Formate Dehydrogenase from the Methane Oxidizer
Methylosinus trichosporium OB3b
DUANE C. YOCH,* YUNG-PIN CHEN, AND MICHAEL G. HARDIN
Department of Biological Sciences, University of South Carolina, Columbia, South Carolina 29208

Received 19 December 1989/Accepted 4 May 1990

Formate dehydrogenase (NAD+ dependent) was isolated from the obligate methanotroph Methylosinus trichosporium OB3b. When the enzyme was isolated anaerobically, two forms of the enzyme were seen on native polyacrylamide gels, DE-52 cellulose and Sephacyrl S-300 columns; they were approximately 315,000 and 155,000 daltons. The enzyme showed two subunits on sodium dodecyl sulfate-polyacrylamide gels. The M, of the α-subunit was 53,800 ± 2,800, and that of the β-subunit was 102,600 ± 3,900. The enzyme (M, 315,000) was composed of these subunits in an apparent α2β2 arrangement. Nonheme iron was present at a concentration ranging from 11 to 18 g-atoms per mol of enzyme (M, 315,000). Similar levels of acid-labile sulfide were detected. No other metals were found in stoichiometric amounts. When the enzyme was isolated anaerobically, there was no cofactor requirement for NAD reduction; however, when isolated anaerobically, activity was 80 to 90% dependent on the addition of flavin mononucleotide (FMN) to the reaction mixture. Furthermore, the addition of formate to an active, anaerobic solution of formate dehydrogenase rapidly inactivated it in the absence of an electron acceptor; this activity could be reconstituted approximately 85% by 50 nM FMN. Flavin adenine dinucleotide could not replace FMN in reconstituting enzyme activity. The Kₜₜₜₜₜₜₜₜₜₜₜₜ of formate dehydrogenase for formate, NAD, and FMN were 146, 200, and 0.02 μM, respectively. “Pseudomonas oxalaticus” formate dehydrogenase, which has physical characteristics nearly identical to those of the M. trichosporium enzyme, was also shown to be inactivated under anaerobic conditions by formate and reactivated by FMN. The evolutionary significance of this similarity is discussed.

Among the large number of microorganisms that have formate dehydrogenase, the NAD-linked enzyme (EC 1.2.1.2) has been isolated from both facultative methylo-
trophic bacteria (3, 13) and several yeast species (20; also reference 4 and references therein). There is a great deal of heterogeneity in the structure and composition of bacterial formate dehydrogenases. They range from molecules with two identical subunits and no prosthetic group, found in facultative methylotrophic strains of Moraxella (30) and in Pseudomonas sp. strain 101 (formerly Achromobacter) (13, 24), to those isolated from the obligate anaerobes Clostridium thermoaceticum (33) and Methanococcus vannelli (20), which have three different metals in their prosthetic group(s). Escherichia coli formate dehydrogenase contains three different subunits, metals, and a heme group (14).

In methylotrophs, formate dehydrogenase is a critical component involved in the oxidative metabolism of methane and methanol. The NADH generated by this enzyme is, in some cases, believed to be the cell's only source of reductant (2). In Methylosinus trichosporium, NAD-linked formate dehydrogenase functions as an in vitro electron donor to methane monoxygenase (32) and indirectly to nitrogenase via a ferredoxin-NAD+ reductase and ferredoxin (9, 10) (Fig. 1). Although formate dehydrogenase plays an important role in the metabolism of nonfacultative methylotrophs (and obligate methanethanotrophs), this enzyme has not yet been isolated from either of these groups of bacteria. In this study we purified the formate dehydrogenase from the obligate methanotroph M. trichosporium and report on its structural and regulatory characteristics.

MATERIALS AND METHODS

Growth conditions. M. trichosporium OB3b cells used for formate dehydrogenase preparations were batch cultured in 9-liter carboys on nitrate-containing medium (11) as described previously (10). In these cultures, the air-methane mixture (5:1) was dispersed by passing it through stone diffusers and stirring the culture slowly with a magnetic bar. Our cultures did not attain a high density (80 to 120 Klett units, no. 66 filter) in these batch cultures and were harvested by centrifugation after 6 days of growth. After harvesting, the cells were immediately frozen (unwashed) in liquid nitrogen until needed.

