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Locations of the Hydrogenases of *Methanobacterium formicicum* after Subcellular Fractionation of Cell Extract

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The F_{420} hydrogenase of *Methanobacterium formicicum* was associated with membranes isolated by sucrose density gradient ultracentrifugation of cell extract. The methyl viologen hydrogenase was present in the soluble fractions. Column chromatography with phenyl-Sepharose CL-4B revealed that the F_{420} hydrogenase was strongly hydrophobic, suggesting that it associates with isolated membranes through hydrophobic interactions.

Two hydrogenases have been reported in H_2-oxidizing methanogens (6, 7, 9, 15). One enzyme (F_{420} hydrogenase) reduces coenzyme F_{420} (F_{420}) and methyl viologen (MV). The other enzyme (MV hydrogenase) reduces MV but not F_{420}. Jin et al. (7) showed that these two hydrogenases in *Methanobacterium formicicum* are distinct enzymes on the basis of subunit composition and peptide mapping. The cellular locations of these enzymes are unclear.

Methanobacterium strain G2R (phenotypically similar to *M. formicicum*) contains a membrane-associated hydrogenase which reduces viologen dyes but not F_{420} (8). The F_{420} hydrogenase is unstable in this organism under the conditions used, as no F_{420}-dependent activity is detected in either the soluble or membrane fraction of cell extract; thus the identity of this membrane-associated hydrogenase could not be determined (8). We have developed methods to stabilize the F_{420} hydrogenase of *M. formicicum* and were thus able to study the location of this enzyme.

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M. formicicum JF-1 (DSM 2639) was cultured with H_2-CO_2 (4:1) and harvested anaerobically as described (11). All subsequent procedures were performed under an atmosphere of O_2-free N_2 as described (10, 11), except that sodium dithionite was not included in buffers. Basal buffer contained 50 mM potassium *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonate (pH 7.5), 10 mM $MgCl_2$ and 2 mM 2-mercaptoethanol.

Although anaerobic procedures were used throughout, both hydrogenases were reversibly inactivated and required reductive reactivation before being assayed. The samples were degassed by eight cycles of alternate evacuation and pressurization with N_2 and then flushed with H_2 for 1 min. They were then made to 48 μM in F_{420} and 30 μM in FAD and incubated in the dark for 1 h at 35°C. Under these conditions, the F_{420} hydrogenase is stabilized toward F_{420}-dependent activity (S. F. Baron and J. G. Ferry, manuscript in preparation).

Hydrogenase was assayed spectrophotometrically at 35°C and pH 7.5 as previously described (10). One unit of activity is the reduction of 1 μmol of F_{420} or MV per min. F_{420} was purified as described (12). Protein was determined by the dye-binding assay (micro-method) of Bradford (3) with bovine serum albumin as the standard.

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Sucrose density gradient ultracentrifugation was employed to separate membranes from soluble proteins in cell extracts of *M. formicicum*. Sucrose solutions with or without Triton X-100 (1%, vol/vol, final concentration) were prepared in basal buffer and layered in polycarbonate centrifuge tubes (16 by 78 mm) fitted with gas-tight screw caps. The gradients consisted of 5.5 ml of 20% (wt/vol) sucrose and 2.0 ml of 30% (wt/vol) sucrose over a 70% (wt/vol) sucrose shelf (1.5 ml). Samples (1.0 ml; 20 mg of protein) of cell extract with or without 1.5% (vol/vol) Triton X-100 were layered onto the gradients and centrifuged in a Beckman 50Ti fixed-angle rotor (90 min; 226,000 × *g*; 5°C).

DEAE-cellulose chromatography was used to remove unbound Triton X-100 and sucrose from hydrogenase samples before hydrophobic interaction chromatography. Fractions from the sucrose gradients were loaded onto a column (1 by 2 cm) of DEAE-cellulose (Whatman DE-52) equilibrated with basal buffer containing 5% (vol/vol) glycerol (buffer A). Unadsorbed material was washed from the column with 11 ml of buffer A. Adsorbed protein was batch eluted with the same buffer (1 ml/min) containing 1 M KCl (buffer B). Recovery of F_{420}-dependent and MV-dependent activity was greater than 70 and 80%, respectively. Hydrophobic interaction chromatography was done as follows. Samples were loaded onto a column (0.9 by 5 cm) of phenyl-Sepharose CL-4B (Pharmacia, Inc.) equilibrated with buffer B. The column was washed with 3.2 ml of buffer B, then with 9.6 ml of buffer A, and finally with a linear gradient of Triton X-100 (0 to 1.5%, vol/vol; 9.6 ml) in buffer A (0.4 ml/min). It was then washed with an additional 9.6 ml of buffer A containing 1.5% (vol/vol) Triton X-100. Fractions of 0.4 ml were collected.

