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Solubilization and Properties of a Particulate Hydrogenase from *Methanobacterium* Strain G2R†

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Mechanical disruption of cells of *Methanobacterium* strain G2R resulted in a 78-fold increase in the specific activity of the hydrogenase as measured by the benzyl viologen reduction assay. Approximately 50% of the activity in disrupted cells was associated with the particulate fraction. Between 69 and 85% of the particulate hydrogenase was released by treatment with the detergents Triton X-100, deoxycholate, and octyl-β-D-glucopyranoside. The relative electrophoretic mobilities of the solubilized and soluble hydrogenases were identical, indicating that G2R possessed a single electrophoretically distinct hydrogenase. The particulate enzyme was inactivated by oxygen and could be reactivated with dithionite or glucose plus glucose oxidase. The enzyme had a pH optimum of 8.5 and resisted heating at 52 but not 77°C. A number of nonspecific dyes, flavin adenine dinucleotide, and riboflavin 5'-phosphate were effective electron acceptors; oxidized nicotinamide adenine dinucleotide, nicotinamide adenine dinucleotide phosphate, and factor 420 were apparently not reduced. Hydrogenase activity was inhibited by *p*-hydroxymercuribenzoate, cyanide, chloroform, and chloramphenicol. The molecular weight of the solubilized enzyme was 900,000, with subunits of molecular weights 38,500, 50,700, and approximately 80,000. It is suggested that, in intact cells of G2R, the large hydrogenase complex is loosely bound to the cell wall or membrane.

Methanogenic bacteria derive energy from the reduction of CO_2 to methane at the expense of molecular hydrogen (22, 44, 45). Some information is available regarding the enzymes and cofactors involved in this reaction (8, 19, 23, 24, 41); however, the mechanism of membrane energization and subsequent coupling to ATP synthesis is unknown. Recently an adenosine triphosphatase activity has been found and linked to the synthesis of ATP in *Methanobacterium thermoautotrophicum* (12).

The initial step in the activation of H_2 in a wide variety of H_2-utilizing organisms is performed by hydrogenase (3, 4, 7, 13, 15, 16, 26, 35, 38, 41), which mediates the reduction of specific cofactors, such as ferredoxin (25), cytochrome c_3 (6, 8), and NAD^+ (36). A similar enzyme has been demonstrated in extracts of *Methanobacterium ruminantium* (41) and *M. thermoautotrophicum* (10, 46). However, in the methanogens a unique cofactor (F_{420}) is implicated (41).

Hydrogenases serve a variety of metabolic functions, determined to some extent by their location in the cell. For example, membrane-bound hydrogenase in *Alcaligenes eutrophus* is involved in membrane energization (31), whereas the corresponding soluble enzyme is required for NADH production (37).

As a preliminary step in the elucidation of the role of hydrogenase in the energy metabolism of the methanogens, the intracellular distribution as well as the physical and biochemical properties of this enzyme were studied by using a strain of *Methanobacterium* which resembles *M. formicicum* (28).

MATERIALS AND METHODS

Strains and growth conditions. *Methanobacterium* strain G2R and *Methanospirillum hungatii* strain GP1 were described previously by Patel et al. (28, 29). *M. thermoautotrophicum* was obtained from R. S. Wolfe. *Methanosarcina barkeri* DSM 800 was obtained from the German Culture Collection, Gottingen, West Germany. Cultures were maintained by weekly transfer into 120-ml serum-stoppered bottles containing 10-ml of SA medium (29) under an atmosphere of CO_2 and H_2 (20:80, vol/vol) at 35°C. *M. thermoautotrophicum* was cultivated at 62°C. Large volumes of cells for the preparation of crude extracts were grown in 12-liter fermentors as described previously (39).

Preparation of cell fractions. Cells were harvested aerobically by using a Sharples centrifuge and washed twice in cold 100 mM *N*-tris(hydroxy-

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methyl)methyl-2-aminoethane-sulfonic acid (TES) buffer, pH 7.1, containing 10 mM $MgSO_4$ (TES/Mg). Cells were broken by two passages through a French pressure cell operated at 12,000 to 16,000 lb/in^2. After centrifugation to remove unbroken cells (4,300 × *g* for 10 min), the extract was centrifuged again at 150,000 × *g* for 90 min. The supernatant was removed, and the pellet was washed twice in cold TES/Mg before being resuspended in the same buffer. The 4,300-×-*g* supernatant is referred to below as the crude extract, the 150,000-×-*g* supernatant is referred to as the soluble extract, and the 150,000-×-*g* pellet is referred to as the particulate fraction. All preparations were stored aerobically in liquid nitrogen.