Formate dehydrogenase assays. Unless otherwise indicated, formate dehydrogenase activity was measured at room temperature (22 to 25°C) by monitoring the reduction of NAD (ε340, 6.2 mM⁻¹ cm⁻¹) with a Perkin-Elmer Lambda 3 spectrophotometer under aerobic conditions. The reaction mixture (in 1 ml) contained 50 mM sodium phosphate buffer (pH 7), 20 mM sodium formate, 0.5 mM NAD, and 2.5 μM flavin mononucleotide (FMN) unless indicated. The reaction was started by the addition of enzyme to the cuvette. One unit of formate dehydrogenase activity was defined as the amount of enzyme that reduced 1 μmol of NAD per min. Specific activities are reported as units per milligram of protein.

Formate dehydrogenase activity was also assayed by monitoring the reduction of 2,6-dichlorophenol indophenol (DCIP). The assay mixture (in a final volume of 2 ml) contained enzyme, 25 mM Tris buffer (pH 7.4), 25 μM DCIP, 2.5 μM phenazine methosulfate, and 20 mM formate. The assays were carried out anaerobically in stoppered cuvettes under an atmosphere of argon by monitoring the reduction of DCIP (ε600, 19.1 mM⁻¹ cm⁻¹).

Purification of formate dehydrogenase. The enzyme was purified anaerobically from 75-g samples of cells that had
been stored in liquid N₂. The buffer used in all of the purification steps was composed of 50 mM sodium phosphate adjusted to pH 7.2, 10% (vol/vol) glycerol, and the proteinase inhibitors benzamidine (1 mM) and phenylmethylsulfonyl fluoride (0.1 mM). This mixture is referred to as the buffer in descriptions of the purification of formate dehydrogenase. When the enzyme was prepared anaerobically, the buffer was degassed by repeated cycles of evacuation and refilling of a side-arm flask with argon. Just before use, glucose (1 g/liter) and glucose oxidase (8 mg/liter) were added to the buffer as an oxygen scavenger system.

Extracts were prepared by anaerobic sonication as described previously (8). Following centrifugation for 20 min at 27,000 rpm, 1 mg of DNase I was added to the supernatant extract. Formate dehydrogenase was collected by passing the crude extract over a bed (approximately 6 cm) of DE-52-cellulose in a 60-cm chromatography column constantly flushed with argon. All the extract was applied to the column at once and maintained under this argon cover. The column was washed with degassed buffer; formate dehydrogenase was eluted as a sharp brown band, followed by a smaller band of activity with buffer containing 0.15 M NaCl. DE-52 chromatography was performed on the major peak of activity by diluting it threefold with buffer and applying it to another column (1.7 by 27 cm). The enzyme was eluted with 200 ml of a 0 to 0.3 M NaCl gradient. Because the enzyme sometimes lost its FMN cofactor at this stage, 1 μM FMN was routinely added to the reaction mixture before these fractions were assayed.

DE-52-cellulose fractions with the highest activity were diluted 1:1 with buffer and applied to a DEAE-Sephadex A50 column (0.9 by 28 cm) that had previously been equilibrated with degassed buffer. The column was eluted with 180 ml of a gradient of NaCl from 0.1 to 0.45 M in buffer. A major peak of activity (60% of the total) usually preceded a minor band at this stage of purification. The minor band (the lower-M₉ formate dehydrogenase species) was not purified beyond this point. The major peak of activity was concentrated on a Diaflow (Millipore Corp.) apparatus with a PM30 membrane. The concentrated enzyme was applied to a Sephacryl S-200 column (0.9 by 53 cm) eluted with anaerobic buffer containing 200 mM NaCl. Most (90%) of the activity was in a high-M₉ form. These fractions were finally applied to a second DEAE-Sephadex A-50 column which was equilibrated and eluted the same as the first. A number of fractions from this column had only minor impurities and were used for subunit analysis.

