JOURNAL OF BACTERIOLOGY, Nov. 1986, p. 688–693
0021-9193/86/110688-06$02.00/0

Vol. 168, No. 2

Purification and Characterization of a Molybdenum-Pterin-Binding Protein (Mop) in *Clostridium pasteurianum* W5

STEPHEN M. HINTON[1]* AND BRENDA MERRITT[2]

Exxon Liaison, Inc., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724,[1] and Exxon Corporate Research Company, Annandale, New Jersey 08801[2]

Received 4 April 1986/Accepted 18 August 1986

A large-scale fractionation scheme purified the major molybdenum(Mo)-binding protein (Mop) from crude extracts of *Clostridium pasteurianum*, with a 10 and 0.2% yield of Mo and protein, respectively. The apparent molecular weight of the purified molybdoprotein is 5,700, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The protein contains 0.7 mol of Mo per mol of protein with a molecular weight of 5,700. Mop, as isolated, has a peak absorbancy at 293 nm. Denaturation and oxidation of the molybdoprotein released multiple pterinlike fluorescent compounds. Mop appears to contain a pterin derivative and Mo, but phosphate analysis indicated that the pterin at the very least is not phosphorylated; phosphorylation is required for functional molybdenum cofactor. All treatments used to release the putative Mo-pterin species from Mop failed to yield a molybdopterin that had detectable molybdenum cofactor activity.

The known metabolic functioning of molybdenum (Mo) involves its participation as a component of two distinct cofactors found in various redox enzymes. The iron-molybdenum cofactor, FeMo-co, is a unique metal cluster in nitrogenase, whereas the molybdenum cofactor (Mo-co) is common to all other known molybdoenzymes (18). It is not known how the nutrient molybdate is biologically processed or chemically transformed into a biological cofactor or catalyst containing Mo. In *Klebsiella pneumoniae*, the products of five genes (*nifB*, *Q*, *N*, *E*, and *V*) are required for the biogenesis, modification, or insertion of FeMo-co into nitrogenase (19). In *Escherichia coli*, the products of five genes (*chlA*, *B*, *D*, *E*, and *G*) are also known to be involved in the biosynthesis of Mo-co (2, 20). Although the two Mo cofactors are distinct, it has been suggested that there may be a common step early in their biogenesis (14, 22).

Several observations indicate that the Mo cofactors are synthesized at one site (free or protein bound) and then inserted into the apoprotein (alternate site synthesis), as opposed to synthesis of the cofactor directly on the apoenzyme. It has been demonstrated (17) that microorganisms constitutively synthesize Mo-co, which is detected as a free cofactor in crude cell lysates. Amy and Rajagopalan (3) identified a 40,000-molecular-weight protein in *E. coli* that binds and appears to stabilize Mo-co. The Mo-co-binding protein has no known enzymatic activity, and attempts to isolate the protein have been frustrated because in vitro Mo-co association with the protein is very labile. Recently, it was shown in *K. pneumoniae* (21) that FeMo-co is synthesized in the absence of the nitrogenase proteins, and the unincorporated FeMo-co appears to be bound to a protein other than the MoFe protein of nitrogenase. These results suggest that molybdenum cofactors or precursor forms of the cofactor may be bound to a carrier protein.

We have been stydying Mo metabolism in *Clostridium pasteurianum* in an attempt to identify and characterize molybdoproteins that may play a role in the biosynthesis of Mo-containing cofactors. A nondenaturing (anaerobic) polyacrylamide gel electrophoresis (PAGE) procedure (10) was used to identify six distinct Mo species in clostridial crude extracts (11). Four of the molybdoproteins have characteristics that suggest that they may play a role in the synthesis of the Mo cofactors. We are interested in characterizing these putative Mo-processing proteins in an attempt to determine how the cell synthesizes the molybdenum cofactors. In this paper, we report a large-scale purification procedure for the major Mo-binding protein (Mop) in *C. pasteurianum*, a protein suspected of being involved in Mo metabolism. A partial biochemical characterization of the purified protein is also presented.

MATERIALS AND METHODS

Organisms, media, and culture conditions. *C. pasteurianum* W5 (wild type) was obtained from L. E. Mortenson. Large-scale cultures of *C. pasteurianum* (160 liters) were grown in medium previously described (10) under N_2-fixing conditions at 37°C and maintained at pH 6.0. The inoculum (6 liters) was the only source of Mo available for growth (final Mo concentration, ~0.3 μM). In the late logarithmic growth phase (optical density at 550 nm, ~2.5 to 3.0) an increase in generation time was observed, indicating a Mo-deficient culture condition (12), at which time excess MoO_4^{-2} was added, resulting in a final concentration ~100 μM. One hour after the Mo supplement, the cells (Mo-pulsed) were harvested, washed, frozen in liquid nitrogen, and stored at −70°C.

