JOURNAL OF BACTERIOLOGY, Jan. 1981, p. 248-256
0021-9193/81/010248-09$02.00/0

Vol. 145, No. 1

In Vitro Activation of Inactive Nitrogenase Component I with Molybdate

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When *Azotobacter vinelandii* was derepressed for nitrogenase synthesis in the presence of WO_4^{2-} rather than MoO_4^{2-}, it synthesized active component II and inactive component I of nitrogenase. This inactive component I could be activated in vitro with the iron-molybdenum cofactor or with MoO_4^{2-}. The latter reaction required adenosine 5'-triphosphate and was inhibited by adenosine 5'-diphosphate. FeMo cofactor and MoO_4^{2-} produced different levels of activation, but there was no evidence that they acted upon different species of demolybdo component I. Rather, it may be that an additional factor necessary for MoO_4^{2-}-mediated activation but not for FeMo cofactor-mediated activation was limiting. Mo was inserted into component I during both FeMo cofactor- and MoO_4^{2-} mediated activations.

The transition element molybdenum has been known to play a key role in N_2 fixation since 1930, when Bortels observed that Mo stimulates growth of *Azotobacter chroococcum* when N_2 is the nitrogen source (4). The importance of Mo in N_2 fixation was understood when nitrogenase was separated into two proteins, and one of these (component I) was found to contain Mo (5). Many of the enzymatic processes of N_2 fixation, including substrate binding and reduction, take place at sites on component I (3), and Mo has been implicated as being essential for these reactions (23).

Azotobacter vinelandii derepressed for nitrogenase synthesis in the presence of WO_4^{2-} rather than MoO_4^{2-} produces an inactive species of component I which can be activated in vitro (14, 20) by adding the iron-molybdenum cofactor (FeMo-co). Previous work showed that this inactive component I can be activated in vivo with MoO_4^{2-}, even in the absence of protein synthesis (13). This implies that an inactive species of FeMo-co is made under these conditions and that cells can insert Mo into the inactive cofactor to activate component I. Despite this, attempts to activate component I with MoO_4^{2-} or with Mo complexes other than FeMo-co in vitro have failed (14).

Inactive species of other molybdoenzymes, such as nitrate reductase (18), sulfite oxidase (11, 12), and formate dehydrogenase (18), have been obtained from a number of organisms grown in the presence of WO_4^{2-}. Two species of inactive sulfite oxidase have been isolated from rats fed WO_4^{2-}; one species contains W, and the other does not (11, 12). The W-containing species accounts for about 30% of the inactive enzyme and can be activated in vitro with MoO_4^{2-}. The W-free species can be activated by adding the molybdenum cofactor (Mo-co) (11) contained by molybdoenzymes other than component I (15).

When *Escherichia coli* is grown on media containing WO_4^{2-}, it synthesizes inactive formate dehydrogenase and nitrate reductase (18), as well as an inactive species of Mo-co that does not seem to be bound to an enzyme (1). Both inactive *E. coli* molybdoenzymes can be activated when cell suspensions are incubated with MoO_4^{2-}. However, neither MoO_4^{2-} nor Mo-co has any effect on the formate dehydrogenase and nitrate reductase activities in cell-free extracts, although ^{99}Mo is incorporated into two proteins with electrophoretic properties similar to those of formate dehydrogenase and nitrate reductase when crude extract is incubated with $^{99}MoO_4^{2-}$. The inactive cofactor obtained from WO_4^{2-}-grown *E. coli* can interact with MoO_4^{2-}- and with inactive nitrate reductase synthesized by *Neurospora crassa* mutant strain Nit-1 to form active nitrate reductase with the same properties as the nitrate reductase synthesized by wild-type *N. crassa* (1).

This paper presents a method for MoO_4^{2-}-mediated activation in vitro of inactive component I obtained from WO_4^{2-}-grown *A. vinelandii*. This reaction provides new information concerning the pathway from MoO_4^{2-} to the synthesis of component I of nitrogenase.

(A preliminary account of this work has ap-

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peared [P. T. Pienkos and W. J. Brill, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, K137, p. 168].)

MATERIALS AND METHODS

Growth of cells. *A. vinelandii* OP (6) is the wild-type strain. Mutant strains UW45, which synthesizes FeMo-co-activatable component I (14), and UW1, which does not synthesize either component I or component II (22), have been described previously. These organisms were grown in a modified Burk medium (24) containing MoO_4^{2-} and WO_4^{2-} at desired levels. Cultures were inoculated with cells that had been depleted of intracellular Mo through at least two cycles of growth in Mo-free medium containing excess NH_4^+ (400 μg of N as ammonium acetate per ml). To prevent the loss of essential micronutrients during growth in this medium, the following trace elements were added (in micrograms per liter of medium): H_3BO_3, 880; $ZnSO_4 \cdot 7H_2O$, 550; $CoCl_2 \cdot 6H_2O$, 200; $MnCl_2 \cdot 4H_2O$, 20; and $CuSO_4 \cdot 5H_2O$, 10.

