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Molybdenum Accumulation and Storage in *Klebsiella pneumoniae* and *Azotobacter vinelandii*

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In *Klebsiella pneumoniae*, Mo accumulation appeared to be coregulated with nitrogenase synthesis. O_2 and NH_4^+, which repressed nitrogenase synthesis, also prevented Mo accumulation. In *Azotobacter vinelandii*, Mo accumulation did not appear to be regulated. Mo was accumulated to levels much higher than those seen in *K. pneumoniae* even when nitrogenase synthesis was repressed. Accumulated Mo was bound mainly to a Mo storage protein, and it could act as a supply for the Mo needed in component I synthesis when extracellular Mo had been exhausted. When *A. vinelandii* was grown in the presence of WO_4^{2-} rather than MoO_4^{2-}, it synthesized a W-containing analog of the Mo storage protein. The Mo storage protein was purified from both NH_4^+ and N_2-grown cells of *A. vinelandii* and found to be a tetramer of two pairs of different subunits binding a minimum of 15 atoms of Mo per tetramer.

Nitrogenase, the enzyme which catalyzes the reduction of N_2 to NH_3, is composed of two dissociable proteins, components I and II. Component I contains the transition element molybdenum, which is thought to be responsible for substrate binding and reduction (24). A cofactor (FeMo-co), containing Mo, Fe, and acid-labile S, has been isolated from component I (21), and there is evidence that it is the active site of nitrogenase (7, 17, 22). FeMo-co is distinct from a similar cofactor (Mo-co) found in nitrate reductase, xanthine oxidase, and other molybdoenzymes (15).

In addition to its catalytic role, Mo seems to control synthesis of nitrogenase in several N_2-fixing organisms (2, 6, 14). However, little is known about the metabolic pathway from extracellular Mo to the synthesis of active component I. In *Clostridium pasteurianum*, Mo is sequestered by a Mo-binding protein (13). Although the role of this protein is not known, it may serve as a Mo storage mechanism or as a precursor of component I.

Species of *Azotobacter* have a high affinity for Mo and an ability to accumulate high intracellular levels of Mo (11). This high affinity is taken advantage of in the use of *A. paspali* as a biological assay for available Mo in soil samples (9).

This work represents a comparative study of the regulation and capacity for Mo accumulation in *Klebsiella pneumoniae* and *A. vinelandii*. Preliminary accounts of this work have appeared (P. T. Pienkos and W. J. Brill, Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, K165, p. 164; P. T. Pienkos and W. J. Brill, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, K77, p. 199).

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MATERIALS AND METHODS

Growth of organisms. *A. vinelandii* OP (4) was grown at 30°C with vigorous aeration in modified Burk medium (25). Cultures were inoculated with cells that had been depleted of intracellular Mo through at least two cycles of growth in Mo-free medium containing ammonium acetate (400 μg of N/ml). Mo accumulation studies were performed by growing cells in 200-ml batches in 1-liter side-arm flasks with fluted bottoms with medium containing NH_4^+ and lacking MoO_4^{2-}. These cells were harvested by centrifugation and suspended in fresh medium in the presence or absence of NH_4^+ with various levels of MoO_4^{2-} or WO_4^{2-}. At selected intervals, 1.0-ml samples were removed for whole-cell acetylene reduction assays (23), 20.0-ml samples were removed, and the cells were harvested, washed with 25 mM Tris-hydrochloride, pH 7.4 (which had been sparged with Ar), and suspended in glass-distilled water for analysis of Mo or W. To obtain enough cell material for purification of the Mo storage protein, cells were grown with vigorous sparging in 15 liters of medium in 5-gallon (ca. 19-liter) vessels.

K. pneumoniae M5a1 was grown in medium described by Yoch and Pengra (26) anaerobically at 30°C. Because this organism does not accumulate measurable levels of Mo when grown on NH_4^+, it was unnecessary to starve cells for Mo before inoculating experimental cultures. Mo accumulation studies with *K. pneumoniae* were identical to those with *A. vinelandii* except that *K. pneumoniae* was grown in 250-ml bottles, and 1.5 h after suspension of cells into fresh medium, L-serine was added to a level of 50 μg/ml to shorten the lag period before appearance of nitrogen-

ase activity (19). Also, 50-ml samples of cultures were removed to provide cells for Mo determination.

Cell densities were measured by viable cell counts or with a Klett-Summerson colorimeter.

Quantitation of Mo, W, and Fe. Mo analysis of whole-cell suspensions or protein solutions was performed with a Perkin-Elmer atomic absorption spectrophotometer equipped with a graphite furnace. Colorimetric assays were used to determine W (5) and Fe (3).