Formate-induced enzyme inactivation of formate dehydrogenase and its reconstitution by FMN. Formate dehydrogenase which was prepared aerobically yielded the active form of the enzyme. It was inactivated in a 50-μl reaction mixture in a stoppered degassed microtube containing 10 μl of glucose (1 g/liter) and glucose oxidase 8 mg and 0.5 mM sodium formate. After 1.5 min, 0.95 ml of the reaction mixture for NAD reduction (with or without FMN) was added to the microtube, and the solution was transferred to a cuvette to monitor NAD reduction. Since the flavin cofactor released from formate dehydrogenase was diluted out in this reaction mixture, it did not have to be removed from the formate-pretreated enzyme in order to demonstrate that the enzyme had been inactivated.

Polyacrylamide gel electrophoresis. Nondissociating discontinuous polyacrylamide tube gel electrophoresis was performed by the method of Hames (18). In this system, the gel buffer was pH 8.8 and the reservoir buffer was pH 8.3. Gels were stained for protein with 0.1% Coomassie blue R-250 in 25% methanol and 10% acetic acid, or for formate dehydrogenase activity by being placed in a solution of formate and triphenyltetrazolium chloride, as described by Yamamoto et al. (33). When activity stains were desired, the upper reservoir buffer (which was enclosed) was first degassed and then bubbled continuously with argon, while the gels were preelectrophoresed for 1 h. To rapidly remove the gels from the tubes, they were frozen in liquid N₂ for a minute to shatter the glass and then placed in test tubes containing degassed activity-staining solution. These tubes were stoppered, evacuated, and refilled with argon.

To determine the M₉ of the formate dehydrogenase subunits, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed with 10% polyacrylamide slab gels and a Laemmli-type discontinuous-system buffer run at pH 8.6 (18).

Other analytical methods. Protein was determined by the Folin-phenol method (26) with bovine serum albumin as the standard. The method of Andrews (1) was used to estimate the M₉ of formate dehydrogenase by the gel filtration method on Sephacryl S-300 which was equilibrated and developed in 0.1 M phosphate buffer, pH 7.2, containing 0.1 M KCl.

Trace metals were determined by inductively coupled plasma emission spectroscopy on a Jarrell-Ash model 965 ICP at the Institute of Ecology, University of Georgia, Athens. The sensitivity for iron was 0.05 ppm. Nonheme (ferrous) iron was also determined by the bathophenantroline method (18). Anaeroba ferredoxin (2 g-atoms of Fe per mol) was used as a standard. Acid-labile sulfide levels were determined by the method of Fogo and Popowsky (15) as modified by Brumby et al. (7).

RESULTS

Different M₉ forms of formate dehydrogenase. When crude extracts were electrophoresed on native disk gels and examined by an activity stain technique, the major band migrated in front of a minor, slower-migrating band of higher M₉ (data not shown). Furthermore, activity profiles from chromatography columns often, but not always, showed two peaks of NAD⁺-reducing activity. For example, on a DE-52-cellulose column, the major activity band eluted with a low concentration of NaCl and a minor peak of activity eluted with a higher salt concentration (Fig. 2). When the main peak from the DE-52-cellulose column was chromatographed on a Sephacryl S-300 column, the major formate dehydrogenase component was the high-M₉ component. We do not know at this time what controls the equilibrium between the two M₉.
forms of formate dehydrogenase, nor are we prepared to say which form predominates in vivo.

Purification of formate dehydrogenase. The purification of formate dehydrogenase from *M. trichosporium*, which resulted in a 130-fold increase in NAD"-reducing activity, is summarized in Table 1. The methods were anaerobic but did not include the use of a glove box. Column fractions were collected in air, evacuated and refilled with argon several times, and stored on ice. Purification under anaerobic conditions was always accompanied by loss of activity, but this activity could be stimulated 4- to 10-fold by the addition of FMN to the reaction mixture. This (reversible) loss of activity was greatest when an oxygen scavenger such as glucose or glucose oxidase was added to the buffers. When the enzyme was purified aerobically, there was a much smaller loss of activity, but it could not be recovered by added FMN. The flavin requirement for this enzyme is discussed in detail below. As shown in Table 1, a large loss in activity occurred on the first DE-52-cellulose column. This loss occurred in both aerobic and anoxic preparations. After the work here had been completed, we found that adding Fe²⁺ [100 μM Fe(III)₃SO₄] and a thiol reducing agent (1 mM cysteine) to the buffer increased the recovery of formate dehydrogenase activity at this step from 20% to approximately 70%. These reagents appear to reconstitute the Fe/S center(s) of formate dehydrogenase, just as they did for the hydrolyase portion of the methane monoxygenase of this organism (16).

