Physiological and Biochemical Characterization of the Soluble Formate Dehydrogenase, a Molybdoenzyme from *Alcaligenes eutrophus*

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Organoautotrophic growth of *Alcaligenes eutrophus* on formate was dependent on the presence of molybdate in the medium. Supplementation of the medium with tungstate lead to growth cessation. Corresponding effects of these anions were observed for the activity of the soluble, NAD⁺-linked formate dehydrogenase (S-FDH; EC 1.2.1.2) of the organism. Lack of molybdate or presence of tungstate resulted in an almost complete loss of S-FDH activity. S-FDH was purified to near homogeneity in the presence of nitrate as a stabilizing agent. The native enzyme exhibited an M_r of 197,000 and a heterotetrameric quaternary structure with nonidentical subunits of M_r 110,000 (α), 57,000 (β), 19,400 (γ), and 11,600 (δ). It contained 0.64 g-atom of molybdenum, 25 g-atom of nonheme iron, 20 g-atom of acid-labile sulfur, and 0.9 mol of flavin mononucleotide per mol. The fluorescence spectrum of iodine-oxidized S-FDH was nearly identical to the form A spectrum of milk xanthine oxidase, proving the presence of a pterin cofactor. The molybdenum-complexing cofactor was identified as molybdopterin guanine dinucleotide in an amount of 0.71 mol/mol of S-FDH. Apparent K_m values of 3.3 mM for formate and 0.09 mM for NAD⁺ were determined. The enzyme coupled the oxidation of formate to a number of artificial electron acceptors and was strongly inactivated by formate in the absence of NAD⁺. It was inhibited by cyanide, azide, nitrate, and Hg²⁺ ions. Thus, the enzyme belongs to a new group of complex molybdo-flavo Fe-S FDH that so far has been detected in only one other aerobic bacterium.

The aerobic facultative chemoautotroph *Alcaligenes eutrophus* is able to grow heterotrophically on various organic substrates or lithoautotrophically with H₂ and CO₂ as the sole sources of energy and carbon. The organism uses formate, which is oxidized to CO₂, as an energy substrate for organoautotrophic growth. Assimilation of CO₂ proceeds via the reductive pentose phosphate cycle (Calvin cycle) (10). During growth on formate, two formate dehydrogenases (FDH) are formed in *A. eutrophus*, a soluble, NAD⁺-dependent enzyme (S-FDH; EC 1.2.1.2) and a membrane-bound enzyme (M-FDH, formate:acceptor oxidoreductase), which is directly linked to the respiratory chain (13, 20). Such a pattern of FDH has only been reported for *Pseudomonas oxalaticus* (16), another aerobic, facultatively chemoautotrophic bacterium.

FDH are widespread in nature and have been isolated from many organisms including plants (42, 55), methylotrophic yeasts (2, 23, 26, 34, 50), aerobic (1, 17, 30, 32, 33, 41, 60) and anaerobic (3, 18, 36, 39, 59) bacteria as well as methanogenic archaea (31, 48). They occur as formate:NAD⁺-oxidoreductases (EC 1.2.1.2) or formate:NADP⁺-oxidoreductases (EC 1.2.1.43 [59]) and as oxidoreductases reacting with cytochromes (EC 1.2.2.1; EC 1.2.2.3), factor F₄₂₀, or other electron acceptors (18, 31, 36, 48, 58). Whereas FDH from methylotrophic yeasts have very similar overall properties, considerable diversity is found for bacterial FDH concerning their size, structure, cofactor and metal content, electron acceptor spectrum, and other features (19).

Among aerobic bacteria, FDH were isolated and characterized from a number of facultative and obligate methylotrophs (1, 17, 30, 32, 33, 60) and from *P. oxalaticus* (41).

According to their main properties, they can roughly be divided into three groups. (i) Soluble, NAD⁺-dependent enzymes that resemble the yeast-type FDH. These enzymes are homodimers, lack cofactors, do not react with artificial electron acceptors, show relatively low affinities for formate, and are not inactivated by oxygen (1, 17). (ii) Soluble, NAD⁺-dependent enzymes that react with redox dyes, have a complex subunit structure, nonidentical subunits, cofactors such as Fe-S centers and/or flavins, and contain biologically active transition metals like molybdenum or tungsten (30, 32, 33, 41, 60), some of them being oxygen-sensitive (41, 60). (iii) Particulate enzymes that do not react with NAD⁺ or NADP⁺ (15, 25).

Here we report on the purification and partial characterization of S-FDH from *A. eutrophus*. Evidence is presented that the organism requires molybdenum during growth on formate and that its S-FDH is a molybdo-flavo Fe-S protein. Thus, the enzyme belongs to a group of complex, NAD⁺-dependent FDH that so far have been found in only few aerobic bacteria and which resemble in some structural and functional features those of FDH from anaerobic prokaryotes.

MATERIALS AND METHODS

Organisms and growth conditions. In this study, *A. eutrophus* H16 (ATCC 17699) and strain HF210, a spontaneous megaplasmid (pHG1)-free mutant of streptomycin-resistant strain HF39, derived from wild-type strain H16 (35), were used. The organisms were grown at 30°C in mineral salts medium (49) supplemented with 1% (vol/vol) trace elements solution SL7 (43) to give a final molybdenum concentration (as Na₂MoO₄ · 2H₂O) of 0.15 μ M, unless stated otherwise. Tungsten (as Na₂WO₄ · 2H₂O) was added at 15 μ M or as

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indicated. Molybdenum-free (Mo-free) medium contained SL7 without added molybdate. For heterotrophic growth, glycerol, fructose, or pyruvate was added as the carbon and energy source at 0.2% (wt/vol); formate was supplemented at 0.2% (wt/vol) for organoautotrophic growth. During growth experiments with formate, cultures were manually titrated with formic acid (20%, wt/vol) to keep the pH value of the medium between 6.8 and 7.4. Growth was observed turbidimetrically with a colorimeter (model 900-3; Klett-Summerson, New York, N.Y.), equipped with filter no. 54. Klett units were transformed into optical density at 436 nm (OD_{436}) values by means of a calibration curve. Lithoautotrophic growth of strain H16 was performed with a gas mixture of hydrogen, carbon dioxide, and oxygen (8:1:1, vol/vol/vol).

Mass cultures were grown in a 10-liter fermentor (B. Braun, Melsungen, Germany) at a constant pH of 6.9, a stirring rate of 500 rpm, and an air supply of 5 liters/min. Titration of pH was done with formic acid (50%, wt/vol). Cells were harvested after the culture had reached an OD_{436} of 7 to 8, washed once at 4°C in 75 mM potassium phosphate (PP) buffer (pH 7.2) containing 10 mM KNO_3 (designated as enzyme buffer), and stored frozen at -20°C until used. Precultures for fermentors were grown as 500-ml suspensions in 2-liter baffled Erlenmeyer flasks with magnetic stirring, with a medium supplemented with 0.1% (wt/vol) fructose and 0.2% (wt/vol) formate.

