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Immunocytochemical Localization of the Coenzyme F_{420}-Reducing Hydrogenase in *Methanosarcina barkeri* Fusaro

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The cytological localization of the 8-hydroxy-5-deazaflavin (coenzyme F_{420})-reducing hydrogenase of *Methanosarcina barkeri* Fusaro was determined by immunoelectron microscopy, using a specific polyclonal rabbit antiserum raised against the homogeneous deazaflavin-dependent enzyme. In Western blot (immunoblot) experiments this antiserum reacted specifically with the native coenzyme F_{420}-reducing hydrogenase, but did not cross-react with the coenzyme F_{420}-nonreducing hydrogenase activity also detectable in crude extracts prepared from methanol-grown *Methanosarcina* cells. Immunogold labeling of ultrathin sections of anaerobically fixed methanol-grown cells from the exponential growth phase revealed that the coenzyme F_{420}-reducing hydrogenase was predominantly located in the vicinity of the cytoplasmic membrane. From this result we concluded that the deazaflavin-dependent hydrogenase is associated with the cytoplasmic membrane in intact cells of *M. barkeri* during growth on methanol as the sole methanogenic substrate, and a possible role of this enzyme in the generation of the electrochemical proton gradient is discussed.

During methanogenesis from methanol and H_2 by resting-cell suspensions of methanol-grown *Methanosarcina barkeri*, ATP synthesis is stringently coupled to an electrochemical proton gradient and a functional DCCD (*N,N'*-dicyclohexylcarbodiimide)-sensitive ATP synthase, indicating a chemiosmotic mechanism for energy conservation (8, 10, 11). The central intermediate of methane formation from methanol is 2-(methylthio)ethanesulfonic acid (CH_3-S-coenzyme M [CoM]) (19, 39, 43) which is reductively demethylated with H_2 to yield CH_4 and 2-mercaptoethanesulfonic acid (CoM-SH) by the multicomponent methyl-coenzyme M methylreductase system (36, 37). Since the free energy change of this reaction ($\Delta G^{0\prime} = -85$ kJ/reaction) is large enough to drive ATP synthesis (9, 19), it has been hypothesized that one or several components of the methylreductase system are membrane associated and involved in proton translocation across the cytoplasmic membrane.

It has been demonstrated recently (16, 22) that the hydrogen-dependent reductive demethylation of CH_3-S-CoM to CH_4, a reaction common to all methanogenic bacteria irrespective of their growth substrate (19, 37), proceeds in two steps:

$$CH_3\text{-S-CoM} + \text{HTP-SH} \rightarrow CH_4 + \text{CoM-S-S-HTP} \quad \text{(reaction 1)}$$

$$\text{CoM-S-S-HTP} + H_2 \rightarrow \text{CoM-SH} + \text{HTP-SH} \quad \text{(reaction 2)}$$

In the first step (reaction 1) the methylreductase catalyzes the reduction of CH_3-S-CoM to CH_4 with L-7-mercaptoheptanoylthreonine phosphate (HTP-SH) as the electron donor (29). The heterodisulfide CoM-S-S-HTP formed is then reduced in the second step to CoM-SH and HTP-SH by a hydrogenase-linked heterodisulfide reductase system (reaction 2), and it has been proposed that the reduction of the mixed disulfide rather than methane formation is linked to proton translocation and thus coupled to energy conservation (16, 21).

Therefore, we decided to determine the ultrastructural location of the coenzyme F_{420}-reducing hydrogenase (F_{420}-hydrogenase) in *M. barkeri* by immunoelectron microscopy, using a specific polyclonal antiserum raised against the homogeneous soluble but hydrophobic enzyme (17). The hydrophobic properties of the protein indicated a possible membrane association in whole cells which could have been lost in the course of cell rupture (34). Immunoelectron microscopy, a powerful method for localizing bacterial and eucaryotic proteins in situ (33–35), revealed that the F_{420}-hydrogenase of *M. barkeri* was predominantly located in the vicinity of the cytoplasmic membrane during exponential growth on methanol as the sole methanogenic substrate, suggesting that the enzyme is membrane associated in intact cells. This result with *M. barkeri* is in agreement with recent findings reported for *Methanococcus voltae* (27) and *Methanobacterium formicicum* (3) and raises the question of whether the deazaflavin-dependent hydrogenase could be involved in the generation of an electrochemical proton gradient across the cytoplasmic membrane.