The specific activity of formate dehydrogenase in crude extracts ranged from 0.12 to 0.3 μmol of NAD⁺ reduced per min per mg of protein. The purification process (Table 1) yielded near homogeneous enzyme having a specific activity of approximately 32 U/mg. Figure 3A, lane 1, is an activity stain of formate dehydrogenase on a native polyacrylamide gel. Lane 2 shows a Coomassie stain of the enzyme following purification; it appears to be greater than 90% pure.

**Molecular mass and subunit structure.** The molecular mass of *M. trichosporium* formate dehydrogenase determined on a calibrated Sephacryl S-300 column (0.9 by 58 cm) was approximately 315 kilodaltons (kDa) (Fig. 4). This value is an average from values for relatively pure samples applied to the column and for a preparation that had only one previous step of purification. The latter sample was used to determine whether the two size species of formate dehydrogenase that often exist early in the purification procedure were comparable in size to the purified components. They seemed to be, as sizes of 309 and 155 kDa were measured.

The 315-kDa form of formate dehydrogenase was demonstrated by electrophoresis in a 10% polyacrylamide gel in the presence of 0.1% SDS to have two subunits (Fig. 3B, lane 3). The α-subunit had a mean molecular weight of 102,600 ± 3,900, and the β-subunit was 54,800 ± 2,800 (five determinations). Since the low- Mr form of formate dehydrogenase also showed the same subunit size pattern (data not shown), this suggests that the enzyme is an αβ heterodimer and that the high- Mr form is an αβ₂ tetramer. The physical and

---

**TABLE 1. Purification of *M. trichosporium* formate dehydrogenase**

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Protein</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vol (ml)</td>
<td>Conc (mg/ml)</td>
</tr>
<tr>
<td>Crude extract</td>
<td>490</td>
<td>2.0</td>
</tr>
<tr>
<td>First DE-52-cellulose</td>
<td>87</td>
<td>1.1</td>
</tr>
<tr>
<td>Second DE-52 cellulose</td>
<td>14</td>
<td>2.0</td>
</tr>
<tr>
<td>DEAE-Sephadex A-50</td>
<td>8</td>
<td>1.0</td>
</tr>
<tr>
<td>First Sephacryl S-200</td>
<td>5.9</td>
<td>0.35</td>
</tr>
<tr>
<td>Second DEAE-Sephacryl A50</td>
<td>2.0</td>
<td>0.09</td>
</tr>
</tbody>
</table>
When mol/mol was the high extent formate, rather than isolated in the deflavo form. The addition of 5 mM sodium thiosulfate to the reaction mixture caused a brown precipitate to form. The amounts of iron, nickel, and molybdenum have been determined using several methods, including atomic absorption spectroscopy and x-ray fluorescence. The metal content of M. trichosporum formate dehydrogenase was found to be 11% iron, 18% nickel, and 2% molybdenum.

The enzyme preparation from M. trichosporum was then used to determine the molecular weight of the formate dehydrogenase. The Sephadex G-100 column (0.9 by 8.0 cm) was eluted with 0.1 M potassium phosphate buffer, pH 7.0, containing 0.1 M KCl. The elution profile showed a single peak at a molecular weight of 170,000.

Table 2 summarizes the composition of formate dehydrogenase.