The *Neurospora crassa nit-1* (allele 345447) mutant was obtained from the Fungal Genetic Stock Center, Humboldt State University Foundation, Arcata, Calif. *Neurospora* mycelia were grown in Vogels liquid medium, as previously described (8). The mycelia were grown for 40 h, harvested, washed, and suspended in Vogels medium containing 0.02 M $NaNO_3$ for 3 h to induce nitrate reductase. The mycelia were harvested, washed, and frozen in liquid N_2 until use.

Purification of Mop. For Mop purification, all manipulations were performed under stringent anaerobic conditions at room temperature. All solutions and reagents were deoxygenated on an inert gas manifold (4) by repeated evacuation and flushing with nitrogen or hydrogen followed

* Corresponding author.

by the addition of dithionite (as $Na_2S_2O_4$; final concentration, 1 mM). Frozen cells (~2 kg [wet weight]) were thawed and suspended in twice the volume (wt/vol) of 25 mM Tris hydrochloride buffer (pH 8.0) containing 2 mM EDTA during the deoxygenating procedure described above. The suspended cells were stirred slowly overnight under a hydrogen atmosphere in the presence of lysozyme (6 g) and DNase (200 mg). The cell lysate was centrifuged for 30 min at 50,000 × *g* at 4°C. The cell-free lysate was passed through a Pellicon Cassette (Millipore Corp.) ultrafiltration cell equipped with a polysulfone filter with a 100,000-molecular-weight exclusion limit. The cassette was set up in a molecular washing configuration. The cell-free lysate was pumped out of an anaerobic closed vessel through the cassette cell, in which proteins with a molecular weight <100,000 pass through the filter into the filtrate catch vessel, and the retained proteins were returned to the closed vessel. The filtrate catch vessel contained a slurry of Whatman DE-52 (~900 g of DEAE-cellulose), which was stirred slowly to keep the anion-exchange resin in suspension. When the volume of the cell-free lysate was approximately 1 liter, the closed vessel was filled with 25 mM Tris hydrochloride buffer (pH 8.0) containing 1 mM dithionite, and the filtration procedure was repeated. Once 24 liters of filtrate were pumped through the cell, the anion-exchange resin in the catch vessel was allowed to equilibrate and settle out of solution. The buffered solution was decanted anaerobically, and the resulting slurry of DE-52 was poured into a chromatographic column (diameter, 7.5 cm). The column was washed with 50 mM Tris hydrochloride buffer (pH 8.0) before Mop was eluted with 0.16 M NaCl in 50 mM Tris hydrochloride buffer. The gold-brown fractions containing Mop were pooled and diluted with 2 volumes (vol/vol) of 25 mM Tris hydrochloride buffer (pH 8.0).

The diluted protein solution was applied to a DEAE-Sepharose column (7.5 by 20 cm), and Mop was eluted in the fractions between 80 and 130 mM NaCl that resulted from a 4-liter linear gradient of NaCl between 50 and 200 mM in 50 mM Tris hydrochloride buffer (pH 8). A 90%-saturated ammonium sulfate (AS) solution was added to the pooled Mop fractions until the solution was 50% saturated with AS. The precipitate formed was removed by centrifugation at 50,000 × *g* for 30 min, and the 50%-saturated AS supernatant (containing Mop) was applied to a phenyl-Sepharose column (2.5 by 15 cm) equilibrated with 50%-saturated AS in 50 mM Tris hydrochloride buffer (pH 8). Mop was eluted from the hydrophobic column with a reverse linear gradient 50 to 0% saturated with AS in 50 mM Tris hydrochloride buffer. The fractions containing Mo (Mop) eluted with ~22%-saturated AS solution.

The fractions containing Mo were pooled and concentrated to ~10 ml with an Amicon ultrafiltration (stirred) cell equipped with a PM 10 membrane. The concentrated protein solution was applied to a Sephacryl S-200 column (5 by 90 cm) equilibrated with 250 mM NaCl in 50 mM Tris hydrochloride buffer (pH 8). Mop was eluted with the same buffer, and the light yellow fractions containing Mo were pooled, concentrated, and pelleted in liquid nitrogen for long-term storage.

Molecular weight of Mop. The molecular weight of Mop was estimated by sodium dodecyl sulfate (SDS)-PAGE from a plot of relative electrophoretic mobility versus the logarithms of the molecular weights of standard proteins and purified Mop.

