

## Molybdopterin Cofactor from *Methanobacterium formicicum* Formate Dehydrogenase

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Received 26 June 1985/Accepted 10 February 1986

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, but after exposure to air it fluoresced with spectra similar to those of described molybdopterin cofactors. Aerobic release from acid-denatured formate dehydrogenase in the presence of  $I_2$  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 the *Neurospora crassa nit-1* mutant.

All known molybdoenzymes except nitrogenase contain a pterin cofactor thought to contain molybdenum (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 the *Neurospora crassa nit-1* mutant (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 fluorescence is present in the molybdenum-containing formate dehydrogenase (FDH) from *Methanobacterium formicicum* (17, 18). Here we 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 Laboratories, Richmond, Calif.), as described by Bradford (4), with bovine serum albumin (Sigma Chemical Co., 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 for 15 min. The boiled enzyme was applied to a Fractogel TSK-HW40S (EM Science, Gibbstown, N.J.) column (1.5 by 32 cm) equilibrated with anoxic 0.1 M ammonium carbonate and 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 FDH by pressure dialysis in an ultrafiltration cell (Amicon Corp., Lexington, Mass.) fitted with a YM30 ( $M_r$  cutoff = 30,000) filter (18). To the enzyme solution (1 ml) was added 9 ml of anoxic 50 mM potassium phosphate buffer (pH 7.5) that contained 10 mM sodium formate followed by concentration to 1 ml under nitrogen. This procedure was repeated five times followed

by five washes with the same buffer, except that sodium formate was excluded.

Cofactors from FDH or xanthine oxidase (XO) were aerobically released by boiling for 20 min at pH 2.5 in the presence of air- $I_2$ -potassium iodide as described by Johnson and Rajagopalan (11). The pterin oxidation products were separated from protein and FAD by gel filtration on a Fractogel TSK-HW40S column (1.5 by 32 cm) 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 in either 1 M ammonium hydroxide for spectral analysis or water for high-performance liquid chromatography (HPLC).

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

Samples were wet ashed as described by Meyer and Rajagopalan (14). The  $P_i$  released was quantitated by the method of Ames (1) with a Hitachi model 100-60 spectrophotometer. An  $A_{820}$  of 0.260 was observed for 10 nmol of  $P_i$ . Ashed  $P_i$  (10 nmol) produced an  $A_{820}$  of 0.240.

**Permanganate oxidation.** The cofactors were aerobically purified as described above. Lyophilized samples were redissolved in 0.1 N sodium hydroxide. After addition of 10 mg of potassium permanganate per ml, the samples were placed in a boiling water bath for 1 h. 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 *N. crassa nit-1* mutant strain. We grew the organism, obtained from John Smarrelli, on Vogel medium to induce synthesis of nitrate reductase (5). Extracts were prepared as described by Amy and Rajagopalan (2) except that phenylmethylsulfonyl fluoride was omitted and all procedures were done anoxically.

The cofactors from FDH (11 mg/ml) and XO (15 mg/ml)

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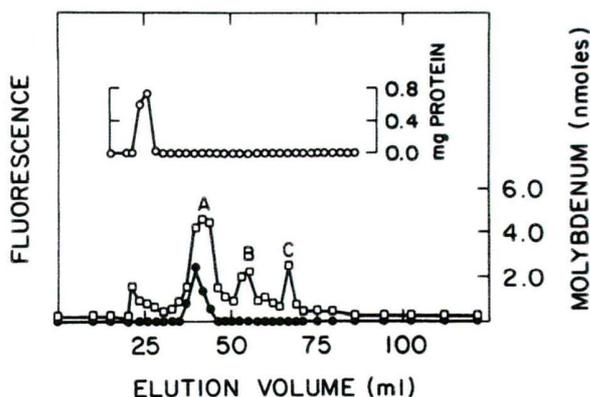


FIG. 1. Anoxic column chromatography of molybdenum and fluorescent compounds released from FDH. Enzyme (1.1 mg) denatured with guanidine was anoxically chromatographed on a Fractogel TSK-HW40S gel filtration column. Samples of fractions were assayed for molybdenum (●) and fluorescence (□) with excitation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

