THE ROLE OF MOLYBDENUM IN THE FORMATE DEHYDROGENASE OF *METHANOBACTERIUM FORMICICUM*

by

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(ABSTRACT)

An examination of oxidation products of the pterin cofactor in the formate dehydrogenase of *Methanobacterium formicicum* revealed that the cofactor is a 6-substituted pterin similar to the molybdopterin of xanthine oxidase. In contrast to the molybdopterin from xanthine oxidase the formate dehydrogenase cofactor was unable to complement the cofactor-deficient nitrate reductase of *Neurospora crassa* mutant *nit*-1 and possessed two phosphate groups.

Incubation of oxidized formate dehydrogenase with cyanide resulted in an irreversible loss of enzyme activity which could not be restored by treatment with sulfide. Equimolar amounts of thiocyanate were released from cyanide-treated formate dehydrogenase suggesting the loss of one terminal sulfur ligand to molybdenum. These results along with electron paramagnetic resonance spectroscopy of the cyanide-inactivated formate dehydrogenase suggest that the the molybdenum ligands of the enzyme are similar to those of xanthine oxidase.

The concentration of molybdenum and the level of formate dehydrogenase activity in extracts of hydrogen-grown *Methanobacterium formicicum* decreased by at least 10-fold when the organism was grown for several transfers in molybdenum-deficient media or with added tungstate. Immunochemical analysis showed that both subunits of the formate dehydrogenase were produced regardless of the growth condition. However the amount of formate dehydrogenase protein decreased more than 10-fold when the amount of molybdenum in the cell was low. The pterin cofactor was present in the inactive enzyme from tungstate-grown cells; however the protein contained less than 0.05 molecules of molybdenum or tungsten per formate dehydrogenase. Messenger RNA specific for the *fdh* gene was detected in high amount in cells grown without added molybdenum and in low amount in cells that contained high amounts of molybdenum. These results suggest that molybdenum is required for the synthesis of a stable formate dehydrogenase and that a molybdenum-dependent repressor may be required for the termination of *fdh* transcription.

FOREWORD

This dissertation consists of eight sections. Section I is a brief introduction. Section II is a review of the literature that pertains to my research. Sections III, IV and V contain the results of the dissertation and are written in publication form. Section VI is a general summary of the results. Section VII cites the literature discussed in Sections I, II and VII. Section VIII is my Curriculum Vitae.

Section III contains methods, results and discussion of the study on cyanide-inactivation of the formate dehydrogenase. These results and electron paramagnetic resonance spectroscopy data of the cyanide treated formate dehydrogenase were published (Barber et al., 1986). The electron paramagnetic resonance data are discussed, but not presented, in section III. Section IV is presented as prepared for publication (May et al., 1986). The material in section V has been prepared for publication and is presented in that form. The titles of the journal articles are listed below.

Barber, M. J., H. D. May, and J. G. Ferry. 1986. Inactivation of formate dehydrogenase from _Methanobacterium formicicum_ by cyanide. Biochemistry 25:8150-8155.

May, H. D., N. L. Schauer, and J. G. Ferry. 1986. Molybdopterin cofactor from *Methanobacterium formicicum* formate dehydrogenase. J. Bacteriol. **166**:500-504.

May, H. D., and J. G. Ferry. 1987. The effect of molybdenum and tungsten on the activity and synthesis of *Methanobacterium formicicum* formate dehydrogenase. Manuscript in preparation for submission to the Journal of Bacteriology.

In addition to the journal articles that are included in the dissertation I have contributed to the following publications while a student in Dr. Ferry's laboratory:

Barber, M. J., L. M. Siegel, N. L. Schauer, H. D. May, and J. G. Ferry. 1983. Formate dehydrogenase from *Methanobacterium formicicum*: electron paramagnetic resonance spectroscopy of the molybdenum and iron-sulfur centers. J. Biol. Chem. **258**:10839-10845.

Baron, S. F., H. D. May, K. C. Terlesky, and J. G. Ferry. 1987. Metabolic regulation in methanogenic bacteria with potential influence on the performance of biomass digesters. Poultry Science, in press.

Shuber, A. P., E. C. Orr, M. A. Recny, P. F. Schendel, H. D. May, and J. G. Ferry. 1986. Cloning, expression, and nucleotide sequence of the formate dehydrogenase genes from *Methanobacterium formicicum*. J. Biol. Chem. **261**:12942-12947.

Dedicated to my Hoosier grandparents:

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SECTION I. GENERAL INTRODUCTION

Methanobacterium formicicum strain JF-1 is a member of a unique group of organisms called the archaebacteria. The archaebacteria have been separated phylogenetically from eubacteria and eucaryotes because of differences in cell wall structures (58), membrane lipids (24,70), coenzymes (29,62,63,109) and sequences of 16s ribosomal RNA (33). Methanogenic bacteria are found in the rumen, human intestines and anaerobic sediments (78,105,106,116). Methanobacterium formicicum was isolated from a benzoate-degrading consortium of sewage sludge (32). It can obtain all of its carbon and energy for growth from formate (97).

The formate dehydrogenase of M. formicicum contains molybdenum and a pterin cofactor (81,100). The enzyme catalyzes the oxidation of formate to CO_2 and reducing equivalents for the production of methane (97,100). This oxidation is the first step in the conversion of formate to methane. To better understand molybdenum biochemistry and the physiology of formate metabolism in M. formicicum, these aspects were studied: 1) the structural environment of molybdenum and the pterin cofactor in the formate dehydrogenase and 2) the role of molybdenum in the synthesis of the enzyme. The results of the research are

presented in this dissertation.

SECTION II. LITERATURE REVIEW

METHANOGENIC BACTERIA

Methanogenic bacteria along with the extreme halophiles and extreme thermophiles belong to a third kingdom called the archaebacteria. This grouping is based upon comparative analysis of 16S rRNA oligonucleotide sequences, which has distinguished the archaebacteria from eubacteria and from eucaryotes (33). Several other unique features of the archaebacteria support this classification. The cell walls of these bacteria contain no peptidoglycan (58). Some cell walls possess pseudomurein, an analog of peptidoglycan that has L-talosaminuronic acid substituted for muramic acid and have α-1,3 instead of β-1,4 glycosidically-linked amino sugars (65,66). Archaebacteria with only glycoprotein, protein or heteropolysaccharide cell walls also have been isolated and characterized (58,59). Membranes of archaebacteria contain ether-linked lipids instead of the ester-linked lipids of eubacteria and eucaryotes (24,70). DNA-dependent RNA polymerases that are structurally more similar to eucaryotic than eubacterial RNA polymerases have been found in methanogenic bacteria (119,120) and tRNA structures from methanogens differ from both eucaryotic and eubacterial tRNAs (38,39). The methanogenic bacteria have

several unique coenzymes such as methanofuran (71,72), methanopterin (63,64,72), coenzyme F_{420} (29), coenzyme F_{430} (62) and coenzyme M (109). More information on the methanogenic bacteria and archaebacteria can be found in review articles by Balch et al. (3), Fox et al. (33) and Woese and Wolfe (115).

Substrates that serve as sole sources of carbon and energy for pure cultures of methanogenic bacteria include H_2/CO_2, formate, acetate, methanol and methylated amines (3,97,105,106). The most studied and best understood methanogenic pathway is the reduction of CO_2 to methane. A simplified illustration of this pathway appears in Figure 1. Carbon dioxide is first transferred to a C-1 carrier, methanofuran, and then is reduced to the formyl level (71,72). The formyl group is passed on to tetrahydromethanopterin, an analogue of tetrahydrofolate, and is reduced to the methylene and then methyl level by reactions similar to those of the tetrahydrofolate pathway (28,31,63). The methyl group is transferred to coenzyme M, another coenzyme of methanogens (109). Methyl coenzyme M is the substrate for the methyl reductase complex which performs the final reduction to methane (30,36,37,86). Hydrogen is the source of electrons at all levels of reduction. When formate is the sole growth substrate, the electrons from its oxidation are used for the reductions.

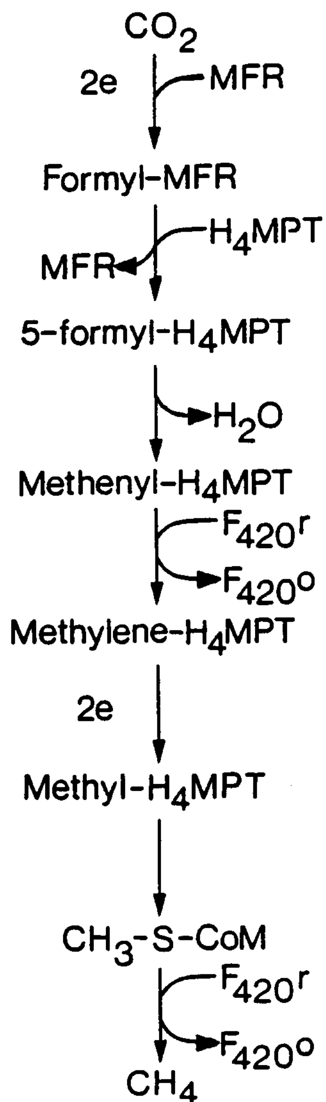


Figure 1. Reduction of CO_2 to CH_4 by methanogenic bacteria. MFR, methanofuran; MPT, methanopterin; F_{420}, coenzyme F_{420}; CoM, coenzyme M; r, reduced form; o, oxidized form.

It is presumed that ATP synthesis is linked to methanogenesis, although the mechanism is not known (61). The reductions involved in methane production probably are part of an electron transport system that leads to the generation of a proton motive force. Several investigators have demonstrated net ATP synthesis in intact cells of methanogenic bacteria by artificial induction of a proton gradient (27,48,49,101).

THE FORMATE DEHYDROGENASE OF *METHANOBACTERIUM FORMICICUM*

Formate dehydrogenase activity has been detected in plants and animals (68,75,80), fungi (13), aerobic bacteria (25,54,83,89), facultative bacteria (35) and strict anaerobes (2,55,60,76,100,117). The formate dehydrogenases of bacteria are involved in anabolic and catabolic pathways. *Clostridium kluyveri* and *Clostridium pasteurianum* reduce CO_2 to formate for cell carbon synthesis (56,110). The homoacetate bacteria *Clostridium formicoaceticum* and *Clostridium thermoaceticum* reduce CO_2 to formate as part of the homoacetate pathway (73,74). These CO_2 reductases are referred to as formate dehydrogenases because their activities are routinely assayed in the direction of formate oxidation. The enterobacteria *Escherichia coli* (35) and *Proteus mirabilis* (67) possess two formate dehydrogenases. One is induced

under anaerobic conditions in the presence of nitrate. This enzyme is involved in the energy-conserving formate-nitrate respiratory pathway (46). The second is part of a formate hydrogenlyase (46,92). The lyase system is thought to allow the organism to maintain an electron balance during fermentative growth by passing electrons from formate to protons. This enzyme is not produced in the presence of nitrate.

The formate dehydrogenase (FDH) of M. formicicum catalyzes the oxidation of formate to CO_2 as the initial step in the conversion of formate to methane (97,100). The enzyme can reduce the dye methyl viologen or a physiological electron acceptor, coenzyme F_{420} (100). Coenzyme F_{420} is a 5-deaza-flavin and is an obligate two electron acceptor. It is found in high amounts in methanogenic bacteria and is thought to be an intermediate in the electron transport chain that is coupled to ATP synthesis (29). Formate dehydrogenase also can reduce FMN and FAD. The apparent K_m's for these cofactors are: F_{420}, 6 µM; FMN, 44 µM; FAD, 98 µM (98). The enzyme cannot reduce $NADP^+$ but formate-dependent reduction of $NADP^+$ can be achieved when FDH is coupled with a coenzyme F_{420}:$NADP^+$ oxidoreductase (98).

Coenzyme F_{420}-mediated formate hydrogenlyase activity has been found in Methanobacterium formicicum (personal

communication). The system consists of the formate dehydrogenase, an F_{420}-reducing hydrogenase and coenzyme F_{420}; it also requires FAD for activity. The function of the formate hydrogenlyase is not known; however it could be involved in the removal of excess reducing potential under stressful conditions. Methanococcus vannielii produces hydrogen from formate when the pH is high (8.8) (107) and Methanobacterium formicicum produces hydrogen from formate when the temperature is high (50°C) (97).

Native FDH of M. formicicum has a molecular weight of 177,000 as determined by gel electrophoresis and gel filtration (100). It is made up of two non-identical subunits with molecular weights of 85,000 and 53,000 as determined by gel electrophoresis under denaturing conditions. From nucleotide sequence data, the molecular weights of the two subunits are calculated to be 75,725 and 43,927, respectively (103). This suggests that the enzyme may not be entirely globular in structure. Amino acid analysis and the nucleotide sequence of the FDH agree with the experimentally-determined stoichiometry of 1,1 (103). Native, active FDH contains (in molar ratios): FAD, 1; molybdenum, 1; zinc, 2; iron, 20; and acid-labile sulfide, 24-29 (100). Formate-dependent reduction of F_{420} requires FAD to be bound to the enzyme (99). Flavin can be removed from FDH by pressure dialysis in buffer that contains

dithionite or formate. Flavin adenine dinucleotide will reconstitute flavin-depleted FDH. Reduced flavin ($FADH_2$) will not reconstitute the flavin-depleted enzyme.

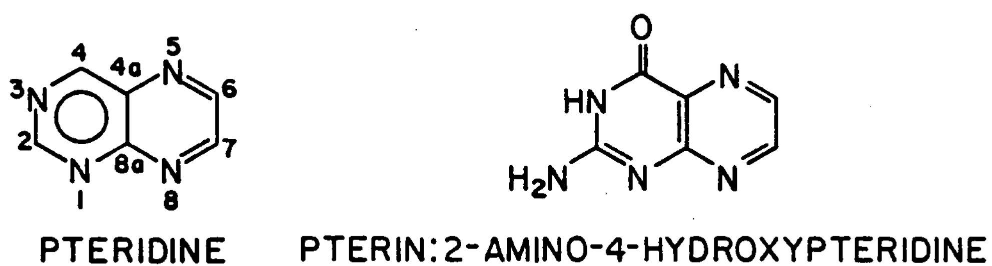
Molybdenum hydroxylases are inhibited by cyanide. My research on the effect of cyanide on the FDH is presented in Section III. Some molybdenum hydroxylases such as xanthine oxidase and xanthine dehydrogenase are irreversibly inhibited by cyanide (114). Removal of the cyanide from the enzyme does not reactivate the enzyme because a terminal sulfur ligand to molybdenum is lost as free thiocyanate (79). Incubation of the desulfo-form of the protein with sulfide reactivates the enzyme. The assimilatory nitrate reductase of _Escherichia coli_ and rat liver sulfite oxidase are reversibly inhibited by cyanide (1). Removal of the cyanide by dialysis reactivates the enzyme because there is no terminal sulfur ligand to the molybdenum.

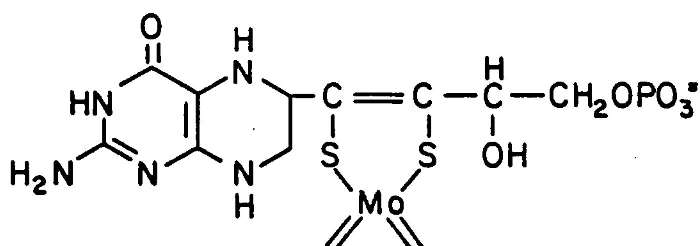
MOLYBDOPTERIN

Two cofactors are known to contain molybdenum. One is the iron-molybdenum cofactor found in nitrogenase (95) and the other is a pterin compound called molybdopterin that is present in xanthine oxidase, nitrate reductase and sulfite oxidase (50). The FDH of _M. formicicum_ was the first FDH shown to have a molybdopterin cofactor (81). My research

on this cofactor is discussed in Section IV. All pterins contain 2-amino-4-hydroxy pteridine and are substituted in the 6 and/or 7 positions (Figure 2). Some pterins function as electron carriers. An example of this is biopterin found in the mammalian liver. Biopterin accepts electrons from a dihydrofolate reductase and donates them to a phenylalanine hydroxylase (96). Tetrahydrofolate and tetrahydromethanopterin contain reduced pteridines and function as carriers of single carbon units. Tetrahydrofolate is used extensively by procaryotes and eucaryotes in biosynthetic as well as energy metabolism (12). Tetrahydromethanopterin, an analog of tetrahydrofolate, has been found only in methanogenic bacteria (see above).

Molybdopterin is a 6-substituted pterin with a 4-carbon side chain. A proposed structure for this compound appears in Figure 2. Under anaerobic conditions molybdopterin released from xanthine oxidase, nitrate reductase or sulfite oxidase can reconstitute the apo-nitrate reductase of *Neurospora crassa* mutant *nit*-1 (50). Due to its extreme lability, the cofactor has never been isolated and characterized while in its native state. All structural information on molybdopterin has come from the study of two oxidized, demolybdo-forms of the cofactor, form A and form B (51). Form A is obtained by boiling the





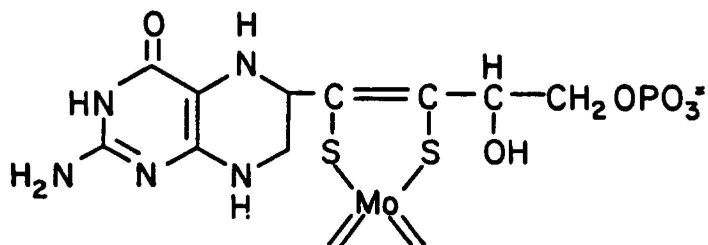
MOLYBDOPTERIN
(PROPOSED STRUCTURE)

Figure 2. Pteridine, pterin and molybdopterin structures.

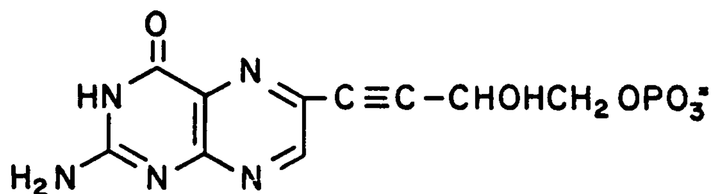
molybdoenzyme at pH 2.5 in the presence of iodine, potassium iodide and air. The fluorescent blue compound is then separated from denatured protein. Form A has a characteristic fluorescence spectrum with excitation/emission maxima of 380 nm/460 nm when in 1 M NH_4OH. Form B is prepared by the same method, except the cofactor is not exposed to iodine and potassium iodide. Form B also fluoresces blue, but with excitation/emission maxima of 390 nm/470 nm when in 1 M NH_4OH.