Purification of S-FDH. All steps were performed at 0 to 4°C. Frozen cells (48 g wet weight) were thawed, suspended in 2.5 volumes of enzyme buffer, and disrupted by two passages through a French pressure cell at 130 MPa (19,000 lb/in²). Unbroken cells and debris were removed by centrifugation at 40,000 × *g* for 35 min. The pellet was discarded, and the supernatant was recentrifuged at 200,000 × *g* for 60 min to sediment particulate cell components. The resulting supernatant is referred to as soluble fraction. The soluble fraction was first brought to 0.25 saturation in ammonium sulfate, centrifuged to remove precipitated material, and subsequently brought to 0.45 saturation in ammonium sulfate. After centrifugation, the protein pellet was redissolved in a few milliliters of enzyme buffer and dialyzed against 40 mM PP buffer (pH 7.0) containing 10 mM KNO_3 (TMAE buffer). The solution was applied to a Fractogel EMD-TMAE 650 (M) column (4 by 9 cm; E. Merck AG, Darmstadt, Germany) equilibrated with TMAE buffer and eluted with six bed volumes of a linearly increasing KCl gradient from 0.05 to 0.5 M in TMAE buffer. S-FDH eluted at about 0.2 M KCl. Protein in active fractions was precipitated at 0.5 saturation in ammonium sulfate, pelleted by centrifugation, dissolved in a few milliliters of enzyme buffer, and dialyzed against 100 mM PP buffer (pH 7.2) containing 10 mM KNO_3 (HIC buffer) and 0.7 M ammonium sulfate. One-half of this protein solution was applied to a Fractogel TSK-Butyl 650 (S) column (1 by 15 cm; Merck) and eluted with seven bed volumes of a linearly decreasing ammonium sulfate gradient from 0.7 to 0 M in HIC buffer. S-FDH eluted at about 0.4 M ammonium sulfate. Due to the limiting column capacity, this step had to be repeated with the second half of the protein solution. Fractions with high S-FDH activity were dialyzed against 20 mM Tris-HCl buffer (pH 7.4) containing 5 mM KNO_3 and applied to a Cibachron Blue-agarose column (Matrex Gel Blue A; 1 by 9 cm; Amicon, Witten, Germany) equilibrated with the same buffer. Under these conditions, S-FDH was not bound to the gel material and eluted in the wash fraction. The latter was directly adsorbed on a Fractogel EMD-DEAE 650 (S) column (1 by 15 cm; Merck),

equilibrated with 40 mM PP buffer (pH 7.1) containing 10 mM KNO_3 (DEAE buffer). The bound protein was then eluted with six bed volumes of a linearly increasing KCl gradient from 0.05 to 0.65 M in DEAE buffer. S-FDH eluted at about 0.25 M KCl. Peak fractions of S-FDH activity were dialyzed against 5 mM PP buffer (pH 7.2) containing 5 mM KNO_3 (HA buffer) and applied to a hydroxylapatite column (0.5 by 7.5 cm; Merck). S-FDH did not adsorb to the column under these conditions, but residual contaminations were removed. The highly purified S-FDH solution showing a brownish color was concentrated (Microsep concentrator; Filtron, Karlstein, Germany) to a final volume of about 1 ml and could be stored frozen at -20°C for several weeks without significant loss of activity. The method of Bradford (11) with bovine serum albumin as the standard protein was used for protein determination.

Activity assays of S-FDH. A standard optical assay of S-FDH activity was performed at 30°C by observing the reduction of NAD^+ at 365 nm ($\epsilon_{365} = 3.43 \text{ mM}^{-1} \text{ cm}^{-1}$). The reaction mixture contained, in a final volume of 1 ml, 75 mM PP buffer (pH 7.7), 40 mM sodium formate, 2 mM NAD^+ , and an appropriate amount of S-FDH. Reactions were usually started by the addition of enzyme solution after 2 min of equilibration. One unit of activity was defined as the amount of enzyme catalyzing the reduction of 1 μmol of NAD^+ or an alternative electron acceptor per min. The following alternative electron acceptors were used at concentrations mentioned in the text: methylene blue ($\epsilon_{601} = 16.3 \text{ mM}^{-1} \text{ cm}^{-1}$), ferricyanide (FeCN ; $\epsilon_{405} = 13.2 \text{ mM}^{-1} \text{ cm}^{-1}$), 2,6-dichlorophenol indophenol (DCPIP; $\epsilon_{600} = 16.3 \text{ mM}^{-1} \text{ cm}^{-1}$), flavin mononucleotide (FMN; $\epsilon_{446} = 11.3 \text{ mM}^{-1} \text{ cm}^{-1}$), flavin adenine dinucleotide (FAD; $\epsilon_{446} = 12.5 \text{ mM}^{-1} \text{ cm}^{-1}$); $NADP^+$ ($\epsilon_{365} = 3.43 \text{ mM}^{-1} \text{ cm}^{-1}$); nitroblue tetrazolium ($\epsilon_{535} = 12.0 \text{ mM}^{-1} \text{ cm}^{-1}$), benzyl viologen (BV; $\epsilon_{550} = 7.78 \text{ mM}^{-1} \text{ cm}^{-1}$), and methyl viologen (MV; $\epsilon_{600} = 12.0 \text{ mM}^{-1} \text{ cm}^{-1}$). Methylene blue, BV, and MV were tested under anaerobic conditions. Potential inhibitors of S-FDH were added to the standard assay mixture at the concentrations mentioned. After 2 min of equilibration of S-FDH with the respective inhibitor at 30°C, the enzyme reaction was started by the addition of sodium formate.

The CO_2 reduction assay was done under anaerobic conditions in reaction buffer (pH 6.8) that had been boiled and subsequently gassed with CO_2 to saturate the buffer with CO_2 (29.3 mM dissolved CO_2 ; in equilibrium this solution contained 84 mM HCO_3^-). An anaerobic solution of 100 mM NADH was prepared in N_2 -gassed water and kept in N_2 -gassed, stoppered tubes. The reaction mixture consisted of 970 μl of CO_2 -saturated 75 mM PP buffer (pH 6.8), 20 μl of NADH, and 10 μl of highly active (69 U mg^{-1}) S-FDH solution. Diaphorase activity of S-FDH was tested anaerobically in 75 mM PP buffer (pH 7.7) in a final volume of 1 ml at 30°C with 2 mM NADH as the electron donor and FeCN , BV, or MV as potential electron acceptors at the concentrations stated. The reaction was started by adding S-FDH to the mixture.

S-FDH activity in whole cells was determined with cells made permeable by cetyltrimethylammonium bromide (CTAB). Cells from small culture volumes (30 to 50 ml) were suspended in enzyme buffer at an OD_{436} of about 100. This suspension was either used directly for activity tests or stored frozen at -20°C. The optical test was performed in a final volume of 1 ml that contained 75 mM PP (pH 7.7), 2 mM NAD^+ , 0.004% (wt/vol) CTAB, and 90 to 150 μg of cell protein. After equilibration for 2 min, the reaction was started by the addition of 40 mM sodium formate. Specific

activity values were calculated by the equation $U \cdot \text{ml}^{-1} \cdot (\text{OD}_{436} \cdot 0.133)^{-1} = U \cdot \text{mg}^{-1}$ of cell protein.

Molecular weight determinations. The relative molecular weight (M_r) of native S-FDH was determined by two methods: (i) nondenaturing gradient polyacrylamide gel electrophoresis (PAGE) (see below) or (ii) gel permeation chromatography (GPC) at 4°C and a flow rate of 0.4 ml/min on a GPC-Diol 500/5 column (1 by 30 cm; Merck) equilibrated with enzyme buffer containing 100 mM NaCl. Thyroglobulin ($M_r = 669,000$), apoferritin (440,000), β -amylase (200,000), ovalbumin (dimer; 90,000), and carbonic anhydrase (dimer; 58,000) were used as calibration proteins (Pharmacia, Freiburg, Germany). Elution was observed at 280 nm.