Hydrogenase assay. Hydrogenase was assayed spectrophotometrically in 3-ml cuvettes fitted with rubber stoppers. The cuvettes were flushed with H_2 scrubbed free of O_2 by passage over a copper mesh at 300°C. A 2-ml amount of H_2-saturated 50 mM Tris (pH 8.5) was added with either 3 μl of 20 mM dithionite or 10 mM glucose plus 39 U of glucose oxidase, to remove traces of oxygen. H_2-saturated benzyl viologen was also added to a final concentration of 2.3 mM; then the rubber cap was fitted onto the cuvette, and the headspace was flushed with H_2. After incubation for 2 min at 25°C, the enzyme was added and the increase in absorbance at 578 nm was recorded over the first 2 min on a Perkin-Elmer recording spectrophotometer. Enzyme activity was expressed as micromoles of benzyl viologen reduced per minute per milligram of protein.

In some experiments H_2 was replaced by O_2-free N_2, and in others Tris buffer was replaced with other buffers as specified. When the effectiveness of electron acceptors was being considered, benzyl viologen was replaced by the appropriate acceptor and the reaction was monitored at the designated wavelength. Inhibitors were tested by preincubating with the enzyme for 5 to 10 min before initiation of the reaction with either benzyl viologen or H_2.

To locate the hydrogenase activity bands on polyacrylamide gels, the gels were incubated for 2 h at 25°C in 10 ml of H_2-saturated 50 mM Tris buffer (pH 8.5) containing 10 mM glucose and 195 U of glucose oxidase. Benzyl viologen was then added to 0.93 mM. Bands of hydrogenase activity started to appear within 20 min. Control gels were run in N_2-saturated buffer.

Protein determinations. Protein was determined by the method of Peterson (30), using bovine albumin as the standard. Samples containing whole cells were heated at 60°C for 30 min before the addition of the Folin reagent.

Solubilization of particulate hydrogenase. Approximately 1.3 mg of G2R particles in 1.0 ml of 10 mM TES (pH 7.1) was diluted with an equal volume of 100 mM octyl-β-D-glucopyranoside (OGP), 2.0% Triton X-100, or 3.0% sodium deoxycholate. After mixing at 25°C for 30 min, the preparations were centrifuged at 150,000 × *g* for 90 min, and the supernatant was removed. The pellets were taken up in 1.0 ml of 10 mM TES (pH 7.1) and extracted again with an equal volume of the detergent. After centrifugation at 150,000 × *g* for 90 min, the individual supernatants were pooled and used as the source of the solubilized enzyme.

Activation of hydrogenase. The activation mixture, in serum-stoppered vials under an atmosphere of H_2, consisted of 1.0 ml of the inactive enzyme at 25°C and either 0.2 to 0.4 mM dithionite or 10 mM glucose plus 62 U of glucose oxidase. Controls were performed with an N_2 atmosphere and with H_2 alone.

Polyacrylamide discontinuous gel electrophoresis. Samples (50 to 100 μl) of either soluble or detergent-solubilized hydrogenase were subjected to electrophoresis in either 4 or 7% acrylamide gels as described by Davis (11). Gels were stained for hydrogenase activity as described above and then decolorized by exposure to oxygen. The gels were then fixed in 12.5% trichloroacetic acid, stained with Coomassie brilliant blue, and destained by diffusion in 5% acetic acid.

SDS-Polyacrylamide gel electrophoresis. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed essentially as described by Weber and Osborn (43).

Bio-Gel 5.0 M chromatography. Hydrogenase solubilized from particles by Triton X-100 was passed through a column (1.75 by 62 cm) of Bio-Gel 5.0 M previously equilibrated with 50 mM Tris (pH 8.5) at 4°C. The enzyme was eluted by downward flow with a pressure head of 35 cm. The flow rate was 5.5 ml/h, and 3.0-ml fractions were collected.

The column was calibrated by using blue dextran, ferritin, catalase, and bovine albumin.