(Sigma) were released under anoxic conditions by the following method. (i) An enzyme solution (5  $\mu$ l) was added to 95  $\mu$ l of 6 M guanidine hydrochloride in 10 mM Tris hydrochloride (pH 8.0) and placed on ice for 5 min. (ii) An enzyme solution (30  $\mu$ l) was added to 120  $\mu$ l of 1.25% (wt/vol) sodium dodecyl sulfate in 125 mM potassium phosphate (pH 7.4), and the mixture was boiled for 1 min. (iii) An enzyme solution (5  $\mu$ l) was added to 90  $\mu$ l 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  $\mu$ l of denatured enzyme solution added to 249  $\mu$ l of *N. crassa nit-1* mutant extract (freshly prepared) and incubated for 30 min at 24°C. The reconstitution mixtures contained, in final concentration, 10 mM sodium ascorbate and 10 mM sodium molybdate. Nitrate reductase activity was assayed by adding 50  $\mu$ l of the reconstitution mixture to 440  $\mu$ l of the assay mixture which contained 0.1 M potassium phosphate (pH 7.4), 10  $\mu$ M 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  $\mu$ l 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 production of nitrite (8). No correction was made for NADPH interference. Color intensity was determined at 540 nm with a Lambda 1 spectrophotometer (The Perkin-Elmer Corp., Norwalk, Conn.).

**HPLC.** Two reverse-phase 5  $\mu$ C18 columns (Bio-Rad) were connected in series for total column dimensions of 400 by 4 mm. The effluent was monitored with a Perkin-Elmer 650-10S fluorescence spectrophotometer fitted with a flowthrough 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 and 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 pterine-6-carboxylic acid (98%) were purchased from Aldrich Chemical Co., Inc. (Milwaukee, Wis.). 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 Co. (Pittsburgh, Pa.). All other chemicals and reagents were obtained from Sigma.

## RESULTS

**Cofactor isolation.** Anoxic gel filtration chromatography of guanidine-denatured FDH resolved three major peaks of material that fluoresced only after exposure to air (Fig. 1). Fluorescence could only be detected 10 h after exposure, and maximum fluorescence intensity was reached after 24 h. Samples not exposed to air showed no fluorescence after 24 h. In two separated 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 (Fig. 2) showed excitation maxima of 385, 325, 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 and 310 nm and the maximum emission to 455 nm. The fluorescence decreased in intensity with decreasing pH (Fig. 3). These properties were similar to those of described molybdopterin cofactors (9–11). The FDH cofac-

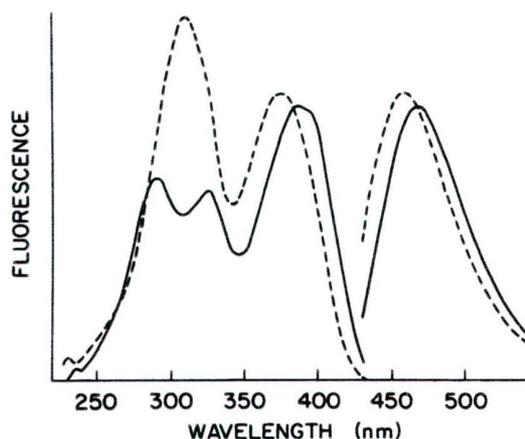


FIG. 2. Fluorescence spectra of the cofactor isolated from FDH. Fractions from peak A (Fig. 1) were pooled and lyophilized. Spectra are of cofactor redissolved in 1 M ammonium hydroxide, approximately pH 11 (—), or water, approximately pH 6 (---), after 24 h of exposure to air. Excitation was at 380 nm, and emission was at 465 nm.

