Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 1636–1640, March 1986
Biochemistry

In vitro synthesis of the iron–molybdenum cofactor of nitrogenase

(nitrogen fixation/*Azotobacter vinelandii*/*Klebsiella pneumoniae*)

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Communicated by Robert H. Burris, November 7, 1985

ABSTRACT **Molybdate- and ATP-dependent *in vitro* synthesis of the iron–molybdenum cofactor (FeMo-co) of nitrogenase requires the protein products of at least the *nifB*, *nifN*, and *nifE* genes. Extracts of FeMo-co-negative mutants of *Klebsiella pneumoniae* and *Azotobacter vinelandii* with lesions in different genes can be complemented for FeMo-co synthesis. Both *K. pneumoniae* and *A. vinelandii* dinitrogenase (component I) deficient in FeMo-co can be activated by FeMo-co synthesized *in vitro*. Properties of the partially purified dinitrogenase activated by FeMo-co synthesized *in vitro* were comparable to those of dinitrogenase from the wild-type organism; e.g., ratios of acetylene- to nitrogen-reduction activities, as well as those of acetylene reduction activities to EPR spectrum peak height at $g = 3.65$, were very similar. *A. vinelandii* mutants UW45 and CA30 have mutations in a gene functionally equivalent to *nifB* of *K. pneumoniae*.**

Nitrogen fixation in *Klebsiella pneumoniae* requires expression of at least 15 genes arranged in seven operons that constitute the *nif* cluster (1, 2). The protein products of most of these genes have been identified and functions have been assigned to them (3–8).

Molybdenum in dinitrogenase (EC 1.18.6.1) (component I) is present in a cofactor (iron–molybdenum cofactor, FeMo-co) containing Fe, Mo, and S (9). FeMo-co has been proposed as the active site for the reduction of N_2, since it restores the activity of certain non-nitrogen-fixing (Nif^-) mutants (4, 9), gives dinitrogenase its characteristic electron paramagnetic resonance (EPR) spectrum, which changes during enzyme turnover (10, 11), and can catalyze acetylene reduction to ethylene (12). *Azotobacter vinelandii* strain UW45, a mutant lacking FeMo-co, has been used extensively to study the cofactor (13). In the best-studied nitrogen-fixing organism, *K. pneumoniae*, the products of at least six genes (other than the two required for structural proteins of dinitrogenase) are required for the synthesis of active dinitrogenase. The genes *mol* (equivalent to *chlD* of *Escherichia coli*) and *nifQ* are required for maximum nitrogen fixation (7, 14–16). Mutations in these genes affect early steps in Mo metabolism leading to the synthesis of FeMo-co (7, 14, 15). Like $NifQ^-$ and $ChlD^-$ strains, $NifV^-$ strains showed high levels of acetylene-reducing activity both *in vivo* and *in vitro* (4). The $NifV^-$ strains efficiently reduce acetylene but not N_2 (17). Recently, it has been suggested that the *nifV* gene product is involved in the final modification of FeMo-co (18, 19). Modification of FeMo-co by the *nifV* gene product is claimed to be essential for efficient nitrogen fixation (18, 19). Mutants with lesions in the genes *nifB*, *nifN*, and *nifE* synthesize a cofactorless dinitrogenase that can be activated *in vitro* by the addition of FeMo-co (4, 9, 20). The protein products of these genes have been identified (4, 8), but there is no information about the processes that they catalyze. Recently, it was shown that biosynthesis of FeMo-co does not require the presence of dinitrogenase subunit proteins (20). In the absence of dinitrogenase structural proteins, FeMo-co accumulates on a different protein, presumably involved in the FeMo-co biosynthesis (20). Very little is known about the pathway of biosynthesis of FeMo-co and its insertion into dinitrogenase.