Table 2. Composition of Formate Dehydrogenase

<table>
<thead>
<tr>
<th>Formate</th>
<th>NAD+</th>
<th>(mg/ml)</th>
<th>Formate</th>
<th>Temperature</th>
<th>pH</th>
<th>Activity</th>
<th>Specific Activity</th>
<th>Enzyme</th>
<th>Formate</th>
<th>NAD+</th>
<th>(mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.00</td>
<td>0.80</td>
<td>3.00</td>
<td>3.20</td>
<td>2.20</td>
<td>2.00</td>
<td>1.50</td>
<td>1.30</td>
<td>4.00</td>
<td>4.00</td>
<td>4.00</td>
<td>4.00</td>
</tr>
<tr>
<td>4.00</td>
<td>0.40</td>
<td>4.00</td>
<td>4.00</td>
<td>4.00</td>
<td>4.00</td>
<td>4.00</td>
<td>4.00</td>
<td>4.00</td>
<td>4.00</td>
<td>4.00</td>
<td>4.00</td>
</tr>
</tbody>
</table>

The enzyme had no activity in the absence of the thiol group, and no chromophore was reported. (4)
was prepared under aerobic conditions, the enzyme was fully active as isolated. However, when this enzyme was purified anaerobically, we found that these preparations lost most of their activity following an early chromatography step but that some of this activity could be recovered by adding low concentrations of FMN to the assay mixture (Fig. 5). The inset shows the kinetics of the FMN stimulatory effect on the activity of one of these fractions. When FMN was added either at the start of or during the reaction, the rate of NAD\(^+\) reduction was linear within 15 s.

When this phenomenon was examined further, it was found that formate dehydrogenase rapidly lost its activity in the presence of formate (Fig. 6A). Under aerobic conditions, the effect of formate was variable, but anaerobically the effect was highly reproducible. The half-life of the activity in the presence of 10 mM formate was approximately 25 s at room temperature. Formate was only effective if NAD was absent; the nonreducible analog, NADP\(^+\), would not substitute for NAD\(^+\) in protecting the enzyme. Much of this NAD-reducing activity could be recovered following anoxic formate treatment by supplementing the reaction mixture with FMN (Fig. 6A, solid circles) but not flavin adenine dinucleotide (FAD) (open triangles). While the recovery of activity was not complete, this may have been due to loss of enzyme activity in the presence of 10 mM formate. For maximum levels of reconstitution, formate could not be added above 0.5 mM and the time of this exposure had to be kept under 2 min. The concentration of formate giving half-maximal inhibition of formate dehydrogenase in 2 min was approximately 8 \(\mu\)M (Fig. 6B). The degree of maximum inhibition was usually about 85\%. Other reductants, such as 2-mercaptoethanol, sodium ascorbate, sodium sulfide, sodium succinate, and dithiothreitol (all at 10 mM), had no inhibitory effect.

The effect of FMN concentration on the reconstitution of formate dehydrogenase activity is shown in Fig. 6C. Inactivation by formate was approximately 90\% before the experiment began, and the reactivation by 50 nM FMN was about
FIG. 7. Formate-induced inactivation of “P. oxalaticus” formate dehydrogenase and its reactivation by FMN. “P. oxalaticus” formate dehydrogenase was obtained from Sigma Chemical Co. (St. Louis, Mo.) and used without further purification (specific activity, <1 U/mg). This enzyme (179 μg) was inactivated under strict anaerobic conditions (in a solution of glucose and glucose oxidase) as described in the text. Traces: a, NAD-reducing activity of the enzyme as purchased; b, activity following inactivation by formate for 2 min; c, activity of formate dehydrogenase from step b to which 1.25 μM FMN had been added.

85%. The saturation of this enzyme with FMN was similar to that obtained with a preparation of enzyme that was active as isolated; its $K_m$ for FMN was 0.015 to 0.03 μM (Table 2). The enzyme used in this experiment (Fig. 6C) had a much higher specific activity than that used in the inactivation experiments (Fig. 6A and B). The near homogeneity of the latter eliminates the possibility that an additional enzyme(s) plays a role in the reversible inactivation of formate dehydrogenase by formate or its reconstitution by FMN.

Dye reduction. Formate dehydrogenase also reduces a number of redox dyes, including DCIP. The role of flavin in dye reduction by this enzyme was tested by comparing rates of bleaching at 600 nm in the presence and absence of FMN. Formate dehydrogenase whose NAD-reducing activity was stimulated 91% by 2 μM FMN showed that the initial rate of DCIP reduction was stimulated only 15% by FMN. The kinetic pattern was, however, quite different in that dye reduction in the absence of added FMN slowed dramatically after 2 min (data not shown), leaving the role of the flavin in dye reduction unclear.