The structures of the two oxidized forms of molybdopterin are presented in Figure 3. Both have a 4-carbon side chain with one phosphate group positioned at the end of the chain. Kruger and Meyer (69) have shown that form A of a molybdopterin found in the carbon monoxide dehydrogenase from *Pseudomonas carboxydoflava* has 2 phosphate groups. They have named this pterin bactopterin. Bactopterin is capable of reconstituting the apo-nitrate reductase of *N. crassa* mutant *nit*-1.

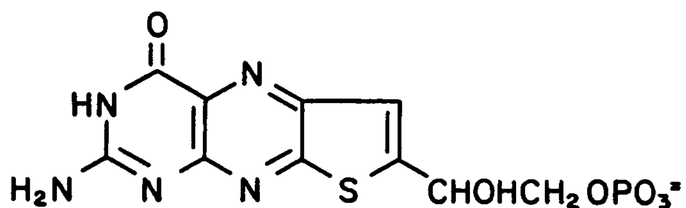
Extended x-ray absorbtion fine structure spectroscopy of the molybdenum-site in xanthine oxidase revealed that the metal is liganded to two sulfurs (donor ligands), one terminal sulfur and one terminal oxygen (16,21). Similar research showed that the molybdenum of sulfite oxidase has two donor sulfur ligands and two terminal oxygen ligands (21). Cyanide inactivation of xanthine oxidase produces a



MOLYBDOPTERIN
(PROPOSED STRUCTURE)



FORM A



FORM B

Figure 3. The proposed structure of molybdopterin and the experimentally determined structures of forms A and B of molybdopterin.

"desulfo" form of the enzyme that has two donor sulfur ligands and two terminal oxygen ligands (4,6). Based upon these results and the known structures of forms A and B of molybdopterin, it is postulated that molybdenum is liganded by two sulfurs to the first and second carbons of the cofactor side chain (Figure 2).

THE ROLE OF MOLYBDENUM IN MOLYBDOENZYME ACTIVITY AND SYNTHESIS

Molybdenum, tungsten and vanadium are transition metals belonging to the Group VI B. They all have nearly identical atomic radii and are present in similar enzymes. Although many enzymes require molybdenum for activity, active tungsten- or vanadium-containing enzymes have been isolated. For example the formate dehydrogenases of *Clostridium formicoaceticum* (73), *C. thermoaceticum* (77) and *C. acidiurici* (113) are more active when they contain tungsten than when they contain molybdenum. An active, vanadium-containing nitrogenase has been isolated from *Azotobacter vinelandii* (41).

The effect molybdenum or tungsten has on the synthesis or stability of molybdoenzymes varies depending upon the enzyme and the organism. Nitrogenase consists of a dinitrogenase and a dinitrogenase reductase. The dinitrogenase contains molybdenum in an iron-molybdenum

cofactor and the dinitrogenase reductase is an iron protein (for reviews of nitrogenase see 90 and 102). Synthesis of either nitrogenase component by the cyanobacterium *Anabaena cylindrica* (42) or by *Plectonema boryanum* (85) is unaffected by the absence of molybdenum or presence of tungsten. *Rhodopseudomonas capsulata* requires molybdenum for the synthesis of stable nitrogenase proteins; however tungsten does not induce synthesis of the enzyme (43).

Under molybdenum-deficient conditions *Klebsiella pneumoniae* has been reported to have low levels of both nitrogenase proteins (18,57). More recently both components were determined to be present in *Klebsiella pneumoniae* regardless if molybdenum or tungsten was present (45). Transcription of the nitrogenase genes (the *nif* HDK operon) in this organism occurs in the presence or absence of molybdenum, but molybdate is required for maximum expression (26).

Azotobacter vinelandii does not produce a stable iron-molybdenum protein unless molybdenum is present (84). A second nitrogen fixation system is encoded for by a separate gene with homology to *nif* H only (10,11,47). This system is active only when the molybdenum concentration is below 100 nM (47). Transcription of both systems is dependent upon molybdenum. When molybdenum concentrations are below 25 nM, genes which code for the iron-molybdenum

protein (*nif* D and *nif* K) are not transcribed (47), but the gene for the alternative nitrogenase is synthesized.

The absence of molybdenum or presence of tungsten does not always affect the synthesis of a stable molybdopterin-containing enzyme. However the level of nitrate reductase protein in cauliflower is low when molybdenum is in limiting concentration (88) and the formate dehydrogenase and nitrate reductase of *Escherichia coli* are synthesized at low levels (approximately 30% maximum) in the absence of molybdenum (34). In *E. coli* the nitrate reductase protein is synthesized in high amounts when tungsten is added to the medium, but the amount of formate dehydrogenase remains low. It is not known whether the messenger RNAs for the enzymes of *E. coli* or cauliflower were produced under these conditions.

Autogenous control of molybdoenzyme synthesis has been demonstrated in *E. coli* (15,91), *Neurospora crassa* (111) and *Klebsiella pneumoniae* (26). Although no direct response to molybdenum was observed, Bonnefoy et al. (15) showed that the autoregulation of the *E. coli* nitrate reductase required the molybdopterin cofactor and the intact structural protein of nitrate reductase. Similarly Tomsett and Garrett (111) showed that the autoregulation of *N. crassa* nitrate reductase was dependent on the structural integrity of the enzyme. Dixon et al. (26) demonstrated

that the molybdenum-iron protein of nitrogenase in K. pneumoniae acted as a positive activator in the regulation of nif H expression (nif H contains the promoter region of the nif HDK operon). This type of autogenous control required both an intact molybdenum-iron protein and molybdate for maximal expression of the operon.

TRANSCRIPTIONAL CONTROL IN ARCHAEBACTERIA

Very little is known about transcriptional control in archaebacteria. Sment and Konisky showed a two-fold increase of mRNA specific to the his A gene of Methanococcus voltae when the gene was derepressed by aminotriazole (104). This suggests that the histidine biosynthetic genes of M. voltae are regulated at the level of transcription. In the absence of aminotriazole the size of the mRNA is 1.5 kb, but when aminotriazole is present the transcript is very large (9 to 10 kb). It is not known whether the size difference is due to processing of the message or if more than one region homologous to the his A gene exists.

Studies of mutants of Halobacterium halobium revealed that bacterioopsin synthesis is controlled at the transcriptional level. Bacterioopsin is the protein moeity of bacteriorhodopsin, the retinal protein that functions as a light-driven proton pump in the purple membranes of H.

halobium (108). The proton translocation results in the generation of a transmembrane electrochemical gradient that can be used to produce ATP. Mutants of H. halobium that do not express the bacterioopsin gene (bop) occur at a very high frequency (10^{-4}) (94). This is due to the transposition of insertion elements into the bop gene. Some of these mutants do not express the gene due to insertions into the structural portion of the gene. Other mutants transcribe the bop gene at a decreased level or not at all when insertions occur in the upstream region. Another gene (brp) is 526 bases upstream of the bop gene and is transcribed in the opposite direction (9). The brp gene is not transcribed when insertion elements enter its coding region. When this occurs the bop gene is not expressed either. Revertants of Brp^- restore the expression of the bop gene (93).

Translational control has not yet been demonstrated in archaebacteria. Methanogenic bacteria have sequences (similar to Shine-Dalgarno sequences) that they may use to identify translation initiation sites on mRNAs (14,22). Interestingly no Shine-Dalgarno regions have been found in the halophiles. More research is needed to determine just how archaebacteria initiate and terminate translation. Translational control in eubacteria has been established (87); however it is not known how extensive it may be. For

more information on the molecular biology of archaebacteria the reader is referred to a recent minireview (23) and to Woese and Wolfe (115).

SECTION III. CYANIDE INACTIVATION OF FORMATE DEHYDROGENASE FROM *METHANOBACTERIUM FORMICICUM*

SUMMARY

Incubation of oxidized formate dehydrogenase with cyanide resulted in an irreversible loss of enzyme activity which could not be restored by treatment with sulfide. Equimolar amounts of thiocyanate were released from cyanide-treated formate dehydrogenase suggesting the loss of one terminal sulfur ligand to molybdenum. Formate dehydrogenase was at least 100-fold more sensitive to cyanide than xanthine oxidase was to cyanide. These results along with electron paramagnetic resonance spectroscopy of the cyanide-inactivated enzymes (3) suggest that the mode of inactivation of formate dehydrogenase is similar to that of xanthine oxidase. The result is the formation of a "desulfo" form of formate dehydrogenase due to the loss of a terminal sulfur ligand of molybdenum. The inability of the enzyme to be reconstituted by sulfide suggests that other differences in the molybdenum environment exist between formate dehydrogenase and xanthine oxidase.

INTRODUCTION

Formate dehydrogenase (FDH) catalyzes the oxidation of formate to CO_2 in aerobic, anaerobic and facultatively anaerobic eubacteria. Among the archaebacteria only methanogenic bacteria have been shown to contain FDH. Approximately 50% of the methanogens examined are capable of utilizing formate as an electron donor for methanogenesis (1). Formate dehydrogenase isolated from Methanococcus vannielii has been shown to contain Se, Mo and Fe/S centers (13).

The FDH purified from Methanobacterium formicicum is an extremely oxygen-sensitive, soluble enzyme that contains FAD, Fe/S centers and a Mo prosthetic group (6,17,18,19). The latter is thought to be present in combination with a substituted or modified pterin similar to molybdopterin (12,15). Molybdopterin, while possibly existing in a number of very similar forms, has been demonstrated to be present in a wide variety of molybdenum-containing enzymes isolated from diverse sources.

With the exception of nitrogenase, which contains molybdenum as part of the iron-molybdenum cofactor (20), molybdenum-containing enzymes are known to be sensitive to inhibition by cyanide with inactivation occurring by either of two possible mechanisms. The oxidized forms of the xanthine-utilizing molybdenum hydroxylases (xanthine

oxidase, xanthine dehydrogenase and aldehyde oxidase) are irreversibly inactivated by cyanide (24). This results in the loss of a terminal sulfur ligand which is released as thiocyanate (14). The molybdoenzymes that do not possess a terminal sulfur ligand, such as sulfite oxidase and nitrate reductase, resist inhibition by cyanide in their oxidized state. In the reduced state all molybdoenzymes are reversibly inhibited by exposure to cyanide. Formate dehydrogenase has previously been shown to be non-competitively inhibited by cyanide with a K_i of 6 μM (17); however the effects of this inhibitor upon the properties of the enzyme's cofactors and site of action have not been studied. In this section I report the results of enzyme inactivation studies to define the mode of inhibition of FDH by cyanide and to facilitate comparison with other molybdenum hydroxylases.

MATERIALS AND METHODS

Cell material. Methanobacterium formicicum strain JF-1 was cultured with either formate or H_2/CO_2 as the sole energy source as previously described (16).

Enzyme preparation and assay. Formate dehydrogenase was anaerobically purified as described by Schauer and Ferry (17). Formate dehydrogenase activity was assayed as described (17) using the formate-dependent reduction of

methyl viologen at 603 nm or coenzyme F_{420} at 420 nm. One unit of activity was the amount of enzyme that reduced 1 µmole of electron acceptor per minute. Xanthine oxidase (Sigma, St. Louis, MO) assays were carried out in 50 mM potassium phosphate buffer, pH 7.5. The oxidation of xanthine (150 µM) in the presence of air was monitored at 295 nm. One unit of activity was the amount of enzyme that oxidized 1 µmole of xanthine (ε_{295} = 12.2 mM^{-1} cm^{-} at pH 7.5) per minute. Both enzyme activities were expressed as units per milligram of protein. Protein was determined by the method of Bradford (8) with bovine serum albumin as standard.

Formate dehydrogenase (6.0 to 12.0 mg) in 50 mM potassium phosphate buffer (pH 7.5) was incubated for 1 hr anaerobically at 23°C in 5 mM KCN in order to liberate thiocyanate. The treated enzyme was taken to dryness by pressure dialysis in an Amicon ultrafiltration apparatus equipped with a YM-30 membrane (Amicon, Lexington, MA). The effluent was tested for thiocyanate by the method of Sorbo (22).

RESULTS

Cyanide inactivation of FDH and xanthine oxidase (XO). The inactivation of FDH by cyanide is shown in Figure 1. Incubation of native enzyme in the oxidized

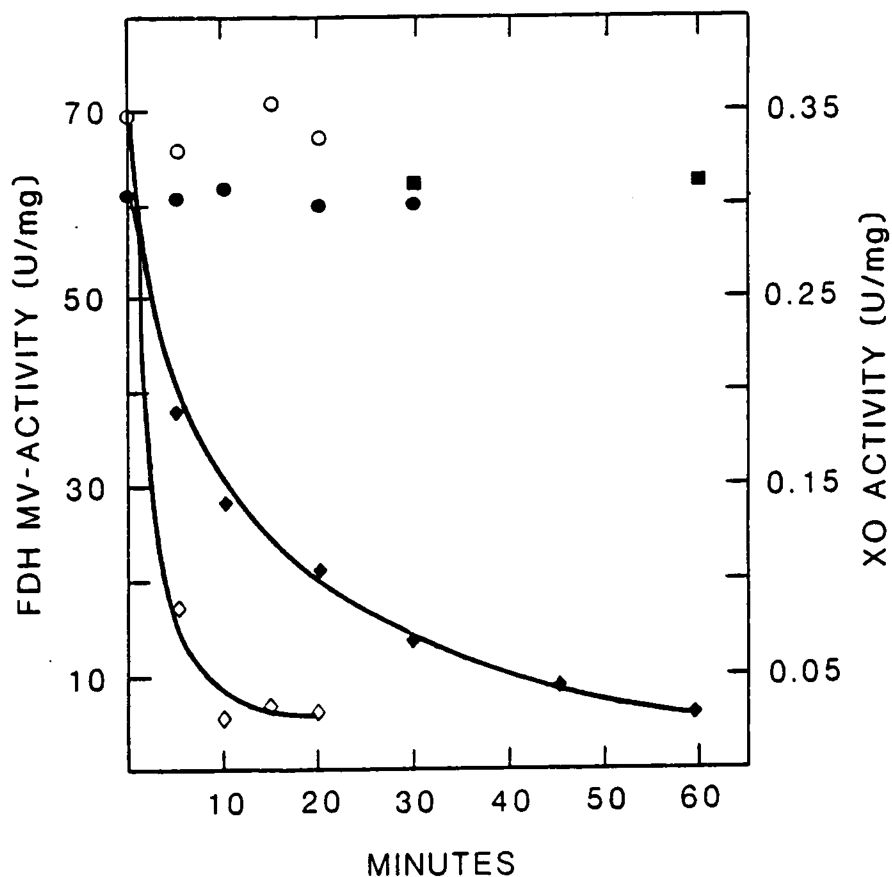


Figure 1. Inactivation of FDH and XO by cyanide. Formate dehydrogenase was incubated with methyl viologen (20 uM) for 25 minutes and then dialyzed. The treated enzyme was incubated in the presence (◇) and absence (○) of KCN (20 uM) in 50 mM potassium phosphate buffer (pH 7.5) under anaerobic conditions. Samples were assayed for formate-dependent reduction of methyl viologen as described in Materials and Methods. Xanthine oxidase (2.2 uM) was incubated in the presence of 20 uM KCN (●), 5 mM KCN (◆) and absence of KCN (■) under the same conditions as described for inactivation of FDH. Xanthine oxidase activity was assayed as described in Materials and Methods.

state at pH 7.5 with excess cyanide resulted in the loss of formate:methyl viologen reductase activity with 50% inactivation after approximately 2 min exposure to 20 µM cyanide. The FDH proved more sensitive to cyanide inhibition than XO. Figure 1 shows 50% inactivation of XO after 10 min incubation with 5 mM cyanide and little inactivation with 20 µM cyanide. Removal of the cyanide from FDH by anaerobic dialysis failed to restore formate-dependent reduction of methyl viologen or coenzyme F_{420} (Table 1). Control samples subjected to identical incubations, except in the absence of cyanide, showed no significant decrease in activity. After inactivation of FDH (6 and 12 mg, respectively) with 5 mM cyanide, 7.8 and 8.1 nmoles of thiocyanate were released per mg of enzyme. Inactivation of XO with 5 mM cyanide released 8.2 and 8.5 nmoles of thiocyanate per mg of functional protein.

Reactivation of cyanide-inactivated enzymes. Attempts to reactivate the cyanide-treated FDH by incubation with sulfide, both in the presence and absence of dithionite and/or sulfide (14,24), proved unsuccessful (Figure 2). However we did observe the expected reactivation of cyanide-inactivated XO when the inactivated enzyme was incubated with sulfide alone or with sulfide and dithionite (Figure 2).

TABLE 1. Inactivation of formate dehydrogenase by cyanide

Reduction of	No KCN	No KCN and dialyzed	KCN (20 μM)	KCN (20 μM) and dialyzed
MV	70.7±6.6	67.5±3.1	6.2±1.1	7.6±1.4
F_{420}	0.51±0.07	0.40±0.04	0.12±0.02	0.11±0.06

Formate dehydrogenase (6 μM) was oxidized by incubation (anaerobic) with methyl viologen (20 μM). Methyl viologen oxidized enzyme was incubated under anaerobic conditions for 20 minutes with KCN (20 μM) in 50 mM potassium phosphate buffer (pH 7.5). Cyanide-inactivated and control samples were anaerobically dialyzed at 4°C for 24 hours against 50 mM potassium phosphate buffer (pH 7.5). Samples were assayed as described in Materials and Methods. All values are given as units of activity per mg of protein (see Materials and Methods). Abbreviations: MV, methyl viologen; F_{420}, coenzyme F_{420}; KCN, potassium cyanide.

