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Composition of the Coenzyme F_{420}-Dependent Formate Dehydrogenase from *Methanobacterium formicicum*

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The coenzyme F_{420}-dependent formate dehydrogenase from *Methanobacterium formicicum* was purified to electrophoretic homogeneity by anoxic procedures which included the addition of azide, flavin adenine dinucleotide (FAD), glycerol, and 2-mercaptoethanol to all buffer solutions to stabilize activity. The enzyme contains, in approximate molar ratios, 1 FAD molecule and 1 molybdenum, 2 zinc, 21 to 24 iron, and 25 to 29 inorganic sulfur atoms. Denaturation of the enzyme released a molybdopterin cofactor. The enzyme has a molecular weight of 177,000 and consists of one each of two different subunits, giving the composition $\alpha_1\beta_1$. The molecular weight of the α-subunit is 85,000, and that of the β-subunit is 53,000. The UV-visible spectrum is typical of nonheme iron-sulfur flavoprotein. Reduction of the enzyme facilitated dissociation of FAD, and the FAD-depleted enzyme was unable to reduce coenzyme F_{420}. Preincubation of the FAD-depleted enzyme with FAD restored coenzyme F_{420}-dependent activity.

The methanogenic bacteria are phylogenetically distant from eubacteria and eucaryotes (10). Consistent with this division, they utilize several unusual cofactors, including the low-potential electron carrier coenzyme F_{420} (8-hydroxy-5-deazaflavin) (6). Several oxidation and reduction reactions are linked to coenzyme F_{420}, including the oxidation of formate catalyzed by formate dehydrogenase, which supplies electrons for the reduction of carbon dioxide to methane (8, 16, 27, 30).

Spectroscopic studies of the formate dehydrogenase from *Methanobacterium formicicum* indicate the presence of molybdenum and iron-sulfur centers (1). Present methods for the purification of this enzyme result in a decrease in the rate of coenzyme F_{420} reduction relative to methyl viologen reduction (27, 28), but preincubation with flavin adenine dinucleotide (FAD) restores coenzyme F_{420}-dependent activity, suggesting that FAD is an essential component (28). Studies have also identified a fluorescent pterin compound associated with the enzyme (27; H. D. May, N. L. Schauer, and J. G. Ferry, submitted for publication). The quantitation of components in coenzyme F_{420}-dependent enzymes is necessary to further understand their function in intramolecular electron transfer. Here we report on the composition and physical properties of the formate dehydrogenase from *M. formicicum* purified to electrophoretic homogeneity by methods that preserve coenzyme F_{420}-dependent activity.

MATERIALS AND METHODS

Cell material. *M. formicicum* JF1 (DSM 2639) was grown in 12-liter batches as described previously (26), except that the basal medium contained the following constituents (grams per liter): $NaHCO_2$, 6.0; NH_4Cl, 1.48; K_2HPO_4, 1.36; KH_2PO_4, 0.90; NaCl, 0.45; $MgSO_4$, 0.045; $CaCl_2 \cdot 2H_2O$, 0.06; $NaCH_3CO_2$, 2.0; Na_2CO_3, 3.0; Na_2MoO_4, 0.024; cysteine hydrochloride, 0.27; Na_2SeO_3, 0.0002; $Na_2S \cdot 9H_2O$, 0.27; $Fe(NH_4)(SO_4)_2$, 0.06; and resazurin, 0.001. Cultures were sparged with H_2-CO_2 (4:1) at 300 ml/min. Cells were harvested in the late log phase at an optical density of 3.0 to 4.5 (550 nm, 1-cm light path). Anoxic harvesting was performed by maintaining the cultures under an atmosphere of H_2-CO_2 (4:1) and using a model LE continuous-flow centrifuge (Carl Padberg, Lehr-Baden, West Germany) operated at 24,000 rpm. The cell paste was immediately frozen and then stored in liquid N_2.