Chemical analysis of Mop. The protein obtained in the final step of purification was analyzed for molybdenum and phosphate by the methods of Clark and Axley (5) and Ames (1), respectively.

Identification of pterinlike chromophore released from Mop. The procedure to release and identify protein-bound molybdopterins was previously described (9). Xanthine oxidase (from buttermilk; Sigma Chemical Co.) and purified Mop (2.0 mg/ml) were denatured with 6 M guanidine hydrochloride in the presence of KI and I_2 (form A) or by boiling the protein in acid (pH 2.5) in the absence of the iodine solution (form B). The chromophore was purified on Sephadex G-25 and QAE-Sephadex, vacuum dried, and suspended before the absorbance and fluorescence spectra were recorded. The purified chromophore (form A) was treated with alkaline permanganate, as previously described (16), which is known to oxidize 6-alkyl pterins to pterin-6-carboxylic acid. Pterin-6-carboxylic acid was isolated from the permanganate oxidation mixture by reverse-phase high-performance liquid chromatography (HPLC) on a Beckman Model 341 chromatograph equipped with a Model 165 UV detector and a Model 154 fluorescence detector. A C_{18} reverse-phase column (0.46 by 25 cm; Alltech Associates, Inc.) was used to separate the pterin derivatives by using isocratic elution (flow rate, 1 ml/min) with 20% methanol containing 0.01 M acetic acid.

Spectral analysis. Absorption spectra were determined with a Kontron Uvikon 810 Spectrophotometer and a Hewlett-Packard 84512A Diode Array Spectrophotometer. Anaerobic experiments were performed with a special cuvette fitted with a serum stopper. The stoppered cuvette was degassed and flushed with nitrogen before the sample was added anaerobically. Fluorescence spectra were determined with a Perkin-Elmer 650-40 Fluorescent Spectrophotometer.

Mo-co assay. Crude extracts of *N. crassa nit-1*, a mutant strain defective in Mo-co synthesis, were prepared by a modified method of Horner (13). Frozen mycelia were ground into a fine powder and suspended in 0.1 M potassium phosphate buffer (pH 7.4) containing 0.17 M NaCl, 1 mM dithiothreitol, 5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 1% (vol/vol) ethanol. The mixture was homogenized and centrifuged, and the supernatant was saved. Protamine sulfate was slowly added (0.019 ml of a 5.3% [wt/wt] solution per ml of supernatant) to the supernatant solution along with 50 mM phenylmethylsulfonyl fluoride in ethanol (1% of the volume) while the solution was continuously stirred for 10 min at 4°C. The solution was centrifuged, and the supernatant was used for detecting a source of Mo-co by reconstitution of NADPH:nitrate reductase activity, as described by Hawkes and Bray (9).

RESULTS

Purification of Mop. Previous studies by Elliott and Mortenson (7) showed that there are two major molybdoproteins in crude extracts of N_2-fixing clostridial cells that can be separated by anion-exchange chromatography, the MoFe protein of nitrogenase and the Mo-binding storage protein (MoB-SP). They proposed that MoB-SP may be involved in the synthesis of molybdenum cofactors. Recently, it was shown that MoB-SP appears to be a mixture of three variants (11). The three variants differ in charge and the amount of Mo bound to the protein. The protein is more anionic in net charge as Mo dissociates from the molecule in the form of a low-molecular-weight (<10,000) Mo species. The results suggest that MoB-SP is an aggregate of a low-molecular-weight Mo protein, designated Mop. We were interested in isolating and characterizing MoB-SP or

Mop to elucidate its role in the biosynthesis of molybdenum cofactors (7, 12).

The large-scale purification scheme developed for the isolation of Mop is a result of two observations. (i) Previous physiological studies (12) showed that there was a substantial (fivefold) increase in Mo proteins (relative to levels under steady-state N_2-fixing conditions) when a Mo-limited growth condition was relieved with relatively high concentrations of molybdate (~100 μM). There is approximately 10 to 20 times the amount of Mo associated with MoB-SP in Mo-pulsed cells compared with that in steady-state nitrogen-fixing cells (12), which made the purification of the protein feasible. (ii) The MoB-SP with an apparent molecular mass of 30 to 50 kilodaltons is the first Mo species which elutes from an anion-exchange column, and when treated with AS, it is converted into a low-molecular-weight Mo protein, Mop, which has stable properties. We determined which column fractions contained Mop by assaying for Mo and evaluated the purification step by the increase in the molybdenum-to-protein ratio. We surveyed the individual proteins in the crude fractions after each purification step by SDS-PAGE. The scheme for large-scale purification of Mop from Mo-pulsed cells of *C. pasteurianum* is shown in Table 1.

