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Purification and Characterization of the Hydrogen Uptake Hydrogenase from the Hyperthermophilic Archaebacterium *Pyrodictium brockii*†

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***Pyrodictium brockii* is a hyperthermophilic archaebacterium with an optimal growth temperature of 105°C. *P. brockii* is also a chemolithotroph, requiring H_2 and CO_2 for growth. We have purified the hydrogen uptake hydrogenase from membranes of *P. brockii* by reactive red affinity chromatography and sucrose gradient centrifugation. The molecular mass of the holoenzyme was 118,000 ± 19,000 Da in sucrose gradients. The holoenzyme consisted of two subunits by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The large subunit had a molecular mass of 66,000 Da, and the small subunit had a molecular mass of 45,000 Da. Colorometric analysis of Fe and S content in reactive red-purified hydrogenase revealed 8.7 ± 0.6 mol of Fe and 6.2 ± 1.2 mol of S per mol of hydrogenase. Growth of cells in $^{63}NiCl_2$ resulted in label incorporation into reactive red-purified hydrogenase. Growth of cells in $^{63}NiCl_2$ resulted in label incorporation into reactive red-purified hydrogenase. Temperature stability studies indicated that the membrane-bound form of the enzyme was more stable than the solubilized purified form over a period of minutes with respect to temperature. However, the membranes were not able to protect the enzyme from thermal inactivation over a period of hours. The artificial electron acceptor specificity of the pure enzyme was similar to that of the membrane-bound form, but the purified enzyme was able to evolve H_2 in the presence of reduced methyl viologen. The K_m of membrane-bound hydrogenase for H_2 was approximately 19 μM with methylene blue as the electron acceptor, whereas the purified enzyme had a higher K_m value.**

The isolation of bacteria that are capable of growth at elevated temperatures has kindled a drive to understand the biochemical basis for extreme thermophily. A better understanding of thermotolerant bacteria is of interest to basic science as well as for use in biotechnological applications (20, 33). One of the promising avenues of research has been in the area of energy metabolism of these unique organisms. Given the extremes of the natural environment of the thermophiles, the ATP-producing electron transport pathways with their associated dehydrogenases may reveal some novel properties when compared with their mesophilic counterparts. Currently, little is understood about the biochemical basis for life at extreme temperatures; however, some avenues of research are beginning to yield results (30).

Hydrogenases catalyze the reversible reaction $H_2 \rightleftharpoons 2H^+ + 2e^-$. Hydrogen metabolism is a very widespread phenomenon in bacteria (2). Most of the research to date has been done in eubacteria; however, advances have been seen in the archaebacteria, especially recently in the methanogens (3, 7, 16–18, 32, 36, 38). Despite the widespread utilization of H_2 in extreme thermophiles and hyperthermophiles, little is known about the properties of hydrogenases in hyperthermophiles (1). (Extreme thermophiles are defined as organisms able to grow at temperatures between 80 and 100°C, and hyperthermophiles are defined as organisms that will grow at 100°C and above.) Until now, the only hydrogenase purified from a hyperthermophile was the evolution-type hydrogenase from *Pyrococcus furiosus* (11). The *P. furiosus* hydrogenase seems to represent a new form of hydrogenase, one that is thermodynamically designed to primarily evolve hydrogen under physiological conditions. In contrast to the *P. furiosus* hydrogenase, the uptake-type hydrogenase from *Pyrodictium brockii* seems to have much more in common with its eubacterial counterparts (31).

P. brockii was originally isolated from the solfatara fields near Volcano, Italy, by Stetter and his colleagues (39, 40). *P. brockii* is a disk-shaped organism with an optimal growth temperature of 105°C. It is a strict anaerobe and grows chemolithotrophically with H_2 and CO_2 (29, 39). In the process of oxidizing H_2, elemental sulfur is reduced to hydrogen sulfide. This form of metabolism has been termed hydrogen-sulfur autotrophy (30, 39).

We report here the purification and characterization of the hydrogen uptake hydrogenase from *P. brockii*. We have investigated this enzyme in terms of metal content, temperature stability, and kinetics of substrate reaction. We have also investigated the role that membranes play in the thermal stability of the hydrogen uptake hydrogenase, since the hydrogenase is normally associated with membranes (31).

