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Purification and Properties of Carbon Monoxide Dehydrogenase from *Methanococcus vannielii*

EDWARD DEMOLL,[1]* DAVID A. GRAHAME,[1] JAMES M. HARNLY,[2] LIN TSAI,[1] AND THRESSA C. STADTMAN[1]

Laboratory of Biochemistry, National Heart, Lung, and Blood Institute, Bethesda, Maryland 20892,[1] and Nutrient Composition Laboratory, Human Nutrition Institute, U.S. Department of Agriculture, Beltsville, Maryland 20705[2]

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Carbon monoxide dehydrogenase was purified to homogeneity from *Methanococcus vannielii* grown with formate as the sole carbon source. The enzyme is composed of subunits with molecular weights of 89,000 and 21,000 in an $\alpha_2\beta_2$ oligomeric structure. The native molecular weight of carbon monoxide dehydrogenase, determined by gel electrophoresis, is 220,000. The enzyme from *M. vannielii* contains 2 g-atoms of nickel per mol of enzyme. Except for its relatively high pH optimum of 10.5 and its slightly greater net positive charge, the enzyme from *M. vannielii* closely resembles carbon monoxide dehydrogenase isolated previously from acetate-grown *Methanosarcina barkeri*. Carbon monoxide dehydrogenase from *M. vannielii* constitutes 0.2% of the soluble protein of the cell. By comparison the enzyme comprises 5% of the soluble protein in acetate-grown cells of *M. barkeri* and approximately 1% in methanol-grown cells.

Carbon monoxide dehydrogenase (CODH) and associated enzymes involved in acetate synthesis from C_1 units have been isolated from clostridia and characterized (17–19). A different form of CODH also has been purified from *Methanosarcina barkeri* (7a, 12). Recently a large protein complex with CODH activity was purified from *Methanosarcina thermophila* (23). The enzymes from these two methanosarcina species were purified from cells grown with acetate as the carbon source and are presumed to have a role in acetate conversion to methane. When these organisms are grown with methanol as the carbon source, they still express CODH activity, albeit at a lower level (11, 16). Cells of *Methanococcus vannielii*, an organism that utilizes formate as a substrate for growth and that is incapable of growth on acetate, also contain appreciable levels of CODH. The isolation and characterization of the enzyme from this source are described in this report. Although the CODH from *M. vannielii* closely resembles the enzyme isolated from acetate-grown cells of *M. barkeri* and differs from the clostridial enzyme, it presumably functions in the synthesis rather than the degradation of acetate.

MATERIALS AND METHODS

Research grade carbon monoxide, minimum purity 99.99%, was purchased from Matheson Gas Products. Coenzyme F_{420} was purified from frozen cells of *M. vannielii* by quaternary aminoethyl and C-18 chromatography, in a manner similar to that previously described (5). 1,1'-Trimethylene-4,4'-dimethyl-2,2'-dipyridylium dibromide was synthesized by alkylation of 4,4'-dimethyl-2,2'-dipyridyl with 1,3-dibromopropane (10).

M. vannielii was cultured and harvested as described previously (7). Protein was estimated by the method of Bradford (4), which is based upon binding of Coomassie blue dye. Protein assays performed by the method of Lowry et al. (14) were subject to interference in the initial steps of the purification. The purity of the enzyme after various chromatographic fractions was determined electrophoretically by the method of Laemmli (13) and by sodium dodecyl sulfate (SDS)-gradient polyacrylamide gel electrophoresis by the Pharmacia Fine Chemicals PhastSystem. Low-molecular-weight standards (lysozyme, 14,400; soybean trypsin inhibitor, 21,500; carbonic anhydrase, 31,000; ovalbumin, 45,000; bovine serum albumin, 66,200; and phosphorylase B, 92,500) were used to calibrate both types of SDS-gels. The molecular weights of native CODH from *M. vannielii* and *M. barkeri* were determined by the method of Hedrick and Smith (9). Native CODH from *M. vannielii* and *M. barkeri* was subjected to electrophoresis on nondenaturing gels of 4.0, 4.5, 5.0, 5.5, 6.0, and 6.5% acrylamide. The Tris-glycine (pH 8.3) system of Davis (6) was used with thyroglobulin (669,000), ferritin (440,000), catalase (232,000), lactate dehydrogenase (140,000), and bovine serum albumin (67,000) as standards. Gels were stained with Coomassie brilliant blue R-250 or G-250 as indicated.