Gel electrophoresis. For nondenaturing gradient PAGE, a gel of 4 to 28% (wt/vol) acrylamide in 200 mM Tris-borate buffer (pH 7.7) containing 10 mM KNO_3 was run at 4°C with 30 mM Tris-borate (pH 7.9) containing 5 mM KNO_3 as the electrode buffer at a constant current of 20 mA for 6 to 7 h. The proteins of the high-molecular-weight marker kit (Pharmacia), including thyroglobulin ($M_r = 669,000$), apoferritin (440,000), catalase (232,000), lactate dehydrogenase (140,000), and bovine serum albumin (67,000) were used as size references.

Denaturing sodium dodecyl sulfate (SDS) discontinuous PAGE at 14% (wt/vol) acrylamide was performed by the method of Laemmli (38). Gradient SDS-PAGE employed gels of 4 to 28% (wt/vol) acrylamide with identical buffers and was run at a constant current of 20 mA at 4°C. For calibration, standard proteins in the M_r range from 14,300 to 170,000 were used (Combithek; Boehringer GmbH, Mannheim, Germany).

Protein staining. Proteins in gels were stained with either Coomassie blue (56) or silver (9). Staining for S-FDH activity after nondenaturing PAGE was done at 30°C under vigorous shaking in a reaction mixture containing 75 mM PP buffer (pH 7.5), 100 mM sodium formate, and 0.04% (wt/vol) nitroblue tetrazolium. The strongly violet activity band occurred after 1 to 2 min. After 10 min, the gel was washed once in distilled water and fixed for another 10 min in 7.5% (wt/vol) acetic acid.

Analytical methods. The absorption spectrum of S-FDH was recorded against a buffer blank on a Uvikon 810 spectrophotometer (Kontron, Eching, Germany) in a solution containing 0.53 mg of purified S-FDH in 1 ml of enzyme buffer. A baseline was stored and automatically subtracted from the measured values. Reduction of S-FDH was achieved by the addition of sodium dithionite or NADH as in Fig. 5.

Nonheme iron was determined by the bathophenanthroline method (6) in apparently homogeneous S-FDH (0.25 and 0.5 nmol) dialyzed against 40 mM Tris-HCl buffer (pH 7.4) containing 10 mM KNO_3 . Determination of molybdenum in the same S-FDH preparation (1.5 nmol) was done by atomic absorption spectroscopy (model 1100 B spectrometer; Perkin-Elmer, Überlingen, Germany). An equal volume of the dialysis buffer was used as a blank. Acid-labile sulfur in S-FDH was determined by the method of Beinert (7). The enzyme (0.1 and 0.2 nmol) used was treated as for the iron assay. The flavin component was liberated from the enzyme by heat treatment of 2 nmol of purified S-FDH in 220 μl of enzyme buffer (pH 7.0). The enzyme was denatured for 30 min at 100°C in a sealed plastic vial in the dark. After cooling for 5 min on ice, precipitated material was centrifuged off to give a colorless pellet and a clear, yellow supernatant which was subjected to high-pressure liquid chromatography (HPLC) and UV-visible spectroscopy analyses. Standard

solutions of FMN, FAD, and riboflavin were used to calibrate a reversed-phase HPLC Nucleosil 100 C18 column (Knauer, Bad Homburg, Germany) at 20°C. The column was eluted by a linearly increasing gradient from 15 to 30% (vol/vol) methanol in 5 mM ammonium acetate buffer (pH 5.9) at a flow rate of 2 ml/min, monitoring the A_{254} . Quantitation of the released flavin was also achieved by determination of the absorption difference between the oxidized and dithionite-reduced states at 450 nm, using an extinction coefficient of $10.6 \text{ mM}^{-1} \text{ cm}^{-1}$ (41).

The iodine oxidation method described by Johnson et al. (28) and Krüger and Meyer (37) was used to liberate form A of the molybdopterin cofactor from 0.5 nmol of purified S-FDH dissolved in 0.3 ml of 40 mM PP buffer (pH 7.2), omitting the gel filtration and ion-exchange chromatography steps. As a positive control, milk xanthine oxidase (Boehringer) was identically treated. Fluorescence spectra were recorded in a Kontron SFM 23/B spectrofluorometer at a fixed excitation wavelength of 380 nm and a fixed emission wavelength of 460 nm.

5'-GMP was identified and quantified by the method of Frunzke et al. (22) after acid hydrolysis of 1.2 mg of purified S-FDH. The protein-free supernatant was analyzed by HPLC on a reversed-phase C_{18} column under isocratic elution conditions, with a Waters 990 photodiode array detector (Millipore, Eschborn, Germany) for recording on-line absorption spectra.

RESULTS

Derepression experiments. S-FDH activity in *A. eutrophus* has previously been shown to be induced by formate (20). This conclusion was confirmed and extended as only formate-grown cells exhibited S-FDH activity ($0.25 U \cdot \text{mg}$ of cell protein $^{-1}$), whereas no activity was detectable after growth on H_2 - CO_2 , fructose, or pyruvate. To address the question whether S-FDH activity, as observed for hydrogenase activities in *A. eutrophus* (21), might be derepressible upon energy limitation after heterotrophic growth, we incubated pyruvate-grown cells for 5 h without the substrate. No S-FDH activity was found in such cells, suggesting that energy limitation did not cause derepression of the enzyme synthesis. Also, growth on a substrate mixture of fructose and glycerol that results in strong derepression of both types of hydrogenases during the second growth phase on glycerol following exhaustion of fructose (21) did not lead to derepression of S-FDH. It is therefore concluded that the hydrogenases and S-FDH are independently regulated.

Effects of molybdate and tungstate on growth. The inhibition of molybdate (Mo)-dependent growth of bacteria by tungstate (W) is a well-known fact (44). It was used to examine a possible Mo requirement of *A. eutrophus* during autotrophic and heterotrophic growth. Cells used to inoculate the test media were pregrown in four successive cultures in Mo-free fructose mineral medium in order to largely deplete the cells of molybdenum. Organoautotrophic growth of plasmid-free strain HF210 on formate (normal doubling time [t_d] of about 3 h) was completely inhibited in the presence of 10 μM W (Fig. 1), a concentration exceeding that of Mo in the standard medium (0.15 μM) by about 67-fold. The inhibition by W was already apparent at 0.1 μM W and increased steadily in a concentration-dependent manner up to full growth cessation (data not shown). Nevertheless, *A. eutrophus* was able to slowly grow with formate ($t_d = 10.5$ h; Fig. 1) in Mo-free medium. We attribute this fact to residual Mo in the medium. In contrast to growth on

FIG. 1. Growth of *A. eutrophus* HF210 in mineral medium on formate (0.2% [wt/vol]) in the presence of 0.15 μ M molybdate (●) (doubling time [t_d] = 3 h), 0.15 μ M molybdate plus 10 μ M tungstate (■), or without molybdate and tungstate added (Δ ; t_d = 10.5 h).

formate, W did not significantly influence either heterotrophic growth of the organism on fructose or pyruvate or lithoautotrophic growth of wild-type strain H16 on H_2 - CO_2 (data not shown).

Effects of molybdate and tungstate on S-FDH activity. To assess a possible correlation between growth inhibition on formate by W and the activity level of S-FDH, we assayed the enzyme in cells induced in the presence or absence of Mo and/or W. For this purpose, strain HF210 was grown on fructose to the log phase (OD_{436} of about 2) and then formate was added to the cultures to induce S-FDH formation. After 5 h of continued growth, S-FDH activity was determined in permeabilized whole cells. Only cells induced in a Mo-supplemented medium without added W displayed high S-FDH activity ($0.312 \text{ U} \cdot \text{mg of cell protein}^{-1}$). Absence of Mo or addition of W caused a dramatic reduction in activity ($0.017 \text{ U} \cdot \text{mg of cell protein}^{-1}$). Thus, a low activity of S-FDH appeared to correlate with growth inhibition on formate in the presence of W.