MATERIALS AND METHODS

Chemicals and reagents. All chemicals were of reagent grade or better and were obtained from Sigma (St. Louis, Mo.), Alfa Chemical (Danvers, Mass.), or J. T. Baker Chemical (Phillipsburg, N.J.). All gases were purchased from Linde Gases (Baltimore, Md.).

Bacterial strain. All experiments were performed with *P. brockii* DSM 2708, originally obtained from Deutsche Sammlung von Mikroorganismen (Göttingen, Germany).

Cell growth. *P. brockii* was grown in continuous culture by modification of the procedure of Brown and Kelly (10, 33a).

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The growth medium used was composed of 25 g of NaCl, 3.5 g of $MgSO_4$, 2.75 g of $MgCl_2$, 0.05 g of NaBr, 0.33 g of KCl, and 2 g of yeast extract per liter. Trace elements were added as described previously (31). An 80% H_2–20% CO_2 mixture was sparged through the culture flask at a rate of 0.1 culture volume per min. *P. brockii* starter cultures were grown in bottles as previously described (29, 31). Cells used in ^{63}Ni labeling experiments were grown in bottles with artificial seawater (31) to which 50 μCi of $^{63}NiCl_2$ (New England Nuclear, Boston, Mass.) was added.

Membrane preparation. Cells were harvested by passing the cell culture through a Whatman no. 1 filter to remove large sulfur particles and then centrifuging at 10,000 × *g* for 20 min at 4°C. The cell pellet was washed in 50 mM MOPS [3-(*N*-morpholine)propanesulfonic acid] (pH 7.0) containing 100 mM NaCl and 2 mM EDTA. Cells from 15 liters of culture were suspended in 10 ml of buffer. After harvesting, the cells were homogenized in an ice-cold ground glass homogenizer and broken by two passages through a French press pressure cell at 20,000 lb/in^2 and 4°C. Membranes were pelleted by centrifugation at 110,000 × *g* for 1 h at 4°C. Membrane pellets from 15 liters of cell culture were suspended in a final volume of 5 to 6 ml of buffer and homogenized in an ice-cold ground-glass homogenizer.

Membranes from ^{63}Ni-grown cells were prepared with the following modifications. Cell cultures were not filtered, but rather cells were harvested directly. Care was taken to avoid as much of the crystalline sulfur as possible. The cell pellet was washed with 50 mM MOPS (pH 7.0) containing 100 mM NaCl. The cells were disrupted by two freeze-thaw cycles, and the membranes were harvested at 110,000 × *g* for 1 h at 4°C. The membranes from 3 liters of cells were suspended in a final volume of 3 ml.

Hydrogenase purification. The purification of *P. brockii* hydrogenase by affinity chromatography was an adaptation of previously published procedures used to purify hydrogenase from two H_2-oxidizing eubacteria (35, 41). Hydrogenase was solubilized from membranes by mixing the membranes 1:1 with 4% (wt/vol) Triton X-100 (made in 50 mM MOPS [pH 7.0]–100 mM NaCl–2 mM EDTA) and stirring thoroughly for 90 min at room temperature. The nonsolubilized material was removed by centrifugation at 110,000 × *g* for 1 h at 4°C.

Solubilized hydrogenase was passed over a 1- by 4-cm column of reactive red 120-agarose (Sigma). The column was washed with 30 ml of 2% Triton X-100 in 50 mM MOPS (pH 7.0)–100 mM NaCl–2 mM EDTA followed by a wash with 30 ml of 50 mM MOPS–400 mM NaCl–2 mM EDTA. Pure hydrogenase was eluted from the column in 15 ml of 50 mM MOPS–1 M NaCl–2 mM EDTA. All remaining protein was removed from the column in 15 ml of 50 mM MOPS–5 M NaCl–2 mM EDTA. The reactive red purification was carried out at room temperature.

^{63}Ni-labeled hydrogenase was purified as described above with the following modifications. The reactive red column contained a 2-ml bed volume, and EDTA was omitted from all of the buffers. The 2% Triton X-100 wash and the 400 mM NaCl wash were done in a volume of 20 ml. The hydrogenase was eluted in 10 ml of buffer.

Protein concentration was measured by the BCA assay (Pierce Chemical, Rockford, Ill.) with bovine serum albumin as a standard.