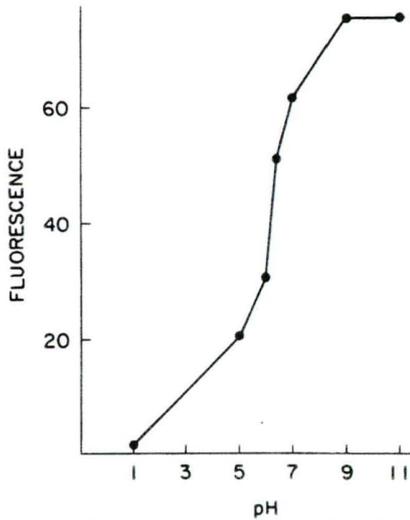


FIG. 3. Effect of pH on fluorescence of the cofactor isolated from FDH. The lyophilized material described in the legend to Fig. 2 was dissolved in water and exposed to air for 24 h. The pH was adjusted with hydrochloric acid or ammonium hydroxide. Relative fluorescence was measured by excitation at 380 nm and emission at 465 nm.

tor 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).

The fluorescence spectra of peaks B and C (Fig. 1) indicated the presence of pterin derivatives (data not

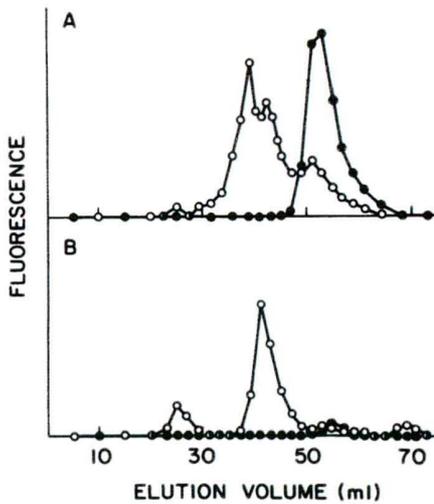


FIG. 4. Gel filtration chromatography of the oxidized cofactors aerobically released from milk XO (A) and FDH (B). The cofactors were released from both enzymes (1 mg of protein) by boiling at pH 2.5 in the presence of air-potassium iodide- $I_2$  and then chromatographed on a Fractogel TSK-HW40S column as described in Materials and Methods. Molybdopterin cofactor fluorescence (O) 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.

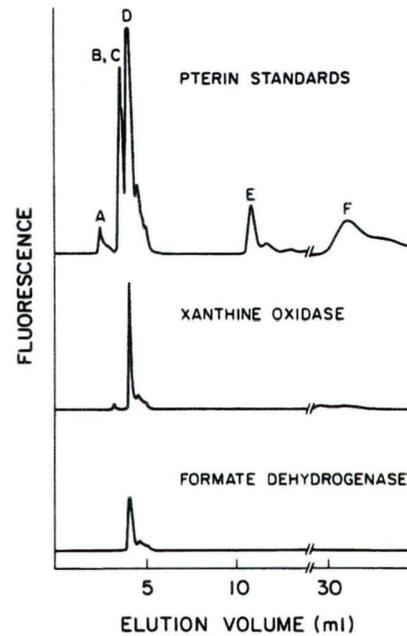


FIG. 5. HPLC elution profiles of pterin standards (top) and permanganate-oxidized cofactors from milk XO (middle) and FDH (bottom). Fluorescence was monitored by excitation at 380 nm and emission at 465 nm. Pterin standards: A, pterin-6,7-dicarboxylic acid; B, 6-methylpterin-7-carboxylic acid; C, pterin-7-carboxylic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

shown). When the enzyme was aerobically denatured and exposed to air for 24 h 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  $I_2$  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 FDH were released in this manner and purified by gel filtration chromatography (Fig. 4). The bulk of pterin oxidation products from both enzymes were separated from denatured protein and FAD. The FAD-free fractions were pooled and analyzed by reversed-phase HPLC, which indicated that a mixture of several fluorescent derivatives was obtained from each cofactor. The excitation and emission spectra of the mixtures from both cofactors were nearly identical to each other and 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, identified as pterin-6-carboxylic acid (Fig. 5). The fluorescence spectra of the compound from the FDH cofactor were nearly identical to the spectra of pterin-6-carboxylic acid (Fig. 6) and those of the compound from the XO cofactor (data not shown). These results suggest 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, and 7-methylpterin standards (Fig. 5). Fluorescence maxima characteristic of the above 7-substituted pterins