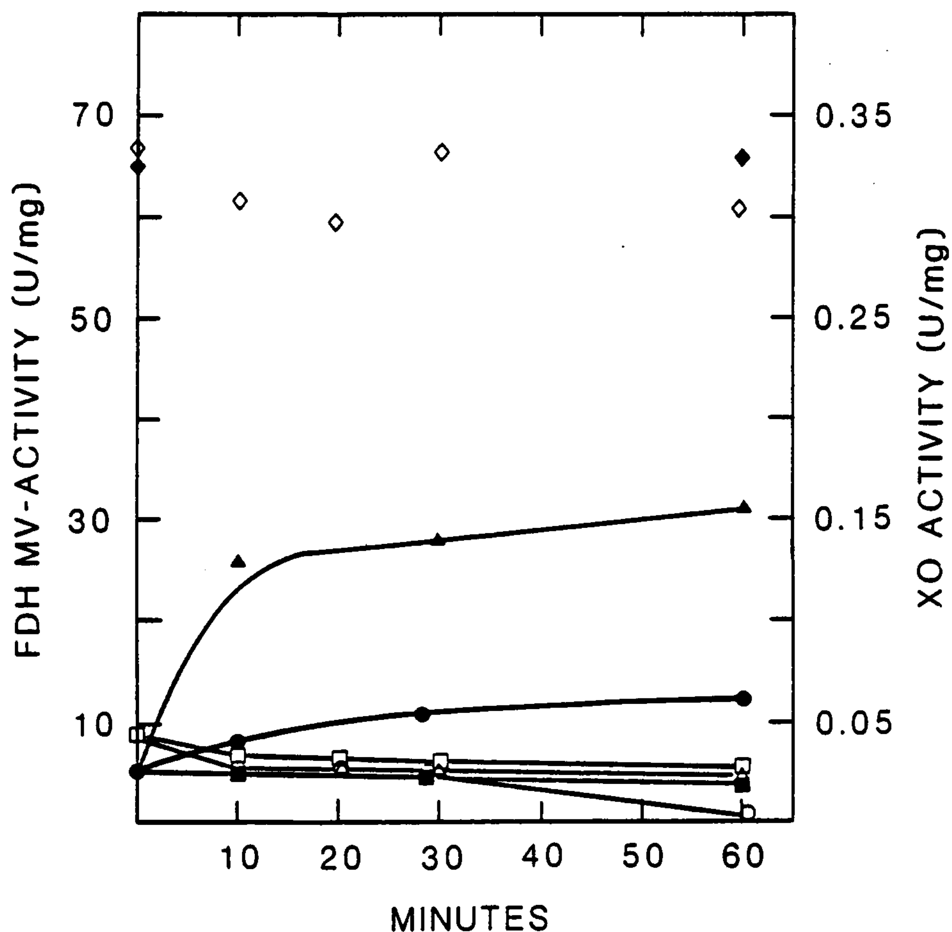


Figure 2. Reactivation of cyanide-treated FDH and XO by sulfide. Cyanide-inactivated FDH (see Figure 1) was incubated in the presence of 1 mM dithionite (□), 1 mM Na_2S (○) and 1 mM dithionite plus 1 mM Na_2S (△) in 50 mM potassium phosphate buffer (pH 7.5) under anaerobic conditions. Aliquots were removed and assayed for formate-dependent reduction of methyl viologen as described in "Materials and Methods". Cyanide inactivated XO (see Figure 1) was incubated in the presence of 1 mM dithionite (■), 1 mM Na_2S (●) and 1 mM dithionite plus 1 mM Na_2S (▲) under the same conditions described for cyanide-inactivated FDH. Untreated and active FDH (◇) and XO (◆) were incubated under the same conditions with no additions.

DISCUSSION

Cyanide inactivation of oxidized molybdenum-containing enzymes XO, xanthine dehydrogenase and aldehyde oxidase has been extensively studied. Cyanide treatment of XO results in the generation of a modified form of the protein referred to as the "desulfo" species. Both chemical (14) and extended x-ray absorption fine structure spectroscopy (7,10) revealed that cyanide treatment results in the extraction of a terminal sulfur ligand of the molybdenum. The sulfur is released as thiocyanate. The alteration in the structure of the Mo center is reflected in an inability of the desulfo enzyme to be reduced by substrate and in changes in the Mo(V) electron paramagnetic resonance (EPR) spectra (4). No effects of cyanide treatment have been observed for any of the other redox cofactors of the enzyme.

Incubation of the purified, oxidized, native FDH with cyanide resulted in the irreversible inhibition of catalytic activity. Based upon a molecular weight of 177,000 for FDH (19), a ratio of 1.4 thiocyanate per cyanide-inactivated enzyme molecule was obtained. However the _fdh_ gene encodes a protein with a calculated molecular weight of 119,652 (21), suggesting that 0.9 thiocyanate was released per enzyme molecule. The detection of stoichiometric amounts of thiocyanate from cyanide-treated

FDH suggests that the enzyme has one terminal sulfur ligand of Mo similar to that of xanthine-oxidizing Mo hydroxylases. The reason that inactive FDH was not reactivated by sulfide is not known. The conditions may not have been optimal for FDH reactivation, but this does not explain the different results for the FDH and XO. Alternatively sulfide reconstitution of the desulfo FDH may not be possible due to the structure of the cofactor. The molybdopterin cofactor is discussed in Section IV.

Partial reduction of the native enzyme with either formate or dithionite resulted in a Mo(V) EPR signal with g-values of $g_1 = 2.020$, $g_2 = 2.006$ and $g_3 = 1.997$ (6). In addition a superhyperfine interaction of two equivalent, strongly-coupled protons was observed. This was repeated in this study; however cyanide inactivation of the oxidized native enzyme resulted in a shift of the Mo(V) EPR signal to a higher field ($g_1 = 2.005$, $g_2 = 1.998$, $g_3 = 1.989$) and a reduction of the superhyperfine splitting (3). A shift to a higher field strength after cyanide treatment has been found for molybdenum hydroxylases that have a terminal sulfur ligand (4). The reduction of the superhyperfine interaction of the protons suggests that a proton in the Mo environment was lost with the terminal sulfur. These results are in agreement with the loss of a terminal sulfur ligand from molybdenum when FDH is inactivated by cyanide.

The EPR g values of both native and cyanide-inactivated formate dehydrogenase are significantly larger than those reported for other molybdenum hydroxylases. The g_{av} of native formate dehydrogenase (g_{av} = 2.008) is the largest encountered for any molybdenum center in a biological system (g_{av} = 1.982 to 1.964) (5,7). However the g values are within the range of 2.025 to 1.949 observed for the principal g values of Mo(V) spectra (9). It is not known why the g values are so high for the molybdenum center of formate dehydrogenase, but it does suggest that the molybdenum domain of formate dehydrogenase is different from that of other molybdoenzymes. The results in Section IV indicate that the side chain of the molybdopterin cofactor of the formate dehydrogenase is not identical to those of other molybdoenzymes. Perhaps this change in structure influences the electron paramagnetic resonance spectrum. It should be noted that arsenite perturbs the Mo(V) spectra of xanthine oxidase and aldehyde oxidase (5). The Mo(V) spectrum of formate dehydrogenase is not affected by arsenite (6).

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SECTION IV. THE MOLYBDOPTERIN COFACTOR FROM FORMATE DEHYDROGENASE OF METHANOBACTERIUM FORMICICUM

SUMMARY

The molybdopterin cofactor from the formate dehydrogenase of *Methanobacterium formicicum* was studied. The cofactor was released by guanidine-denaturation of homogeneous enzyme which also released greater than 80% of the molybdenum present in the enzyme. The anoxically-isolated cofactor was nonfluorescent; however it fluoresced with spectra similar to that of described molybdopterin cofactors after exposure to air. Aerobic release from acid-denatured formate dehydrogenase in the presence of iodine (I_2) and potassium iodide produced a mixture of fluorescent products. Alkaline permanganate oxidation of the mixture yielded pterin-6-carboxylic acid as the only detectable fluorescent product. The results showed that the cofactor from formate dehydrogenase contained a pterin nucleus with a 6-alkyl side chain of unknown structure. Covalently bound phosphate was also present. The isolated cofactor was unable to complement the cofactor-deficient nitrate reductase of *Neurospora crassa* mutant *nit*-1.

INTRODUCTION

All known molybdoenzymes, with the exception of nitrogenase, contain a pterin cofactor (9,16). Molybdopterin cofactors are present in enzymes with a wide diversity of functions and are found in both eucaryotes and eubacteria. The cofactors studied have in common a pterin nucleus with a 6-alkyl side chain and all are able to complement the cofactor-deficient nitrate reductase of *Neurospora crassa* mutant *nit*-1 (9).

Methanogenic bacteria are phylogenetically distant from eubacteria and eucaryotes (7) and contain unique cofactors which include 7-substituted pterins (13,19), such as methanopterin (12). A cofactor with pterin-like fluorescence is present in the molybdenum-containing formate dehydrogenase (FDH) from *Methanobacterium formicicum* (17,18). Here I describe the properties of this cofactor.

MATERIALS AND METHODS

Anoxic purification of the molybdopterin cofactor. FDH was purified to homogeneity as previously described (18). Protein was determined with protein dye reagent (Bio-Rad, Richmond, CA) according to Bradford (4) with bovine serum albumin (Sigma, St. Louis, MO) as the protein standard. Enzyme in 50 mM potassium phosphate buffer (pH

7.5) was made 6 M in guanidine-hydrochloride and boiled 15 min. The boiled enzyme was applied to a 1.5 cm x 32 cm Fractogel TSK-HW40S (EM Science, Gibbstown, NJ) column equilibrated with anoxic 0.1 M ammonium carbonate and was eluted with the same buffer. The strictly anoxic manipulations and preparation of anoxic buffer solutions used to exclude air at each step were as previously described (17). Column fractions were collected and stored as frozen pellets in liquid nitrogen.

Aerobic purification of cofactors. Bound flavin adenine dinucleotide (FAD) was removed from the FDH by pressure dialysis in an Amicon (Danvers, MA) ultrafiltration cell fitted with a YM30 (M_r=30,000 cutoff) filter (18). To the enzyme solution (1 ml) was added 9 ml of anoxic 50 mM potassium phosphate buffer (pH 7.5) containing 10 mM sodium formate. This mixture was concentrated to 1 ml under nitrogen. The procedure was repeated 5 times followed by 5 washes with anoxic 50 mM potassium phosphate buffer (pH 7.5)

Cofactors from FDH or xanthine oxidase (XO) were aerobically released by boiling for 20 min at pH 2.5 in the presence of air, iodine (I_2) and potassium iodide according to Johnson and Rajagopalan (11). The pterin oxidation products were separated from protein and FAD by gel filtration on a 1.5 cm x 32 cm Fractogel TSK-HW40S column

equilibrated with 0.1 M ammonium carbonate and eluted with the same solution. The fluorescent FAD-free fractions were pooled and lyophilized. The residue was redissolved either in 1 M ammonium hydroxide for spectral analysis or in water for high performance liquid chromatography (HPLC) analysis.

Phosphate determination. The cofactor was aerobically isolated from FAD-depleted FDH as described above, except the dialysis buffer was replaced with 10 mM Tris (pH 7.6) to remove unspecifically bound Pi from the enzyme. The lyophilized cofactor was redissolved in water and the residual unspecific Pi was removed by the method of Nielsen and Lehninger (15).

Samples were wet-ashed according to the procedure of Meyer and Rajagopalan (14). The Pi released was quantitated according to the method of Ames (1) using a Hitachi model 100-60 spectrophotometer (Tokyo, Japan). An absorbance of 0.260 at 820 nm was observed for 10 nanomoles of Pi. Ashed Pi (10 nanomoles) gave an absorbance of 0.240 at 820 nm.

Permanganate oxidation. The cofactors were aerobically purified as described above. Lyophilized samples were redissolved in 0.1 N sodium hydroxide. After the addition of 10 mg/ml potassium permanganate, the samples were placed in a boiling water bath for one hour. The reaction was stopped by the addition of 95% ethanol and

the precipitated manganese dioxide was removed by centrifugation. Samples for HPLC analysis were applied directly to the column.

Cofactor activity. Cofactor activity was assayed by complementation of nitrate reductase in extracts of the cofactor-deficient Neurospora crassa mutant strain nit-1. This strain, obtained from John Smarrelli, was grown on Vogel's medium to induce synthesis of nitrate reductase (5). Extracts were prepared according to Amy and Rajagopalan (2), except phenylmethylsulfonyl fluoride was omitted. All procedures were performed anoxically.

The cofactors from FDH (11 mg/ml) and XO (15 mg/ml) (Sigma) were released under anoxic conditions by the following methods: (i) 5 µl of an enzyme solution was added to 95 µl of 6 M guanidine-hydrochloride in 10 mM Tris-hydrochloride (pH 8.0) and placed on ice for 5 min, (ii) 30 µl of an enzyme solution was added to 120 µl of 1.25% (w/v) sodium dodecyl sulfate in 125 mM potassium phosphate (pH 7.4) and the mixture was boiled for 1 min, or (iii) 5 µl of an enzyme solution was added to 90 µl of 0.1 M sodium chloride in 0.1 M potassium phosphate (pH 7.4), the pH was lowered to 2.3 with 2 M hydrochloric acid and the mixture was placed on ice for 2 min. The pH of each sample was adjusted to 7.0 with 4 M sodium hydroxide. Reconstitution mixtures contained 1 µl of denatured enzyme solution added

to 249 µl of <u>Neurospora</u> <u>crassa</u> mutant <u>nit</u>-1 extract (freshly prepared) and were incubated for 30 min at 24°C. The reconstitution mixtures contained a final concentration of 10 mM sodium ascorbate and 10 mM sodium molybdate. Nitrate reductase activity was assayed by adding 50 µl of the reconstitution mixture to 440 µl of the assay mixture. The assay mixture contained 0.1 M potassium phosphate, pH 7.4; 10 µM FAD; 10 mM sodium molybdate; 5 mM sodium sulfite (freshly prepared); 10 mM sodium molybdate and 10 mM sodium nitrate. The reaction was initiated with 10 µl of 10 mM NADPH and then incubated for 30 min at 24°C. Nitrate reductase activity was assayed by the diazo-coupling colorimetric assay which measures the production of nitrite (8). No correction was made for NADPH interference. The color intensity was determined at
540 nm with a Perkin-Elmer Lambda 1 spectrophotometer (Oak Brook, IL).

HPLC. Two reverse phase 5 µ C18 columns (Bio-Rad) were connected in series for total column dimensions of 400 mm x 4 mm. The effluent was monitored with a Perkin-Elmer 650-10S fluorescence spectrophotometer fitted with a flow-through cell. The mobile phase was 10 mM potassium phosphate buffer at pH 7.0 and was applied to the column with a Bio-Rad 1330 HPLC pump at 0.5 ml/min at a pressure of 116 kPa. Pterin standards were dissolved in 0.1 N

sodium hydroxide before analysis.

Spectra. Absorption spectra were recorded on a Perkin-Elmer 552 double beam spectrophotometer. Fluorescence spectra were obtained with a Perkin-Elmer model 650-10S fluorescence spectrophotometer. Fluorescence spectra were not corrected.

Molybdenum determinations. Molybdenum was quantitated by atomic absorption spectroscopy as previously described (19).

Chemicals. Except where otherwise stated, XO was a gift from Michael Barber. Guanidine-hydrochloride (99%) and pterin-6-carboxylic acid (98%) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Methanopterin was a gift from J. C. Escalante-Semerena. The following were gifts from J. A. Keltjens: 6-methylpterin-7-carboxylic acid, 7-methylpterin-6-carboxylic acid and 7-methylpterin. Pterin-6,7-dicarboxylic acid was produced from 6-methylpterin-7-carboxylic acid by alkaline permanganate oxidation. Pterin-7-carboxylic acid was produced from 7-methylpterin by alkaline permanganate oxidation. HPLC grade ammonium carbonate was purchased from Fisher Scientific (Pittsburgh, PA). All other chemicals and reagents were obtained from Sigma.

RESULTS

Cofactor isolation. Anoxic gel filtration chromatography of guanidine-denatured FDH resolved 3 major peaks of material that fluoresced only after exposure to air (Figure 1). Fluorescence could not be detected before 10 hours of exposure and maximum fluorescence intensity was reached after 24 hours. Samples not exposed to air showed no fluorescence after 24 hours. In two separate experiments, peak A also contained 81 and 93% of the molybdenum present in the enzyme. Since sodium molybdate also eluted in the same position as peak A, it was not possible to determine whether molybdenum was associated with this material.

The fluorescence spectra of the pooled peak A fractions (Figure 2) showed excitation maxima of 385 nm, 325 nm and 290 nm, and a maximum emission of 465 nm at approximately pH 11. Lowering the pH to approximately 6 shifted the excitation maxima to 375 nm and 310 nm and the maximum emission to 455 nm. The fluorescence decreased in intensity with decreasing pH (Figure 3). These properties were similar to those of described molybdopterin cofactors (9,10,11). The FDH cofactor was distinct from methanopterin which fluoresces maximally near pH 3.5 with an excitation maximum at 350 nm and an emission maximum at 440 nm (12).

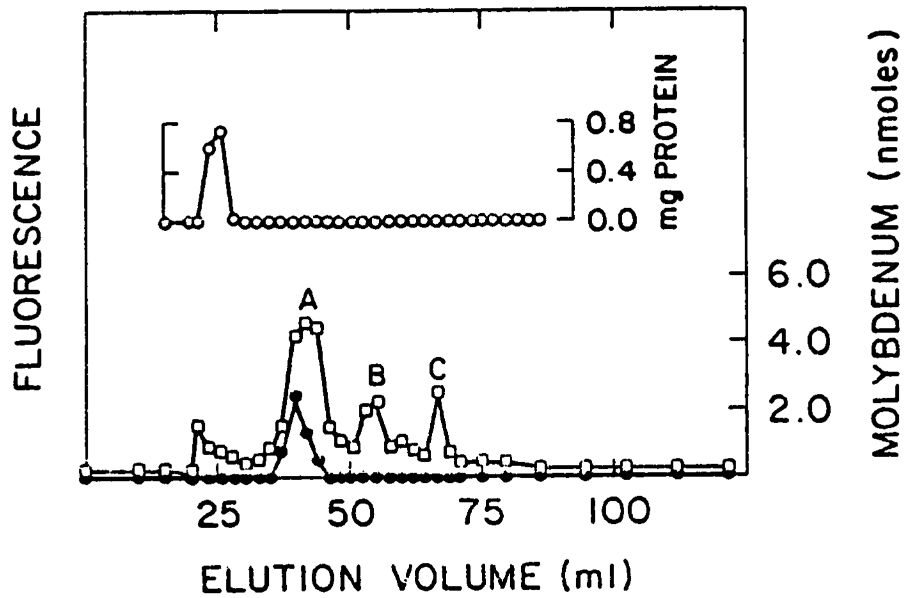

Figure 1. Anoxic column chromatography of molybdenum and of fluorescent compounds released from formate dehydrogenase. Enzyme (1.1 mg) denatured with guanidine was anoxically chromatographed on a Fractogel TSK-HW40S gel filtration column. Samples of fractions were assayed for molybdenum (●) and fluorescence (□) with excitation at 380 nm and emission at 465 nm after 24 hours exposure to air.