All solutions used for enzyme isolation and assay were either made with water equilibrated with the N_2 atmosphere of the Anaerobic Facility or were deoxygenated by sparging with nitrogen. Completeness of deoxygenation was assessed by the inability of the solutions to oxidize reduced methyl viologen.

Preparation of the cell supernatant. The enzyme purification steps were performed during 4 successive days in the Anaerobic Facility in the Laboratory of Biochemistry at the National Heart, Lung, and Blood Institute. Unless otherwise indicated, all procedures were carried out at room temperature. Frozen cells were thawed in 10 mM Tris hydrochloride (pH 8.1)–2 mM $Na_2S_2O_4$ in a 1:2 ratio (wt/vol) and disrupted by sonication at 0°C. One volume of an anaerobic mixture of acetone in 10 mM Tris hydrochloride (pH 8.1)–2 mM $Na_2S_2O_4$ (30%, vol/vol) was added to the sonicate, and the resulting 15% acetone solution was then centrifuged at 30,000 × *g* for 30 min at room temperature. The supernatant (256 ml) was decanted and saved. The pellet, which contained no detectable CODH, was discarded.

DEAE-cellulose chromatography. The sonic extract (256 ml) was applied to a column (5.0 by 16 cm) of DE-52 cellulose (Whatman Ltd.) equilibrated with 40 mM Tris hydrochloride (pH 8.1)–2 mM $Na_2S_2O_4$. After the column was washed with 120 ml of the equilibration buffer, the

* Corresponding author.

adsorbed CODH was eluted in a 1,200-ml linear gradient of 0 to 0.5 M NaCl in the equilibration buffer.

Phenyl-Sepharose chromatography. Partially purified CODH from the DEAE chromatographic step was brought to 0.75 M in ammonium sulfate by the addition of solid crystals. This solution was then applied to a column (2.5 by 12.5 cm) of phenyl-Sepharose CL-4B (Pharmacia), which had been equilibrated with 0.75 M $(NH_4)_2SO_4$–50 mM Tris hydrochloride (pH 8.1)–2 mM $Na_2S_2O_4$. After application of the sample the column was washed with an additional 30 ml of the equilibration buffer. Protein was eluted with 515 ml of a decreasing linear gradient of 0.70 M $(NH_4)_2SO_4$–50 mM Tris hydrochloride (pH 8.1)–2 mM $Na_2S_2O_4$ to 40 mM Tris hydrochloride (pH 8.1)–2 mM $Na_2S_2O_4$.

Hydroxylapatite chromatography. The pool of fractions, which contained CODH from the phenyl-Sepharose chromatographic step, was diluted with 1 volume of 40 mM Tris hydrochloride (pH 8.1)–2 mM $Na_2S_2O_4$ and applied to a column (2.5 by 13.5 cm) of Bio-Gel HTP (Bio-Rad Laboratories), which had been equilibrated with 40 mM Tris hydrochloride (pH 8.1)–2 mM $Na_2S_2O_4$. A 585-ml linear gradient from 0 to 0.6 M K_2HPO_4 in 40 mM Tris hydrochloride (pH 8.1)–2 mM $Na_2S_2O_4$ was used to elute the CODH.

Concentration and storage of CODH. The pool of fractions from the hydroxylapatite step was brought to 0.60 M in ammonium sulfate and then loaded onto a column (2 by 2.5 cm) of phenyl-Sepharose, which had been equilibrated with 0.75 M ammonium sulfate–50 mM Tris hydrochloride (pH 8.1)–2 mM $Na_2S_2O_4$. CODH was eluted from the column with 5 mM K_2HPO_4–5 mM Tris hydrochloride (pH 8.1)–2 mM $Na_2S_2O_4$. The concentrated solution of the enzyme was then frozen by dripping into liquid nitrogen. Frozen CODH was stored at −60°C. For various experiments frozen pellets of the enzyme were transported in liquid nitrogen to the Anaerobic Facility, where they were then thawed and used. The enzyme apparently retained full activity throughout these procedures.