The cell-free lysate was passed through the Pellicon Ultrafiltration Cassette to separate the low-molecular-weight Mo proteins (i.e., Mop) from the high-molecular-weight molybdoenzymes (formate dehydrogenase and the molybdenum-iron protein of nitrogenase) and to reduce the amount of protein for ion-exchange chromatography. Approximately 85% of the contaminating Mo was separated from the fractions containing Mop after passage through the first anion-exchange column (batch DE-52). The apparent molecular mass of Mop at this point in the purification scheme was between 30 and 50 kilodaltons, as determined by ultrafiltration. The addition of AS resulted in either the dissociation of a Mop aggregate or the dissociation of Mop from another (binding) macromolecule, with the resulting observed monomer having an apparent molecular weight greater than 5,000 but less than 10,000, as determined by ultrafiltration membrane cutoff criteria. After hydrophobic and molecular-sieve chromatography, the protein was homogeneous, as determined by SDS-PAGE. The procedure for purification of Mop from clostridial crude lysates resulted in approximately a 53- and 500-fold enrichment with respect to the Mo/protein ratio and protein, respectively. From 1.8 kg of wet cell paste, ~375 mg of Mop was obtained, pelleted, and stored in liquid nitrogen.

Biochemical characterization of Mop. The estimated molecular weight of purified Mop is 5,700 as determined by SDS-PAGE (Fig. 1). Chemical analysis showed that the protein contains approximately 0.7 mol of Mo per mol of protein with a molecular weight of 5,700. There were only trace amounts of phosphate (Mo/P ratio, ~100:1). The UV absorbancy of the native protein as isolated (anaerobically) is shown in Fig. 2. Upon a brief exposure (~15 min) to air, the peak absorbance at 293 nm remained unchanged but there was a small increase in the absorbance of the air-oxidized protein in the 400-nm range. Chemical oxidation (with ferricyanide) of the native protein (data not shown) produced an even greater increase in the relative absorbancy in the 300- to 400-nm range. These results are typical of pterins as they are oxidized.

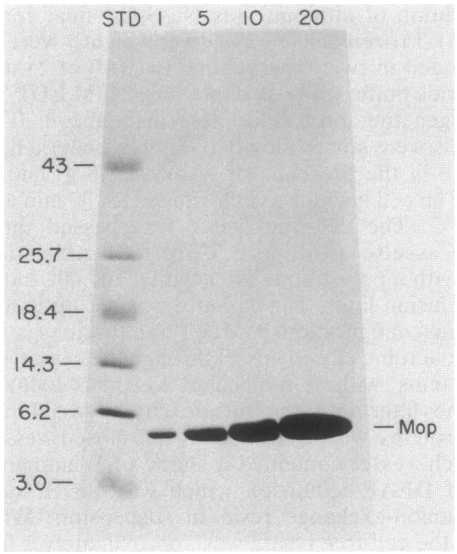

FIG. 1. Electrophoretic analysis of purified Mop from *C. pasteurianum*. The lanes (from left to right) contained 1, 5, 10, and 20 μg of purified Mop which was analyzed to determine the state of purity and the effective electrophoretic mobility by SDS-PAGE. The lane on the far left contained molecular mass standards (STD) (in kilodaltons) as indicated. The proteins were resolved in a linear pore gradient (12 to 22.5% acrylamide) and stained with Coomassie blue. The relative electrophoretic mobility of Mop indicates that its apparent molecular weight is 5,700.

TABLE 1. Scheme for purification of Mop from *C. pasteurianum*

Step	Amt (%[a]) of:		Mo/protein ratio (μmol/g)	Purification (fold)[b]
	Mo (μmol)	Protein (g)		
Crude lysate	510 (100)	200 (100)	2.5	1
Cell-free lysate	340 (80)	160 (67)	2.1	0.8
Ultrafiltration/batch ion exchange	64 (12.5)	90 (45)	0.7	0.3
DEAE-Sepharose Phenyl-Sepharose	56 (11)	20 (10)	2.8	1.1
Sephacryl S-200	50 (10)	0.375 (0.19)	133	53

[a] Percent recovery.
[b] Fold purification with respect to Mo/protein ratio.