Preparation and handling of crude extracts. Crude extracts were prepared anaerobically by the osmotic shock technique (18). Acetylene reduction assays (23), anaerobic column chromatography (20), and in vitro activation of inactive component I from *A. vinelandii* mutant strain UW45 and of inactive nitrate reductase from *Neurospora crassa* mutant strain Nit-1 (15) have been described. Protein was quantitated by the biuret reaction (10). DEAE-cellulose columns were washed with linear gradients of NaCl in 25 mM Tris-hydrochloride, pH 7.4, and the fractions were analyzed for NaCl concentration with a conductivity meter.

Polyacrylamide gel electrophoresis. The technique for performing nondenaturing slab gel electrophoresis has been described (20). Sodium dodecyl sulfate (SDS)-gel electrophoresis used a 3% polyacrylamide stacking gel and a 10% polyacrylamide separating gel (12).

RESULTS

Mo accumulation in *A. vinelandii* and *K. pneumoniae*. Early attempts to elucidate the role of Mo in N_2 fixation led to the observation that Mo is accumulated in *A. vinelandii* regardless of the nitrogen source (11). This implies that Mo accumulation in this organism is not coregulated with nitrogenase synthesis as it appears to be in *C. pasteurianum* (8). We compared the levels of intracellular Mo in cells that had been derepressed for nitrogenase synthesis with those of repressed cells. NH_4^+-grown, Mo-starved cells of *A. vinelandii* were suspended in media containing increasing concentrations of MoO_4^{2-} in the presence and absence of NH_4^+. After 3 h of incubation, samples of these cultures were removed for whole-cell nitrogenase assays and Mo analysis. Nitrogenase activity was detectable only in cells suspended in media lacking NH_4^+ but containing MoO_4^{2-}, and Mo accumulation occurred in both the presence and absence of NH_4^+ (Fig. 1). A concentration of 0.2 μM MoO_4^{2-} was sufficient for full expression of nitrogenase activity, but Mo accumulation increased far beyond that level. Raising the concentration of MoO_4^{2-} in the growth medium to 1.0 mM led to an intracellular Mo concentration 25 times higher than was required for the synthesis of a full complement of component I.

When the same experiment was done with *K. pneumoniae*, two differences were immediately

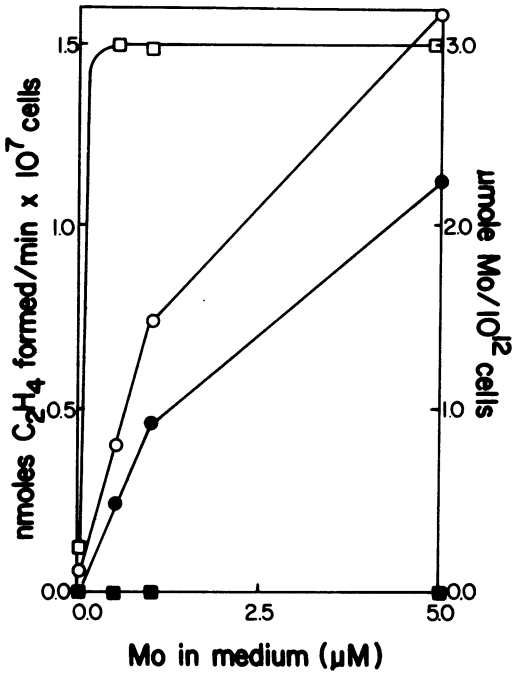

FIG. 1. *Mo accumulation and nitrogenase activity in A. vinelandii incubated with increasing levels of MoO_4^{2-}. Mo-starved cells were grown to late log phase in medium lacking Mo but containing NH_4^+. The cells were collected by centrifugation and suspended in media containing increasing levels of MoO_4^{2-} in the presence or absence of NH_4^+. After 3 h of vigorous shaking at 30°C, 1.0-ml culture samples were removed for acetylene reduction assays and 20.0-ml samples were centrifuged; the pellets were washed with 25 mM Tris-hydrochloride, pH 7.4, centrifuged, and suspended in 1.0 ml of glass-distilled water for Mo analysis by atomic absorption spectroscopy. Symbols: ●, Mo accumulation in media with NH_4^+; ○, Mo accumulation in media without NH_4^+; ■, nitrogenase activity in media with NH_4^+; □, nitrogenase activity in media without NH_4^+.*

evident (Fig. 2). First, Mo accumulation was observed only in cells that were suspended in NH_4^+-free media. Second, the level of intracellular Mo was much lower in this organism than in *A. vinelandii*. Like *A. vinelandii*, *K. pneumoniae* did not exhibit nitrogenase activity in the presence of NH_4^+ or when starved for MoO_4^{2-}.