Purification of formate dehydrogenase. Strictly anoxic manipulations and anoxic buffer solutions were used to exclude O_2 in every step of the purification as described previously (27). All steps were performed at 4°C. The anoxic buffer, 50 mM potassium phosphate (pH 7.5), also contained 5% (wt/vol) glycerol, 10 mM sodium azide, 10 μM FAD, and 2 mM 2-mercaptoethanol. The cell suspension (130 g of thawed cell paste in 260 ml of buffer) was passed through a French pressure cell (SLM-Aminco, Urbana, Ill.) at 1,405 kg/cm^2 and then centrifuged at 100,000 × *g* for 30 min. To the supernatant solution was added solid ammonium sulfate to 60% saturation (4°C) under strictly anoxic conditions as described previously (27). The solution was adjusted to pH 7.5 by the addition of 10% (wt/vol) ammonium hydroxide. Precipitated protein was removed by centrifugation at 100,000 × *g* for 30 min. The supernatant solution that contained the enzyme was applied to a DEAE-cellulose (Whatman DE-53) column (5 by 15 cm) equilibrated with buffer 60% saturated with ammonium sulfate (25°C). The column was developed at pH 7.5 with a 1.2-liter linear gradient of ammonium sulfate decreasing from 40 to 20% saturation. Formate dehydrogenase-containing fractions with the greatest specific activity were selected, concentrated by ultrafiltration through a YM-30 membrane (30,000-M_r cutoff; Amicon Corp., Lexington, Mass.), and loaded onto a phenyl–Sepharose CL-4B (Pharmacia, Inc., Piscataway, N.J.) column (2.5 by 20 cm) equilibrated with buffer 20% saturated with ammonium sulfate (6°C). The column was developed at pH 7.5 with a linear gradient of ammonium sulfate decreasing from 15 to 0% saturation. Formate dehydrogenase-containing fractions were combined and loaded directly onto a hydroxylapatite column (2.5 by 20 cm) (Calbiochem-Behring, La Jolla, Calif.). The column was developed at pH 7.5 with a linear gradient of potassium phosphate increasing from 0.05 to 0.3 M. Formate dehydro-

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genase-containing fractions were combined and concentrated to 5 ml by ultrafiltration as described above.

Unbound, exogenously added FAD was routinely removed from the purified enzyme by anoxic pressure dialysis under O_2-free N_2 in an Amicon ultrafiltration apparatus equipped with a YM-30 membrane. To the enzyme solution (5 ml) was added 5 ml of FAD-free anoxic buffer, followed by anoxic concentration to 5 ml. The enzyme was filtered in this manner 10 times. Native FAD-depleted formate dehydrogenase was prepared by removing the remaining bound FAD by the same pressure dialysis procedure, except that 0.5 mM formate or 1 mM sodium dithionite was added to the FAD-free buffer.

Analytical methods. (i) Formate dehydrogenase assay. Formate dehydrogenase was assayed as described previously (27) by monitoring the formate-dependent reduction of coenzyme F_{420} at 420 nm or of methyl viologen at 603 nm. One unit of activity was the amount of enzyme that reduced 1 μmol of electron acceptor per min, and the specific activity was expressed as units per milligram of protein. Coenzyme F_{420} was purified from *M. formicicum* as described previously (28).

(ii) Protein determinations. Protein was determined by the method of Bradford (3) with homogeneous formate dehydrogenase as the protein standard. The standard was prepared by first concentrating the enzyme solution fivefold under O_2-free N_2 (60 lb/in^2) and then adding anoxic distilled water to the original volume. The enzyme was filtered in this manner six times, and the retentate was dried under vacuum to a constant weight. The residue was weighed with a Cahn model 7500 electrobalance and dissolved in 50 mM potassium phosphate (pH 7.5).

(iii) Electrophoresis. Native polyacrylamide slab gel electrophoresis was performed with the Tris-asparagine (Sigma Chemical Co., St. Louis, Mo.) buffer system at pH 8.5 as described previously (4), except that 10 mM sodium azide, 2 mM dithiothreitol, and 5% (wt/vol) glycerol were included in the electrode buffer and 5 μmol of Tris-thioglycolate (Sigma) was added to the samples (5). Gels were stained for protein with Coomassie blue G-250 as described previously (25). Gels were stained for enzymatic activity as described previously (27) by incubation in 5 ml of the coenzyme F_{420} assay reaction mixture, which also contained 20 mM sodium formate. Incubation was carried out for 20 min at 25°C under an atmosphere of O_2-free N_2. The formate dependence of the activity stain was determined by incubating duplicate gels in assay mixture without formate. Activity-stained gels were photographed under long-wave UV light. Formate dehydrogenase activity was indicated by a dark band of the nonfluorescent, reduced form of coenzyme F_{420} against a fluorescent background of oxidized coenzyme.

The subunit composition and subunit M_r were determined by sodium dodecyl sulfate (SDS) polyacrylamide slab gel electrophoresis with 12% gels by the method of Weber and Osborn (31). Gels were stained for protein with Coomassie blue R-250. Densitometer scanning was performed with a Zeineh (LKB Instruments, Inc., Rockville, Md.) Soft LASER scanning densitometer.

(iv) Amino acid analysis. The amino acid analysis of formate dehydrogenase was performed by Lowell H. Ericsson (AAA Laboratory, Mercer Island, Wash.). Acid hydrolysis was done in 6 N HCl at 110°C for 24, 48, and 96 h. Cysteine was determined as cysteic acid after performic acid oxidation and acid hydrolysis; the amount was calculated from the cysteic acid-alanine ratio. Tryptophan was determined by 48-h alkaline hydrolysis at 135°C by the method of Hugli and Moore (13), and the amount was calculated from the trytophan-histidine ratio.