Hydrogenase assay. Hydrogen uptake activity was determined amperometrically (25, 42, 43) with the modifications previously described (31). All assays were done at 90°C in 50 mM MOPS (pH 7.0)–100 mM NaCl–2 mM EDTA. The assay itself takes only 1 to 2 min. Saturating levels of methylene blue (200 μM) were used as the terminal electron acceptor unless otherwise noted.

SDS-polyacrylamide gel electrophoresis. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was done on 10% polyacrylamide gels by the method of Laemmli (21). SDS molecular weight markers were purchased from Sigma and Bio-Rad (Rockville Center, N.Y.). Silver staining was done by the method of Morrissey (27).

Sucrose gradients. Molecular mass estimates of purified hydrogenase were made by using the linear sucrose gradient centrifugation method of Martin and Ames (26). An 11-ml 5 to 20% sucrose gradient was formed in an SW41 tube (Beckman). Approximately 300 μg of reactive red-purified hydrogenase was mixed in 1% sucrose and layered on the top of the gradient. The final sample volume was 0.5 ml. Samples were centrifuged at 135,000 × *g* for 16 h at 18°C in a Beckman SW41 rotor. Fractions were collected by piercing the bottom of the centrifuge tube and collecting 0.5-ml fractions. Fractions were assayed for hydrogenase activity as described above. Standards used for molecular weight estimation were phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, and soybean trypsin inhibitor (Bio-Rad). The molecular mass of hydrogenase was estimated against each of these standards and then all values were averaged as described by Martin and Ames (26). The standard deviation given was obtained from all of the calculated values used in the molecular mass estimation.

Metals analysis. Iron was determined colorometrically with *o*-phenanthroline by the method of Lovenberg et al. (24). Acid-labile sulfur was analyzed by the procedure of Chen and Mortenson (12). Sodium sulfide standards were made as described by Beinert (8). Ferredoxin from *Clostridium pasteurianum* was used as an internal control in both assays and was kindly provided by M. W. W. Adams. The nickel content of purified hydrogenase was measured by growing *P. brockii* in bottles in the presence of 50 μCi of $^{63}NiCl_2$ (New England Nuclear). The amount of radioactivity incorporated was measured by using a Rack Beta Scintillation Counter (LKB Instruments, Rockville, Md.) and 4 ml of Opti-Fluor (Packard Instrument Co., Downers Grove, Ill.) per vial.

RESULTS

Hydrogenase purification. Previous efforts have shown that reactive red is an effective method for the aerobic purification of some eubacterial hydrogenases (35, 41). Since earlier studies had indicated both a biochemical and a structural similarity between the eubacterial *Bradyrhizobium japonicum* hydrogen-uptake hydrogenase and the archaebacterial *P. brockii* hydrogenase (31), purification with reactive red after detergent solubilization was attempted. Nearly complete solubilization of hydrogen uptake activity from membranes by 2% Triton X-100 was achieved (Table 1). All purification steps were done aerobically, because previous studies (31) showed that exposure to O_2 did not significantly damage the activity in crude extracts.

The vast majority of the solubilized hydrogenase activity bound to the reactive red column, and a wash of 400 mM NaCl was sufficient to remove most protein but still leave the majority of the hydrogenase activity bound to the column. Hydrogenase activity could then be removed from the column by eluting with 1 M NaCl. It should be noted that the final yield of purified hydrogenase varied between different batches of cell culture (final yields from 10% to about 100%

TABLE 1. Purification of *P. brockii* hydrogen uptake hydrogenase by reactive red chromatography

Fraction[a]	Sp act (μmol of H_2 consumed/min/mg of protein)	Fold purification	Activity recovered (μmol/min)	% of crude extract
Membranes	0.93	1	62.8	100.00
Solubilized	28.1	30.2	58.9	93.8
1	0.21	0.23	0.08	0.13
2	0.31	0.33	0.16	0.25
3	12.0	12.9	0.77	1.2
4	189.5	203.2	9.73	15.5
5	129.5	139.5	0.72	1.1

[a] Fraction 1 is the flowthrough from loading the solubilized material. Fraction 2 is a 100 mM NaCl–2% Triton X-100 wash. Fraction 3 is a 400 mM NaCl wash. Fraction 4 has the purified hydrogenase eluted in 1 M NaCl. Fraction 5 is a 5 M NaCl wash.

have been seen). This variation in yield could often be explained by the vigorousness of mixing during solubilization and indicates that the solubilized enzyme is stable during purification.