Materials. Bio-Gel 5.0 M was obtained from Bio-Rad Laboratories. Standards for SDS electrophoresis (14,300 to 71,500 daltons) were obtained from BDH Biochemicals. All other reagents were of the highest purity available.

RESULTS

Distribution and specific activities of hydrogenase. Whole cells, as well as crude, soluble, and particulate fractions, of *Methanobacterium* strain G2R were tested for hydrogenase activity (Table 1). Dramatic increases in specific activity were observed after French pressure cell disruption of the whole cells. Also, these data show that approximately 50% of the total activity found in crude extracts was associated with the particulate fraction, although lower values

TABLE 1. *Distribution and specific activity of hydrogenase*[a]

Fraction	Sp act (μmol/min per mg)	Distribution (%)
Whole cells	0.27	
Crude extract	30.6	
Soluble fraction	28.5	52.7
Particulate fraction	29.6	47.3

[a] All fractions were activated by preincubation at 25°C for 30 min with 0.4 mM dithionite under H_2. The assay mixture (2.0 ml), under an H_2 atmosphere, consisted of 50 mM Tris (pH 8.5), 2.3 mM benzyl viologen, 0.02 mM dithionite, and 1 to 5 μg of protein.

were obtained for some preparations.

Solubilization of the particulate hydrogenase. Microorganisms in which the hydrogenase activity is equally distributed between the particulate and soluble fractions often possess two or more electrophoretically distinct enzymes (1). To establish the number of hydrogenases present in extracts of strain G2R, it was necessary to solubilize the particulate enzyme. Two extractions of the particles with either 50 mM OGP, 1.0% Triton X-100, or 1.5% deoxycholate effectively removed 69 to 85% of the total recoverable hydrogenase activity and 54 to 62% of the total protein (Table 2). With all of the detergents, the total activity recovered was in excess of 100%. Activation of solubilized enzymes due to removal of lipid by detergents has been reported previously (20).

Solubilized preparations stored in liquid N_2 without prior removal of the solubilizing agent suffered no loss of activity for up to 1 month. These preparations were routinely diluted at least 10-fold before activation, because as little as 6 mM OGP prevented glucose oxidase-mediated activation (data not shown). In addition, when the enzyme was stored aerobically overnight at 4°C in the presence of 30 mM OGP, less than 10% of the activity (compared with a control lacking the detergent) could be recovered upon activation.

OGP-solubilized hydrogenase was subjected to polyacrylamide discontinuous electrophoresis on gels containing 4 and 7% acrylamide. The gels were assayed for hydrogenase activity, and then restained for protein (Fig. 1). The majority of the hydrogenase activity that developed within 1 h after benzyl viologen addition (Fig. 1, gels a and b) was associated with a protein band (band E) having an R_f value of 0.33 on 4% acrylamide gels. A faint band of activity ran with the dye front. Other faint bands of activity could be detected but were not reproducibly present in all gels. Activity bands did not appear when N_2 replaced H_2 in the reaction mixture.

When 7% acrylamide gels were used (Fig. 1, gels c and d), it was found that the major hydrogenase band (E) had penetrated the stacking but not the running gel. The faint activity band, hereafter referred to as the fast-moving enzyme, had an R_f value of 0.5.

The activity banding patterns observed in both 4 and 7% gels were unaffected by the inclusion of 30 mM OGP in the running gel or, conversely, by the removal of OGP from the sample by dialyzing against TES/Mg before electrophoresis. Similar activity patterns on 4% gels were produced with undialyzed preparations solubilized by either Triton X-100 or sodium deoxycholate.

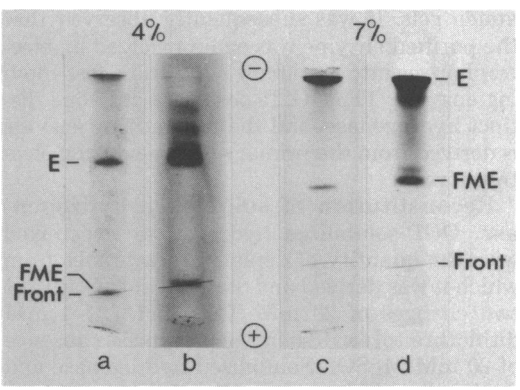

FIG. 1. *Polyacrylamide discontinuous gel electrophoresis of OGP-solubilized hydrogenase. Acrylamide gels (4 and 7%) were stained for hydrogenase activity (gels b and d) and for protein (gels a and c). E, The major band of hydrogenase activity; FME, fast-moving enzyme.*