(v) Metal and inorganic sulfur determinations. Iron and zinc were determined by atomic absorption spectroscopy on an Instrumentation Laboratories model 351 spectrometer. Atomization was accomplished with an air-acetylene flame. Samples were diluted into the working range with 2 M nitric acid. Molybdenum was also determined by atomic absorption spectroscopy, except that the spectrometer was fitted with an Instrumentation Laboratories model 555 graphite furnace. Samples were dried under a stream of N_2 (75°C), digested for 1 h in 1 M nitric acid (75°C), and then dried again under N_2. Samples were diluted into the working range with 10 mM nitric acid. Subsamples were injected directly into the graphite furnace and dried at 75°C for 25 s and at 100°C for 25 s. Pyrolysis was done at 750°C for 30 s and at 950°C for 30 s. Atomization was done at 2,800°C for 5 s. Formate dehydrogenase samples and standards for neutron activation analysis were placed in polyethylene bags and irradiated for 4 h at a flux of 5×10^{13} neutrons cm^{-2} s^{-1}, followed by a 180° rotation and irradiation for 2 h at the same flux. Samples and standards were counted at a distance of 20 cm after 6 days and at 1 cm after approximately 6 weeks with a high-resolution germanium detector (GAMMA-X-1) (11). The uncertainty in concentration was one standard deviation, based on counting statistics.

Inorganic sulfide was determined by the method of Beinert (2) in 0.5-ml culture tubes fitted with serum stoppers (7 by 15 mm).

(vi) Analysis for bound flavin. Bound flavin was released by boiling (15 min) formate dehydrogenase solutions previously freed of exogenously added FAD. The boiled samples were centrifuged to remove precipitated protein, and the supernatant solutions were then analyzed by reconstitution of apo-D-amino-acid oxidase activity (9), which is specific for FAD. Apo-D-amino-acid oxidase was prepared by the method of Massey and Curti (20). FAD and flavin mononucleotide (FMN; Sigma) standards were purified by reverse-phase high-performance liquid chromatography as described previously (18); this method was also used to analyze the boiled enzyme supernatant solutions for the presence of released FMN.

(vii) Spectra. Absorption spectra were measured in cells of 1-cm path length with a Perkin-Elmer model 552 double-beam spectrophotometer. Excitation and emission spectra were measured with a Perkin-Elmer model 650-10s spectrofluorometer.

RESULTS

Purification of formate dehydrogenase. A representative summary of the purification of the formate dehydrogenase from *M. formicicum* is shown in Table 1. The ratio of coenzyme F_{420}-dependent activity to methyl viologen-dependent activity after the final step (0.081) was nearly identical to the ratio in the crude extract (0.077), indicating no selective loss of coenzyme F_{420}-dependent activity during the purification. In the final step, the formate dehydrogenase eluted from the hydroxylapatite column with a constant specific activity (±1 U/mg of protein) across one symmetrical protein peak. Although the specific activity was decreased, this step removed contaminating proteins. Further chromatography of the purified enzyme (3.5 mg) through a Sephacryl S-300 (Pharmacia) column (1.5 by 20 cm) failed to increase the specific activity of the starting material. No significant loss in coenzyme F_{420}-dependent activity occurred when the enzyme was freed of exogenously added

TABLE 1. Purification of formate dehydrogenase from *M. formicicum*

Step	Total protein (mg)	Sp act (μmol of acceptor reduced/min per mg of protein) assayed with:		Activity assayed with coenzyme F_{420}	
		Coenzyme F_{420}	Methyl viologen	Total U (μmol of coenzyme F_{420} reduced/min)	% Recovery
Crude extract	7,800	1.2	15.6	9,360	100
$(NH_4)_2SO_4$ supernatant	2,600	4.7	57.9	12,220	130
DEAE-cellulose	590	12.4	153	7,315	78
Phenyl-Sepharose CL-4B	26.5	41.2	507	1,092	12
Hydroxylapatite	18.0	36.2	447	652	7

FAD. In contrast, partial purification with sodium dithionite and without FAD in the buffer solution results in a 175-fold decrease in the ratio of coenzyme F_{420}-dependent activity to methyl viologen-dependent activity (28).

The formate dehydrogenase was purified to homogeneity, as judged by nondissociating polyacrylamide gel electrophoresis. Only one band was detected on gels stained for protein (Fig. 1, lane B), and this band was enzymically active when assayed with coenzyme F_{420} (Fig. 1, lane A).