Reactive red-purified hydrogenase ran anomalously on conventional gel filtration columns; therefore molecular mass of the purified hydrogenase was determined by sucrose gradients. On 5 to 20% sucrose gradients, the majority of the hydrogen uptake activity migrated at an approximate molecular mass of 118,000 ± 19,000 Da. The most active sucrose gradient fractions were run on SDS-polyacrylamide gels. Silver staining of those gels showed that *P. brockii* hydrogenase consists of two subunits (Fig. 1, lane 1). The large subunit ran at approximately 66,000 Da, and the smaller subunit ran at approximately 45,000 Da. All sucrose gradient fractions that contained hydrogenase activity contained only the two bands shown in Fig. 1, lane 1. Although additional bands could be seen on some SDS-polyacrylamide gels of reactive red-purified hydrogenase (not subjected additionally to gradient purification), most SDS gels from reactive red-purified enzyme also looked like that shown in lane 1 of Fig. 1. The data from the silver-stained gels together with the molecular mass estimates from the sucrose gradients suggest that the *P. brockii* hydrogenase is an alpha 1, beta 1 dimer.

Despite the high growth temperature of *P. brockii*, purified hydrogenase is still sensitive to thermal destruction. This

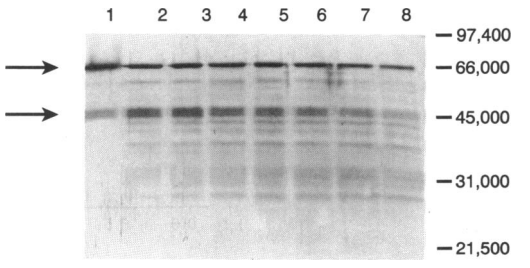

FIG. 1. Purified hydrogenase from reactive red affinity chromatography and subsequent sucrose gradient centrifugation. The two arrows mark the 66,000- and 45,000-Da subunits. The lane 1 sample was placed in SDS sample buffer with β-mercaptoethanol but was not boiled. The samples in lanes 2 through 8 were boiled in SDS sample buffer with β-mercaptoethanol for the following times (lanes): 2, 20 min; 3, 40 min; 4, 60 min; 5, 80 min; 6, 100 min; 7, 120 min; 8, 140 min. About 5 μg of hydrogenase protein was loaded per lane. The numbers on the right are molecular mass standards given in daltons.

TABLE 2. Comparison of membrane-bound and purified hydrogenase for ability to couple H_2 oxidation to various electron acceptors[a]

Electron acceptor	E_0' (mV)	Relative activity (%)	
		Membrane bound	Purified
Methylene blue (200 μM)	11	100	100
Phenazine methosulfate (400 μM)	80	40	8
Ferricyanide (1.5 mM)	360	30	21
DCIP (200 μM)	217	55	24
Cytochrome *c* (100 μM)	250	6	3
FMN (1 mM)	−122	2	11
Benzyl viologen (1 mM)	−360	5	21
Methyl viologen (1 mM)	−440	2	<1

[a] Relative activity is hydrogen uptake activity in comparison to that with methylene blue. The data from the membrane-bound hydrogenase have been published previously (31) and are presented only for comparison. Acceptors are at saturating concentrations, and each value is the average of three amperometric determinations. The 100% value for membranes was 2.9 μmol of H_2 consumed per min per mg of protein. The 100% value for the purified enzyme was 275 μmol of H_2 consumed per min per mg of protein. The standard reduction potentials (E_0) were taken from reference 23. DCIP, Dichloroindophenol; FMN, flavin mononucleotide.

destruction could be readily visualized by running boiled samples on SDS-polyacrylamide gels. When the purified enzyme was boiled for increasing amounts of time in SDS sample buffer, degradation products appeared fairly rapidly (Fig. 1). The appearance of these new bands coincided with the disappearance of the 66- and 45-kDa bands.

Purified hydrogenase showed aberrant SDS-polyacrylamide gel mobilities that were dependent upon the presence or absence of β-mercaptoethanol in the sample buffer. In samples not treated with β-mercaptoethanol, an additional silver staining band appeared at an approximate molecular mass of 94,000 Da (data not shown). In addition, the bands at 66,000 and 45,000 Da were much more diffuse. Since only two sharp bands were seen when β-mercaptoethanol was included in the sample buffer, the 94,000-Da band seen in the absence of β-mercaptoethanol may represent the holoenzyme. This result also suggests that disulfide bonds play a role in the structural integrity of the hydrogenase holoenzyme.