Another method for correlating the control of Mo accumulation with the control of nitrogenase synthesis was to compare the time courses of these two processes. Mo-starved cells were suspended in medium containing 0.2 μM MoO_4^{2-} in the presence and absence of NH_4^+. At intervals, samples were removed for nitrogenase assays and Mo quantitation. Figure 3 shows the time

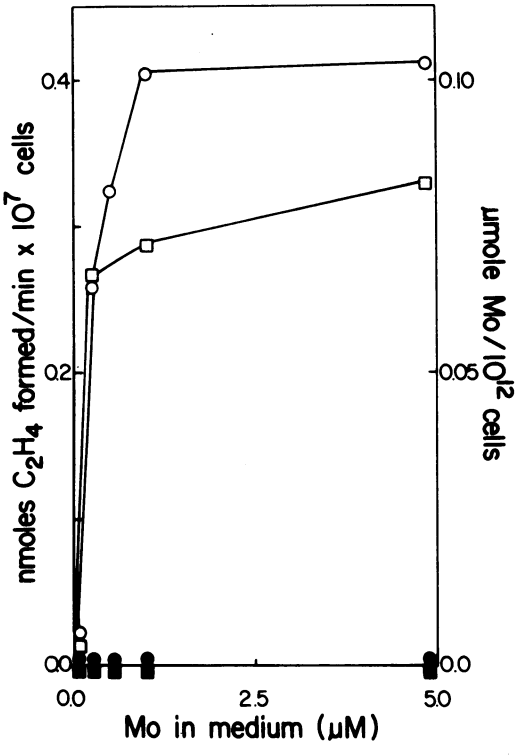

FIG. 2. *Mo accumulation and nitrogenase activity in K. pneumoniae incubated with increasing levels of MoO_4^{2-}. Cells were grown to late log phase in Mo-free medium containing NH_4^+ and then collected by centrifugation and suspended in media containing increasing levels of MoO_4^{2-} in the presence or absence of NH_4^+. After being incubated anaerobically at 30°C for 1.5 h, 1.0 ml of 1% serine was added to each 200-ml culture, and the cultures were incubated for an additional 4.5 h. At the end of this incubation, 1.0-ml culture samples were removed for acetylene reduction assays, and 50-ml samples of cultures were prepared for atomic absorption spectroscopy as described in the legend of Fig. 1. Symbols are as in Fig. 1.*

course of development of nitrogenase activity and Mo accumulation in *A. vinelandii*. Mo accumulation was very rapid, having begun before cells would be harvested in the centrifuge. At 0.2 μM MoO_4^{2-} in the medium, accumulation went to completion within 1 h, and the extracellular MoO_4^{2-} was exhausted a full hour before the first appearance of nitrogenase activity. Cells suspended in medium containing NH_4^+ demonstrated an Mo accumulation pattern almost identical to that of NH_4^+-starved cells, but no nitrogenase activity was observed.

When Mo-starved cells of *K. pneumoniae* were transferred to medium containing 0.2 μM MoO_4^{2-}, only the cells in NH_4^+-free medium accumulated detectable levels of Mo (Fig. 4). Again, it was observed that the level of Mo accumulation was lower in this organism than in *A. vinelandii*; only 25% of the extracellular MoO_4^{2-} was accumulated by *K. pneumoniae* compared with 100% for *A. vinelandii*. Mo accumulation was not rapid in *K. pneumoniae*; rather, it closely followed the development of nitrogenase activity.

K. pneumoniae is a facultative aerobe, but it can only fix N_2 anaerobically (19). We examined Mo accumulation under aerobic and anaerobic conditions. Oxygen not only repressed nitrogenase synthesis, but also completely inhibited Mo accumulation (data not shown). Adding chloramphenicol to inhibit protein synthesis had a similar effect on *K. pneumoniae* (Table 1). Chloramphenicol-treated cells were unable to synthesize nitrogenase and unable to accumulate Mo. When cells of *A. vinelandii* were treated with chloramphenicol, however, synthesis of nitrogenase was completely inhibited but Mo accumulation was unaffected. In fact, the intracellular Mo concentration was even higher in the

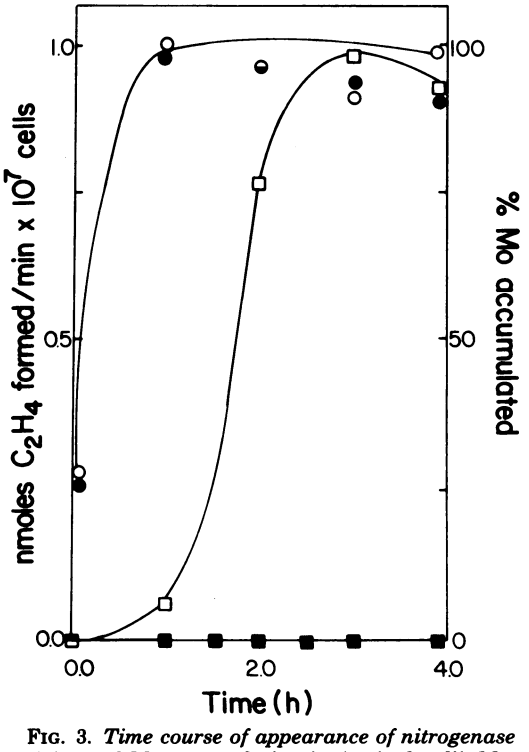

FIG. 3. *Time course of appearance of nitrogenase activity and Mo accumulation in A. vinelandii. Mo-starved, NH_4^+-grown cells were suspended in media containing 0.2 μM MoO_4^{2-} in the presence or absence of NH_4^+. At intervals, samples were taken for nitrogenase activity and Mo determination as described in the legend of Figure 1. Symbols are as in Fig. 1.*