Analysis of pterinlike chromophore associated with Mop. A pterinlike fluorescent chromophore was isolated from Mop by using standard procedures (16) to release, oxidize, and purify protein-bound molybdopterins (Fig. 3). The UV absorbancy of the chromophore from Mop and the molybdopterin from xanthine oxidase (isolated under two different conditions [forms A and B] [16]) is shown in Fig. 4. Purification by anion-exchange chromatography of form A of the pterinlike chromophore released from Mop (Fig. 3) and the molybdopterin from xanthine oxidase showed that more than one fluorescent species was released from each protein; one eluted from QAE-Sephadex with 0.01 N acetic acid and the other eluted with 0.01 N HCl. HPLC of the fluorescent material eluting off the anion-exchange column with acetic and hydrochloric acids showed that there were several fluorescent species in each fraction (Fig. 5). Permanganate oxidation of the purified pterins eluting off the anion-exchange column with HCl reduced the number of fluorescent species detected by HPLC, with an enrichment of the peak coeluting with pterin-6-carboxylic acid.

Xanthine oxidase had detectable cofactor activity under all conditions (i.e., acid, SDS, and guanidine hydrochloride) used to release the molybdenum cofactor from the protein.

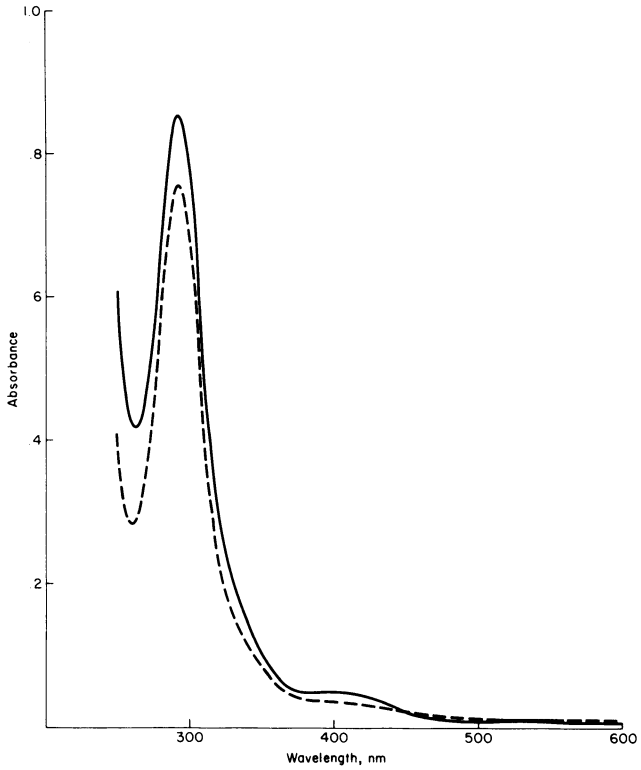

FIG. 2. Absorption spectra of Mop as isolated anaerobically (– – –) and air oxidized (——).

Under no conditions, however, did we find cofactor activity for the Mo-pterin released from Mop.

DISCUSSION

Previously, we proposed that during the biosynthesis of Mo cofactors (i.e., FeMo-co and Mo-co) there may be stable intermediates or precursors in Mo processing, possibly molybdoproteins, because *C. pasteurianum* accumulated Mo before we detected the derepression of molybdoenzyme activity (11). By equilibrium labeling *C. pasteurianum* cells with ^{99}Mo during their shift from growth on ammonia to dinitrogen-fixing conditions, we detected four Mo species which incorporated ^{99}Mo either simultaneously with or just before the appearance of the molybdoenzymes, formate dehydrogenase and the molybdenum-iron (MoFe) protein of nitrogenase. The most intriguing candidates suspected to be involved in Mo processing were the low-molecular-weight Mo species and MoB-SP. Elliott and Mortenson (7) identified MoB-SP (which was named for its putative function) as the major molybdoprotein (other than the MoFe protein) in crude extracts of N_2-fixing clostridial cells. Recently, it was demonstrated that MoB-SP appears to exist as a mixture of three size and charge variants, and the form of Mo dissociating from MoB-SP appears to be a low-molecular-weight species (11). The evidence suggests that MoB-SP is either an aggregate of the low-molecular-weight Mo species or is bound to another macromolecule. Initial attempts to purify MoB-SP were frustrated by Mo dissociating from the protein, which resulted in an apparent increase in charge and a decrease in molecular size (S. Hinton, unpublished results). Partially purified fractions of MoB-SP (pure with respect to molybdoproteins) when treated with high concentrations of AS, yield a small-molecular-size (<10 kilodaltons) molybdoprotein with stable properties, which we named Mop. We are determining the relationship of MoB-SP and Mop.