Electron acceptors. H_2 uptake hydrogenases from aerobic H_2-oxidizing bacteria characteristically can couple H_2 uptake to the reduction of positive potential electron acceptors. The ability to use these acceptors is usually strikingly similar for the membrane-bound enzyme and the purified enzyme (28). Methylene blue is commonly used as the standard (100%) in such experiments. The ability of purified *P. brockii* hydrogenase to use a variety of artificial electron acceptors differs somewhat from that seen (31) for the membrane-bound enzyme (Table 2). Almost all of the electron acceptors tested had a reduced ability to support hydrogen uptake with the purified enzyme as compared with the same enzyme in membranes. The exception is that the low-potential dyes benzyl viologen and flavin mononucleotide could act as better acceptors for the purified hydrogenase than for the membrane-bound enzyme.

One unexpected result was that purified hydrogenase could evolve hydrogen at a rate of approximately 8 nmol of H_2 per min per μg of protein in the presence of 1 mM reduced methyl viologen. Hydrogen evolution is a characteristic not seen for the *P. brockii* membrane-bound enzyme (31). Reduced benzyl viologen was unable to elicit hydrogen

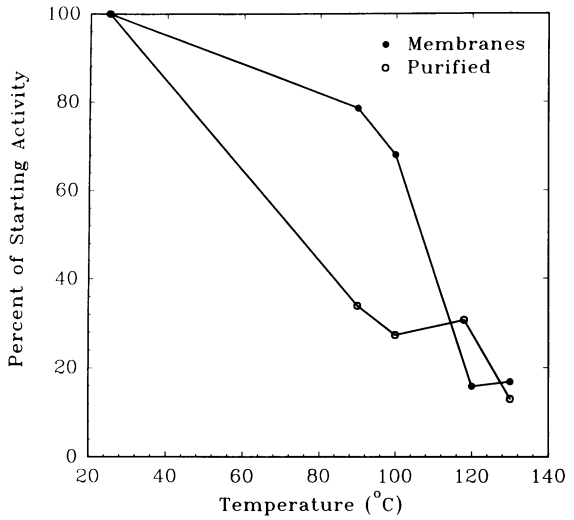

FIG. 2. Hydrogen uptake activity loss with increasing temperatures. Samples were incubated in a tightly stoppered Eppendorf tube for 15 min at the temperatures shown and then immediately assayed for hydrogen uptake activity with 200 μM methylene blue at 90°C.

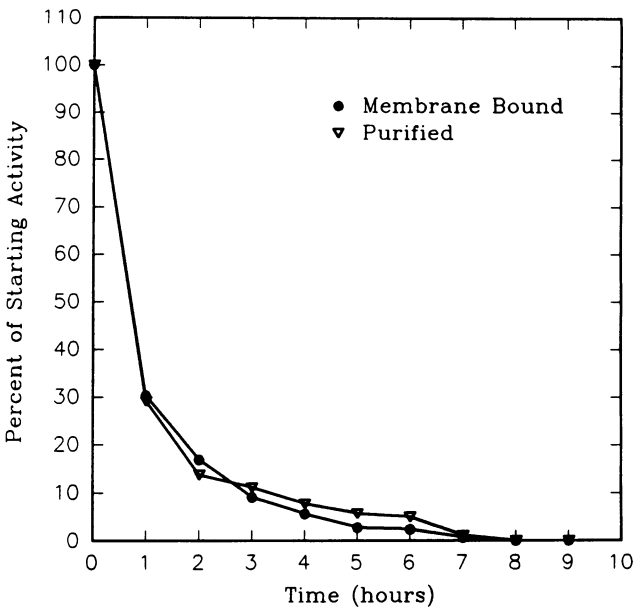

FIG. 3. Stability of hydrogenase over time at 98°C. Samples were incubated for the indicated amount of time and then immediately assayed for hydrogen uptake activity with 200 μM methylene blue at 90°C.

evolution for either the purified (data not shown) or the membrane-bound enzyme (31).