TABLE 2. *Solubilization of the particulate hydrogenase*

Treatment[a]	Hydrogenase[b]	Sp act (μmol/min per mg)[c]	Distribution of recovered activity (%)	Total activity recovered (%)	Total protein solubilized (%)
None		22.2	100.0	100.0	
Triton X-100 (1.0%)	S	36.4	85.0	300.0	62
	P	10.2	15.0		
DOC (1.5%)[d]	S	41.0	69.0	270.0	62
	P	18.4	31.0		
OGP (50 mM)	S	21.1	77.0	121.6	54
	P	6.3	23.0		

[a] Particles were treated twice with the indicated reagent and mixed at 25°C for 30 min; fractions were separated by centrifugation at 150,000 × *g* for 90 min, and each fraction was activated and assayed as described in Table 1, footnote *a*.

[b] S, Solubilized; P, particulate.

[c] The specific activities for the solubilized fractions were calculated by using the concentration of particulate protein from which they were derived.

[d] DOC, Sodium deoxycholate.

When the soluble hydrogenase obtained after French pressure cell lysis was electrophoresed under the same conditions, the primary enzyme (R_f = 0.33, 4% gel) and the fast-moving enzyme (R_f = 0.5, 7% gel) were both observed. Thus, on the basis of electrophoretic mobility, no differences exist between the soluble and detergent-solubilized hydrogenases.

The fast-moving enzyme detected in all solubilized or soluble preparations could be a distinct hydrogenase produced in small quantities by the cell or merely an electrophoretic artifact. To test the latter possibility, the primary enzyme band (E) was removed from a number of 4% gels that had been loaded with Triton X-100-solubilized hydrogenase. The enzyme was extracted from the gel fragments into a small volume of 10 mM TES (pH 7.1), filtered to remove pieces of acrylamide, concentrated under a stream of N_2, and electrophoresed again on both 4 and 7% acrylamide gels. It was subsequently observed that the purified enzyme was again resolved by electrophoresis into a primary band and a fast-moving enzyme. Thus, G2R possesses only one distinct hydrogenase, and the fast-moving enzyme is derived from the primary enzyme during electrophoresis.

Reconstitution of solubilized hydrogenase. OGP-solubilized hydrogenase was mixed with the quantity of depleted membranes from which it was derived and dialyzed at 4°C against two changes of 20 mM TES (pH 7.1)–1 mM dithiothreitol for 18 h in the presence or absence of 20 mM $MgSO_4$. Solubilized hydrogenase and depleted membranes were dialyzed separately as controls. Under these conditions, virtually all of the OGP should be removed from the enzyme (5). The membrane and soluble fraction were separated by centrifugation, and the distribution of hydrogenase was determined. It was found that removal of the detergent by dialysis in the presence or absence of $MgSO_4$ did not lead to rebinding of the hydrogenase to the membrane.

Properties of the particulate hydrogenase. (i) Oxygen sensitivity. The particulate fraction, isolated and stored under H_2, exhibited low levels of hydrogenase activity. This was attributed to trace amounts of oxygen in the enzyme preparation. When prepared and stored aerobically, the particles were completely devoid of activity; however, activity was restored by the addition of reducing agents, including dithionite or glucose plus glucose oxidase (Fig. 2). At the specified times, samples of reduced enzyme were removed and assayed for hydrogenase activity. Maximum activity in the presence of dithionite was obtained after 1 h, whereas 2 to 4 h were required for activation by glucose oxidase. The

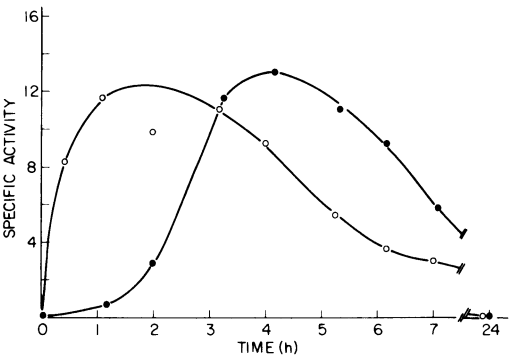

FIG. 2. *Activation of membrane-bound hydrogenase by dithionite or glucose oxidase. After activation, samples were removed at intervals and assayed as described in Table 1, footnote a. Specific activity is expressed as micromoles of benzyl viologen reduced per minute per milligram of protein. Symbols: ○, dithionite activated; ●, glucose-glucose oxidase activated.*

enzyme could also be activated by 10 mM $FeSO_4$, the activation curve resembling that for glucose oxidase. Dithionite, a strong reducing agent, activated more rapidly than glucose oxidase; however, the maximum specific activities obtained were similar for both methods. H_2 alone was ineffective in activating the enzyme. Activation by both dithionite and glucose plus glucose oxidase took place under N_2 as effectively as under H_2.