After ultracentrifugation of the sucrose gradients, a membrane fraction was present in a narrow, light brown, translucent band at the top of the 70% sucrose shelf. Electron micrographs (not shown) of this material confirmed the presence of membrane vesicles. This band was absent in the sucrose gradients loaded with cell extract which had been treated with Triton X-100 to solubilize membranes. Contaminating whole cells and cell debris formed a pellet.

After ultracentrifugation of untreated extract, 58% of the F_{420}-dependent activity was detected in the membrane fraction (I, Fig. 1A), and the remaining 42% was distributed between the soluble proteins (III) and a broad band (II) situated between the membrane and soluble fractions.

After ultracentrifugation of Triton X-100-treated extract, no F_{420}-dependent activity was present at the top of the 70% sucrose shelf (Fig. 1B); instead, the activity increased near

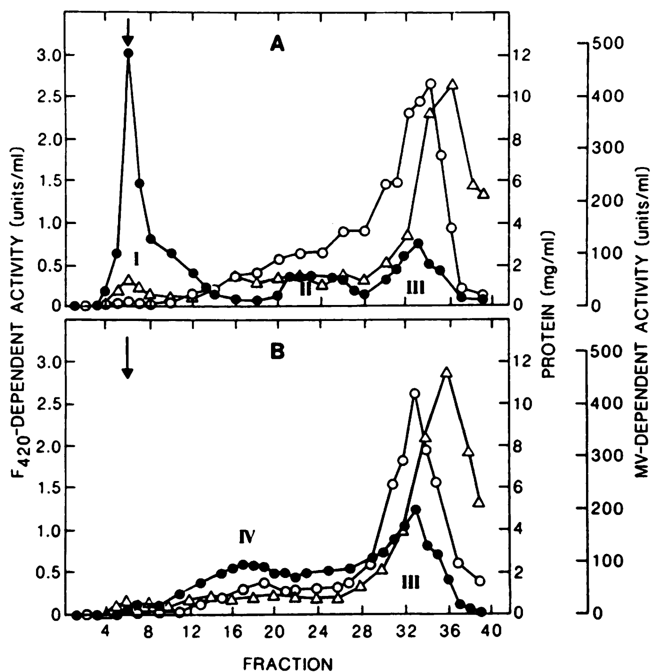

FIG. 1. Separation of the F_{420} hydrogenase and MV hydrogenase of *M. formicicum* by sucrose density gradient ultracentrifugation. (A) Cell extract was centrifuged through sucrose gradients containing no Triton X-100. (B) Cell extract containing 1.5% (vol/vol) Triton X-100 was centrifuged through sucrose gradients containing 1% (vol/vol) Triton X-100. Symbols: ●, F_{420}-dependent activity; ○, MV-dependent activity; △, protein. Arrow denotes the top of the 70% sucrose shelf. Roman numerals denote peaks of hydrogenase activity described in the text. Fractions (0.25 ml) are numbered from the bottom of the tube.

the middle of the gradient (IV) and in the soluble fractions (III). The total F_{420}-dependent activity recovered throughout the sucrose gradient without Triton X-100 (4.6 U) was nearly the same as that with Triton X-100 (4.8 U) and in both cases was greater than 85% of that loaded. Thus, all of the membrane-associated F_{420}-dependent activity (I, Fig. 1A) could be accounted for in fractions III and IV (Fig. 1B) after solubilization of membranes.

These results show that the F_{420} hydrogenase of *M. formicicum* is associated with isolated membranes, and they suggest that the enzyme may be located on membranes in the intact cell. The F_{420} hydrogenases studied to date are monomers which form aggregates of up to M_r 800,000 (5–7, 14), including the enzyme from *M. formicicum* JF-1 for which aggregates never exceed M_r 790,000 (unpublished data). Therefore, fractions II and III (Fig. 1A) may represent aggregates and monomers, respectively, of the F_{420} hydrogenase which may have dissociated from the membranes. The carbon monoxide dehydrogenase complex of *Methanosarcina thermophila* (M_r 10^6) (13) sediments to the top of the 30% sucrose layer when ultracentrifuged under the same conditions used in this study (K. Terlesky, personal communication). Therefore, the membrane-associated F_{420} hydrogenase (I, Fig. 1A) does not represent free aggregates large enough to sediment to the same position as membranes in sucrose gradients.

When hydrogenase from solubilized membranes was chromatographed on the hydrophobic interaction column, the F_{420}-dependent activity eluted as a single peak at the end of a linear gradient of Triton X-100 (Fig. 2A). This peak coincided with a peak of MV-dependent activity, but no other peaks of activity were observed. Similarly, when the soluble fractions were chromatographed over the same column, the F_{420}-dependent activity eluted in a single peak at the end of the Triton X-100 gradient and was accompanied by a peak of MV-dependent activity (Fig. 2B). However, the majority of the MV-dependent activity (79%) eluted in two peaks well resolved from the F_{420}-dependent activity, indicating that they represented the MV hydrogenase. These results show that the F_{420} hydrogenase is more hydrophobic than the MV enzyme and suggest that the F_{420} hydrogenase associates with isolated membranes through hydrophobic interactions.