This paper reports conditions for molybdate-dependent *in vitro* synthesis of FeMo-co and reconstitution of nitrogenase activity.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. *K. pneumoniae* mutant strains UN1655 (*nifB4691*), UN1688 (*nifN4724*), UN1100 (*nifE4420*::Mu), UN1089 (*nifD4409*::Mu), and UN1661 (*nifD4697*) have been described (21). *A. vinelandii* mutant strains UW45, UW10, UW6, and UW38 have been described previously (13, 22–24). *A. vinelandii* strain CA30, a mutant that can be activated *in vitro* upon addition of FeMo-co, was kindly provided by P. E. Bishop. Growth and derepression of nitrogenase in mutant strains of *K. pneumoniae* (6, 7) and *A. vinelandii* (22, 25) have been described previously. When molybdenum-free medium was required, Na_2MoO_4 was omitted, ultrapure chemicals were used, and all glassware was treated with 4 M HCl and washed with glass-distilled water (7). Molybdenum-free media were prepared in glass-distilled water. When stated, mutant strains of *K. pneumoniae* and *A. vinelandii* were derepressed for nitrogenase synthesis in the presence of 50 μM and 1 mM tungstate, respectively (20). Cultures for tungsten growth experiments were prepared by at least two cycles of growth in molybdenum-free medium, containing 28 mM ammonium acetate, to deplete intracellular molybdenum (7, 26).

Preparation of Crude Extracts. All buffers used throughout the procedure were sparged with purified nitrogen for approximately 30 min. These buffers were further deoxygenated on a gassing manifold by repeated evacuation and flushing with argon (purified through a heated copper catalyst) with constant mixing (6). These buffers contained 1.7 mM sodium dithionite, added just before use.

K. pneumoniae cells were suspended in 0.1 M Tris·HCl buffer, pH 7.4, at 1 g of cell paste per 2 ml of buffer containing 5–10 μg of deoxyribonuclease I per ml. The cells were broken anaerobically with a French pressure cell at 16,000 pounds/inch^2 (110 MPa) and centrifuged at 21,000 × *g* for 40

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Abbreviation: FeMo-co, iron–molybdenum cofactor.

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min at 0–4°C under an argon atmosphere (6). The supernatant solution was transferred to an argon-filled bottle with a syringe rinsed with anaerobic buffer and flushed with argon. The crude extract was stored at −20°C. Extracts from *A. vinelandii* mutant strains were prepared by osmotic shock (25).

ATP-Generating System. The ATP-generating system (25) contained 4.2 mM ATP, 50 mM phosphocreatine, 8.3 mM $MgCl_2$, and 0.33 mg of creatine kinase per ml of 25 mM Tris·HCl buffer, pH 7.4. Dithionite solution (0.1 M) was prepared anaerobically in 0.013 M NaOH. ATP-generating solution and dithionite were mixed in a ratio of 3:1, after deoxygenating the ATP-generating solution by repeated evacuation and flushing with argon purified through a heated copper catalyst. This mixture is referred to as ATP/dithionite throughout this manuscript. Unless otherwise stated, MoO_4^{2-} (10 μl of 1 mM) added to the FeMo-co synthesis reactions was mixed just before use with 200 μl of ATP/dithionite to remove traces of O_2 present in molybdate solution.

FeMo-co Synthesis *in Vitro* and Nitrogenase Assay. The reactions were carried out in 9-ml serum vials (1.5-ml final reaction volume). The vials were sealed with serum stoppers and repeatedly evacuated and filled with purified argon. Three hundred microliters of 25 mM Tris·HCl, pH 7.4, containing 1.7 mM dithionite was added to each vial, the vials were agitated, and the buffer was removed after 10–15 min. One hundred microliters of 25 mM Tris·HCl buffer, pH 7.4, was added to each vial, followed by 200 μl of each of the mutant extracts used for complementation. When different aliquots of extracts were used, the volume was adjusted to 0.5 ml with 25 mM Tris·HCl buffer, pH 7.4. Ten microliters of 1 mM Na_2MoO_4 and 200 μl of ATP/dithionite were added to each vial and the reaction mixtures were incubated at room temperature for 30 min, unless otherwise specified. After this incubation, 800 μl of ATP/dithionite and 20 μl of purified dinitrogenase reductase (30 μg of protein) were injected in each vial. The vials were brought to atmospheric pressure by piercing the serum stopper with a hypodermic needle, 0.5 ml of acetylene was injected, and the vials were incubated with shaking at 30°C for 15 min. The reaction was terminated by injecting 0.1 ml of 4 M NaOH, and the ethylene formed was measured with a Packard gas chromatograph with a Porapak N (Waters Associates) column (6).