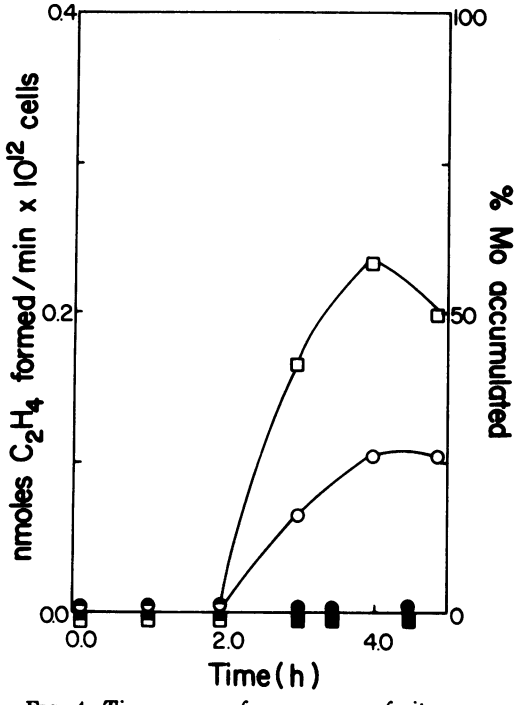

FIG. 4. *Time course of appearance of nitrogenase activity and Mo accumulation in K. pneumoniae. Mo-starved, NH_4^+-grown cells were suspended in media containing 0.2 μM MoO_4^{2-} in the presence or absence of NH_4^+. The cultures were treated as described in the legend of Fig. 2, and samples were taken at intervals and assayed for nitrogenase activity and Mo content. Symbols are as in Fig. 1.*

treated cells than in the control cells because both cultures had exhausted the extracellular MoO_4^{2-}, but chloramphenicol stopped cell division so that the Mo was divided among fewer cells.

Mo storage protein. The ability of *A. vinelandii* to accumulate high levels of Mo suggested that this Mo was being stored and could be used for synthesis of component I in the event of exhaustion of extracellular Mo. This hypothesis was tested by growing cells in 200-ml cultures in media containing NH_4^+ in the presence and absence of 1.0 μM MoO_4^{2-}. The MoO_4^{2-}-grown cells were resuspended in NH_4^+-free, MoO_4^{2-}-free medium, and the MoO_4^{2-}-starved cells were divided into two batches and suspended into NH_4^+-free media in the presence and absence of MoO_4^{2-}. Samples were taken periodically and tested for acetylene reduction activity in vivo. The cells that went from Mo-free to Mo-free medium were unable to synthesize active nitrogenase, but the cells that went from medium containing MoO_4^{2-} (and had therefore accumulated intracellular Mo) to Mo-free medium demonstrated as much nitrogenase activity as did the cells transferred from Mo-free medium to medium containing MoO_4^{2-} (data not shown).

Crude extracts were prepared from cells that had been grown on various levels of MoO_4^{2-} in the presence and absence of NH_4^+, and these extracts were fractionated on anaerobic DEAE-cellulose columns. Figure 5 shows the Mo elution profiles of the fractionation of extracts of cells grown in the presence of 0.1, 0.2, and 50.0 μM MoO_4^{2-}. All of the Mo from cells grown in media containing NH_4^+ was eluted in a single peak at an NaCl concentration of 0.15 M. Two Mo peaks were detected from cells grown on N_2 as nitrogen source. One peak, eluted with 0.2 M NaCl, corresponded to component I, and it was the only Mo detected from cells grown in the limiting MoO_4^{2-} concentration of 0.1 μM. When the concentration of MoO_4^{2-} in the growth medium was increased, a second Mo peak appeared at the same NaCl concentration as the stored Mo found in NH_4^+-grown cells. The increase in extracellular MoO_4^{2-} above 0.1 μM had no effect either on the level of Mo found in the component I peak or on the level of nitrogenase activity. When similar experiments were done with *K. pneumoniae*, the only Mo peak eluted corresponded to component I (S. Klevickis, unpublished data). This agreed with the observation that *K. pneumoniae* accumulates much less Mo than *A. vinelandii* and is evidence that this organism has no Mo storage capabilities.