Purification of S-FDH. Since S-FDH activity in cell extracts of *A. eutrophus* prepared in unsupplemented buffers was unstable, resulting in an almost complete loss after 2 days, various compounds were tested for a stabilizing effect. Nitrate, azide, and NAD^+ proved to be most effective, with 10 mM nitrate (as KNO_3) increasing the half-life of S-FDH at 4°C to about 14 days. Thus, 10 mM KNO_3 was routinely included in all buffers to maintain the stability of S-FDH. The enzyme was aerobically purified 150-fold from the soluble cell fraction at an activity yield of just 14%, a result of collecting only the most active fractions and of a continuous slow inactivation of the enzyme. From 48 g of wet cells, 1.9 mg of nearly pure S-FDH with a specific activity of $80.2 \text{ U} \cdot \text{mg}^{-1}$ was obtained (Table 1). The pure enzyme had a brownish color and later proved to be an Fe-S protein (see below).

The copurification of a protein that was eventually identified as phosphoribulokinase (53) rendered the enrichment of S-FDH more difficult. Anion-exchange chromatography on TMAE-Fractogel actually yielded two distinct peaks of FDH activity, one of which also contained phosphoribulose kinase. This activity pattern was not altered by use of other separation methods like hydrophobic interaction chromatography, GPC, or density gradient centrifugation and led to

TABLE 1. Purification of S-FDH from the soluble cell fraction of *A. eutrophus* HF210

Step	Total protein (mg)	Total act ^a (U)	Sp act (U/mg)	Purification (fold)	Recovery (%)
Soluble fraction	2,075	1,098	0.53	1.0	100
Salt precipitate	1,027	1,163	1.13	2.1	106
TMAE-Fractogel	91	921	10.1	19	84
Butyl-Fractogel	32.5	503	15.5	29	46
Blue-agarose	6.9	340	49.3	93	31
DEAE-Fractogel	3.2	233	72.9	138	21
Hydroxylapatite	1.9	152	80.2	152	14

^a Activity determined by the S-FDH standard assay.

considerable losses in yield. Only by affinity adsorption chromatography on Cibachron Blue-agarose could phosphoribulose kinase be removed, and no further splitting of FDH activity peaks was observed.

Molecular weight. The M_r of native S-FDH was determined by GPC and by nondenaturing gradient PAGE. S-FDH eluted from the gel column in a single symmetrical protein peak coinciding with the enzyme activity. An average M_r of 197,000 was calculated from three different experiments. The results suggested the presence of only one native form of the enzyme with respect to its size. After three separate runs, nondenaturing gradient PAGE analysis gave an M_r of 177,000 (Fig. 2), which agreed fairly well with that determined by GPC. Activity staining of both the soluble fraction and the purified enzyme revealed only one FDH activity band showing the same M_r as the purified enzyme after protein staining (Fig. 2). The value obtained by GPC was considered to represent the true native M_r , because it nearly matched the sum of the subunit sizes (see below).

Subunit sizes and structure. SDS gradient PAGE experiments disclosed four types of subunits as constituents of purified S-FDH: α (M_r = 110,000), β (M_r = 57,000), γ (M_r = 19,400), and δ (M_r = 11,600) (Fig. 3). Their sizes add up to a calculated M_r of 198,700 for the holoenzyme, which is in excellent agreement with the data obtained by GPC. Apparently, S-FDH has a heterotetrameric ($\alpha\beta\gamma\delta$) structure. To confirm that the four polypeptides were enzyme subunits, purified S-FDH was subjected to nondenaturing gradient PAGE, cut off from the gel, electroeluted under nondenaturing conditions, and finally reelectrophoresed by SDS-PAGE. Again, the four polypeptides were observed, corroborating the conclusion. The subunits were present in stoichiometric amounts, as judged by spectrophotometric scanning of

FIG. 2. Gradient PAGE (4 to 28% [wt/vol] acrylamide) of S-FDH under nondenaturing conditions. Lanes: a, 8 μ g of protein from the soluble fraction of *A. eutrophus* HF210, activity staining; b, 1 μ g of purified S-FDH, activity staining; c, 2 μ g of purified S-FDH, Coomassie blue staining. Numbers on left show the sizes (in kilodaltons) of reference proteins.

FIG. 3. SDS-PAGE (14% [wt/vol] acrylamide) of purified S-FDH. Lanes: a, 1.0 μ g of reference proteins; b, 0.8 μ g of S-FDH. Silver staining. Numbers refer to the sizes (in kilodaltons) of the reference proteins.

Coomassie blue-stained SDS-polyacrylamide gels (data not shown). A relative molar ratio for $\alpha:\beta:\gamma:\delta$ of 1.0:1.1:1.3:1.1 was found, thus confirming the heterotetrameric structure of the S-FDH.

Catalytic properties. The pH dependence of S-FDH activity was determined to be in the range from pH 5.7 to 9.6 with overlapping buffer systems. In PP buffer, optimal activity was obtained at pH 7.7 that shifted slightly to pH 7.9 in Tris-HCl buffer. The enzyme showed a broad plateau of high activity in the range between pH 6.9 and 8.3. Below pH 5.0 and above pH 10.0, S-FDH was completely inactive. Optimal pH stability of S-FDH was found at pH 7.5 in the presence of 10 mM KNO_3 . Above pH 8.0, stability decreased rapidly. The temperature optimum of the enzyme was at 45°C, above which rapid inactivation occurred.

S-FDH exhibited the expected strict substrate specificity since only formate but not formaldehyde, methanol, ethanol, oxalate, acetate, lactate, malate, or maleate was used as an electron donor. Various potential artificial electron acceptors, covering a broad range of redox potentials, were tested, and a number of them, including BV and MV, were reduced by the enzyme (Table 2), a property which is common only to FDH of the more complex type (30, 41, 48, 59) but not to yeast FDH. None of the other natural electron acceptors tested could replace NAD^+ , pointing at a strict electron acceptor specificity *in vivo*. Substrate saturation kinetics of S-FDH for both formate and NAD^+ displayed simple

TABLE 3. Inhibitory effects of various compounds on the activity of S-FDH^a

Compound	Final concn (mM)	% Inhibition
NaN_3	1.0	58
	5.0	86
KNO_3	10.0	38
KCN	0.01	20
	0.1	52
	1.0	100
HgCl_2	0.01	100
2-Mercaptoethanol	1.0	0
Dithioerythreitol	1.0	0
Methanol	1,000	0

^a All assays were run at 10 mM sodium formate. The uninhibited partially purified S-FDH had a specific activity of 28.3 U $\cdot$ mg⁻¹.

Michaelis-Menten patterns. K_m values of 3.3 mM for formate and 0.09 mM for NAD^+ were obtained.

The enzyme also showed diaphorase activity with high-potential as well as low-potential electron acceptors and NADH as the electron donor, which suggested the presence of a flavin component (see below). Specific diaphorase activities (units per milligram of protein) determined at 2 mM NADH and the following electron acceptor concentrations were as follows: 1.56 with FeCN (2 mM), 38.62 with BV (1 mM), and 17.20 with MV (2.5 mM). The potential capacity of the enzyme to catalyze the reduction of CO_2 (29.7 mM dissolved CO_2) or HCO_3^- (84 mM) was tested at pH 6.8 with NADH (2 mM) as the electron donor under anaerobic conditions. No reaction was detected during an incubation period of 40 min.