Nitrogenase synthesis was derepressed by growing cells in medium containing limiting NH_4^+ (100 μg of N per ml), allowing them to exhaust all NH_4^+, and then incubating them for an additional 3.5 h. Unless otherwise stated, cells were derepressed for nitrogenase synthesis in the presence of either 1.0 μM Na_2MoO_4 or 5.0 μM Na_2WO_4. Cells used for autoradiography were grown in the absence of Mo or W and were allowed to exhaust all NH_4^+. Then, 5.5 μM $^{185}WO_4^{2-}$ (specific activity, 100 Ci/mol) or 0.2 μM $^{99}MoO_4^{2-}$ (specific activity, 750 Ci/mol) was added, and the cells were incubated for an additional 3.5 h. Because of the short half-life of ^{99}Mo, labeled cells were broken immediately after harvest and used for electrophoresis and preparation of FeMo-co.

Preparation and handling of crude extracts. Crude extracts were prepared by suspending cells in 5 volumes of 25 mM Tris-hydrochloride (pH 7.4) containing 0.3 mg of sodium dithionite per ml and then breaking them anaerobically with a French pressure cell. For one experiment, extract was prepared by osmotic shock (17). Acetylene reduction assays (21) and anaerobic column chromatography (19) have been described previously. Concentrated FeMo-co made from crystalline component I was prepared by V. K. Shah. ^{99}Mo-labeled FeMo-co was prepared from a crude extract of $^{99}MoO_4^{2-}$-grown cells in the manner described previously for the preparation of FeMo-co from component I (20). Protein was measured by the Biuret reaction (10).

Molybdenum determination. Quantitation of Mo was performed by the colorimetric method of Clark and Axley (7).

Polyacrylamide gel electrophoresis. Anaerobic polyacrylamide slab gel electrophoresis (19) was performed with an Ortec pulsed-power electrophoresis apparatus (Ortec Inc., Oak Ridge, Tenn.). Samples were prepared anaerobically by incubating 100 μl of crude extract (4 to 5 mg of protein) with 0.1 μg of DNase and 0.1 μg of RNase in 25 mM tris-hydrochloride (pH 7.4) containing 0.4 M glycerol. Various combinations of an ATP-generating system, MoO_4^{2-}, and FeMo-co were added, and the volume was brought to 0.2 ml with 25 mM Tris-hydrochloride (pH 7.4). After a 30-min incubation at room temperature, 10-μl samples (0.2 to 0.25 mg of protein) were removed for electrophoresis, and the remainder of the solution was tested for acetylene reduction activity.

Immediately after electrophoresis, the gels were frozen on a block of dry ice and placed directly against a piece of Kodak X-OMat X-ray film (Eastman Kodak, Rochester, N.Y.). The films were exposed for an appropriate length of time at −80°C and then developed. After autoradiography, the gels were stained for protein with Coomassie brilliant blue R-250 in 50% trichloroacetic acid and destained with one wash in 7% acetic acid, followed by several washes in 7% acetic acid–30% ethanol (16).

Scintillation counting. A gel containing ^{185}W was positioned over its developed autoradiograph, and the radioactive areas were cut out. The label was eluted by incubating the gel fragments in a solution containing 10 ml of scintillation fluid and 0.3 ml of Protosol (New England Nuclear Corp., Boston, Mass.) for 18 h at 37°C. Radioactivity was measured with a Packard scintillation counter.

ATP-generating system. The ATP-generating system (21) contained 8.3 mM $MgCl_2$, 4.2 mM ATP, 50 mM creatine phosphate, and 0.33 mg of creatine kinase per ml in 25 mM Tris-hydrochloride (pH 7.4). Unless otherwise stated, MoO_4^{2-}-mediated activation of component I was carried out in a 0.2-ml volume including 40 μl of this ATP-generating system. Acetylene reduction assays were performed in 1.0-ml volumes containing 0.6 ml of the ATP-generating system.

Reagents. All reagents used were of ultrapure or analytical grade and were available commercially. It was essential to use only reagents of the highest quality for growth media to minimize contamination with Mo or W. Glass-distilled water and acid-cleaned glassware were used throughout. $Na_2{}^{99}MoO_4$ was obtained from Union Carbide Corp., Tuxedo, N.Y., and $Na_2{}^{185}WO_4$ was obtained from Amersham Corp., Arlington Heights, Ill.

RESULTS

Activation of inactive component I of nitrogenase with MoO_4^{2-}. When *A. vinelandii* is derepressed for nitrogenase synthesis in the presence of WO_4^{2-}, it yields 60 to 80% of the amount of component I protein normally obtained from MoO_4^{2-}-grown cells (13). The level of component I activity in WO_4^{2-}-grown cells was 2 to 5% of the level in MoO_4^{2-}-grown cells; it is likely that this residual activity was due to trace levels of contaminating Mo in the growth medium, because *A. vinelandii* has a very high affinity for Mo. Even when cells were grown in the presence of 100 μM WO_4^{2-}, the addition of 20 nM MoO_4^{2-} caused a threefold increase in component I activity. Therefore, it appears that the residual activity was due to Mo-containing component I and that demolybdo component I is completely inactive.

Nitrogenase requires ATP for activity; thus, acetylene reduction assays are done in the presence of an ATP-generating system (9). Even though earlier unsuccessful attempts to activate inactive component I with MoO_4^{2-} employed acetylene reduction assays and an ATP-generating system (14), we could activate inactive component I by preincubating a crude extract from WO_4^{2-}-grown cells and MoO_4^{2-} with a small volume of ATP-generating system (40 μl, compared with 600 μl for the usual nitrogenase assay) before adding substrates and proceeding with the acetylene reduction assay. As Fig. 1 shows, the addition of MoO_4^{2-} and the ATP-generating system increased the nitrogenase activity sixfold compared with residual activity, and the addition of MoO_4^{2-} alone had no effect. Under these same conditions, FeMo-co-mediated activation of component I yielded activity which was about 30-fold higher than residual activity.