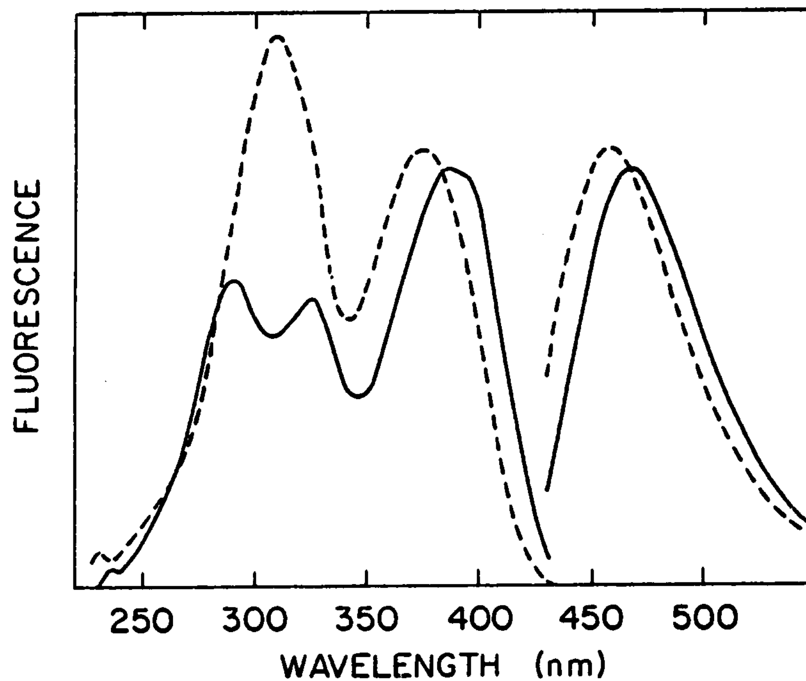

Figure 2. Fluorescence spectra of the formate dehydrogenase cofactor. Fractions from peak A (Figure 1) were pooled and lyophilized. Spectra are of cofactor redissolved in 1 M NH_4OH (——) or water (---). Spectra were taken after the samples had been exposed to air for 24 hours. Excitation was set at 380 nm and emission was set at 465 nm.

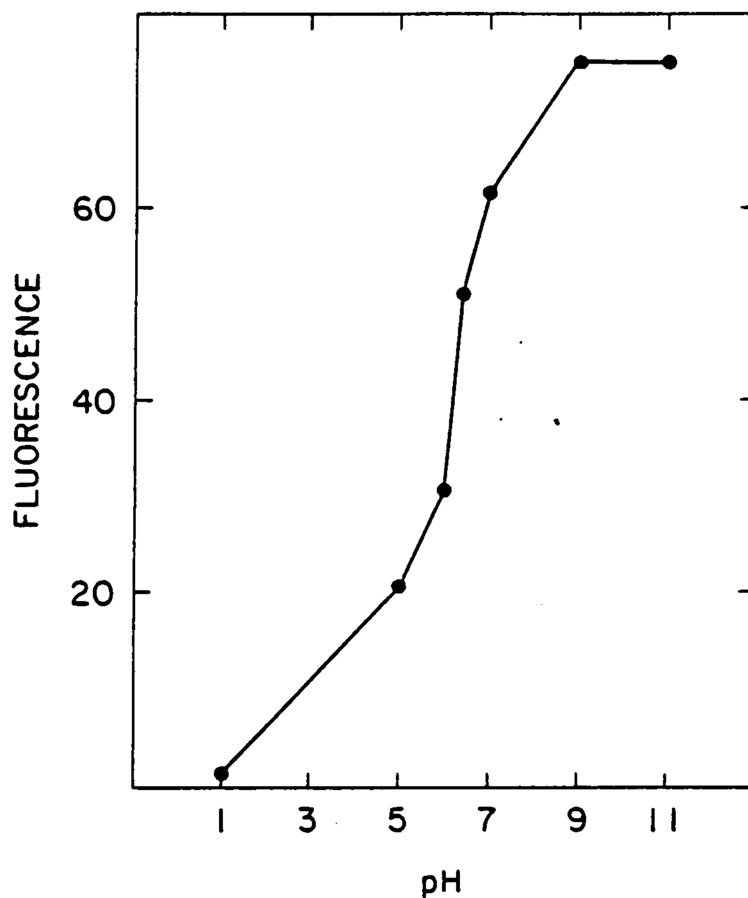

Figure 3. Effect of pH on fluorescence of the formate dehydrogenase cofactor. The lyophilized material described in Figure 3 was dissolved in water and exposed to air for 24 hours. The pH was adjusted with hydrochloric acid or ammonium hydroxide. Relative fluorescence was measured at an excitation of 380 nm and emission of 465 nm.

The fluorescence spectra of peaks B and C (Figure 1) indicated the presence of pterin derivatives (data not shown). When the enzyme was aerobically denatured and exposed to air for 24 hours before anoxic gel filtration, at least four peaks with pterin fluorescence were resolved in addition to peak A. These results suggested that the cofactor was unstable and that degradation occurred during isolation.

Properties of the pterin nucleus. Boiling XO aerobically at pH 2.5 in the presence of potassium iodide and iodine (I_2) releases a form of the cofactor that is converted to pterin-6-carboxylic acid by alkaline permanganate oxidation (9,10). The cofactors from XO and the FDH were released in this manner and purified by gel filtration chromatography (Figure 4). The bulk of pterin oxidation products from both enzymes were separated from denatured protein and FAD. The FAD-free fractions from each cofactor were pooled and analyzed by reversed-phase HPLC which indicated that a mixture of several fluorescent derivatives were obtained from each. The excitation and emission spectra of the mixtures from both cofactors were nearly identical to each other and to the published spectra for form A of the cofactor from XO (10,11). Alkaline permanganate oxidation of the products from both cofactors produced one fluorescent compound, detectable by HPLC and

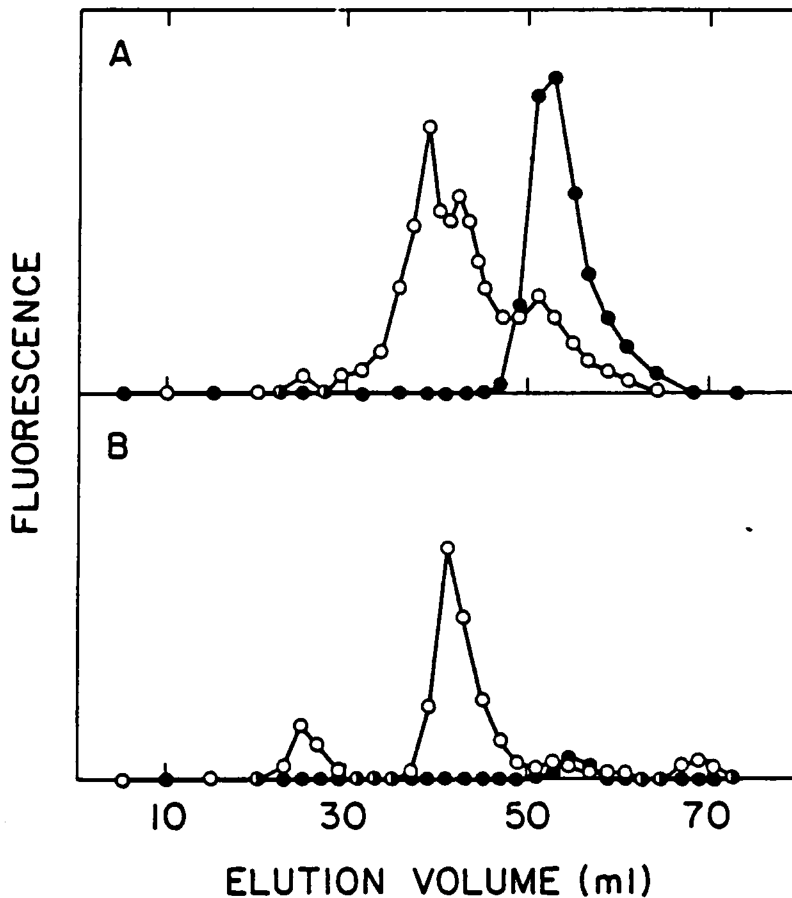

Figure 4. Gel filtration chromatography of milk xanthine oxidase cofactor (A) and formate dehydrogenase cofactor (B). The cofactors were released from both enzymes (1.0 mg of protein) by boiling at pH 2.5 in the presence of air, potassium iodide and I_2 and then chromatographed on a Fractogel TSK-HW40S column as described in Materials Methods. Molybdopterin cofactor fluorescence (○) was monitored with excitation at 380 nm and emission at 465 nm. Flavin fluorescence (●) was monitored with excitation at 445 nm and emission at 520 nm.

identified as pterin-6-carboxylic acid (Figure 5). Pterin-6-carboxylic acid and the FDH cofactor permanganate oxidation product coeluted when chromatographed together (data not shown). The fluorescence spectra of the compound from the FDH cofactor were nearly identical to the spectra of pterin-6-carboxylic acid (Figure 6) and to the spectra of the compound from the XO cofactor (data not shown). These results suggested that the pterin from the FDH cofactor contained a 6-alkyl side chain of unknown structure. The fluorescent permanganate oxidation product from the FDH cofactor was well resolved from the pterin-6,7-dicarboxylic acid, 6-methylpterin-7-carboxylic acid, pterin-7-carboxylic acid, or 7-methylpterin standards (Figure 5). Fluorescence maxima characteristic of the above 7-substituted pterins were absent in the spectra of the permanganate-oxidized formate dehydrogenase cofactor. These results suggested that carbon 7 of the pterin nucleus was not substituted with an alkyl group or a methyl substituent.

Phosphate determination. Figure 4 shows that residual FAD was separated from the FDH cofactor by TSK-HW40S gel filtration. No absorbance or fluorescence characteristic of FAD was detected in the cofactor-containing fractions. Before ashing and after removal of residual unspecific Pi, no Pi was detected in cofactor samples, which indicated

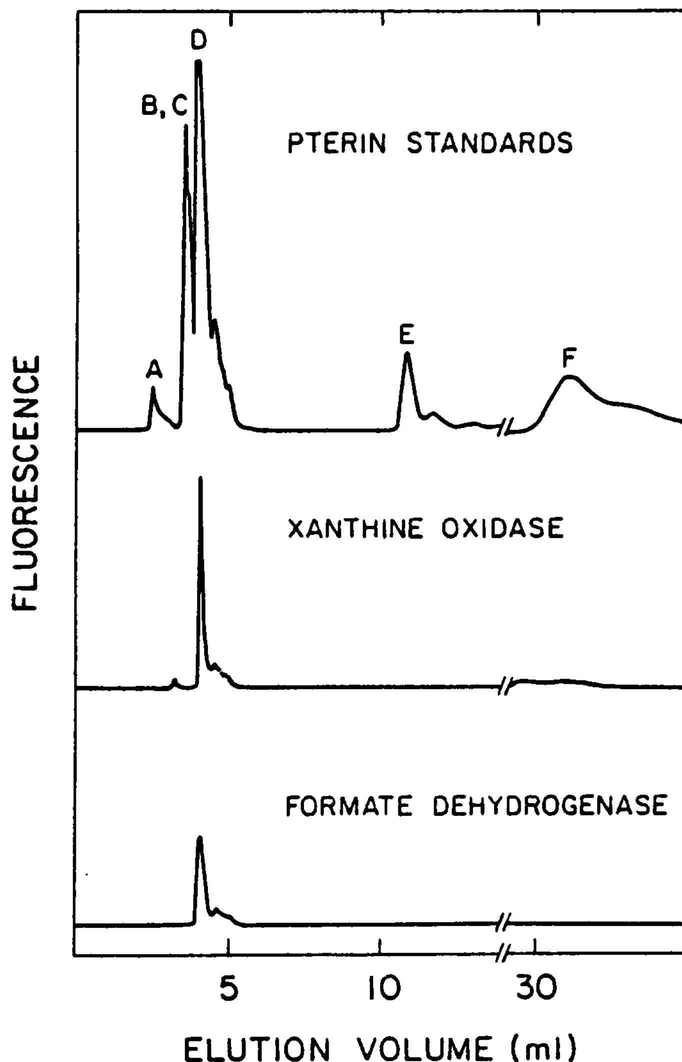

Figure 5. HPLC elution profiles of pterin standards (top) and of permanganate oxidized cofactors from milk xanthine oxidase (middle) and formate dehydrogenase (bottom). Fluorescence was monitored by excitation at 380 nm and emission at 465 nm. Pterin standards: A, pterin-6,7-dicarboxylic acid; B, 6-methylpterin-7-carboxylic acid; C, pterin-7-carboxylic acid; D, pterin-6-carboxylic acid; E, biopterin; F, 7-methylpterin.

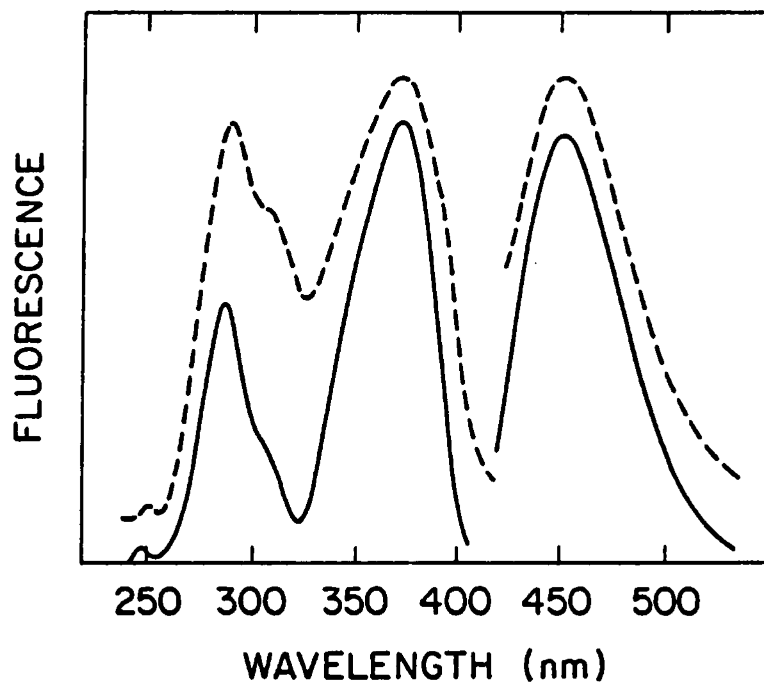

Figure 6. Fluorescence spectra of pterin-6-carboxylic acid (——) and the alkaline permanganate oxidation product of the formate dehydrogenase cofactor (---). The flavin-free fractions (Figure 4) were pooled and the samples were oxidized as described in Materials and Methods. Excitation was set at 365 nm and emission was set at 450 nm. Both samples were in 1.0 M NH_4OH.

that only covalently bound Pi remained. After removal of unspecific Pi and ashing, 16.4 nmol of Pi was detected in cofactor isolated from 1.0 mg of protein (calculated from the mean of 8 determinations of cofactor isolated from 0.34 - 0.68 mg of protein). Based on a MW of 177,000 for the FDH (18), a total of three Pi were obtained per enzyme molecule. It is estimated that the FDH contains one molybdopterin cofactor since the enzyme contains one molybdenum center (3,18). These results suggest that covalently bound Pi was associated with the pterin cofactor. The extreme instability of the cofactor excluded quantitation of the Pi per cofactor; however, the results suggest that more than one Pi per molybdenum center is likely.

Activity. The molybdopterin cofactors from XO and FDH, released by three different methods, were tested for the ability to complement the cofactor-deficient nitrate reductase of the Neurospora crassa mutant nit-1 (Table 1). Although good activity was obtained with cofactor released from XO (Sigma), no significant activity was obtained with the cofactor released from FDH. Cofactor preparations from both enzymes contained approximately the same 380 nm absorbance and fluorescence intensity at 465 nm (in 1 M NH_4OH). Good activity was also obtained when cofactor was released from a 1:1 mixture of the two enzymes. This

TABLE 1. Complementation of Neurospora crassa mutant nit-1 nitrate reductase with cofactors from formate dehydrogenase and xanthine oxidase.

Release[a]	Mean nmol ±SD of nitrite (no. of trials) produced by:[b]		
	XO	FDH	XO + FDH
6 M guanidine hydrochloride	24.6±2.8(7)	0.6±1.0(7)	26.9±1.4(3)
1% sodium dodecyl sulfate	32.4±1.8(3)	0.0±0.0(3)	
pH 2.3	6.3±1.0(3)	0.5±0.7(3)	

[a] See Materials and Methods.

[b] Produced in 30 minutes by 50 µl of N. crassa mutant nit-1 extract reconstituted with 1 µl of denatured enzyme solution (see Materials and Methods). No nitrite was detected in controls when enzyme was omitted from the reconstitution mixture. Abbreviations: XO, xanthine oxidase; FDH, formate dehydrogenase.

indicated that neither the FDH nor the cofactor inhibited complementation. No activity was obtained with the FDH cofactor when the procedure was performed aerobically.

DISCUSSION

The results presented here indicate that the FDH from M. formicicum contained a molybdopterin cofactor. Similar to all other molybdopterins studied, this cofactor contained bound phosphate and was a 6-alkyl substituted pterin. The cofactor was released by denaturation of the protein, indicating that it was noncovalently bound. The presence of this cofactor in FDH from an organism phylogenetically distant from both eubacteria and eukaryotes demonstrates the universality of molybdopterin cofactors.

Unlike methanopterin, a cofactor in the pathway of carbon dioxide reduction to methane (6,12), carbon 7 of the FDH cofactor was not methylated. These results indicate that both 6,7-substituted and 6-substituted pterins exist in methanogenic bacteria. The native structure of the 6-alkyl side chain of molybdopterin cofactors is unknown, but a structure is proposed based upon the characterization of fluorescent metal-free oxidation products of the cofactor from XO (10,11). The cofactor from FDH was unstable. Using strictly anoxic procedures, the cofactor

was isolated in a nonfluorescent form. However whether purified in the presence of air or under strictly anoxic conditions, more than one degradation product was formed. Thus the native structure of the FDH cofactor side chain was not determined. All molybdopterin cofactors studied are able to complement the cofactor-deficient nitrate reductase except the FDH cofactor. This apparent anomaly is unexplained. The native structure of the FDH cofactor may not be significantly different from other molybdopterins, but the FDH cofactor may have been modified upon release from the enzyme. Other hypotheses would be based upon structural differences in the native 6-alkyl side chains of the cofactors. Also the molybdenum electron paramagnetic resonance signal of the native FDH from *M. formicicum* is different from that of all other molybdoenzymes studied in having two g values, g_1 and g_2, and a g_{av} value greater than 2.0 (3). Further research is necessary to determine the native structures from the nonoxidized, metal-containing, native cofactors from *M. formicicum* FDH and other molybdoenzymes.