Assay of CODH. The assay used throughout the purification procedure consisted of measurements of CO-dependent reduction of 2,3,5-triphenyltetrazolium chloride catalyzed by the enzyme. The standard assay mix (1 ml) contained 2 mM 2,3,5-triphenyltetrazolium chloride in 0.1 M potassium phosphate (pH 7.7). The assay was performed at room temperature and was preceded by a 1-min sparge of the assay mix with CO. The reaction was initiated by the addition of 5 μl of enzyme. All reactions were performed in sealed tubes under an atmosphere of CO to preclude reduction of the dye by hydrogenases present in the initial crude enzyme fractions (the atmosphere of our Anaerobic Facility contains 1% hydrogen). Reactions were terminated by injection of 1 ml of acetone (not deoxygenated) at 0°C. The stoppered tubes containing the reduced dye were kept on ice before measurement of the dissolved red formazan by absorbance at 500 nm. Under these conditions 1 μmol of the formazan has an absorbance at 500 nm of 1.112. One unit of enzyme activity is defined as 1.0 μmol of 2,3,5-triphenyltetrazolium chloride reduced per min.

Atomic absorption analysis. Metal determinations were made with a prototype multielement atomic absorption spectrometer (8). A graphite furnace atomizer (model HGA-500; Perkin-Elmer Corp., Ridgefield, Conn.) was employed with platform atomization at 2,700°C for all elements. The sweep gas was argon with stopped flow during atomization. The instrument was calibrated with standards prepared in 5% HNO_3. Previous recovery studies have shown the suitability of acid standards for Tris hydrochloride-buffered solutions (J. M. Harnly and D. L. Garland, Methods Enzymol., in press).

pH optimum. The reaction was allowed to proceed for 1 min but otherwise was performed as described above. The buffer consisted of 50 mM [2(*N*-morpholino)ethanesulfonic acid], 50 mM {1,3-bis[tris(hydroxymethyl)-methylamino] propane}, and 50 mM potassium phosphate.

RESULTS

In initial experiments the viscosity of crude sonic extracts and the presence of colloidal suspensions of insoluble sulfides interfered with chromatographic procedures employing DEAE-cellulose or phenyl-Sepharose. Addition of a 30% acetone solution to the sonic extract before centrifugation alleviated the sulfide problem, although final concentrations of acetone greater than approximately 20% caused the precipitation of CODH. When the acetone step was not performed, the recovery of CODH activity in the supernatant was significantly less. DEAE chromatography proved to be an effective first step for isolation of CODH, since the enzyme was one of the first proteins to elute from the column after initiation of the gradient. The enzyme was eluted midway in the gradient in the phenyl-Sepharose chromatographic step and at the end of the gradient as a virtually homogeneous component in the hydroxylapatite step. The homogeneity of the protein peak containing CODH from this last step was verified by SDS-polyacrylamide gel electrophoresis (Fig. 1). From the overall purification obtained it is estimated that CODH represents approximately 0.2% of the soluble protein of *M. vannielii* cells (Table 1).

Molecular weight determinations. Experiments performed with the Laemmli system (13) indicated that the protein from *M. vannielii* is composed of two nonidentical subunits of molecular weights 86,000 and 17,000 (Fig. 1). These apparent values are slightly less than those obtained for the *M. barkeri* enzyme (91,000 and 19,000, respectively). However, these differences were not as obvious when the SDS-gradient gel system of analysis was used. The molecular weights estimated from mobilities in the SDS-gradient gel for the CODH subunits were the same for both enzymes, 89,000 and 21,000. Furthermore, analysis of the native proteins on nondenaturing gels (Fig. 1) indicated that the enzyme from *M. barkeri* had a greater net negative charge. The molecular weights of the native proteins, CODH from *M. vannielii* and CODH from *M. barkeri*, are essentially identical (Fig. 2). The molecular weight obtained from the nondenaturing gels for CODH was 220,000. If an $\alpha_2\beta_2$ structure for CODH is presumed, then the values from the SDS-gradient gel also would predict a molecular weight of 220,000 for the *M. vannielii* enzyme as well as the *M. barkeri* enzyme.