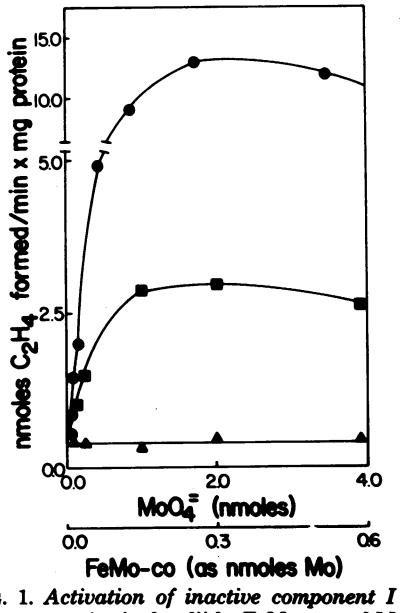

FIG. 1. *Activation of inactive component I from WO_4^{2-}-grown A. vinelandii by FeMo-co and MoO_4^{2-}. A 50-μl amount of an extract (1.8 mg of protein) from cells grown and derepressed for nitrogenase synthesis in the presence of 5.0 μM WO_4^{2-} was incubated anaerobically in an 8.5-ml serum vial with FeMo-co or Na_2MoO_4 in the presence or absence of 40 μl of the ATP-generating system; 25 mM Tris-hydrochloride (pH 7.4) was added to bring the volume to 0.2 ml. After a 30-min preincubation at room temperature, 0.2 ml of 100 mM sodium dithionite, 0.6 ml of the ATP-generating system, and 22 μmol of acetylene were added, and nitrogenase assays were performed. Symbols: ●, extract incubated with the ATP-generating system and FeMo-co; ■, extract incubated with the ATP-generating system and MoO_4^{2-}; ▲, extract incubated with MoO_4^{2-} alone.*

The level of activation by MoO_4^{2-} never exceeded 20% of the level of activation by FeMo-co, and attempts to increase the level of activation by changing the concentrations of the constituents of the ATP-generating system or of dithionite (added to all buffers and reagents to protect nitrogenase and FeMo-co from oxygen inactivation) or by adding $FeCl_3$ or $MnCl_2$ failed. Inactive component I from strain UW45 could be activated by FeMo-co, but the addition of MoO_4^{2-} even in the presence of the ATP-generating system had no effect on enzyme activity (data not shown).

ATP requirement for MoO_4^{2-}-mediated activation of component I. It was surprising to observe that preincubation of the crude extract with MoO_4^{2-} and the ATP-generating system could result in activation of inactive component I, whereas incubation of the extract, MoO_4^{2-}, and the ATP-generating system during the acetylene reduction assay had no effect on component I activity. When an extract of WO_4^{2-}-grown cells and a saturating level of MoO_4^{2-} were titrated with varying amounts of the ATP-generating system (keeping the volume constant at 0.2 ml), activation peaked when the amount of the ATP-generating system added was about 40 μl and then declined (Fig. 2). In a normal acetylene reduction assay, 0.6 ml of the ATP-generating system is used in a 1.0-ml assay; thus, the concentration of the ATP-generating system is three times higher than the optimal level for activation by MoO_4^{2-} (21). At this higher level, activation was greatly inhibited.

As Fig. 3 shows, MoO_4^{2-}-mediated activation of inactive component I also occurred in the presence of Mg-ATP, although the level of activation was somewhat lower than the maximum activity obtained by the addition of the ATP-generating system. The addition of ADP inhibited activation of component I with MoO_4^{2-} and Mg-ATP, although it had no effect on nitrogenase activity per se because it was rapidly converted to ATP by the action of the creatine kinase in the ATP-generating system that was added for the acetylene reduction assay. The inhibitory effect was caused by ADP and not Mg^{2+} because addition of 40 nmol of Mg^{2+} had no effect on MoO_4^{2-}-mediated activation in the presence of Mg-ATP. MoO_4^{2-}-mediated activation of component I was ATP specific; Mg-CTP, Mg-UTP, and Mg-GTP did not stimulate activation of component I.

Nonadditive activation by FeMo-co and MoO_4^{2-}. We showed above (Fig. 1) that the level of activation of inactive component I from WO_4^{2-}-grown cells by MoO_4^{2-} was about 20% of the level of activation by FeMo-co. The effects

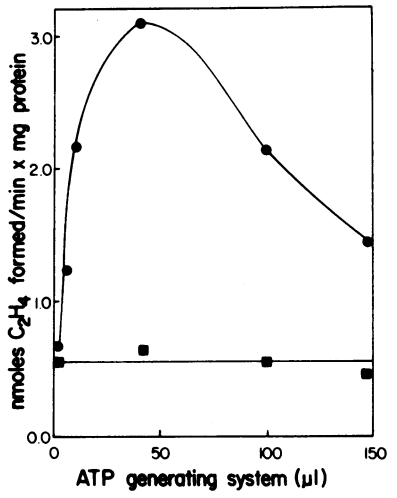

FIG. 2. *Effect of ATP-generating system on MoO_4^{2-}-mediated activation of component I. A 50-µl amount of an extract (1.8 mg of protein) from WO_4^{2-}-grown cells was preincubated anaerobically for 30 min in the presence and absence of 1.0 nmol of MoO_4^{2-} with varying levels of the ATP-generating system. The reaction volume was adjusted to 0.2 ml by adding 25 mM Tris-hydrochloride (pH 7.4). After preincubation, dithionite, the ATP-generating system, and acetylene were added for acetylene reduction assays. Symbols: ●, extract incubated with MoO_4^{2-} and the ATP-generating system; ■, extract incubated with the ATP-generating system alone.*