Mop from N_2-fixing *C. pasteurianum* cells was purified to homogeneity as determined by SDS-PAGE after a 53- and 500-fold enrichment with respect to the Mo/protein ratio and protein, respectively. Mop has an apparent molecular weight of 5,700, as determined by gel electrophoresis (Fig. 1). The protein contains 0.7 mol of Mo per mol of protein with a molecular weight of 5,700. The Mo environment has been shown by extended X-ray absorption fine-structure analysis to be unusual in comparison with that of most other molybdoenzymes in that the Mo has no sulfur ligands (S. Cramer and S. Hinton, unpublished results). However, the Mo environment (tri-oxo species) in Mop was quite similar to the site in clostridial formate dehydrogenase (6), an end product of the Mo processing pathway.

Mop as isolated (anaerobically) had an absorption peak of 293 nm, and air-oxidized Mop showed a slight increase in absorbance in the 400-nm range. After denaturation of the molybdoprotein in the presence of chemical oxidants (KI and I_2), pterinlike fluorescent compounds appeared that separated from the protein. Johnson and Rajagopalan (15) showed that the Mo-co isolated from various molybdoenzymes (but not of course from the MoFe protein of nitrogenase) is a 6-substituted pterin of undefined structure. HPLC of the fluorescent material released from the protein showed that there are multiple fluorescent oxidation products, which is typical for the decay of 6-substituted pterins (15). Alkaline permanganate oxidation of the 6-substituted pterin mixture released from the protein is known to reduce the number of fluorescent products as a result of oxidation of the side chain generating pterin-6-carboxylic acid. Treating the isolated pterin derivative from Mop under these conditions led to the appearance of fewer fluorescent products, but the data do not allow us to conclude that pterin-6-carboxylic acid appeared. Nevertheless, the evidence indicates that Mop con-

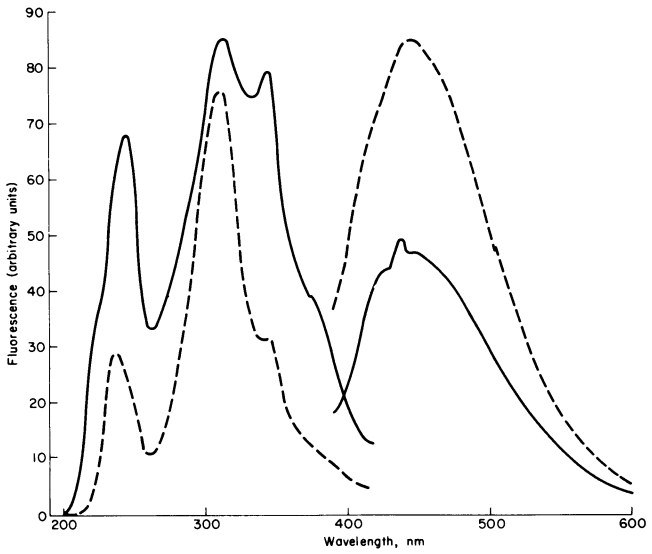

FIG. 3. Fluorescence spectra of chromophore released from Mop. Shown are the fluorescence spectra of the pterin derivatives (form A) isolated from Mop eluting off the anion-exchange column with 0.01 N acetic acid (– – –) and 0.01 N HCl (——).

tains or binds a pterin derivative. If the Mo and pterin are associated, the complex may be related to Mo-co.

Various treatments were used to release the putative Mo-pterin species from Mop to determine if it was able to reconstitute the activity of Mo-co-deficient nitrate reductase. With all standard treatments used to release protein-bound Mo-co, Mop failed to yield a molybdopterin that was capable of reconstituting nitrate reductase activity. It is noteworthy that the proposed structure of Mo-co is a reduced pterin ring with a four-carbon side chain containing an enedithiol and a terminal phosphate ester (16). Mop appears to contain a reduced pterin, because upon air oxidation there is an increase in absorbance in the 400-nm range, which is typical for pterins undergoing oxidation. We have evidence that Mop binds a pterin derivative, but it does not appear to be phosphorylated. The molybdenum-to-phosphate ratio is 1:1 in catalytically active Mo-co, whereas Mop has only trace amounts of phosphate (Mo/P ratio, >100:1). Apparently, the pterin moiety bound to Mop, at the very least is not phosphorylated, which may account for it being inactive in the Mo-co reconstitution assay. We are attempting to activate the benign Mo-pterin species associated with Mop.

In conclusion, we can speculate about a physiological role for Mop. Previous studies (12) showed that Mop appears to be expressed under growth conditions requiring molybdoenzymes (i.e., N_2-fixing conditions), as well as under conditions where molybdoenzyme activity was detected at extremely low levels. We have suggested that Mop is involved in Mo metabolism, and we now propose that it contains an inactive form of Mo-co awaiting phosphorylation and activation during the biosynthesis of molybdoenzymes. However, we have no direct evidence to support such a hypothesis. Further work on the structure and activation of the Mo-pterin species and on the relationship of Mop and MoB-SP is required to explain the role of Mop in Mo metabolism.