Nitrogen reduction (25) and anaerobic column chromatography (27) have been described previously. Protein concentrations were determined by the method of Bradford (28), using serum albumin as a standard. Dinitrogenase (27) and FeMo-co (9) were purified as described in the references. Dinitrogenase reductase from the second DEAE-cellulose column (27) was further purified by passage through a Sephadex G-100 column (2.5 × 86 cm) in 0.1 M NaCl in 25 mM Tris·HCl buffer, pH 7.4. EPR spectroscopy was performed as reported (10, 11), at microwave frequency of 9.27 GHz and with modulation frequency of 100 kHz. Samples were maintained at 26K with liquid helium boil-off for cooling.

ATP, phosphocreatine, hexokinase, glucose-6-phosphate dehydrogenase, glycerol, Tris base, and deoxyribonuclease I were obtained from Sigma. Creatine kinase was obtained from Miles Laboratories. DEAE-cellulose was a Whatman DE-52 (microgranular) product. Ultrapure molybdate and tungstate were obtained from Research Organic/Inorganic Chemical (Sun Valley, CA). All other chemicals were of ultrapure or analytical grade available commercially.

RESULTS AND DISCUSSION

Complementation of Crude Extracts of FeMo-co-Negative Mutants. When a crude extract of a *K. pneumoniae* and an *A. vinelandii* mutant strain defective in the biosynthesis of FeMo-co were mixed and incubated for 30 min before the acetylene-reduction assay was initiated by injecting ATP/dithionite and acetylene, substantially more activity was observed in certain combinations (Table 1). Extracts of UN1688 ($NifN^-$) and UN1100 ($NifE^-$) mutants of *K. pneumoniae* generated significant activity when mixed with extracts of *A. vinelandii* strains UW45 or CA30. On the other hand, extracts of UN1655 ($NifB^-$) failed to complement UW45 or CA30 extracts. Extracts of *A. vinelandii* mutants UW45 and CA30 did not complement with each other. Our results suggest that *A. vinelandii* strain UW45 and CA30 are defective in the gene equivalent to *nifB* of *K. pneumoniae*.

Requirements for FeMo-co Synthesis. Pienkos *et al.* (26) reported that inactive dinitrogenase in wild-type *A. vinelandii* derepressed for nitrogenase synthesis in the presence of tungstate could be activated *in vitro* by molybdate and ATP. However, they could not activate inactive dinitrogenase by adding molybdate and ATP to the extracts of UW45. We were also unable to activate inactive dinitrogenase by molybdate and ATP/dithionite in the extracts of FeMo-co-negative mutants of either $NifB^-$, $NifN^-$, or $NifE^-$ *K. pneumoniae* strains or *A. vinelandii* (UW45 and CA30). On the other hand, addition of molybdate and ATP/dithionite to the mixture of extracts of UW45 ($NifB^-$) and UN1100 ($NifE^-$) generated substantial activity (Table 2). Similar results were obtained with a mixture of UW45 ($NifB^-$) and UN1688 ($NifN^-$), while UW45 plus UN1655 ($NifB^-$) failed to generate activity. Our results demonstrate that the complementation of UW45 extract with the *nifB* gene product is essential for molybdate- and ATP-dependent synthesis of FeMo-co *in vitro*. It can be seen from the data that omission of any one of the components of the assay system eliminated the ability to generate FeMo-co activity (Table 2). About 25% activity is observed even when ATP is omitted during preincubation. However, ATP-generating solution is added during the subsequent acetylene reduction assays and FeMo-co synthesis during this period cannot be avoided. The requirement of ATP for FeMo-co synthesis is further demonstrated by the data reported in a later section (*Partial Purification and Characterization of Dinitrogenase Activated by FeMo-co Synthesized* in Vitro). A 15-min exposure of UN1100 ($NifE^-$) extract to air resulted in loss of molybdate- and ATP/dithionite-dependent FeMo-co synthesis activity, suggesting that the *nifB* gene product supplied by this extract is sensitive to oxygen. In a reconstitution experiment, dinitrogenase defi-