of MoO_4^{2-} and FeMo-co on activation of component I were not additive (Table 1), in contrast to the effects of MoO_4^{2-} and Mo-co on activation of inactive sulfite oxidase (11, 12). FeMo-co plus MoO_4^{2-} produced approximately the same level of activation as the level obtained with FeMo-co alone. In the case of inactive sulfite oxidase, the order of addition of MoO_4^{2-} and Mo-co is essential to show the additive effect. With inactive component I, the order of addition made no difference; MoO_4^{2-} plus FeMo-co never produced higher activity than FeMo-co alone.

Additional factor required. FeMo-co-mediated activation of inactive component I from extracts of mutant strain UW45 or WO_4^{2-}-grown wild type does not appear to require additional factors or enzymes (V. K. Shah, unpublished data). The first suggestion that an additional factor was required for activation by MoO_4^{2-} came from a comparison of the levels of activation produced in extracts prepared by osmotic shock and French pressure cell disruption (Table 2). Both extracts were prepared from cells obtained from the same culture. Because the concentration of proteins other than nitrogenase was higher in the French pressure cell extract than in the osmotic shock extract, it was more meaningful to express nitrogenase activity in terms of volume rather than protein concentration. Activation of inactive component I with FeMo-co produced identical levels of acetylene reduction activity in the two extracts, indicating that the concentration of nitrogenase in the extracts was the same, but a difference in the levels of MoO_4^{2-}-activated component I implied that

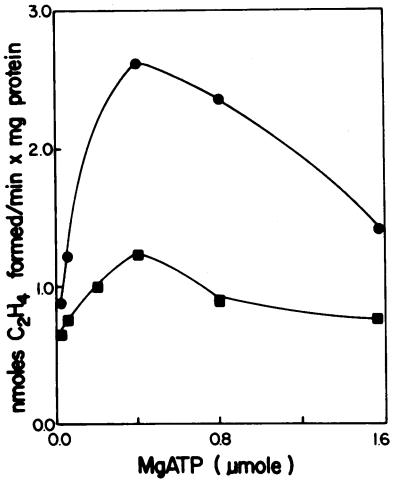

FIG. 3. *Inhibition of MoO_4^{2-}-mediated activation of component I by ADP. A 50-µl amount of extract (1.8 mg of protein) from WO_4^{2-}-grown cells was preincubated anaerobically for 30 min with 2 nmol of MoO_4^{2-} and varying levels of Mg-ATP in the presence and absence of ADP. Activation mixtures were brought to 0.2 ml with 25 mM Tris-hydrochloride (pH 7.4). After preincubation, acetylene reduction assays were performed. Symbols: ●, no ADP added to activation mixture; ■, 40 nmol of ADP added to activation mixture.*

TABLE 1. *Nonadditive effects of MoO_4^{2-} and FeMo-co*

Addition(s)	Nitrogenase sp act[a]
None	0.5
ATP-generating system	0.5
ATP-generating system + MoO_4^{2-}	2.6
ATP-generating system + FeMo-co	13.7
ATP-generating system + MoO_4^{2-} + FeMo-co	11.9

[a] Nitrogenase specific activity is expressed as nanomoles of C_2H_4 formed per minute per milligram of protein. Activations were carried out in the manner described in the legend to Fig. 1. A crude extract of WO_4^{2-}-grown cells was first titrated with MoO_4^{2-} and FeMo-co to determine the amounts necessary to produce maximum activation. Samples of extract were then incubated with the indicated combinations of 40 µl of ATP-generating system, MoO_4^{2-}, and FeMo-co. After 30-min incubations, acetylene reduction assays were performed.

some other factor(s) was limiting in the osmotic shock extract; this factor(s) was presumably lost with the cell debris.

Further evidence that another factor was required for MoO_4^{2-}-mediated activation of component I came from gel filtration chromatography. An extract from WO_4^{2-}-grown cells was fractionated on an anaerobic Sephadex G-25 column. Component II and inactive component I were collected together in a brown band immediately after the void volume. The component I in this fraction could be activated with FeMo-co but not with MoO_4^{2-} (Table 3). However, the addition of subsequent fractions to the nitrogenase fraction partially restored the capacity for MoO_4^{2-}-mediated activation of component I. Most of this unidentified factor was eluted in the first 10-ml fraction collected after the colored nitrogenase fraction, and the tail of the peak was collected in the next 10-ml fraction, but there was no activity in the third or later fractions. The addition of the later fractions plus MoO_4^{2-} and the ATP-generating system to the nitrogenase fraction had no effect on the component I activity. Similar data were obtained when the experiment was repeated with a Sephadex G-75 column (data not shown). Based on elution profiles, the factor necessary for MoO_4^{2-}-mediated activation of component I had an estimated molecular weight of 2,000 to 4,000. This factor was very labile even when maintained anaerobically, and it was necessary to test for activity immediately after the fractions were eluted from the column. This prevented further characterization of the factor.