MATERIALS AND METHODS

Organism and growth conditions. *M. barkeri* Fusaro (DSM 804) was obtained from the Deutsche Sammlung von Mikroorganismen, Braunschweig, Federal Republic of Germany (FRG). The strain was grown anaerobically in 1-liter glass bottles containing 500 ml of complex medium with 200 mM methanol under an atmosphere of 80% N_2–20% CO_2 as described before (23), and growth was followed by measuring the total gas production with lubricated glass syringes.

Preparation of crude extracts. Cells were harvested in the mid-logarithmic phase of growth by aerobic centrifugation for 20 min at 6,800 × *g* and 4°C in a Sorvall GS-3 rotor and washed once in 100 ml of 50 mM Tris hydrochloride (pH 7.5) containing 0.1 mM phenylmethylsulfonyl fluoride (buffer A). The packed cell material was then resuspended in buffer A in

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a ratio of 1 g of wet cells per 3 ml of buffer. After the addition of a few crystals of DNase, the cells were disrupted by three passages through a French pressure cell (Aminco) at 121.3 MPa and cell debris was removed by low-speed centrifugation as described previously (17).

Preparation of antiserum. A sample of 290 μg of electrophoretically homogeneous F_{420}-hydrogenase (holoenzyme) suspended in 0.5 ml of 20 mM Tris hydrochloride (pH 7.5) containing 0.1 mM phenylmethylsulfonyl fluoride (buffer B) was emulsified with an equal volume of Freund's complete adjuvant and injected subcutaneously into a male New Zealand White rabbit. After 2 weeks the rabbit was boosted once with the same amount of enzyme in 0.5 ml of buffer B emulsified with an equal volume of Freund's incomplete adjuvant. The rabbit was bled 6 weeks later, and the blood was allowed to clot overnight at 4°C. The serum was collected after centrifugation of 1.5-ml blood samples in a table top centrifuge (Biofuge A; Heraeus Sepatech GmbH, Osterode, FRG) at 4,500 rpm for 15 min. The serum was stored in multiple aliquots at −70°C.

Electrophoresis and immunoblotting. Separation and analysis of native samples of crude extracts and of homogeneous F_{420}-hydrogenase in nondenaturing linear 4 to 20% polyacrylamide gradient gels were routinely performed in triplicate flanked by lanes with molecular mass standard protein mixtures as described previously (17). After electrophoresis the gel was sliced into three strips. The first strip, composed of one lane with crude extract and a second lane with purified F_{420}-hydrogenase, was stained for methyl viologen-reducing hydrogenase activity. The second and the third strip each consisted of one lane with crude extract, a second lane with homogeneous hydrogenase, and a third lane with molecular mass standard proteins. The second strip was stained with Coomassie brilliant blue for proteins, while the proteins of the third strip were electrophoretically transferred onto a nitrocellulose (NC) membrane (BAS 83, 0.2-μm pore size; Schleicher & Schüll), which had been backed by a second NC membrane to trap proteins passing through the first sheet. The electrotransfer was carried out in a temperature-controlled LKB 2005 Transphor Electroblotting Unit filled with 5 liters of 40 mM Tris–40 mM boric acid (pH 8.2)–1 mM EDTA containing 10% (vol/vol) methanol at 0.5 A and 4°C for 8 to 16 h (6, 42). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of denatured protein samples was performed in 12% polyacrylamide gels with 5% stacking gels by the general procedure of Laemmli as specified previously (17). Prior to electrophoresis the protein samples were denatured for 45 min in 3% (wt/vol) SDS–100 mM 1,4-dithioerythritol at room temperature. Samples of denatured proteins were routinely run in duplicate flanked by lanes with appropriate molecular mass standard protein mixtures. After electrophoretic separation of the polypeptides, the gel was sliced into two strips. Each strip included one sample lane and a second lane with molecular mass reference proteins. The polypeptides of one strip were stained for proteins with Coomassie brilliant blue, while the polypeptides of the other strip were electrophoretically transferred to an NC membrane as described before, except that the electrotransfer was carried out in 25 mM Tris hydrochloride (pH 8.3) containing 20% (vol/vol) methanol at 0.5 A and 4°C for 9 h. After electrotransfer the NC membranes were washed in 50 mM Tris hydrochloride (pH 7.4)–0.2 M NaCl (Tris-buffered saline) for 15 min at room temperature, blotted dry between two sheets of chromatography paper (3MM; Whatman), and then stored at −20°C until immunological probing. To check the elution efficiencies of the proteins out of the native and denaturing polyacrylamide gels during electrotransfer, the blotted gels were always stained for proteins with Coomassie brilliant blue (17).