Cyanide anions and Hg^{2+} cations were the most powerful inhibitors of S-FDH activity among the compounds tested for an inhibitory potential (Table 3). Cyanide gave a time- and concentration-dependent inhibition pattern when applied in micromolar concentrations. The enzyme activity was not restored within 20 min after addition of either 20 mM Na_2S or 20 mM FeCN. Reducing compounds such as 2-mercaptoethanol or dithioerythreitol had no effect on enzyme activity at the concentrations tested. Likewise, methanol, an inhibitor of enzymes containing a molybdopterin cofactor (14, 40, 45), did not affect the activity. Although azide and nitrate stabilized S-FDH activity, both anions caused significant inhibition of the enzyme. The inhibition by nitrate could not fully be overcome by an excess of formate. A mixed competitive-noncompetitive type of inhibition by nitrate (K_i of 15 mM) with respect to formate was observed (data not shown).

Reactivation of formate-inactivated enzyme by FMN. When S-FDH was preincubated for 2 min at 30°C with 40 mM sodium formate, it lost its activity, as the subsequent addition of NAD^+ yielded no reaction (Fig. 4). However, upon further addition of micromolar concentrations of FMN, reduction of NAD^+ slowly started to accelerate and proceed linearly after 3 to 4 min (Fig. 4). The extent of reactivation was dependent on the concentration of FMN added. FAD could not replace FMN, even during prolonged incubation for 20 min. Nevertheless, addition of FMN to the inactive mixture containing FAD restored enzyme activity to the same extent as that seen at the corresponding concentration of FMN in the absence of FAD.

UV-visible spectrum. The spectrum of the brownish, air-oxidized S-FDH revealed a broad region of absorption

TABLE 2. Relative activities of S-FDH with various potential electron acceptors

Electron acceptor ^a	$E^{\circ'}$ (mV)	Final concn (mM)	Relative act (%) ^b
FeCN	+360	2	10
DCPIP	+217	0.1	54
NBT	+50	0.2	74
MB	+11	0.1	194
FAD	-219	0.3	0
FMN	-219	0.3	0
NADP ⁺	-320	2	0
NAD^+	-320	2	100
BV	-360	5	113
MV	-446	5	74

^a Specific activity of partially purified S-FDH measured with the natural acceptor NAD^+ (28.3 U $\cdot$ mg⁻¹) was taken as 100%.

^b NBT, nitroblue tetrazolium; MB, methylene blue.

FIG. 4. Reactivation experiments with purified S-FDH inactivated by formate. S-FDH was inactivated by incubation in PP buffer (pH 7.7) in the presence of 40 mM sodium formate at 30°C for 2 min. Subsequent addition of 2 mM NAD^+ did not yield a reaction. Further addition of FMN at the various micromolar concentrations indicated led to a partial reactivation of the enzyme. The original activity of noninactivated enzyme (reaction started by addition of enzyme) is shown by the dashed line.

between 320 and 650 nm with maxima at 375, 422, and 570 nm and a shoulder at 453 nm (Fig. 5). Possibly the absorption peak at 422 nm was due to Fe-S centers in the enzyme, and those at 375 and 453 nm might originate from a flavin group. Bleaching the enzyme with either NADH or dithionite lead to a colorless protein solution and decreased absorption in the spectral range of interest.

Metal and cofactor determination. Molybdenum determination by atomic absorption spectroscopy revealed 0.64 g-atom/mol of enzyme. The determination of the iron content yielded 25 g-atom/mol of enzyme, and the content of acid-labile sulfur amounted to 20 g-atom/mol of enzyme. Heat or acid treatment of the purified enzyme resulted in a clear, yellow supernatant which contained a flavin compound as judged by fluorescence spectroscopy. The flavin was clearly identified as FMN by HPLC analysis (data not shown). Quantitation of these data gave 0.9 mol of FMN/mol of S-FDH. Additional quantitation by calculating the difference in A_{450} between oxidized and reduced flavin resulted in 0.92 mol of FMN/mol of S-FDH.

Upon iodine oxidation, a fluorescent compound was liberated from S-FDH that exhibited form A excitation and

FIG. 5. UV-visible absorption spectra of purified S-FDH in an air-oxidized state (solid line), reduced by 50 nM NADH (dashed line), or reduced by the addition of crystalline dithionite to 1 ml of enzyme solution (dotted line). Spectra were recorded with the enzyme (0.53 mg · ml⁻¹) in 40 mM PP buffer (pH 7.2) containing 10 mM KNO_3 and 200 mM KCl, against a buffer blank.

FIG. 6. Fluorescence spectra of form A of the molybdopterin cofactors isolated after iodine oxidation of purified S-FDH (solid line) or milk xanthine oxidase (dashed line), recorded in 1 N NH_4OH . Excitation spectra (on the left) were taken at an emission wavelength of 460 nm; emission spectra (on the right) were taken at an excitation wavelength of 380 nm.

emission spectra very similar to those obtained from identically treated milk xanthine oxidase (Fig. 6), an enzyme known to harbor a molybdopterin cofactor (57). Excitation maxima occurred at 276, 364, and 385 nm, and an emission maximum occurred at 456 nm. Thus, these results provide evidence that S-FDH is a molybdoenzyme containing a molybdopterin cofactor.

By reversed-phase HPLC analysis of the acid-hydrolyzed S-FDH, a single compound was detected at 13.8 min that showed the typical absorption spectrum of 5'-GMP (Fig. 7). Quantitation revealed 0.71 mol of 5'-GMP per mol of S-FDH. These data indicate that S-FDH contains a molybdopterin guanine dinucleotide as the molybdenum cofactor.

DISCUSSION

Effects of metals on S-FDH. Our present results demonstrated that *A. eutrophus* required Mo (i) during organoautotrophic growth on formate and (ii) in formation of active S-FDH, with W being an antagonist of Mo. A comparable inhibition of formate-dependent growth by W was also observed with *P. oxalaticus* (data not shown). An induction of NAD^+ -linked FDH activity by formate in the presence of Mo and inhibition of the induction by W has already been reported for the aerobic, facultative chemoautotroph *Paracoccus denitrificans* (12), but further features of this latter FDH are unknown. We ascribe the slow growth of *A. eutrophus* on formate in Mo-free medium to residual Mo originating from impurities of other medium components. A Mo concentration as low as 0.1 nM is sufficient to allow formation of active Mo-dependent enzymes in *Escherichia coli* (44). In this context, the second FDH, M-FDH, present in *A. eutrophus* has to be considered. This enzyme might (i) also require Mo for activity and be inhibited by W, (ii) be inhibited by W although it has no Mo requirement, or (iii) not be inhibited by W but its activity alone might not be sufficient for energy conservation and concomitant NADH formation from formate. Known effects of W on Mo-requiring bacterial cultures are (i) inhibition of Mo uptake into the cells, as described for the anaerobe *Clostridium pasteurianum* (24), and (ii) replacement of Mo by W within the molybdopterin cofactor (46) of Mo-dependent enzymes, thus rendering the holoenzyme inactive (FDH_N from *E. coli* [51]).

FIG. 7. Identification and quantitation of 5'-GMP by reversed-phase HPLC after acid hydrolysis of S-FDH, recorded at 270 nm. The inset shows the absorption spectrum of the material eluted at 13.8 min.