Incorporation of ^{185}W and ^{99}Mo into component I. Cells of *A. vinelandii* were derepressed for nitrogenase synthesis in the presence of $^{185}WO_4^{2-}$ in an attempt to show that Mo in the form of MoO_4^{2-} or FeMo-co could displace W in inactive component I. A crude extract from $^{185}WO_4^{2-}$-grown cells was treated with MoO_4^{2-} and FeMo-co in the manner described above for activation, and then samples were removed for anaerobic polyacrylamide gel electrophoresis. The remainder of each sample was tested for acetylene reduction activity to be sure that activation had occurred. Figure 4A shows that a crude extract from MoO_4^{2-}-grown cells had a major band (Fig. 4A, lane b) corresponding to purified component I (lane a). Inactive component I from WO_4^{2-}-grown cells (Fig. 4A, lane c) seemed to have a slightly lower electrophoretic mobility than active component I. Treatment of an extract of $^{185}WO_4^{2-}$-grown cells with two concentrations of FeMo-co caused increases in component I activity and shifts in electrophoretic mobility (Fig. 4A, lanes d and e). Even though

TABLE 2. *Activation of component I in osmotic shock and French press extracts*

Extract	Addition	Nitrogenase activity[a]
French press	None	21.9
French press	MoO_4^{2-}	102.4
French press	FeMo-co	426.7
Osmotic shock	None	17.1
Osmotic shock	MoO_4^{2-}	47.8
Osmotic shock	FeMo-co	427.9

[a] Nitrogenase activity is expressed as nanomoles of C_2H_4 formed per minute per milliliter of crude extract. A sample of WO_4^{2}-grown cells was divided into two batches, which were broken in the same volume of 25 mM Tris-hydrochloride (pH 7.4) by the French pressure cell method and by the osmotic shock method. Extracts were treated with the ATP-generating system and with MoO_4^{2} or FeMo-co in the manner described in the legend to Fig. 1. The values reported represent the maximum levels of activation.

TABLE 3. *Reconstitution of MoO_4^{2-}-mediated activation of component I*

Prepn	Addition(s)	Nitrogenase sp act[a]
Crude extract	None	0.8
Crude extract	FeMo-co	10.5
Crude extract	ATP-generating system + MoO_4^{2-}	4.0
Nitrogenase fraction[b]	None	0.5
Nitrogenase fraction	FeMo-co	6.1
Nitrogenase fraction	ATP-generating system + MoO_4^{2-}	0.6
Nitrogenase fraction	Mo insertion factor	0.5
Nitrogenase fraction	Mo insertion factor + ATP-generating system + MoO_4^{2-}	1.4

[a] Nitrogenase specific activity is expressed as nanomoles of C_2H_4 formed per minute per milligram of protein.

[b] A 2-ml amount of crude extract from WO_4^{2-}-grown cells was loaded onto an anaerobic Sephadex G-25 column (10 by 500 mm) run in 25 mM Tris-hydrochloride (pH 7.4), and fractions were collected anaerobically. The nitrogenase fraction was collected (7 ml) as a brown band eluted immediately after the void volume. Subsequent colorless fractions were collected in 10-ml volumes and tested for Mo insertion factor activity by incubating 100 μl of each colorless fraction with 50 μl of the nitrogenase fraction, the ATP-generating system, and MoO_4^{2-}, as described in the legend to Fig. 1. The value reported is for the first colorless fraction, which contained the peak Mo insertion factor activity. The second colorless fraction also contained activity, but the subsequent fractions had none.