After ultracentrifugation of either untreated or Triton X-100-treated cell extract, the soluble fractions (III, Fig. 1) contained most of the MV-dependent activity, suggesting that the MV hydrogenase was present in these fractions. Three observations support this conclusion: (i) the ratio of F_{420}-dependent to MV-dependent activity in the most active fraction of I (Fig. 1A) was 385-fold greater than that in the most active fraction of III (Fig. 1A); (ii) the total MV-dependent activity (about 600 U) in fraction III was similar regardless of the presence of Triton X-100; (iii) hydrophobic interaction chromatography of the soluble fractions resolved distinct hydrogenases, one capable of reducing F_{420} and MV

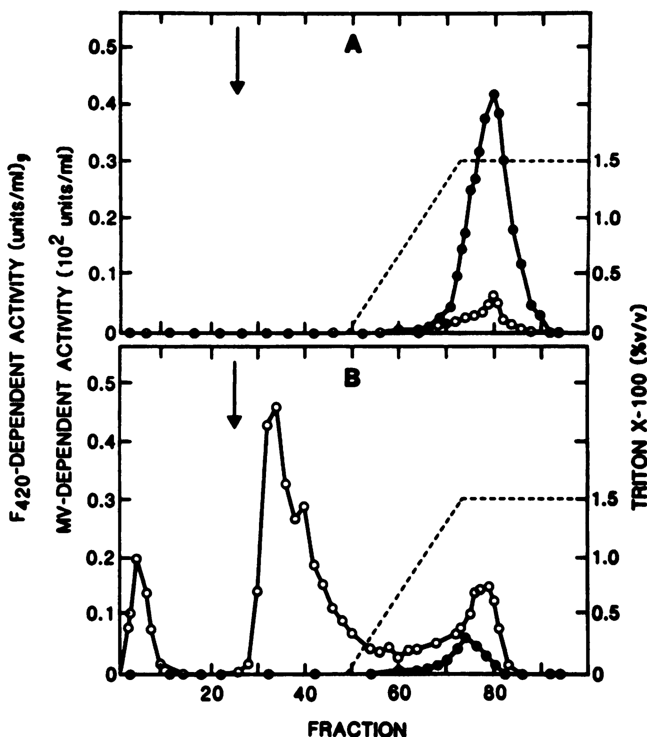

FIG. 2. Hydrophobic interaction chromatography of the F_{420} hydrogenase and MV hydrogenase of *M. formicicum*. (A) Triton X-100-solubilized membranes (fractions 3 to 14 from the sucrose gradient in Fig. 1A) were passed over a DEAE-cellulose column and then chromatographed on a column of phenyl-Sepharose CL-4B as described in the text. (B) As for panel A, except fractions 28 through 39 from the sucrose gradient in Fig. 1A were used and were not treated with Triton X-100 before DEAE-cellulose chromatography. Symbols: ●, F_{420}-dependent activity; ○, MV-dependent activity; ------, Triton X-100 gradient. The arrow denotes the start of washing with 9.6 ml of buffer A (see text).

and the other capable of reducing MV but not F_{420} (Fig. 2B). These results do not necessarily suggest that the MV hydrogenase is a soluble enzyme in vivo, since it could be loosely bound to the membrane and become dislodged during preparation of cell extract or sucrose gradient fractionation.

The synthesis of ATP in *Methanosarcina barkeri* and *Methanosphaera stadtmanae* is driven by a proton motive force generated across the membrane by methanogenesis from methanol and H_2 (1, 2), and membrane preparations from the H_2-oxidizing methanogen, *Methanobacterium thermoautotrophicum*, carry out H_2-driven ATP synthesis (4). Our results indicate that the F_{420} hydrogenase of *M. formicicum* is associated with membranes and therefore may be involved in ATP synthesis in this and other H_2-oxidizing methanogens.

The F_{420} hydrogenases of *Methanobacterium* spp. are reported to be unstable (8, 9). The F_{420} hydrogenase from *M. formicicum* JF-1 contains bound FAD required for reduction of F_{420} (9). Hydrophobic interaction chromatography converts the enzyme to a deflavo species which reduces MV but not F_{420}; preincubation of the enzyme with FAD restores the F_{420}-dependent activity (9). Similarly, when the enzyme is reactivated by incubation under H_2 with F_{420}, it loses all F_{420}-dependent activity, unless FAD is present, and retains most of the MV-dependent activity (Baron and Ferry, in preparation). In this study, the reactivation mixture contained 30 μM FAD, which stabilized reactivated F_{420} hydrogenase and reconstituted any deflavo enzyme present. The ratio of F_{420}-dependent to MV-dependent activity of the peak in Fig. 2A was 21-fold greater than that of the peak of F_{420}-dependent activity in Fig. 2B. The reason for this is unknown; perhaps the dissociated form of the F_{420} hydrogenase is irreversibly converted to deflavo enzyme, while the conversion of the membrane-associated form is reversible.

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