Metals analysis. All known hydrogenases are iron-sulfur proteins (1, 2), and most contain nickel as well (19). Purified *P. brockii* hydrogenase was assayed for the presence of iron, sulfur, and nickel. Colorometric data suggest that there are 6.2 ± 1.2 atoms of S and 8.7 ± 0.6 atoms of Fe per 110,000-Da dimer. When *P. brockii* was grown in the presence of $^{63}NiCl_2$, the purified hydrogenase contained detectable amounts of radioactivity. We obtained 1,505 cpm in approximately 20 μg of purified enzyme (see Materials and Methods). This procedure does not address whether nickel plays an active role in hydrogen activation or is merely a structural component of the enzyme. The amount of Ni per molecule of hydrogenase was not estimated, since calculations based upon the presence of ^{63}Ni are unreliable due to the low specific activity of commercially available $^{63}NiCl_2$.

Stability of hydrogenase. Evidence from SDS gels (Fig. 1) suggested that despite the high growth temperature of *P. brockii*, the hydrogenase enzyme could be damaged (at least degraded) by exposure to high temperatures. To address the thermostability of *P. brockii* hydrogenase, two experiments were performed. In both experiments, the lability of the membrane-bound hydrogenase and purified hydrogenase were compared to ascertain what, if any, role the membrane plays in hydrogenase thermostability. In the first experiment, *P. brockii* hydrogenase was exposed to a high temperature for 15 min and immediately assayed in an effort to judge its upper limit of thermotolerance (Fig. 2). At physiological temperatures (95 to 110°C), it seemed that the membranes did help to stabilize the hydrogenase structure. At 90°C the pure enzyme lost approximately 68% of the initial activity; however, the membrane-bound enzyme only lost approximately 20%. At temperatures above 110°C, however, the membranes were no longer able to stabilize the structure of hydrogenase.

In the second experiment, hydrogenase was incubated at 98°C for up to 9 h. Time points were taken to quantitate the remaining hydrogen uptake activity (Fig. 3). In contrast to the first experiment, the membranes seemed not to offer any protection to the hydrogenase in the longer-term experiments. The membrane-bound hydrogenase and the purified enzyme were inactivated at approximately the same rate.

Hydrogen uptake kinetics. Membrane-bound hydrogenase showed a K_m for hydrogen of approximately 19 μM with 200 μM methylene blue as an electron acceptor (Fig. 4). Purified hydrogenase had a much higher K_m (approximately 70 μM), although this number varied greatly between different prep-

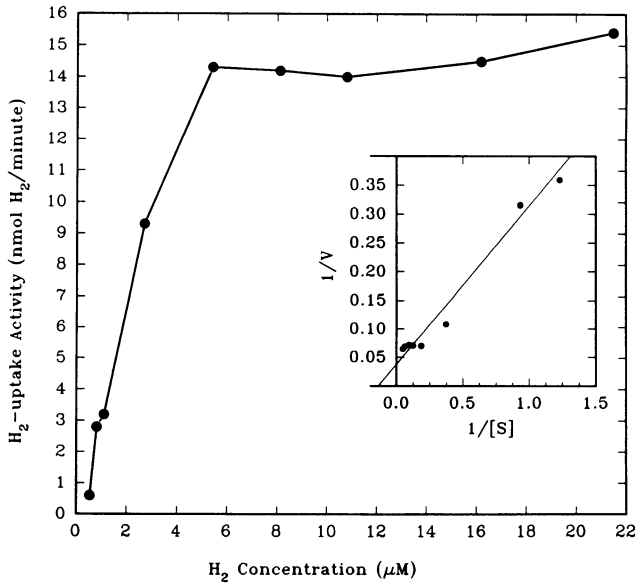

FIG. 4. Hydrogen uptake kinetics of membrane-bound hydrogenase. All assays were done at 90°C with methylene blue as the electron acceptor. K_ms were determined by double-reciprocal plotting.

arations of purified hydrogenase (data not shown). Nevertheless, the data indicate that the membranes do play an important role in the structural integrity of *P. brockii* hydrogenase.