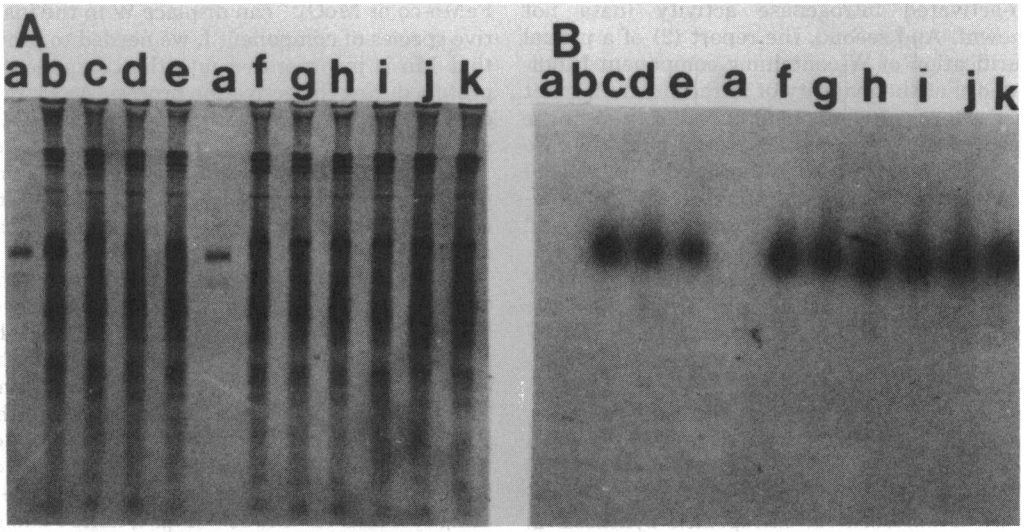

FIG. 4. *Polyacrylamide gel electrophoresis and autoradiography of an extract from $^{185}WO_4^{2-}$-grown cells. Crude extract was prepared from cells which had been grown in the presence of 5.5 μM $^{185}WO_4^{2-}$. Samples of extract were prepared anaerobically for electrophoresis by incubating them with glycerol, RNase, DNase, and different combinations of the ATP-generating system, MoO_4^{2-}, and FeMo-co. After 30 min of incubation at room temperature, 10 μl of each sample (approximately 0.3 mg of protein) was loaded onto the gel, and the remainder of each sample was assayed for acetylene reduction activity to insure that activation had occurred. After electrophoresis was completed, the gel was frozen against a flat piece of dry ice and placed against X-ray film for 60 h. After the film was developed, the gel was thawed and stained. (A) Stained polyacrylamide gel. (B) Autoradiograph. Lanes a, Pure component I; lanes b, extract from MoO_4^{2-}-grown cells; lanes c, extract from $^{185}WO_4^{2-}$-grown cells plus ATP-generating system plus FeMo-co (0.14 nmol of Mo); lanes d, extract from $^{185}WO_4^{2-}$-grown cells plus ATP-generating system plus FeMo-co (0.55 nmol of Mo); lanes e, extract from $^{185}WO_4^{2-}$-grown cells plus ATP-generating system plus FeMo-co (1.10 nmol of Mo); lanes f, untreated extract from $^{185}WO_4^{2-}$-grown cells; lanes g, extract from $^{185}WO_4^{2-}$-grown cells plus ATP-generating system; lanes h, extract from $^{185}WO_4^{2-}$-grown cells plus 5.0 nmol of MoO_4^{2-}; lanes i, extract from $^{185}WO_4^{2-}$-grown cells plus ATP-generating system plus 1.0 nmol of MoO_4^{2-}; lanes j, extract from $^{185}WO_4^{2-}$-grown cells plus ATP-generating system plus 5.0 nmol of MoO_4^{2-}; lanes k, extract from $^{185}WO_4^{2-}$-grown cells plus ATP-generating system plus 10.0 nmol of MoO_4^{2-}.*

the sample in Fig. 4A, lane e, was fully activated, the component I band shifted position only to a point intermediate between active component I and inactive component I. Treatment of crude extract with the ATP-generating system alone (Fig. 4A, lane g), with MoO_4^{2-} alone (lane h), or with the ATP-generating system and MoO_4^{2-} (lanes i through k) had no effect on the electrophoretic mobility of component I, although the samples receiving both the ATP-generating system and MoO_4^{2-} demonstrated the expected levels of activation. Similar gels run with extracts of unlabeled WO_4^{2-}-grown cells produced identical results. The component I in extracts of mutant strain UW45 could also be activated with FeMo-co. The component I in an untreated extract of strain UW45 had the same electrophoretic mobility as the component I of untreated WO_4^{2-}-grown wild-type cells, and treatment with FeMo-co caused the same shift in the electrophoretic mobility of the component I of strain UW45 as it did with the component I of WO_4^{2-}-grown cells. Attempts to enhance the differences in electrophoretic mobility by changing the conditions of electrophoresis either had no effect or lowered the resolution of the protein bands.

The autoradiograph produced by the ^{185}W in the gel (Fig. 4B) gave very surprising results. It appeared to confirm an earlier report that inactive component I from WO_4^{2-}-grown *A. vinelandii* contains W (2). However, neither treatment with FeMo-co nor treatment with MoO_4^{2-} (which produced the expected levels of activation) had any effect on the amount of ^{185}W in the component I regions of the gel. We were skeptical of these results for two reasons. First, when the radioactive areas of the gel were cut out and the ^{185}W was eluted and counted, the amount of ^{185}W present was one order of magnitude higher than was expected from the component I concentration estimated by the FeMo-

co-activated nitrogenase activity (data not shown). And second, the report (2) of a partial purification of W-containing component I indicated that the majority of intracellular W is not in the form of component I; rather, it is in the form of the W-containing analog of the Mo storage protein which is synthesized in cells of *A. vinelandii* that are both repressed and derepressed for nitrogenase synthesis (Pienkos and Brill, J. Bacteriol., in press).

Therefore, it seemed possible that the ^{185}W bands on the autoradiograph corresponded not to inactive component I, but rather to ^{185}W bound to the storage protein. To test this, wild-type cells were grown in the presence of $^{185}WO_4^{2-}$ and excess NH_4^+ (to repress nitrogenase synthesis), and cells of the mutant strains UW1 (which does not synthesize either component) and UW45 (which has a defect in FeMo-co synthesis) were derepressed for nitrogenase synthesis in the presence of $^{185}WO_4^{2-}$. Extracts of these cells were run on a gel, and the autoradiographic patterns were compared with the pattern of an extract of wild-type cells derepressed for nitrogenase synthesis in the presence of $^{185}WO_4^{2-}$. All of these extracts produced ^{185}W bands in the region of component I on the gel (Fig. 5), indicating that the ^{185}W bands were not due to component I.

Because we were unable to show that Mo as FeMo-co or MoO_4^{2-} can displace W in the inactive species of component I, we needed to prove that Mo is incorporated into the component I protein during the activation process. An extract of WO_4^{2-}-grown cells was treated with $^{99}MoO_4^{2-}$ or $Fe^{99}Mo$-co prepared from an extract of $^{99}MoO_4^{2-}$-grown cells. Again, electrophoresis was performed with samples of treated and untreated extracts, and the gel was used to expose an autoradiograph.