The DEAE-cellulose fractionation of *A. vinelandii* extracts suggested that both NH_4^+- and N_2-grown cells store excess Mo in the same form, but it was not clear if the stored Mo was in the form of MoO_4^{2-} or in a higher-molecular-weight form such as an inorganic polymer or molybdoprotein. This question was answered by fraction-

TABLE 1. *Effects of chloramphenicol on Mo accumulation*[a]

Organism	Chloramphenicol	Nitrogenase sp act[b]	Mo (μmol/10^{12} cells)
A. vinelandii	−	0.59	0.59
A. vinelandii	+	0.00	0.71
K. pneumoniae	−	0.27	0.09
K. pneumoniae	+	0.00	0.00

[a] Mo-starved NH_4^+-grown cells of *A. vinelandii* and *K. pneumoniae* were suspended in media containing 0.2 μM MoO_4^2 and lacking NH_4^+ in the presence and absence of 100 μg of chloramphenicol per ml. After normal derepression of nitrogenase synthesis, samples were taken for nitrogenase assays and Mo determinations.

[b] Nanomoles of C_2H_4 formed per minute × 10^7 cells.

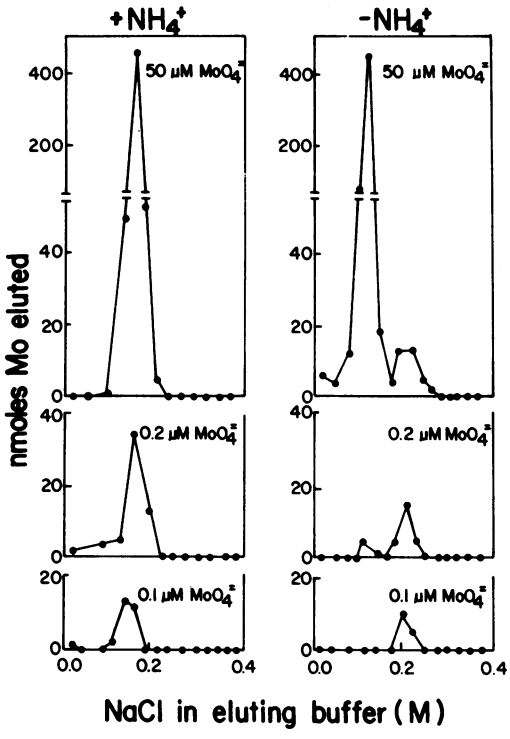

FIG. 5. *Mo elution profiles from DEAE-cellulose columns. Cultures of A. vinelandii were grown to late log phase in the presence of 0.1, 0.2, and 50.0 μM MoO_4^{2-} with NH_4^+ or N_2 as the nitrogen source. The cells were harvested by centrifugation, washed with cold, 25 mM Tris-hydrochloride, pH 7.4, which had been sparged with Ar, and then broken anaerobically by osmotic shock. Samples of each extract (200 mg of protein) were fractionated anaerobically on DEAE-cellulose columns (17 by 140 mm) eluted with NaCl gradients made with 50 ml mM Tris-hydrochloride and 50 ml of 0.5 NaCl in 25 mM Tris-hydrochloride. Five-milliliter fractions were collected and analyzed for Mo by atomic absorption.*

ation of crude extract of NH_4^+-grown cells on an anaerobic Sephadex G-75 column. This column easily distinguished the stored Mo present in the crude extract from MoO_4^{2-} (Fig. 6) and indicated that Mo is stored as a molecule of at least 40,000 daltons. A sample of *A. vinelandii* extract was then treated with protease and fractionated on the same Sephadex G-75 column. In the protease-treated extract, the stored Mo was broken down and eluted as a low-molecular-weight compound.

Samples of stored Mo collected from a DEAE-cellulose column were tested for FeMo-co and Mo-co activity by determining if they could activate the inactive component I obtained from *A. vinelandii* mutant strain UW45 or the inactive nitrate reductase obtained from *N. crassa* mutant strain Nit-1 (15). Neither untreated samples nor samples mixed with dilute HCl could serve as a source of either molybdenum cofactor.

Tungsten storage in *A. vinelandii*. Substitution of W for Mo has been performed frequently in efforts to elucidate the role of Mo in molybdoenzymes (16). A W-containing species of component I was partially purified from WO_4^{2-}-grown cells of *A. vinelandii*, and it was observed that the component I contained only a small proportion of the total intracellular W (1). This suggested that *A. vinelandii* can accumulate W just as it does Mo. Extracts of WO_4^{2-}-grown cells were fractionated in the same manner as the extracts of MoO_4^{2-}-grown cells to de-