Molecular weight and subunit composition. The M_r of the native formate dehydrogenase was estimated by two methods to be 177,000. Electrophoresis in polyacrylamide gels with several porosities showed the M_r to be 177,000 (Fig. 2). The M_r determined by gel filtration through a calibrated Sephacryl S-300 column was 176,000 (Fig. 3). The enzyme dissociated into two protein bands when electrophoresed in the presence of SDS (Fig. 1C). The M_r of the slower-moving band (α-subunit) was 85,000, and the M_r of the faster-moving band (β-subunit) was 53,000 (Fig. 4). Densitometer scans of gels showed that the α-subunit constituted 63% of the total protein and that the β-subunit constituted 37%. Based on relative protein concentrations and on subunit molecular weights, we estimated that the native enzyme contained one each of two different subunits (α,β configuration).

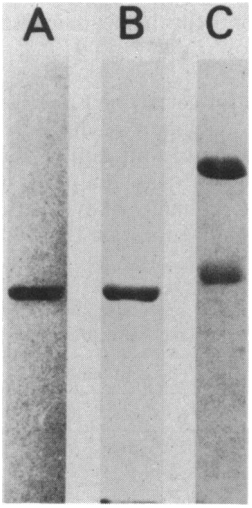

FIG. 1. Polyacrylamide slab gel electrophoresis of formate dehydrogenase in the absence and presence of SDS. (Lane A) Native enzyme (45 μg) stained for activity by the formate-dependent reduction of coenzyme F_{420} as described in Materials and Methods: (lane B) same as in lane A but stained for protein with Coomassie blue G-250; (lane C) enzyme (25 μg) electrophoresed in the presence of SDS and stained for protein with Coomassie blue R-250.

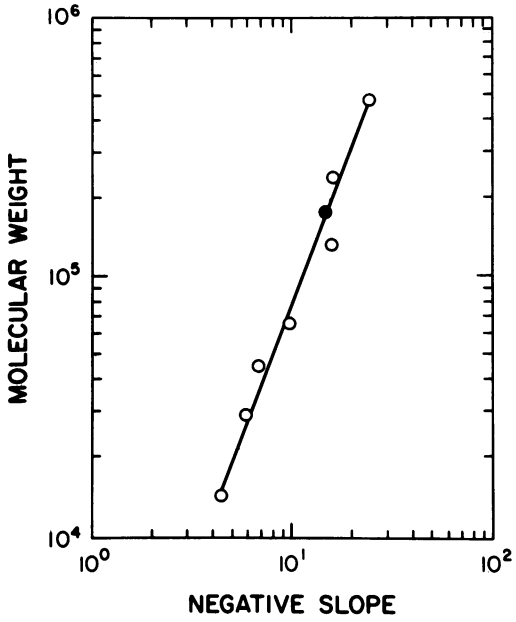

FIG. 2. Molecular weight determination of native formate dehydrogenase by polyacrylamide gel electrophoresis. The relative electrophoretic mobilities of protein standards and formate dehydrogenase were determined by the method of Hedrick and Smith (12) in nondenaturing gels with acrylamide concentrations of 4.5, 5.5, 6.5, 7.5, 8.5, and 9.5% (wt/vol) and with Tris-glycine buffer at pH 9.5. The log of the relative mobilities was plotted against the gel concentration by the method of Neville (23). The molecular weights of the proteins were plotted as a function of the negative slope. Symbols: ○, M_r standards from Sigma (urease tetramer, 480,000; urease dimer, 240,000; bovine albumin dimer, 132,000; bovine albumin monomer, 66,000; ovalbumin, 45,000; carbonic anhydrase, 29,000; lactalbumin, 14,200; ●, formate dehydrogenase.

Components. The formate dehydrogenase was analyzed for metal and inorganic sulfur content (Table 2). Based on an M_r of 177,000 for the native enzyme, the results indicated 21 to 24 mol of iron, 2 mol of zinc, 1 mol of molybdenum, and 25 to 29 mol of inorganic sulfur present per mol of enzyme. Neutron activation analysis indicated that the biologically active metals selenium, tungsten, and cobalt were not present in stoichiometrically significant amounts. Other elements detectable by neutron activation analysis but not present in significant amounts included chromium, cadmium, rubidium, barium, strontium, mercury, and cesium.

After exogenously added FAD was removed, boiling of the formate dehydrogenase released 1.0 ± 0.1 mol of bound FAD per mol of enzyme (M_r, 177,000) assayable with FAD-specific apo-D-amino-acid oxidase. Reverse-phase high-performance liquid chromatography confirmed that FAD was released, but FMN was not detected.