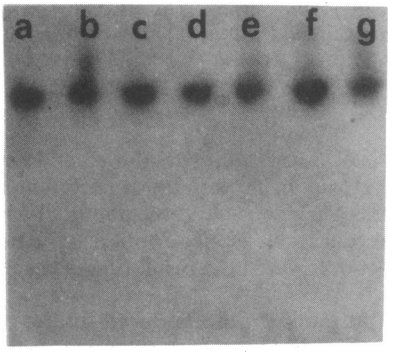

FIG. 5. *Presence of ^{185}W in extracts of cells lacking component I. Crude extracts were prepared anaerobically from cells repressed or derepressed for nitrogenase synthesis in the presence of 5.5 μM $^{185}WO_4^{2-}$. Samples of extracts were prepared, and electrophoresis and autoradiography were carried out as described in the legend to Fig. 4. Lane a, Extract from wild type repressed by NH_4^+ in $^{185}WO_4^{2-}$; lane b, extract from wild type derepressed in $^{185}WO_4^{2-}$; lane c, extract from wild type derepressed in $^{185}WO_4^{2-}$ plus FeMo-co; lane d, extract from UW1 derepressed in $^{185}WO_4^{2-}$; lane e, extract from UW1 derepressed in $^{185}WO_4^{2-}$ plus FeMo-co; lane f, extract from UW45 derepressed in $^{185}WO_4^{2-}$; lane g, extract from UW45 derepressed in $^{185}WO_4^{2-}$ plus FeMo-co.*

The autoradiograph is shown in Fig. 6. The crude extract from $^{99}MoO_4^{2-}$-grown cells (Fig. 6, lanes a) and purified component I (lanes b) provided reference markers on the gel and the autoradiograph. $Fe^{99}Mo$-co (Fig. 6, lanes j) and $^{99}MoO_4^{2-}$ (lanes d) did not produce spots on the autoradiograph because they were eluted out of the gel during electrophoresis. An extract of WO_4^{2-}-grown cells treated with the ATP-generating system and $^{99}MoO_4^{2-}$ (Fig. 6, lanes f and g) or with $Fe^{99}Mo$-co (lanes h and i) produced ^{99}Mo bands in the component I region of the gel. The incorporation of ^{99}Mo bands into component I was the result of the activation process since the addition of $^{99}MoO_4^{2-}$ to an extract of WO_4^{2-}-grown cells in the absence of the ATP-generating system had no effect either on component I activity or on the incorporation of ^{99}Mo into component I protein (Fig. 6, lanes e). Incorporation of ^{99}Mo into component I protein was proportional to the level of activation, as judged by the intensity of the ^{99}Mo bands on the autoradiograph. Doubling the concentration of $^{99}MoO_4^{2-}$ or $Fe^{99}Mo$-co resulted in a doubling of the activation of component I and an approximate doubling of the intensity of the ^{99}Mo bands.

DISCUSSION

When *A. vinelandii* is derepressed for nitrogenase synthesis in the presence of WO_4^{2-} rather than MoO_4^{2-}, it synthesizes inactive component I which can be activated in vitro by FeMo-co (14, 20) or by MoO_4^{2-}. The latter reaction has a specific requirement for ATP and is inhibited by ADP. It is not yet known whether ATP is necessary for an energy-requiring step in Mo processing or whether component I must interact with a component II-ATP complex (25) for activation to occur. Spectroscopic evidence has indicated that the Mo in component I is not bound to oxo ligands (8), and so it is clear that MoO_4^{2-} must be processed and Mo must be inserted into a demolybdo species of FeMo-co to activate component I.

Activation of inactive component I is similar to activation of inactive sulfite oxidase obtained from rats fed WO_4^{2-} (11, 12) in that the level of activation produced by MoO_4^{2-} is consistently