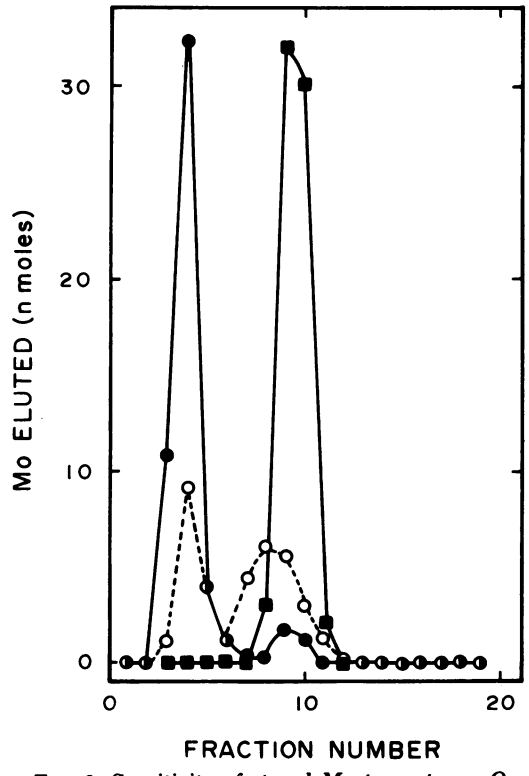

FIG. 6. *Sensitivity of stored Mo to protease. Osmotic shock extract of cells grown in the presence of NH_4^+ and 2.0 μM MoO_4^{2-} containing 26.3 mg of protein per ml was divided into two samples. One sample received protease at 0.5 U/ml; the other sample was untreated. Both samples were incubated anaerobically at 30°C for 12 h, and then 1.0-ml samples were fractionated on a Sephadex G-75 column (17 by 140 mm) which was washed anaerobically with Tris-hydrochloride at 5°C. Three-milliliter fractions were collected and tested for Mo by atomic absorption spectroscopy. An anaerobic solution of Na_2MoO_4 in Tris-hydrochloride was fractionated similarly on the same column. Symbols: ●, untreated extract; ○, protease-treated extract; ■, MoO_4^{2-}.*

termine if a W-containing analog of the Mo storage protein was synthesized. Figure 7 shows the W elution pattern from DEAE-cellulose columns used to fractionate extracts of cells grown in medium containing 5.0 μM WO_4^{2-}. The cells in one culture were derepressed for nitrogenase synthesis by allowing them to exhaust all NH_4^+; the other culture contained excess NH_4^+ to repress nitrogenase synthesis. As was the case with MoO_4^{2-}-grown cells, repressed cells produced only one W-containing compound which was eluted with 0.1 M NaCl, and the extract of derepressed cells yielded two W peaks: one eluted with 0.1 M NaCl, and the other eluted with 0.2 M NaCl. It is likely that the latter peak corresponded to the inactive W-containing species of component I. Treatment of extract of WO_4^{2-}-grown cells with protease resulted in elution of a low-molecular-weight form of W, indicating that W is stored by *A. vinelandii* in a protein-bound form just as Mo is (Fig. 8).

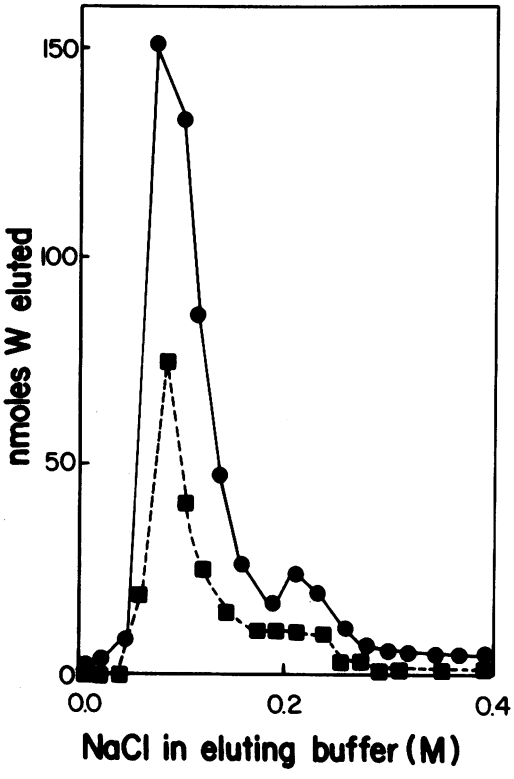

FIG. 7. *Elution profiles of stored W from DEAE-cellulose columns. Two cultures of A. vinelandii were grown in the presence of 5 μM WO_4^{2-}. One culture was derepressed for nitrogenase synthesis by growing it in limiting NH_4^+. In the other culture, nitrogenase synthesis was repressed by excess NH_4^+. Both cultures were harvested, and the cells were broken by osmotic shock. The extract of repressed cells contained 21.7 mg of protein per ml; the extract of derepressed cells contained 18.8 mg of protein per ml. Four-milliliter samples of both extracts were fractionated on DEAE-cellulose columns as described in the legend of Fig. 5. Quantitation of W was performed colorimetrically. Symbols: ●, extract of derepressed cells; ■, extract of repressed cells.*

Purification of the Mo storage protein. The Mo storage protein was purified from cells of *A. vinelandii* that had been grown in medium containing NH_4^+ and 10 μM MoO_4^{2-}. The presence of the Mo storage protein was monitored throughout purification by following the Mo concentration by atomic absorption spectroscopy and testing samples on a Sephadex G-75 column to insure that we were following the high-molecular-weight protein and not a breakdown product. The Mo storage protein was unstable throughout purification whether it was maintained anaerobically or aerobically, and so attempts were made to purify the protein rapidly and to maintain it at 0 to 5°C.