Table 1. Acetylene reduction activity with mixtures of crude extracts of FeMo-co-negative mutants

Source of extract*	Ethylene formed,† nmol
UN1655 ($NifB^-$)	0.3
UN1688 ($NifN^-$)	1.4
UN1100 ($NifE^-$)	3.2
UW45	2.9
CA30	0.6
UW45 + UN1655	2.9
UW45 + UN1688	49.6
UW45 + UN1100	64.6
CA30 + UN1655	0.6
CA30 + UN1688	23.0
CA30 + UN1100	46.2
UW45 + CA30	2.9

*Portions (0.2 ml) of each extract used for the reaction contained 2.9–4.3 mg of protein.
†Acetylene reduction assays were carried out for 15 min at 30°C.

Table 2. Requirements for FeMo-co synthesis

Condition	Ethylene formed,* nmol
Complete system†	223.9
− ATP/molybdate	6.7
− Molybdate	14.7
− ATP	56.1
− UW45(W)‡	4.0
− UN1100	1.5
− UW45(W) + UW45(W) oxidized for 15 min	112.5
− UN1100 + UN1100 oxidized for 15 min	7.7
− UN1100 + UN1688	189.3
− UN1100 + UN1655	2.3

*Acetylene reduction assays were carried out for 15 min at 30°C.
†Complete system contained 0.2 ml of UW45(W) extract (2.7 mg of protein), 0.2 ml of UN1100 extract (4.3 mg of protein), and other components as described in *Materials and Methods*. Portions of UN1688 and UN1655 extracts used in the assays contained 3.6 and 4.3 mg of protein, respectively.
‡UW45(W) represents the strain grown with tungstate instead of molybdate.

cient in FeMo-co was still activatable by FeMo-co, showing that loss of activity was not due to denaturation of activatable dinitrogenase. On the other hand, UW45 extract exposed to air for 15 min still produced approximately 50% of the molybdate- and ATP/dithionite-dependent activity. These data suggest that the essential components supplied by this extract are not as oxygen sensitive as the *nifB* gene product.

Dependence of FeMo-co Synthesis on Extract Concentration. Synthesis of FeMo-co activity was dependent on the amount of UW45 and UN1100 extracts in the reaction mixtures (Table 3). The activity generated is proportional to the amount of UN1100 extract, suggesting that some component from NifB$^-$ extract is limiting in the assays. Consistent with this, there is no dramatic increase in activity with 2.1 and 2.7 mg of protein of UW45 extract.

Effect of Time of Preincubation on the FeMo-co Synthesis. Data on the effect of preincubation time on the FeMo-co synthesis are presented in Fig. 1. Thirty-minute preincubation produced 3 times more FeMo-co activity compared to the assay carried out without preincubation. It should be noted that FeMo-co synthesis during the time course of the acetylene-reduction assay cannot be avoided. There was no significant increase in the activity when the reaction mixtures were preincubated for 45 min instead of 30 min. It is likely that the reaction mixture becomes limiting in some component required for FeMo-co synthesis.