The data obtained so far for *A. eutrophus* do not allow conclusions to be drawn as to the mechanism of the W effect on S-FDH. Nevertheless, the fact that W-supplemented cells still contained about 5% of maximal S-FDH activity could imply the overwhelming presence of a molybdopterin cofactor in which W replaced Mo, leading to inactive holoenzyme molecules of S-FDH. Selenium (applied as 1 μ M selenite) did not affect organoautotrophic growth and S-FDH activity of the organism (data not shown). This was also observed for FDH activity in *P. oxalaticus* (41). In several anaerobic bacteria and archaea, selenite enhances growth and/or FDH activity (31, 47, 59). The FDH of these organisms are selenium enzymes.

Properties of S-FDH. Nitrate proved to be the most effective stabilizer of S-FDH activity during purification. The nitrate and azide anions, both being inhibitors of the enzyme, are thought to be transition-state analogs of formate during catalysis by FDH (8) and were reported to stabilize FDH from various bacteria (3, 32, 36, 48). Stabilization of S-FDH from *A. eutrophus* by nitrate as well as by azide was enhanced in the presence of NAD^+ . The determined K_m of the enzyme for formate is more than 1 order of magnitude higher than in similarly structured FDH, whereas the K_m for NAD^+ is in good agreement with values of other FDH (30, 41, 60). The reason for the high K_m for formate is not clear, although 0.1 mM nitrate from the enzyme stock solution was carried over into the assay mixtures. However, the simple Michaelis-Menten saturation kinetics for both formate and NAD^+ argue against a substantial influence of the nitrate on the calculated K_m values.

The most striking feature of the S-FDH from *A. eutrophus* is its complex subunit structure. Four types of subunits (α through δ) were found, closely resembling the subunit composition of the FDH from the aerobic methanotroph *Methylosinus trichosporium* OB3b (30). However, the *A. eutrophus* enzyme seems to be a heterotetramer ($\alpha\beta\gamma\delta$), whereas the native form of the FDH from *M. trichosporium* apparently has an $(\alpha\beta\gamma\delta)_2$ structure ($M_r = 400,000$ [30]). An earlier work on the latter enzyme reported an $(\alpha\beta)_2$ structure ($M_r = 315,000$ [60]), the same as for the S-FDH from *P. oxalaticus*

(41). Nevertheless, the M_r values determined for the α (110,000) and β (57,000) subunits of S-FDH from *A. eutrophus* are very similar to those from *M. trichosporium* and *P. oxalaticus*. All three enzymes differ markedly in their structure from the FDH of *Moraxella* sp. (α_2 ; $M_r = 98,000$ [1]) and *Pseudomonas* sp. strain 101 (α_2 ; $M_r = 80,000$ [17]; formerly designated as methylotrophic bacterium strain 1 or *Achromobacter parvulus*).

The measured value of 0.64 g-atom of molybdenum per mol of S-FDH points at a content of 1 g-atom of molybdenum per mol in the fully active enzyme. An amount of 0.71 mol of 5'-GMP per mol of S-FDH is similar to the molybdenum content found, also suggesting one mol of MGD per mol of native enzyme. A partial loss of MGD during purification is probably responsible for the lower molybdenum and 5'-GMP contents of the S-FDH preparation. In the FDH from *M. trichosporium* (30), a fairly comparable amount of 1.5 g-atom of molybdenum per mol of enzyme [$(\alpha\beta\gamma\delta)_2$ structure] was determined. However, no molybdenum was detected in the S-FDH from *P. oxalaticus* (41). The enzymes from *A. eutrophus* and *M. trichosporium* (30) also showed similar contents of iron (about 25 g-atom) and acid-labile sulfur (about 20 g-atom) per mol of enzyme, when referred to an $\alpha\beta\gamma\delta$ structure, and a very similar optical spectrum. These data indicate the presence of multiple Fe-S clusters in the S-FDH of *A. eutrophus* in the same manner as they occur in the *M. trichosporium* enzyme (30). Relative to the $(\alpha\beta)_2$ structure of the enzyme from *P. oxalaticus* and from *M. trichosporium* OB3b according to reference 60, the specific iron and sulfide contents in the former enzymes are about twice of those in the latter FDH. It can be speculated that the enzyme preparations from *P. oxalaticus* and *M. trichosporium* (60), which were done anaerobically, either had lost some of their iron and sulfur or a considerable amount of iron and sulfur must be located in the two smaller subunit species detected in the FDH preparations from *A. eutrophus* and *M. trichosporium* (30). Another striking difference between these two groups of FDH preparations concerns their specific activity values, which might relate to the number of redoxactive Fe-S centers. Whereas the enzyme preparations

from *A. eutrophus* and *M. trichosporium* (30) displayed high specific activities of 80 and 98 U · mg⁻¹, respectively, those from *P. oxalaticus* and *M. trichosporium* (60) had only about 32 U · mg⁻¹.

Addition of formate in the absence of NAD⁺ inactivates FDH from various aerobic bacteria to different extents (17, 33, 41, 60). The S-FDH from *A. eutrophus* was found to be completely inactivated by formate, a probable consequence of the release of the FMN from the enzyme. In the presence of NAD⁺, the inactivation was greatly diminished but remained detectable. The rate of NAD⁺ reduction in the standard FDH assay was linear only during the first 1 to 2 min with a subsequent progressive decline. When FMN was added to the reaction mixture, the reaction proceeded linearly for several minutes. Accordingly, formate-inactivated S-FDH could partially be reactivated by FMN, but not by FAD. This is equivalent to findings observed for the enzymes from *P. oxalaticus* (41) and *M. trichosporium* (60). FMN (1 mol/mol) was unambiguously shown to be the flavin component of S-FDH from *A. eutrophus*. The enzyme from *P. oxalaticus* was reported to contain 2 mol of FMN per mol (41). Two moles of an unidentified flavin cofactor are also present in the enzyme from *M. trichosporium* (30). The FDH ($\alpha\beta$ structure) from the archaeon *Methanobacterium formicicum* contains 1 mol of FAD (48).

Irreversible inhibition of air-oxidized S-FDH was achieved by treatment with cyanide, which is a well-known inhibitor of all known molybdoenzymes (4, 5, 14), except the iron-molybdenum cofactor-containing nitrogenases (52). S-FDH was also inhibited by azide and nitrate, which are known transition metal ligands (54). For nitrate, a mixed competitive-noncompetitive inhibition with respect to formate was detected, and formate did not completely abolish nitrate inhibition. It may be assumed that nitrate interacts with or near the formate-binding domain at the active site of S-FDH. Methanol usually inhibits molybdoenzymes in which Mo occurs in the redox state (V), which is attained by a one-electron reduction of Mo(VI) during turnover (5, 14, 45). However, methanol had no significant inhibitory effect on the S-FDH of *A. eutrophus*.

The absorption spectrum of oxidized S-FDH revealed maxima typical of Fe-S centers and of a flavin component and showed a nearly identical overall shape to that from the FDH of *M. trichosporium* (30). In contrast to the latter enzyme and other FDH, treatment of S-FDH with formate in the range from 0.5 to 100 mM produced only very little change in absorption (data not shown). Obviously, the redox-active centers responsible for the absorption were not significantly reduced when formate was applied in the absence of NAD⁺, although the enzyme was inactivated under these conditions. Complete reduction, however, was achieved upon treatment with NADH in the absence of formate. These findings might be due to only a minor reduction of the redox center(s) by formate in the absence of NAD⁺ in vitro. On the contrary, NADH is able to reduce the Fe-S center(s), probably via the FMN.

In agreement with its physiological function, purified S-FDH from *A. eutrophus* did not catalyze the reduction of CO₂ or bicarbonate, with NADH as the electron donor. Similarly, the FDH from *M. trichosporium* (30) and the N-FDH from *Pseudomonas* sp. strain 101 (32) showed no reverse reaction, unlike the enzyme from the anaerobe *Clostridium thermoaceticum* (59) that has a role in CO₂ reduction.