Atomic absorption analysis. CODH preparations from *M. vannielii* contained 2.0 ± 0.1 g-atoms of Ni and 16 ± 7 g-atoms of Fe per mol of enzyme. Zn was detected in some samples at levels as high as 20 g-atoms per mol of enzyme. However after treatment with 0.1 M EDTA and subsequent desalting by repeated ultrafiltration, the Zn content was lowered to less than 0.4 g-atom per mol of enzyme with no apparent loss of activity. A similar decrease in apparent Zn content was also observed with the *M. barkeri* CODH after dialysis against EDTA (7a). Neither Mo nor Co was detected in CODH from *M. vannielii*.

Properties of the enzyme. A relatively high pH optimum, approximately 10.5, of the enzyme was determined when the standard assay was used and the reduction was allowed to proceed for 1 min (Fig. 3). As reported for the enzymes from

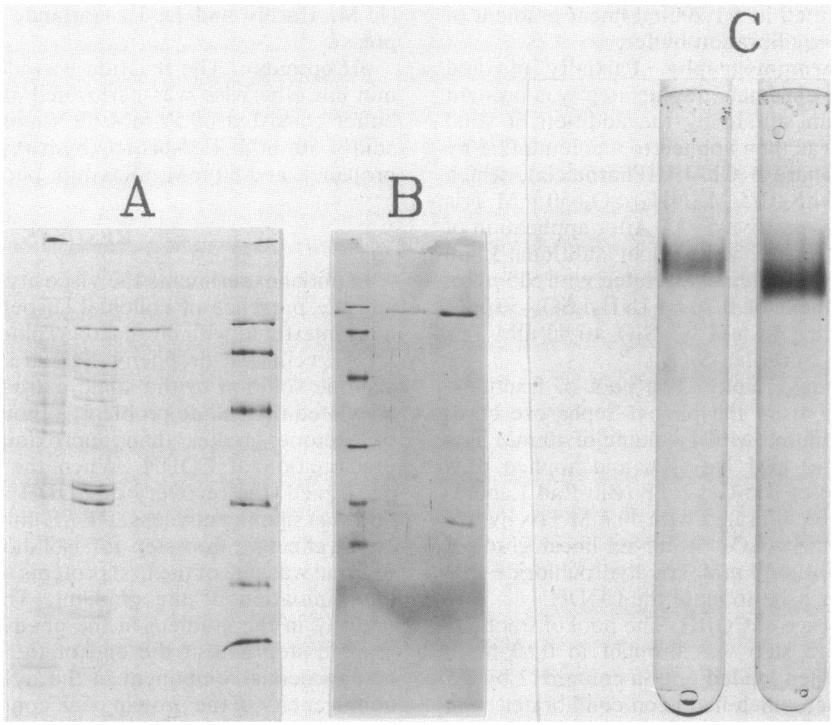

FIG. 1. Polyacrylamide gel electrophoresis. (A) The 12% SDS gels were run employing the Laemmli system (13) as described in Materials and Methods and stained with Coomassie brilliant blue G-250 in perchloric acid. Lanes from left to right were loaded with pooled fractions from the following: DEAE cellulose, phenyl-Sepharose, hydroxylapatite, vacant lane, and molecular weight standards. The poorly staining small subunit is not detectable here. (B) SDS-gel electrophoresis as in A. Lanes from left to right were loaded with molecular weight standards, CODH from *M. barkeri*, and CODH from *M. vannielii*. (C) Nondenaturing polyacrylamide gels. Left, CODH from *M. vannielii*; Right, CODH from *M. barkeri*. Procedures are described in Materials and Methods.

M. barkeri (7a) and *M. thermophila* (23), CODH from *M. vannielii* catalyzed the CO-dependent reduction of methyl viologen, FAD, and FMN but did not reduce the coenzyme F_{420}. As observed with the enzyme from *M. barkeri* (7a), CODH from *M. vannielii* reduced clostridial ferredoxin with CO as electron donor and did not reduce NAD^+ or $NADP^+$. Additionally we observed that both the *M. barkeri* and *M. vannielii* enzymes catalyzed the CO-dependent reduction of the viologen 1,1′-trimethylene-4,4′-dimethyl-2,2′-dipyridylium dibromide (E_0' = −656 mV [1]). Preliminary results indicate that the reduced form of this dye acts as electron donor in the reduction of CO_2 to CO catalyzed by CODH from *M. vannielii*.