Activation of *K. pneumoniae* and *A. vinelandii* Dinitrogenase Deficient in FeMo-co by FeMo-co Synthesized *in Vitro*. From

Table 3. Dependence of the FeMo-co synthesis on the amount of complementary extracts

UW45(W) extract, mg of protein	UN1100 extract, mg of protein	Ethylene formed,* nmol
2.7	—	1.7
2.7	1.1	42.0
2.7	2.2	98.6
2.7	3.2	142.2
2.7	4.3	192.4
2.1	4.3	189.1
1.4	4.3	182.6
0.7	4.3	129.2
—	4.3	4.1

*Acetylene reduction assays were carried out for 15 min at 30°C.

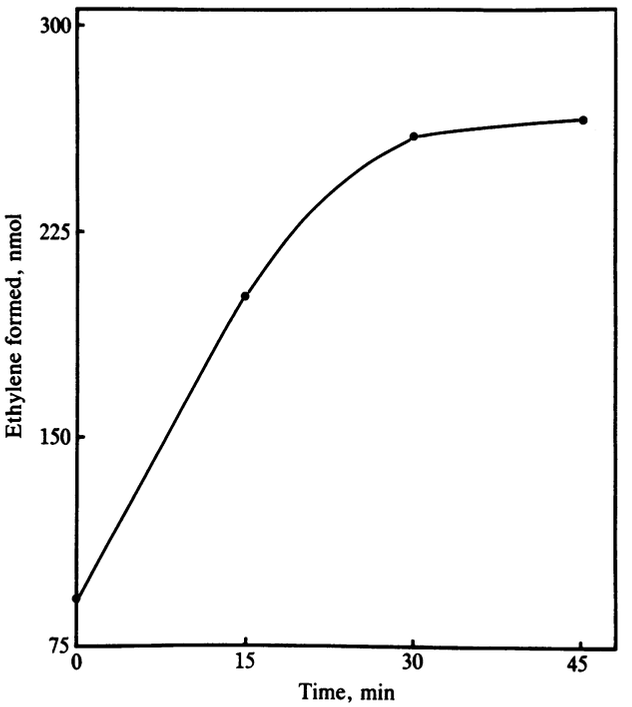

FIG. 1. Effect of period of preincubation on the FeMo-co synthesis. Each assay mixture contained 0.2 ml of UW45 extract (2.5 mg of protein), 0.2 ml of UN1100 extract (4.5 mg of protein), and other components described in *Materials and Methods*. Acetylene reduction assays were carried out for 15 min at 30°C subsequent to preincubation.

the data presented above, it is not possible to judge whether it is *K. pneumoniae* or *A. vinelandii* dinitrogenase deficient in FeMo-co, or both, that is being activated by FeMo-co synthesized *in vitro*. To resolve this, we used *K. pneumoniae* and *A. vinelandii* strains with mutations in the structural genes of dinitrogenase (21, 24). Dinitrogenase in these mutants cannot be activated by FeMo-co *in vitro* (4, 13, 20). These mutants synthesize FeMo-co *in vivo* (ref. 20; unpublished data) and thus should supply *nifB*, *nifN*, and *nifE* products *in vitro*. To overcome the problem of preformed FeMo-co in these mutants, we derepressed them in the medium containing tungstate instead of molybdate (13, 20, 26). Extracts of tungstate-derepressed cells were used as the source of *nifB*, *nifN*, and *nifE* products to complement *in vitro* FeMo-co synthesis reactions.

Inactive dinitrogenase from *K. pneumoniae* mutants with lesions in the *nifB*, *nifN*, or *nifE* genes can be activated by the FeMo-co synthesized *in vitro* upon complementation with *A. vinelandii* mutant extract (Table 4). Similarly, inactive dinitrogenase in the extract of *A. vinelandii* mutant strain UW45 can be activated by FeMo-co synthesized upon addition of the *K. pneumoniae* mutant extract. These results demonstrate that inactive dinitrogenase from both *A. vinelandii* and *K. pneumoniae* can be activated by FeMo-co synthesized *in vitro*.