DISCUSSION

Both the metal content and the subunit composition of purified *P. brockii* hydrogenase place it in the nickel-iron-sulfur (NiFeS) category of hydrogenases. This category of hydrogenases is structurally much more conserved than the FeS hydrogenases (1). All known NiFeS hydrogenases have at least two subunits that seem to represent a core NiFeS hydrogenase (1). In this respect the apparent alpha 1, beta 1 structure of *P. brockii* hydrogenase fits nicely into the NiFeS category, despite the fact that the bacterium itself is only distantly related to the hydrogenase-containing organisms studied most often. The amount of Fe and S present in the purified *P. brockii* hydrogenase suggests that two 4Fe-4S clusters are present. This would be consistent with the Fe and S content of some other NiFeS hydrogenases (19). However, *P. brockii* hydrogenase has far less Fe and S than the hydrogenase from the heterotroph *P. furiosus* (11). This difference of Fe and S content may reflect the vastly different physiological roles these two hydrogenases play in their respective metabolisms. Given the presence of unusual FeS clusters in *P. furiosus* hydrogen evolution hydrogenase and ferredoxin (11, 13, 14), further studies such as electron paramagnetic resonance spectroscopy are necessary to determine the exact structure of the *P. brockii* hydrogenase FeS clusters as well as to address the role of Ni in this enzyme.

Many of the membrane-bound NiFeS hydrogenases are thought to be involved in electron transport-dependent energy generation (28), since most are linked to respiratory chains. Most of these enzymes are also essentially irreversible; that is, they do not evolve (or evolve very little) H_2 even in the presence of reduced low-potential artificial electron donors. In this respect, the *P. brockii* hydrogenase presents some contradictions. The membrane-bound form of the enzyme is unable to evolve H_2; however, once the enzyme is purified, appreciable H_2 evolution can occur in the presence of reduced methyl viologen. This suggests that in its native conformation (membrane bound), the *P. brockii* hydrogen uptake hydrogenase is fairly similar to the eubacterial NiFeS hydrogenases; however, enough structural differences are present to allow the *P. brockii* hydrogenase to be reversible to a small extent once it has been purified. This is consistent with earlier data suggesting that the *P. brockii* hydrogenase was similar in structure to, but not an exact copy of, the *B. japonicum* hydrogen uptake hydrogenase (31). It remains to be seen whether these structural differences between the *P. brockii* hydrogenase and mesophilic hydrogenases are responsible for the thermostability of the *P. brockii* enzyme.

The thermostability data present some interesting conclusions. First, the initial stability of the hydrogenase with respect to temperature is enhanced by its association with the membrane. This is probably due to hydrophobic interactions between the membrane and the hydrogenase, resulting in the stabilization of the final structure. Despite this interaction, the membranes are not able to protect hydrogenase from long-term thermal damage. Considering work with other hyperthermophilic proteins, the *P. brockii* hydrogenase appears not to be particularly thermostable. For example, the hydrogenase from *P. furiosus* has a half-life of 2 h at 100°C (11), and the ferredoxin from *P. furiosus* is also thermostable, losing only 10% of its activity in a hydrogen-evolution assay after 24 h at 98°C (4). In addition, the α-glucosidase from *P. furiosus* has a half-life of 48 h at 98°C (15). The reason for *P. brockii* having so unstable an enzyme is not clear, especially considering that the hydrogenase is required for the primary mode of growth and the optimal growth temperature is 105°C. The fact that both the membrane-bound form and the purified enzyme were inactivated at the same rate suggests that the thermal instability is inherent in the protein structure and is not due to other elements such as proteases (9).

Kinetically, the *P. brockii* hydrogenase has an affinity for H_2 that falls in the middle of the known eubacterial hydrogenases. The nitrogen-fixing eubacteria have hydrogenases with the lowest K_m for H_2. For example, purified *B. japonicum* hydrogen uptake hydrogenase has a K_m for H_2 of 1.4 μM (5, 6). However, the purified hydrogenase from *Alcaligenes eutrophus* (a non-nitrogen-fixing bacterium) has a K_m of 32 μM with 200 μM methylene blue as an electron acceptor (34). Similarly, the membrane-bound hydrogenase from *Paracoccus denitrificans* has a K_m of 26 μM when in the membranes and a K_m of 20 μM after solubilization (37).

The *P. brockii* uptake-type hydrogenase seems to be fairly closely related to the eubacterial uptake-type hydrogenases, in both its physiological and physical characteristics. The presence of such a conserved enzyme in the thermophiles may be of taxonomic interest, especially given the unsettled phylogenetic status of the thermophiles (22, 44). At the least, this study provides new enzymological and biochemical data enabling us to compare the diverse ways in which bacteria deal with the activation of the simplest of all substrates, namely, H_2. Further research into the differences between the *P. brockii* hydrogenase and its mesophilic counterparts will hopefully elucidate some of the structural requirements and components that are the basis of thermophily.

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