Based on an M_r of 177,000 for the native formate dehydrogenase, the amino acid residues per mole of enzyme were as follows: alanine, 149; arginine, 71; asparagine, 181; cysteine, 33; glutamine, 175; glycine, 130; histidine, 33; isoleucine, 96; leucine, 106; lysine, 99; methionine, 56;

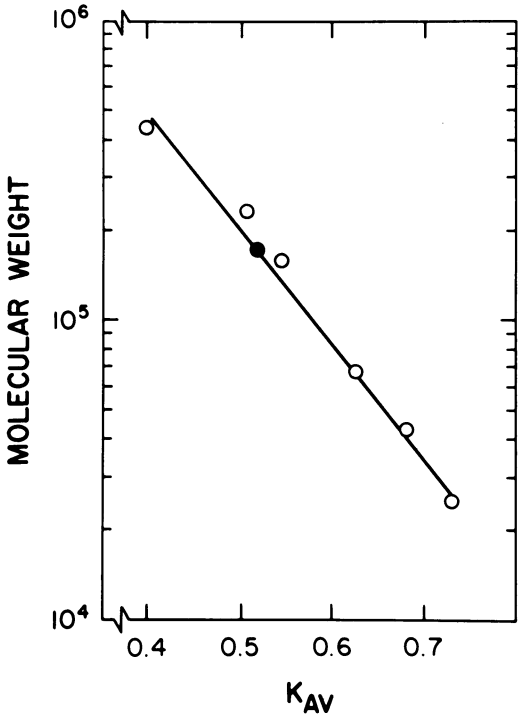

FIG. 3. Molecular weight determination of native formate dehydrogenase by gel filtration chromatography. Gel filtration was done on a Sephacryl S-300 (Pharmacia) column (0.9 by 60 cm) developed with anoxic buffer supplemented with 0.1 M KCl. Activity was monitored by the formate-dependent reduction of methyl viologen. Symbols: ○, M_r standards from Sigma (ferritin, 440,000; catalase, 232,000; aldolase, 158,000; bovine albumin, 66,000; ovalbumin, 45,000; chymotrypsinogen, 25,000); ●, formate dehydrogenase. K_{AV}, $(V_e - V_o)/(V_t - V_o)$ where V_o equals void volume, V_t equals total volume, and V_e equals elution volume.

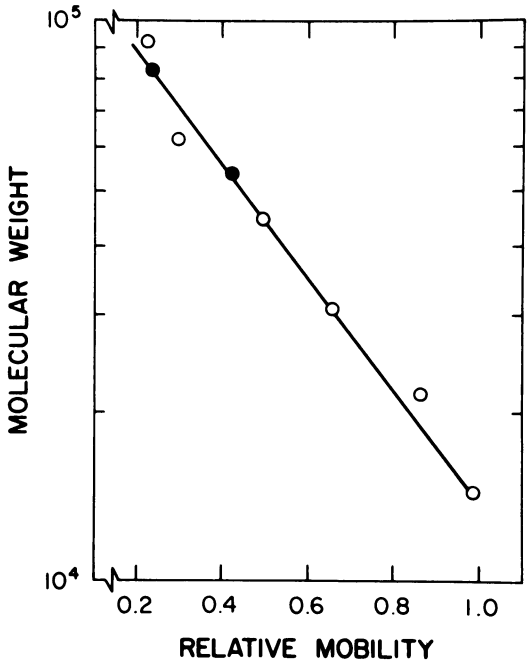

FIG. 4. Determination of subunit molecular weights by SDS slab gel electrophoresis. The relative mobilities of protein standards and formate dehydrogenase were determined as described in Materials and Methods. Symbols: ○, M_r standards from Sigma (phosphorylase *b*, 92,500; bovine albumin, 66,200; ovalbumin, 45,000; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500; lysozyme, 14,400); ●, formate dehydrogenase.

phenylalanine, 51; proline, 82; serine, 65; threonine, 87; tryptophan, 26; tyrosine, 49; and valine, 113. The results indicated a preponderance of acidic amino acids over basic amino acids. The ratio of uncharged polar to nonpolar amino acids was 0.74.

A fluorescent compound with spectra highly characteristic of pterin derivatives (Fig. 5) was also released from the enzyme previously depleted of bound FAD. Fluorescence required 48 h to fully develop after exposure to air. Maximal excitation occurred at 400 nm, and maximal emission occurred at 472 nm. The excitation and emission spectra were similar to those of form B of molybdopterin cofactors isolated from several molybdoenzymes (15) and pterin components recently isolated from other formate dehydrogenases (19, 33). Based on the molybdenum content (Table 2) and assuming 1 atom of molybdenum per molecule of pterin, the results indicated that there was 1 mol of molybdopterin per mol of formate dehydrogenase (M_r, 177,000).