To determine the molecular masses of the immunoreactive polypeptides on the NC membranes, the shrinkage of the polyacrylamide gels and of the membranes during electrotransfer had to be accounted for (4). Therefore, the frozen NC membranes were allowed to thaw for 15 min at room temperature and the immobilized proteins on the membranes were visualized by staining with Ponceau S (44). The locations of the molecular mass standard proteins were marked and, after destaining, the NC membranes were incubated for 1 h at room temperature in Tris-buffered saline containing 5% (wt/vol) bovine serum albumin and 0.05% (vol/vol) Tween 20 (blocking buffer) to saturate unspecific binding sites. Then the membranes were incubated for 1 h in blocking buffer containing anti-F_{420}-hydrogenase antiserum at a dilution of 1:40,000. After three 10-min washes in blocking buffer, the blots were incubated for 1 h in the same buffer containing goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase at a dilution of 1:10,000. The immunoblots were washed three times for 10 min each in blocking buffer, followed by a 10-min wash in 0.1 M diethanolamine (pH 9.6)–1 mM $MgCl_2$ (reaction buffer). The anti-F_{420}-hydrogenase antibodies bound to antigens on the NC membranes were detected by the slightly modified procedure described in reference 7, using 5-bromo-4-chloro-3-indolyl phosphate and 4-Nitro Blue Tetrazolium as substrates in 20 ml of reaction buffer.

Immunoelectron microscopy. *M. barkeri* cells from the mid-logarithmic growth phase were fixed for 12 h at room temperature under anaerobic conditions by addition of formaldehyde and glutardialdehyde to the growth medium to give final concentrations of 2 and 0.5% (vol/vol), respectively. The cells were sedimented by centrifugation and washed aerobically in 100 mM cadodylate (pH 7.2). After immobilization with 2% (wt/vol) low-melting agarose in the above buffer at 32°C, the cells were dehydrated with increasing concentrations of ethanol and decreasing temperature to, finally, −35°C in a Cryocask cooling unit (GBF, Braunschweig, FRG). At this temperature the cells were embedded in Lowicryl K4M as described by Roth et al. (35), and the samples were polymerized in the cooling unit for 24 h at −35°C and 6-W UV light immersed in 100% ethanol as coolant. Ultrathin sections (120 to 150 nm) were picked up on nickel grids (300 mesh) and, before labeling with protein A-$gold_{10\ nm}$, prepared by the method of Frens (18), sections were treated with 0.05% (vol/vol) Tween 20 and 0.25% (wt/vol) aqueous skim milk powder to prevent unspecific antibody binding (33). Immunogold labeling was done with a specific rabbit antiserum raised against the F_{420}-hydrogenase from *M. barkeri* for 1 to 2 h at room temperature, followed by a 30-min incubation with protein A-gold. Labeled sections were analyzed with a Zeiss CEM 902 transmission electron microscope at 80 kV, using the electron spectroscopic imaging mode at electron energy losses of 40 eV with an energy width of 20 eV or the global bright-field mode.

Protein determination. Protein concentrations were determined by the Bradford protein assay, using bovine serum albumin as standard (12).

Materials. Premixed protein molecular mass standards were purchased from Pharmacia, Freiburg, FRG. Phenylmethylsulfonyl fluoride, methyl viologen, 2,3,5-triphenyltetrazolium chloride, Ponceau S, bovine serum albumin (fraction V), Tween 20, 5-bromo-4-chloro-3-indolyl phosphate

(*p*-toluidine salt), and 4-Nitro Blue Tetrazolium chloride were obtained from Sigma Chemie GmbH, Deisenhofen, FRG. Nitrocellulose membranes were from Schleicher & Schüll GmbH, Dassel, FRG. Alkaline phosphatase-conjugated AffiPure goat anti-rabbit immunoglobulin G (heavy and light chains) was purchased from Dianova GmbH, Hamburg, FRG. All reagents for PAGE were from Bio-Rad Laboratories GmbH, Munich, FRG. Gases were obtained from Messer Griesheim, Düsseldorf, FRG. All other chemicals were of reagent grade and purchased from Merck, Darmstadt, FRG.