Reducive inactivation and FMN reconstitution of “Pseudomonas oxalaticus” formate dehydrogenase. Because the name Pseudomonas oxalaticus describes a strain of bacteria that is not, in fact, a member of the genus Pseudomonas (17), it is described as “species incertae sedis” in the eighth edition of Bergey’s Manual (12), and it is customary that the name appear in quotation marks. The formate dehydrogenase from “P. oxalaticus” is very similar in its physical properties to that of M. trichosporium in that both are FeS flavoproteins with the same molecular weight. This prompted us to look for other similarities, specifically the effect of formate pre-reduction on NAD-reducing activity. When a partially purified preparation of the “P. oxalaticus” enzyme was preincubated with formate under anoxic conditions, most of the activity was lost when it was subsequently assayed for NAD reduction (Fig. 7, trace b). The anoxic conditions were essential for the inactivation process, and the addition of FMN rapidly restored this activity (Fig. 7, trace c). These conditions are identical to those required for the reductive inactivation of M. trichosporium formate dehydrogenase.

**DISCUSSION**

The NAD-dependent formate dehydrogenase from M. trichosporium has been purified and shown to be a nonheme iron protein whose activity is FMN dependent. Much of the huge loss of activity in the first step of purification (Table 1) can be prevented if Fe$^{2+}$ and a thiol-reducing agent are added to the cell extract. Although glycerol in the buffer also helps, Fe$^{2+}$ and cysteine are the best stabilizing agents yet found for this enzyme.

The enzyme is composed of two types of polypeptide chains ($M_r$ 102,600 and 53,800) which aggregate to produce two different molecular weight forms. The predominant form appears to have an $M_r$ of approximately 315,000, a size consistent with that expected of an αβ$_2$ tetramer. This value (315 kDa) is considerably smaller than the 480 to 490 kDa reported previously (D. R. Jollie and J. D. Lipscomb, Abstr. 6th Int. Symp. Microbial Growth on C$_1$ Compounds, 1987, p. 227). These differences might be explained by the fact that a small variability in $V_s$ of the high-molecular-weight protein standards can make a big difference in the slope of the standard curve and, therefore, the $M_r$ of an unknown. Data points from duplicate runs of the standards, ferri- and thyroglobulin, were therefore plotted individually in Fig. 4. A standard curve constructed from chromatographing protein standards on a Bio-Gel A-1.5m column (2.5 by 50 cm) eluted with buffer containing 10% glycerol and 300 mM NaCl proved to be completely unsatisfactory because ferri-ent (440 kDa), one of the key standards, always eluted in a volume indicative of a 100-kDa protein. In sum, in our hands, the $M_r$ of M. trichosporium formate dehydrogenase is smaller than reported by the Minnesota group, but is nearly identical to that of a similar enzyme from “P. oxalaticus” (28).

Electrophoresis of crude extracts or enzyme from the initial DE-52-cellulose column usually showed one main band of activity with a minor band of higher molecular weight which developed more slowly, presumably because of its lower concentration. When the extracts were applied to columns, however, a very different picture emerged in that a high-$M_r$ form of formate dehydrogenase was dominant. The formate dehydrogenase from “P. oxalaticus,” which is otherwise strikingly similar in composition and subunit size to that of M. trichosporium (Table 2), shows a size pattern that is just the opposite in that the low-$M_r$ form of the enzyme appeared during purification (28). These workers believe that this enzymatic reaction, because it did not take place in pure enzyme preparations. We are not prepared to say which $M_r$ form is found in vivo, since extraction and electrophoresis may influence the aggregation of this enzyme.