ACKNOWLEDGEMENTS

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SECTION V. EFFECT OF MOLYBDENUM AND TUNGSTEN ON THE SYNTHESIS OF FORMATE DEHYDROGENASE IN *METHANOBACTERIUM FORMICICUM*

SUMMARY

The effect of molybdate and/or tungstate on the activity and synthesis of the formate dehydrogenase of *Methanobacterium formicicum* was investigated. Growth on hydrogen was unaffected by the concentration of molybdate in the growth medium. Metabolism of formate was inhibited by more than 10-fold when the organism was grown in the presence of tungstate. This inhibition was relieved when tungstate-grown cultures were supplemented with molybdate and allowed to grow. Crude extracts of cells grown in molybdenum-deficient media or with added tungstate contained low amounts of molybdenum and had severely decreased formate dehydrogenase activity. Western blot analysis showed that both subunits of the formate dehydrogenase were produced regardless of the growth condition, but that the level of cross-reacting material decreased when the amount of molybdenum in the cell was low. In order to better quantitate the amount of formate dehydrogenase cross-reacting material, an indirect enzyme-linked immunoassay was used to assay extract protein that had been fractionated by fast protein liquid

chromatography. By this method extracts of cells grown with 10^{-6} M molybdate were shown to contain greater than 10-fold more cross-reacting material than cells grown for several transfers in molybdenum-deficient media. Extracts of cells grown with 10^{-6} M or 10^{-3} M tungstate had approximately the same amount of cross-reacting material as cells grown with 10^{-6} M molybdate. This inactive protein was purified and shown to have the same subunit composition as active enzyme. A fluorescent compound with spectra identical to form A of molybdopterin was released from the inactive enzyme, but the protein contained less than 0.05 molecules of molybdenum or tungstate per formate dehydrogenase. A 462 base fragment of the fdh A gene, specific for one region on the M. formicicum chromosome, was used as a probe for the detection of formate dehydrogenase messenger RNA. The transcript was detected in high amount when molybdenum was limiting and in low amount when the molybdenum supply was high.

INTRODUCTION

Methanobacterium formicicum can use formate or H_2 and CO_2 as the sole energy and carbon source (1,39). The formate dehydrogenase (FDH) activity of this organism is similar in either hydrogen- or formate-grown cells (39). The FDH utilizes methyl viologen or the physiological

electron acceptor coenzyme F_{420} (40,41,42). The FDH consists of two subunits (molecular weights of 85,000 and 53,000) in a 1:1 ratio and contains molybdenum (3,42). May et al. (29) demonstrated that the FDH possesses a pterin cofactor similar, but not identical, to molybdopterin. Molybdenum is thought to be associated with this cofactor. Molybdopterin cofactors are present in molybdoenzymes other than nitrogenase. Nitrogenase has an iron-, sulfur- and molybdenum-containing cofactor (37).

There are many reports of inactive enzymes being synthesized when molybdenum is not available or when tungsten is present in the growth medium (9,10,15,17,19,23,32). Immunoassays have shown that the levels of molybdoproteins are low in certain organisms grown under molybdenum-deficient conditions (14,16,31,33). Molybdenum is required for the transcription of the *nif* HDK genes of nitrogenase in *Azotobacter vinelandii* (20). In addition molybdenum represses the synthesis of an alternative nitrogen fixation system in *A. vinelandii* (5,6,20). Molybdenum has no regulatory effect on the synthesis of nitrogenase in the cyanobacteria *Anabaena cylindrica* (15) and *Plectonema boryanum* (32). The nitrogenase genes in *Klebsiella pneumoniae* are expressed in the presence or absence of molybdenum (19,26); however molybdenum stimulates expression (12). The nitrate

reductases of Escherichia coli and Neurospora crassa contain molybdenum in association with a molybdopterin cofactor (21). Gene expression for both of these enzymes is autogenously controlled (7,47). This control is dependent on the structural integrity of the enzymes, and in the case of the E. coli nitrate reductase, the molybdopterin cofactor is required. This suggests a role for molybdenum in the synthesis of these molybdoenzymes, although the mechanism of action is not fully understood.

Regulation of enzyme synthesis in the methane-producing bacteria has not been extensively studied. Sment and Konisky suggest that the histidine biosynthetic pathway in Methanococcus voltae is transcriptionally controlled (45). Here we report on the effect of molybdenum or tungsten on the growth of M. formicicum along with the effects that these metals have on the activity, synthesis and/or stability of the FDH.

MATERIALS AND METHODS

Cell growth. Methanobacterium formicicum strain JF-1 was grown in culture tubes (16 mm x 150 mm, Belco Glass, Inc., Vineland, NJ) that contained 5 ml of medium. Tubes were sealed with a butyl rubber stopper secured with an aluminum crimp collar (2). Cultures were grown under an atmosphere of $H_2:CO_2$ (80:20) at 40°C. Growth was followed

at 550 nm with a Spectronic 20 spectrophotometer (Bausch & Lomb, Inc., Rochester, NY). One optical density unit corresponds to a cell mass of 0.75 g (dry weight) per liter (38). All glassware for tube or fermentor cultures was acid-cleaned in concentrated sulfuric acid before use.

The basal medium (molybdenum-deficient) was made as described with the following constituents (grams per liter): NH_4Cl_3, 1.48; K_2HPO_3, 1.36; KH_2PO_3, 0.90; NaCl, 0.45; $MgSO_4$, 0.045; $CaCl_2$, 0.06; CH_3COONa, 2.0; Na_2CO_3, 3.0; cysteine-HCl, 0.27; Na_2SeO_3, 0.0002; Na_2S, 0.27; $Fe(NH_4)_2SO_4$, 0.01; resazurin, 0.001. A vitamin solution and a trace mineral solution (no molybdenum or tungsten) were added (both at 1% vol/vol) (50). Sodium formate (0.5% wt/vol) was added to the medium in case formate was needed for full induction of FDH. Large-scale growth of *M. formicicum* was done in 10-liter batches as described (39). Cultures were sparged with $H_2:CO_2$ (80:20) at 300 ml per min. Cells were harvested during log phase with an OD_{550nm} near 2.0. Anoxic harvesting was performed by maintaining the culture under an atmosphere of $H_2:CO_2$ or N_2 and using a continuous flow centrifuge (Cepa, Lehr-Baden, West Germany) operated at 24,000 revolutions per min. The cell paste was frozen immediately and stored in liquid N_2.

Preparation of crude extracts. The crude extracts were prepared as described (39). The anoxic buffer was 50

mM potassium phosphate (pH 7.5) and also contained 10 mM NaN_3 and 2 mM 2-mercaptoethanol. The cell suspension (10 g thawed cell paste:20 ml buffer) was passed through a French pressure cell (SLM-Aminco, Urbana, IL) at 1405 kg/cm^2 followed by centrifugation at 10,000 x g for 20 min. The supernatant solution was stored in liquid N_2.

FDH purification and assay. FDH was purified as described (41). Purity of the enzyme samples was routinely checked by densitometry of SDS polyacrylamide gels (9%). Electrophoresis was performed according to the methods of Laemmli (28). The enzyme preparations used were never less than 90% pure. Activity was assayed by following the formate-dependent reduction of methyl viologen or coenzyme F_{420} with a Perkin-Elmer Lambda 1 spectrophotometer (Perkin-Elmer, Oak Brook, IL). Reaction conditions and components have been described (40). Protein was detected with protein dye reagent (Bio-Rad, Richmond, CA) according to Bradford (8) with bovine serum albumin (Sigma, St. Louis, MO) as the protein standard.

Molybdenum and tungsten assays. Molybdenum was detected by atomic absorption spectrophotometry on a Varian Instruments model 475 spectrophotometer (Varian Instruments, Palo Alto, CA) as described (42). The molybdenum standard was purchased from Fisher Scientific (Springfield, NJ). Tungsten was assayed according to

Cardenas and Mortenson (11). Sodium tungstate (Fisher Scientific) was used as a standard.

Methane assays. A Varian Instruments model 2440 gas chromatograph equipped with a flame ionization detector was used to assay methane. The column was 0.32 cm x 182.88 cm stainless steel and contained 80/100 mesh silica gel (Supelco, Bellefonte, PA). The column oven was operated at 100°C and N_2 was the carrier gas. Purified methane (Airco, Industrial Gas Division, Research Triangle Park, NC) was used as a standard. Chromatographic data were integrated and concentrations of methane calculated with a Varian Instruments CDS-111 data system.

Preparation of antibody against FDH. Rabbits were immunized subcutaneously with 0.5 mg (0.5 ml) of purified FDH mixed with an equal volume of Freund's complete adjuvant (Calbiochem-Behring Corp., LaJolla, CA). The rabbits were given subcutaneous booster inoculations each week for 6 weeks with the same amount of enzyme mixed with Freund's incomplete adjuvant (Sigma). Antisera were collected, chilled at 4°C, centrifuged at 12,000 x g for 20 min and filter-sterilized through a 0.2 µ Acrodisc disposable filter (Gelman Sciences, Ann Arbor, MI).

Indirect enzyme linked immunoassay (ELISA). The following buffers were used in the ELISA (grams per liter): 1) carbonate buffer (pH 9.6): Na_2CO_3, 1.59;

$NaHCO_3$, 2.93; NaN_3, 0.2; 2) phosphate-buffered saline Triton X-100 (PBS-T) (pH 7.4): NaCl, 8.0; KH_2PO_4, 0.2; Na_2HPO_4-$7H_2O$, 2.2; KCl, 0.2; NaN_3, 0.2; Triton X-100, 0.5 ml/liter; 3) diethanolamine buffer (pH 9.8): diethanolamine 97 ml/liter; NaN_3, 0.2; $MgCl_2$-$6H_2O$, 0.1. Deionized water was used to make all buffers. The carbonate buffer was stored at 24°C and was used within 2 weeks. PBS-T was stored at 4°C and diethanolamine buffer was stored at 24°C in a dark bottle.

Material to be assayed was appropriately diluted in carbonate buffer and was incubated overnight at 37°C in polystyrene microtiter wells (250 µl per well, Immulon I plates, Dynatech Laboratories, Inc., Alexandria, VA). The wells were washed once with 0.5% gelatin and 0.5% casein (Sigma) in PBS and then 5 times with PBS-T. Antiserum was diluted 1:5000 in PBS-T and was incubated in the wells for 1 h at 37°C (250 µl per well). The wells were washed 5 times with PBS-T. Anti-rabbit IgG alkaline phosphatase conjugate was diluted 1:1000 in PBS-T and was incubated in the wells for 1 h at 37°C (250 µl per well). The wells were again washed 5 times with PBS-T. Phosphatase substrate (p-nitrophenyl phosphate disodium, Sigma) in diethanolamine buffer (1 mg of substrate per ml of buffer) was incubated in the wells for 30 min at 37°C (200 µl per well). The reaction was stopped by the addition of 20 µl

of 5 N NaOH to each well at 30 min. Absorbance due to the phosphatase assay was read at 405 nm on a ELIA reader (Fisher Scientific). When quantitating FDH protein from FPLC chromatography (see below), dilutions for the ELISA were done so as to obtain absorbance values between 0.2 and 0.9.

Western blot analysis. Crude extract components were separated by SDS polyacrylamide gel electrophoresis in a 9% gel according to the methods of Laemmli (28). The proteins were transferred to nitrocellulose by electroelution in a Hoeffer Transfor Unit (Hoeffer Scientific Instruments, San Francisco, CA). Electroelution was carried out overnight at 1.0 amp. The transfer buffer was 25 mM Tris-HCl, 192 mM glycine, 20% (v/v) methanol (pH 8.3). The blot was incubated for 1.5 hours in 0.5% casein/0.5% gelatin in PBS-T. It was then incubated for 1.5 hours with FDH antibody in PBS-T (1:5000) followed by incubation for 1.5 hours with ^{125}I-conjugated anti-rabbit antibody in PBS-T. The blot was washed three times in PBS-T (for 20 min each) and then dried. All incubations and washes were done at 37°C. X-ray film (XAR film, Kodak, Rochester, NY) was exposed to the blot for 16 hours.

Fast protein liquid chromatography (FPLC). Crude extract (approximately 20 mg of protein/ml) was filtered through a 0.2 μ filter (Gelman). Two mg of the filtered

sample was applied to a Mono Q HR 5/5 ion exchange column (Pharmacia, Piscataway, NJ) equilibrated with 50 mM potassium phosphate, pH 7.6. During sample loading the column was washed with equilibration buffer for 6 min (1.5 Mpa) at a rate of 0.5 ml/min. A 0.0 to 1.0 M KCl gradient was then applied for 30 min. This was followed by two 6-min washes using 1.0 and 2.0 M KCl. The high salt buffer consisted of 2.0 M KCl in 50 mM potassium phosphate, pH 7.6. All buffers were filtered through a 0.2 µ nylon filter (MicroSep, Honeoye Falls, NY) and degassed. All FPLC buffers and equipment were placed in a Coy anaerobic chamber (Coy Laboratory Products, Inc., Ann Arbor, MI) and sample preparations and collections were performed in the chamber. Pharmacia P-500 pumps, a GP-250 gradient programer, a single path UV-1 monitor and a Frac 100 fraction collector were used.

RNA isolation. Total RNA was isolated as described by Krol et al. (27). All buffers, glassware, pipette tips, Eppendorf tubes, etc. used for RNA isolation or Northern analysis were treated with 0.001% diethylpyrocarbonate (DEP) and autoclaved. Approximately 0.1 g (wet weight) of cell paste was resuspended in 0.5 ml lysis buffer (50 mM Tris-HCl, pH 8.0; 20 mM EDTA; 1% SDS), 0.5 ml phenol (1000 phenol:140 m-cresol:0.4 8-hydroxyquinoline w/w/w) and 1 µl of DEP. This was mixed and then centrifuged at 12,000 x g

for 5 min. The aqueous layer was phenol-extracted once more and recentrifuged. The NaCl concentration of the aqueous phase was made 0.1 M and 2 volumes of cold 95% ethanol were added. The sample was placed in a -20°C freezer overnight and then centrifuged. The ethanol was decanted and the RNA pellet was dried under a vacuum. The pellet was resuspended in 20 µl of 50 mM Tris-HCl (pH 8.0) containing 20 mM EDTA. The concentration and purity of RNA was determined by absorbance at 260 nm and the 260 nm/280 nm absorbance ratio, respectively.

Plasmid DNA used for specific probes. A DNA fragment known to contain *fdh* A and *fdh* B was isolated from a *M. formicicum* gene library by Shuber et al. (44). The *fdh* gene and its flanking regions were subcloned into pUC-18. The new plasmid was designated pUCfd-18 and was harvested from *Escherichia coli* grown to stationary phase. The plasmid was harvested and purified by the method of Birnboim and Doly (4). Restricted plasmid DNA was separated in 0.8% agarose. The separation buffer was 10 mM Tris-HCl, 10 mM boric acid, 0.1 mM EDTA, pH 8.0. The fragments were cut from the gel and electroeluted (Model UEA Unidirectional Electroelutor, International Biotechnologies, Inc., New Haven, CN) into 20 mM Tris-HCl, 5 mM NaCl, 0.2 mM EDTA, 10 M ammonium acetate, pH 8.0. After 3 washes in isobutanol the fragment was precipitated

in 95% ethanol.

Northern blots and hybridization. Northern dot blot analysis was performed according to Thomas (46). Total RNA (2.5 µg/µl) was denatured in 1.0 M glyoxal/10 mM sodium phosphate (pH 7.0) by heating for 1 hour at 50°C. The samples were diluted in sterile water and spotted onto nitrocellulose that had been equilibrated in 20 x SSC (3.0 M sodium chloride; 0.3 M sodium citrate, pH 7.0). Northern gel blot analysis was done as described by McMaster et al. (30). Total RNA was denatured in 1.0 M glyoxal/10 mM sodium phosphate (pH 7.0) and 50% DMSO by heating at 60°C for 15 min. Denatured RNA (10 µg) was electrophoresed in a 1.1% agarose gel. The RNA was electroeluted onto GeneScreen hybridization membranes (New England Nuclear, Boston, MA). Transfer was carried out for 2 hours at 0.25 amp followed by 2 more hours at 1 amp in 25 mM sodium phosphate buffer (pH 6.5).

The pre-hybridization solution contained 5 x SSC, 0.01% SDS, 50% deionized formamide, 50 mM sodium phosphate buffer (pH 7.5), 0.2% DEP, 5 x Denhardt's solution (1 x Denhardt's contains 0.1% each of ficoll, polyvinylpyrollidone and BSA) and 10.0 ng/ml of denatured salmon sperm DNA. The salmon sperm DNA was boiled for two minutes and then placed on ice before being added to the mixture. Pre-hybridization was carried out at 42°C for

12-18 hours. Specific, denatured DNA probe was added to the above mixture for hybridization. The probe DNA was nick-translated with (alpha-^{32}P) dCTP (3000 Ci/mmol, New England Nuclear) according to the instructions of the BRL Nick Translation Kit (Bethesda Research Laboratories, Gaithersburg, MD). Hybridization was carried out at 37°C for 12-18 hours. The filters were washed 3 times in 2 x SSC containing 0.1% SDS at 23°C for 5 min followed with two 30-minute washes at 50°C in the same solution. Washing temperatures of 37, 42, 50, and 65°C were tested. All temperatures gave similar results. The filters were then washed in 0.3 x SSC containing 0.1% SDS at 23°C for 5 min. The filters were dried and x-ray film (Kodak) was exposed to them for 18-30 hours at -70°C.

DNA isolation and Southern hybridization. Total genomic DNA was isolated from frozen cells of _M_. _formicicum_ by phenol extraction. The DNA was extracted with phenol 4 times, with chloroform once and then precipitated with ethanol. The isolated DNA was treated with DNAase-free RNAase for 6 hours at 37°C and digested with the appropriate restriction enzyme. The digested DNA was then fractionated in a 0.7% agarose gel. The DNA was electrophoretically transferred onto GeneScreen hybridization transfer membranes (New England Nuclear) according to the methods suggested by the manufacturer.

Pre-hybridizations and hybridizations were performed as described for Northern hybridizations.

RESULTS

Effect of molybdate and tungstate on the growth of *Methanobacterium formicicum*. Growth on hydrogen was not affected by the concentration of molybdate or tungstate in the medium (Figure 1). We observed no decrease in FDH activity after 5 successive transfers of the organism into medium containing no added formate (data not shown). Therefore hydrogen-grown cultures were used to study the effects of molybdenum or tungsten on FDH synthesis and activity.