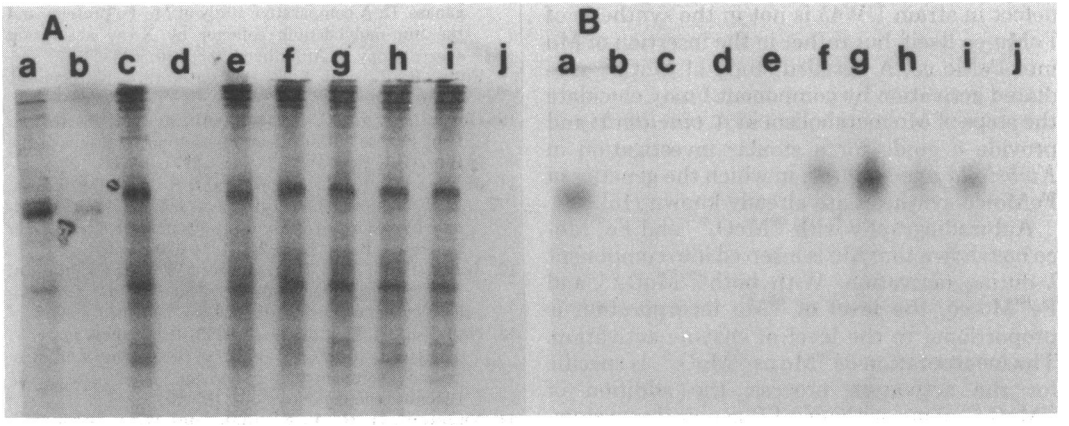

FIG. 6. *Incorporation of ^{99}Mo into inactive component I. Crude $Fe^{99}Mo$-co was prepared by acidification and N-methylformamide extraction of a crude extract from cells derepressed for nitrogenase synthesis in the presence of 0.2 μM $^{99}MoO_4^{2-}$ (750 μCi/mol). Samples of extract were prepared, and electrophoresis and autoradiography were carried out as described in the legend to Fig. 4, except that the autoradiograph was exposed for 7 days. (A) Stained polyacrylamide gel. (B) Autoradiograph of the same gel. Lanes a, Extract from $^{99}MoO_4^{2-}$-grown cells; lanes b, pure component I; lanes c, untreated extract from WO_4^{2-}-grown cells; lanes d, 0.5 nmol of $^{99}MoO_4^{2-}$ (0.3 μCi); lanes e, extract from WO_4^{2-}-grown cells plus 0.5 nmol of $^{99}MoO_4^{2-}$ (0.3 μCi); lanes f, extract from WO_4^{2-}-grown cells plus ATP-generating system plus 0.5 nmol of $^{99}MoO_4^{2-}$ (0.3 μCi); lanes g, extract from WO_4^{2-}-grown cells plus ATP-generating system plus 1.0 nmol of $^{99}MoO_4^{2-}$ (0.6 μCi); lanes h, extract from WO_4^{2-}-grown cells plus 5 μl of $Fe^{99}Mo$-co (approximately 0.2 μCi); lanes i, extract from WO_4^{2-}-grown cells plus 10 μl of $Fe^{99}Mo$-co (approximately 0.4 μCi); lanes j, 5 μl of $Fe^{99}Mo$-co (approximately 0.2 μCi).*

lower than the level of activation produced by the cofactor (FeMo-co for component I, Mo-co for sulfite oxidase). With demolybdo sulfite oxidase, Mo-co and MoO_4^{2-} produce an additive effect, and it has been shown that two separate species exist—one that can be activated only by Mo-co and one that can be activated only by MoO_4^{2-}. However, the addition of both FeMo-co and MoO_4^{2-} to a crude extract of WO_4^{2-}-grown cells of *A. vinelandii* does not have greater effect on component I activity than the addition of FeMo-co alone. Either inactive component I exists as only one activatable species, or FeMo-co can activate both species and MoO_4^{2-} can activate only one.

A different explanation for the two levels of activation produced by MoO_4^{2-} and FeMo-co may lie in the limitation of a factor necessary for MoO_4^{2-}-mediated activation but not for FeMo-co-mediated activation. Such a factor can be separated from component I by removing membrane proteins with centrifugation of an osmotic shock extract or by gel filtration chromatography of a French pressure cell extract. The latter technique removes a low-molecular-weight factor that may be required in stoichiometric amounts with component I to allow complete activation. The size and lability of this factor suggest the possibility that it may be a demolybdo FeMo-co precursor which may interact with Mo to produce active FeMo-co, thereby activating component I. This may be similar to the free species of Mo-co found in *E. coli* (1).

It has been reported that inactive component I from WO_4^{2-}-grown *A. vinelandii* contains W (2), and our results with polyacrylamide gel electrophoresis neither confirmed nor refuted this since the component I region of the gel was obscured by the presence of another W-containing molecule with an electrophoretic mobility similar to that of component I. Because this W-containing compound contained one order of magnitude more W than would be expected for component I, it is likely that it is the W-containing analog of the Mo storage protein (Pienkos and Brill, in press). Further work will be required to separate component I from the storage protein and prove conclusively that it contains W which can be displaced by activation with MoO_4^{2-} or FeMo-co. Unfortunately, the W analog of component I is very labile.

An unexpected result of the electrophoresis of the extracts of WO_4^{2-}-grown cells was the change in electrophoretic mobility brought on by FeMo-co but not by MoO_4^{2-}. We do not yet have an adequate explanation for this phenomenon, but it may supply a clue for a detailed study of the activation process. The observation that inactive component I from mutant strain UW45 has electrophoretic properties identical to those of the component I from WO_4^{2-}-grown wild-type cells is an interesting one. This may indicate that the

defect in strain UW45 is not in the synthesis of FeMo-co itself, but rather in the insertion of Mo into FeMo-co. A detailed study of MoO_4^{2-}-mediated activation by component I may elucidate the steps of Mo metabolism in *A. vinelandii* and provide a guide for a similar investigation in *Klebsiella pneumoniae*, in which the genetics of FeMo-co synthesis are already known (16).

Autoradiography with $^{99}MoO_4^{2-}$ and Fe^{99}Mo-co has shown that Mo is inserted into component I during activation. With both $^{99}MoO_4^{2-}$ and Fe^{99}Mo-co, the level of ^{99}Mo incorporation is proportional to the level of enzyme activation. The incorporation of ^{99}Mo as $^{99}MoO_4^{2-}$ is specific for the activation process; the addition of $^{99}MoO_4^{2-}$ alone, with no ATP-generating system, results in neither Mo incorporation nor a change in enzyme activity.

ACKNOWLEDGMENTS

This research was supported by the College of Agricultural and Life Sciences, University of Wisconsin, Madison, and by Public Health Service grant GM22130 from the National Institute of General Medical Sciences.

We thank V. K. Shah for supplying FeMo-co and purified component I and for his valuable suggestions.

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