Spectral properties. The anoxic (as prepared) formate dehydrogenase was brown in color and had an absorption maximum at 275 nm, with a shoulder at 325 nm. The visible absorbance increased from 600 to 350 nm. A brief exposure of the enzyme solution to air resulted in no significant loss in activity, but the spectrum showed additional absorbance in a broad double peak, with maxima at approximately 380 and 435 nm (Fig. 6). The A_{275}/A_{380} ratio was 3.9, and the A_{275}/A_{435} ratio was 4.6. The A_{380} and A_{435} decreased upon reduction of the enzyme with the substrate sodium formate (Fig. 6), and the decrease was not enhanced by subsequent reduction with sodium dithionite (data not shown). The difference spectrum between air-oxidized and formate-reduced enzymes showed a maximum at 435 nm, with shoulders at 380 and 325 nm (Fig. 6, inset). The absorbances in these regions may have been influenced by nonheme iron and FAD. The absorption coefficients for the air-oxidized formate dehydrogenase were as follows: $\varepsilon_{450}^{mM} = 50.9$, $\varepsilon_{400}^{mM} = 57.5$, $\varepsilon_{380}^{mM} = 62.3$, and $\varepsilon_{280}^{mM} = 290$.

FAD requirement for coenzyme F_{420}-dependent activity. No significant loss in coenzyme F_{420}-dependent activity relative to methyl viologen-dependent activity occurred when the formate dehydrogenase was dialyzed in the absence of formate (Fig. 7). Continued dialysis of the enzyme with buffer containing 0.5 mM formate (Fig. 7, arrow) resulted in

TABLE 2. Metal and inorganic sulfur content of formate dehydrogenase from *M. formicicum*

Component	Amt (nmol/mg of protein)in prepn:	
	I	II
Fe	118 ± 10[a]	133.9 ± 0.04[b]
Zn	8.8 ± 0.5[a]	11.2 ± 0.5[b]
Mo	6.2 ± 0.5[a]	5.5 ± 0.35[b]
W		0.03[b]
Se		0.02[b]
Co		0.003[b]
S	141 ± 5[c]	163 ± 5[c]

[a] Determined by atomic absorption spectroscopy.
[b] Determined by neutron activation analysis.
[c] Determined by chemical analysis.

an 11-fold loss in coenzyme F_{420}-dependent activity. Preincubation of the FAD-depleted enzyme with 17 μM FAD for 5 min at 35°C restored the coenzyme F_{420}-dependent specific activity from 0.2 to 2.2, with no significant change in the methyl viologen-dependent activity. Reduced FAD ($FADH_2$) or FMN did not replace FAD. Similar results were obtained when dithionite (1 mM) replaced formate as the reductant (data not shown). An absorbance typical of FAD was present in the visible spectrum of denatured enzyme previously dialyzed with dithionite-free buffer solution. In contrast, denatured enzyme previously dialyzed in the presence of dithionite had a featureless spectrum indicating the loss of bound FAD. These results indicated that formate or dithionite facilitated the dissociation of bound FAD from the formate dehydrogenase and, as previously suggested (29), showed that this flavin was required for the reduction of coenzyme F_{420} by the enzyme.

DISCUSSION

Previous studies showed that the coenzyme F_{420}-dependent activity of formate dehydrogenase from *M. formicicum* is unstable (27, 28). Here we described the properties of this enzyme anoxically purified to homogeneity by methods that stabilized the coenzyme F_{420}-dependent activity. The enzyme contained, in relative molar amounts, 1 molybdenum, 2 zinc, 21 to 24 iron, and 25 to 29 inorganic sulfur atoms and 1 molecule of FAD. The enzyme had an M_r of 177,000 and contained one each of two different subunits with M_rs of 85,000 and 53,000. The formate dehydrogenase contained no significant amounts of tungsten or selenium. Growth of the organism on formate and synthesis of the formate dehydrogenase by H_2-CO_2-grown cells was dependent on the presence of sodium molybdate in the growth medium, and sodium tungstate did not replace sodium molybdate (H. D. May, N. L. Schauer, and J. G. Ferry, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, I80, p. 159). These results suggest that *M. formicicum* synthesized only a molybdenum-containing formate dehydrogenase that did not require selenium. In contrast, *Methanococcus vannielii* synthesizes two formate dehydrogenases, an enzyme of M_r 105,000 that contains one molybdenum atom and a high-molecular-weight complex that contains tungsten and selenocysteine, when the organism is grown in tungsten-supplemented medium (16). The formate dehydrogenase from *M. formicicum* contained 1 mol of molybdenum per mol of enzyme. A pterin component was isolated from the enzyme, suggesting a molybdopterin. The properties of this molybdopterin are described elsewhere (May et al., submitted).