The effect of tungsten on the metabolism of formate by hydrogen-grown cultures is shown in Figure 2. The rate of formate-dependent methanogenesis decreased in response to increasing amounts of tungstate present in the media. Methanogenesis by cultures grown with 10^{-3} M molybdate was not affected (data not shown). Similar results were obtained when formate was the only source of carbon and energy (data not shown). The addition of up to 10^{-3} M molybdate (at the time formate was added) to cultures grown in the presence of 10^{-3} M tungstate did not overcome the inhibition after 10 hours of incubation. However after 3 days growth on hydrogen, the cultures incubated with

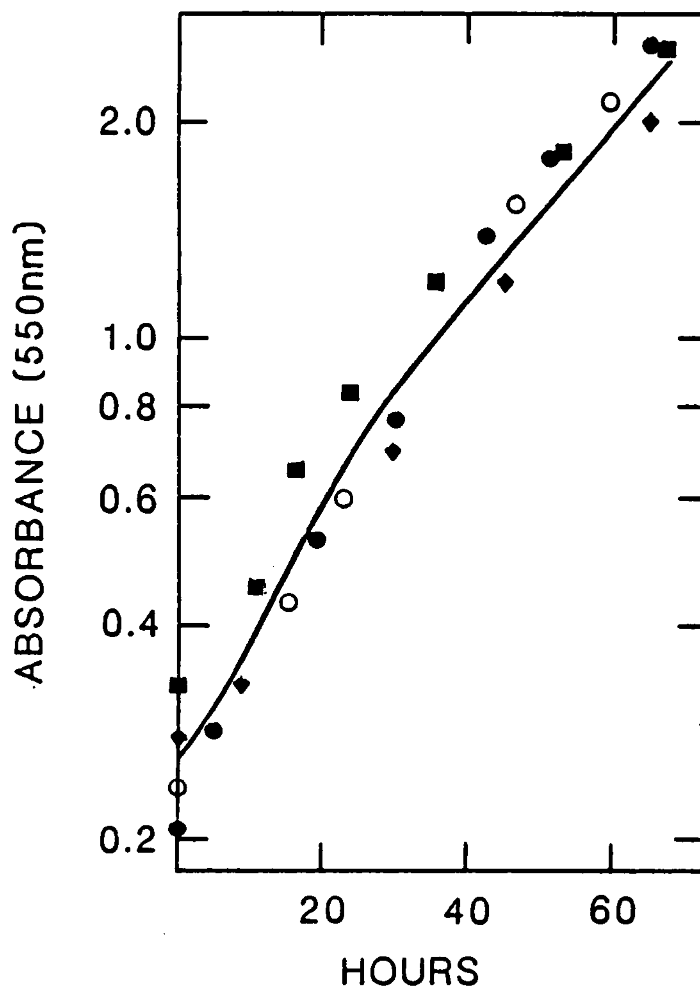

Figure 1. Growth of *Methanobacterium formicicum* on hydrogen and carbon dioxide. The cultures were grown with the following amounts of molybdate or tungstate: (■), with 10^{-4} M molybdate; (○), with 10^{-6} M molybdate; (●) with no added molybdate (after five transfers); (◆), with 10^{-5} M tungstate.

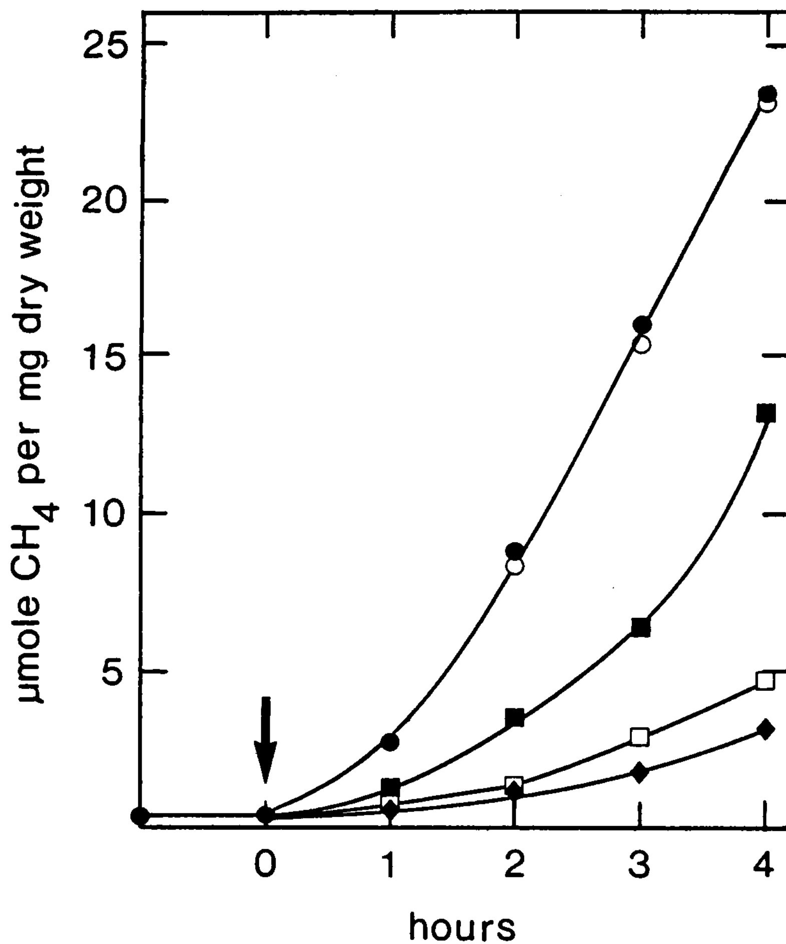

Figure 2. Formate-dependent methanogenesis by hydrogen-grown Methanobacterium formicicum. Hydrogen-grown cultures (in sealed tubes) were placed in a nitrogen environment. After methanogenesis had ceased, 0.5% sodium formate was added to each culture (arrow). The cultures were grown on hydrogen and carbon dioxide with the indicated amounts of tungstate. Symbols: (●), no added tungstate; (○), 10^{-6} M tungstate; (■), 10^{-5} M tungstate; (□), 10^{-4} M tungstate; (◆), 10^{-3} M tungstate. Results are reported as the mean values from triplicate cultures.

molybdate and tungstate produced more methane from formate than cultures grown with high amounts of tungstate and low amounts of molybdate (Figure 3). These results suggest that molybdenum and tungsten compete for molybdenum-requiring processes in M. formicicum.

Effect of molybdate and tungstate on FDH activity. Table 1 shows that by adjusting the molybdate concentration of the growth media, the molybdenum concentrations of cell extracts varied by nearly 100-fold. Formate dehydrogenase activity decreased with decreasing concentrations of molybdenum detected in extracts. Maximum activity was obtained when the extracts contained greater than 0.3 nmol molybdenum per mg protein. Approximately a 15-fold decrease in activity occured when the molybdenum concentration dropped below 0.07 nmol per mg protein. When tungstate was added to media at equimolar or 1000-fold greater concentrations than that of molybdate, the incorporated molybdenum decreased by more than 10-fold (table 2). Formate dehydrogenase activity was approximately 50-fold lower in these cells. High levels of tungsten were present in extracts of cells grown in the presence of 10^{-3} M tungstate.

Immunochemical analysis of crude extracts. Crude extracts were analyzed in a Western blot using antibody to the FDH. Figure 4 shows that both subunits to the FDH were

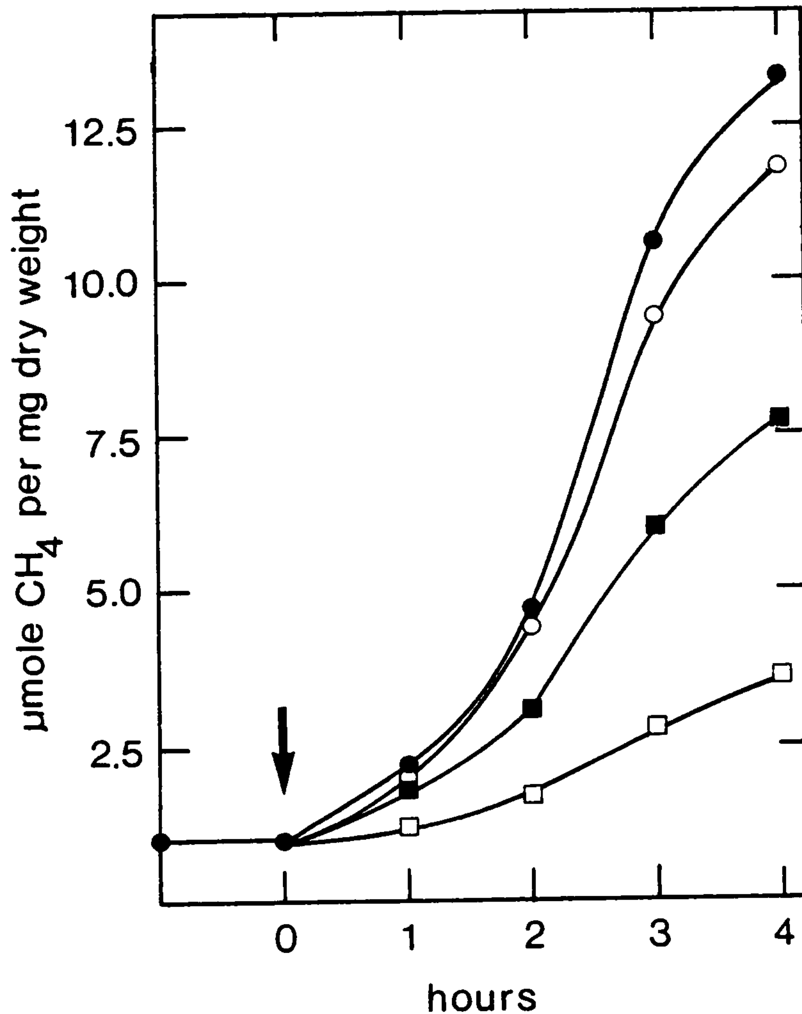

Figure 3. Formate-dependent methanogenesis by hydrogen-grown Methanobacterium formicicum. Hydrogen-grown cultures (in sealed tubes) were grown with 10^{-5} M tungstate. After two days of growth, the indicated amounts of molybdate were added to the cultures. The cultures were then incubated in a nitrogen environment with 0.5% formate. Increasing concentrations of molybdate were not accompanied by increasing methane production. The cultures were then grown on hydrogen for three more days. Hydrogen was replaced by nitrogen and 0.5% formate was added (arrow) after methanogenesis had ceased. Symbols: (□), 10^{-6}M molybdate; (■), 10^{-5} M molybdate; (○), 10^{-4} M molybdate; (●), 10^{-3} M molybdate. Results are reported as the mean of values from triplicate cultures.

TABLE 1. Molybdenum concentration and formate dehydrogenase activity in crude extracts of _Methanobacterium formicicum_.

Fermentor* culture	Mo in cell extract	Activity assayed with:	
		MV	F_{420}
	(nmol/mg protein)	(U/mg protein)	
A	2.22±.49	1.80±.29	1.07±.06
B	0.51±.10	1.64±.36	1.02±.06
C	0.29±.03	1.61±.30	0.73±.05
D	0.18±.03	0.72±.10	0.36±.05
E	0.11±.01	0.57±.12	0.31±.04
F	0.07±.01	0.12±.03	<0.01
G	0.03±.01	0.05±.01	<0.01

* Cultures A-C were grown in media supplemented with 10^{-4} M, 10^{-5} M and 10^{-6} M molybdate, respectively. Cultures D-G were in media with no added molybdate and are arranged in decreasing order of the amount of molybdenum present in the extracts. The first culture was inoculated with a culture grown in media that contained 10^{-6} M added molybdate. Abbreviations: Mo, molybdenum; W, tungsten; MV, methyl viologen; F_{420}, coenzyme F_{420}; U, units of activity (1 unit = 1 µmole of electron acceptor reduced per minute per ml of reaction mix). All values are given as the mean ± the standard deviation (minimum of 4 determinations).

TABLE 2. Molybdenum and tungsten concentrations and formate dehydrogenase activity in crude extracts of Methanobacterium formicicum.

Additions to the medium		Metal in extract		Activity assayed with:	
$MoO_4^{=}$	$WO_4^{=}$	Mo	W	MV	F_{420}
(M)		(nmol/mg protein)		(U/mg protein)	
10^{-6}	---	0.29±.03	<0.5	1.61±.30	0.73±.05
10^{-6}	10^{-3}	0.03±.01	8.9±.8	0.03±.01	<0.01
---	10^{-6}	0.03±.01	<0.5	0.03±.01	<0.01

Abbreviations: $MoO_4^{=}$, molybdate; $WO_4^{=}$, tungstate; Mo, molybdenum; W, tungsten; MV, methyl viologen; F_{420}, coenzyme F_{420}; U, units of activity (1 unit = 1 µmole of electron acceptor reduced per minute per ml of reaction mix). All values are given as the mean ± the standard deviation (minimum of 4 determinations).

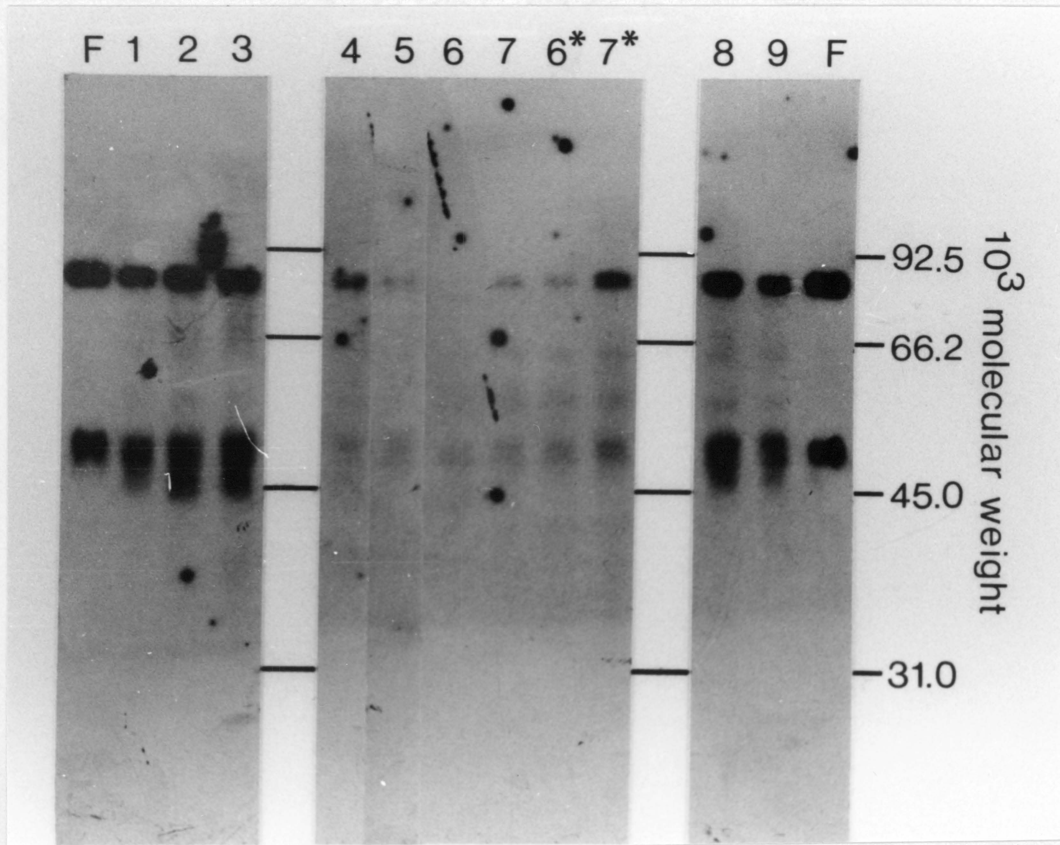

Figure 4. Western blot analysis of crude extracts of <u>Metahanobacterium formicicum</u>. Lanes 1-3 were loaded with 10 ug of extract protein from cells containing 2.22 to 0.29 nmoles of molybdenum per mg protein, respectively. Lanes 4-7 were loaded with 10 ug of extract protein from cells containing 0.18 to 0.03 nmoles of molybdenum per mg protein, respectively. Lanes 6* and 7* were loaded with 50 ug of extract protein from cultures 6 and 7. Lanes 8 and 9 were loaded with 10 µg of extract protein from cells grown with 10^{-3} M and 10^{-6} M tungstate, respectively. Lanes designated by an F were loaded with 0.5 ug of purified formate dehydrogenase. Cultures for lanes 1-3 were grown with added molybdate (10^{-4} M to 10^{-6}M). Cultures for lanes 4-7 were grown in series with no added molybdate. A culture grown with 10^{-6} M molybdate was used as the starting inoculum (10%).

synthesized whether or not molybdate or tungstate was added to the growth medium. The figure also shows that the amount of cross-reacting material produced decreased when M. formicicum was grown with no added molybdenum. The levels of cross-reacting material in cells grown with added molybdate or tungstate were approximately the same.

To better estimate the amounts of FDH protein in crude extracts, an indirect enzyme-linked immunoassay (ELISA) was developed. The assay detected at least 5 ng of the enzyme (Figure 5) and the assay was linear between 5 and 12 ng of FDH. For undetermined reasons the ELISA's detection of FDH in crude extract was inhibited. When added to the extract homogeneous enzyme was not fully detected by the assay. However FDH activity (MV) and cross-reacting material could be quantitatively recovered after fractionation of extracts on an FPLC Mono Q ion exchange column. Cells containing low levels of molybdenum (Figure 6, middle) had a lower amount of FDH cross-reacting material than cells which contained high amounts of molybdenum (Figure 6, top). Furthermore the protein peak (absorbance at 280 nm) that coincides with FDH activity and cross-reacting material was absent in extracts from molybdenum-deficient cells. Although cells grown in the presence of tungstate contained negligible FDH activity (Table 2), a protein peak and high amounts of cross-reacting material eluted in the same

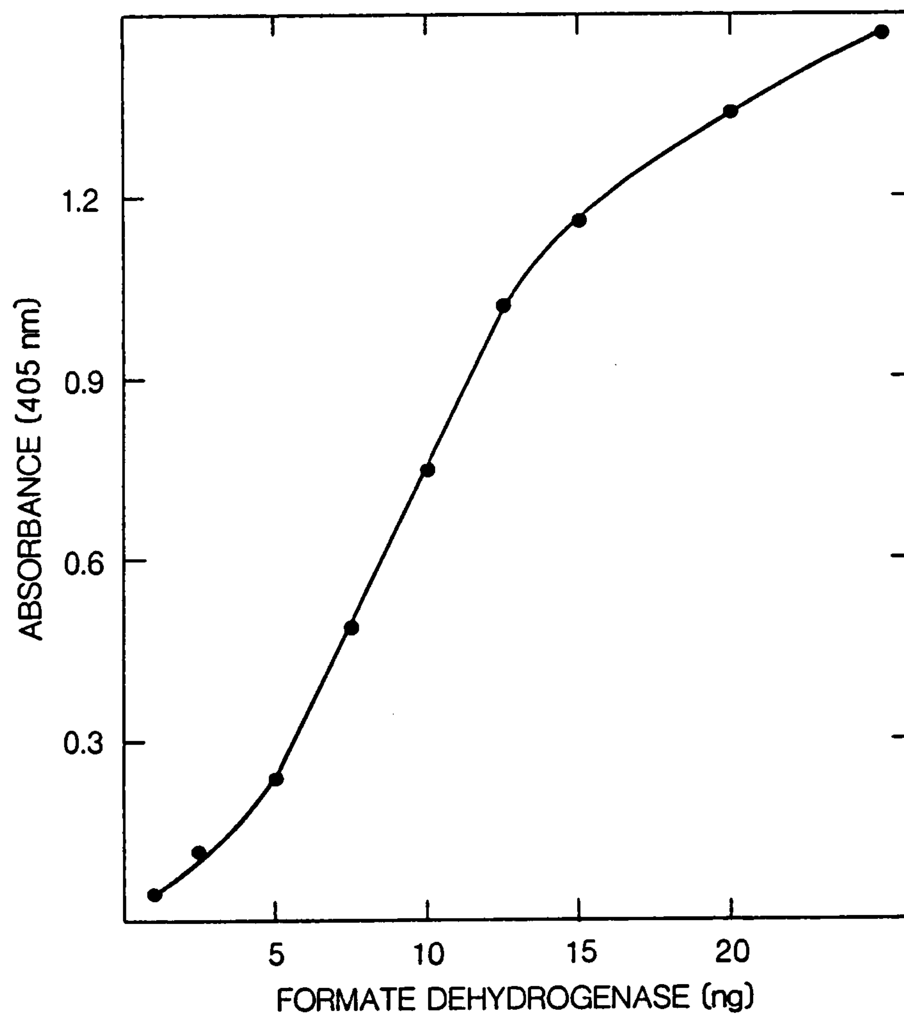

Figure 5. Detection of purified formate dehydrogenase in the enzyme linked immunosorbent assay (ELISA).