Once maximum activity was reached, the activated (or reduced) enzyme rapidly became inactive at 25°C (Fig. 2) or at 4°C (data not shown). Storage of the reduced enzyme for 24 h at 25°C resulted in a complete loss of activity, whereas 30% of the activity remained if incubation took place at 4°C. Rapid inactivation could not be reversed by the addition of fresh dithionite or glucose oxidase. Activation by glucose oxidase in the presence of 1 mM $FeSO_4$, 0.2 mM dithiothreitol, 1 mM EDTA, 0.15 mM riboflavin 5′-phosphate, boiled crude extract, or 10 mM EDTA plus 10 mM dithiothreitol, and 1.7 M glycerol failed to protect the enzyme from rapid inactivation.

The particulate enzyme could be stored for 24 h at 4°C in the oxygenated form without loss of activity. The enzyme was routinely stored in liquid N_2 and remained stable over a 4-month period. For most experiments, hydrogenase was activated with dithionite or glucose plus glucose oxidase in TES/Mg and used within 30 min of reaching maximum activity.

(ii) pH optimum. The effect of pH and buffer type on the glucose oxidase-activated particulate hydrogenase was studied by using buffers (50

mM) and pH ranges as follows; TES, pH 6.5 to 7.5; *N,N*-bis(2 hydroxyethyl)-2-aminoethanesulfonic acid, pH 7 to 9; Tris, pH 8 to 9; and glycylglycine, pH 8.5 to 9.5. Maximum activity was observed in the pH range of 8.5 to 9.0. Similar results were found for the dithionite-activated enzyme.

(iii) Effect of electron acceptors. A number of different electron acceptors were tested for their ability to substitute for benzyl viologen. The nonspecific dyes methyl viologen, methylene blue, and 2,6-dichlorophenol-indophenol replaced benzyl viologen in the reaction (Table 3). Methylene blue had the highest affinity of any of the dyes for the enzyme. Flavin adenine dinucleotide and riboflavin 5′-phosphate also acted as effective electron acceptors, with K_m values of 0.008 and 0.01 mM, respectively. The maximum velocities obtained with the flavins, however, were significantly lower than those of the dyes. Potassium ferricyanide, $NADP^+$, and NAD^+ (each at 1 mM), as well as F_{420} (absorbance at 420 nm, 1.2), were not reduced by the hydrogenase.

(iv) Effect of inhibitors. A variety of compounds were tested for their ability to inhibit the benzyl viologen-linked particulate hydrogenase. As Table 4 shows, carbonyl cyanide *p*-trifluoromethoxy-phenylhydrazone, dicyclohexylcarbodiimide, 2-bromoethane sulfonate (a coenzyme M analog [17]), and NaN_3 did not inhibit. However, *p*-hydroxymercuribenzoate at a concentration of 0.025 mM inhibited the hydrogenase by 92%, suggesting that sulfhydryl groups are involved in the active site. Chloroform, a competitive inhibitor of methane synthesis (44), also inhibited the hydrogenase activity.

TABLE 3. *Effect of various electron acceptors on hydrogenase activity*[a]

Electron acceptor[b]	K_m (mM)	V_{max} (μmol/min per mg)
Benzyl viologen	0.310	2.270
Methyl viologen	0.290	1.300
Methylene blue	0.004	0.930
2,6-Dichlorophenol-indophenol	0.500	0.940
Flavin adenine dinucleotide	0.080	0.120
Riboflavin 5′-phosphate	0.010	0.015

[a] Particulate hydrogenase was activated and assayed as described in Table 1, footnote *a*, with the appropriate acceptor replacing benzyl viologen.