It was assumed early in this study that because formate dehydrogenase contained nonheme iron, we would have higher success in purifying it anaerobically. A comparative aerobic isolation to homogeneity has not been done to confirm this assumption; however, we know that aerobic preparations of partial purity do not require added FMN and are stable at −20°C for at least a week under aerobic conditions and longer in liquid N$_2$. The decision to isolate formate dehydrogenase anaerobically did, however, lead to the interesting observation that FMN stimulated the NAD-reducing activity of this enzyme following the second chromatography step and thereafter. The greater the precautions to exclude air (such as the addition of glucose and glucose oxidase to the buffers), the quicker the enzyme required FMN during the course of the purification. This characteristic is not peculiar to the enzyme from M. trichosporum, since the formate dehydrogenase from “P. oxalaticus” releases FMN in the presence of either formate or sulfide (plus 1.2 M ammonium sulfate) (28) and that from M. formicicum releases FAD during purification (31). This phe-
of the reductive dissociation of the FAD is also seen in the \( F_{420} \)-reducing hydrogenase from \( M. \) formicicum (5) and the hydrogenase of \( Aklaligenes \) eutrophus (29) yielding the inactive deflavo enzymes. We have now shown (Fig. 7) that the flavin is reductively dissociated from "\( P. \) oxalat\-icus" formate dehydrogenase under the exact conditions used for the enzyme from \( M. \) trichosporium.

The photoheterotroph \( Rhodopseudomonas \) palustris, when adapted to grow on formate, was shown to have a formate dehydrogenase that was equally stimulated by either FMN or FAD (34). \( M. \) trichosporium formate hydrogenase was recently reported to contain a flavin that had characteristics of neither FMN nor FAD (D. R. Jollie and J. D. Lipscomb, Abstr. 6th Int. Symp. Microbial Growth on \( C_1 \) Compounds, p. 227). If this flavin nucleotide is not FNM, it is readily replaced by FMN after it is released from the enzyme in a reducing environment (Fig. 6A and C). Consistent with this observation, Kawamura et al. (25) showed that FNM greatly stimulated formate- (and NAD-) dependent \( H_2 \) evolution in extracts of \( M. \) trichosporium and \( Methylomonas \) albus, a process involving formate dehydrogenase and hydrogenase coupled by NAD. FAD and riboflavin were completely ineffective in these reconstitution processes (Fig. 6A and reference 25).

In regard to the isolated flavin from the \( M. \) trichosporium enzyme possibly having properties of neither FMN nor FAD, there is precedent for a modified flavin being isolated from \( Megasperhaera \) (formerly \( Pseudoptextroptocus \)) \( elsenii \) NADH dehydrogenase (27). In this case, the isoalloxazine ring of FAD was modified. However, when treated with phosphodiesterase, it proved to be similar enough to FMN to reconstitute an FMN-specific apoflavodoxin. A ring modification might explain why added FMN reconstitutes \( M. \) trichosporium formate dehydrogenase activity but the isolated flavin does not appear to coelute with FMN.

The similarity of "\( P. \) oxalat\-icus" and \( M. \) trichosporium formate dehydrogenases dictates a closer look at the former in regard to its characteristics and classification. "\( P. \) oxalat\-icus" is capable of growth on formate but not on methanol as its sole source of carbon and energy. It has peritrichous flagella, which by definition excludes it from the genus \( Pseudomonas \), and suggests that it belongs with the genus \( Alcaligenes \) (17). "\( P. \) oxalat\-icus" \( OX1 \) has recently been shown to share 50% homology at the DNA level with \( Alcaligenes \) eutrophus \( H20 \) and phenotypic similarity of more 90% with this organism (22), making them fairly closely related. The high degree of biochemical similarity between the formate dehydrogenases of \( M. \) trichosporium and "\( P. \) oxalat\-icus" has some interesting evolutionary implications.

The first is that these two organisms are phylogenetically related, but there is no independent evidence for this (other than their similarity with regard to formate dehydrogenase). By current numerical taxonomy standards, "\( P. \) oxalat\-icus" is actually believed to be highly divergent from the methylotherps, such as the genus \( Methanomonas \) (21). A second possibility that might explain the apparent similarity between these two enzymes is that formate dehydrogenases, in order to carry out their function, must have a highly specific structure, which therefore must be highly conserved. This is not true, however, since there is a high degree of variability among the enzymes isolated from different species (see Introduction). The third possibility to explain the similarity of the two enzymes is that they evolved in parallel and that their similarity is explained by some intracellular physiological factors that put identical selective constraints on the evolution of the formate dehydrogenase genes in these two organisms.

ACKNOWLEDGMENT

This work was supported in part by a grant from the National Science Foundation (DMB-8419927) to D.C.Y.

LITERATURE CITED