RESULTS

Characterization of the polyclonal antiserum. Gel electrophoretic analysis of aerobically prepared crude extracts of methanol-grown cells of *M. barkeri* in native linear polyacrylamide gradient gels revealed five bands of methyl viologen-reducing hydrogenase activity (17). Two of these bands could be attributed to the F_{420}-hydrogenase, which existed in two forms with molecular masses of 198 and of 845 kDa (Fig. 1A, lanes 3 and 4), the latter probably a tetramer of the 198-kDa species. When higher dilutions of comparable crude extract preparations were subjected to gel electrophoretic analysis followed by activity staining with tetrazolium salts, only two prominent bands of methyl viologen-reducing hydrogenase activity were detectable and their molecular masses were estimated to be 198 and 64 kDa (Fig. 1A, lanes 1 and 2). Only a very faint band could be detected at the position of the 845-kDa form and virtually no bands at locations below 64 kDa.

The polyclonal antiserum raised against the holoenzyme of the homogeneous F_{420}-hydrogenase of *M. barkeri* reacted specifically with the 198- and 845-kDa species of the deazaflavin-dependent enzyme (Fig. 1B), but not with the coenzyme F_{420}-nonreducing hydrogenase (MV-hydrogenase) of 64 kDa also detectable in these extracts by activity staining (cf. Fig. 1A, lane 2, with Fig. 1B, lane 1). Even with a 28-fold amount of crude extract proteins per lane (33 μg), no cross-reaction was observed between antiserum and 64-kDa hydrogenase (data not shown). Since the antiserum contains antibodies against all three subunits of the F_{420}-hydrogenase (Fig. 2, lane 2), it appears very unlikely that the 64-kDa MV-hydrogenase is a decomposition product, eventually a homo- or a heterodimer of the 33- and/or the 30-kDa subunit of the holoenzyme which has retained the methyl viologen-reducing hydrogenase activity. However, we cannot completely preclude that such a dimer with a very high specific methyl viologen-reducing hydrogenase activity exists, for the antigen concentration could be below the detection limit of our immunoassay. The sensitivity of the assay was determined for all three subunits after electrophoretic separation of serial dilutions of SDS-denatured homogeneous F_{420}-hydrogenase followed by electrotransfer of the peptides to an NC membrane and immunological probing as described in Materials and Methods. The detection limits were 14 ng of protein for the 48-kDa, 10 ng of protein for the 33-kDa, and 36 ng of protein for the 30-kDa subunit.

Immunoelectron microscopy. Using the specific anti-F_{420}-hydrogenase antiserum at dilutions between 1:400 and 1:600, application of the postembedding protein A-colloidal gold technique on ultrathin sections of methanol-grown *M. barkeri* cell aggregates from the mid-logarithmic growth phase revealed that gold particles were predominantly located in the vicinity of the cytoplasmic membrane(s) (Fig. 3A). Moreover, most of the gold tags were distinctly aligned in two parallel rows on the opposite sides of the thick common cell wall separating two adjacent cells in the pseudosarcina-shaped cell aggregates characteristic for *M. barkeri* (Fig. 3A, large circles). A quantitative evaluation of 21 cells from this and several similar electron micrographs showed that 88% (standard deviation, ±4.7%) of the gold label was found in the immediate vicinity of the cell membrane, while only 12% (standard deviation, ±4.7%) was scattered in the cytoplasm. Thin sections incubated with preimmune serum (Fig. 3B) or with protein A-gold complexes alone (data not shown) exhibited little or no labeling. However, in some regions of common cell wall between adjacent cells, the gold particles seemed to be diffusely located within the cell wall rather than in or at the cell membrane (Fig. 3A, small circles). This diffuse label within the cell wall could be due to gold marker scattering around the antigenic epitope (33), surface relief formation during ultrathin sectioning of the plastic-embed-