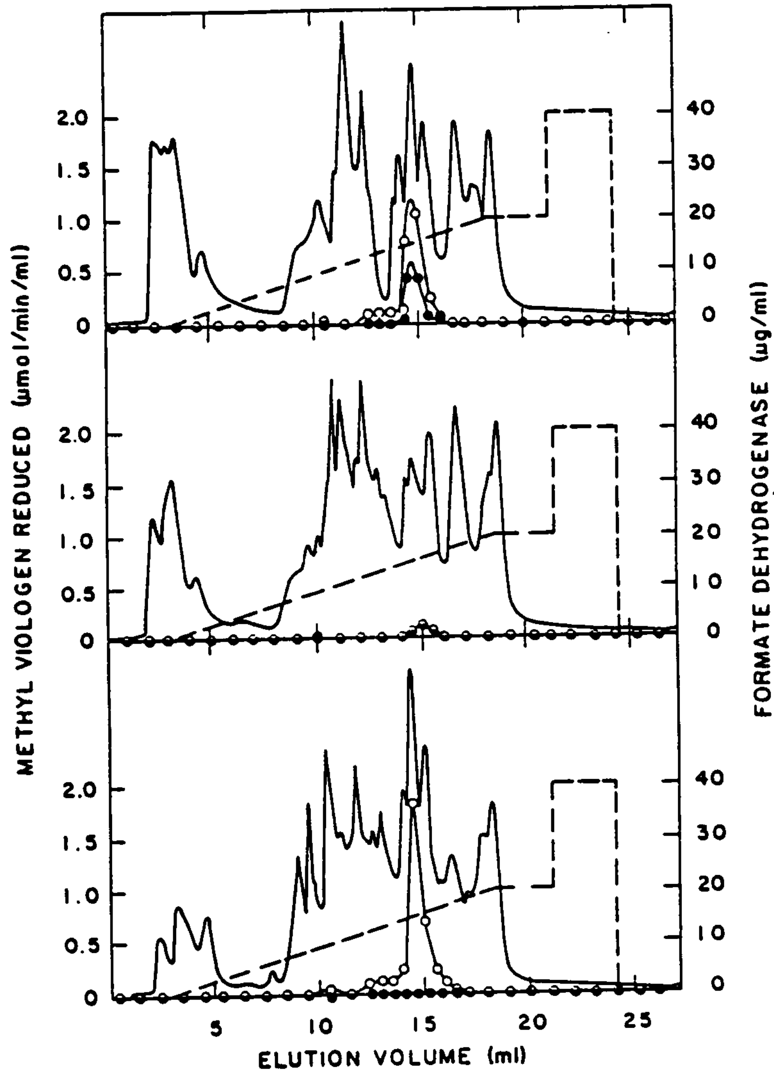

Figure 6. FPLC-fractionated extracts of Methanobacterium formicicum. Top: 2.0 mg of extract protein from cells grown with 10^{-6} M molybdate (cells contained 0.29 nmole molybdenum per mg protein). Middle: 2.0 mg of extract protein from cells grown with no added molybdate (cells contained 0.03 nmole molybdenum per mg protein). Bottom: 2.0 mg of extract protein from cells grown with 10^{-3} M tungstate (cells contained 8.9 nmoles tungsten per mg protein). Symbols: (●), formate-dependent reduction of methyl viologen; (O), relative ug of formate dehydrogenase detected in the ELISA (relative to purified formate dehydrogenase); (——), absorbance at 280 nm; (---), KCl gradient (0.0-1.0 M, 1.0-2.0 M, 2.0-1.0 M).

position as active FDH (Figure 6, bottom). The amount of cross-reacting material in the extracts was estimated as µg of FDH (relative to the reaction of homogeneous FDH in the ELISA) per mg of extract protein. The results given in table 3 show that the extracts containing low amounts of molybdenum had 10-fold less cross-reacting material than extracts of cells that contained high amounts of molybdenum or tungsten.

Formate dehydrogenase from tungstate-grown *M. formicicum*. Formate dehydrogenase was purified from cells grown with 10^{-3} M tungstate. The low amount of FDH activity in the extract of these cells was lost during the first purification step and therefore the ELISA was used to follow the inactive protein throughout purification. Analysis of the purified inactive FDH by SDS-polyacrylamide gel electrophoresis showed that the protein contained the same subunits as active FDH (Figure 7). However denaturation of the enzyme released a fluorescent compound with an excitation/emission spectrum identical to that of the molybdopterin cofactor from active FDH (Figure 8). The relative amount of fluorescence due to cofactor from inactive FDH enzyme was approximately the same as that from cofactor released from active enzyme (data not shown). Analysis for molybdenum and tungsten revealed that neither metal was present in the protein in significant amount

TABLE 3. ELISA-detected formate dehydrogenase in FPLC-fractionated extracts of *Methanobacterium formicicum*.

Additions to the medium		Metal in cell extract[a]		Cross-reacting material[b]
$MoO_4^{=}$	$WO_4^{=}$	Mo	W	
(M)		(nmol/mg protein)		(µg/mg protein)
10^{-5}	---	0.51±.10	<0.5	31.3
10^{-6}	---	0.29±.03	<0.5	24.3
---	---	0.07±.01	<0.5	2.0
---	---	0.03±.01	<0.5	1.7
10^{-6}	10^{-3}	0.03±.01	8.9±.8	30.5
---	10^{-6}	0.03±.01	<0.5	25.1

[a] Values are given as the mean ± the standard deviation (minimum of 4 determinations).

[b] Values are given as the mean of 2-3 determinations.

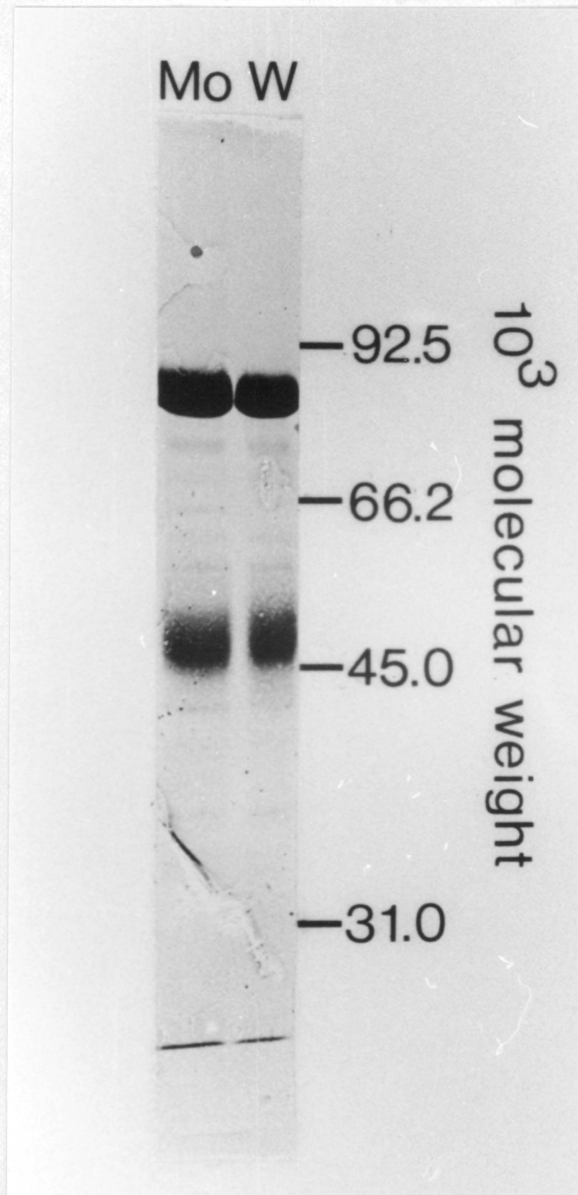

Figure 7. SDS-polyacrylamide gel fractionation of formate dehydrogenase from Methanobacterium formicicum. Mo, 40 ug of formate dehydrogenase from cells grown with 10^{-6} M molybdate; W, 40 ug of formate dehydrogenase from cells grown with 10^{-3} M tungstate.

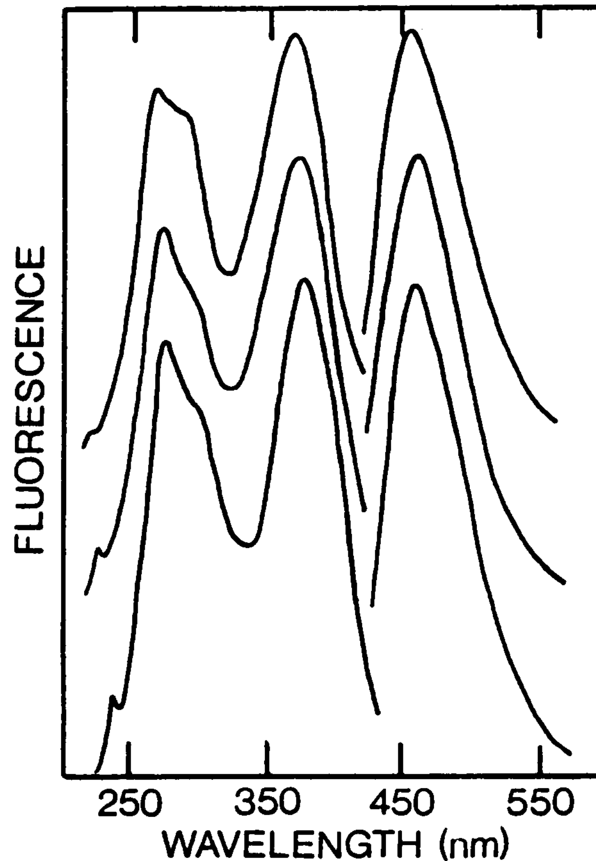

Figure 8. Excitation/emission spectra of fluorescent compounds from xanthine oxidase (top), active formate dehydrogenase (middle) and inactive formate dehydrogenase (bottom). Active formate dehydrogenase was purified from cells grown with molybdate and inactive enzyme was purified from cells grown with tungstate. Samples were treated with iodine and potassium iodide according to the methods of Johnson and Rajagopalan (19) in order to obtain form A of the molybdoOpterin cofactor. All samples were in 1.0 M NH_4OH. Excitation was set at 380 nm and emission was set at 465 nm.

(<0.05 mole per mole of FDH).

Effect of molybdate or tungstate on transcription of the *fdh* gene. *Methanobacterium formicicum* chromosomal DNA was digested by several restriction enzymes and was then assayed by Southern hybridization using a 462 bp *Sna* B1 to *Bgl* II DNA fragment. Restriction site analysis showed one *Sph* 1 site, one *Sna* B1 site and three *Nco* 1 sites within the 10.6 kb insert (Figure 9). From this it was predicted that the probe used would hybridize to one 3.0 kb DNA fragment from an *Nco* 1 digest, one 2.6 kb fragment from an *Nco* 1/*Sph* 1 double digest and one 2.4 kb fragment from a *Nco* 1/*Sna* B1 double digest if only one region of the chromosome was homologous to the probe. The results in Figure 10 confirm this prediction.

The results from Northern dot blot hybridizations of total RNA from cells grown with varying amounts of molybdate or tungstate are shown in Figure 11. Overall the amount of *fdh* mRNA increased when the amount of molybdenum in the cells decreased. Approximately 2 to 10-fold more *fdh* mRNA was found in molybdenum-deficient cells than in cells containing high amounts of molybdate. Cells grown with tungstate had levels of *fdh* message that were between the levels discovered in cells grown with and without molybdate. Total RNA from cells grown with and without added molybdate was fractionated by agarose gel

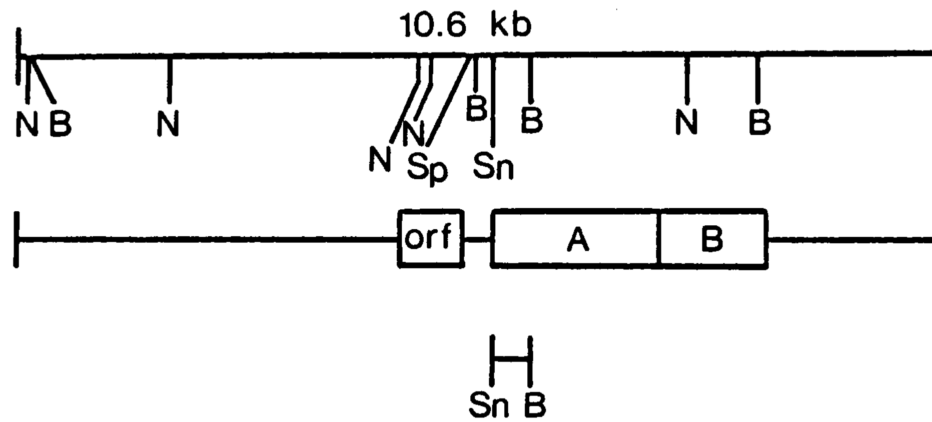

Figure 9. Restricition map of Methanobacterium formicicum DNA. Methanobacterium formicicum DNA was inserted into plasmid pUCfd-18 (see Materials and Methods). Symbols: (A), fdh A; (B), fdh B; orf, open reading frame. Symbols of restriction enzymes: Sp, Sph 1; Sn, Sna B1; N, Nco 1; B, Bgl II.

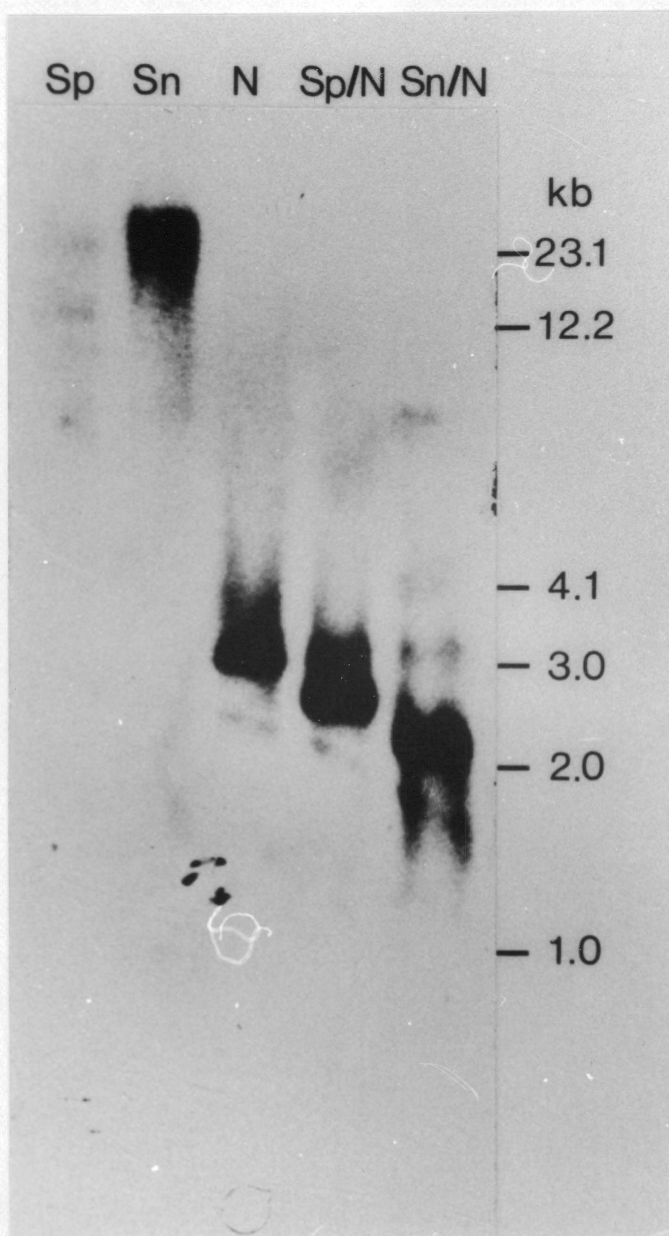

Figure 10. Southern hybridization of digested *Methanobacterium formicicum* total DNA. Symbols for restriction enzymes: Sp, *Sph* 1; Sn, *Sna* B1; N, *Nco* 1; Sp/N, double digest of *Sph* 1 and *Nco* 1; Sn/N, double digest of *Sna* B1 and *Nco* 1.