[b] Changes in absorbance were monitored at the following wavelengths: benzyl viologen, 578 nm (ϵ = 8.65 mM^{-1} cm^{-1}); methyl viologen, 578 nm (ϵ = 9.7 mM^{-1} cm^{-1}); methylene blue, 660 nm (ϵ = 72.6 mM^{-1} cm^{-1}); 2,6-dichlorophenol-indophenol, 610 nm (ϵ = 19.1 mM^{-1} cm^{-1}); flavin adenine dinucleotide, 450 nm (ϵ = 8.3 mM^{-1} cm^{-1}); and riboflavin 5′-phosphate, 445 nm (ϵ = 8.3 mM^{-1} cm^{-1}).

TABLE 4. *Effect of inhibitors on G2R membrane-bound hydrogenase*[a]

Inhibitor[b]	Concn (mM)	Reagent used to start reaction	Sp act (μmol/min per mg)	% of control
None		Benzyl viologen	33.0	100
FCCP	0.05	Benzyl viologen	34.8	106
DCCD	0.12	Benzyl viologen	33.0	100
NaN_3	1.00	Benzyl viologen	31.9	97
2-Bromo-ethane sulfonate	1.0	Benzyl viologen	27.3	83
PHMB	0.025	Benzyl viologen	2.8	9
CAP	0.25	Benzyl viologen	5.2[c]	16
KCN	1.0	Benzyl viologen	19.1[c]	58
None		H_2	21.3	100
CAP	0.25	H_2	3.4[c]	16
KCN	1.0	H_2	13.7	64
Chloroform	0.19	H_2	9.4	44

[a] Particulate hydrogenase was activated under H_2 with 0.4 mM dithionite and assayed as described in Table 1, footnote *a*.

[b] Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxy-phenylhydrazone; DCCD, dicyclohexylcarbodiimide; PHMB, *p*-hydroxymercuribenzoate.

[c] Denotes activity between 2 and 5 min after initiation of the reaction.

Some differences in inhibition kinetics occurred in response to the electron donor used. When benzyl viologen was added to initiate the reaction, neither chloramphenicol (CAP), an inhibitor of methane synthesis (19, 24), nor KCN had an effect on the initial rate of hydrogenase activity. However, between 2 and 5 min after benzyl viologen addition, considerable inhibition was produced by both compounds. When the reaction was initiated with H_2 (Table 4), KCN was effective within the first 2 min. CAP, on the other hand, had no effect on the initial rate of activity, but maximum inhibition was again observed between 2 and 5 min.

(v) Molecular weight. Hydrogenase solubilized by Triton X-100 was passed through a Bio-Gel 5 M column (exclusion limit, 5,000,000 daltons). The molecular weight, determined to be approximately 900,000, agreed roughly with the electrophoretic data where the hydrogenase was unable to enter 7% acrylamide gels (exclusion limit, ~1,000,000 daltons). Fractions from the Bio-Gel 5 M column containing enzyme activity were subjected to electrophoresis on 4 and 7% gels. Both the primary enzyme and the fast-moving enzyme gave banding patterns essentially identical to those illustrated above (Fig. 1).

(vi) Subunit composition of hydrogenase. The primary enzyme purified earlier from 4% acrylamide gels was subjected to SDS electro-

phoresis. Three bands were obtained, with molecular weights of 38,500, 50,700, and approximately 80,000.

(vii) Comparison of the particulate and solubilized hydrogenases. Some particulate hydrogenases undergo modification of physical and biochemical properties upon solubilization (2, 4). Therefore, a number of properties of the OGP-solubilized hydrogenase were compared with those of the particulate enzyme. Neither particulate nor solubilized enzyme was affected by heating in TES/Mg in the inactive form at 52°C for up to 90 min; however, both suffered complete loss of activity after 15 min at 77°C. The solubilized and particulate hydrogenases were similar with respect to pH optima and to inhibition by KCN, CAP, and *p*-hydroxymercuribenzoate (data not shown).

Electrophoretic mobilities of the soluble hydrogenases from other methanogens. As noted above, particulate (solubilized) and soluble forms of hydrogenase from *Methanobacterium* strain G2R gave identical electrophoretic mobilities. However, significant differences were observed when the soluble hydrogenases from several methanogenic species were compared. When 4% acrylamide gels and 10 to 50 μg of protein per gel were used, the R_f values for the major activity bands (where 1.0 represents the dye front) were as follows: *Methanobacterium* strain G2R, 0.31; *M. hungatii* GP1, 0.23; *M. thermoautotrophicum,* 1.0; and *M. barkeri,* 0.0.