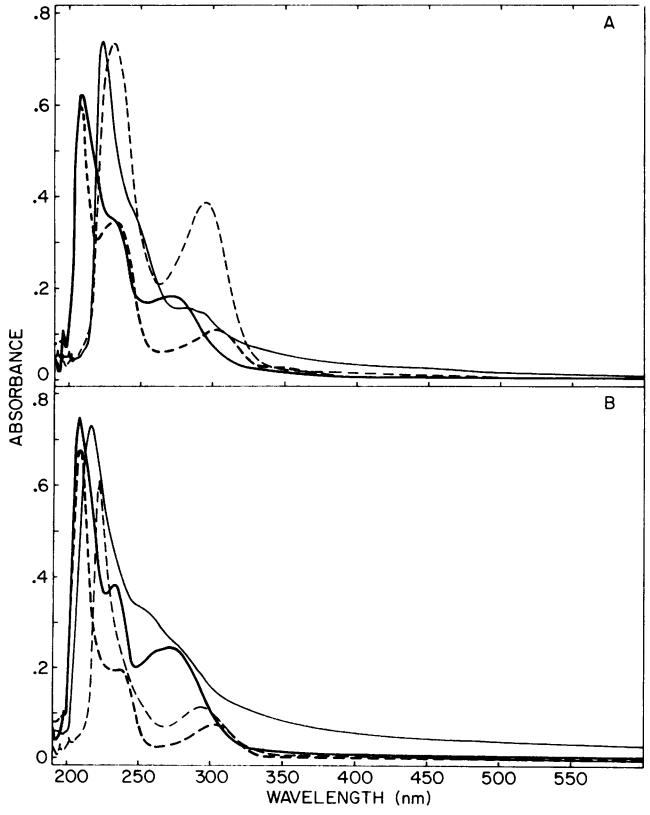

FIG. 4. Absorption spectra of form A (A) and form B (B) of pterin derivatives isolated from Mop and xanthine oxidase. The absorption spectra of the pterin derivatives isolated from Mop were recorded in 0.1 N HCl (thick solid line) and 0.1 N NaOH (thin solid line). The absorption spectra of the pterin derivatives isolated from xanthine oxidase were also recorded in 0.1 N HCl (thick broken line) and 0.1 N NaOH (thin broken line).

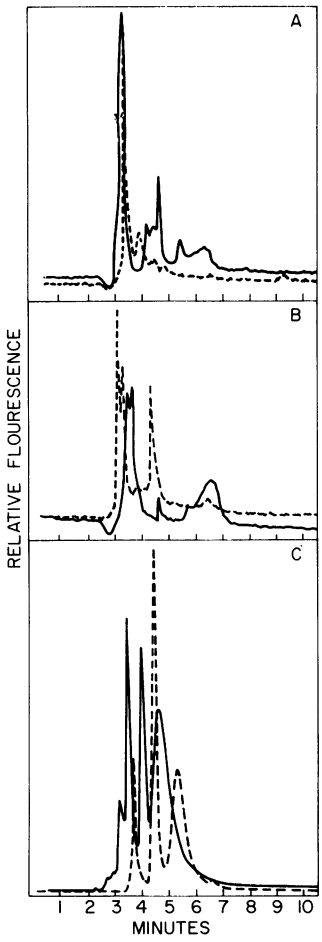

FIG. 5. HPLC elution profiles of fluorescent species released from Mop (——) and xanthine oxidase (– – –). Profiles are shown for the fluorescent material (form A) eluted off the anion-exchange column with 0.01 N acetic acid (A) and 0.01 N HCl (B) and for the fluorescent material oxidized with permanganate (eluted with HCl) (C). Pterin-6-carboxylic acid coeluted with the fluorescent products in the peak at 4.5 min.

ACKNOWLEDGMENTS

We thank Rick Bare for assisting in the purification of the protein. We also thank Dan Lundell and Ed Stiefel for reviewing the manuscript.

LITERATURE CITED

1. **Ames, B. N.** 1966. Assay of inorganic, total phosphate and phosphatases. Methods Enzymol. **8**:115–118.