A summary of purification of the Mo storage

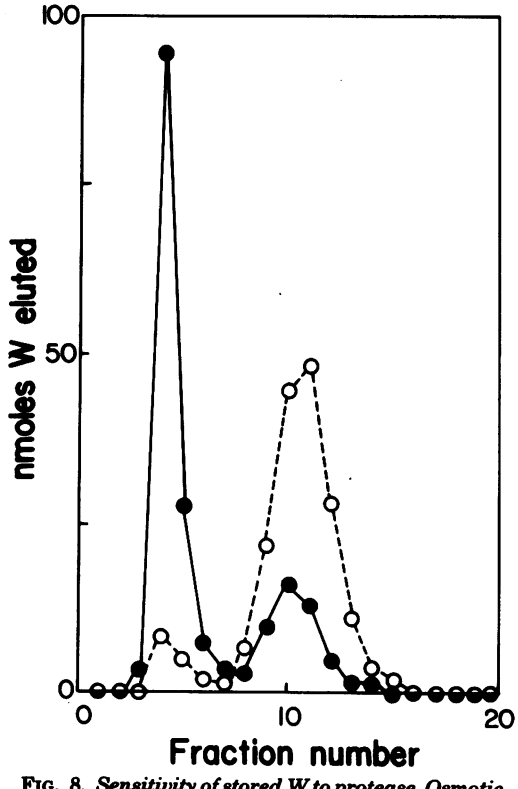

FIG. 8. *Sensitivity of stored W to protease. Osmotic shock extract (containing 23.6 mg of protein per ml) from cells grown in medium containing NH_4^+ and 1.0 μM WO_4^{2-} was divided into two samples and treated as described in the legend of Fig. 6. Symbols: ●, untreated extract; ○, protease-treated extract.*

TABLE 2. *Purification of the Mo storage protein*

Prepn	Vol (ml)	Total protein (mg)	Total Mo (nmol)	nmol of Mo/ mg of protein	% Recovery
Crude extract	140	1,172	10,220	8.7	100
DEAE fraction	70	82.7	4,382	53.0	42
20–50% ammonium sulfate cut	3	24.3	1,593	65.6	16
Sephadex G-100 fraction	22	3.2	516	161.2	5

protein is given in Table 2. Crude extract was prepared in 25 mM Tris-hydrochloride, pH 7.4, and 140 ml was loaded on a DEAE-cellulose column (2.5 by 25 cm) that had been equilibrated with Tris-hydrochloride. The column was washed with 1 bed volume of 50 mM NaCl in Tris-hydrochloride, and the Mo storage protein was eluted with 50 mM NaCl in 50 mM imidazole buffer, pH 6.5. The peak Mo storage protein fractions were treated with 20% ammonium sulfate, and the precipitate was discarded. The supernatant solution was made 50% with ammonium sulfate, and the Mo storage protein was precipitated. It was resuspended in 3.0 ml of imidazole buffer and loaded on a Sephadex G-100 column (2.5 by 90 cm) which was washed with imidazole buffer. The peak fractions were pooled and tested for purity by nondenaturing and SDS-polyacrylamide gel electrophoresis (Fig. 9). One protein band was visible on the native gel, but two bands of approximately equal intensity were seen on the SDS-gel, indicating that the Mo storage protein consists of two different subunits. When SDS-gels were run with purified Mo storage protein obtained from N_2-grown cells, two protein bands were seen with the same mobility as those of NH_4^+-grown cells (data not shown). Therefore, Mo is stored by the same system in both N_2 and NH_4^+-grown cells of *A. vinelandii*.

The molecular weights of the subunits were estimated to be 21,000 and 24,00 by comparison with the electrophoretic mobilities of protein standards. The elution profiles of native Mo storage protein on Sephadex and Sepharose columns indicated that the molecular weight was on the order of 100,000 (data not shown), and so it would appear that the Mo storage protein is a tetramer of two different subunits with a molecular weight closer to 90,000. We used this estimation to calculate that 14.5 atoms of Mo are bound to each molecule of Mo storage protein. Attempts to bind more Mo to the storage protein by incubating the purified preparation with 1.0 mM MoO_4^{2-} failed. The peak fraction of purified Mo storage protein was tested for the presence of Fe by reaction with α,α-dipyridyl (3), and the test was negative, indicating that the concentration of Fe was less than 5 nmol/ml compared with an Mo concentration of 84 nmol/ml in that fraction.