CODH from *M. vannielii* is extremely oxygen-labile, and no means of reactivation of the oxygen-inactivated enzyme has been found. Even in a nitrogen atmosphere, with the O_2 concentration at less than 5 ppm, the pure enzyme lost a significant amount of its activity within 18 h, unless dithionite (initially 2 mM) was included in the buffer. Although dithionite addition was apparently unnecessary for retention of activity during the first two steps of the purification, it was routinely included in buffers. Since the atmosphere of the Anaerobic Facility is approximately 1% hydro-

TABLE 1. Purification of CODH

Purification step	Protein[a] (mg)	U[b]	Sp act (U/mg)	Purification (fold)	Yield (%)
Crude sonicate	5,850	1,919	0.328	1.00	100
Supernatant	4,350	1,976	0.454	1.38	103
DEAE	373	1,317	3.53	10.76	68.6
Phenyl-Sepharose	52.8	1,300	24.6	75.0	67.7
Hydroxylapatite	7.2	1,367	190.0	579	71.2

[a] Protein was measured as described in Materials and Methods.
[b] Assay for CODH was performed as described in Materials and Methods.

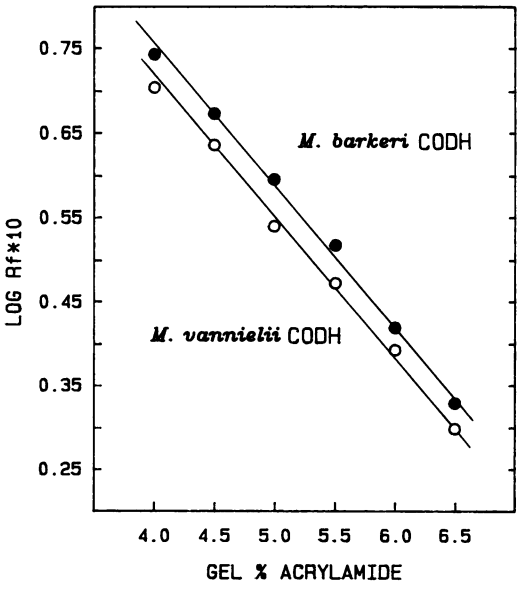

FIG. 2. Native molecular weight determinations of CODH from *M. vannielii* and *M. barkeri*. Procedures are described in Materials and Methods.

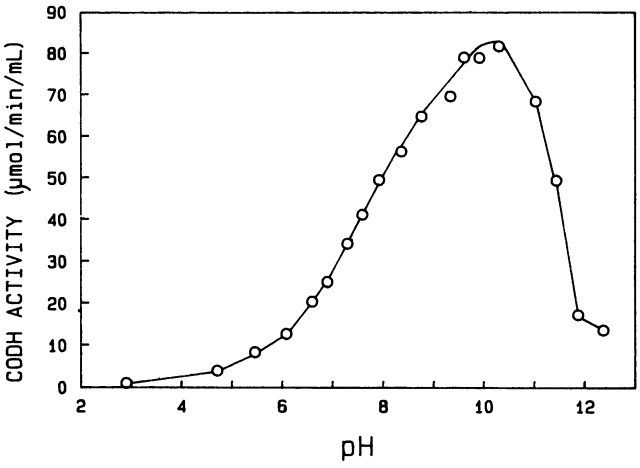

FIG. 3. Dependence of *M. vannielii* CODH activity on pH. The enzyme was assayed as described in Materials and Methods.

gen, the impure enzyme undoubtedly was protected from oxidation by hydrogenases in the preparations. The enzyme activity in the standard assay was unaffected by 20 mM EDTA.

Spectral measurements. The electronic absorption spectrum of CODH from *M. vannielii* exhibited a broad shoulder, which is characteristic of iron-sulfur proteins, between 375 and 600 nm. This portion of the spectrum appeared identical to that reported for the enzymes from *M. thermophila* (23) and *M. barkeri* (12).

Only a single signal at $g = 2.003$, with a width of about 10 G, was observed by electron paramagnetic resonance spectroscopy at 77 K for CODH from *M. vannielii* with or without added CO.