2. **Amy, N. K.** 1981. Identification of the molybdenum cofactor in chlorate-resistant mutants of *Escherichia coli*. J. Bacteriol. **148**:274–282.
3. **Amy, N. K., and K. V. Rajagopalan.** 1979. Characterization of molybdenum cofactor from *Escherichia coli*. J. Bacteriol. **140**:114–124.
4. **Burgess, B. K., D. B. Jacobs, and E. I. Stiefel.** 1980. Large-scale purification of high activity *Azotobacter vinelandii* nitrogenase. Biochim. Biophys. Acta **614**:196–209.
5. **Clark, L. J., and J. H. Axley.** 1955. Molybdenum determination in soils and rocks with dithiol. Anal. Chem. **27**:2000–2003.
6. **Cramer, S. P., C.-L. Liu, L. E. Mortenson, J. T. Spence, S.-M. Liu, I. Yamamoto, and L. G. Liungdahl.** 1985. Formate dehydrogenase molybdenum and tungsten sites—observation by EXAFS of structural differences. J. Inorg. Chem. **23**:119–124.
7. **Elliott, B. K., and L. E. Mortenson.** 1977. Molybdenum storage component from *Clostridium pastuerianum*, p. 205–217. *In* W. Newton, J. R. Postgate, and C. Rodriguez-Barrueco (ed.), Recent developments in nitrogen fixation. Academic Press, Inc. (London), Ltd., London.
8. **Garrett, R. H., and A. Nason.** 1969. Further purification and properties of *Neurospora* nitrate reductase. J. Biol. Chem. **244**:2870–2882.
9. **Hawkes, T. R., and R. C. Bray.** 1984. Quantitative transfer of the molybdenum cofactor from xanthine oxidase and from sulphide oxidase to the deficient enzyme *nit*-1 mutant of *Neurospora crassa* to yield active nitrate reductase. Biochem. J. **219**:481–493.
10. **Hinton, S. M., and L. E. Mortenson.** 1985. Anaerobic multiphasic gel electrophoresis of the molybdoproteins in crude extracts of *Clostridium pastuerianum*. Anal. Biochem. **145**:222–229.
11. **Hinton, S. M., and L. E. Mortenson.** 1985. Identification of molybdoproteins in *Clostridium pastuerianum*. J. Bacteriol. **162**:477–484.
12. **Hinton, S. M., and L. E. Mortenson.** 1985. Regulation and order of involvement of molybdoproteins during synthesis of molybdoenzymes in *Clostridium pastuerianum*. J. Bacteriol. **162**:485–493.
13. **Horner, R. D.** 1983. Purification and comparison of *nit*-1 and wild-type NADPH-nitrate reductase of *Neurospora crassa*. Biochim. Biophys. Acta **744**:7–15.
14. **Imperial, J., R. A. Ugalde, V. K. Shah, and W. J. Brill.** 1985. Mol^- mutants of *Klebsiella pneumoniae* requiring high levels of molybdate for nitrogenase activity. J. Bacteriol. **163**:1285–1287.
15. **Johnson, J. L., B. E. Hainline, and K. V. Rajagopalan.** 1980. Characterization of the molybdenum cofactor of sulfite oxidase, xanthine oxidase, and nitrate reductase. J. Biol. Chem. **255**:1783–1786.
16. **Johnson, J. L., and K. V. Rajagopalan.** 1982. Structural and metabolic relationship between the molybdenum cofactor and urothione. Proc. Natl. Acad. Sci. USA **79**:6856–6860.
17. **Ketchum, P. A., and R. S. Swarin.** 1973. *In vitro* formation of assimilatory nitrate reductase: presence of the constitutive component in bacteria. Biochem. Biophys. Res. Commun. **52**:1450–1456.
18. **Pienkos, P. T., V. K. Shah, and W. J. Brill.** 1977. Molybdenum cofactors from molybdoenzymes and *in vitro* reconstitution of nitrogenase and nitrate reductase. Proc. Natl. Acad. Sci. USA **74**:5468–5471.
19. **Roberts, G. P., T. MacNeil, D. MacNeil, and W. J. Brill.** 1978. Regulation and characterization of protein products coded by the *nif* (nitrogen fixation) genes of *Klebsiella pneumoniae*. J. Bacteriol. **136**:267–279.
20. **Stewart, V., and C. H. MacGregor.** 1982. Nitrate reductase in *Escherichia coli* K-12: involvement of *chlC*, *chlE*, and *chlG* loci. J. Bacteriol. **151**:788–799.
21. **Ugalde, R. A., J. Imperial, V. K. Shah, and W. J. Brill.** 1984. Biosynthesis of iron-molybdenum cofactor in the absence of nitrogenase. J. Bacteriol. **159**:888–893.
22. **Ugalde, R. A., J. Imperial, V. K. Shah, and W. J. Brill.** 1985. Biosynthesis of the iron-molybdenum cofactor and the molybdenum cofactor in *Klebsiella pneumoniae*: effect of sulfur source. J. Bacteriol. **164**:1081–1087.