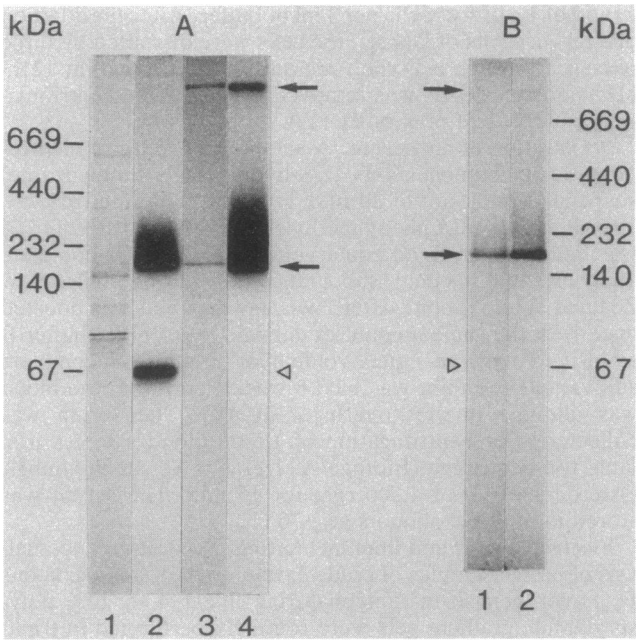

FIG. 1. Gel electrophoretic and immunological analysis of native F_{420}-hydrogenase of *M. barkeri* Fusaro. Triplicate samples of crude extracts and homogeneous F_{420}-hydrogenase were separated by electrophoresis in a native linear 4 to 20% polyacrylamide gradient gel; then the gel was sliced into strips and analyzed as described in Materials and Methods. (A) Protein staining with Coomassie brilliant blue (lanes 1 and 3) and staining for methyl viologen-reducing hydrogenase activity with tetrazolium salts (lanes 2 and 4). Lanes 1 and 2, Crude extract, 4.8 and 2.4 μg of protein, respectively; lanes 3 and 4, homogeneous F_{420}-hydrogenase, 2.2 μg and 140 ng of protein, respectively. (B) Immunoblot probed with the anti-F_{420}-hydrogenase antiserum. Lane 1, Crude extract, 1.2 μg of protein; lane 2, homogeneous F_{420}-hydrogenase, 70 ng of protein. The locations of the molecular mass standard proteins and their masses are indicated to the left of the gels and to the right of the corresponding blots. The size marker mixture contained, in order of decreasing molecular mass, thyroglobulin, ferritin, catalase, lactate dehydrogenase, and bovine serum albumin. Arrows point to the positions of the two forms of F_{420}-hydrogenase and open triangles indicate the locations of MV-hydrogenase. The apparent differences in total length and the minor deviations in the location of the polypeptide bands between gel and blot reflect the shrinkage of the polyacrylamide gel strip and of the NC membrane during electrotransfer (4).

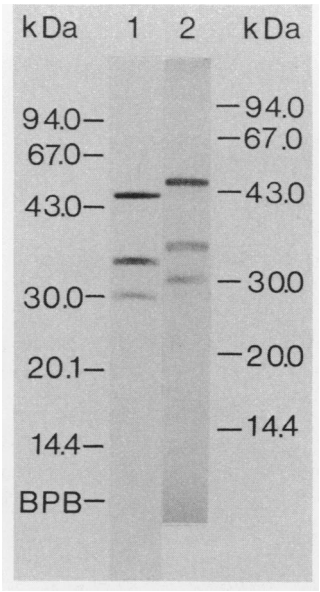

FIG. 2. Gel electrophoretic and immunoblot analysis of denatured homogeneous F_{420}-hydrogenase of *M. barkeri* Fusaro. Homogeneous F_{420}-hydrogenase was denatured and the polypeptides were separated by electrophoresis in a 12% SDS-polyacrylamide gel followed by staining for proteins with Coomassie brilliant blue (lane 1, 3.5 μg of protein) or by immunostaining with the anti-F_{420}-hydrogenase antiserum (lane 2, 1.2 μg of protein). The locations of the molecular mass standard proteins and their masses are indicated to the left of the gel and to the right of the corresponding blot. The mixture of reference proteins used for SDS-PAGE contained, in order of decreasing molecular mass, phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, and α-lactalbumin. The apparent differences in total length and the deviations in the location of the polypeptide bands between gel and blot reflect the shrinkage of the polyacrylamide gel strip and of the NC membrane during electrotransfer (4). BPB, Bromophenol blue.