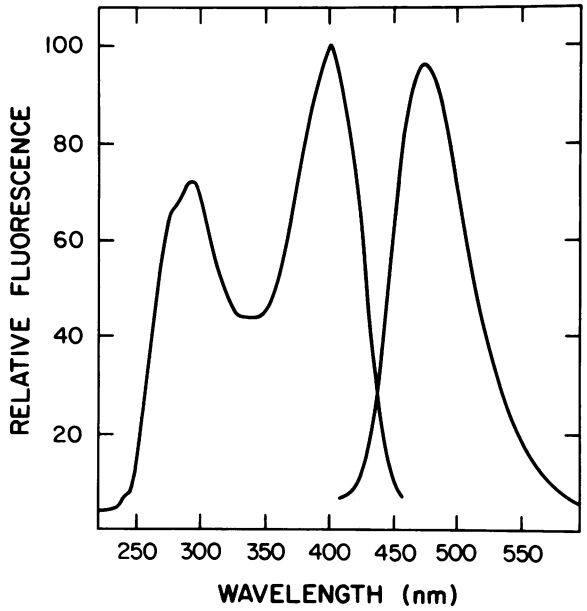

FIG. 5. Fluorescence spectra of the pterin component released from formate dehydrogenase. Bound FAD was released from 0.3 mg of formate dehydrogenase as described in Materials and Methods. The FAD-depleted enzyme solution was made 6 M in guanidine hydrochloride under anoxic conditions. Pterin was separated from denatured protein by anoxic ultrafiltration through a YM-30 membrane as described in Materials and Methods. The filtrate containing pterin was exposed to air, incubated for 48 h, and adjusted to pH 11 with 0.1 M ammonium hydroxide before spectroscopic analysis.

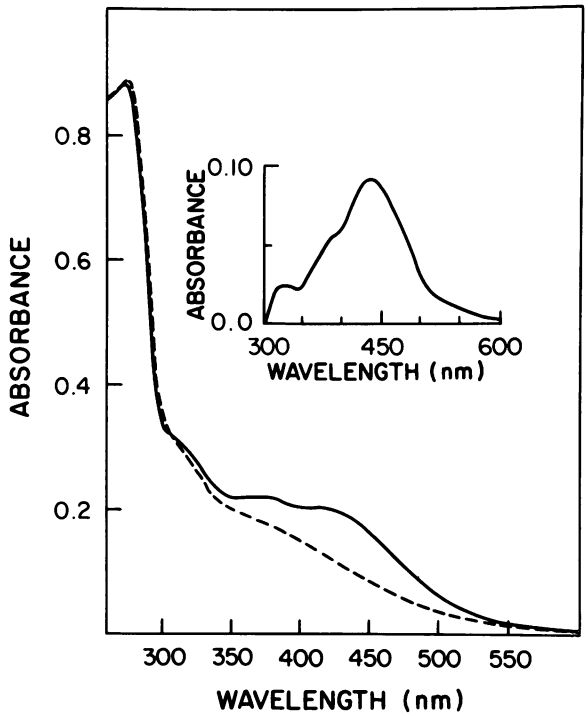

FIG. 6. UV-visible absorption spectra of formate dehydrogenase before and after reduction with sodium formate.(——) Spectrum of 0.425 mg of enzyme in 0.7ml of 50 mM potassium phosphate buffer at pH 7.5. The enzyme solution was exposed to air for 1 min and then degassed with N_2 to remove O_2 before analysis. (-----) Spectrum after the addition of 5 μmol of sodium formate to the air-oxidized enzyme. The inset shows the difference spectrum between air-oxidized and formate-reduced formate dehydrogenase.

The formate dehydrogenase contained 21 to 24 mol of iron per mol of enzyme, and the spectral characteristics were typical of enzymes that contain iron-sulfur clusters. The enzyme was highly enriched in equivalent amounts of iron, inorganic sulfur, and cysteine, suggesting the presence of multiple iron-sulfur clusters. When freed of exogenously added FAD, the formate dehydrogenase retained 1 mol of bound FAD per mol of enzyme, with no significant loss in coenzyme F_{420}-dependent activity. These results suggest that no additional components were required for the formate-dependent reduction of coenzyme F_{420} by the pure enzyme. As for many iron-sulfur flavoproteins, the visible spectrum of the native formate dehydrogenase did not have an absorbance characteristic of FAD. The FAD absorbance