The enzymes from *M. vannielii* and *M. barkeri* were tested for their abilities to carry out the reaction [1-^{14}C]acetyl coenzyme A + CO $\rightleftarrows$ ^{14}CO + acetyl coenzyme A. Although the clostridial enzyme can catalyze this exchange of ^{14}C, neither CODH from *M. barkeri* nor the enzyme from *M. vannielii* catalyzed the exchange of ^{14}C from [1-^{14}C]acetyl coenzyme A to ^{14}CO when the experiment was performed as described for the clostridial system (20). Moreover, addition of the *M. barkeri* CODH to reaction mixtures containing the clostridial CODH neither stimulated nor inhibited the exchange reaction (D. A. Grahame and S. W. Ragsdale, unpublished results).

DISCUSSION

The levels of CODH expressed in *M. barkeri* and *M. thermophila* are increased markedly when acetate replaces methanol as the carbon source for growth (11, 23); this has been interpreted to indicate a requirement for CODH in acetate conversion to CH_4 and CO_2. The fact that the enzyme is expressed at lower levels in methanol-grown cells does not necessarily imply a role for CODH under these growth conditions. However, a role for CODH in acetate synthesis in methanogens, possibly in a manner similar to that described for clostridia (17), is supported by the fact that the enzyme is present in *M. vannielii*, which does not use acetate as a substrate for growth (22) and consequently would have no apparent need for an acetate-cleaving enzyme activity.

The pure CODH from *M. vannielii* has the same subunit composition and molecular weight and similar metal content as the enzyme from acetate-grown *M. barkeri*, suggesting that the same enzyme could be involved in both the synthesis and breakdown of acetate in these organisms. The values obtained for Ni in CODH from *M. vannielii* were very reproducible (±10%), in contrast to those for iron, which varied greatly for different preparations (±45%). Zn has been reported in CODH preparations from *M. barkeri* (7a), *Methanosarcina thermophila* (23), *Clostridium thermoaceticum* (18), *Acetobacterium woodii* (19), *Pseudomonas carboxydovorans* (15), and *Rhodospirillum rubrum* (2). However Zn is a common contaminant in biological samples. Moreover, its almost complete elimination from the *M. vannielii* enzyme, and to a lesser extent from the *M. barkeri* enzyme, by EDTA treatment and dialysis resulted in no apparent loss of enzyme activity. Therefore a role for Zn in CODH from methanogens is not well supported.

A protein complex isolated from *M. thermophila* was reported to contain five subunits with molecular weights 89,000, 71,000, 60,000, 58,000, and 19,000 as determined by SDS-polyacrylamide gel electrophoresis (23). The 89,000- and 19,000-molecular-weight polypeptides, which were not detected in extracts of methanol-grown cells of *M. thermophila*, are similar in size to the two subunits of CODH of *M. barkeri* and *M. vannielii*. It is possible that the 89,000- and 19,000-dalton polypeptides were not detected in methanol-grown *M. thermophila* because CODH is expressed at a very much lower level in the absence of acetate. In *M. barkeri* CODH constitutes approximately 5% of the soluble cell protein when the organism is grown on acetate (12). With methanol as the sole carbon source, the level of the enzyme is about one-fifth of that present in the acetate-grown cells (11).

The enzyme complex from *M. thermophila* shows an electron paramagnetic resonance spectrum at 113 K that is nearly identical to that exhibited by the clostridial CODH (21, 23), whereas the enzyme from *M. vannielii* showed no such spectrum at 77 K. The electron paramagnetic resonance spectra of the enzymes from *M. barkeri* (J. A. Krzycki, L. E. Mortensen, and R. C. Prince, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, I51, p. 173) and *R. rubrum* (2) also differ from that of clostridial CODH. Furthermore, the enzymes from *M. barkeri*, *M. vannielii*, and *R. rubrum* (2) failed to catalyze the exchange of carbon monoxide with the carboxyl carbon of acetyl coenzyme A that is a characteristic property of the clostridial system (20). These results are consistent with the conclusion that the $\alpha_2\beta_2$ moiety of the CODH isolated from *M. vannielii* and *M. barkeri* requires additional protein components for catalysis of the acetate synthesis or acetate cleavage reactions. Apparently, for CODH activity as assayed by CO-dependent reduction of dyes (7a, 12), only the $\alpha_2\beta_2$ enzyme complex, containing the 89,000- and 21,000-dalton subunits, is required.

The reported absence (3) of detectable amounts of CODH in methanogens that require acetate supplements for growth on carbon dioxide and hydrogen provides further evidence for a role of the enzyme in acetate metabolism rather than in the overall pathway of carbon dioxide reduction to methane.

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