ded cells (data not shown [13]), or a small-range distortion of the cellular border line by pressing and shearing during the sectioning process. Since treatment of the cells with osmium tetroxide during fixation has been omitted so as not to destroy antigenicity of the hydrogenase molecules, the typical appearance of the cytoplasmic membrane as a black-white-black double track is not found. However, applying electron energy loss spectroscopy with the electron microscope in its imaging mode (5) at a spectrometer setting of 40 eV, the cytoplasmic membrane with the immunolabeled antigenic sites could be visualized as a weak bright shining line between the dark cell wall matter and the cytoplasm (Fig. 4, open arrows) in some regions of the thin section. These results indicate that the F_{420}-hydrogenase is a membrane-associated enzyme in *M. barkeri* cells during exponential growth on methonal as the sole methanogenic substrate.

Unfortunately, we cannot decide at which side of the membrane the enzyme is located since we could not produce protoplasts from *M. barkeri* due to the unavailability of lytic enzymes for the degradation of the heteropolysaccharide cell wall (15), and so far we have also been unsuccessful in obtaining protoplasts from substrate-depleted cultures as described for *M. barkeri* FR-19. In addition, the resolution limits of the immunogold technique due to gold marker scattering around the antigenic epitope (33) do not allow any reliable conclusion with respect to the sidedness of the hydrogenase from the results of our labeling experiments (Fig. 3A and 4).

DISCUSSION

The energy metabolism of methanogenic bacteria has probably been most extensively studied in methanol-grown cells of *M. barkeri* Fusaro, primarily during methane formation from a substrate mixture of methanol and hydrogen, and all experimental data published to date indicate a chemiosmotic mechanism for energy conservation involving an electrochemical proton gradient (19). Moreover, it has been concluded that the electron transfer from H_2 to CH_3-S-CoM drives the translocation of protons across the cytoplasmic membrane (11), since the acidification of the medium by resting-cell suspensions of *M. barkeri* could not be detected in the presence of 2-bromoethanesulfonic acid, a potent inhibitor of the methylreductase reaction (reaction 1), or when H_2 was replaced by N_2 or air. This interpretation is strongly supported by data obtained in labeling experiments under similar experimental conditions (10), for under an atmosphere of H_2 whole cells of *M. barkeri* oxidized only 1% of the added [^{14}C]methanol to $^{14}CO_2$ but reduced 80% to $^{14}CH_4$, precluding a significant contribution to the generation of the proton electrochemical gradient by methanol oxidation. Since the electrons for the reduction of CH_3-S-CoM during methane formation from methanol and H_2 apparently originate from the oxidation of hydrogen (10, 11), it was extremely intriguing to determine the cytological localization of the F_{420}-hydrogenase in whole cells of *M. barkeri*. This enzyme has been shown to constitute 1.6% of the total cellular protein in methanol-grown cells, suggesting an important role in the metabolism of this methanogenic bacterium (17). The unambiguous localization of 88% of the F_{420}-hydrogenase molecules in the vicinity of the cytoplasmic membrane(s) in *M. barkeri* (Fig. 3A and 4) indicates that this enzyme is membrane associated during exponential growth on methanol and might be involved in the generation of the electrochemical proton gradient.

Until recently the search for a membrane-associated component of the methyl-coenzyme M methylreductase system had been concentrated on the immunocytochemical localization of the true methylreductase, formerly designated component C, in various methanogenic bacteria (19). Although the methylreductases of *Methanococcus voltae* (31) and *Methanobacterium thermoautotrophicum* (2) were localized in the vicinity of the cytoplasmic membrane, the enzymes of *M. barkeri* (19, 30) and *M. mazei* (41) were found to be scattered in the cytoplasm. Moreover, it has recently been demonstrated (32) that ATP synthesis in crude vesicle preparations of the methanogenic strain Gö1 is uncoupler and DCCD sensitive and stringently coupled to the electron transfer from H_2 to the heterodisulfide CoM-S-S-HTP as electron acceptor (reaction 2), but is not affected by 2-bromoethanesulfonic acid (reaction 1). These data strongly suggest that at least one component of the hydrogenase-linked heterodisulfide system must be membrane associated and be involved in proton translocation, while a membrane-bound methylreductase is not required.