DISCUSSION

Mechanical disruption of *Methanobacterium* strain G2R yielded both particulate and soluble forms of the hydrogenase. Detergents solubilized most (70 to 85%) of the particulate enzyme. The detergent-solubilized and the soluble hydrogenase activities displayed identical mobilities on 4% acrylamide gels, suggesting a single species of the enzyme. A number of faint activity bands appeared in some preparations, as reported for the hydrogenases of other microorganisms (1).

The apparent solubilization of the hydrogenase during mechanical breakage and the ease of solubilization by detergents suggest a loose association with the membrane or cell wall. Also, removal of the detergent from solubilized G2R hydrogenase by dialysis in the presence of depleted membranes and Mg^{2+}, an effective procedure for reconstitution of membrane enzymes (32), failed to remove significant amounts of hydrogenase from the supernatant. This loose association was not unexpected since it has been demonstrated that, in most cases, all of the enzymes and electron carriers for methane synthesis are easily released by mechanical breakage of the cells (18, 33; for alternate cases, see references 12 and 34). The absence of any significant differences in heat stability, pH optimum, and inhibitor sensitivity between the particulate and solubilized enzymes suggests that no major structural changes take place during solubilization, an observation that is in agreement with the loose association between the enzyme and the particles.

Association of a hydrogenase with the membrane suggests a possible role in membrane energization (31). Indeed, it has been shown that the *Paracoccus denitrificans* hydrogenase is physically aligned across the membrane (38) and may therefore take part in a vectorial transfer of protons (40). The ease with which the enzyme is solubilized and the absence of significant hydrogenase activity (benzyl viologen assay) in whole cells of G2R suggest that the hydrogenase does not span the membrane.

Hydrogenases differ widely in their sensitivity to oxygen. Some are completely and irreversibly inactivated by exposure to oxygen but retain full activity when stored anaerobically (14). Others are inactivated by oxygen but can be reactivated under reducing conditions (36). The latter enzymes are very unstable when stored anaerobically. It was postulated that reversible inactivation was due to oxygenation of the enzyme, whereas irreversible inactivation was ascribed to complete oxidation of the enzyme (31). We have found that the G2R hydrogenase was oxygenated during aerobic preparation and that the addition of dithionite or glucose oxidase resulted in the rapid restoration of activity. The activated enzyme was extremely unstable, however, and could not be stabilized by the addition of a number of protective agents.

The G2R hydrogenase, like the enzyme from many other strains, reacts readily with artificial electron acceptors and flavins. The observation that F_{420} was not reduced by the particulate hydrogenase is surprising since other workers have demonstrated the H_2-dependent reduction of F_{420} in crude extracts of other methanogens (41, 46). The reason for this discrepancy is unknown. Preliminary studies on F_{420} reduction by crude extracts of G2R prepared under H_2 gave negative results, suggesting that the inability of the particulate enzyme to react with F_{420} was not due to its initial exposure to oxygen.

The particulate hydrogenase was inhibited by CAP and chloroform, both potent inhibitors of methanogenesis (19, 24, 44). The data presented were consistent with CAP acting as an electron acceptor in the benzyl viologen assay (27). The mechanism of chloroform inhibition remains to be elucidated.

We have reported here a molecular weight for

the G2R hydrogenase of approximately 900,000. Molecular weights of 52,000 (42), 60,000 (9), 89,500 (21), and 205,000 (36) have been reported for other hydrogenases. SDS electrophoresis of the high-molecular-weight enzyme isolated from polyacrylamide gels showed three bands having molecular weights of 38,500, 50,700, and approximately 80,000. The hydrogenase of *Desulfovibrio gigas*, with a molecular weight of 89,000, consisted of two unequal subunits having molecular weights of 62,000 and 26,000 (21). Conceivably, the G2R hydrogenase could consist of different subunits that aggregate to form the 900,000-dalton enzyme. Alternatively, the high-molecular-weight complex may consist of several separate enzymes.

Differences in the electrophoretic mobilities of bacterial hydrogenases have been considered as a possible taxonomic guide (1). We have shown that the soluble hydrogenases from several species of methanogens possessed strikingly different mobilities. Further studies on the methanogen hydrogenases should shed light on the extent of evolutionary divergence within this ancient group of microorganisms.

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