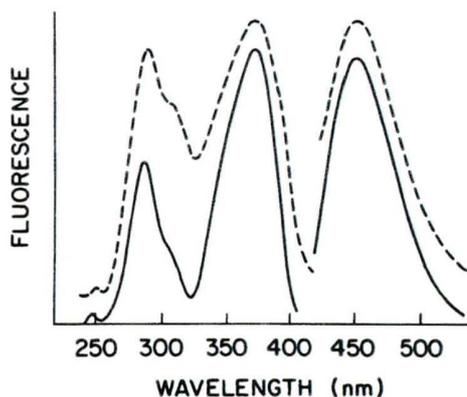


FIG. 6. Fluorescence spectra of the alkaline permanganate oxidation product from the FDH cofactor (---) and pterin-6-carboxylic acid (—) in 1 M ammonium hydroxide. The FAD-free fractions (Fig. 4) were pooled, and samples were oxidized as described in Materials and Methods. Excitation was at 365 nm and emission at 450 nm.

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, including 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  $P_i$ , no  $P_i$  was detected in cofactor samples, which indicated that only covalently bound  $P_i$  remained. After removal of unspecific  $P_i$  and ashing, 16.4 nmol of  $P_i$  was detected in cofactor isolated from 1.0 mg of protein (calculated from the mean of eight determinations of cofactor isolated from 0.34 to 0.68 mg of protein). Based on an  $M_r$  of 177,000 for FDH (18), a total of three  $P_i$  were obtained per enzyme molecule. It is estimated that FDH contains one molybdopterin cofactor, since the enzyme contains one molybdenum center (3, 18). Since the enzyme was free of flavin, these results suggest that covalently bound  $P_i$  was associated with the pterin cofactor. The instability of the cofactor excluded quantitation and therefore determination of the amount of  $P_i$  per cofactor; however, the results suggest that more than one  $P_i$  per molybdenum center is likely.

**Activity.** The molybdopterins cofactors from XO and FDH, released by three different methods, were tested for the ability to complement the cofactor-deficient nitrate reductase of the *N. crassa nit-1* mutant (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  $A_{380}$  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, which indicated that neither FDH nor the cofactor inhibited complementation. No activity was obtained with the FDH cofactor when the procedure was done aerobically.

#### DISCUSSION

The results presented here indicate that FDH from *M. formicicum* contained a molybdopterin cofactor. Similar to

TABLE 1. Complementation of *N. crassa nit-1* mutant nitrate reductase with cofactors from FDH and milk XO

Release <sup>a</sup> method	Mean ( $\pm$ SD) nmol of nitrite (no. of trials) produced by: <sup>b</sup>		
	XO	FDH	XO + FDH
6 M guanidine hydrochloride	24.6 $\pm$ 2.8 (7)	0.6 $\pm$ 1.0 (7)	26.9 $\pm$ 1.4 (3)
1% Sodium dodecyl sulfate	32.4 $\pm$ 1.8 (3)	0.0 $\pm$ 0.0 (3)	
pH 2.3	6.3 $\pm$ 1.0 (3)	0.5 $\pm$ 0.7 (3)	

<sup>a</sup> See Materials and Methods.

<sup>b</sup> Produced in 30 min by 50  $\mu$ l of *N. crassa nit-1* mutant extract reconstituted with 1  $\mu$ l of denatured enzyme solution (see Materials and Methods). No nitrite was detected in controls when enzyme was omitted from the reconstitution mixture.

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, which indicated that it was noncovalently bound. The presence of this cofactor in FDH from an organism phylogenetically distant from both eubacteria and eucaryotes demonstrates the universality of molybdopterins 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 molybdopterins cofactors is unknown, but a structure is proposed based on 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, we isolated the cofactor 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 molybdopterins 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 those of other molybdopterins, but the FDH cofactor may have been modified upon release from the enzyme. Other hypotheses would be based on structural differences in the native 6-alkyl side chains of the cofactors. It is important to note that the molybdenum electron paramagnetic resonance signal of 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  $g_{avg}$  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.

#### ACKNOWLEDGMENTS

We thank David Brown for assistance in obtaining cell material. This work was supported by grant PCM-8409558 from the National Science Foundation and by basic research grant 5082-260-0710 from the Gas Research Institute.

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