Effect of Tungstate and Vanadate on Molybdate-Mediated FeMo-co Synthesis. The antagonistic effect of tungstate on molybdate metabolism in nitrogen-fixing organisms has been well documented (26, 30–33). We examined the effect of tungstate on molybdate-dependent synthesis of FeMo-co *in vitro* (Table 5). Addition of 100 nmol of tungstate to the reaction mixture did not significantly inhibit molybdate (10 nmol)-dependent FeMo-co synthesis. Approximately 33% activity was still observed even after adding a 100-fold excess of tungstate (1 μmol) to the FeMo-co synthesis reaction. These data show that molybdate is preferentially utilized for

Table 4. Activation of *K. pneumoniae* and *A. vinelandii* dinitrogenase deficient in FeMo-co by FeMo-co synthesized *in vitro*

Source of activatable component I	Addition	Ethylene formed,* nmol
UN1655 (NifB⁻)	UW6(W)†	120.1
UN1688 (NifN⁻)	UW6(W)	140.8
UN1100 (NifE⁻)	UW6(W)	168.5
UW45(W)	UN1089(W)	94.7
UW45(W)	UN1661(W)	85.4
UW45(W)	UW6(W)	159.3
UN1655	—	0.3
UN1688	—	1.3
UN1100	—	2.4
UW45(W)	—	1.2
—	UW6(W)	0.4
—	UN1089(W)	0.3
—	UN1661(W)	0.0

Portions (0.2 ml) of *A. vinelandii* extracts used for the reaction contained 2.4–3.1 mg of protein, and *K. pneumoniae* extracts contained 3.6–4.3 mg of protein. The strains grown with tungstate instead of molybdate are indicated by (W).
*Acetylene reduction assays were carried out for 15 min at 30°C.
†Similar results were obtained when UW10(W) or UW38(W) extracts were used instead of UW6(W) extract.

FeMo-co synthesis, even in the presence of 10- to 100-fold excess of tungstate. Studies with nitrogen-fixing organisms have demonstrated that molybdate is preferentially utilized over tungstate (26, 31, 33). In *K. pneumoniae*, molybdate transport is competitively inhibited by tungstate, which exhibits an apparent K_i identical to the K_m for molybdate (ref. 14; unpublished data). Therefore, the discriminatory step does not occur during transport. It seems that the enzyme(s) involved in FeMo-co synthesis metabolizes molybdate preferentially over tungstate. Approximately 68% activity was still observed even after adding 100-fold excess of vanadate (1 μmol) to the FeMo-co synthesis reaction, while the same amount of sulfate did not inhibit the reaction.

Effect of Tetrathiomolybdate and Chloramphenicol on Molybdate-Dependent FeMo-co Synthesis. Tetrathiomolybdate is known to block the FeMo-co binding to FeMo-co-deficient dinitrogenase (34). Addition of 25 nmol of tetrathiomolybdate to the FeMo-co synthesis reaction mixture inhibited FeMo-co synthesis by 85%. Even after the addition of 25 nmol of tetrathiomolybdate to these reaction mixtures, FeMo-co-activatable dinitrogenase remained that could be activated by FeMo-co. If FeMo-co synthesis had not been affected by tetrathiomolybdate, 80–90% activity would have been observed in these assays. Thus, it seems that the reaction(s) for FeMo-co synthesis is sensitive to inhibition by tetrathiomolybdate.

Chloramphenicol, but not tetracycline, prevented molybdate-dependent restoration of nitrogenase activity in *K. pneumoniae in vivo* (35). The authors suggested that the inhibition by chloramphenicol may be a specific effect on molybdate uptake or processing by the cell. Addition of 200 μg of chloramphenicol to the FeMo-co synthesis reactions did not inhibit molybdate-dependent synthesis of FeMo-co activity.