Molybdopterine cofactor. Purified S-FDH liberated a compound upon iodine oxidation that revealed fluorescence

spectra very similar to those of the form A compound from milk xanthine oxidase. The appearance of form A in a protein preparation definitely proves the existence of a molybdopterine cofactor (46). Thus, our findings are the first direct proof of a molybdopterine cofactor contained in an FDH from an aerobic bacterium, although the data obtained with the FDH from *M. trichosporium* strongly suggest the existence of the cofactor in that enzyme (30). The well-coinciding determined values of 0.64 g-atom of molybdenum and 0.72 mol of 5'-GMP per mol of S-FDH suggest a ratio of 1 mol of intact molybdopterine guanine dinucleotide per mol of native enzyme. This type of cofactor in the *A. eutrophus* enzyme, molybdopterine guanine dinucleotide is identical to the one already known from FDH of the facultatively anaerobic bacterium *E. coli* (29, 46) and the obligate anaerobe archaeon *M. formicicum* (27), which leads to the interesting question about molybdopterine guanine dinucleotide as the possibly common type of molybdenum cofactor in prokaryotic FDH.

Comparing the overall properties of the S-FDH from *A. eutrophus* and of the enzymes from *M. trichosporium* and *P. oxalaticus* with those of FDH from other organisms, these complex enzymes resemble FDH from anaerobic and facultatively anaerobic bacteria or from archaea rather than those from other aerobic bacteria (1, 17, 33). Nearly all the former enzymes share the involvement of one or more biologically active metal species bound to a pterin cofactor and to Fe-S clusters, a feature that is absent in the simpler-structured FDH from aerobic bacteria.

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REFERENCES

- Asano, Y., T. Sekigawa, H. Inukai, and A. Nakazawa. 1988. Purification and properties of formate dehydrogenase from *Moraxella* sp. strain C-1. *J. Bacteriol.* 170:3189-3193.
- Avilova, T. V., O. A. Egorova, L. S. Ioanesyan, and A. M. Egorov. 1985. Biosynthesis, isolation and properties of NAD-dependent formate dehydrogenase from the yeast *Candida methylca*. *Eur. J. Biochem.* 152:657-662.
- Axley, M. J., D. A. Grahame, and T. C. Stadtman. 1990. *Escherichia coli* formate-hydrogen lyase. Purification and properties of the selenium dependent dehydrogenase component. *J. Biol. Chem.* 265:18213-18218.
- Barber, M. J., H. D. May, and J. G. Ferry. 1986. Inactivation of formate dehydrogenase from *Methanobacterium formicicum* by cyanide. *Biochemistry* 25:8150-8155.
- Bauder, R., B. Tshisuaka, and F. Lingens. 1990. Microbial metabolism of quinoline and related compounds. VII. Quinoline oxidoreductase from *Pseudomonas putida*: a molybdenum-containing enzyme. *Biol. Chem. Hoppe-Seyler* 371:1137-1144.
- Beinert, H. 1978. Micro methods for the quantitative determination of iron and copper in biological material. *Methods Enzymol.* 54:435-445.
- Beinert, H. 1983. Semi-micro methods for analysis of labile sulfide and of labile sulfide plus sulfane sulfur in unusually stable iron-sulfur proteins. *Anal. Biochem.* 131:373-378.
- Blanchard, J. S., and W. W. Cleland. 1980. Kinetic and chemical mechanisms of yeast formate dehydrogenase. *Biochemistry* 19:3543-3550.
- Blum, H., H. Beier, and H. J. Gross. 1987. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* 8:93-99.
- Bowien, B., U. Windhövel, J.-G. Yoo, R. Bednarski, and B. Kusian. 1990. Genetics of CO₂-fixation in the chemoautotroph