Probably the F_{420}-hydrogenase is a peripheral and not an integral membrane protein, for the enzyme has been purified as a soluble protein with hydrophobic properties in the absence of a detergent (17). Subsequently, the hydrogenase could be bound to either the external or the internal surface of the membrane by noncovalent interactions with integral membrane proteins (1, 26). These protein-protein interac-

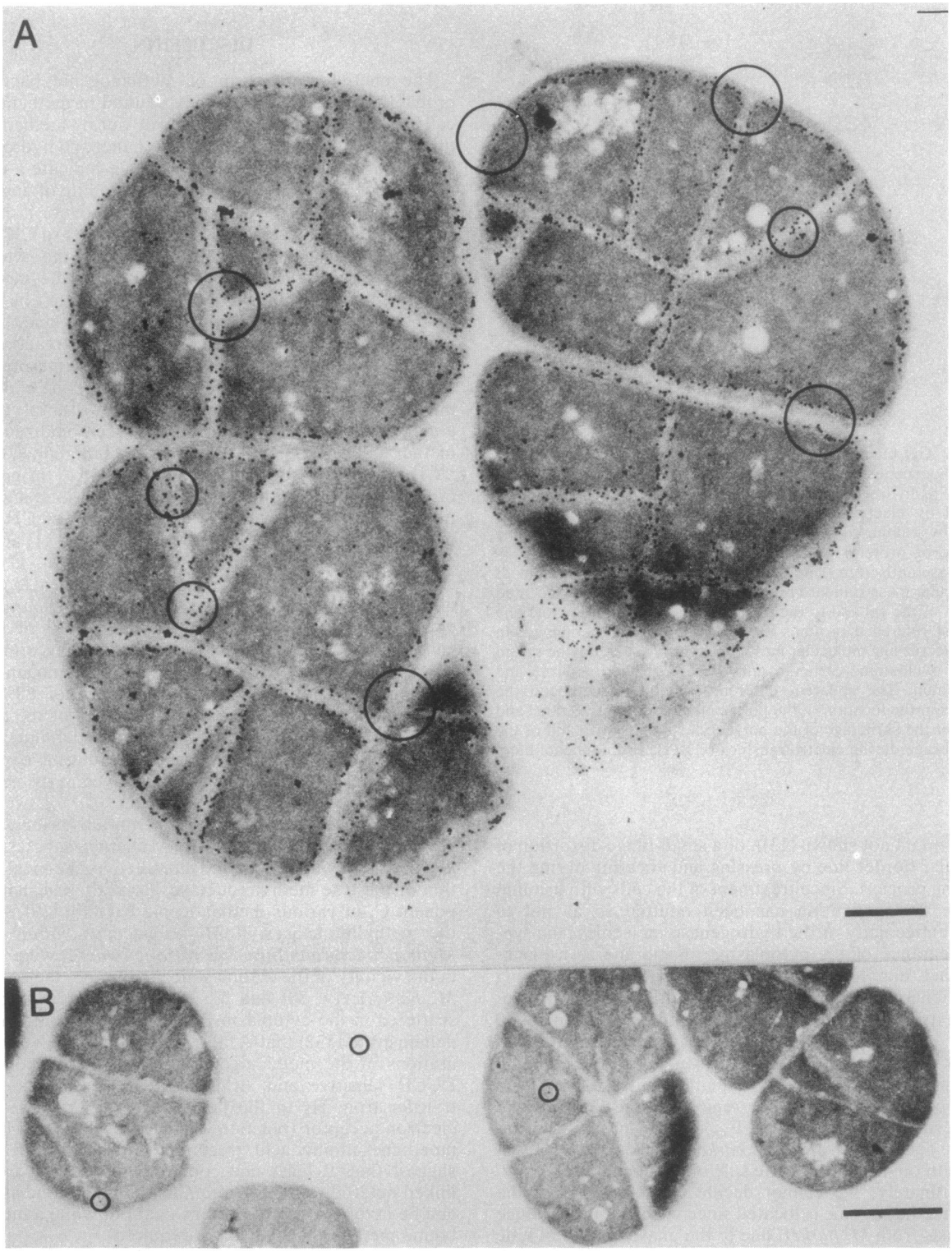

FIG. 3. Immunogold labeling of ultrathin sections of methanol-grown *M. barkeri* cells from the mid-logarithmic growth phase with anti-F_{420}-hydrogenase antiserum. (A) Survey of *M. barkeri* cells at low magnification. Large circles indicate gold tags aligned along the cytoplasmic membranes; small circles point out areas of diffuse marker binding or aligned binding within the cell wall. Bar, 0.5 μm. (B) *M. barkeri* cells incubated with preimmune serum instead of anti-F_{420}-hydrogenase antiserum. Bar, 1 μm. The cells had been fixed under anaerobic conditions with 2% (vol/vol) paraformaldehyde–0.5% (vol/vol) glutardialdehyde for 12 h at room temperature.