Partial Purification and Characterization of Dinitrogenase Activated by FeMo-co Synthesized *in Vitro*. To isolate and characterize active dinitrogenase, the FeMo-co synthesis reaction (identical to the complete system reported in Table 2) was carried out with 8 ml of extract from each mutant. The ATP-generating system was omitted from the control experiment, and glucose (100 μmol) and hexokinase (25 units) were added to eliminate ATP present in the extracts. The reaction

Table 5. Effect of tungstate, vanadate, and sulfate on the molybdate-dependent synthesis of FeMo-co activity

Addition	Activity, %
None	100
Tungstate, 100 nmol	91.5
Tungstate, 1000 nmol	32.6
Vanadate, 100 nmol	92.3
Vanadate, 1000 nmol	67.6
Sulfate, 100 nmol	98.8
Sulfate, 1000 nmol	94.8
− molybdate	3.2

Acetylene reduction assays were carried out for 15 min at 30°C. Activity of 100% represents 178.2 nmol of ethylene formed per assay. Portions (0.2 ml) of UW45(W) and UW38(W) extracts contained 2.4–2.6 mg of protein. Molybdate (10 nmol) was added to all assays except the minus molybdate control.

mixture was incubated at 30°C for 45 min and was applied to anaerobic DEAE-cellulose columns (0.75 × 25 cm) as reported previously (27). Columns were washed with 1 bed volume each of 0.1 M and 0.15 M NaCl in 25 mM Tris·HCl buffer, pH 7.4. Dinitrogenase fractions were eluted with 0.3 M NaCl in 25 mM Tris·HCl buffer, pH 7.4, and analyzed for acetylene- and nitrogen-reduction activities in the presence of excess of purified dinitrogenase reductase (27). Dinitrogenase fraction (2.4 mg of protein) from the extracts incubated in the presence of molybdate and ATP/dithionite showed acetylene- and nitrogen-reduction activities of 517 and 132 nmol per assay, respectively (an acetylene-to-nitrogen reduction ratio of 3.9). These results clearly demonstrate that the inactive dinitrogenase activated by FeMo-co synthesized *in vitro* is as effective as native dinitrogenase for reduction of nitrogen. It has been suggested that the *nifV* gene product is involved in final modification of FeMo-co and that this modification of FeMo-co by the *nifV* gene product is essential for effective nitrogen fixation *in vivo* (18, 19). From our data, it seems that any such modification of FeMo-co by the *nifV* gene product occurs during the synthesis of FeMo-co *in vitro*.

Dinitrogenase fraction from the control experiment (carried out without ATP-generating system) showed less than 2% acetylene-reduction activity compared to dinitrogenase activated by FeMo-co synthesized in the presence of the ATP-generating system. Therefore, ATP is required for FeMo-co synthesis. Pienkos *et al.* (26) had reported the requirement of ATP for molybdate-dependent activation of inactive dinitrogenase in extracts of *A. vinelandii* wild-type derepressed in the presence of tungstate instead of molybdate.

Dinitrogenase fractions from both the columns were also analyzed by EPR spectroscopy (10, 11). The dinitrogenase fraction from the reaction carried out in presence of ATP/dithionite had an EPR spectrum similar to that exhibited by crystalline dinitrogenase from the wild type. Dinitrogenase from the reaction carried out in the absence of the ATP-generating system failed to show the dinitrogenase spectrum. The ratios of acetylene reduction activities and EPR spectrum peak heights at $g = 3.65$ of crystalline dinitrogenase from the wild type and the enzyme activated by FeMo-co synthesized *in vitro* were the same. These results suggest that inactive dinitrogenase activated by FeMo-co synthesized *in vitro* is very similar to native dinitrogenase.