- Alcaligenes eutrophus*. FEMS Microbiol. Lett. **87**:445–450.
11. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
 12. Burke, K. A., K. Calder, and J. Lascelles. 1980. Effects of molybdenum and tungsten on induction of nitrate reductase and formate dehydrogenase in wild type and mutant *Paracoccus denitrificans*. Arch. Microbiol. **126**:155–159.
 13. Chandra, T. S., and Y. I. Shetna. 1975. Oxalate and formate metabolism in *Alcaligenes* and *Pseudomonas* species. Antonie van Leeuwenhoek Int. J. Gen. Microbiol. **41**:465–477.
 14. Coughlan, M. P. 1980. Aldehyde oxidase, xanthine oxidase and xanthine dehydrogenase: hydroxylases containing molybdenum, iron-sulfur and flavin, p. 119–185. In M. P. Coughlan (ed.), Molybdenum and molybdenum containing enzymes. Pergamon Press, Oxford.
 15. Deyhle, R. R., and L. L. Barton. 1977. Nicotinamide adenine dinucleotide-independent formate dehydrogenase in *Mycobacterium phlei*. Can. J. Microbiol. **32**:125–130.
 16. Dijkhuizen, L., M. Knight, and W. Harder. 1978. Metabolic regulation in *Pseudomonas oxalaticus* OX1. Arch. Microbiol. **116**:77–83.
 17. Egorov, A. M., T. V. Avilova, M. M. Dikov, V. O. Popov, Y. V. Rodionov, and I. V. Berezin. 1979. NAD-dependent formate dehydrogenase from methylotrophic bacterium, strain 1. Purification and characterization. Eur. J. Biochem. **99**:569–576.
 18. Enoch, H. G., and L. Lester. 1982. Formate dehydrogenase from *Escherichia coli*. Methods Enzymol. **89**:537–543.
 19. Ferry, J. G. 1990. Formate dehydrogenase. FEMS Microbiol. Rev. **87**:377–382.
 20. Friedrich, C. G., B. Bowien, and B. Friedrich. 1979. Formate and oxalate metabolism in *Alcaligenes eutrophus*. J. Gen. Microbiol. **115**:185–192.
 21. Friedrich, C. G., B. Friedrich, and B. Bowien. 1981. Formation of enzymes of autotrophic metabolism during heterotrophic growth of *Alcaligenes eutrophus*. J. Gen. Microbiol. **122**:69–78.
 22. Frunzke, K., L. Gremer, P. Hofmüller, J. Tachil, D. Willbold, and O. Meyer. (Universität Bayreuth). 1993. Personal communication.
 23. Hou, C. T., R. N. Patel, A. I. Laskin, and N. Barnabe. 1982. NAD-linked formate dehydrogenase from methanol grown *Pichia pastoris*. Arch. Biochem. Biophys. **216**:296–305.
 24. Hughes, M. N., and R. K. Poole. 1989. Metals and microorganisms, p. 131–132. Chapman & Hall, Ltd., London.
 25. Izumi, Y., H. Kanzaki, N. Kato, and H. Yamada. 1988. Dichlorophenolindophenol-linked formate dehydrogenase of the methanol-utilizing *Mycobacterium gastri* MB19. FEMS Microbiol. Lett. **56**:277–280.
 26. Izumi, Y., H. Kanzaki, S. Morita, H. Futazuka, and H. Yamada. 1989. Characterization of crystalline formate dehydrogenase from *Candida methanolica*. Eur. J. Biochem. **182**:333–341.
 27. Johnson, J. L., N. R. Bastian, N. L. Schauer, J. G. Ferry, and K. V. Rajagopalan. 1991. Identification of molybdopterin guanine dinucleotide in formate dehydrogenase from *Methanobacterium formicicum*. FEMS Microbiol. Lett. **77**:213–216.
 28. Johnson, J. L., B. E. Hainline, K. V. Rajagopalan, and B. H. Arison. 1984. The pterin component of the molybdenum cofactor. Structural characterization of two fluorescent derivatives. J. Biol. Chem. **259**:5414–5422.
 29. Johnson, J. L., L. W. Indermaur, and K. V. Rajagopalan. 1991. Molybdenum cofactor biosynthesis in *Escherichia coli*. J. Biol. Chem. **266**:12140–12145.
 30. Jollie, D. R., and J. D. Lipscomb. 1991. Formate dehydrogenase from *Methylosinus trichosporium* OB3b. Purification and spectroscopic characterization of the cofactors. J. Biol. Chem. **266**:21853–21863.
 31. Jones, J. B., and T. C. Stadtman. 1981. Selenium-dependent and selenium-independent formate dehydrogenases of *Methanococcus vannielii*. J. Biol. Chem. **256**:656–663.
 32. Karzanov, V. V., Y. A. Bogatsky, V. I. Tischkov, and A. M. Egorov. 1989. Evidence for the presence of a new NAD-dependent formate dehydrogenase in *Pseudomonas* sp. 101 cells grown on a molybdenum-containing medium. FEMS Microbiol. Lett. **60**:197–200.
 33. Karzanov, V. V., C. M. Correa, Y. G. Bogatsky, and A. I. Netrusov. 1991. Alternative NAD-dependent formate dehydrogenases in the facultative methylotroph *Mycobacterium vaccae* 10. FEMS Microbiol. Lett. **81**:95–100.
 34. Kato, N., M. Kano, Y. Tani, and K. Ogata. 1974. Purification and characterization of formate dehydrogenase in a methanol-utilizing yeast, *Kloeckera* sp. no. 2201. Agric. Biol. Chem. **38**:111–116.
 35. Kortlücke, C., and B. Friedrich. 1992. Maturation of membrane-bound hydrogenase of *Alcaligenes eutrophus*. J. Bacteriol. **174**:6290–6293.
 36. Kröger, A., E. Winkler, A. Innerhofer, H. Hackenberg, and H. Schagger. 1979. The formate dehydrogenase involved in electron transport from formate to fumarate in *Vibrio succinogenes*. Eur. J. Biochem. **94**:465–475.
 37. Krüger, B., and O. Meyer. 1986. The pterin (bactopterin) of carbonmonoxide dehydrogenase from *Pseudomonas carboxydoflava*. Eur. J. Biochem. **157**:121–128.
 38. Laemmli, U. K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
 39. Liu, C.-L., and L. E. Mortenson. 1984. Formate dehydrogenase of *Clostridium pasteurianum*. J. Bacteriol. **159**:375–380.
 40. Meyer, O. 1982. Chemical and spectral properties of carbon monoxide:methylene blue oxidoreductase. J. Biol. Chem. **257**:13333–13341.
 41. Müller, U., P. Willnow, U. Ruschig, and T. Höpner. 1978. Formate dehydrogenase from *Pseudomonas oxalaticus*. Eur. J. Biochem. **83**:485–498.
 42. Peacock, D., and D. Boulter. 1970. Kinetic studies of formate dehydrogenase. Biochem. J. **120**:763–769.
 43. Pfennig, N., and H. G. Trüper. 1981. Isolation of members of the families Chromatiaceae and Chlorobiaceae, p. 279–289. In M. P. Starr, H. Stolp, H. G. Trüper, A. Ballows, and H. G. Schlegel (ed.), The prokaryotes. Springer-Verlag, KG, Berlin.
 44. Pinsent, J. 1954. The need for selenite and molybdate in the formation of formic dehydrogenase by members of the *Coli-aerogenes* group of bacteria. Biochem. J. **57**:10–16.
 45. Poels, P. A., B. W. Groen, and J. A. Duine. 1987. NAD(P)-independent aldehyde dehydrogenase from *Pseudomonas testosteroni*. Eur. J. Biochem. **166**:575–579.
 46. Rajagopalan, K. V., and J. L. Johnson. 1992. The pterin molybdenum cofactor. J. Biol. Chem. **267**:10199–10202.
 47. Sawers, G., J. Heider, and A. Böck. 1991. Expression and operon structure of the *sel* genes of *Escherichia coli* and identification of a third selenium-containing formate dehydrogenase isoenzyme. J. Bacteriol. **173**:4983–4993.
 48. Schauer, N. L., and J. G. Ferry. 1986. Composition of the coenzyme F₄₂₀-dependent formate dehydrogenase from *Methanobacterium formicicum*. J. Bacteriol. **165**:405–411.
 49. Schlegel, H. G., H. Kaltwasser, and G. Gottschalk. 1961. Ein Submersverfahren zur Kultur wasserstoffoxidierender Bakterien: Wachstumsphysiologische Untersuchungen. Arch. Mikrobiol. **38**:209–222.
 50. Schlensog, V., and A. Böck. 1990. Identification and sequence analysis of the gene encoding the transcriptional activator of the formate hydrogen lyase system of *Escherichia coli*. Mol. Microbiol. **4**:1319–1327.
 51. Scott, R. H., and J. A. DeMoss. 1976. Formation of the formate-nitrate electron transport pathway from inactive components in *Escherichia coli*. J. Bacteriol. **126**:478–486.
 52. Shah, V. K., R. A. Ugalde, J. Imperial, and W. J. Brill. 1984. Molybdenum in nitrogenases. Annu. Rev. Biochem. **53**:231–257.
 53. Siebert, K., P. Schobert, and B. Bowien. 1981. Purification, some catalytic and molecular properties of phosphoribulokinase from *Alcaligenes eutrophus*. Biochim. Biophys. Acta **658**:35–44.
 54. Thauer, R. K., G. Fuchs, and B. Käufer. 1975. Reduced ferredoxin:CO₂ oxidoreductase from *Clostridium pasteurianum*. Effects of ligands to transition metals on the activity and the

- stability of the enzyme. Hoppe-Seyler's Z. Physiol. Chem. **356**:653–662.
55. Uotila, L., and M. Koivusalo. 1979. Purification of formaldehyde and formate dehydrogenases from pea seeds by affinity chromatography and *S*-formylglutathione as the intermediate of formaldehyde metabolism. Arch. Biochem. Biophys. **196**:33–45.
56. Weber, K., and M. Osborn. 1969. The reliability of the molecular weight determination by dodecylsulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. **244**:4006–4012.
57. Wootton, J. C., R. E. Nicolson, J. M. Cock, D. E. Walters, J. F. Burke, W. A. Doyle, and R. C. Bray. 1991. Enzymes depending on the pterin cofactor: sequence families, spectroscopic properties of molybdenum and possible cofactor-binding domains. Biochim. Biophys. Acta **1057**:157–185.
58. Yagi, T. 1979. Purification and properties of cytochrome *c*-553, an electron acceptor for formate dehydrogenase of *Desulfovibrio vulgaris*, Miyazaki. Biochim. Biophys. Acta **548**:96–105.
59. Yamamoto, I., T. Saiki, and L. G. Ljungdahl. 1983. Purification and properties of NADP-dependent formate dehydrogenase from *Clostridium thermoaceticum*, a tungsten-selenium-iron protein. J. Biol. Chem. **258**:1826–1832.
60. Yoch, D. C., Y.-P. Chen, and M. G. Hardin. 1990. Formate dehydrogenase from the methane oxidizer *Methylosinus trichosporium* OB3b. J. Bacteriol. **172**:4456–4463.