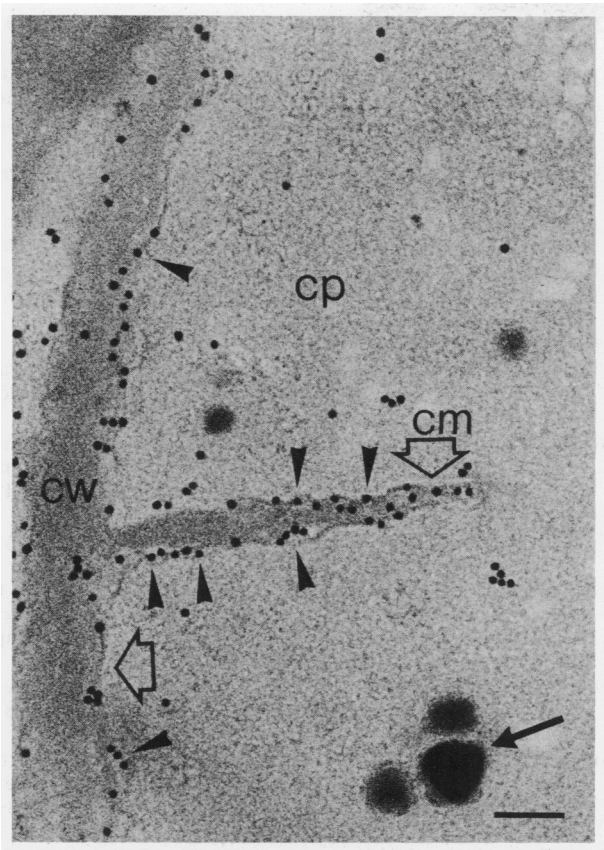

FIG. 4. Identification of the cytoplasmic membrane by imaging electron energy loss spectroscopy with the electron microscope at the cell borders between adjacent cells. An ultrathin section of methanol-grown *M. barkeri* cells after immunogold labeling with anti-F_{420}-hydrogenase antiserum is shown. Contrast-differentiation of the cytoplasmic membrane (cm) which is visible as a weak bright line (open arrows) contouring the cytoplasm (cp) along the dark cell wall (cw). Small arrowheads indicate gold tags aligned along the cytoplasmic membrane. Arrow marks a hole in the section which appears completely black. The spectrometer setting is 40 eV at an energy width of 20 eV. Ultrathin sections are nonosmicated and poststained with 4% (wt/vol) uranylacetate. Bar, 0.1 μm.

tions are apparently lost upon cell rupture due to changes in ionic strength and/or in pH of the surrounding medium, thus releasing the enzyme from the membrane. As a peripheral membrane protein the F_{420}-hydrogenase cannot function as a primary proton pump like bacteriorhodopsin (20), for it lacks the transmembrane proton channel which is prerequisite for a proton pump. However, irrespective of a location at the outer or inner surface of the membrane, the enzyme could initiate a hydrogen-dependent electron transport chain coupled to the reduction of the heterodisulfide CoM-S-S-HTP and thus participate in the generation of the electrochemical proton gradient detectable in *M. barkeri* (16, 19, 21, 26). Yet, one has to consider the possibility that one of the MV-hydrogenases also detectable in methanol-grown *M. barkeri* in addition to the F_{420}-hydrogenase (Fig. 1A, lane 2) (17) could couple the oxidation of H_2 with the heterodisulfide reductase system as indicated by results obtained for *Methanobacterium thermoautotrophicum* (22).

Further research will be necessary to clarify the physiological roles of the hydrogenases (14, 36, 38) found in *M. barkeri* (17) and other methanogens (24, 25, 28, 40, 45) and to identify the proteins involved in the generation of the electrochemical proton gradient required for energy conservation by a chemiosmotic process.

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