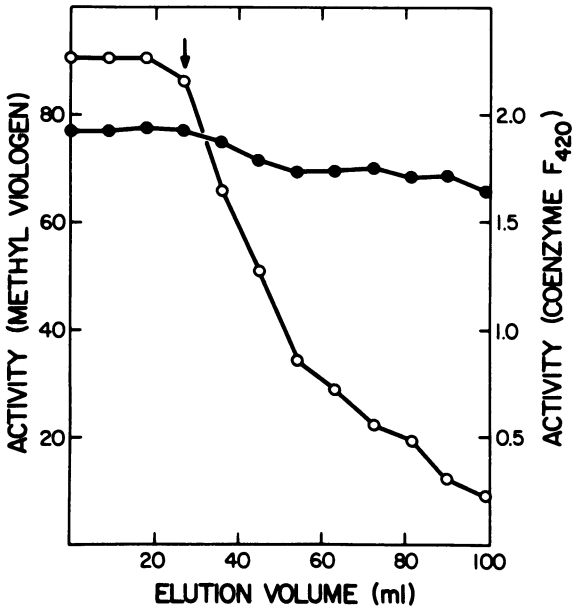

FIG. 7. Pressure dialysis of formate dehydrogenase in the absence or presence of sodium formate. Purified formate dehydrogenase (9.5 mg) was concentrated to a volume of 10 ml over an Amicon YM-30 (30,000-M_r cutoff) membrane filter and assayed for coenzyme F_{420}-dependent activity, methyl viologen-dependent activity, and protein. The solution was further concentrated to a volume of 1 ml, followed by the addition of FAD-free buffer to a total volume of 10 ml. Enzyme activity and protein were again measured. The enzyme was washed a second time in this manner. The solution was again concentrated to 1 ml, and FAD-free buffer containing 0.5 mM sodium formate (arrow) was added to the dialysis unit before enzyme activity and protein concentration were measured. The enzyme was washed an additional eight times in formate-containing FAD-free buffer. Activities are given in units per milligram of protein. Symbols: ●, methyl viologen-dependent activity; ○, coenzyme F_{420}-dependent activity.

was probably masked by the absorbances of the multiple iron-sulfur clusters. However, the maximum at 435 nm (Fig. 6) may be attributable to the 445-nm maximum of FAD shifted to a lower wavelength as a result of association with the enzyme. The presence of dithionite or formate facilitated the dissociation of bound FAD from the enzyme, and the results showed that the bound FAD was required for the reduction of coenzyme F_{420}. A possible function for FAD is the mediation of electron transfer between one-electron centers, such as molybdopterin and iron-sulfur clusters, and the obligate two-electron (hydride ion) acceptor coenzyme F_{420} (14, 22, 28). FAD is present in the coenzyme F_{420}-reducing hydrogenase from *Methanobacterium thermoautotrophicum* (14) and is required for the reduction of coenzyme F_{420} by the hydrogenase from *M. formicicum* (22). These results also indicate that the presence of FAD and the absence of dithionite in all buffer solutions stabilized the coenzyme F_{420}-dependent activity during purification of the formate dehydrogenase by the methods reported here.

The formate dehydrogenase from *M. formicicum* contained approximately 2 mol of zinc per mol of enzyme. A zinc requirement for catalytic activity was not investigated, and zinc could have associated with the enzyme during purification. However, the cellular zinc content of 10 species of methanogens is higher in the species that utilize formate, and zinc concentrations are positively correlated with the molybdenum content of the formate-utilizing species (29). Hypothetically, zinc could participate in a hydroxylation mechanism for the oxidation of formate; zinc, a Lewis acid, could stabilize hydroxyl ions, facilitating a nucleophilic attack at the carbon atom of formate. Zinc could also possibly act as a structural component or facilitate the reduction of FAD (24).

The methanogenic bacteria are phylogenetically distant from eubacteria and eucaryotes, and this division is thought to have been an early evolutionary event (10). Thus, a comparison of independently evolved formate dehydrogenases from these diverse groups is expected to identify conserved structures that may indicate essential functions for this enzyme. A diversity of physiological electron acceptors exists for the formate dehydrogenases studied from the eubacteria and methanogenic bacteria. The enzymes from the strictly anaerobic eubacteria *Clostridium thermoaceticum* (33), *Wolinella succinogenes* (17), *Clostridium pasteurianum* (19), and *Desulfovibrio vulgaris* (32) utilize $NADP^+$, quinones, ferredoxin, and cytochrome *c*-553, respectively. The facultative anaerobe *Escherichia coli* contains a membrane-bound formate dehydrogenase that reduces coenzyme Q (7). The enzyme from the aerobe "*Pseudomonas oxalaticus*" reduces NAD^+ (21). Formate dehydrogenases from the methanogenic bacteria are linked to coenzyme F_{420} (8, 16, 27, 30). This diversity of organisms and electron acceptors is also evident in the composition of the enzymes. The formate dehydrogenases studied (7, 17, 19, 33) contain either molybdenum or tungsten, except for the enzyme from "*P. oxalaticus*." All contain iron and inorganic sulfur, but only the enzymes (7, 17) from *E. coli* and *W. succinogenes* also contain heme iron. Among the eubacteria, only the formate dehydrogenase from "*P. oxalaticus*" contains flavin (FMN); as for the formate dehydrogenase from *M. formicicum*, the inactive deflavo-enzyme is reactivated by preincubation with FMN (21), consistent with the proposed function of flavin as an electron donor for the obligate two-electron acceptor NAD^+. As for the *M. formicicum* enzyme reported here, reducing conditions were required to remove the FMN component (21).

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