane 3). These data provide further evidence that the A. vinelandii extract contains a physiological reductant(s) that functions in the in vitro synthesis of FeMo-co. From the results presented here, however, it is not clear if a stoichiometric amount of reductant is required for each molecule of FeMo-co synthesized or if the reductant is merely required to reduce the proteins involved in FeMo-co biosynthesis (e.g. NIFNE and dinitrogenase reductase) to the redox state in which they are active in the biosynthesis of FeMo-co.

Stability of the Components Involved in the in Vitro Synthesis of FeMo-co—To more definitively demonstrate that the lack of FeMo-co synthesis observed in the absence of DTH (when using the oxidized extract) is due to the requirement of reductant for FeMo-co synthesis and is not the result of the instability of the components required for FeMo-co biosynthesis in the absence of DTH, the following assays were performed. The components required for FeMo-co synthesis (molybdate, homocitrate, MgATP, oxidized extract of strain UW45, and NifB-co) were incubated in the absence of DTH for the standard 30-min incubation time. At this time, DTH was added to the assays and the reactions were incubated for an additional 30 min to allow FeMo-co synthesis and insertion into apodinitrogenase to occur. Comparison of the activities of the assays presented in Table II, line d, to which DTH was added after the 30 min
Table III

| DTH present during FeMo-co synthesis | Activitya |
|--------------------------------------|-----------|
| nmol                                |           |
| 0                                   | 0.06      |
| 10                                  | 0.19      |
| 100                                 | 0.97      |
| 200                                 | 1.34      |
| 500                                 | 2.37      |
| 1,300                               | 3.03      |

a In vitro FeMo-co synthesis assays were performed as described in Table I. Indigo carmine-oxidized extract of UW45 was used. DTH was added to the reactions from a 1 (1 and 10 nmol), 10 (100, 200, and 500 nmol), or 100 nmol (700 and 1,300 nmol) stock solution prepared in 0.1 M Tris-HCl (pH 8).

b Activity is expressed as nmol \( \text{C}_2\text{H}_4 \) formed per min/mg protein.

preincubation period, and line e, which contained DTH from the start of the preincubation phase, shows that almost identical in vitro FeMo-co synthesis activities were detected whether DTH was added after the preincubation phase or was present from the start of the reaction. These results demonstrate that the components required for FeMo-co synthesis are stable during the incubation in the absence of DTH. Independent assays to monitor the NIFNE and dinitrogenase reductase activities of the oxidized UW45 extract following preincubation in the absence of reductant confirmed that both of these components are stable (data not shown). The stability of the components required for FeMo-co synthesis in the absence of DTH further suggests that the lack of dinitrogenase activity observed when DTH is excluded from the preincubation phase of the assay (that contains oxidized extract) is due to the lack of FeMo-co synthesis in the absence of reductant.

Amount of DTH Required to Support in Vitro FeMo-co Synthesis—The amount of DTH required to support in vitro FeMo-co synthesis was investigated. The typical in vitro FeMo-co synthesis assay contains 1,300 nmol of DTH during the preincubation phase. The indigo carmine-oxidized extract was used to test the requirement for DTH and the results presented in Table III demonstrate that 700 nmol of DTH supported maximum in vitro FeMo-co synthesis activity. However, when the amount of DTH was reduced to 500 nmol, a substantial reduction in activity was observed. It is likely that a significant amount of the DTH added to the assay was consumed by non-nif components present in the oxidized extract. It will be interesting to further investigate the requirement of reductant when a purified in vitro FeMo-co biosynthesis system is available.

Other Reductants in in Vitro FeMo-co Synthesis—Ti(III) citrate has been reported to serve as a source of reductant for a number of enzyme-catalyzed reduction reactions, including nitrogenase-catalyzed reduction reactions (17, 18), and was therefore tested in the in vitro FeMo-co synthesis system. Ti(III) citrate supported the synthesis of FeMo-co. Approximately 80 nmol Ti(III) citrate added to the preincubation phase of the assay supported an activity of approximately 5 nmol \( \text{C}_2\text{H}_4 \) formed per min/assay. The addition of increasing amounts of Ti(III) citrate to the assay resulted in an inhibition of activity. This inhibition might be due to the increasing amounts of citrate added as the amount of reductant is increased. Citrate can be utilized by the FeMo-co biosynthetic machinery to produce an aberrant form of FeMo-co that is less efficient in the reduction of \( \text{C}_2\text{H}_4 \) to \( \text{C}_2\text{H}_6 \) (25).