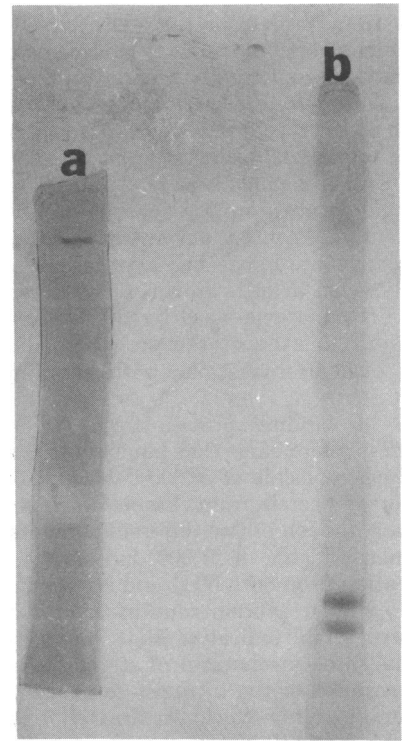

FIG. 9. *Electrophoresis of purified Mo storage protein. Samples of purified Mo storage protein obtained as the peak fraction from the Sephadex G-100 column were prepared for electrophoresis. Then 1.4 μg of protein was loaded on the nondenaturing gel (a) and 2.3 μg of protein was loaded on the SDS-gel (b). After electrophoresis was carried out, both SDS-gels were stained with Coomassie brilliant blue R250.*

DISCUSSION

In *K. pneumoniae*, Mo accumulation is tightly regulated and quite likely under the control of *nif* gene expression. The presence of NH_4^+ or O_2 (both of which repress nitrogenase synthesis) completely inhibits Mo accumulation. Also, the level of intracellular Mo correlates well with the level of nitrogenase so that the kinetics of Mo accumulation and nitrogenase synthesis are al-

most identical. These observations are quite similar to those of Mo accumulation in *C. pasteurianum* (8), but differ with our observations of *A. vinelandii*, in which Mo accumulation appears to be unregulated. Cells accumulate high levels of Mo whether they are grown on N_2 or NH_4^+. The concentration of intracellular Mo increases with the concentration of extracellular MoO_4^{2-} and can reach a level more than 25 times higher than needed for the synthesis of a full complement of component I. However, Mo accumulation is not simply a passive process because *A. vinelandii* can rapidly exhaust the growth medium of MoO_4^{2-} and thus must expend energy to hold it against a concentration gradient. Unlike *K. pneumoniae*, *A. vinelandii* requires no de novo protein synthesis for Mo accumulation; essential permeases and storage mechanisms are apparently synthesized constitutively, even in cells grown in the absence of MoO_4^{2-}. This is evidenced by the ability of *A. vinelandii* to exhaust the growth medium of MoO_4^{2-} even after treatment with chloramphenicol.

The Mo-binding protein from *C. pasteurianum* is a monomer that binds up to 6 atoms of Mo per molecule of 50,000 daltons (13). The Mo storage protein from *A. vinelandii* is made up of a pair each of two different subunits with molecular weights of 21,000 and 24,000. After purification from both NH_4^+ and N_2-grown cells, the Mo storage protein contains identical subunit structures, indicating that *A. vinelandii* uses the same mechanism of storage of excess Mo regardless of the nitrogen source. Purified Mo storage protein has approximately 15 atoms of Mo bound to it. No Fe was found in the Mo storage protein. WO_4^{2-}-grown cells of *A. vinelandii* make a W-containing analog of the Mo storage protein. This W-containing protein has been detected autoradiographically by electrophoresis of crude extracts of $^{185}WO_4^{2-}$-grown cells of *A. vinelandii* (P. T. Pienkos, S. Klevickis, and W. J. Brill, manuscript submitted for publication).

The Mo storage protein is not a source of FeMo-co to activate inactive component I from *A. vinelandii* mutant strain UW45 or Mo-co to activate inactive nitrate reductase from *N. crassa* mutant strain Nit-1. The Mo storage protein was unstable throughout the purification process, releasing Mo as a low-molecular-weight compound (possibly MoO_4^{2-}). This instability may have been an artifact of the purification techniques, but it is possible that Mo release is an inherent feature of this protein which may be required to provide an immediate source of Mo for component I synthesis. Because of this instability, the number of Mo atoms per Mo storage protein in vivo may be much greater than 15.

Mo-starved cells of *A. vinelandii* are unable to synthesize component I (14); therefore, MoO_4^{2-} may play a regulatory role in component I synthesis. However, cells that had stored Mo while nitrogenase synthesis was repressed were able to synthesize component I in the absence of MoO_4^{2-}. This suggests that Mo bound to the Mo storage protein rather than MoO_4^{2-} might act in a regulatory role for component I synthesis. A mutant strain of *A. vinelandii*, UW71, has a defect in Mo accumulation (16). This strain may be useful in attempts to understand the regulation of expression of *nif* genes in *A. vinelandii*.

A. vinelandii seems unique in its ability to store high levels of Mo even under conditions where Mo is unnecessary for growth. A possible explanation for this characteristic is that *A. vinelandii* may have evolved in habitats with large fluxes in the concentration of Mo. Under such circumstances, it would be advantageous to store Mo in times of plenty so that it would be available in times of scarcity.

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