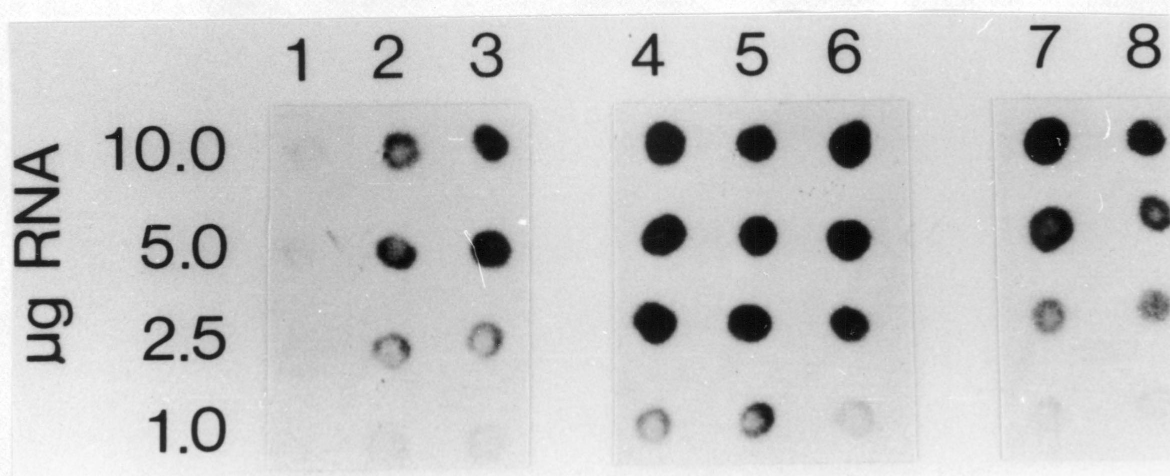

Figure 11. Northern dot blot hybridization of total RNA from Methanobacterium formicicum. Lanes 1-3 were loaded with RNA from cells containing 2.22 to 0.29 nmoles of molybdenum per mg protein, respectively. Lanes 4-6 were loaded with RNA from cells containing 0.18 to 0.03 nmole of molybdenum per mg protein, respectively. Lanes 7 and 8 were loaded with RNA from cells grown with 10^{-3} M and 10^{-6} M tungstate, respectively. Cultures for lanes 1-3 were grown with added molybdate (10^{-4} M to 10^{-6} M) and cultures for lanes 4-6 were grown in a series with no added molybdate. A culture grown with 10^{-6} M molybdate was used to inoculate the series (10% inoculum).

electrophoresis and hybridized with the same DNA probe from fdh A (Figure 12). The size of the message was >12.0 kb regardless of the condition. The same result was observed with a DNA probe from fdh B (personal communication). DNAase-free RNAase treatment of RNA from all samples resulted in no hybridization. Hot phenol extractions, chloroform extractions, isobutanol extractions, isoamyl alcohol extractions and treatment with proteinase K did not alter the migration of the message. To better determine the size of the transcript, Northern hybridizations were performed with DNA fragments upstream and downstream of the fdh gene. A 180 bp DNA fragment that is 3.9 kb upstream of fdh A hybridized with a 2.7 kb and 1.4 kb message (personal communication). No hybridization with the large transcript was observed. A DNA fragment downstream of fdh B (to the end of the insert) hybridized only to the large transcript.

DISCUSSION

In contrast with *Methanobacterium formicicum*, the growth of some methanogenic bacteria on hydrogen is dependent upon molybdenum or tungsten. Schonheit et al. (43) showed that *Methanobacterium thermoautotrophicum* requires molybdenum for growth on hydrogen. *Methanobacterium wolfei* and *Methanoplanus endosymbiosis*

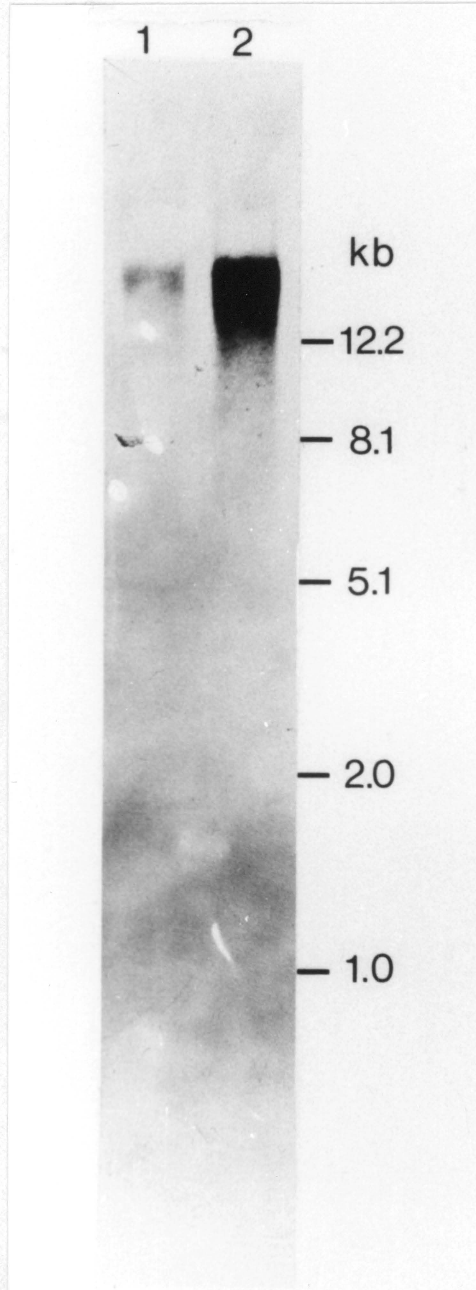

Figure 12. Northern gel blot analysis of total RNA from Methanobacterium formicicum. (1), 10 ug of RNA from cells containing 0.51 nmole of molybdenum per mg protein (cells grown with 10^{-5} M molybdate). (2), 10 ug of RNA from cells containing 0.03 nmole of molybdenum per mg protein (grown with no added molybdate).

require tungsten for growth on hydrogen (48,49). Although my results show that M. formicicum did not require either metal for growth on hydrogen, metabolism of formate was inhibited by tungsten. The inhibition could be relieved if the tungstate-grown culture was supplemented with molybdate and allowed to resume growth. Without growth the inhibition continued. Not only does this show a competition between the metals for molybdenum-requiring processes, but it suggests that the inactive FDH produced could not be quickly activated by the addition of molybdate. Perhaps new protein synthesis is required in order to recover FDH activity.

The molybdenum requirement for the synthesis of FDH in Methanobacterium formicicum is similar to the requirement that several organisms have for the full expression of molybdoenzymes. Examples of such enzymes are the iron-molybdenum dinitrogenase of Azotobacter vinelandii (31), the nitrogenase proteins of Rhodospeudomonas capsulata (16), the nitrate reductase of cauliflower (33) and the formate dehydrogenase and nitrate reductase of the formate-nitrate respiratory pathway in Escherichia coli (14). The full expression of FDH in tungstate-grown M. formicicum is similar to the synthesis of nitrate reductase in tungstate-grown E. coli (14), but differs from the synthesis of formate dehydrogenase in E. coli (14) and

nitrogenase in R. capsulata (16). The latter two enzymes are not synthesized at optimal levels when tungstate is added to the growth medium. The results also show that the FDH in tungstate-grown M. formicicum was inactive and did not contain molybdenum or tungsten. The opposite is true for Methanococcus vannielii which requires tungsten for growth on formate and produces an active, tungsten-containing formate dehydrogenase (24,25).

The fdh gene in M. formicicum was transcribed even when the molybdenum concentration in the cells was low. Therefore the low levels of FDH in these cells was not due to a lack of fdh mRNA. Without molybdenum the FDH apoprotein may be unstable; however the existence of metal-free inactive FDH in tungstate-grown cells suggests that incorporation of the metal into the apoenzyme is not required for enzyme stability. This does not eliminate the possibility that molybdenum or tungsten is required for cofactor synthesis and/or cofactor insertion into the protein. Insertion of the cofactor may stabilize the FDH. The discovery of cofactor in the inactive FDH of tungstate-grown cells supports this hypothesis.

The low levels of fdh mRNA in M. formicicum containing high amounts of molybdenum suggest that a molybdenum-dependent protein may repress transcription. Proteins that could act as such a repressor have been

isolated from other organisms. A low molecular weight (5700 daltons) molybdenum- and pterin-containing protein has been isolated from extracts of *Clostridium pasteurianum* (18). Molybdenum storage proteins could also act as repressors. Proteins in *Clostridium pasteurianum* (13), *Klebsiella pneumoniae* (36) and *Azotobacter vinelandii* (36) have this potential. The molybdenum storage protein in *A. vinelandii* is a tetramer of two pairs of different subunits that bind at least 15 atoms of molybdenum per tetramer (36).

Another explanation for the low levels of mRNA in *M. formicicum* containing high concentrations of molybdenum is that the *fdh* gene is under autogenous control with the FDH holoenzyme acting as a repressor. This would be similar to the autoregulation of nitrate reductase synthesis in *Escherichia coli* (35) and *Neurospora crassa* (47). Although no direct response to molybdenum was observed, Pascal et al. (35) showed that the autoregulation of *E. coli* nitrate reductase requires the molybdopterin cofactor. Similarly *N. crassa* nitrate reductase negatively controls its own synthesis. A structurally intact enzyme is required for this autoregulation.

Another molybdoenzyme proposed to be autogenously controlled is the molybdenum-iron protein of nitrogenase. The *nif* HDK operon codes for the structural components of

nitrogenase and nif H contains the promoter region for this operon (34). Dixon et al. demonstrated with nif::lac fusions that both molybdate and the molybdenum-iron protein are required for maximal expression of nif H in Klebsiella pneumoniae (12). This suggests that the molybdenum-iron protein along with molybdenum acts as a positive activator in the autogenous control of the operon. In agreement with the results of Dixon et al., Jacobson et al. (20) showed that the nif HDK operon in Azotobacter vinelandii is not transcribed in the absence of molybdenum. An alternative nitrogenase in A. vinelandii is encoded for by a separate gene with homology to nif H (6,20). This gene is not transcribed unless the molybdenum concentration in the medium drops below 1.0 µM (20).

The size estimate for the fdh mRNA is somewhat surprising. It could be that the transcript is being held up by some contaminate which was left untouched by the different extractions we attempted. It is also possible that secondary structures caused the mRNA to migrate slowly in the gel. However a colleague in this laboratory (Dr. P. Patel) has shown that the transcription start site is 3.9 kb upstream of fdh A and that the termination site is at least 2.0 kb downstream from the end of fdh B. Based upon these results the transcript containing the fdh gene must be at least 10.0 kb long. This is not the only large

transcript produced by methanogenic bacteria. Sment and Konisky reported that in the presence of aminotriazole, the transcript of the *his* A gene from *Methanococcus voltae* was at least 10.0 kb long (45).

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SECTION VI. GENERAL SUMMARY

The purpose of this study was to investigate the molybdenum center of the formate dehydrogenase from Methanobacterium formicicum. This was done in two parts: the first was concerned with the structural environment of the metal and the other with the role of molybdenum in the stability and synthesis of the enzyme.

MOLYBDOPTERIN

Other molybdoenzymes contain either the iron-molybdenum cofactor or molybdopterin. The former is an iron-, molybdenum- and sulfide-containing cluster only found in nitrogenase (19,95,102,118). Molybdopterin is a pterin substituted in the 6 position by a 4-carbon side chain (50-52). One phosphate molecule is present at the end of the side chain (52). From extended x-ray absorbtion fine structure spectroscopy it is hypothesized that molybdenum binds to the side chain through sulfur linkages (16). One other sulfur and one oxygen are terminally liganded to the metal (16,21). Under anaerobic conditions molybdopterin released from active enzyme can reconstitute apo-nitrate reductase of Neurospora crassa mutant nit-1 (50). Due to the extreme lability of molybdopterin (especially to oxygen) the structure of the active cofactor

has not been elucidated. Therefore the structure described above has been determined by the investigation of oxidized cofactor.

The molybdopterin cofactor from the formate dehydrogenase of *M. formicicum* did not reactivate the apo-nitrate reductase (Section IV). An examination of oxidation products of the cofactor revealed that the cofactor is a 6-substituted pterin. The fluorescence of the oxidized cofactor was very similar to that of the molybdopterin of other molybdoenzymes. The *fdh* gene (103) encodes a protein with a calculated molecular weight of 119,652. Based upon this molecular weight, flavin-free formate dehydrogenase contained 2 phosphates per enzyme. This suggests that the phosphates are associated with the molybdopterin since no other phosphate-containing cofactors are present in the enzyme.

The above results suggest that the side chain of the formate dehydrogenase cofactor is different from the side chain of other molybdopterins. Purification of the cofactor under anoxic conditions prevented the pterin from fluorescing; however several forms of the pterin were produced. Therefore a definitive study on the structure of the non-oxidized cofactor was not possible.

The oxidized forms of xanthine-utilizing molybdenum hydroxylases (xanthine oxidase, xanthine dehydrogenase,

aldehyde oxidase) are irreversibly inactivated by cyanide (114). Cyanide treatment of these oxidized forms causes the loss of a terminal sulfur ligand from the molybdenum (79). The sulfur is lost as thiocyanate. Addition of sulfide to the inactivated enzymes under reduced conditions will reconstitute the enzymes (79). Oxidized forms of molybdoenzymes (e.g. sulfite oxidase and nitrate reductase) that do not possess a terminal sulfur ligand are not inhibited by cyanide (1). When in the reduced state all molybdopterin-containing enzymes are reversibly inhibited by cyanide (i.e. removal of the cyanide reactivates the enzyme).

In Section III the effects of cyanide on the formate dehydrogenase of *M. formicicum* are described. Oxidized formate dehydrogenase (by incubation with methyl viologen) was irreversibly inactivated by cyanide; however activity could not be restored by incubating the enzyme with sulfide. Based on the calculated molecular weight of the formate dehydrogenase (103) 0.9 thiocyanate was released per enzyme molecule. This suggests that the enzyme possesses one terminal sulfur ligand to molybdenum. It is possible that the sulfur released as thiocyanate was not specific for molybdenum. However the inactivation altered the electron paramagnetic resonanace spectroscopic properties of the molybdenum center of the enzyme. Partial

reduction of the native enzyme gave a signal with the following g values: $g_1 = 2.020$, $g_2 = 2.006$ and $g_3 = 1.997$. In the presence of cyanide these values were shifted to a higher field ($g_1 = 2.005$, $g_2 = 1.998$ and $g_3 = 1.989$) and the complexity of the superhyperfine splitting pattern was reduced. The Mo(V) epr signal of xanthine oxidase also shifts to a higher field when the enzyme is inactivated by cyanide.

The g values of both native and cyanide-inactivated formate dehydrogenase are significantly larger than those reported for other molybdenum hydroxylases. The g_{av} of native formate dehydrogenase ($g_{av} = 2.008$) is the largest encountered for any molybdenum center in a biological system ($g_{av} = 1.982$ to 1.964) (4,6,40,112). However the g values are within the range of 2.025 to 1.949 observed for the principal g values of Mo(V) spectra (17). It is not known why the g values are so high for the molybdenum center of formate dehydrogenase, but it does suggest that the molybdenum domain of formate dehydrogenase is different from that of other molybdoenzymes. The results in Section IV indicate that the side chain of the molybdopterin cofactor of the formate dehydrogenase is not identical to those of other molybdoenzymes. Perhaps this change in structure influences the electron paramagnetic resonance spectrum. Also arsenite perturbs the Mo(V) spectra of

xanthine oxidase and aldehyde oxidase (7), but does not affect the Mo(V) spectrum of formate dehydrogenase (8).

FORMATE DEHYDROGENASE SYNTHESIS AND MOLYBDENUM

Stable forms of molybdoenzymes are synthesized in the absence of molybdenum (18,20,42,44,45,53,85). However *Rhodopseudomonas capsulata* and *Azotobacter vinelandii* do not produce stable iron-molybdenum proteins of nitrogenase unless molybdenum is present (43,84). In *Escherichia coli* a dissimilatory nitrate reductase and a formate dehydrogenase catalyze the energy-conserving reduction of nitrate by formate (46). The synthesis of these enzymes decreases approximately 3-fold when the organism is grown under molybdenum-deficient conditions (34). Transcription of the nitrogenase genes in *A. vinelandii* (the *nif* HDK operon) occurs only in the presence of molybdenum (47). A second nitrogen fixation system is encoded for by a separate gene with homology to *nif* H only (47). This system is transcribed only when the molybdenum concentration is below 100 nM). A direct effect by molybdenum on the transcription of molybdopterin-containing enyzymes has not been demonstrated.

The formate dehydrogenase activity of *Methanobacterium formicicum* decreased by more than 30-fold when the organism was grow under molybdenum-deficient

conditions (Section V). Immunological assays demonstrated that under these conditions, the level of formate dehydrogenase protein had decreased by at least 10-fold. Cells grown with tungsten produced normal levels of formate dehydrogenase, but the enzyme was inactive. This inactive enzyme had the same subunit composition as active enzyme and contained the molybdopterin cofactor, but possessed no molybdenum or tungsten. High amounts of messenger RNA to the fdh gene were present in cells grown under molybdenum-deficient conditions. Therefore the low quantity of formate dehydrogenase in these cells was due to a post-transcriptional requirement for molybdenum. Since the inactive formate dehydrogenase of cells grown with tungsten was stable and metal-free, it appears that molybdenum was not directly required for stability. The metal could be required for insertion of cofactor, thereby indirectly stabilizing the enzyme.

The low levels of fdh mRNA in M. formicicum grown with molybdenum suggest that a molybdenum-dependent repressor may limit transcription under these conditions. The repressor could be formate dehydrogenase, which autogenously controls the fdh gene. Autogenous control of molybdoenzyme synthesis has been observed in E. coli (15,91), Neurospora crassa (111) and Klebsiella pneumoniae (26). Although no direct response to molybdenum was

observed, Bonnefoy et al. (15) showed that the autoregulation of the E. coli nitrate reductase required the molybdopterin cofactor and the intact structural protein of nitrate reductase. Similarly Tomsett and Garrett (111) showed that the autoregulation of N. crassa nitrate reductase was dependent upon the structural integrity of the enzyme. Dixon et al. (26) demonstrated that the molybdenum-iron protein of nitrogenase and molybdate were required for maximal expression of nif HDK. This suggests that the iron-molybdenum protein autoregulates its synthesis as a positive activator.

Although autoregulation appears to play a significant role in molybdoenzyme synthesis, more research is needed to confirm the regulatory mechanism of the fdh gene in M. formicicum. Very little is known about transcriptional control in archaebacteria. Sment and Konisky showed a two-fold increase of mRNA specific to the his A gene of Methanococcus voltae when the gene was derepressed by aminotriazole (104). In the absence of aminotriazole the size of the mRNA is 1.5 kb, but when aminotriazole is present the transcript is very large (9 to 10 kb). The results in Section V show that the mRNA that is complementary to the fdh gene also is very large (at least 10 kb). It will be interesting to see what this transcript contains and just how its synthesis is regulated.

SECTION VII. LITERATURE CITED

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