The abilities of NADH and dithiothreitol to fulfill the requirement for reductant in the in vitro synthesis of FeMo-co were also investigated. NADH supported the synthesis of FeMo-co in assays that included indigo carmine-oxidized cell-free extracts. Assays that contained 50 nmol of NADH and 500 nmol of DTH exhibited almost identical activities. NADH has been used as a physiological electron donor for acetylene reduction with crude extracts (26). Dithiothreitol was ineffective in the FeMo-co synthesis system. Ineffectiveness of dithiothreitol to recover activity of the oxidized extracts suggests that the loss of activity upon oxidation is not the result of oxidation of a free sulfhydryl group on apodinitrogenase (27) but rather the loss of a source of reducing equivalents.

Possible Roles for Reductant in the in Vitro Synthesis of FeMo-co—The results presented here suggest that reductant is required for in vitro FeMo-co biosynthesis when indigo carmine-oxidized extract is the source of the proteins required for FeMo-co synthesis. The role of reductant in the in vitro synthesis of FeMo-co is currently unclear. The reductant might simply reduce a protein(s) required in the reaction to the state in which it is active in the FeMo-co synthesis assay. The initial reduction of such a protein might be the only requirement for reductant in the in vitro synthesis of FeMo-co. Alternatively, there might be a stoichiometric requirement for reductant for each molecule of FeMo-co synthesized. One possible function of the reductant is to reduce the molybdenum added to the assay to the form of molybdenum that is incorporated into the FeMo-co. The molybdenum added to the assay (as \( \text{Na}_2\text{MoO}_4 \)) is in the +6 oxidation state. Spectroscopic analysis of the molybdenum atom in FeMo-co suggests that the molybdenum atom is in the more reduced +4 oxidation state (28). Assays that contain an extract of molybdenum-grown A. vinelandii require reductant for in vitro FeMo-co synthesis, although they are not dependent on added \( \text{Na}_2\text{MoO}_4 \) (data not shown). From these observations we deduce that the reductant is not solely required in an initial step of processing or reducing the molybdenum atom to a form that can be used in FeMo-co synthesis because this processing step should have occurred in vivo in molybdenum-grown A. vinelandii cells. The ability of A. vinelandii to sequester significant amounts of molybdenum is attributed to the molybdenum storage protein (24). Although the oxidation state of molybdenum that is associated with the molybdenum-storage protein is unknown, the results presented in Fig. 1 suggest that incorporation of molybdenum (added as \( \text{Na}_2\text{MoO}_4 \)) into the molybdenum storage protein does not require reductant. It is possible, however, that the reductant is required to reduce molybdenum associated with the molybdenum-storage protein prior to its incorporation into FeMo-co. Perhaps the reductant is required to reduce some other component(s) involved in FeMo-co synthesis or a precursor(s) of FeMo-co.

Neither flavodoxin nor ferredoxin I is required for in vitro FeMo-co synthesis in DTH-free extracts. A. vinelandii flavodoxin and ferredoxin I have each been implicated as members of the electron transport chain to nitrogenase (2, 29). If either flavodoxin or ferredoxin I is the physiological reductant functioning in the in vitro synthesis of FeMo-co (in reductant-free extract that is not oxidized), it was hypothesized that a requirement for reductant might be observed in reactions that contained extract of flavodoxin and/or ferredoxin I. Neither flavodoxin nor ferredoxin I was tested in the in vitro FeMo-co synthesis assay (data not shown). A. vinelandii is able to fix \( \text{N}_2 \) in the absence of either or both flavodoxin and ferredoxin I and thus at least one other protein can also function in vivo as an electron donor to nitrogenase (30).

The results presented here suggest that FeMo-co activation of apodinitrogenase might be more efficient when the apodini-
trogenase is in a reduced state(s). Pierik et al. (21) have shown that oxidized dinitrogenase prepared as in this study contains the P-clusters in a mixture of oxidation states, and such a mixture of oxidation states is consistent with the results presented in this paper. The oxidation state of the apodinitrogenase might affect the interaction of apodinitrogenase with the 

in assays that utilize the oxidized extract is simply to reduce the protein(s) that is known to be required for the insertion of FeMo-co into apodinitrogenase. Alternatively, the oxidation state of the apodinitrogenase might affect the interaction of apodinitrogenase with FeMo-co itself. Ensign et al. (31) demonstrated that the reduced but not the oxidized apo-carbon monoxide dehydrogenase from Rhodospirillum rubrum was able to accept nickel into its active site, and it is possible that a similar situation occurs for the insertion of FeMo-co into apodinitrogenase.

Conclusion—A requirement for reductant in the in vitro FeMo-co synthesis system has been established. The addition of reductant, however, is only required if extract of A. vinelandii is chemically oxidized prior to addition to the assay. This observation suggests that a physiological reductant that is able to function in FeMo-co biosynthesis is present in extracts of A. vinelandii or that the reductant requirement in assays that utilize the oxidized extract is simply to reduce the protein(s) required for FeMo-co biosynthesis to the appropriate redox state(s). DTH, Ti(III) citrate, and NADH were found to satisfy the requirement for reductant in the in vitro FeMo-co synthesis assay.

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