Further Characterization of Protein Factors Involved. All of our FeMo-co synthesis experiments used at least one extract from a mutant strain of *A. vinelandii*. Attempts to replace the extract of *A. vinelandii* strain UW45 (NifB⁻) with *K. pneumoniae* NifB⁻ strains or generate FeMo-co activity by mixing *K. pneumoniae* mutant extracts were unsuccessful. Since *A. vinelandii* is very effective in scavenging molybdenum and it

synthesizes a Mo-storage protein even during growth in a medium containing ammonium (7, 29), it seemed possible that this protein was the essential factor from *A. vinelandii* extracts. However, addition of the extracts of *A. vinelandii* wild type (grown in ammonium-containing medium) to *K. pneumoniae* NifB$^-$ + NifN$^-$ or NifB$^-$ + NifE$^-$ complementation assays failed to generate any FeMo-co activity. Further, addition of the extracts of *A. vinelandii* wild type (grown in ammonium-containing medium) to *K. pneumoniae* NifB$^-$ + UW45 complementation reaction did not significantly stimulate/inhibit FeMo-co synthesis. Thus, the enzyme/factor contributed by the extracts of *A. vinelandii* mutant strains is not synthesized constitutively but its synthesis is dependent upon derepressing conditions. In *K. pneumoniae*, dinitrogenase activity can be restored by the addition of molybdate to Mo-starved cells in the absence of protein synthesis (7, 35). Our attempts to activate the inactive dinitrogenase by molybdate and ATP in extracts of Mo-starved *K. pneumoniae* wild type were unsuccessful. It is possible that some enzyme, such as the *nifQ* gene product, involved in the processing of molybdate is very labile in *K. pneumoniae* and is inactivated during preparation of extracts.

A. vinelandii mutant strain UW111 synthesizes inactive dinitrogenase that can be activated *in vitro* by the addition of FeMo-co. Extracts of strain UW111 generated Mo-dependent FeMo-co activity when mixed with extracts of strain UW45. These data suggest that mutation in strain UW111 is not in the *nifB* gene but possibly in the *nifN* or *nifE* gene. Addition of UW111 extracts to *K. pneumoniae* NifB$^-$, NifN$^-$, or NifE$^-$ extracts failed to generate Mo-dependent FeMo-co activity. Addition of UW111 extracts to NifB$^-$ + NifN$^-$ or NifB$^-$ + NifE$^-$ complementation reactions also failed to generate any FeMo-co activity. From these data it seems that *nifEN* enzyme product (possibly Mo-free FeMo-co precursor) accumulates in *A. vinelandii* strain UW45 but not in *K. pneumoniae* NifB$^-$ mutants. The reasons for inability of *K. pneumoniae* extracts to generate Mo-dependent FeMo-co activity *in vitro* should become apparent as more precise information on the reactions involved becomes available.

FeMo-co synthesis *in vitro* provides a method to assay the products of *nifB*, *nifQ*, and *nifV* genes, and possibly *nifEN* enzyme product. It may be possible to assay one or more of these gene products individually by measuring ATPase activity, since FeMo-co biosynthesis requires ATP. Once the gene products involved in FeMo-co biosynthesis are purified, it will be possible to synthesize FeMo-co by using a completely defined system.

We thank C. Felix and W. Antholine of the National Biomedical ESR Center (Milwaukee, WI) for their help in performing EPR spectroscopy. The technical assistance of Claire Knocke is greatly appreciated. We thank Gary Roberts and R. H. Burris for critical reading of the manuscript. This research was supported by the College of Agricultural and Life Sciences, University of Wisconsin–Madison, a McKnight Foundation grant to the Center for the Study of Nitrogen Fixation, and Public Health Service Grant GM22130. J.I. was the recipient of a postdoctoral fellowship from the Ministerio de Educacion y Universidades of Spain. R.A.U. was a Fellow of the Consejo Nacional de Investigaciones Cientificas y Tecnicas, Republica Argentina. P.W.L. is a Harry and Evelyn Steenbock Career Development Award Recipient. EPR studies were supported by National Institutes of Health Grant RR-01008 to the National Biomedical ESR Center.

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